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Etude de l'effet d'apports exogènes de silicium et / ou de proline dans l'amélioration de la tolérance de la symbiose *Medicago* - *Ensifer meliloti* aux contraintes salines

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Résumé

Les effets du silicium (Si) et/ou de la proline sur l'amélioration de la tolérance à la contrainte saline ont été étudiés chez deux espèces de luzerne, *Medicago sativa* L. et *M. truncatula* Gaertn. Deux variétés marocaines de *M. sativa* L., *Oued Lmalah* (*OL*) et *Demnate 201* (*Dm*) et une variété européenne *NS Mediana ZMS V* (*NS Med*) originaire de la Serbie ont été utilisées. Les expériences ont été menées aux différents stades de développement sous conditions contrôlées. Les résultats ont montré que la salinité réduit le taux de germination et la viabilité de l'embryon et inhibe la mobilisation des réserves des embryons en particulier chez la variété *NS Med*. Cette restriction de la germination est concomitante à un stress oxydant reflété par des fortes accumulations en malonyldialdehyde (MDA) et en peroxyde d'hydrogène (H_2O_2) et une toxicité ionique manifestée par un rapport K^+/Na^+ très réduit. Cependant, le traitement des graines par le Si induit une forte accumulation de la proline et améliore la germination, la viabilité des embryons et la mobilisation des réserves. Le Si induit également une forte activité de la catalase (CAT) et du superoxyde dismutase (SOD) et réduit la teneur en MDA et en H_2O_2. Au stade plante, la salinité réduit la croissance des plantes et la nodulation chez toutes les variétés étudiées. Cette restriction de la croissance est associée à une diminution significative de la teneur des feuilles en chlorophylles, de la fluorescence chlorophyllienne (F_v/F_m) et de la conductance stomatique. La salinité réduit la teneur des plantes en azote et en K^+ et augmente celle en Na^+. La comparaison entre les trois variétés ciblées montre que la variété européenne *NS Med* est significativement la plus affectée. L'apport exogène de Si ou de proline induit une accumulation significative des solutés compatibles telles que la proline, la glycine betaïne et les sucres solubles et améliore l'activité antioxydante de la SOD, la CAT, l'ascorbate peroxydase et la glutathionne réductase. Par rapport aux plantes non traitées, le Si améliore aussi la teneur relative en eau, réduit le niveau de stress oxydant et rétablit par conséquent la croissance ainsi que l'activité photosynthétique des plantes stressées. De même, chez la plante modèle *M. truncatula*, l'application de la proline et de Si module l'expression des gènes codant des enzymes du métabolisme de la proline telles que la Pyroline-5-carboxylate synthétase 1 (P5CS1), la P5CS2, l'Ornithine aminotransférase, la Proline déshydrogénase 1 et la P5C déshydrogénase ainsi que le gène *Low silicon 2* codant un transporteur du Si. L'apport exogène de Si et/ou de proline montre des effets améliorateurs plus importants quand ces deux molécules sont appliquées séparément chez *M. sativa*, alors que l'application combinée des deux molécules est plus bénéfique pour *M. truncatula*. Nos résultats démontrent que l'apport exogène de proline et/ou du Si présente un intérêt évident pour le développement d'une agriculture durable. L**e** développement de biostimulants à base de proline ou Si est fortement recommandé pour améliorer la productivité des cultures sous contraintes environnementales.

Mots clés: *Medicago; Ensfer meliloti*; Salinité; Silicium; Proline; Germination; Croissance; Photosynthèse; Métabolisme de la proline; Transporteur de silicium.

Abstract

The effects of silicon (Si) and/or proline on the tolerance to salt stress were investigated in alfalfa *Medicago sativa* L. and *Medicago truncatula* Gaertn. Two Moroccan *M. sativa* varieties, *Oued Lmalah* (*OL*) and *Demnate 201* (*Dm*), and a European *M. sativa* variety *NS Mediana ZMS V* (*NS Med*) originating from Serbia were used. Experiments were carried out at different stages of development. Results showed that salt stress reduced seed germination and embryo viability and inhibited reserve mobilization, particularly in the *NS Med* variety. The restricted germination is concomitant with an oxidative stress reflected by high levels of malonyldialdehyde (MDA) and hydrogen peroxide (H_2O_2) and ionic toxicity indicated by lower K^+/Na^+ ratio. However, Si supply induces a significant accumulation of proline and improves seed germination, embryo viability and reserve mobilization. Si also triggers high catalase (CAT) and superoxide dismutase (SOD) activities and reduces MDA and H_2O_2 contents. During plant growth, salinity reduces plant growth and nodulation in all of the varieties. Growth restriction is accompanied by a significant decrease in leaf chlorophyll content, chlorophyll fluorescence (*Fv/Fm*) and stomatal conductance. Salinity also reduces plant nitrogen and K^+ and increases Na^+. Among the three alfalfa varieties, the European variety *NS Med* is the most affected. Exogenous supply of Si and proline results in a considerable accumulation of some compatible solutes, such as proline, glycine betaine and soluble sugars together with an enhanced antioxidant enzyme activity, such as SOD, CAT, ascorbate peroxidase and glutathione reductase. This improved leaf relative water content, reduced oxidative stress and therefore restores growth and photosynthetic activity of salt-stressed plants. Similarly, using the model legume *M. truncatula*, the application of proline and Si modulates the expression of genes encoding enzymes of proline metabolism, such as Pyroline-5-carboxylate synthetase 1 (P5CS1), P5CS2, Ornithine aminotransferase, Proline dehydrogenase 1, and P5C dehydrogenase as well as *Low silicon 2* gene encoding a silicon transporter. In conclusion, separate application of proline and Si is more beneficial for improving *M. sativa* salt tolerance while the combined application of the two molecules is beneficial for *M. truncatula*.

Keywords: *Medicago; Ensifer meliloti;* Salinity; Silicon; Proline; Germination; Growth; Photosynthesis; Proline metabolism; Si transporter.

ملخص

في هده الدراسة، تم اختبار تأثير السليسيوم و البرولين على تحسين مقاومة الفصة (*Medicago sativa*) لتأثير الملوحة. تم استعمال بذور نوعين من الفصة المغربية (الواد المالح ودمنات 201) ونوع أوروبي من صربيا (*NS Mediana ZMS V*). التجارب تمت في ظروف جد محكمة وخلال مختلف مراحل النمو. اصفرت النتائج على ان الملوحة (200mM NaCl) قد اترت سلبا على انبات البدور، قابلية الجنين للحياة وقدرته على استهلاك المخزون الطاقي خصوصا لدى النوع الأوروبي. التأثير السلبي للملوحة على انبات البدور كان مرفق بأكسدة الأغشية ناتجة عن انتاج كميات كبيرة من الماء الأوكسيجيني وامتصاص كبير لأيون الصوديوم. على خلاف ذلك، إضافة السليسيوم في الوسط أدى الى انتاج كميات كبيرة من البرولين مما أدى الى تحسين انبات البدور، قابلية الجنين للحياة وقدرته على استهلاك المخزون الطاقي. إضافة السليسيوم أدى كذلك الى تحسين نشاط بعض الأنزيمات متل السيبر أوكسيد ديسموتاز والكطلاز مما ادى الى تحسين جودة الأغشية والنقص من انتاج الماء الأوكسيجيني. خلال مرحلة النمو، اترت الملوحة (120-200 mM) سلبا على نمو الفصة وعلى اشتغال العقد لدى جميع الأنواع المدروسة. ضعف النمو تحت تأثير الملوحة كان مرفق بكميات جد صغيرة من اليخضور علاوتا عن اشعاع ضوئي (F_v/F_m) وتبادلات غازية جد ضعيفة. تأثير الملوحة كان أيضا جد واضح من خلال التأثير على كمية البوتاسيوم والنيتروجين. المقارنة بين أنواع الفصة المدروسة ابانت على ان النوع الأوروبي هو النوع الأقل مقاومة للملوحة في حين أن النوع المغربي (الواد المالح) هو النوع الأكثر مقاومة. إضافة السليسيوم او البرولين في الوسط أدى الى انتاج كميات كبيرة من السكريات الدائبة و البرولين و لغليسين بيطايين إضافة الى نشاط جد عالي لبعض الأنزيمات متل السيبوريكسيد ديسموطاز, الكاطالاز, الأسكوربات بيروكسيداز و الغليتاتيون بيروكسيداز. هذه الاستجابة أدت الى تحسين مستوى نسبة الماء في الأوراق وانخفاض معدل ضغط الأكسدة مما أدى الى تحسين نمو نباتات الفصة وقدرتهم على الحفاض على مستوى عال من التركيب الضوئي. نتائج هده الدراسة ابانت كذلك على ان التأتير الإيجابي لسيليسيوم ولبرولين تم عن طريق التأتير على اشتغال الجينات المراقبة لانتاج البرولين (*pyroline-5-carboxylase synthetase 1* (*P5CS1*), *P5CS2*, l'*ornithine aminotransférase,* la *proline déshydrogénase 1* et la *P5C déshydrogénase*), بالإضافة للجينات المراقبة لامتصاص و تنقل السليسيوم (*Low silicon 2*). المقارنة بين العلاجات المستعملة ابانت على أن إضافة السيليسوم والبرولين كل لوحده لها تأتير جد ملحوض على تعزيز مقاومة الفصة (*M. sativa*) للملوحة، في حين المقاومة العالية للملوحة للنبتة النموذج (*M. truncatula*) لوحضت تحت الإضافة المشتركة للاثنين.

الكلمات المفتاح: *Medicago*، ملوحة، سليسيوم، برولين، انبات، نمو، مقاومة، التركيب الضوئي، استقلاب البرولين، تنقل السليسيوم

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- **El Moukhtari A**, Séverine P, Crilat E, Carol P, Farissi M, Savoure A. Proline and silicon-mediated alleviation of salinity stress in *Medicago truncatula* by regulating proline metabolism and silicon transporters related genes. En preparation.

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Introduction générale

Les Fabacées ou légumineuses représentent une composante très importante dans la plupart des systèmes agricoles (Semba et al. 2021). Elles sont très riches en protéines, en fibres et en éléments minéraux (Tas et Shah 2021; Multari et al. 2015). Ces plantes ont la capacité de réduire l'azote atmosphérique en ammoniac au sein de nodosités racinaires grâce à leur symbiose avec les rhizobia du sol (Raza et al. 2020). Cette symbiose fournit l'azote nécessaire pour les Fabacées et contribue ainsi à l'amélioration du bilan azoté du sol au profit des autres plantes associées au système de culture mixte ou en rotation (Duchene et al. 2017). Ainsi, la symbiose entre les rhizobia et les Fabacées peut, lorsqu'elle fonctionne bien, permettre aux agriculteurs de limiter les coûts liés à l'utilisation de fertilisants chimiques et la pollution de l'environnement (Farissi et al. 2014).

La luzerne (*Medicago* sp) est l'une des Fabacées fourragères la plus cultivée dans les régions méditerranéennes grâce à ses nombreuse caractéristiques agronomiques (Lesins et Lesins 2012; Hammani et al. 2009). Elle représente une source importante des protéines pour les vaches laitières, les ovins, les bovins et d'autres animaux (Radovic et al. 2009). L'importance agro-écologique de la luzerne est d'autant plus élevée quand elle se manifeste par la fixation biologique de l'azote atmosphérique (FBN) en symbiose avec l'espèce de rhizobia *Ensifer meliloti* (anciennement appelée *Sinorhizobium meliloti*). Au stade floraison, la FBN liée à la symbiose *M. sativa-Ensifer meliloti* est estimée à 300 kg par hectare par an (Bruning et Rozema 2013). De même, grâce à son système racinaire pivotant, la luzerne peut absorber l'eau à une profondeur d'au moins 3 m, ce qui lui donne la capacité de se développer dans les régions où les conditions environnementales sont difficiles (El-Ramady et al. 2020). Ce système racinaire pivotant peut aussi fractionner le sol et amélioré sa texture (Mouradi et al. 2018).

Dans l'agriculture marocaine, la luzerne *M. sativa* représente la première plante fourragère. Sa culture occupe environ 455 000 ha, soit 25% de la superficie totale consacrée aux cultures fourragères (Mouradi et al. 2018). Les agriculteurs utilisent généralement des populations locales qui ont évolué selon un processus évolutif complexe dû à la sélection naturelle et artificielle associée à des flux géniques intraspécifiques engendrant une variabilité génétique très importante (Farissi et al. 2014). Ces populations sont caractérisées par leur adaptation aux conditions locales (Farissi et al. 2011; Mouradi et al. 2018). Cependant, la richesse de ce patrimoine génétique est actuellement de plus en plus menacée par plusieurs facteurs environnementaux, telles que la sécheresse, la contamination des sols par les métaux lourds et la salinisation des sols par les eaux d'irrigation (Bouizgaren, 2007).

La salinité est l'un des facteurs abiotiques majeurs qui contraint la production des cultures en générale et celle des Fabacées en particulier (Van zelm et al. 2020; Faghire et al. 2013). Cette

contrainte provoque à la fois un abaissement du potentiel hydrique du sol et une toxicité ionique (Munns et Tester 2008). De plus, à cause des changements climatiques et de l'irrigation, la salinité augmente à travers le monde (Deinlein et al. 2014). Dans le bassin méditerranéen, caractérisé par des précipitations irrégulières et une forte transpiration, la salinité affecte 20% des terres arables (Safdar et al. 2019). La salinité affecte la germination des graines, la croissance des plantes, la photosynthèse qui conduit à une baisse de la productivité et du rendement (Nadeem et al. 2019). De plus, cette contrainte agit sur la survie et la prolifération des rhizobia au niveau du sol et la rhizosphère, elle perturbe le processus d'infection, et affecte directement le fonctionnement des nodosités racinaires (Kirova et Kocheva 2021).

Pour faire face aux contraintes abiotiques, notamment la salinité, les plantes adoptent des mécanismes de tolérance. Ainsi, l'accumulation de solutés organiques compatibles représente l'un de ces mécanismes de tolérance (Bargaz et al. 2015). La proline représente l'un des osmolytes les plus fréquemment accumulés chez les plantes pour limiter les effets de la contrainte saline (Alvarez et al. 2021). Il a été démontré que, lors d'une contrainte saline, la proline est synthétisée essentiellement au niveau du cytosol à partir du glutamate *via* l'action successive de la Pyrroline-5-carboxylate synthétase (P5CS) et la Pyrroline-5-carboxylate réductase (P5CR) (Szabados et Savouré 2010). D'autres études ont démontré que chez les Fabacées, la proline est synthétisée *via* la voie de l'ornithine (AbdElgawad et al. 2015). En effet, à travers une transamination, l'Ornithine aminotransférase (δOAT) convertit l'ornithine et le α-ketoglutarate en glutamate et en pyrroline-5-carboxylate (P5C). Ce dernier sera réduit par la suite en proline sous l'action de la P5CR. Le catabolisme de cet acide aminé se produit dans la mitochondrie sous l'action successive de la Proline déshydrogénase (ProDH) et de la Pyrroline-5-carboxylase déshydrogénase (P5CDH) en utilisant respectivement le FAD et le NAD^+ comme cofacteur et en produisant le P5C et le glutamate (Szabados et Savouré 2010). D'autre part, des études antérieures ont montré que l'apport exogène de proline a des effets bénéfiques sur les plantes exposées aux contraintes salines (Ashraf et Foolad 2007). Elle améliore la germination des graines, la croissance, la photosynthèse, la FBN et la productivité des plantes exposées à la contrainte saline (Meena et al. 2019).

Le silicium (Si), deuxième élément le plus abondant dans la croute terrestre, a souvent été décrit pour ses effets protecteurs contre les contraintes abiotiques (Debona et al. 2017). Au cours d'une contrainte saline, le Si a montré des effets très positifs sur la germination des graines, la photosynthèse, l'ajustement osmotique, la stabilité membranaire, le métabolisme oxydatif et la FBN (Rizwan et al. 2015; Etesami et Adl 2020; Putra et al. 2021). Cependant, les mécanismes d'action de cet élément sont encore à élucider et plusieurs hypothèses ont été proposées. Une

première hypothèse suggère que le Si se polymérise à la surface des tissus en formant une barrière mécanique (Coskun et al. 2019). Une seconde hypothèse postule que le Si pourrait induire des modifications métaboliques dans les cellules végétales, ce qui suggère que le Si peut activer directement ou indirectement des réactions de défense chez la plante par notamment la régulation de l'activité des enzymes, l'induction de l'accumulation des solutés compatibles et de l'expression de gènes cibles (Al Murad et al. 2020). Cependant, ces effets dépendent généralement de la capacité des plantes à accumuler le Si, qui dépend de la présence de transporteurs spécifiques connus sous le nom low silicon transporter (LSi) (Coskun et al. 2021). Dans ce contexte, cette thèse de doctorat vise comme objectif principal l'évaluation de l'impact de l'apport exogène du Si et de la proline dans l'amélioration de la tolérance de la symbiose luzerne-rhizobia à la contrainte saline, et de tester si la combinaison des deux molécules renforce la tolérance des plantes à cette contrainte. Nous nous sommes intéressés aux effets de ces molécules sur la mobilisation des réserves et la viabilité de l'embryon au cours de la germination, la photosynthèse, la nutrition minérale, l'osmorégulation, le métabolisme oxydatif et la fixation biologique de l'azote atmosphérique. A l'échelle moléculaire, le rôle du Si et de la proline dans la tolérance de la symbiose luzerne-rhizobia à la contrainte saline a été étudié à travers la régulation de l'expression des gènes *P5CS1, P5CS2, OAT, P5CDH* et *ProDH1* qui codent des enzymes impliquées dans le métabolisme de la proline, *LSi2* qui code pour un transport de Si et d'autres en relation avec la tolérance à la contrainte osmotique tels que *la Dehydrin 2* (*DHN2*).

Chapitre I

Synthèse bibliographique

I. Généralités sur les légumineuses

Les Fabacées constituent la troisième des plus grandes familles des plantes à fleurs (Zhu et al. 2005). Elles comportent plus de 750 genres et plus de 20.000 espèces différentes (Ratnayake et al. 2001; Tas et Shah 2021). Parmi celles-ci, environ 60 espèces ont été domestiquées, dont le soja, le haricot, le pois chiche et la lentille (Boukid et al. 2019). C'est une famille qui présente une grande importance économique et écologique (Rochester et al. 2001). A l'échelle mondiale, les Fabacées occupent aujourd'hui avec 180 millions d'hectares en surface cultivée, soit environ 15% des terres cultivées (Morel et al. 2012). Au Maroc, la culture des Fabacées représente 5% de la surface agricole utile, avec une production moyenne annuelle de 332.000 tonnes (Mouradi et al. 2018).

Les Fabacées jouent un rôle très important dans le système agricole, grâce à leur capacité d'établir une symbiose fixatrice d'azote atmosphérique (FSN) avec les Rhizobia (Semba et al. 2021). Cette famille de plantes constitue aussi une part très importante de l'alimentation humaine depuis environ 8.000 ans (Tas et Shah 2021). Les Fabacées sont très riches en glucides et en protéines, et offrent également des teneurs importantes en lipides, fibres, éléments minéraux et vitamines pour l'alimentation humaine et animale (Multari et al. 2015). On distingue deux types de Fabacées, les Fabacées à graines (fève, pois chiche, haricot, etc.) et les Fabacées fourragères.

Au sein des Fabacées fourragères on distingue des espèces annuelles comme *Trifolium alexandrinum* L., *T. incarnatum* L., *Trigonella foenumgraecum* L., *Medicago polymorpha* L., *Medicago truncatula* Gaertn (McCartney et Fraser 2010), et des espèces pérennes (vivaces) telles que *T. pratense, T. repens, Onobrychis* spp, *Lotus corniculatus, M. sativa* L. (Carlsson et Huss-Danell 2003; Şakiroğlu 2021). L'espèce *M. sativa* subsp. *sativa* est la principale Fabacées vivace cultivée dans la plupart des régions tempérées, en particulier là où les systèmes agricoles reposent largement sur la production du fourrage (Annicchiarico et al. 2015).

I-1 Présentation de la luzerne

La luzerne est une plante herbacée appartenant au genre *Medicago* sp. Ce genre regroupe plus de 60 espèces d'intérêt agronomique dont les deux tiers sont des espèces annuelles, mais aussi des espèces pérennes (Şakiroğlu et İlhan 2021). Les espèces annuelles tels que *M. arabica, M. lupulina* et *M. truncatula* ont récemment connu un regain d'intérêt grâce à leur utilisation en rotation avec les céréales (Avci 2021; Prosperi et al. 2001). Au sein de genre *Medicago* sp., il existe aussi des espèces pérennes dont la plus répandue et la plus connue *M. sativa* (Prosperi et al. 2001).

I-1-1 **Caractéristiques botaniques**

- **La luzerne cultivée *M. sativa***

M. sativa L. ou la luzerne cultivée est une plante pérenne, allogame, entomophile, tétraploïde (2n=4x=32 chromosomes) avec un génome de 800 à 1000 Mbp et dont le fruit est une gousse (Flajoulot et al. 2005; Şakiroğlu et İlhan 2021; Biazzi et al. 2019). Cette espèce est la plus cultivée dans le monde grâce à ces nombreux avantages agronomiques. Elle possède un système racinaire pivotant et profond qui peut aller jusqu'à deux mètres et qui lui permet d'améliorer la structure du sol (El-Ramady et al. 2020). La racine porte des nodosités au sein desquelles se déroule la fixation symbiotique de l'azote atmosphérique (FSN) grâce à l'établissement d'une symbiose avec un grand nombre d'espèces de Rhizobia dont la plus connue est *Ensifer meliloti* (anciennement appelée *Sinorhizobium meliloti*) (Mouradi et al. 2018; El-Ramady et al. 2020). Les tiges sont dressées et glabres, parfois paludes. Un pied de *M. sativa* peut porter un très grand nombre de tiges. Les feuilles sont généralement trifoliées. L'espèce *M. sativa* présente des fleurs hermaphrodites de couleur violette, groupées en inflorescence sous forme de grappes courtes et pyramidales avec 15 à 30 fleurs (Birouk et al. 1997).

- **L'espèce modèle *M. truncatula***

M. truncatula est une plante annuelle, autogame et diploïde (2n=2x=16). Elle possède un génome de faible taille de 550 Mpb, et se caractérise par un temps de génération de trois mois, ce qui la rend intéressante pour les analyses moléculaires (Rose 2008). Un autre avantage de *M. truncatula* est sa transformation facile par *Agrobacterium rhizogenes* et *A. tumefaciense* (Boisson-Dernier et al. 2001; Rose 2008). De plus, des cartes génétiques ont été établies par plusieurs laboratoires (Cook 1999; Ané et al. 2008). Les deux principaux génotypes de *M. truncatula* actuellement utilisés en laboratoire sont A17 issu du cultivar Jemalong et R-108-1 issu de l'écotype 108-1 (Ané et al. 2008; Journet et al. 2001).

En raison de son cycle de vie court, son génome diploïde de faible taille, une importante production de graines et sa bonne réponse aux transformations génétiques, *M. truncatula* a été proposée en 1990 comme une plante modèle pour étudier l'interaction Fabacées-rhizobia (Barker et al. 1990). Le génome de *M. truncatula* présente une forte synténie avec plusieurs autres Fabacées, notamment la luzerne cultivée (*M. sativa*) et le pois (Zhu et al. 2005; Aubert et al. 2006). Cette similarité permet ainsi le transfert des connaissances sur cette plante modèle vers d'autres Fabacées. Ces avantages sont mis à profit pour des études de génomique fonctionnelle et structurale en vue de l'identification de gènes agronomiques intéressants, ainsi

que l'étude des interactions symbiotiques Fabacées-rhizobia (Cook 1999; Catoira et al. 2000; Ané et al. 2008).

I-1-2 Culture de la luzerne

La luzerne est la plante fourragère la plus cultivée dans le monde, avec une superficie de plus de 30 millions ha (Singer et al. 2017). A l'échelle mondiale, l'Amérique du nord, le Canada, l'Australie, l'Argentine, L'Inde, l'Italie, la Nouvelle Zélande, la France et la Russie sont les principaux producteurs mondiaux de luzerne (El-Ramady et al. 2020). Au Maroc, la luzerne cultivée appartient à l'espèce *M. sativa* L. (Lesins et Lesins 2012). Sa culture occupe 80.500 ha, soit environ 22,7% de la superficie consacrée aux cultures fourragères dont 40% en systèmes irrigués (Hammani et al. 2009). Elle constitue la seule espèce fourragère cultivée dans les oasis et les palmeraies des vallées du Ziz, du Drâa et du Dadès et elle est la principale plante fourragère dans les régions de Tadla et Haouz (Hammani et al. 2009; Bouazzama et al. 2015). Au Maroc, les agriculteurs utilisent généralement des populations locales qui ont évolué selon un processus évolutif complexe où interviennent notamment la sélection naturelle et humaine et des flux géniques intraspécifiques engendrant sur des années une variabilité génétique très importante (Farissi et al. 2014). Ces populations sont caractérisées par leur adaptation aux conditions locales (Mouradi et al. 2018) et elles contribuent fortement au développement socio-économique des familles locales en tant que principale source de nutrition du bétail (Farissi et al. 2011, 2013). Bouizgaren (2012) définit trois zones de culture de la luzerne au Maroc:

- Les zones irriguées au nord de l'Atlas, telles que celles de Tadla et du Haouz, présentant respectivement 24.000 ha et 19.000 ha.
- Les oasis sahariennes et les palmeraies des vallées du Ziz, du Drâa et du Dadès, au sud de l'Atlas où la luzerne constitue la seule espèce fourragère cultivée. Les superficies de ces zones atteignent 7.300 ha et 9.400 ha respectivement au niveau de Tafilalet et d'Ouarzazate.
- Les montagnes de l'Atlas avec principalement la zone de Béni Mellal, d'Azillal, de Khénifra et du Haut Atlas ''demnate''.

I-1-3 **Exigence pédoclimatique de la luzerne**

Une culture de luzerne productive à long terme dépend en premier lieu d'un taux de germination élevé. Ainsi, toute contrainte, biotiques ou abiotiques, affectant ce stade critique pourrait avoir des conséquences néfaste sur le rendement (Hamidi et Safarnejad 2010; Štrbanović et al. 2016). La luzerne peut se développer dans des conditions climatiques variées allant des régions arides aux régions tropicales. Elle peut se développer jusqu'à une altitude de 2.500 m (El-Ramady et

al. 2020). L'intervalle d'irrigation moyen de la luzerne est de 7 à 10 jours (El-Ramady et al. 2020). Cependant grâce à son système racinaire profond, la luzerne peut supporter une sécheresse avec un intervalle d'irrigation de 25 à 30 jours (El-Ramady et al. 2020). Elle fructifie dès que la pluviométrie dépasse 400 mm an^{-1} (Hammani et al. 2009). De plus, même si la luzerne supporte une température de l'ordre de 37 °C et minimale de l'ordre de 8 à 9 °C, la température pour un développement optimal est de l'ordre de 25 °C (Janati 1990). Quant au pH du sol, les plantes de luzerne affectionnent les sols neutres à pH compris entre 6,5 et 7, profonds et secs avec un drainage interne adéquat (Bouizgaren 2007; Peters et al. 2005). La luzerne préfère les sols fertiles riches en phosphore, potassium, calcium et en oligo-éléments (Janati 1990; Bouizgaren 2007; Ottman 2010). De plus, comme la luzerne possède un système racinaire pivotant, les sols profonds favorisent son développement, ce qui lui permet d'occuper une grande surface de sol et par conséquent une meilleure absorption de l'eau et des éléments nutritifs (Haynes 1980).

I-1-4 Intérêts agronomique et écologique de la luzerne

La luzerne joue un rôle important dans l'agriculture mondiale et dans le développement durable. Elle présente un grand nombre d'intérêts agronomiques et environnementaux (Venkateshwaran et Ané 2011). La luzerne constitue une source majeure de vitamines et de fibres de qualité pour l'alimentation animale et humaine (Tableau 1) (Gawel 2012, 2017). Elle représente aussi une source importante de protéines pour le bétail et constitue aussi un élément nutritif de base pour les vaches laitières, les bovins, les ovins, les chèvres et d'autres classes d'animaux domestiques (Radovic et al. 2009). De plus, grâce à un système racinaire pivotant, la luzerne améliore la texture du sol, limite son érosion et permet de limiter la perte en nitrates par lessivage (Broderick et al. 2001). De même, les feuilles de la luzerne peuvent constituer une source importante de matière organique, ce qui permet d'améliorer la fertilité du sol (Suttie 2000). L'importance agronomique d'une culture de luzerne réside également dans la capacité de cette espèce à fixer des grandes quantités de N_2 grâce à son association avec les rhizobia. Cette fixation de l'azote peut aller jusqu'à 300 kg ha^{-1} an^{-1} (Bruning et Rozema 2013). Grace à cette relation symbiotique, la luzerne peut apporter une quantité d'azote voisine de 30 à 50 kg ha^{-1} an^{-1} dans le cas de rotation de culture (Franche et al. 2009). Les caractéristiques agronomiques réduisent l'utilisation des intrants chimiques expansifs et polluants pour l'environnement (Farissi et al. 2014).

Tableau 1: Valeur nutritionnelle des feuilles de luzerne (El-Ramady et al. 2020)

Elément	Valeur
Energie (Kcal 100 g^{-1})	344
Protéine (g kg^{-1})	600
Lipide (g kg^{-1})	225
Carbohydrate (g kg^{-1})	125
Fibre (g kg^{-1})	10
Carotène (mg kg^{-1})	867
Thiamine (mg kg^{-1})	5
Riboflavine (mg kg^{-1})	5
Niacine (mg kg^{-1})	242
Acide folique (mg kg^{-1})	0.33
Acide ascorbique (mg kg^{-1})	22
Calcium (mg kg^{-1})	18650
Fer (mg kg^{-1})	990

II. Les contraintes salines

II-1 Définition

La salinité est définie par un excès de sels minéraux solubles dans l'eau d'irrigation ou dans la solution du sol (Farissi et al. 2014). Ces sels sont représentés en grande partie par la combinaison de trois cations (Ca^{2+}, Mg^{2+} et Na^{+}) et trois anions (Cl^{-}, SO_4^{2-} et HCO_3^{-}) (Safdar et al. 2019). Historiquement, les sols ont été classés comme salins, sodiques ou salins-sodiques en fonction de la concentration totale en sel et des rapports Na^{+}/Ca^{2+} et Na^{+}/Mg^{2+} dans le sol (Yadav et al. 2011). En général, les chlorures de sodium (NaCl) sont les sels les plus fréquents et représentent plus de 90% des sels (Farissi et al. 2014; Dudley 1994).

II-2 L'origine de la salinité

Deux types de salinité ont été décrits: salinité primaire et salinité secondaire. La salinité primaire est le résultat de l'accumulation de sels sur de longues périodes par des processus naturels dans le sol ou les eaux souterraines (Parihar et al. 2015; Yadav et al. 2011; Safdar et al. 2019). Elle est provoquée par deux processus naturels (Parihar et al. 2015). Le premier est l'altération des matériaux parentaux contenant des sels solubles. Les processus d'altération décomposent les roches et libèrent des sels solubles de divers types, principalement des chlorures de sodium, de calcium et de magnésium et, dans une moindre mesure, des sulfates et des carbonates. Le second est le dépôt de sel océanique transporté par le vent et la pluie.

La salinisation secondaire résulte des activités humaines qui modifient l'équilibre hydrologique du sol entre l'eau appliquée (irrigation ou pluie) et l'eau utilisée par les cultures (Yadav et al. 2011). Le défrichement et le remplacement de la végétation pérenne par des cultures annuelles,

l'irrigation excessive, l'utilisation d'une eau d'irrigation riche en sels, un drainage insuffisant ou l'utilisation excessive d'engrais sont les principales origines de la salinité secondaire.

II-3 Effets des contraintes salines sur les plantes

La contrainte saline n'affecte pas seulement la qualité et les propriétés physico-chimiques du sol, mais affecte aussi la production agricole. En général, les plantes qui poussent dans un environnement salin font face à trois contraintes (Van zelm et al. 2020; Munns et Tester 2008; Parida et al. 2004; Safdar et al. 2019):

- Une sécheresse physiologique: l'augmentation de la concentration en sels de la solution du sol entraîne une réduction du potentiel osmotique de celui-ci. L'eau devient alors moins disponible pour les plantes.
- Une toxicité ionique: les ions salins peuvent causer des toxicités en interférant avec les processus physiologiques et biochimiques de la plante à cause de leur accumulation en grandes quantités.
- Une déficience nutritionnelle: les ions responsables de la salinité entrent en compétition avec d'autres ions indispensables à la croissance et au développement des plantes entrainant l'apparition des carences et des déséquilibres importants.

L'effet de la salinité sur la plante peut être observé à différents stades de développement.

II-3-1 Effet de la contrainte saline sur la germination des graines

La germination des graines représente l'un des stades critiques dans le cycle de vie des plantes (Ali et Elozeiri 2017). Elle conditionne le développement ultérieur de la jeune plante dans son milieu et probablement sa productivité (Ali et Elozeiri 2017). La contrainte saline représente l'un des principaux facteurs limitant la germination des graines chez nombreuses espèces (Rajabi Dehnavi et al. 2020 ; Ludwiczak et al. 2021 ; Debez et al. 2018). La germination dépendante de la présence d'eau, d'oxygène et d'une température favorable, la salinité en induisant une contrainte osmotique diminue la capacité de la graine à absorber l'eau qui engendre un retard ou une inhibition complète de la germination (Zhang et al. 2010). De plus, l'influence de la salinité peut se manifester par des effets toxiques des ions sodium et chlorure sur la germination des graines (Farissi et al. 2011; Ghoulam et Fares 2001). A travers un effet osmotique et/ou une toxicité ionique, la contrainte saline induit souvent une germination irrégulière et une émergence non synchronisée des plantules, induisant un faible peuplement et un rendement réduit (Farissi et al. 2014). Chez *Suaeda salsa* et *Phragmites australis*, Xiao et al. (2016) ont rapporté que l'exposition des graines à des concentrations élevées de NaCl réduit

significativement le pourcentage et la vitesse de germination avec une réduction de la longueur de la radicule. Dans le même sens, Ghavami et Ramin (2007) ont montré que l'imbibition des graines de *Silybum marianum* avec une solution saline réduit significativement le pourcentage de germination, le taux de germination et l'émergence des jeunes plantes avec une augmentation dans le temps nécessaire pour la germination de 50% des graines. De plus, chez *Pancratium maritimum*, la contrainte saline affecte négativement la division cellulaire et le métabolisme de l'embryon des graines en germination en réduisant l'activité des enzymes hydrolytiques de l'albumen telles que les amylases, les protéases, les RNases et les phosphatases (Elsayed et al. 2018). De même, Liu et al. (2018) ont démontré chez *Oryza sativa* que la contrainte saline induit un déséquilibre hormonal en augmentant la balance acide abscissique/acide gibbérellique conduisant à une dormance plus longue.

II-3-2 Effet de la contrainte saline sur la croissance des plantes

Plusieurs études ont démontré que la contrainte saline a un effet néfaste sur la croissance et le développement des plantes (Van Zelm et al. 2020; Yang et Guo 2018). Selon Sandhu et al. (2017), la contrainte saline provoque chez la luzerne une réduction de la biomasse sèche, la hauteur des plantes et le nombre de tiges. De même, chez la betterave, Ghoulam et al. (2002) ont montré que l'exposition des plantes à 200 mM NaCl induit une réduction significative du nombre de feuilles, de la surface foliaire, des biomasses fraiches et sèche des parties aérienne et racinaire. La salinité a un effet négatif sur le nombre de gousses par plante, le nombre de graines par gousse, le poids de mille graines et augment le pourcentage des graines stériles (Mahmood et al. 2009).

Selon Van Zelm et al (2020), la salinité affecte tous les processus physiologiques de la plante. En effet, la diminution du contrôle de l'état hydrique (Meng et al. 2018), le déséquilibre ionique (Hu et Schmidhalter 2005), le ralentissement de la synthèse protéique, la perturbation de la stabilité des structures membranaires, l'inhibition des activités enzymatiques (Shahid et al. 2020), les changements dans l'extensibilité de la paroi cellulaire (Munns 2002), la réduction de la capacité photosynthétique (Huang et al. 2019) et l'altération du métabolisme hormonal (Shahid et al. 2020) sont parmi les principales causes de la réduction de la croissance des plantes en réponse aux contraintes salines.

II-3-3 Effet de la contrainte saline sur l'état hydrique de la plante

La disponibilité en eau des tissus en croissance devient un facteur limitant en conditions salines en induisant une sécheresse physiologique même en présence d'eau dans le sol (Manchanda et

Garg 2008). L'absorption de l'eau par les plantes exposées à la contrainte saline devient donc importante. Selon Neumann et al. (1988), la contrainte saline inhibe l'expansion foliaire à travers la réduction de la turgescence. En outre, Munns (2002) a rapporté que la présence de concentrations élevées en sel dans le sol induit une contrainte osmotique réduisant par conséquent l'assimilation de l'eau par les racines ainsi que son transport vers les parties aériennes. Par conséquent, Santander et al. (2021) ont montré une augmentation du potentiel osmotique foliaire accompagné par une réduction significative de la teneur relative en eau (TRE) des feuilles de *Lactuca sativa* sous contrainte saline. La réduction de la TRE foliaire en présence du sel a été rapportée également chez *Medicago sativa* (Farissi et al. 2018), *Artemisia absinthium, A. vulgaris, Chenopodium album, Salsola komarovii, Sanguisorba minor* (Calone et al. 2021) et *Cucumis melo* (Akrami et Arzani 2018).

II-3-4 Effet de la contrainte saline sur l'absorption des nutriments

La salinité affecte négativement l'équilibre nutritionnel des plantes qui résulte généralement d'une perturbation de l'assimilation, le transport et la distribution des nutriments (Farooq et al. 2018). En effet, l'une des conséquences les plus dommageables de la contrainte saline est la substitution de K^+ par Na^+ engendrant un rapport Na^+/K^+ élevé (Basu et al. 2021). La présence du sel dans le milieu de culture inhibe également l'absorption du phosphore, de l'azote, du magnésium, du calcium et du fer par les racines ainsi que leur transport vers les parties aériennes (Miura et al. 2013; Wang et al. 2021; Alpaslan et al. 1998). De même chez les Fabacées, la contrainte saline réduit la quantité de N_2 fixée par les Rhizobia (Farissi et al. 2014). Cependant, cette contrainte entraine une accumulation de certains ions bivalents comme le manganèse, le cuivre et le zinc, qui sont délétères pour le métabolisme de la plante (Zaghdoud et al. 2021; Hasanpour et al. 2015). Des études antérieures ont montré, qu'en plus de déséquilibres ioniques, la contrainte saline inhibe l'activité de certaines enzymes clés impliquées dans l'assimilation des nutriments notamment la nitrate réductase (Ghoulam et al. 2002), les phosphatases acides (D'Souza et Devaraj 2010) et les phytases (Nasri et al. 2012).

II-3-5 Effet de la contrainte saline sur la photosynthèse

La photosynthèse est l'un des processus physiologiques majeurs des plantes. L'effet de la contrainte saline sur ce processus se manifeste essentiellement par la réduction du nombre et de la taille des stomates (Kwon et al. 2019). Cela perturbe les échanges gazeux des feuilles en réduisant l'assimilation de CO_2, ce qui influence directement l'activité de la RuBisCO ainsi que d'autres enzymes notamment la nitrate réductase et la saccharose phosphate synthétase (Pan et

al. 2021). Chez la luzerne, il a été démontré également que l'exposition des plantes à 200 mM NaCl pour une durée de 30 jours induit une augmentation significative de la teneur des feuilles en Na^+ (Farissi et al. 2018). Cette augmentation de Na^+ est négativement corrélée avec la teneur des feuilles en pigments photosynthétiques notamment les chlorophylles a et b ainsi les teneurs en chlorophylles totales. Alamri et al. (2020) ont démontré que la salinité, en particulier le Na^+ induit l'activité de certaines enzymes dégradant les chlorophylles telles que la chlorophyllase, la Chl-degrading peroxidase et la pheophytinase. La contrainte saline réduit également la photosynthèse nette, le rendement quantique F_v/F_m, la transpiration et la conductance stomatique chez plusieurs espèces de plantes dont le *Spartina pectinata*, *Distichlis spicata*, *Bolboschoenus maritimus*, *Z. mays* et *Cucumis sativus* (Maricle et Maricle 2018; Yang et Lu 2005; Shu et al. 2012).

II-3-6 Effet de la contrainte saline sur l'établissement de la symbiose Fabacées-Rhizobia

L'effet de la contrainte saline sur l'établissement de la symbiose Fabacées-Rhizobia se manifeste généralement à différents niveaux. Avant l'établissement de la symbiose, la salinité réduit le nombre de poils racinaires et les sites d'infections des racines (Zahran et Sprent 1986; Manchanda et Garg 2008). La contrainte saline peut également affecter les Rhizobia en réduisant leur survie, leur ineffectivité et leur développement ainsi que leur distribution dans la rhizosphère (Zahran 1999). Des teneurs élevées en sel peuvent aussi altérer l'expression des gènes *Nod* et par conséquent des enzymes impliquées dans la synthèse des facteurs Nod affectant ainsi le dialogue moléculaire entre le micro et le macro-symbionte (Dardanelli et al. 2008; Shankar et al. 2021). D'autre part, en présence de *Bradyrhizobium japonicum*, les poils absorbants de soja exposé à la contrainte saline présentent peu de courbure ou de déformation suggérant une réduction de la capacité des Rhizobium à synthétiser les facteurs Nods (Zahran 1999). Après l'établissement de la symbiose, la contrainte saline réduit le nombre et la taille des nodosités, le nombre des bactéroïdes à l'intérieur des nodosités et par conséquent le fonctionnement des nodosités (Bruning et al. 2013; Tu 1981; Egbichi et al. 2014). La contrainte saline a également des effets négatifs sur la teneur des nodosités en leghémoglobine et sur l'activité de la nitrogénase (Mhadhbi et al. 2011; Wani et al. 2017). Tous les effets négatifs de la salinité sur la symbiose Fabacées-Rhizobia présentés ci-dessus expliquent en partie la réduction de la FBN sous contrainte saline.

II-3-7 Effet de la contrainte saline sur la fixation symbiotique de l'azote

La FSN chez les Fabacées est généralement très sensible à la contrainte saline (Zahran 1999; Saadallah et al. 2001). Selon Soussi et al (1999), la performance de la fixation de l'azote atmosphérique est réduite en réponse à la contrainte saline. En effet chez *Glycine max*, la contrainte saline induit par 100 mM NaCl réduit l'activité nitrogénase des nodosités par 23% (Dolatabadian et al. 2012). De plus, l'exposition des plantes de *Lotus japonicus* à la contrainte saline affecte négativement la teneur des nodosités en leghémoglobine et l'activité de la phosphoenolpyruvate carboxylase et la malate déshydrogénase réduisant par conséquent la respiration nodulaire et le métabolisme du carbone chez les bactéries (López Lluch 2008; Farghire et al. 2011). La réduction de l'activité nitrogénase, de la teneur des nodosités en leghémoglobine, de la respiration nodulaire et du métabolisme carboné des bactéroïdes a un effet direct sur la FSN des Fabacées.

III. Méthodes utilisées pour améliorer la tolérance des plantes à la contrainte saline

De nombreuses stratégies ont été développées pour améliorer la tolérance des plantes à la contrainte saline. La sélection et les croissements conventionnels sont parmi les stratégies les fréquentes pour la création des variétés tolérantes à la contrainte saline (Ashraf et Akram 2009). Cependant, cette approche nécessite beaucoup de temps et souvent des gènes indésirables sont transférés en combinaison avec les gènes d'intérêt. La modification génétique des plantes s'est avérée très efficace et efficiente par rapport à la sélection conventionnelle (Nadeem et al. 2020). Par exemple, les plantes transgéniques *G. max* qui surexpriment le gène *StP5CS* isolé de *Solanum torvum* montrent une grande tolérance à la contrainte saline (Zhang et al. 2015). Cependant, l'utilisation de plantes génétiquement modifiées n'est pas acceptée dans la plupart des pays du monde. D'autres méthodes telles qu'un apport exogène de certaines molécules, glucose et nitrate par exemple, ont été montrées avoir des effets positifs sur la germination, la croissance et la productivité de plantes exposés à la contrainte saline (Atia et al. 2009; Hu et al. 2012). Cependant, ces méthodes ont été considérées comme étant coûteuses et polluantes (Farissi et al. 2014).

Le développement des nouvelles approches moins couteuses et respectueuses de l'environnement sont recommandées. Dans cette perspective, l'apport exogène de silicium (Si) et de proline ont été montrés avoir des effets bénéfiques sur la tolérance des plantes aux contraintes abiotiques notamment la contrainte saline (Souri et al. 2020; Ben Rejeb et al. 2012).

III-1 Rôle de l'apport exogène de silicium dans l'amélioration de la germination des graines sous contraintes abiotiques (Article 1).

La germination des graines est considérée comme le stade le plus critique dans le cycle de vie des plantes à cause de la sensibilité élevée des graines aux contraintes abiotiques notamment la sécheresse, la salinité, les métaux lourds, etc. Par ailleurs, une germination rapide et synchronisée des graines, en particulier dans des environnements contraints, est nécessaire pour une croissance uniforme des plantules et, par conséquent, pour le rendement des cultures. Le silicium (Si) est devenu une des approches les plus prometteuses pour améliorer la germination des graines, en particulier dans des conditions défavorables. Cette synthèse bibliographique vise à décrire les mécanismes induits par le Si au cours de la germination des graines en particulier dans les environnements défavorables. L'effet du Si sur la viabilité des embryons et la mobilisation des réserves des graines en germination a été rapporté. Nous avons également développé le rôle de l'apport exogène de Si dans l'augmentation de l'activité d'enzymes hydrolytiques et dans la balance hormonale des graines lors de leur germination sous contraintes abiotiques. Les effets bénéfiques de Si sur la stabilité membranaire, le métabolisme oxydant et la modulation de l'expression des gènes chez les graines exposées à des contraintes abiotiques ont été évalués de manière critique.

Ces données bibliographiques font l'objet d'une revue soumise pour publication.

How silicon alleviates the effect of abiotic stresses during seed germination: A review.

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Abstract

Rapid synchronized seed germination is desirable to ensure seedling establishment and improve crop yield. However, abiotic stresses from drought, salinity, and heavy metals have a negative impact on seed germination. The application of silicon (Si) has emerged as a promising approach for improving seed germination, especially under unfavorable conditions. However, the mechanisms of Si action have not been systematically studied in germinating seeds under conditions of abiotic stress. Considering the potential importance to sustainable agriculture, here we review recent findings of how seeds of numerous species, including several important crops, respond to Si treatment under abiotic stress. Exogenous Si has multiple effects on embryo viability, reserve mobilization, hormone/enzyme activity, membrane integrity, antioxidant metabolism, and regulation of gene expression in seed germination.

Keywords: Silicon, salinity, drought, heavy metals, seed germination, tolerance mechanisms.

I Introduction

Seed germination is the crucial stage when individual progeny of plant sexual reproduction become established (Waterworth et al. 2019) and the environment of the future growth and development of the plant is determined (Hubbard et al. 2012; Ali and Elozeiri 2017). Germination is naturally very sensitive to abiotic stresses, including those caused by heavy metals, salinity, and drought (Dehnavi et al. 2020; Farissi et al. 2011; Ghavami and Ramin 2007; Seneviratne et al. 2017; Xiao et al. 2016). Abiotic stresses either delay or entirely prevent seed germination through osmotic stress and/or ionic toxicity (Debez et al. 2018; Haghighi et al. 2012; Farissi et al. 2011). Some abiotic stresses also increase reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) in seeds (Bailly 2019), that may be produced during desiccation, germination, and aging (Pulido et al. 2009). In imbibed seeds, it has been reported that the metabolic activity in the different cell compartments such as NADPH oxidase activity in the plasma membrane, respiratory activity in mitochondria, purine catabolism in peroxisomes and lipid metabolism in glyoxysomes contribute to ROS production (Bailly 2004; Gomes and Garcia 2013). ROS disturb cellular metabolism by causing the peroxidation of proteins, lipids, and nucleic acids leading to seed deterioration and germination failure (Parvin et al. 2019). The exposure of seeds to salinity, drought, or heavy metals therefore induces modifications in protein structure, which diminishes hydrolytic enzyme activities so mobilization of seed reserves is compromised (Farissi et al. 2011; Hameed et al. 2021; Kranner and Colville 2011; Liu et al. 2018; Muscolo et al. 2014; Seneviratne et al. 2017).

Si is the second most abundant element after oxygen in the earth's crust. Exogenous application of Si has improved seed germination and embryo viability under various abiotic stresses (El Moukhtari et al. 2021; Lamsaadi et al. 2022; Rizwan et al. 2015; Siddiqui et al. 2020). An example of Si improving the seed viability of *Medicago sativa* L. under salt stress is illustrated in Fig. 1. Under drought, salinity, or heavy metal stresses, the addition of Si was found to improve seed germination rates in various plant species by influencing reserve mobilization, membrane integrity, ROS detoxification, antioxidant system, phytohormone metabolism, and gene expression (Ayed et al. 2021; Delavar et al. 2017; Gou et al. 2020; Khan and Gupta 2018). The beneficial effects of exogenous Si on plant tolerance to various abiotic stresses have been the subject of several reviews (El Moukhtari et al. 2021; Rizwan et al. 2015; Siddiqui et al. 2020) covering effects on plant growth, photosynthesis, water nutrition, and productivity of different crops under various abiotic stresses. Here we focus on reviewing the effect of exogenous Si on seed germination under abiotic stresses. First, we briefly highlight

how different abiotic stresses affect seed germination, purposely considering evidence from a wide range of species. Then within this framework, the possible modes of Si action on seed germination are critically reviewed. Future areas of research to deepen our understanding of the mechanisms triggered by Si during seed germination are discussed.

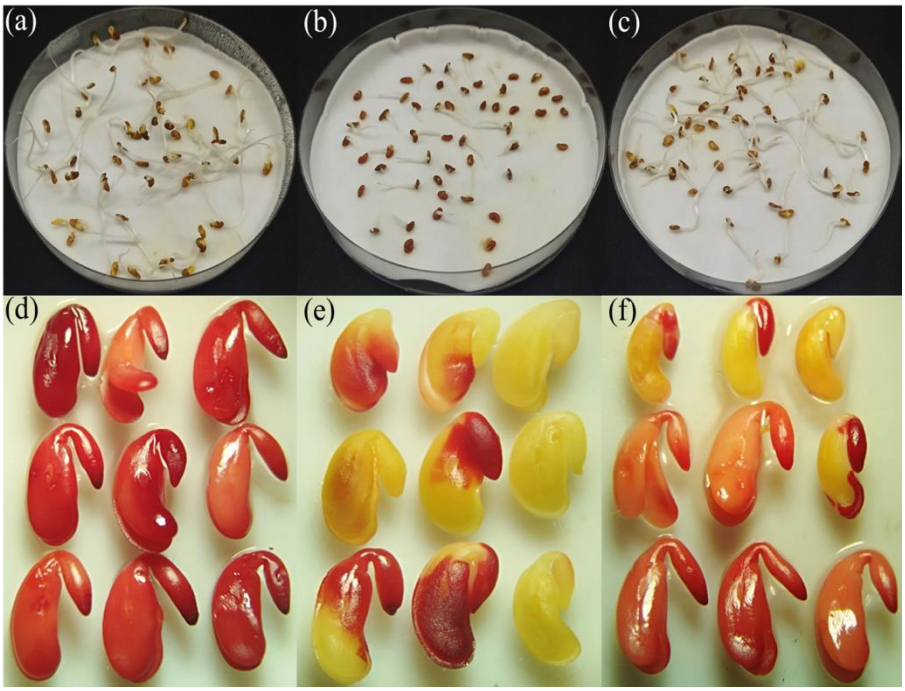

Fig. 1 Germination (a, b, and c) and embryo viability (d, e, and f) of seeds of the Moroccan *Medicago sativa* L. Demnate 201 variety treated with water (a, d), with 200 mM NaCl (b, e), or with 200 mM NaCl and 3 mM silicon in the form of $CaSiO_3$ (c, f). The germination rates were (a) 100%, (b) 51%, and (c) 85% after seven days of treatment. Embryo viability was evaluated after 12 hours of treatment using the 2,3,5 triphenyltetrazolium chloride (TTC) method in which viable embryos are stained red due to the reduction of TTC by respiratory activity of the cells. Images were taken under a binocular magnifying glass with 10x magnification.

II Relationship between seed dormancy and germination

Seed formation itself is a key strategy adopted by plants, especially annuals, to overcome unfavorable environments (Boesewinkel and Bouman 1984). Mature seeds are spread from the mother plant into the soil, where they germinate or stay dormant. Seeds can sense the surrounding environment and cannot germinate in the absence of appropriate environmental conditions (Baskin and Baskin 2004; Finch-Savage and Footitt 2017). Environmental factors such as temperature, nitrate, light, water, oxygen, smoke, and allelochemicals may all form an

integrative signal to determine whether and when seeds germinate or stay dormant (Graeber et al. 2012; Sano and Marion-Poll 2021; Yan and Chen 2020).

Seed dormancy is an orchestrated process during which the germination of viable seeds is blocked or delayed to withstand unfavorable environmental conditions (Finkelstein et al. 2008), which is key for the survival of the offspring. Dormancy is established during seed maturation and reaches a maximum level in freshly harvested seeds (Nakabayashi and Leubner-Metzger 2021). Seed dormancy therefore counters the phenomenon of preharvest sprouting, which is a major detrimental issue in cereal production (Tai et al. 2021). Abscisic acid (ABA) initiates and maintains dormancy (Koornneef et al. 2002). Carles et al. (2002) found that ABA-deficient *A. thaliana* seeds (*abi4-1*, *abi5-2* and *abi5-5*) germinated under osmotic stress, even with as much as 175 mM NaCl or 300 mM mannitol in the growth medium, but the seedlings died 7 days after germination. This example shows that ABA serves to inhibit germination in environmental conditions that are too extreme for the proper establishment of the seedlings. In contrast, it is well-known that gibberellic acid (GA) breaks seed dormancy and triggers seed germination (e.g. Stejskalová et al. 2015). For example, seeds of the GA biosynthesis *A. thaliana* mutant *ga1-3* only germinate if exogenous GA is applied (Hauvermale and Steber 2020). A key step in the germination process is the rupture of seed coat by the emerging radicle and GA stimulates radicle growth by mediating the activation of cell division in the embryonic root apical meristem (Ravindran and Kumar 2019). In tomato and Arabidopsis, GA promotes embryo growth and weakens the structures surrounding the embryo (Yamaguchi and Kamiya 2001). GA also stimulates the synthesis and translation of specific mRNAs, particularly those encoding α-amylase, a hydrolytic enzyme required for the digestion of seed reserves needed for germination (Ali and Elozeiri 2017; Muralikrishna and Nirmala 2005).

III Overview of the effects of abiotic stresses on seed germination

III-1 Drought stress

Water is a major factor necessary for the imbibition and germination of seeds (Bradford 2017). Drought decreases external water potential, limiting the seed's ability to take up water and impairing germination (Balestrazzi et al. 2011; Campos et al. 2020). This effect has been observed in numerous Fabaceae species, including *Medicago sativa* (Han et al. 2017; Wang et al. 2009), *Glycine max* (Wijewardana et al. 2018, 2019), and *Lens culinaris* (Aniat-ul-Haq and Agnihotri 2010). Water stress was found to reduce several positive seed germination traits in some crop species of the Poaceae, Solanaceae, Asteraceae and Brassicaceae families. For

example, germination percentage, germination rate, and vigor index are lowered by water stress in *Triticum aestivum* (Kizilgeci et al. 2017), *Hordeum vulgare* (Barati et al. 2015), *Oryza sativa* (Ali et al. 2020), *Solanum lycopersicum* (Galviz-Fajardo et al. 2020), *Helianthus annuus* (Toscano et al. 2017) and *Brassica napus* (Xiong et al. 2018). According to Llanes et al. (2015), drought stress can influence seed germination by changing endogenous plant growth regulator levels. For example, water deficit mediated by PEG treatment triggered an increase in ABA and a decrease in GA in *Trifolium repens*, impairing seed germination (Hassan et al. 2021). When less GA is available under water shortage, reserve mobilization is compromised, because the synthesis of various hydrolytic enzymes is not stimulated (Bewley 2001). In fact, Muscolo et al. (2014) and Hameed et al. (2021) reported that water deficiency significantly impaired the activities of α- amylase, β-amylase and proteases in the endosperm of lentil and wheat seeds, respectively, which consequently hampered seed reserve mobilization. In *Medicago truncatula* water deficit also alters embryo respiration and seed viability (Balestrazzi et al. 2011). In drought-stressed wheat, a decrease in germination rate appeared to result from the induction of oxidative stress, where a higher level of malonyldialdehyde (MDA) was noted together with an increase in H_2O_2 and O_2^- (Guo et al. 2017).

III-2 Salinity stress

Salinity causes serious abiotic stress, severely hindering seed germination and delaying seedling emergence. When seeds are germinated on filter paper, salinity affects indexes such as percentage, rate, and speed of germination of various plant species, including *Sorghum bicolor* (Dehnavi et al. 2020), *M. sativa* (Farissi et al. 2011), *Silybum marianum* (Ghavami and Ramin 2007), *Suaeda salsa* and *Phragmites australis* (Xiao et al. 2016), *Buchloe dactyloides* and *Bouteloua gracilis* (Zhang et al. 2012). Salinity is even reported to delay the germination of some halophytes such as *Cakile maritime* (Debez et al. 2004, 2018), *Salicornia europaea* (Calone et al. 2020), *Spartina maritima*, and *Spartina densiflora* (Infante-Izquierdo et al. 2019) as the times for 50% germination of viable seeds (T_{50}) are longer when the level of salt stress is high.

Salinity inhibits seed germination through various metabolic changes. In addition to the effects of osmotic stress, salinity may induce ionic toxicity due to an excess of Na^+ and Cl^- (de Oliveira et al. 2013). Salinity drastically decreased the activities of α- and β-amylase in *Amaranthus caudatus* (BiaŁecka and Kępczyński 2009), with negative consequences on seed reserve mobilization (Farissi et al. 2011). A similar effect was observed in *O. sativa*, where

salinity reduced the expression of the α-amylase gene and the activity of the enzyme (Liu et al. 2018). Similarly, catabolism of starch and lipids during *Cucumis sativus* seed germination was inhibited and delayed under salinity (Zhang et al. 2017). In *Pancratium maritimum* seeds, salinity affects cell division and causes DNA fragmentation, micronucleus formation, and chromosomal abnormalities (Mohamed et al. 2018). Salt can also disturb the homeostasis of phytohormones leading to failure of seed germination (Miransari and Smith 2014). Moreover, either through osmotic stress and/or ionic toxicity, salinity mediates an over-accumulation of ROS, leading to damage of nucleic acids, lipids, proteins, and carbohydrates which limits seed germination (Ibrahim 2016).

III-3 Heavy metals

Heavy metals have been shown to negatively influence seed germination and seedling growth of various plant species (El Rasafi et al. 2020; Seneviratne et al. 2017). There have been numerous descriptions of cadmium (Cd) delaying or completely inhibiting germination. For example, wheat grain germination was reduced by 47% at 0.12 mM Cd (Guilherme et al. 2015), while no *O. sativa* grains germinated at 1 mM Cd (Ahsan et al. 2007). Mercury (Hg), another toxic metal, is known to suppress seed germination and seedling growth. In *Vigna radiata*, 7 mM Hg reduced seed germination, seedling elongation, and dry weight accumulation by 42%, 70%, and 47%, respectively, compared to the control (Muhammad et al. 2015). Copper (Cu) also has a harmful impact on seed germination, even at low concentrations. For example, 10 µM Cu reduced seed germination of *H. vulgare*, *T. aestivum*, and *O. sativa* by more than 7%, 39%, and 63%, respectively (Mahmood et al. 2007). When *M. sativa* seeds were sown on solid media, 24% fewer seeds germinated when treated with 40 ppm nickel (Ni) (Aydinalp and Marinova 2009). Lead (Pb) inhibits seed germination in various plant species, including *L. culinaris* (Cokkizgin and Cokkizgin 2010). When 4.5 mM Pb was applied to lentil seeds, the germination percentage and vigor index were 67% and 83% lower than controls without Pb, respectively, and the mean germination time was 17% longer. Seed germination inhibition by other metalloids has been reported, including aluminum (Al) (Rodrigues et al. 2019), arsenic (As) (Mridha et al. 2021), and chromium (Cr) (Khan et al. 2020).

How heavy metal stress prevents seed germination is a complex biochemical process. Heavy metals can block aquaporins by binding to a cysteine residue close to the pore of the protein (Daniels et al. 1994) thereby impeding water uptake by seed cells and limiting seed imbibition (Cardoso et al. 2015; Lefèvre et al. 2009). Similar effects have been observed in *Pisum sativum*

under $HgCl_2$ and $ZnCl_2$ stresses (Kshetrimayum et al. 2017). Furthermore, enzymes participating in reserve mobilization, such as α- and β-amylases, proteases, leucine amino peptidases as well as cysteine-, aspartic-, and metallo-proteases, are altered by heavy metals, resulting in lower enzyme activity that has a negative effect on germination (Seneviratne et al. 2017). Heavy metal stress may also alter endogenous phytohormone homeostasis in seeds (Seneviratne et al. 2017). In *Cicer arietinum*, the negative effect of Pb or zinc (Zn) stress on seed germination was due to an increase in ABA and a decrease in GA content (Atici et al. 2005). Heavy metals can also reduce seed germination by inducing oxidative stress, reducing cell viability, and altering embryo growth (Gonzalez-Mendoza et al. 2009; Li et al. 2005; Sethy and Ghosh 2013).

To summarize, abiotic stresses, including drought, salinity and heavy metals, have a significant negative impact on seed germination of various species of plants. The negative effects include altered ion and water uptake, reduced reserve mobilization, modifications of proteins/enzymes and oxidative stress resulting in reduced seed viability and vigor (Table 1).

Table 1 Effects of abiotic stresses on germinating seeds of different plant species compared to non-stressed controls

Abiotic stress			Plant species	Effects on germination	References
Type of stress	**Agent**	**Stress level**			
Drought stress	Mannitol	-0.3, -0.6, -1.2 and -2.4 MPa	*Glycine max*	Lower germination indexes Shorter hypocotyl and root More abnormal seedlings	Machado Neto et al. (2004)
	PEG	15%	*Brassica napus*	Lower germination indexes Lower seed vigor index More ROS accumulation More lipid peroxidation More non-enzymatic antioxidants Increased activity of antioxidant enzymes More ABA	Xiong et al. (2018)
	PEG	10%	*Gossypium hirsutum*	Lower germination indexes Increased ABA/GA ratio Less α-amylase More ROS accumulation More lipid peroxidation Delayed water absorption capacity of testa	Bai et al. (2020)
	PEG	13.6 and 20.4%	*Hordeum vulgars*	Lower germination indexes Fewer primary roots Shorter maximum root length Shorter shoots	Barati et al. (2015)
	PEG	0.5-2.5 MPa	*Juglans regia*	Lower germination indexes Less water in radicle and plumule More lipid peroxidation Increased activity of antioxidant enzymes More antioxidant compounds More ABA, SA and JA Less GA and IAA More polyamines	Lotfi et al. (2019)

PEG	10-21%	*Lens culinaris*	Lower germination indexes Less water in germinating seeds Less α-amylase More proline	Muscolo et al. (2014)
PEG	-0.40, -0.50 and -0.75 MPa	*Lens culinaris*	Lower germination percentage Reduced seedling survival Shorter root and shoot	Aniat-ul-Haq and Agnihotri (2010)
PEG	35%	*Medicago sativa*	Lower germination indexes More ROS accumulation More lipid peroxidation Increased activity of antioxidant enzymes	Wang et al. (2009)
PEG	20%	*Oryza sativa*	Lower germination indexes Less water in seeds More ROS accumulation More lipid peroxidation More ABA Upregulated *OsNCED3* expression Upregulated SOD and CAT related gene expression	Liu et al. (2019)
PEG	-0.3, -0.6 and -0.9 Mpa	*Oryza sativa*	Lower germination indexes Shorter plumules Shorter roots Shorter seedlings	Ali et al. (2020)
PEG	17%	*Trifolium repens*	Lower germination indexes Less cytokinin More ABA Less β-amylase Less total amylase More starch and soluble sugar More ROS accumulation More lipid peroxidation Increased CAT activity	Hassan et al. (2021)

Heavy metals	$AlCl_3$	30 mmol L^{-1}	*Triticum aestivum*	Lower germination indexes Lower amylase and esterase activity More ROS accumulation More lipid peroxidation	Zhang et al. (2010)
	$AlCl_3$	2 mmol L^{-1}	*Oryza sativa*	Lower germination indexes Lower GA/ABA ratio Downregulation of the expression of ABA catabolism genes (*OsABA8ox*$_1$ and *OsABA8ox*$_2$) Lower α- and β-amylase activity More lipid peroxidation Increased antioxidant enzyme activity	Xu et al. (2017)
	Cd	100-500 µM	*Brassica napus*	Induced ROS accumulation Induced lipid peroxidation Ultrastructural changes in cells of germinating seeds	Ali et al. (2015)
	$CdCl_2$	5 mM	*Phaseolus vulgaris*	Lower germination indexes Reduced embryo growth Lower α-amylase and invertase activity Induced lipid peroxidation	Sfaxi-Bousbih et al. (2010)
	$CdCl_2$	10-30 mg L^{-1}	*Trigonella foenum-graecum*	Lower germination indexes	Espanany et al. (2015)
	$CdCl_2$	1-5 mM	*Triticum aestivum*	Lower germination indexes Shorter coleoptile and radicles Lower amylase activity Induced lipid peroxidation	Hu et al. (2015)
	$CuSO_4$	100-500 µM	*Hordeum vulgare*	Lower germination indexes Shorter radicles Lower *α*- and *β*-amylase, acid phosphatase, and alkaline phosphatase activities Induced lipid peroxidation	Kalai et al. (2014)

				Induced proline accumulation	
	Cr^{3+}	50-800 mg L^{-1}	*Arabidopsis thaliana*	Lower germination indexes Less embryo viability Increased seed coat permeability	de Silva et al. (2021)
	Cr (VI)	100 µM	*Triticum aestivum*	Lower germination indexes Lower α-amylase activity Fewer free amino acids Induced ROS accumulation Induced lipid peroxidation	Lei et al. (2021)
	$K_2Cr_2O_7$	30-300 mg L^{-1}	*Lepidium sativum*	Lower germination indexes	Pavel et al. (2013)
	$PbCl_2$	0.5-4.5 mM	*Lens culinaris*	Lower germination indexes Shorter plumules and radicles	Cokkizgin and Cokkizgin (2010)
	Pb $(NO_3)_2$	0.05-1 g L^{-1}	*Triticum aestivum*	Induced lipid peroxidation, Increased proline accumulation Lower α-amylase activity Increased antioxidant enzyme activities	Lamhamdi et al. (2011)
Salinity	NaCl	150-300 mM	*Arabidopsis thaliana*	Lower germination indexes Induced ROS accumulation Induced lipid peroxidation Less embryo viability	Luo et al. (2021)
	NaCl	150 mM	*Gossypium hirsutum*	Lower germination indexes Less α-amylase and β-galactosidase Lower GA/ABA ratio Less melatonin	Chen et al. (2021)
	NaCl	150 mM	*Gossypium hirsutum*	Lower germination indexes Downregulated ABA catabolism (*CYP707A2*) and GA biosynthesis (*GA20ox1*) genes Upregulated ABA biosynthesis (*NCED2*, *NCED5* and *NCED9*) and GA catabolism (*GA2ox1*) genes	Kong et al. (2017)

NaCl	50 and 100 mmol dm^{-3}	*Gossypium hirsutum*	Lower germination indexes Lower α-amylase activity Less reduced-sugar Less total amino acids	Ashraf et al. (2002)
NaCl	100 mM	*Lactuca sativa*	Lower germination indexes Lower phosphatase and phytase activities	Nasri et al. (2012)
NaCl	200 mM	*Limonium bicolor*	Reduced germination indexes Lower GA/ABA ratio Lower amylase and α-amylase activities Decreased *GA20ox*, *GA3ox* and *CYP707A1* gene expression Increased *NCED1* and *NCED3* gene expression More Na^+ and Cl^-	Li et al. (2019)
NaCl	50-200 mM	*Medicago sativa*	Lower germination indexes Less reserve mobilization Ionic toxicity	Farissi et al. (2011)
NaCl	100-200 mM	*Sorghum bicolor*	Lower germination indexes Lower salinity tolerance index Higher stress susceptibility index	Dehnavi et al. (2020)
NaCl	200 mM	*Trigonella foenum- graecum*	Lower germination indexes Less seed reserve mobilization Reduced seed viability Induced ROS accumulation Induced lipid peroxidation Ionic toxicity	Lamsaadi et al. (2022)
NaCl	60 and 120 mM	*Triticum aestivum*	Lower germination indexes Less water uptake Lower α-amylase activity	El-Hendawy et al. (2019)
NaCl	120 mM	*Oryza sativa*	Lower germination indexes Less bioactive GA	Liu et al. (2018)

			Lower α-amylase activity Downregulated α-amylase gene expression	
NaCl	50-200 mM	*Pancratium maritimum*	Lower germination indexes Affects cell division DNA fragmentation, micronucleus formation, and chromosomal abnormalities	Mohamed et al. (2018)
Na_2CO_3	50 mM	*Oryza sativa*	Lower germination indexes Less bioactive GA Lower α-amylase content	Li et al. (2019)
$NaHCO_3$	90 mmol L^{-1}	*Cucumis sativus*	Lower germination indexes Shorter hypocotyl and radicles Lower α- and β-amylase activity Increased lipid peroxidation	Sun and Luo (2014)

PEG, polyethelene glycol; GA, gibberellic acd; ABA, abscisic acd; Na_2CO_3, sodium carbonate; $NaHCO_3$, sodium hydrogen carbonate; Pb $(NO_3)_2$, lead (II) nitrate; $PbCl_2$, lead chloride; Cr(VI), chromium hexavalent; Cr^{3+}, chromium; $CuSO_4$, copper sulfate; $CdCl_2$, cadmium chloride; Cd, cadmium; $AlCl_3$, aluminum chloride.

IV Si improves seed germination under abiotic stresses

Si has repeatedly been found to have a positive effect on seed germination under abiotic stress (e.g. Rizwan et al. 2015; Siddiqui et al. 2020), and several of the reports are listed in Table 2. Elemental Si increases seed germination percentage, germination index, and seedling vigor of *C. sativus* exposed to stress caused by 200 mM NaCl (Gou et al. 2020). Investigations by Almutairi (2016) indicate that 1 mM nano-silicon increased germination percentage and germination rate of *S. lycopersicum* seeds under different NaCl concentrations (150-200 mM). In a different study by Zhang et al. (2015), germination rate, germination index, and vitality indexes of *Glycyrrhiza uralensis* seeds grown under salt stress (150 mM NaCl) were all improved upon Si treatment. In *Trigonella foenum-graecum* seeds germinating under NaCl induced salinity stress**,** the application of $CaSiO_3$ improved the germination percentage, germination speed, velocity index, germination energy, peak value and vitality index, all in a shorter mean germination time (Lamsaadi et al. 2022). Alsaeedi et al. (2017) showed that Si in the form of nano-silica restricted Na^+ uptake by *Phaseolus vulgaris* seeds resulting in a final germination percentage and germination rate that were 19.7% and 22.6% higher, respectively, than Na^+-stressed *P. vulgaris* seeds not supplied with Si. Toxicity of NaCl on germination of *T. aestivum* (Azeem et al. 2015), *Zea mays* (Naguib and Abdalla 2019), and *L. sativa* (Alves et al. 2020) was alleviated by Si application.

Si added as an exogenous compound was also beneficial as it improved seed germination under other abiotic stresses, such as drought and heavy metal stress (Arif et al. 2021). Under drought stress, Ayed et al. (2021) reported that Si in the form of sodium metasilicate ($Na_2SiO_3H_2O$) increased germination percentage, germination, and seedling vigor indexes of *T. turgidum* by 22%, 62%, and 39%, respectively. Furthermore, under 100 µM Cd stress, SiO_2 supplements improved *Phyllostachys edulis* seed germination (Emamverdian et al. 2021). In *O. sativa*, 5 mM silicic acid increased germination percentage under 150 µM As stress from 56% to 78% (Khan and Gupta 2018). In seed germination experiments with *Z. mays*, the toxic effects of Al were alleviated by exogenous application of Si (Delavar et al. 2017). Si in the form of Na_2SiO_3 can also augment seed germination in *C. sativus* under 3-phenylpropionic acid induced stress (Bu et al. 2018) and in *C. melo* under autotoxicity stress (Zhang et al. 2020). The studies above indicate that Si has an amelioratory effect on the germination of seeds grown under various abiotic stressors.

Table 2 Potential modes of Si action during seed germination under various abiotic stresses

Plant species	Si			Stress		Mode of action	References
	Form	Technique	Concentration	Type	Level		
Cucumis melo	Na_2SiO_3	Soaking	2 mM	MPWE	20 mg L^{-1}	Germination traits Reserve mobilization Gene expression Antioxidant activities Membrane integrity	Zhang et al. (2020)
Cucumis sativus	Na_2SiO_3	Soaking	2 mM	3-PPA	2 mM	Germination traits Reserve mobilization Gene expression	Bu et al. (2018)
Cucumis sativus	Na_2SiO_3	Soaking	0.3 mM	NaCl	200 mM	Germination traits Reserve mobilization Membrane integrity ROS detoxification Antioxidant activities Phytohormones Gene expression	Gou et al. (2020)
Lactuca sativa	Ca_2SiO_4	Priming	0.05-0.1 mM	NaCl	50 mM	Germination traits Antioxidant activities Membrane stability	Alves et al. (2020)
Lathyrus odoratus	nano-Si	Priming	20 mg L^{-1}	NaCl	21.60 dS m^{-1}	Germination traits	El-Serafy et al. (2021)
Lens culinaris	Na_2SiO_3	Soaking	2 mM	PEG	18%	Germination traits Reserve mobilization Antioxidant activities ROS detoxification Membrane stability	Biju et al. (2017)
Oryza sativa	$H_4SiO_2^-$	Priming	5 mM	As	150 uM	Germination traits Antioxidant activities Gene expression	Khan and Gupta (2018)

Oryza sativa	SiO_2	Priming	350 mg L^{-1}	PEG	-0.9 Mpa	Germination traits Antioxidant activities Membrane stability	Gana Ali et al. (2021)
Phaseolus vulgaris	nano-Si	Soaking	300 mg L^{-1}	Na^+	1-5 g L^{-1}	Germination traits Nutrient homeostasis	Alsaeedi et al. (2017)
Phyllostachys edulis	nano-Si	Soaking	200 µM	Cd	100 µM	Germination traits	Emamverdian et al. (2021)
Solanum lycopersicum	Na_2SiO_3	Soaking	0.5 mM	PEG	10%	Germination traits Antioxidant activities ROS detoxification Membrane integrity	Shi et al. (2014)
Solanum lycopersicum	nano-Si	Soaking	0.5-3 mM	NaCl	150-200 mM	Germination traits Gene expression	Almutairi (2016)
Trigonella foenum-graecum	$CaSiO_3$	Soaking	3 mM	NaCl	200 mM	Germination traits Reserve mobilization Embryo viability Antioxidant activities ROS detoxification Membrane integrity Compatible osmolytes K^+/Na^+ ratio	Lamsaadi et al. (2022)
Triticum aestivum	K_2SiO_3	Priming	1.5 mM	NaCl	up to 20 dS m^{-1}	Germination traits	Feghhenabi et al. (2020)
Triticum turgidum	Na_2SiO_3	Priming	15-20 mg L^{-1}	PEG	150 g L-1	Germination traits	Ayed et al. (2021)
Zea mays	Na_2SiO_3	Soaking	2 mM	NaCl	75 mM	Germination traits Phytohormones Reserve mobilization	Delavar et al. (2017)
Zea mays	Na_2SiO_3	Soaking	2 mM	Al	10 mM	Germination traits Phytohormones Reserve mobilization	Delavar et al. (2017)

Zea mays	nano-Si	Priming	10 mg L^{-1}	NaCl	150 mM	Germination traits Embryo respiration Reserve mobilization Antioxidant activities Phytohormones Membrane integrity ROS detoxification	Naguib and Abdalla (2019)

MPWE: Melon plant water extract; PEG: Polyethelene glycol; 3-PPA: 3-phenyl-propionic acid

V Si treatment enhances seed reserve mobilization under abiotic stress

Si application improves the germination of seeds under abiotic stress conditions by modulating reserve mobilization (Fig. 2). For example, in drought-stressed germinating seeds of *Eleusine coracana*, Mundada et al. (2021) recently found that Si increased the breakdown of glucose to acetyl CoA via pyruvate, which was diverted to lipid biosynthesis instead of the tricarboxylic acid cycle. This is good evidence that Si has a role in lipid metabolism. Additionally, Si augmented the activity of α-amylase, β-amylase, and α-glucosidase in osmotically stressed seeds of *L. culinaris* (Biju et al. 2017). Similar findings were reported in *C. sativus* (Ning et al. 2020). In wheat, seed priming with Si nanoparticles increased amylase activity under drought stress (Rai-Kalal et al. 2021). Similarly, in *C. sativus* under salt stress, application of 0.3 mM sodium silicate alleviated the negative effects of 200 mM NaCl and significantly increased α-amylase activity (Gou et al. 2020). Seed priming with nano-silica improved embryo respiration by increasing aldolase and isocitrate lyase activities (Naguib and Abdalla 2019). Under Al stress, the activity of amylase was 45% higher in Al-stressed seed with $NaSiO_3$ compared to Al-stressed seed without the addition of Si (Delavar et al. 2017). In a study of *C. melo* seeds, the application of Si increased α-, β- and total-amylase activities under autotoxicity stress (Zhang et al., 2020). Exogenous Si may therefore alleviate the negative effects of abiotic stresses on seed germination by promoting reserve mobilization, but more detailed studies, including testing for embryo viability and monitoring gene expression and the activity of other enzymes related to reserve mobilization, are required.

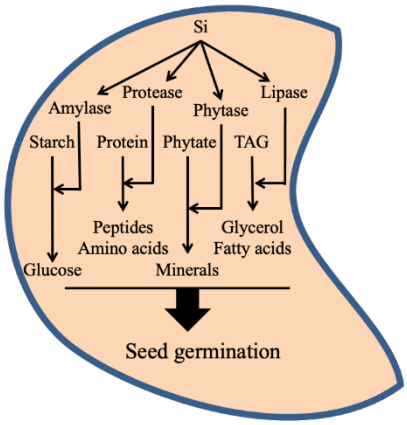

Fig. 2 Proposed effects of Si on seed reserve mobilization during seed germination under abiotic stresses. TAG, triacylglycerol

VI Exogenous Si mediates seed germination by modulating phytohormone balance

The effects of Si on phytohormone synthesis in stressed plants during vegetative growth have been reviewed by El Moukhtari et al. (2021) and Rizwan et al. (2015). Only a few reports show a link between Si treatment and plant hormone metabolism during seed germination (Fig. 3). A recent study demonstrated that seed priming with nano-silica increased *Z. mays* seed germination under 150 mM NaCl stress, and this was correlated with a higher GA/ABA ratio (Naguib and Abdalla 2019). The authors also revealed that changes in hormone metabolism were sufficient to explain the effect of Si on seed germination, particularly under unfavorable conditions. Another interesting study demonstrated that under 75 mM NaCl or 10 mM Al stress, *Z. mays* seeds that were soaked in 2 mM Na_2SiO_3 solution contained more GA and less ABA compared to the control, leading to a higher germination rate (Delavar et al. 2017). According to Gou et al. (2020), the increased GA in NaCl-treated *C. sativus* treated with Si was the result of downregulated expression of GA catabolism gene *GA2ox*, while the decreased ABA was the result of downregulated expression of ABA anabolism genes such as *NCED1* and *NCED2*. The combined effect was to raise the GA/ABA ratio to the level needed for dormancy release and germination. In PEG-stressed seeds of *E. coracana*, the application of Si modulates jasmonic acid (JA) synthesis during germination, especially under stressed conditions (Mundada et al. 2021). These results provide evidence that Si is effective in improving seed germination through the modulation of GA, ABA, and JA metabolism. Further investigations are necessary to understand the mode of action of Si and any signaling crosstalk in modulating phytohormones in seeds during abiotic stress.

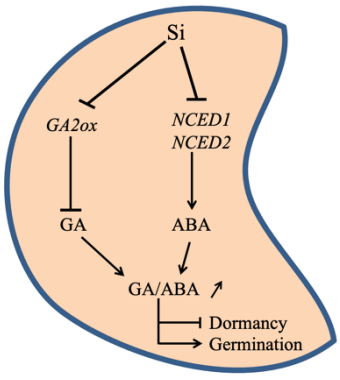

Fig. 3 Proposed effects of exogenous Si application on phytohormone balance during seed germination under abiotic stresses. *NCED, nine-cis-Epoxycarotenoid dioxygenases*; *GA2ox, Gibberellin 2-oxidases*; GA, gibberellic acid; ABA, abscisic acid

VII Si enhances antioxidant machinery and improves integrity, functionality, and stability of membranes during seed germination

During environmental stresses, the generation of ROS is enhanced (Nadarajah 2020). At high levels, ROS causes proteins, lipids, and nucleic acid peroxidation, hence causing cell damage and death (Hasanuzzaman et al. 2020). Maintaining membrane integrity and functionality during environmental change is a challenge for sessile plants (Rogowska and Szakiel 2020). Antioxidative defense systems, including both enzymatic and non-enzymatic antioxidants, play a major role preventing ROS build-up in cells (Kasote et al. 2015). Si has been reported to lower ROS activity and restore damaged plant membranes during seed germination by enhancing the antioxidant system (Debona et al. 2017; Mostofa et al. 2021). In drought-stressed *S. lycopersicum* (Shi et al. 2014) and *L. culinaris* (Biju et al. 2017), Na_2SiO_3 increased the activity of ascorbate peroxidase (APX), peroxidase (POX), catalase (CAT), and superoxide dismutase (SOD), consequently decreasing how much H_2O_2, O_2^-, and malondialdehyde was generated, with less lipid peroxidation as a result. Parallel evidence using the Evans Blue method is that under 200 mM NaCl *C. sativus* seed membrane integrity was improved by Si-treatment (Gou et al. 2020). The latter effect was linked to the ability of Si to limit ROS generation by modulating the activity of SOD, POX, CAT and APX. Similar findings were reported by Naguib and Abdalla (2019), who showed that the decreases in both lipid peroxidation and ROS accumulation in salt-stressed *Z. mays* seeds were associated with the higher activities of SOD, POX, and polyphenol oxidase (PPO) in response to nano-silica applied as a priming agent. Again as a priming agent, Si stimulated the activities of antioxidant enzymes, particularly CAT, decreasing lipid peroxidation in Cd-stressed lettuce seeds (Pereira et al. 2021). It is clear from the above studies that Si improves seed germination under multiple abiotic constraints by enhancing enzymatic and non-enzymatic antioxidant activities and thus maintaining the integrity and stability of membranes. Currently studies related to the molecular mechanisms and the metabolic pathways of Si-mediated stress tolerance are still limited.

VIII Si modulates gene expression during seed germination

The complex process of seed germination is governed by the expression of specific sets of genes (Rajjou et al. 2012). Si has been reported to regulate transcript levels of various abiotic stress-related genes during seed germination. The example of 0.3 mM Na_2SiO_3 downregulating the expression of *NCED1* and *NCED2* encoding nine-cis-epoxycarotenoid dioxygenases and *GA2ox* encoding gibberellin 2-oxidase was already cited (Gou et al. 2020). In another case, as

ABA is known to activate *Mitogen-activated protein kinase* (*MAPK*) expression (Lu et al. 2002), the lower levels of ABA due to Si treatment (Delavar et al. 2017) may be the reason *MAPK2* and *MAPK3* are downregulated in Si-treated salt-stressed *S. lycopersicum* (Almutairi 2016). ABA was also shown to regulate the expression of *Ethylene response factor* (*ERF*), a key regulator in abiotic stress (Müller and Munné-Bosch 2015). In *S. lycopersicum*, the application of Si downregulates the expression of *Ethylene response factor 5 (ERF5)*, and this was associated with lower levels of ABA (Almutairi 2016). Moreover, *ERF* controls *Respiratory burst oxidase* (*RBOH*) (Yao et al. 2017), and the reduction of *ERF5* expression in salt-stressed *S. lycopersicum* in response to Si treatment downregulates *RBOH1* (Almutairi 2016).

The accumulation of compatible osmolytes, including proline, is one of the most common strategies to overcome adverse environmental conditions (El Moukhtari et al. 2020). This response is mediated by several genes, including *delta-1-Pyrroline5-carboxylate synthetase* (*P5CS*) (Szabadoz and Savouré 2010). In a study by Almutairi (2016), the application of Si increased the expression of *P5CS* in *S. lycopersicum* during NaCl-induced salinity stress. This suggests that Si may be involved in mediating osmotic adjustment in germinating seeds during osmotic stress. Another mechanism of plant resistance to salt stress is to decrease Na^+ uptake and accumulation (Bargaz et al. 2015) through the action of the Na^+/H^+ antiporters of the plasma membrane (Rizwan et al. 2015). To investigate how Si controls Na^+ uptake by the seeds, Almutairi (2016) studied the effect of Si on the expressions of *Na^+/H^+ antiporter 6* (*NHX6*) in salt-stressed seeds of *S. lycopersicum*. The results showed that Si under salt stress up-regulated this gene, preventing Na^+ uptake by the seeds. Abscisic Acid-Responsive Element-Binding Protein (AREB) is required for the establishment of *Arabidopsis* seedlings (Sharma et al. 2011). During salinity stress, Si-treatment upregulated *AREB* expression in *S. lycopersicum* as compared to controls (Almutairi 2016). Acid phosphatase and esterases belonging to a group of hydrolytic enzymes that catalyze the hydrolysis of the seed reserve are required for the germination process (Kantharaju and Murth 2014; Senna et al. 2006). In Cd-stressed lettuce, the use of Si as a priming agent triggered the expression of genes associated with esterase and acid phosphatase (Pereira et al. 2021). The upregulation of *Low silicon 2* (*OsLSi2)* and *OsLSi6* genes and the downregulation of the *OsLSi1* gene, which all encode Si-transporters, were reported in *O. sativa* supplemented with Si under 150 µM As stress (Khan and Gupta 2018). These findings indicate that *O. sativa* seeds may take Si up from the soil via OsLsi2 and/or OsLSi6 transporters. Moreover, seven days after rice seed imbibition, Si upregulated genes

encoding Nitrate reductase (*NR*), Nitrite reductase (*NiR*), Glutamine synthetase (*GS*), Glutamate synthase (*GOGAT*), high affinity Nitrate transporter protein (*NRT2*), high affinity Ammonium transporter protein (*AMT1*), high affinity Phosphate transporters 1 and 2 (*PHT1*, *PHT2*), Acid phosphatases (*APase*), Potassium channel protein (*KAT1*) and Potassium transporter protein (*HAK10*) during As induced stress (Khan and Gupta 2018). In *C. sativus*, the *AMY* and *BMY* genes encoding α-amylase and β-amylase, respectively, were upregulated after 36h of stress from 10% PEG in the presence of Si, indicating that Si contributes to reserve mobilization of the embryo (Ning et al. 2020). In line with this, the positive effects of 2 mM Si on *C. sativus* under toxicity induced by 2 mM 3-phenylpropionic acid were attributed to the higher *AMY* and *BMY* transcript levels (Bu et al. 2018).

IX Si uptake by germinating seeds

Si has been reported to be taken up by plants in three different modes: active, passive, and rejective (Rizwan et al. 2015; Zhu and Gong 2014). Si assimilation by plants from soil was reported to be facilitated by the specialized Si-transporters Lsi1 and Lsi2 (Coskun et al. 2021). *Lsi1* was first discovered in *O. sativa* by Ma et al. (2006), where the suppression of its expression resulted in less Si uptake. Soon after another gene named *Lsi2* was identified in a rice mutant which accumulates less Si than the wild type and does not display any deleterious symptoms when grown in the presence of germanium, a toxic analog of Si (Ma et al. 2007). Other Si transporters, Lsi3 and Lsi6, have since been documented (Ahire et al. 2021). Most of the studies have investigated the uptake of Si by plants during vegetative stages, showing particularly that Si transporters are localized in roots. However, there is little information on the presence and role of functional Si-transporters in seeds. Strong evidence comes from a report by Khan and Gupta (2018) who demonstrated that Si supplementation to *O. sativa* seeds germinating under As stress upregulated the expression of *Lsi1*, *Lsi2* together and *Lsi6* (analog to *Lsi1*). These findings indicate that seeds express Si-transporter genes potentially allowing Si uptake.

X Investigation of Si application for improvement of seed germination

Numerous reports have documented a beneficial effect of Si on seed germination of several plant species, particularly under unfavorable conditions (Ayed et al. 2021; Pereira et al. 2021; Sun et al. 2021). Si has the potential to induce various physiological changes inside seeds, but the effects on seed germination may depend on how Si is applied. To date, the most widespread method is to soak seeds in a solution with an optimal concentration of Si (Haghighi et al. 2012;

Zhang et al. 2020). For example, the germination percentage of *L. culinus* seeds under 18% PEG stress was higher if they were soaked in 2 mM Na_2SiO_3 (Biju et al. 2017). The authors suggested that the induction of seed reserve mobilization mediated the improved seed germination. Under salinity stress, the improved germination percentage observed for *G. uralensis* seeds soaked in low Si concentrations was mediated by better membrane integrity and antioxidant systems (Zhang et al. 2015). Soaking *C. sativus* seeds in 0.3 mM $NaSiO_3$ increased seed germination under 200 mM NaCl by triggering the expression of some genes related to dormancy release and seed germination (Gou et al., 2020). Soaking seeds with Si was also reported to increase seed germination by modulating plant hormone metabolism, especially ABA and GA (Delavar et al. 2017).

Priming seeds with Si is another method (Khan and Gupta 2018) that is widely used to synchronize the germination of individual seeds in a population, resulting in enhanced, faster, and more vigorous germination (Marthandan et al. 2020; Sharma et al. 2014). With this method, Si generally enhances the metabolic processes in the seed, which makes it efficient in responding more quickly to abiotic stress (Abdel Latef and Tran 2016; Hameed et al. 2013). For example, *Triticum turgidum* seeds primed with sodium metasilicates showed improved germination parameters, including germination percentage, germination index, and seedling vigor index under drought stress compared to unprimed seeds (Ayed et al. 2021). Consistent with this, Alves et al. (2020) observed that seed priming with 0.05 mM Ca_2SiO_4 increased the germination percentage and germination rate index of *Lactuca sativa* exposed to 50 mM salinity stress. According to Pereira et al. (2021), Si priming affects seed metabolism at both physiological and molecular levels. The authors demonstrated that primed seeds showed an improved antioxidant response and upregulation of stress alleviation genes. The positive impact of seed priming with Si was also reported in *O. sativa* under As stress (Khan and Gupta 2018).

Si has also been used as a coating agent (Rufino et al. 2017). This method is generally used to protect seeds against biotic stress (Pedrini et al. 2017). For example, Nguyen et al. (2016) showed that coating soybean seeds with Ag/SiO_2 nano-composites provided antifungal activity against *Fusarium oxysporium* and *Rhizoctonia solani*. The authors also showed that seed coating with Si was able to increase soybean seed germination to 100% in the presence of *F. oxysporium* and to between 98% to 100% in the presence of *R. solani*. Additionally, under normal non-stressed conditions, Corlett et al. (2014) demonstrated that Si-coated barley seeds exhibited a greater emergence speed index than that of the non-coated control without affecting the physiological quality of the seed. The authors hypothesized that seed coating with Si would

be a promising way to protect barley seeds against pathogens without affecting the rate of seedling emergence nor the physiological quality of the seed. When seeds of *Z. mays* were coated with 20 mg H_2SiO_3 kg^{-1}, the established plants showed better growth and yield in terms of plant height, cob length and diameter, thousand-grain weight, and grain yield, more photosynthetic pigments, and enhanced enzymatic and non-enzymatic antioxidant activities under 120 mM NaCl stress compared to those from uncoated seeds (ur Rehman et al. 2020).

XI Conclusions and future perspectives

In this review, we intended to bring together evidence of how Si mediates seed germination under abiotic stresses. The literature shows that the application of Si improves seed germination, reduces germination time, and synchronizes germination under abiotic stressors. Si likely ameliorates seed germination under abiotic stresses through different modes of action, passing by ion and water uptake, reserve mobilization, phytohormone balance, reduction in oxidative stress by enhancing the activities of antioxidant defense system, and upregulation of gene expression (Fig. 4).

Previous investigations showed that Si is beneficial to plant abiotic stress tolerance (Debona et al. 2017; El Moukhtari et al. 2021; Rizwan et al. 2015), but the mechanisms of Si action are still unclear. Some authors hypothesized that Si can protect plant by forming a mechanical barrier (Coskun et al. 2019). Others suggested that soluble Si can be taken up by the roots and act as a secondary messenger to modulate defense responses (Coskun et al. 2019; Fauteux et al. 2005; Van Bockhaven et al. 2013). However, these hypotheses assume plants absorb Si from the soil solution. In roots, Si is taken up from the soil solution by an influx transporter encoded by *Lsi1* (for review see Ma and Yamaji 2015). However, to date, little is known about active Si transporters in seeds. Therefore, more studies are needed to identify and characterize Si transporter activity in seeds to understand better the mechanisms underlying the role of Si during seed germination.

Several studies showed the beneficial concentration-dependent effects of various compounds of Si, including $CaSiO_3$, $NaSiO_3$, K_2SiO_3, and silicon nanoparticles, on seed germination (El Moukhtari et al. 2021; Rizwan et al. 2015; Siddiqui et al. 2020). However, according to Voogt and Sonneveld (2001), silicate salts, like $NaSiO_3$ and K_2SiO_3, used as sources of Si in growth media, are highly alkaline and if not carefully handled, may cause precipitation issues. Furthermore, many authors have reported that plants absorb Si from soil or nutrient solutions only in the form of $Si(OH)_4$ (Coskun et al. 2019). Therefore, it would be

interesting to conduct more studies using different Si forms at varying doses to determine the optimal Si form and concentration that farmers could use to improve seed germination under abiotic stress.

In summary, the application of Si is a promising strategy to improve seed germination under abiotic stresses, but more specific research is needed in different crop species to show how Si acts in diverse agricultural contexts with optimal modes of application.

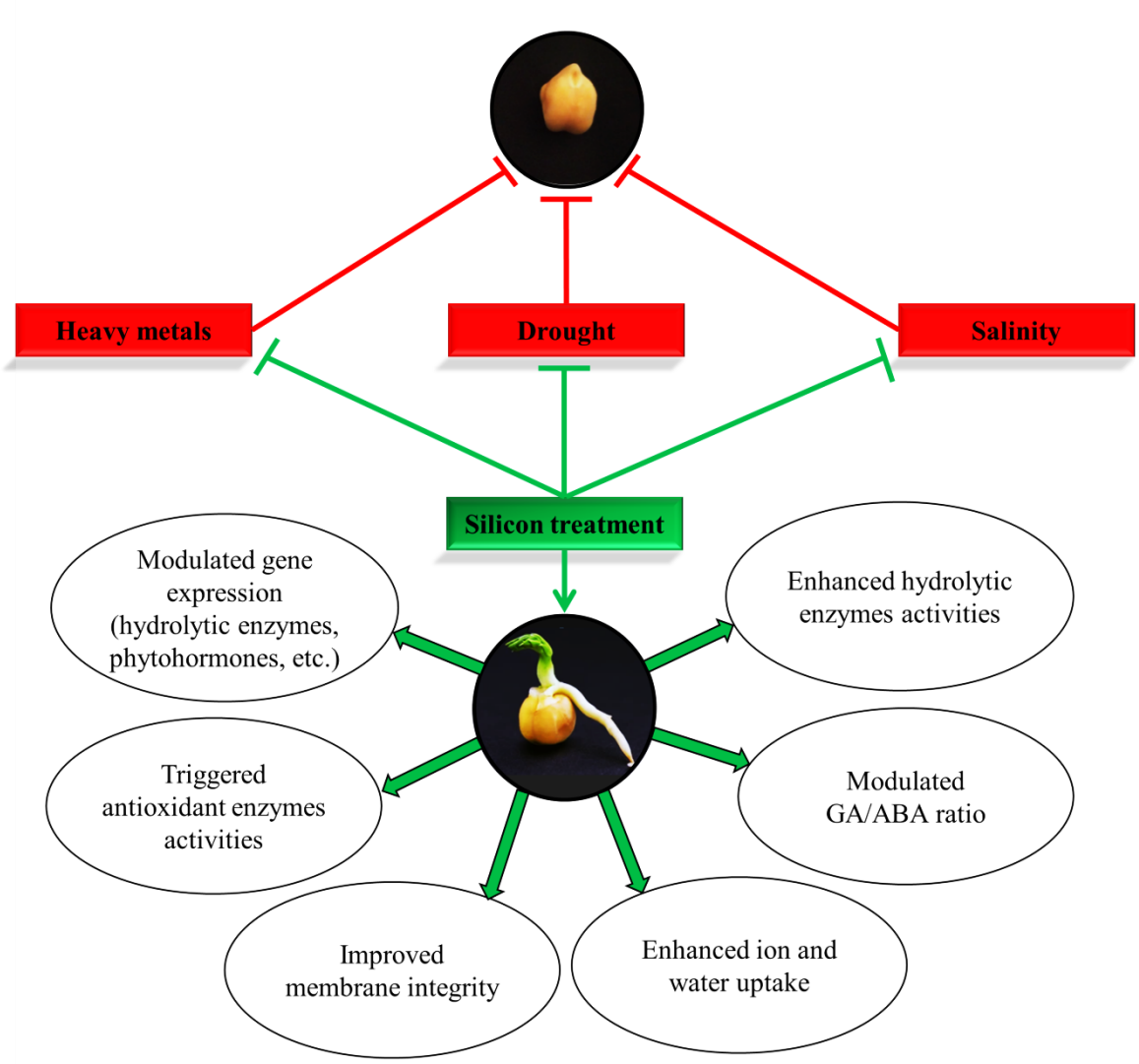

Fig. 4 Schematic summarizing the modes of action of silicon during seed germination under different abiotic stresses. Silicon upregulates hydrolytic enzyme activities, modulates phytohormone homeostasis, enhances ion and water uptake, induces antioxidant enzyme activities and improves membrane integrity, and upregulates the expression of abiotic stress tolerance genes. GA, gibberellic acid; ABA, abscisic acid

Statements and Declarations

Author Contributions

Conceptualization: A.E.M and A.S.; Literature review: A.E.M.; Original draft: A.E.M. and M.K.; Review editing: A.S., M.F., C.C., C.A., and W.Z.; Funding acquisition: A.S., M.F., and C.A.; Supervision: A.S. and M.F.; All authors read and approved the final manuscript.

Competing Interests

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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III-2 Rôle de l'apport exogène de silicium dans la tolérance de la symbiose Fabacées-Rhizobia à la contrainte saline (Article 2).

Le Si est le deuxième élément le plus abondant dans la croute terrestre après l'oxygène. Il a été démontré que l'utilisation du Si exogène représente l'une des stratégies les plus bénéfiques pour pallier au problème de la salinité. De même, chez les Fabacées, le Si a montré des effets positifs sur la fixation symbiotique de l'azote atmosphérique. Nous présentons dans cette partie l'état de l'art en se focalisant sur les effets du Si dans l'amélioration de la tolérance des Fabacées à la contrainte saline. Ainsi, ses effets sur la croissance des plantes, la photosynthèse et la productivité des Fabacées ont été détaillés. Également, le rôle de l'apport exogène du Si dans l'établissement de la symbiose Fabacées-Rhizobia, son fonctionnement sous contrainte saline ainsi que les mécanismes de tolérance induits par l'apport exogène du Si ont été analysés de manière critique.

Ces données bibliographiques font l'objet d'une publication intitulée ‘‘Exogenous silicon application promotes tolerance of legumes and their N2 fixing symbiosis to salt stress’’ dans la revue Silicon.

REVIEW PAPER

Exogenous Silicon Application Promotes Tolerance of Legumes and Their N_2 Fixing Symbiosis to Salt Stress

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Abstract
Legumes, the second-most-important crop family, are a key source of biological nitrogen in agriculture and potentially contribute to sustainable cropping systems. Nevertheless, most legumes are salt sensitive, especially during biological nitrogen fixation (BNF). Therefore, improving legume growth and symbiosis efficiency under this constraint constitutes a great challenge to meet the increasing food demands and to protect soils from negative impacts of chemical fertilizers. In this perspective, silicon (Si) has been found to mitigate salt stress effect and improve legume development at the overall developmental stages. Whether direct or indirectly, Si counteracts salt stress effects on seed germination, plant growth and nodulation. The improvement of water uptake and nutrient homeostasis, the modification of gas exchange, the regulation of phytohormone and compatible solute biosynthesis and the regulation of the antioxidant metabolism under salinity are the key mechanisms evoked by plants upon Si treatment. Furthermore, during rhizobial symbiosis, Si has been shown to induce nodule formation and act on nodule functionality by increasing bacteroids and symbiosomes number, nitrogenase activity and leghemoglobin content under salinity. Here, we reviewed recent progress related to the role of exogenous Si in improving legume salt tolerance and highlighted the mechanisms through which Si could mediate salt tolerance. The needs of future research for better understanding how Si can promote salt tolerance in legumes are also addressed.

Keywords Beneficial element · Salinity · Legumes · Nodulation · Biological nitrogen fixation · Photosynthesis

Highlights
- Silicon (Si) is a beneficial element for enhancing legume plant growth and productivity under salt stress.
- Application of Si improves biological nitrogen fixation by enhancing nitrogenase activity and nodule functionality.
- Si alleviates salt-mediated osmotic stress by up-regulating water uptake and compatible solutes accumulation.
- Si reduces oxidative stress under salinity by activating enzymatic and non-enzymatic antioxidant defense system.
- Si alleviates salt toxicity by regulating nutrient homeostasis.

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1 Introduction

Soil salinization and inappropriate irrigation management practices have devastating impacts on legume crops. To date, approximately 20 % of the total arable land is salt-affected [1]. Furthermore, because of the global warming and climatic changes, this threat is predicted to be more severe for the near future [2]. The effect of salt stress on plants including legumes has been comprehensively reviewed and this effect can be observed at overall development stages [3–5]. Indeed, during germination, salinity has been reported to inhibit hydrolytic enzyme activities and to reduce seed reserve mobilization, which in return reduces or completely inhibits seed germination [6, 7]. Salinity has also been reported to reduce leaf area, stomatal conductance, chlorophyll fluorescence and chlorophyll content, which directly reduce the rate of photosynthesis [8–11]. Moreover, the deleterious effect of salt on plants is also mediated through the generation of reactive oxygen species (ROS). At high concentration, ROS were reported to have a damaging effect on cell structure and normal metabolism by

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causing lipids, protein and nucleic acid peroxidation [12, 13]. Salinity reduces legume growth by causing ionic toxicity and nutrient deficiencies [1].

Legumes represent an important source of proteins for both humans and livestocks. Thus, grain legumes are vital component of local diet in developing countries. Likewise, in the agro-ecosystem, legumes play a key role in balancing soil nitrogen (N) content through its biological nitrogen fixation (BNF) with soil rhizobia. This symbiotic interaction limits or reduces the use of chemical fertilizers which are expansive and unfriendly for the environment [14–17]. Legumes are also used in intercropping or rotation with cereals, as the other plants can benefit from the N fixed by theme in symbiosis with rhizobia [18–20]. Moreover, since legumes were demonstrated to be able to reduce greenhouse gas emissions [21], they are widely used in intercropping with cereal, as the intensification of cereal based-cropping system aggravates the greenhouse gas emissions [22]. Additionally, comparing to the monoculture, cereals and legumes have shown higher yield and seed quality when they were cultivated in intercropping [23].

To ensure increase in food demands, it is necessary to increase the global food production by 38 % and 57 % by 2025 and 2050 respectively [24]. Legume species are considered as salt sensitive. Therefore, this imposes more pressure on the use of alternative approaches to maintain food supplies and in the same time on the exploitation of the salt affected land. In this context, the exogenous supplementation of silicon (Si) has been reported to be one of the promising strategies to overcome salt stress effect on plants.

Si, second most abundant element in the earth crust, has got much attention these last few years. It is well documented that Si could improve the plant tolerance to biotic and abiotic stressors [25–28]. Its beneficial effects depend on the plants ability to absorb it from the soil solution. Thus, since some legume species have been characterized as plants without Si transporter, they were considered as Si-rejective [29], which make it difficult to understand how this element improves legumes salt-tolerance. Furthermore, Si should not be considered as essential for plants [26, 29, 30], based on the three criteria of the essentiality of elements established by Arnon and Stout [31]. An element is not considered essential unless (a) its deficiency makes it impossible for the plant to complete its vegetative or reproductive stage, (b) specific symptoms will appear under its deficiency and this can be prevented or corrected only by its supply and (c) it is directly involved in the plant nutrition. Besides, based on the classification of Epstein and Bloom [32], an element should be considered essential if it fulfills either one or both of the following criteria: (a) the element is part of a molecule that is an integral aspect of the plant's structure or metabolism and (b) when compared to plants with lower deficit, the plant can be so severely deficient in the element that it demonstrates anomalies in growth, development, or reproduction. Accordingly, Si will be considered as an essential element for higher plants since its supply confers many physiological and biochemical changes, including plant growth and productivity, photosynthesis, water uptake, nutrient homeostasis, etc. [33–35]. More than that, it has been found that plants without Si tend to grow abnormally showing for example less chlorophyll, leaf senescence and death, growth inhibition and oxidative stress under abiotic stresses, while adding Si helps them to grow up normally [36]. According to Ma et al. [37], Si is the only nutrient that is not harmful when it is excessively accumulated in plants.

Regardless its uptake and being essential in higher plants, exogenous Si has been reported to alleviate salt stress toxicity and to improve seed germination of various plant species including legumes [38]. Furthermore, in salt stressed Mung bean, exogenous Si application increased chlorophyll content, stomatal conductance, transpiration rate, and net photosynthesis [39]. As NaCl caused osmotic stress, applied Si to salt stressed plants induced the accumulation of compatible solutes to counteract water flow from legumes [40–43]. Si application has been reported to increase the activity of some antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) and reduced ROS content and lipid peroxidation [42–45]. Thus, although beneficial Si application in improving legume tolerance to some abiotic stresses have been the subject of a couple of reviews these last years [29, 46, 47], the mechanisms by which Si improves legume tolerance to salt stress is steel poorly understood. Here we review recent developments on Si-induced salt stress tolerance in model and cultivable legume species and examines the unreviewed information regarding some morphological and physiological changes in salt-stressed legumes. Mechanisms of detoxifications triggered in salt stressed-legumes under Si treatment are also highlighted. The applications for future research are discussed.

2 Si Uptake, Transport and Accumulation in Higher Plants

In the soil, Si content varies from 50 to 400 g Si Kg^{-1} [33]. Likewise, about 50-70 % of soil mass is SiO_2, which make it the second abundant element, after oxygen, in the earth's crust [48]. However, in contrast to the high abundance of Si in soil, very low amount of Si is available to be directly used by plants because of its low solubility in the soil solution [33, 49]. Generally, all soil grown plants contain Si with an equivalent concentration or more than those of macronutrients (N, P and K) [50]. However, even though all plants were reported to be contained Si in their tissue, the content of plant on it is generally species dependent. It can reach up to 10 % of the total dry weight of plants based on their Si absorption capabilities [51].

According to Handreckt and Jonest [52], plants were classed as high Si accumulator, intermediate or non-accumulator based on their content on Si. However, another classification proposed by Takahashi et al. [53] make possible to group plants into three groups: active, passive or rejective; based on the mechanism by which plant root assimilate Si from the soil solution. In addition, others like Liang et al. [34] and Henriet et al. [54] have reported the coexistence of both active and passive transport within the same plant.

In active accumulators such as sugarcane, rice and wheat, Si accumulation was reported to represent 1.5–10 % of total dry weight of shoots [26]. For other plant species like cucumber and melon described as passive accumulators, their shoots have been reported to contain Si between 0.5 % and 1.5 % of total dry weight [26, 34]. Si content in legume shoots do not exceed 0.5 % of total dry weight [50]. Therefore legumes are classified as Si rejective [29]. However, other authors like Liang et al. [34] and Guntzer et al. [49] have described soybean, a seed legume, as passive accumulator of Si. Likewise, *Vigna radiata* has been recently reported as a Si accumulator [43].

Plant takes up Si from the soil solution in the uncharged form of ortho-silicic acid ($Si(OH)_4$) [30, 55–57], which is present in the soil solution at 0.1 mM to 0.6 mM when pH is below 9 [58]. In addition to $Si(OH)_4$, Zhu and Gong [36] reported that plants could absorb Si from soil solution in the form of SiO_2. It has been widely reported that Si assimilation by plants involved influx and efflux transporters [59], encoded by two genes named *Lsi1* and *Lsi2* respectively [36]. *Lsi1* was shown to encode an aquaporin as an influx transporter [36, 58], which ensures Si transport from the soil solution to the root cells [26]. *Lsi2* encodes a local plasma membrane transporter, which ensures Si movement from exodermal cells to the apoplast [60]. In addition, Ma et al. [58] have also reported that Si translocated to the aerial parts through the xylem using transpiration water flux. Interestingly, based on sequence homology with the rice Si transporter, the model legume *Medicago truncatula* has been reported to have one gene for Lsi1 and one gene for Lsi2, which make this plant a mild Si accumulator [61]. Moreover, recently Nawaz et al. [62] reported that *M. truncatula* have two homologous *Lsi2* genes. Accordingly, *Phaseolus vulgaris* has also been considered as mild Si accumulator, as it also contains one gene for Lsi1 and two genes for Lsi2 [61]. Moreover, Deshmukh et al. [63] demonstrated that soybean assimilate Si through an influx transporter encoded by two genes named, $GmNIP_{2-1}$ and $GmNIP_{2-2}$ and transcriptomic analysis has shown that the expression of those two genes was higher in both roots and shoots. Other legumes such as *Trifolium pretense*, *Vigna unguiculata*, *Glycine max* and *Cicer arietinum* have also been reported to have homologs of *Lsi2* gene [62].

3 The Effect of Exogenous Si on Legume Seed Germination Under Salt Stress

Germination is a critical process in the life cycle of seed plants. This stage is very sensitive to abiotic stressors particularly salinity causing osmotic stress accompanied with ionic toxicity [7]. According to Farissi et al. [64], the effect of salt stress on germination of *M. sativa* seeds was reflected by its delay or its complete inhibition. Only few studies have reported some effects of exogenous Si on legume seed germination under salt stress. For example, Zhang et al. [38] demonstrated that 2 mM of exogenous potassium silicate (K_2SiO_2) improved germination rate, germination index and vitality index of *Glycyrrhiza uralensis* under 150 mM NaCl. Similarly, Alsaeedi et al. [65] showed that 300 mg L^{-1} of nanosilica (NS) is able to increase the final germination percentage and germination speed of *P. vulgaris* seeds by 19.7 % and 22.6 % respectively under 5 g Na^+ L^{-1} and vigor index by 144.6 % under 4 g Na^+ L^{-1}. The authors also showed that germination time was decreased from 6.43 under 4 g Na^+ L^{-1} to 5.83 when the seeds were treated with 300 mg L^{-1} of NS, reflected 9 % of reduction. Otherwise, priming of *Triticum aestivum* L. seeds with 30 mM of sodium silicate (Na_2SiO_3) restored seed germination to 100 % under 120 mM NaCl [66]. In the same line, *Lactuca sativa* L. seed priming with 0.1 mM of calcium silicate (Ca_2SiO_4) has been recently reported by Alves et al. [67]. Authors found that Si mediates seed germination under 50 mM NaCl highlighted by high germination percentage and germination rate index. According to Biju et al. [68], Si improves lentil seed germination under drought stress through an increase of hydrolytic and antioxidant enzymatic activities. In addition, as seed germination is modulated by gibberellic acid (GA) / abscisic acid (ABA) ratio [69, 70], Si has been reported to mediate *Cucumis sativus* L. seed germination under high level of salt stress (up to 250 mM NaCl) by inhibiting the expressing of *GA20ox* catabolism gene as well as those responsible for ABA anabolism such as *NCED1* and *NCED2* [71]. However, mechanisms by which Si-mediated legume seed germination under salt stress is still poorly understood and detail studies under these conditions are needed for better understanding the mechanism by which Si improves this key process.

4 Effect of Si on Growth, Biomass and Yield of Legumes Under Salt Stress

Legumes are cultivated mainly for forage or grain production. Because they are salt sensitive, their biomass under salt stress

is severely affected, particularly when their growth depends on BNF. Improving legumes growth and productivity by using alternative approach under abiotic stresses is of such interest. Si was shown to enhance plant growth at different agronomical, morphological and physiological levels [72]. In *M. sativa*, Meng et al. [73] tested the effect of 2 mM Na_2SiO_3 supplementation to the soil solution on 200 mM NaCl tolerance. They found that exogenous Si can mitigate the adverse effect of NaCl on *M. sativa* growth by greater shoot and root dry weight by 16 % and 11 % respectively. Similar results have been found by Lee et al. [74] on *G. max*, where the addition of 2.5 mM Na_2SiO_3 under 80 mM of NaCl significantly increased shoot and root lengths, plant fresh weight and plant dry weight by 18 %, 11 %, 33 % and 9 % respectively relative to salt-stressed plant without Si supplementation. Furthermore, in salt-stressed cowpea and kidney beans, 1 mM $CaSiO_3$ improved root dry weight, stem dry weight, shoot dry weight and whole plant dry weight in the two evaluated plant species [75]. Wu et al. [42] found that 1 mM Na_2SiO_3 could alleviate *Onobrychis viciaefolia* damage caused by 100 mM NaCl and improve plant fresh and dry weight as well as the number of leaves. In addition to the number of leaves, Si was also found to be able to delay the premature leaf senescence under abiotic stresses including salt stress [8]. Moreover, adding 4 mM of K_2SiO_3 to *C. arietinum* was found effective in alleviating the negative effects of salt on shoot dry weight, root dry weight and seed yield [41]. In the same plant species, 0.5 or 1 mM of exogenous Na_2SiO_3 has been confirmed to be able to increase grain yield under salt stress [76]. In the line with this, foliar spray of diatomite to *Vicia faba* alleviated the negative effect of salt and improved pod number, pod dry weight, seed number and seed dry weight and the effect was more obvious when Si was applied at 1000 ppm [77]. In a similar study conducted by Kardoni et al. [78] on *V. faba*, Na_2SiO_3 counteracted the negative effects of a wide range of salt treatment (1, 2, 3, 4 and 5 ds m^{-1}) and improved grain yield and 100-seed weight. Adding Si to salt-stressed *P. vulgaris* resulted in an increase of seed number, 100-seed weight and yield [79]. Above findings strongly suggested that depending to the form of applied Si and the severity of stress, exogenous Si increased legume plant growth and productivity under salt-induced stress (Fig. 1).

5 Si Balances Legume Minerals Uptake Under Salty Conditions

Salinity has devastating impacts on legume plant nutrient uptake. As mineral nutrient uptake plays an important role in plant development, maintaining ion homeostasis by regulating their uptake, transport and translocation is essential for plant not only to survive under salt stress but also to continue its growth, development and productivity [4]. Investigations on the effect of exogenous Si on plant nutrition has revealed its crucial role in restoring ion homeostasis under salt stress, as observed in several forage and grain legumes plant species [47]. In *C. arietinum*, Garg and Bhandari [41] reported that 0.4 mM K_2SiO_3 application increased the accumulation of each of N, P, Mg and K contents under salt stress. Accordingly, Si application increased the content of salt stressed *T. alexandrinum* on K, Ca, Mg and P by 46 %, 56 %, 45 % and 70 % respectively as compared to the Si-untreated stressed plants. Similarly, in a study conducted by Hellal et al. [77] on *V. faba*, the content of P and K in both shoot and seed were gradually decreased as salt increased in soil solution, but SiO_2 application as foliar spray reduced this effect. Furthermore, 1 mM of K_2SiO_3 improved the tolerance of *M. sativa* to 120 mM NaCl and increased Ca^{2+} content in roots and Mn^{2+} content in leaves [80]. Increased uptakes of K^+ and Ca^{2+} following application of Si were also reported in salt-stressed cowpea, kidney bean [75] and in *V. radiata* [43].

Mechanisms by which exogenous Si could increase soil P availability and its uptake by plants include (i) a decrease in P sorption in soil [81] (ii) a better Pi uptake by roots by increasing root exudation of some organic acids like malate and citrate [82], (iii) an increase in soil P availability by augmenting soil pH [81], (iv) and upregulation of some plant genes involved in P uptake particularly during P starvation [82].

The above studies strongly suggested that exogenous Si alleviates salt stress in legumes by improving uptake of some nutrients. However, for better understanding the mechanisms involved in this process, studies on some enzymes involved in nutrient assimilation such as nitrate reductase, phytase and phosphatase as well the regulation of their expression are essential to get a better understanding of the Si effect.

6 Exogenous Si Maintained Plant Water Balance in Salt-stressed Legumes

Reduction of leaf relative water content (RWC) is one, among other, of the most physiological traits that serve as an osmotic stress index [33]. Si has been reported to be able to maintain RWC in plants growing in saline environments [40, 83–85]. In 4.5 g SiO_2 Kg^{-1} soil treated-*T. alexandrinum*, RWC was raised in the salt-sensitive genotype from 62.40 to 81.03 % in the presence of 3000 ppm NaCl indicating an improvement rate of 32 % of RWC [86]. In contrast for the salt-tolerant genotype, the improvement rate was only 17 %. In addition, Mahmood et al. [39] found that RWC was 1.37- and 1.44-fold higher in salt-stressed mung bean treated respectively with either 1 or 2 kg K_2SiO_3 ha^{-1} as a foliar spray than in the absence of Si. In the same line, 4 mM K_2SiO_3 significantly alleviated the negative effect of 100 mM NaCl stress raising RWC in both salt-tolerant-HC 3 and salt sensitive-CSG 9505 genotypes of *C. arietinum* [41]. In the same way, 0.6 g K_2SiO_3

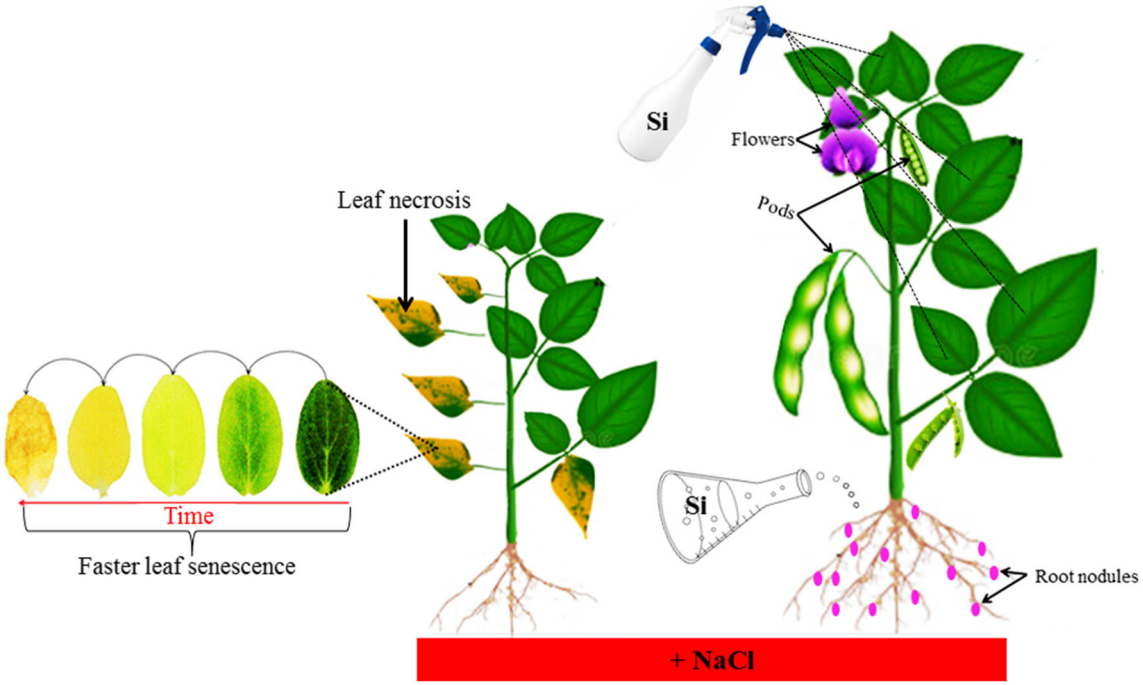

Fig. 1 Proposed effects of exogenous silicon treatment on legume plants growth and productivity under salt stress conditions. Abbreviations: NaCl, sodium chloride, Si, silicon

kg^{-1} soil increased the RWC and reduced the leaf water potential in two-year-old *G. uralensis* plants under different salt concentrations (6 and 9 g NaCl Kg^{-1} soil) and after different time of treatment [87]. Improving plant RWC under salt stress after Si supplementation has also been documented in *V. radiate* [43].

Maintaining water content under osmotic stress is a complicated process resulting from a balance between water uptake and water loss by transpiration. According to Zhu and Gong [36], silicic acid polymerizes and precipitates forming an opal phytolith that prevents water loss. Similarly, Coskun et al. [26] documented that Si prevents water loss under osmotic stress by its deposition in cuticles. On other hand, Si has been reported to modulate aquaporin-related gene expression, which is particularly important to improve water homeostasis and balance particularly under water stress [88]. Furthermore, Si could improve cellular osmotic potential through synthesis of compatible solutes (See Section 7). Studies behind these findings are still needed particularly at the molecular level for better understanding the possible mechanisms induced by Si to mediate osmotic stress tolerance.

7 Exogenous Si Mediates the Biosynthesis of Compatible Solutes in Salt-stressed Legumes

Osmotic adjustment through the accumulation of compatible solutes is one of the adaptive salt-tolerance strategies adopted by legumes particularly during N_2-fixing process [89]. Indeed, sucrose, fructose and glucose contents were increased by 20–30 % in root of 100 mM salt-stressed *Onabrychis viciaefolia* plants when treated with 1 mM Na_2SiO_3 as compared to salt-stressed control plants [42]. Likewise, in *G. uralensis*, Zhang et al. [87, 90, 91] reported an increase in the content of soluble sugars as a response to Si addition under salt conditions. In the same way, Ahmad et al. [43] documented that Si improved *V. radiata* salt stress tolerance by an increase in glycine betaine content. According to Garg and Singh [92], exogenous application of K_2SiO_3 was also able to modulate trehalose metabolism in pigeon pea nodules by on the one hand improving trehalose 6-phosphate synthase and trehalose 6-phosphatase activities and on the other hand inhibiting the activity of trehalase, which results in high nitrogenase activity, leghemoglobin and N content under cadmium (Cd) and zinc stresses.

Proline accumulation is recognized as a tolerance index because its concentration has been shown to be generally higher in salt tolerant than in salt sensitive plants. However, toxic effects of proline when applied exogenously have been reported [93]. According to Zhang et al. [90, 91], treatment of salt-stressed *G. uralensis* by Si led to the accumulation of proline. Similarly, *V. radiata* and *O. viciaefolia* plants exposed to salt stress have higher proline contents when treated with exogenous Si [42, 43]. In contrast, applied 4.5 g SiO_2 Kg^{-1} soil to salt stressed *T. alexandrium* plants resulted in lowering proline content by 26 % as compared to Si untreated salt-stressed *T. alexandrium* plants [86]. Moreover, in a study

carried out by Lee et al. [74], the authors found that the application of $CaSiO_3$ at 2.5 mM to the soil solution of 80 mM NaCl-stressed *G. max* reduced proline content as compared to Si-untreated salt-stressed plants. Negative correlation between Si addition and proline content was also reported by Mahmood et al. [94] and Zamani et al. [76] in salt stressed Mung bean and *C. arietinum* respectively.

Above studies clearly indicate that Si might enhance salt tolerance in legumes by involving osmolytes production and provide the evidence that Si plays a crucial role in osmotic adjustment.

8 Si-mediated Biosynthesis of Phytohormones in Salt-stressed Legumes

Phytohormones are compounds produced in very low concentrations but able to regulate a variety of cellular processes and plant responses to changing environmental conditions including salinity [95, 96]. For example, excessive concentrations of some ions like Na^+ and Cl^- have been shown to induce a change in the endogenous level of plant growth hormones [97]. It was shown that salt stress increases the level of ABA in *P. vulgaris*, which inhibits the transport of both Na^+ and Cl^- to the shoot [98]. In *G. max*, Lee et al. [74] showed an increase in ABA level under 80 mM NaCl conditions, however when exogenous Si was applied, a lower ABA content was measured. Similar results were also reported by Zhang et al. [87] on *G. uralensis*. The authors demonstrated that K_2SiO_3-treatement contracted the effect of NaCl on ABA and decreased its endogenous level. In addition, exogenous application of GA was found to be useful to contract the devastating impact of salt stress on *V. radiate* [99]. In this line, Lee et al. [74] tested the effect of 2.5 mM Na_2SiO_3 treatment on *G. max* tolerance to 80 mM NaCl through GA regulation. Results indicated that GA_1, GA_4, GA_{12}, GA_{19} and GA_{24} levels were decreased upon salt stress, while the supplementation of Si significantly increased their levels. In a similar study, applied K_2SiO_3 mediated *G. uralensis* salt tolerance by increasing GA_3 level [87]. Indolacetic acid (IAA) accumulation has also been reported as one of the key responses of salt tolerance [96], and its exogenous application has been recommended as a crucial strategy to alleviate the adverse effect of salt on plants [100]. In *G. uralensis*, Zhang et al. [87] demonstrated that IAA was decreased upon salt stress but increased in response to Si treatment.

9 Exogenous Si Improves Photosynthesis in Legumes Under Salt Stress Conditions

Salinity was found to reduce leaf area and gas exchange as well as chlorophyll synthesis leading to a decrease of photosynthesis and as a result plant growth and productivity [4, 10]. Si has been largely reported to have positive effects on plant growth under changing environments and these effects have been confirmed to be often associated with the ability of Si to improve photosynthesis [47, 101]. Mahmood et al. [39] conducted a study on mung bean exposed to salt stress, and they found that spraying K_2SiO_3 (1 and 2 kg ha^{-1}) on 10- and 30-day-old plants importantly resulted in higher net photosynthesis, chlorophyll and carotenoids contents, stomatal conductance and transpiration rate compared to untreated salt-stressed control. In a similar study conducted recently by Meng et al. [73], 2 mM Na_2SiO_3 supplementation to *M. sativa* was found to mitigate the inimical impact of 200 mM NaCl by increasing various photosynthetic attributes including chlorophyll content, net photosynthesis, stomatal conductance and transpiration rate. In addition, after one month of 60 mM NaCl treatment, leaf area, stomatal conductance and net photosynthesis were increased respectively by 26 %, 37 % and 28 % in *P. vulgaris* plants when they were treated exogenously by 1.5 mM K_2SiO_3 [102]. Under salt conditions, Si was also reported to increase stomatal number and improves RuBisCO activity and as a result internal CO_2, which supports a key role of Si in photosynthetic activity [41, 43, 75]. Beneficial effects of Si on chlorophylls under salt stress has been studied by Alamri et al. [8] in *Brassica juncea*. They found that Si could increase the activity of some chlorophyll synthesis enzymes including δ-aminolevulinic acid dehydratase and porphobilinogen deaminase and inhibits those responsible on its degradation, such as chlorophyllase, chlorophyll-degrading peroxidase and pheophytinase. The roles of Si on photosynthesis indexes in salt-stressed legume plants are summarized in Table 1.

10 Biological Nitrogen Fixation is Enhanced by Si Under Salt Stress Conditions

BNF in legumes has proved in many studies to be limited under salt stress, because of the high salt sensibility of nodulation process and nitrogenase activity [106, 107]. Exogenous Si application has been reported as one of the most effective strategies to improve legumes nodulation and N_2 fixation particularly under stressed conditions [46, 47]. Indeed, improving BNF by exogenous Si under salt stress has been shown by Kurdali et al. [108] on *Sesbania aculeata.* An increase of 39 % of the amount of N fixed was noted in plant treated with

Table 1 Roles of exogenous Si in legume photosynthesis improvement under salt stress conditions

Legume species	Substrate	Salt stress		Exogenous silicon			Specific functions	References
		Level	Duration (day)	Form	Application way	Level		
Cicer arietinum	Sand : loam	60, 80 or 100 mM	65	K_2SiO_3	Growth solution	4 mM	Increased total Chl and RuBisCo activity.	[41]
Cowpea	Hydroponic conditions	40 mM	Not shown	$CaSiO_3$		0.5 or 1 mM	Increased Pn, E, gs, Ci and total Chl.	[75]
Glycyrrhiza uralensis	Field conditions	3.12, 5.46 or 7.81 dS m^{-1}	Not shown	K_2SiO_3	Foliar spray	1 or 2 kg ha^{-1}	Increased Chl a, gs, E.	[39]
	Sandy loam soil	6 g Kg^{-1}	90 70 and 110		Add to the soil	0.1 g Kg^{-1}	Increased Pn, E and gs. Increased Chl a, Chl b, Chl a+b and Chl a/Chl b ratio.	[103]
Glycine max	Hydroponic conditions	100 mM	1 and 2	Not shown	Growth solution	2 mM	Increased total Chl, Ci, gs, E, Pn, Pn/Ci ratio and Pn/E ratio.	[104]
Kidney bean		40 mM	Not shown	$CaSiO_3$		0.5 or 1 mM	Increased Pn, E, gs, Ci and total Chl.	[75]
Medicago sativa	Soil	50, 100 or 200 mM	28	Na_2SiO_3		2 mM	Increased Chl, Pn, gs and E.	[73]
Phaseolus vulgaris	Peat: vermiculite	30 or 60 mM	23 and 30	K_2SiO_3		1.5 mM	Increased gs and Pn.	[102]
	Ion-free sand	150 mM	30		Foliar spray	6 mM	Increased Pn, E, Chl a, Chl b and carotenoids.	[105]
Trifolium alexandrium	Soil	2000 or 3000 ppm	75	SiO_2	Growth solution	1.5, 3 and 4.5 g Kg^{-1}	Increased Pn and total Chl.	[86]
Vicia faba		2.84, 6.03 or 8.97 dS m^{-1}	45 and 90		Foliar spray	250, 500 or 1000 ppm	Increased Chl a, Chl b and carotenoids.	[77]
Vigna radiata	Sand : vermicompost	50 or 100 mM	29	Na_2SiO_3	Growth solution	2 mM	Increased FS, Chl a, Chl b, Total Chl, Carotenoids, Fv/Fm, QPSII, qp, NPQ, A, E and gs.	[43]

Abbreviations: A, CO_2 assimilation; Chl, chlorophyll; Ci, intercellular CO_2; E, transpiration rate; FS, frequency of stomata; NPQ, non-photochemical quenching; Pn, net photosynthesis

both NaCl and Si as compared to salt stressed control. Additionally, using the acetylene reduction assay, Putra et al. [109] found that the activity of nitrogenase in *M. truncatula* root nodules was boosted by more than 85 % upon Si-treatment, which potentially reflects an increase in N fixation mediated by rhizobia. Similarly, under cadmium and zinc toxicity, exogenous application of 300 mg K_2SiO_3 kg^{-1} soil to *Cajanus cajan* resulted in an increase of nodule number, nitrogenase activity, leghemoglobin concentration and as a consequence an increase of N content [92]. Furthermore, applied Si to the unstressed *G. max* (BRS- MG 800 A cultivar) resulted in an increase of the root nodule number, nodule size and leaf N content by 82 %, 38 % and 18 % respectively when compared to Si-untreated plants [110]. Similarly, in *V. unguiculata*, Nelwamondo and Dakora [111] reported that Si promotes nodule formation as well as their function. According to Putra et al. [46], improved nodule activity in response to Si application might be related to the ability of Si to accelerate exchanges of solutes and gasses between the soil and the plant. In addition, Si has also been reported by Nelwamondo et al. [112] to have a positive structural effect inside nodules by increasing the number of bacteroids and symbiosomes. Studies also reported that Si accumulates in nodules and increases cell wall thickness [46]. Si-increased nodulation could be explained by the increase of the infection sites [108, 113]. Another explanation of the increased number of nodules upon Si treatment is the fact that silicification might affect some key symbiotic signals such as flavonoid compounds required for symbiosis establishment [46, 114]. More interestingly, by analyzing rhizospheric soil of *G. max* plants, Shamshiripour et al. [115] indicated that Si has also the potential to increase populations of bacteria such as silicate-solubilizing bacteria population as well as microbial biomass and respiration rate. The above studies suggested that exogenous Si may represent an effective strategy to improve BNF under salt stress (Fig. 2), but detail mechanisms are still not clear.

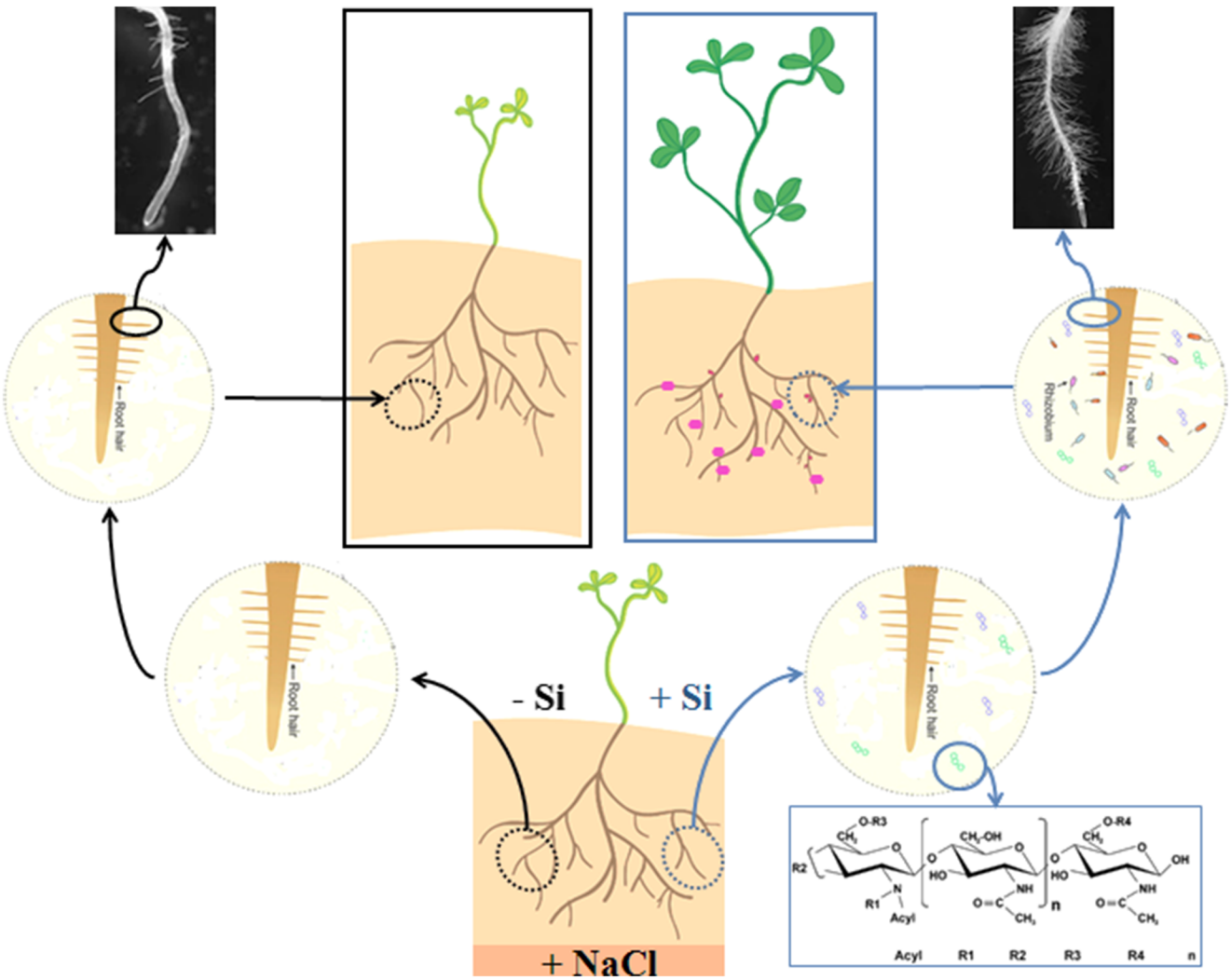

Fig. 2 Proposed representation of how does silicon (Si) treatment promote legume root nodulation under salt stress. Si addition modulates isoflavonoids secretion involving in free-living rhizobia attraction which in return secrete some biochemical substance (Nod factors) leading to root nodulation

11 Exogenous Si Reduces Ion Toxicity in Legumes Under Salt Stress Conditions

Decrease of the Na^+ uptake and/or its sequestration into vacuole represent a crucial adaptive strategy used by plants to increase their tolerance to salt stress [116]. When *C. arietinum* was treated with exogenous K_2SiO_3, a decrease in Na^+ uptake was observed leading to an increase of K^+/Na^+ ratio [41]. In the same line, Shahzad et al. [117] showed in *V. faba* that 1 mM Na_2SiO_3 was able to reduce leaf Na^+ content by 22 % and Cl^- by 14 % with an increase in K^+/Na^+ ratio. Similarly, when applied exogenously to salt stressed plants, Si induced a decrease of Na^+ and an increase of K^+ in shoot and leaves of *M. sativa* and of *T. alexandrinum* as compared to salt-stressed plants [80, 86]. Recently, Zhang et al. [91] presented a key role of Si in the reduction of salt toxicity in *G. uralensis* by reducing the Na^+ uptake and increasing K^+/Na^+ ratio. In *V. unguiculata* and *P. vulgaris*, Murillo-Amador et al. [75] showed a low shoot/root Na^+ ratio for both species. Furthermore, in *P. vulgaris*, Zuccarini [102] reported that Si reduced Na^+ in the leaves more than in the roots. These results demonstrate that Si not only reduced the uptake of Na^+ by roots, but also its translocation to aerial parts.

Several studies have reported a key role of the Na^+/H^+ antiporter in maintaining Na^+ homeostasis under salt stress by sequestrating it in vacuole or its exclusion from the cytosol [36, 89]. Two Na^+/H^+ antiporters were reported, SOS1 localized in the plasma membrane and NHX in the tonoplaste [72]. In salt-stressed *Zea mays*, 1.5 mM of exogenous $Si(OH)_4$ upregulated both *SOS* and *NHX* transcript levels under 40 mM NaCl and decreased root Na^+ content [118]. As K^+/H^+ symporter plays an important role in maintaining K^+ homeostasis, Si was also reported to increase the activity of this symporter under salt stress, which increases K^+ content in plants helping to maintain osmotic homeostasis [119]. These findings could explain the fact that Si decreased Na^+ and Cl^- and increased K^+ in salt stressed legumes (Fig. 3).

Contrary to the above studies, Romero-Aranda et al. [83] reported that 2.5 mM K_2SiO_3 application has no effect on Na^+ and Cl^- contents in NaCl-stressed *Lycopersicon esculentum*. These results showed that Si-reduced osmotic stress under salinity does not always depend on the effect of Si in reducing Na^+ and Cl^- contents. In the other hand, Si-mediated osmotic adjustment by inducing compatible solute biosynthesis under salt stress (See Section 7) is one of the most important strategies to cope with salinity-mediated osmotic stress. In the line with this, the addition of 2.5 mM K_2SiO_3 to the soil solution of salt stressed-*L. esculentum* plants resulted in a decrease of leaf water potential leading to an increase in plant water content [83]. Similarly, Tuna et al. [84] showed a better relative water content in leaves of salt-stressed barley with Si treatment. Si

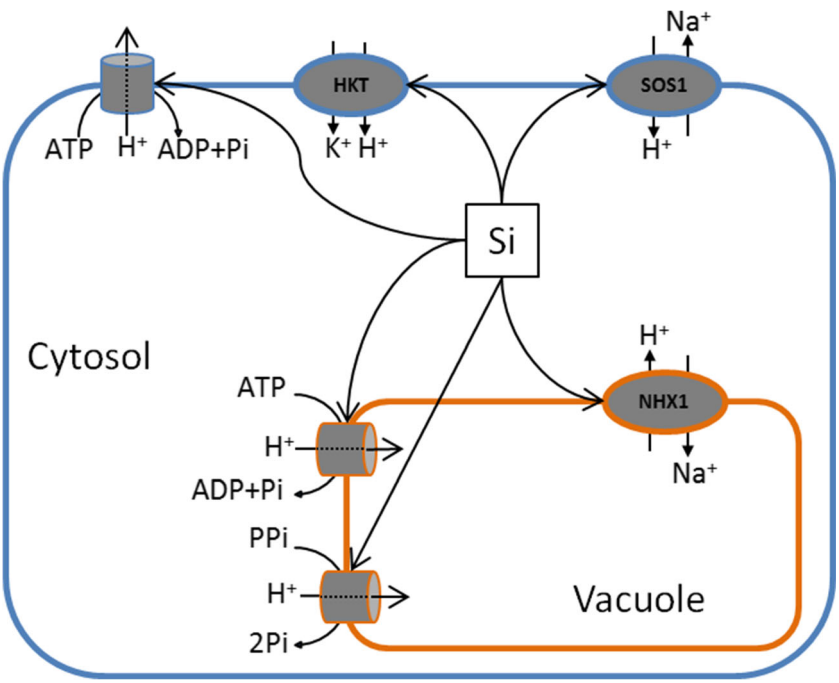

Fig. 3 Proposed mechanisms of the action of silicon (Si) in the reduction of salt toxicity in legumes. Abbreviations: Si, silicon; SOS1; salt overly sensitive 1; HKT, high-affinity K^+ transporters; NHX1, sodium/hydrogen exchanger 1

increased plant water content under salt stress could explain the important role of Si in improving salt tolerance by diluting salt concentration in the cells.

12 Exogenous Si Reduced ROS Production and Membrane Damage in Salt-stressed Legumes

Salt stress triggers ROS accumulation, leading to an oxidative stress and membrane damage [13]. Malonyldialdehyde (MDA), reflecting membrane lipid peroxidation, is widely used as an oxidative stress and membrane integrity indicator [44, 120]. Incorporation of 2 mM Na_2SiO_3 to the growth medium of salt-stressed *M. sativa* (50-200 mM) significantly reduced leaf contents of superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and MDA [73]. In *T. alexandrinum*, SiO_2 increased membrane stability index from 62.4 to 80.9 in helaly cultivar and from 74.3 to 82.6 in Sarw1 cultivar under severe salinity stress (3000 ppm) [86]. Similar findings were reported by Mahmood et al. [39] who showed that electrolyte leakage was reduced by 24 % in salt-stressed mung bean as a response to 2 kg K_2SiO_3 ha^{-1} applied as a foliar spray. Likewise, exposure of *G. uralensis* to NaCl increased MDA content and membrane permeability in plants after 150 days of treatment and this was found to be mitigated by 0.6 g K_2SiO_3 Kg^{-1} soil supplementation [87]. The same was found by Wu et al. [42] in *O. viciaefolia* treated with 1 mM Na_2SiO_3 under 100 mM NaCl. Exogenous Si-mediated membrane stability under salt stress was also reported in *V. radiata* [43], Cowpea and kidney bean [75].

13 Exogenous Si Reduces Oxidative Stress in Salt-stressed Legumes

Under abiotic stress, plants set up various non-enzymatic and enzymatic mechanisms to detoxify the excess of ROS. Studies showed that Si could alleviate salinity-induced oxidative stress by increasing the content of non-enzymatic scavengers such as ascorbic acid (AsA), alkaloids, flavonoids and carotenoids and by inducing the activity of enzymatic systems including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione S-transferase (GST) [72, 121]. Indeed, Meng et al. [73] reported that exogenous application of 2 mM Na_2SiO_3 to the growth medium of *M. sativa* exposed to 200 mM NaCl for 28 days significantly decreased the content of H_2O_2 and O_2^- and this decrease was significantly correlated with an increase in SOD, peroxidase (POD) and CAT activities. Zhang et al. [91] showed that K_2SiO_3 addition to *G. uralensis* treated with NaCl for 110 days reduced O_2^- and H_2O_2 content, increased some non-enzymatic antioxidants like AsA and glutathione (GSH) as well as activities of some antioxidant enzymes such as APX, CAT, GPX and POD. Similar findings have been documented by Zhang et al. [90] on the same plant species, where AsA and GSH contents was increased and the activities of APX, SOD, POD and CAT were improved by 1 mM of K_2SiO_3 under 100 mM NaCl treatment. In addition, in a study conducted by Wang et al. [45] on 120 mM NaCl-treated *M. sativa*, supplementation of 1 mM K_2SiO_3 to the growth medium significantly increased the activity of APX in roots, shoots and leaves. In the same work, the authors showed an increase in the activities of CAT and POD in leaves and shoots respectively. In agreement with these results, Ahmad et al. [43] reported an increase in the activities of SOD, CAT, APX and GR in *V. radiata* as a response to salt stress induced by 100 mM NaCl application and these enzymatic activities were further boosted in salt-stressed plants when they were treated with 3 mM of exogenous Na_2SiO_3. These studies highlithed that Si could reduce oxidative stress in legumes under salinity by inducing both enzymatic and non-enzymatic antioxidant activities (Fig. 4; Table 2). In addition to these findings, studies on antioxidant related gene expression in response to Si application particularly under abiotic stress would be interesting for better understanding of the mechanisms by which Si-mediated oxidative stress tolerance in legumes.

14 Effect of Si Fertilizers Under Field Conditions

Previous investigations evidenced the positive effects of Si on plant growth and productivity, and especially under changing environments [25]. However, the majority of studies have been conducted greenhouses under controlled conditions, which may not reflect the effect of Si under field conditions. For example, to improve the tolerance of *Z. mays* to Cd stress under field conditions, Wang et al. [124] used different Si forms including Si-calcium (CaSi), Si-potash (KSi), semi-finished product of Si-potash (SKSi) and Na_2SiO_3 fertilizers. Here the authors demonstrated that 9000 kg CaSi ha^{-1}, 900 kg KSI ha^{-1} and 900 SKSi ha^{-1} reduced Cd concentrations in plants by up to 71.5 %, 42 % and 40.7 % respectively compared with the control. Conversely, as compared to control, Na_2SiO_3 induced a slight increase in plant Cd concentration, which impaired *Z. mays* plants growth. However, in another interesting study conducted by Taha et al. [125] in *T. aestivum* grown in salt-stressed soil, 30 mM Na_2SiO_3 foliar spray increased significantly plant growth, photosynthesis,

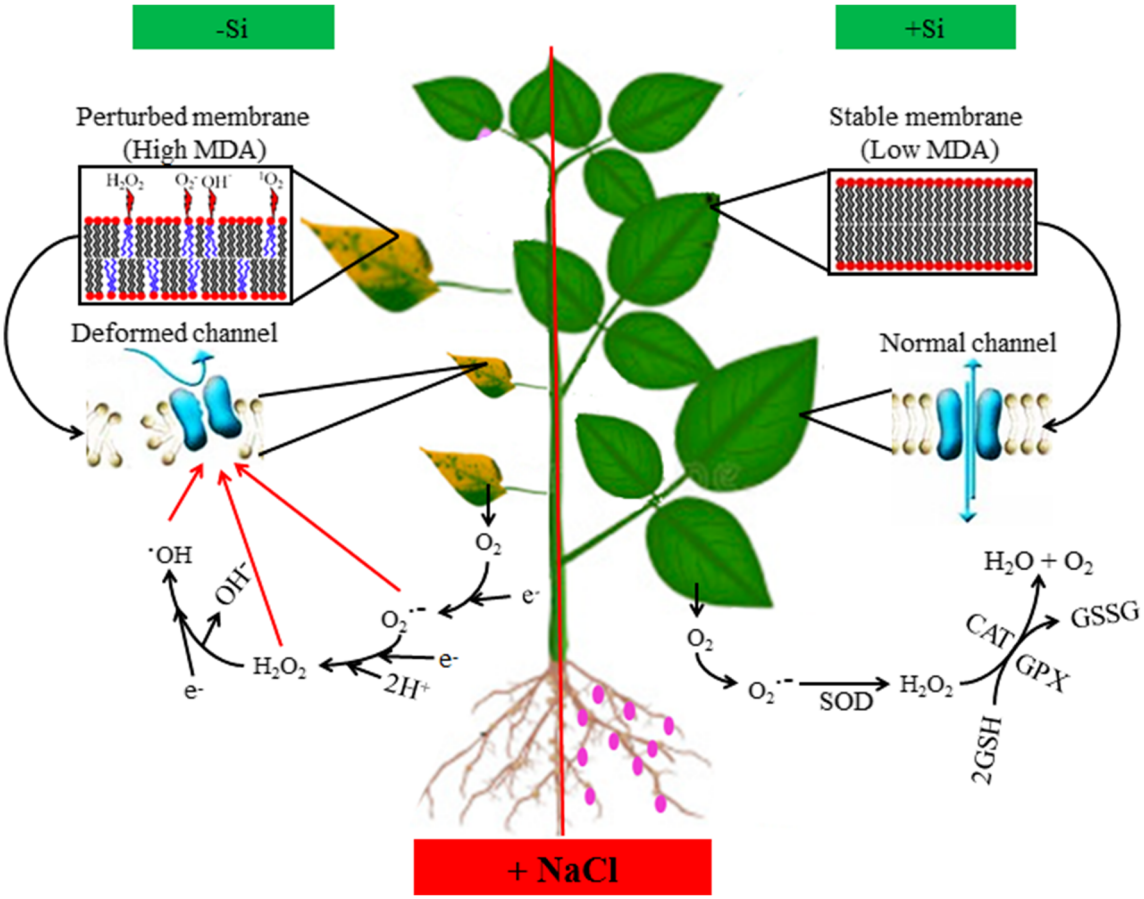

Fig. 4 Schematic overview of Si-mediated oxidative stress regulation in legume plants under salt stress condition. Salt stress induced an overproduction of reactive oxygen species (ROS) leading to a loss of membrane integrity, while Si addition helps in ROS scavenging by increasing antioxidant enzymes activities. Abbreviations: Si, silicon; MDA, malonyldialdehyde; O_2, oxygen; e-, electron; $O_2^{\cdot-}$. superoxide anion; H^+, proton; H_2O_2, hydrogen peroxide; $^{\cdot}OH$, hydroxyl radical; SOD, superoxyde dismutase; CAT, catalase; GPX, glutathione peroxidase; GSH, reduced glutathione; GSSH, oxidized glutathione; NaCl, sodium chloride

performance index, membrane stability index, RWC, compatible solutes and enzymatic and non-enzymatic antioxidants system. In addition, using 150 mg $CaSiO_3$ kg^{-1} soil, as a sustainable strategy, improved significantly plant height, number of tillers, number of grains per spike, 1000-grain weight, harvest index and K^+/Na^+ ratio of *T. aestivum* grown in salt-affected soil [126]. Moreover, when sprayed on the leaves at a rate of 2 kg ha^{-1}, K_2SiO_3 increased stomatal conductance, transpiration rate, RWC, photosynthetic pigments, and salt tolerance index, alleviating as a result the negative effects of salinity on *V. radiata* growth and production [39]. Similarly, when applied exogenously at 300 kg ha^{-1}, Si fertilizer (with 62.9 % of SiO_2) improved *O. sativa* tolerance to water stress and the effect was dose and timing dependent [127]. In the line with these results, application of K_2SiO_3 at the rate of 12 kg ha^{-1} resulted in a higher yield and biomass in *T. aestivum* plants grown under drought stress [128]. Likewise, in a calcareous grey desert soil, SiO_2 supplementation to the plow layer (20 cm in depth) at the rate of 600 kg ha^{-1} increased both yield and fruit quality of table *Vitis vinifera* [129]. More interestingly, since 2014, about 13 field trials have been conducted in Morocco in sugar beet treated with Agrisilica (26 % of Si) fertilizer during sowing at doses of 150, 200, 250, and 300 kg ha^{-1} [130]. Results indicated that sugar beet yield was systematically increased up to 40 % with the increase of fertilizer dose, and an increase in the sugar yield by 4.8 Mg ha^{-1} was observed when Si fertilizer was applied at 250 kg ha^{-1}. Moreover, $CaSiO_3$ supplementation to the growth medium or K_2SiO_3 foliar spray were reported to reduce *Phakopsora pachyrhizi* pathogenesis in *G. max* by 43 and 36 % respectively [131]. Si fertilizers were also reported to improve growth and productivity of other crops including corn, rapeseed, potato, meadows, berry, vegetables, orchards and ornamental plants under both normal and stressed conditions [132]. Above studies clearly indicated that Si could be used as a fertilizer to improve crops growth and productivity under field conditions, and the effect is depending to the form, the optimal concentration and the application way of Si. Therefore, for better understanding of the effect of Si on crop developments under field conditions, more

Table 2 Putative roles of exogenous silicon in legume salt stress tolerance in relation to antioxidant defense system induction

Legume species	Substrate	Salt stress		Exogenous silicon			Antioxidant responses to salt stress	References
		Level	Duration (day)	Form	Application way	Level		
Acacia gerrardii	Sand: perlite: peat	200 mM	56	K_2SiO_3	Growth solution	2 mM	Increased AsA, SOD, POD, CAT, APX and GR	[122]
Glycine max	Hydroponic condition	100 mM	1	Not shown		2 mM	Decreased APX, CAT and GSH	[104]
Glycyrrhiza uralensis	Sand	50 mM	10, 20, 30	K_2SiO_3		1, 2, 4 or 6 mM	Increased SOD and POD	[123]
	Filter paper	50, 100 or 150 mM	10			1, 2, 4, 6 or 8 mM	Increased SOD	[38]
	Sandy loam	6 g Kg^{-1}	70, 110			0.1 g Kg^{-1}	Increased AsA, GSH, APX, CAT, GPX and POD.	[91]
	Filter paper	100 mM	10			1 mM	Increased AsA, GSH, APX, CAT, SOD and POD.	[87]
Medicago sativa	Hydroponic condition	120 mM	15				Increased APX, CAT and POD	[45]
	Soil	50, 100 or 200 mM	28	Na_2SiO_3		2 mM	Increased CAT, SOD and POD	[73]
Phaseolus vulgaris	Ion-free sand	150 mM	30	K_2SiO_3	Foliar spray	6 mM	Increased glutathione, APX, CAT, SOD, POX and GR.	[105]
Vigna radiata	Sand : vermicomp-ost	50 or 100 mM	29	Na_2SiO_3	Growth solution	3 mM	Increased APX, CAT, GR and SOD	[43]

Abbreviations: AsA, ascorbic acid; APX, ascorbate peroxidase; CAT, catalase; GR, glutathione reductase; GSH, glutathione; SOD, superoxyde dismutase; POD, peroxidase

studies using different Si forms and at varied doses are recommended.

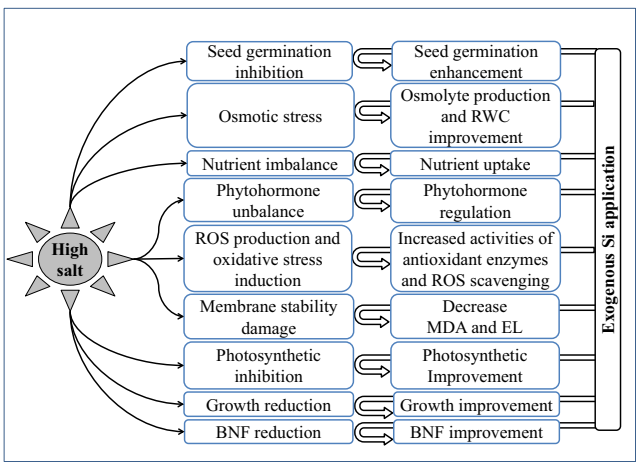

Fig. 5 Summary of the main effect of exogenous silicon (Si) application on legume salt tolerance. Abbreviations: Si, silicon; RWC, relative water content; ROS, reactive oxygen species; MDA, Malonyldialdehyde; EL, electrolyte leakages; BNF, biological nitrogen fixation

15 Conclusions

Legumes are important crop and they provide important nutrient source for human food and animal feed. They also fix atmospheric N with rhizobia, which allows them to ensure their N nutrition without any chemical fertilizers. However, legumes and their symbioses are very sensitive to abiotic stress, with a particular high sensitivity to salt stress. As salt stress causes both osmotic stress and ionic toxicity, it represents one of the major threats to legumes and its effect is observed at the different growth stages. Several works reported that exogenous Si was beneficial in improving plant salt-stress tolerance. Based on recent knowledge, Si showed a positive effect on legume seed germination, plant growth, development and productivity under salt stress (Fig. 5). Exogenous Si has also been confirmed to mediate BNF under diverse abiotic stress including salt stress. This is mediated by the positive effect of Si on nodule formation and size, leghemoglobin content and nitrogenase activity. Exogenous Si has also positive effects inside the nodules by increasing bacteroids and symbiosome number. Thus, it is clear from this review that Si has beneficial impact on legumes and its application under salt stress is recommended as a sustainable way for increasing growth, development, BNF and productivity of legumes.

16 Future Perspectives

Studies showed that Si uptake is an important factor for Si-induced plant tolerance to biotic and abiotic stresses [25, 26, 33, 36]. It is well documented that Si-uptake is related to the presence of some specific transporter called Lsi which ensure its transport from the soil solution into the plant cells [36]. Likewise, some plant family such as legumes has been considered as Si rejective, because the majority of the species belonging to this family does not have Lsi transporter [29]. However, although this plant family was considered as Si-rejective, Si application showed intriguing beneficial effects on plant growth of several legume species [42, 43, 74]. Furthermore, in some species like *G. max*, Si-induced salt stress tolerance was reported to be related to the presence of some specific Si-transported encoded by two genes, $GmNIP_{2-1}$ and $GmNIP_{2-2}$, which make this plant species a Si accumulator [63]. In addition, *M. truncatula* and *P. vulgaris* have been reported to have genes encoding for Lsi1 and Lsi2. *M. truncatula* was considered as mild Si accumulator [61]. Therefore, more studies on other legume species are required to identify new Si transporters and to better understand and clarify the ability of legumes to absorb Si.

Na^+ rejection from the cytosol or its compartmentalization into vacuole has been documented in several reports to be one of the most important strategies to overcome salt stress in plant [89]. Na^+/H^+ antiporters encoded by *SOS1* gene in the plasma membrane or by *NHX1* in the tonoplaste have been reported to have a crucial role in maintaining Na^+ homeostasis under salt conditions [36, 119]. Exogenous Si reduces Na^+ content in salt-stressed maize and this effect has been reported to be related to the ability of Si to upregulate the *SOS1* and *NHX1* gene expression [118]. Increasing K^+ content in plant under osmotic stress will help plants to maintain osmotic and cellular homeostasis [36, 133]. Its transport in plants was reported to be mediated by a high-affinity K^+ transporter (HKT) [134, 135]. In legumes, several studies have shown that Si reduced Na^+ and increased K^+ content [41, 117]. However, to date, no one has investigated the effect of Si on *SOS1*, *NHX1* and *HKT* gene expression in salt-stressed legumes. Therefore, it would be interesting to study how exogenous Si could induce salt tolerance in legumes through *SOS1*, *NHX* and *HKT* gene expression.

In agro-ecosystem, BNF through specific activity of nitrogenase represents one of the most important interests of legumes-rhizobia symbiosis [14, 17]. Some reports indicated that Si improves symbiosis establishment under salt stress [108]. However, to our knowledge, the effect of Si on nitrogenase activity under salt stress has not been studied yet. To better understand mechanisms by which exogenous Si improves BNF, the effect of Si on nitrogenase activity and on its gene expression particularly under salt stress will be important to be focused.

Maintaining water relationship in plant under osmotic stress is an important prospect for salt tolerance. Aquaporins, group of water channels, are known to mediate plant water uptake, and a positive correlation between aquaporins gene expression and tolerance to salt stress was observed in some plant species such as *Eutrema salsugineum* [136]. In some plant species like sorghum and cucumber, Si was reported to enhance water uptake under salt stress by upregulating aquaporins gene expression [85, 137]. However its effect on legume aquaporin related-gene expression is still poorly understood. Therefore, it would be interesting to investigate the effect of Si application on legume aquaporins gene expression particularly under salt stress.

Proline is known to play an important role in plant tolerance to diverse abiotic stress [93, 138, 139]. According to Szabados and Savouré [140], proline helps plant to maintain osmotic homeostasis, represent a ROS scavenger and N source. Studies reported in this review showed that the effect of Si on proline is species-dependent manner. Therefore, detail studies on the effect of exogenous Si on the expression of proline metabolism related-genes; *pyroline-5-carboxylase synthase* (*P5CS*), *Ornithine-δ-aminotransferase* (*OAT*) and *Proline dehydrogenase* (*PDH*); in legumes particularly under salt stress are needed to better understanding the effect of Si on proline metabolism.

Besides proline, the accumulation of glycine betaine and polyamines in legumes is a widespread response to salt stress [89, 141, 142]. Previous investigations showed that Si could alleviate salt stress in legumes through glycine betaine accumulation [43]. Furthermore, in *C. sativus*, Si-treatment increased salt tolerance highlighted by high polyamines content [143]. However, the specific mechanisms linking Si treatment with glycine betaine and polyamines accumulation in legumes under salt stress are not well understood. Therefore, further investigations are required particularly at the molecular level for a deeper understanding of the connection between Si treatment and glycine betaine and polyamines accumulation under salt stress.

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Data Availability Not applicable.

Code Availability Not applicable.

Declarations

Conflict of Interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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III-3 Rôle de l'apport exogène de la proline dans la tolérance des plantes cultivées sous contrainte saline (Article 3).

La proline est un acide aminé multifonctionnel nécessaire pour la synthèse protéique et le métabolisme primaire des plantes. Sa synthèse s'effectue généralement au niveau du cytosol et des chloroplastes à partir du glutamate sous l'action séquentielle de la Pyrroline-5-carboxylate synthétase (P5CS) et la P5C réductase (P5CR). D'autre part, la voie de l'ornithine représente une voie alternative pour la synthèse de la proline *via* l'activité de l'Ornithine aminotransférase (δOAT) qui convertit l'ornithine et le α-cétoglutarate en P5C et en glutamate. Le catabolisme de la proline a lieu dans la mitochondrie sous l'action séquentielle de la Proline déshydrogénase (ProDH) et de la Pyrroline-5-carboxylate déshydrogénase (P5CDH). Des études antérieures suggèrent que la proline pourrait jouer un rôle déterminant dans la tolérance des plantes aux contraintes abiotiques. De même, son apport exogène a été rapporté comme étant une stratégie efficace pour pallier l'effet de contraintes abiotiques telles que la déficience nutritionnelle, la contrainte hydrique et la contrainte saline. Ainsi, nous nous sommes intéressés à résumer l'état de l'art sur l'effet de l'apport exogène de la proline dans l'amélioration de la tolérance des plantes à la contrainte saline. L'amélioration de la germination des graines, la croissance, la photosynthèse, la fixation biologique de l'azote atmosphérique et la productivité des plantes sous contrainte saline en réponse à l'ajout de proline exogène ont été rapportées. Les mécanismes stimulés par la proline exogène pour renforcer la tolérance des plantes à la contrainte saline ont été évalués de manière critique.

Ces données bibliographiques ont fait l'objet d'une publication intitulée ‘‘How does proline treatment promote salt stress tolerance during crop plant developement ?’’ dans la revue Frontiers in Plant Science.

How Does Proline Treatment Promote Salt Stress Tolerance During Crop Plant Development?

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Soil salinity is one of the major abiotic stresses restricting the use of land for agriculture because it limits the growth and development of most crop plants. Improving productivity under these physiologically stressful conditions is a major scientific challenge because salinity has different effects at different developmental stages in different crops. When supplied exogenously, proline has improved salt stress tolerance in various plant species. Under high-salt conditions, proline application enhances plant growth with increases in seed germination, biomass, photosynthesis, gas exchange, and grain yield. These positive effects are mainly driven by better nutrient acquisition, water uptake, and biological nitrogen fixation. Exogenous proline also alleviates salt stress by improving antioxidant activities and reducing Na^+ and Cl^- uptake and translocation while enhancing K^+ assimilation by plants. However, which of these mechanisms operate at any one time varies according to the proline concentration, how it is applied, the plant species, and the specific stress conditions as well as the developmental stage. To position salt stress tolerance studies in the context of a crop plant growing in the field, here we discuss the beneficial effects of exogenous proline on plants exposed to salt stress through well-known and more recently described examples in more than twenty crop species in order to appreciate both the diversity and commonality of the responses. Proposed mechanisms by which exogenous proline mitigates the detrimental effects of salt stress during crop plant growth are thus highlighted and critically assessed.

Keywords: salinity, proline, plant development, photosynthesis, biological nitrogen fixation, nutrient uptake, water nutrition, antioxidants

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INTRODUCTION

Salinity is a major abiotic stress that severely affects crop plant growth and development from seed germination to harvest. In recent years, increasing deleterious effects on agricultural productivity have been observed especially in arid and semiarid regions where rainfall is low and evapotranspiration is high (Jha et al., 2019). It is estimated that more than 7% of total land and almost 20% of arable land are affected by salinity with affected areas increasing at an annual rate of

1–2% (Zhu and Gong, 2014; Rizwan et al., 2015). It is indeed predicted that more than 50% of arable land will be rendered unproductive by 2050 due to the levels of salt stress induced in crops (Vinocur and Altman, 2005; Bianco and Defez, 2009). This trend coincides with the increasing challenge of ensuring global food security, so it is even more urgent to be able to exploit more arable land and increase crop productivity even in infertile soil by developing efficient and tolerant crops able to grow in salty conditions (Rengasamy, 2006; Safdar et al., 2019). Thus, new alternative approaches to allow crops to efficiently tolerate salt stress are needed. Indeed, the use of exogenous compounds, which are both ecofriendly and easily available, such as silicon (Zhu and Gong, 2014; Rizwan et al., 2015), trehalose (Nounjan et al., 2012), glycine betaine (Hossain and Fujita, 2010), and proline (Hoque et al., 2008; Deivanai et al., 2011; Wani et al., 2016), is a sustainable approach to overcoming the negative effects of salt stress on seed germination, plant growth, and productivity.

Proline is the most common endogenous osmolyte accumulated under various abiotic stresses including salinity (Szabados and Savouré, 2010; Slama et al., 2015). When applied as an exogenous compound to crops, proline can improve salt tolerance (Heuer, 2010). For example, in salt-stressed *Zea mays*, foliar application of proline increased plant growth with a positive effect on yield characteristics (Alam et al., 2016). The beneficial effects of exogenous proline application on salt stress tolerance has been the subject of several reviews. For example, Ashraf and Foolad (2007) focused on the effect of exogenous proline on seed germination, seedling growth and Na^+/K^+ ratio. More recently Meena et al. (2019) considered some beneficial effects of exogenous proline on plant tolerance to varying environments. Some of the latest progress in the subject addresses aspects related to ionic toxicity reduction, biological nitrogen fixation, and salt tolerance related-gene expression. Therefore, this review integrates this most recent research with current thinking on proline and plant salt tolerance in the context of some key developmental stages of crop growth.

IMPACTS OF SALINITY ON DEVELOPMENTAL PHYSIOLOGY OF CROP PLANTS

With the exception of halophytes, which represent 1–3% of the flowering plants, most plants, and especially crops, are salt-sensitive during their life cycle. Salt stress reduces plant growth and productivity (for review see van Zelm et al., 2020) and may be a direct effect due to the accumulation of Na^+ and Cl^- or an indirect effect due to water deprivation (Ghars et al., 2008; Safdar et al., 2019).

Seed germination may be drastically affected by salinity in both glycophytes and halophytes (Ghoulam and Fares, 2001; Liu et al., 2018). Salinity inhibits *Lens culinaris* seed germination by disturbing hydrolytic enzyme activities such as α-amylase, β-amylase and α-glucosidase (Sidari et al., 2008). Salt-inhibited *Medicago sativa* seed germination is correlated with the inhibition of the seed reserve mobilization (Farissi et al., 2011). Furthermore, salinity is known to inhibit seed germination by disturbing the homeostasis of plant growth regulators such as abscisic acid and gibberellic acid, the two of the main phytohormones participating in the regulation of germination (Holdsworth et al., 2008; Skubacz et al., 2016). Salinity causes secondary stress, known as oxidative stress, when reactive oxygen species (ROS) accumulate in cells. At high levels, ROS disturb normal metabolism by peroxidating proteins, lipids, and nucleic acids (Hayat et al., 2012; Farissi et al., 2014). Salinity-induced oxidative stress and membrane damage during germination and seedling growth have been described in several plant species and explain some of the deleterious effects of salt stress on seed germination (Wang et al., 2010; Zhang et al., 2015). The proposed effects of salinity on plant during seed germination are summarized in **Figure 1**.

As the radicle and later the roots emerge, the presence of salt triggers osmotic stress which makes water uptake more difficult. In addition, high salt concentrations in soil disrupt mineral nutrition leading to ion imbalance in the cells. Accumulation of excess sodium in plant cells has a toxic effect as it leads to precipitation or partial denaturation of proteins, phytohormone imbalances, generation of ROS, and changes in membrane permeability (**Figure 2**). Salinity was also reported to affect N metabolism at different steps including N uptake, NO_3^- reduction and NH_4^+ assimilation by disturbing the activities of the main enzymes involved in nitrogen metabolism such as nitrate reductase, nitrite reductase, glutamate synthetase, and glutamate synthase (Ashraf et al., 2018).

As the seedling establishes itself to become autotrophic, photosynthesis, essential for growth, is very vulnerable to salt stress (**Figure 2**) (Safdar et al., 2019). Numerous studies have reported that photosynthesis is suppressed by salinity in several plant species. Smaller leaf area, fewer photosynthesis pigments, lower quantum efficiency of photosystem II (*Fv/Fm*) and less gas exchange were reported under salty conditions, which clearly contributed to the reduction observed in length and biomass of both shoots and roots (Ben Ahmed et al., 2011; Wani et al., 2016). Likewise, salinity was reported to induce the activity of some enzymes that degrade chlorophyll (Jamil et al., 2007). As a consequence of chlorophyllase induction, the total amount of chlorophyll decreases and chloroplast structure is disturbed, which directly influence photosynthesis rate and hence plant growth. Under osmotic stress, plants close their stomata to prevent water loss by transpiration (Yang et al., 2006). However, this mechanism also limits the assimilation of CO_2, which then slows the photosynthesis rate and limits plant growth and productivity. High levels of salt may also affect cell division (Munns and Tester, 2008).

Legume–rhizobium symbiosis is a specific relationship established between legumes and nitrogen-fixing bacteria such as rhizobia. During this mutualistic symbiosis, inside a newly formed organ called a nodule, rhizobia are able to provide enough nitrogen to the host legume through the specific activity of nitrogenase and in return they receive a variety of

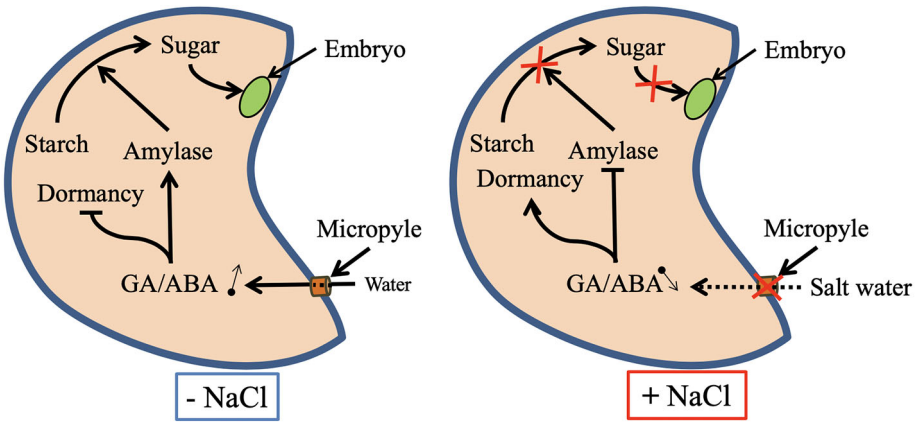

FIGURE 1 | Proposed mechanisms for the regulation of seed dormancy release and seed reserve mobilization under salt-stressed and unstressed conditions. Abbreviations: ABA, abscisic acid; GA, gibberellic acid.

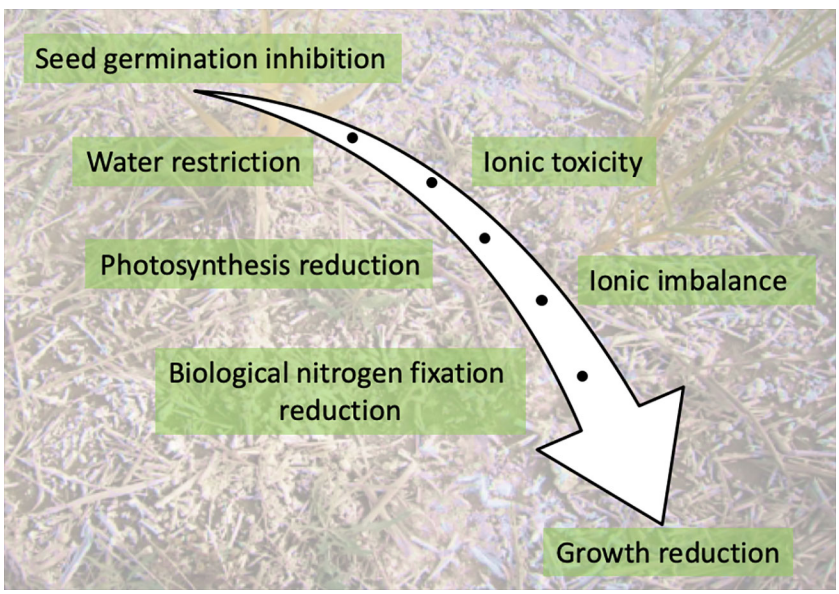

FIGURE 2 | Key impacts of salinity on a developing plant.

carbon-based compounds from photosynthates and some micronutrients *e.g.*, Fe, S, Mo (Checcucci et al., 2017). Legume–rhizobium symbiosis represents one of the main ecological processes in the agroecosystem due to its benefits on soil fertility (Farissi et al., 2014). However, this symbiotic process is drastically limited by salt stress, affecting both the micro- and macro-symbiont. Indeed, depending on their sensitivity, salinity affects the survival and distribution of rhizobia in the soil (Zahran, 1999). Salinity was also reported to inhibit legume–rhizobium symbiosis establishment by reducing the number of root hairs containing infection threads (Zahran and Sprent, 1986). In addition, if symbiosis is already established, salinity decreases symbiotic performance by reducing leghemoglobin synthesis and nitrogenase activity (López et al., 2008). Studies also showed that salt stress limits the supply of carbon sources to the bacteroids by reducing the activity of phosphoenolpyruvate carboxylase and malate dehydrogenase, and so the number of bacteroids inside the nodule (López et al., 2008). The main effects of salt stress on legume–rhizobium symbiosis are summarized in **Figure 3**.

PROLINE METABOLISM IN PLANTS

In higher plants, biosynthesis of proline occurs *via* two pathways depending on the relative availability of the alternative

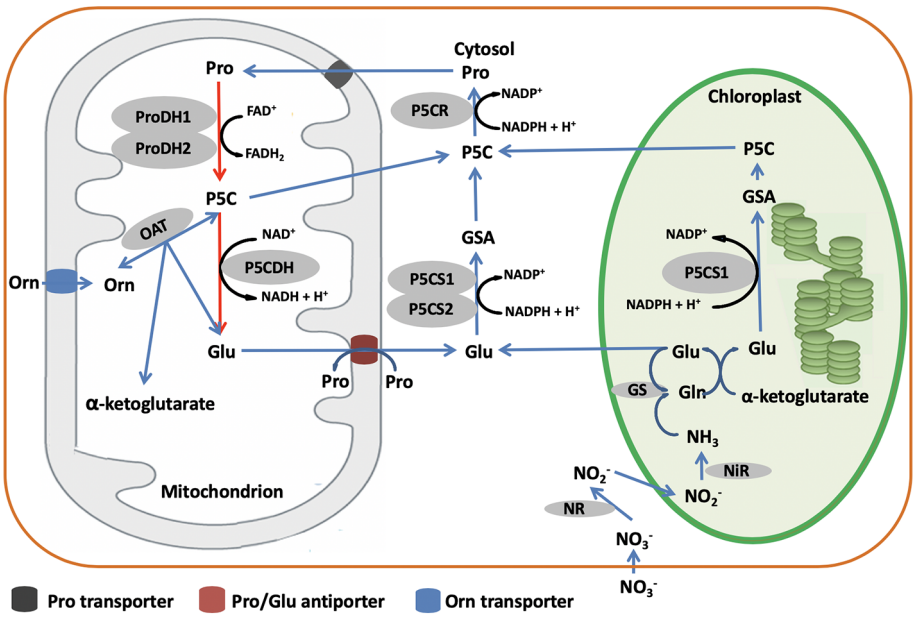

FIGURE 3 | Proposed proline metabolism pathways in higher plants. Biosynthesis (blue lines) and catabolism (red lines) are shown. Abbreviations: Pro, proline; Glu, glutamate; Orn, ornithine; NR, nitrate reductase; NiR, nitrite reductase.

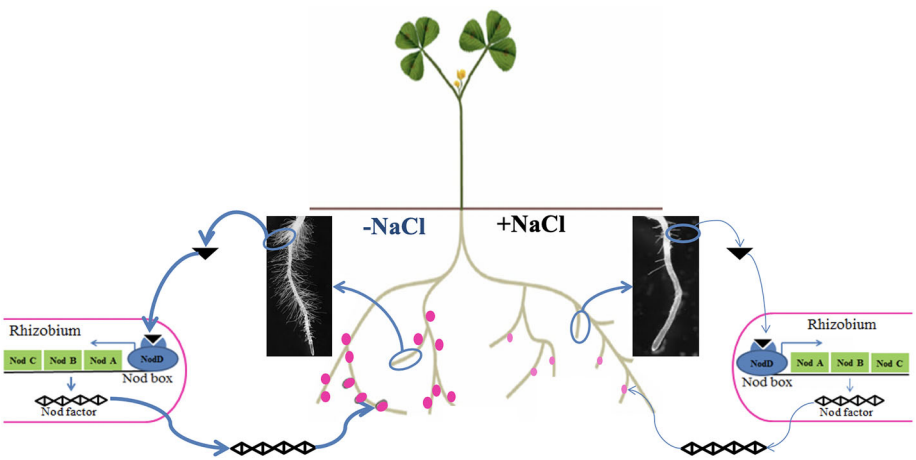

FIGURE 4 | Conceptual illustration of the effect of salt stress on the establishment and functioning of legume–rhizobium symbiosis. Under nitrogen deficiency, without salt stress the legume emits many (bold arrow) chemical cues in the form of isoflavonoids (black triangle) to specifically attract rhizobia, which in turn secrete Nod factors allowing recognition between the two partners and formation of functional nodules (dark pink dots). However, salt stress negatively affects the legume–rhizobium dialogue by reducing isoflavonoid secretion (thin arrow) leading to less nodulation (number and biomass) and nodule function (pale pink dots).

substrates, glutamate (Glu) and ornithine (Orn) (**Figure 4**). The Glu pathway starts with pyrroline-5-carboxylate synthetase (P5CS) that uses ATP and NAD(P)H+H^+ to reduce Glu to glutamate-semialdehyde (GSA), which spontaneously converts to pyrroline-5-carboxylate (P5C) (Szabados and Savouré, 2010). Then, P5C is reduced to proline by the action of P5C reductase (P5CR) using NADPH and H^+ (Szabados and Savouré, 2010). In most plant species, P5CS is encoded by two genes, *P5CS1* and *P5CS2*, while P5CR is encoded by only one gene (Szabados and Savouré, 2010). However, in some species like *Medicago truncatula*, P5CS is possibly encoded by three genes (Kim and Nam, 2013; Nguyen et al., 2013). The Orn pathway has mostly been considered as an alternative pathway for proline biosynthesis. Ornithine-δ-aminotransferase (OAT) transaminates Orn to produce GSA and P5C, which is then reduced to proline by the action of P5CR (Mansour and Ali, 2017). According to You et al.

(2012), transgenic lines of rice constitutively overexpressing *OAT* produce higher levels of proline than wild type, pointing to a more pivotal role of the Orn pathway in proline biosynthesis.

Although the genes and enzymes involved in proline biosynthesis have been well studied, the preferential use of Glu or Orn as substrate is still unclear. Some authors have reported that the preferred pathway is dependent on the developmental stage, the Orn pathway having a particularly crucial role in seedling development (Schmid et al., 2005; Hayat et al., 2012). Others however, have documented that the pathway preference is species-dependent. Indeed, AbdElgawad et al. (2015) noted that the Glu pathway involving P5CS and P5CR is predominant in grass, while the Orn pathway with OAT and P5CR is predominant in legumes. This difference may be related to the N nutritional status. In fact, Delauney et al. (1993) found that OAT is nitrogen-dependent. Another aspect to consider is the environmental control over which proline biosynthetic pathway is used. Zhen and Ma (2009) showed that P5CS activity (Glu pathway) increased upon salt stress treatment, while OAT activity (Orn pathway) appeared not to be affected, suggesting that the Glu pathway rather than the Orn pathway plays a more significant role in proline accumulation during osmotic regulation. In *Vigna aconitifolia*, Delauney et al. (1993) showed that salt stress induced the accumulation of *P5CS* mRNA while *OAT* mRNA levels were suppressed. Lei et al. (2016) and Mansour and Ali (2017) confirmed that the accumulation of proline under salt stress is related to the up-regulation of *P5CS* (Glu pathway) genes and down-regulation of *Proline dehydrogenase* (*PDH*) genes. In comparison, Funck et al. (2008) demonstrated that OAT is localized in mitochondria and it is not essential for proline biosynthesis.

For catabolism, proline is converted back to Glu in the mitochondria by the sequential action of PDH and P5C dehydrogenase (P5CDH). Although the Nomenclature Committee of The International Union of Biochemistry and Molecular Biology (IUBMB) recommended the name glutamate γ-semialdehyde dehydrogenase (GSALDH), an enzyme name derived from its substrate, for the second enzyme of proline catabolism, the name P5CDH is kept in the review for clarity for the research community. PDH oxidizes proline to P5C which is converted to Glu by P5CDH using NAD^+ as electron acceptor (Servet et al., 2012; Schertl et al., 2014). In some plant species including *Arabidopsis thaliana*, *Nicotiana tabacum*, and *M. sativa*, PDH is encoded by two genes, whereas P5CDH is encoded by a single gene (Miller et al., 2005; Ribarits et al., 2007; Servet et al., 2012).

INFLUENCES OF PROLINE METABOLISM ON PHYSIOLOGICAL AND BIOCHEMICAL PROCESSES

Proline has been widely reported to be a multifunctional amino acid that acts at different plant growth stages (Szabados and Savouré, 2010). Indeed, proline metabolism plays a key role in the oxidative pentose phosphate pathway (OPPP) by generating $NAD(P)^+$ in the cytosol (Signorelli, 2016). Since the OPPP is involved in triggering seed germination, it is believed that proline metabolism has a beneficial effect on seed germination (Kavi Kishor and Sreenivasulu, 2014). When stomata are closed under osmotic stress to avoid water losses by transpiration, CO_2 assimilation is limited (Yang et al., 2006). This phenomenon reduces carbon fixation and NAD(P)H consumption by the Calvin cycle and leads to accumulation of ROS by electrolyte leakage. However, proline biosynthesis requires the oxidation of two $NAD(P)H^+$ molecules to $NADP^+$ (**Figure 4**), which helps to reduce NAD(P)H and recycle $NAD(P)^+$. Furthermore, the oxidation of NAD(P)H to $NADP^+$ during proline biosynthesis increases $NADP^+$ which will be reduced in the pentose phosphate pathway to $NAD(P)H^+$ generating one molecule of CO_2 (**Figure 5**). Thus, the CO_2 generated allows carbon reduction to continue under stressful conditions, while the NAD(P)H will be used in proline biosynthesis to prevent ROS production (Verslues and Sharma, 2010; Signorelli et al., 2015). Proline was also reported to contribute to photosynthesis improvement by protecting RuBisCo activity and mitochondrial

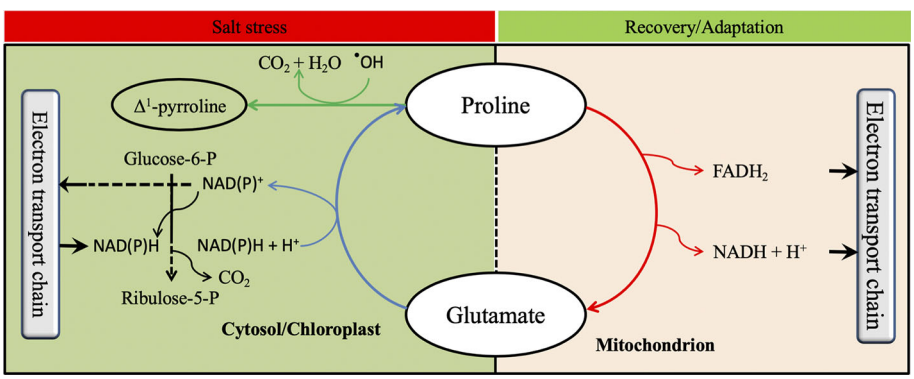

FIGURE 5 | Possible influence of proline metabolism on physiological and biochemical processes. The oxidative pentose phosphate pathway is shown by black lines, the proline anabolic pathway with blue lines, the proline catabolic pathway with red lines, and proline scavenging reactive oxygen species with green lines.

electron transport chain complex II (Solomon et al., 1994; Hamilton and Heckathorn, 2001). Furthermore, proline anabolism allows plants to adjust their osmotic homeostasis which helps to restore plant water content particularly under osmotic stress (Misra and Gupta, 2005). Proline metabolism has also been documented to play an important role during biological nitrogen fixation (BNF) particularly under stressed conditions. Indeed, a high positive correlation between the expression of *StP5CS* and two nodulation-related genes and one leghemoglobin gene was reported by Ren et al. (2018). Likewise, proline catabolism was reported to provide energy to the bacteroids during the BNF (Kohl et al., 1988) suggesting that both proline anabolism and catabolism improved BNF efficiency. In addition, proline has been reported in several studies to play a role in non-enzymatic antioxidant activities (Matysik et al., 2002; Signorelli et al., 2015). These proposed roles of proline metabolism in key physiological and biochemical are illustrated in **Figure 5**.

EFFECT OF EXOGENOUS PROLINE DURING SALT STRESS

Exogenous Proline Application and Proline Metabolism Under Salt Stress

Many studies show that salt stress triggers the induction of genes involved in proline biosynthesis, which leads to proline accumulation (Armengaud et al., 2004; Kim and Nam, 2013; Nguyen et al., 2013). According to Székely et al. (2008), knocking out the function of P5CS in *A. thaliana* indicates a key role for this enzyme in plant salt tolerance because the *p5cs1* plants are hypersensitive to salt. Exogenous application of proline can effectively improve tolerance of plants to salt stress through the regulation of endogenous proline metabolism, partly achieved through differential expression of specific proline-related genes. For example, de Freitas et al. (2018) demonstrated that foliar application of proline to *Z. mays* resulted in a decrease in P5CS activity and an increase in PDH under salt stress. Similar results in salt stressed *Sorghum bicolor* were reported more recently (de Freitas et al., 2019). Adding exogenous proline led to a decrease in P5CS activity in both stressed and unstressed *Eurya emarginata*, but to an increase in PDH activity only in unstressed plants (Zheng et al., 2015). Under salt stress, *Triticum aestivum* seed priming with exogenous proline significantly decreased the content of proline and P5C with a reduction in the activity of P5CS, while PDH activity was significantly increased (Rady et al., 2019). The effect of exogenous proline on *PDH* expression was also reported by Kiyosue et al. (1996) and Nakashima et al. (1998) in *A. thaliana*. Deuschle et al. (2001) reported that, in addition to PDH, exogenous proline increased *P5CDH* transcript levels, and suggested that this response may protect plants against proline toxicity. However, other authors like Nounjan et al. (2012) have shown that applying exogenous proline significantly increased expression of *P5CS* and *P5CR* in salt-stressed *Oryza sativa*.

Effect of Proline Treatment on Seed Germination Under Salt Stress

Seed germination is one of the most critical stages in the plant life cycle (Hubbard et al., 2012) because it is very sensitive to abiotic stress. In particular, salt stress causes osmotic stress that limits seed water absorption and ion toxicity due to the high accumulation of Na^+ and Cl^- (Murillo-Amador et al., 2002; Farissi et al., 2011). In recent years, there have been numerous papers about the effect of exogenous compounds like hormones, mineral elements, and amino acids in alleviating salinity stress during seed germination (Atia et al., 2009; Dallali et al., 2012; Rizwan et al., 2015; Coskun et al., 2016). However, the effect of exogenous proline on seed germination under salt stress is poorly understood as only a few studies have been published. Deivanai et al. (2011) demonstrated that exogenous proline had a positive concentration-dependent effect on seed germination under salt stress. Application of 1 mM proline alleviates the negative effect of 400 mM NaCl, but 100 mM proline did not have a significant effect. Similarly, 50 mM proline treatment improved seed germination of two cultivars of *S. bicolor* under salt conditions (Nawaz et al., 2010). Therefore exogenous proline application at suitable concentrations may alleviate the negative effect of salt stress by regulating cellular osmotic balance, but detailed studies behind these data are still needed to better understand the molecular mechanisms involved.

Effects of Proline Treatment on Plant Growth and Biomass Under Salt Stress

It is well documented that certain concentrations of exogenous proline regulate different aspects of plant growth and development under salt stress including rises in biomass and productivity (Huang et al., 2009; Nawaz et al., 2010; Nounjan et al., 2012; Wu et al., 2017). Addition of exogenous proline improved the growth of calli from two *Medicago sativa* cultivars upon salt stress, but dry weight and proline contents between the two were different with a better salt tolerance correlated with higher proline accumulation (Ehsanpour and Fatahian, 2003). Khan et al. (2014) tested the effects of 30 and 60 mM proline applied as a foliar spray to *Helianthus annuus*, concentrations that induced tolerance to 60 and 120 mM NaCl. They found that exogenous proline mitigates the salt stress effects on plant growth as proven by longer shoots and roots, and greater fresh and dry weights of shoots and roots, and this positive effect was more pronounced at the lower proline concentration (30 mM). Similarly, Wani et al. (2016) reported that a foliar spray of 20 mM proline alleviates the negative effects of salt stress on *Brassica juncea* by increasing lengths and fresh and dry masses of both shoots and roots, and the area of leaves. In addition, exogenous proline supply significantly increased plant height and number of roots in salt stressed *O. sativa* (Teh et al., 2016). Likewise, application of proline increased dry mass of leaves and roots and their soluble protein contents in salt stressed *Z. mays* (de Freitas et al., 2018). In some cases, exogenous proline stimulates yield under salt stress. Exogenous proline increased fresh and dry biomasses, grain yield and 1000-grain weight of salt-stressed *T. aestivum* (Rady et al., 2019). In salt-stressed *Z.*

mays, foliar-applied proline increased the number of seeds per plant, total grain weight and the 100-grain weight (Alam et al., 2016). In general, exogenous application of proline increased plant growth and productivity under salt-induced stress but the underlying mechanisms, probably linked to some hormonal regulation, still remain elusive.

Exogenous Proline Alters Stress-Responsive Gene Expression Under Salt Stress

Evidence for the mechanisms by which exogenous proline improves plant salt tolerance is still scarce. In order to gain some insight into such mechanisms at the gene level, Nounjan et al. (2012) studied the effect of exogenous proline on the expression of proline metabolism-related genes *P5CS* and *P5CR* as well as genes encoding antioxidant enzymes, superoxide dismutases (*Cu/ZnSOD, MnSOD*), ascorbate peroxidase (*CytAPX*), and catalase (*CatC*), in salt-stressed *O. sativa* seedlings. Results showed that after six days of salt treatment, exogenous proline upregulated *P5CS* and *P5CR* transcript levels. Likewise, the genes encoding antioxidant-related enzymes were upregulated by exogenous proline added to the salt-stressed rice plants. In a different study on salt-stressed *N. tabacum*, exogenous proline was found to increase transcript levels of genes encoding SOD, cationic peroxidase (POX) and CAT (Hoque et al., 2008). To understand more about the mechanistic role of gene regulation in exerting the effect of exogenous proline as plant salt tolerance, additional genetic experiments are required to particularly investigate the expression of genes related to the transport and translocation of Na^+ and Cl^-. More needs to be known about the relationship between the addition of proline and the expression of aquaporin-related genes under salt stress.

Exogenous Proline Influences Plant–Water Relations Under Salt Stress

Much research has documented how exogenous proline substantially alleviates salt stress by increasing leaf water potential, water content and restoring water use efficiency (**Table 1**). In *Brassica juncea*, Wani et al. (2016) noted that the leaf water potential was reduced under salt stress, but 20 mM proline applied as a foliar spray completely reversed the loss in water potential. Similarly, Huang et al. (2009) demonstrated that, under saline conditions, exogenous proline could alleviate the growth inhibition of salt-sensitive *Cucumis sativus*, and this was accompanied with leaves having higher water content. Studying salt-stressed *O. europaea* plants, Ben Ahmed et al. (2011) found that the relative water content is 1.05 and 1.09-fold higher under 25 and 50 mM of exogenous proline, respectively, than in the absence of proline. In the same way, 20 mM exogenous proline significantly alleviated the negative effects of 200 mM NaCl and raised the leaf water content in *Eurya emarginata* (Zheng et al., 2015). The role of exogenous proline in maintaining higher plant water content under salinity was also reported in *Onobrychis viciifolia* (Wu et al., 2017) and *S. bicolor* (de Freitas et al., 2019).

Many authors have suggested that the increase in water content and water potential of leaves in response to exogenous proline under salt stress could be because the proline triggers the accumulation of some organic and inorganic compounds such as proline, glycine betaine, soluble sugars and K^+ that help plants adjust their cellular osmotic potential and hence maintain higher water content (Ben Ahmed et al., 2011; Nounjan et al., 2012; Khan et al., 2014; Zheng et al., 2015). Another possibility is that maintaining a favorable water content under osmotic stress may be attributed to the regulation of the expression of root aquaporin genes in response to exogenous proline. These possible mechanisms for mediating osmotic stress tolerance and improving plant water content need to be studied in more detail at the molecular level.

Exogenous Proline Balances Mineral Nutrient Uptake and Assimilation Under Salt Stress

Salinity not only increases Na^+ and Cl^- in plants but also induces decreases in Ca^{2+}, K^+, Mg^{2+}, NO_3^-, S, and other essential nutrients leading to overall nutrient deficiency (Manchanda and Garg, 2008; Farissi et al., 2014). The positive effects of exogenous proline on plant tolerance to salt stress have been linked to increased assimilation of nutrients in many studies. Abdelhamid et al. (2013) reported that exogenous proline application increased P, K, NO_3^- and NO_2^- contents in *Phaseolus vulgaris* under different levels of salinity (three fields with electrical conductivities of 1.84, 6.03, or 8.97 dS m^{-1}). Similarly, exogenous proline increased leaf N, Ca^{2+} and K^+ contents in *Cucumis melo* exposed to stress from 150 mM salt (Kaya et al., 2007). Also under salty conditions, exogenous proline increased Ca^{2+} and K^+ in *S. bicolor* (de Freitas et al., 2019) and *O. europaea* (Ben Ahmed et al., 2011). Alam et al. (2016) suggested that exogenous proline may increase the uptake of N, P, K^+ and S in *Z. mays* under salinity. As well as nutrient uptake, the activities of some enzymes involved in nutrient assimilation are triggered by exogenous proline under salty conditions. Nitrate reductase is one of the most important enzymes involved in nitrogen assimilation and exogenous proline stimulates its activity in *H. annuus* (Khan et al., 2014) and *C. melo* (Yan et al., 2011) exposed to salt stress. Recently, Teh et al. (2016) reported that exogenous proline alleviated the negative effects of salt stress and enhanced nitrate reductase and Glu synthase activities in *O. sativa*. Some authors have suggested that proline may provide a good way to store and recycle nitrogen under stress conditions (Heuer, 2010; Szabados and Savouré, 2010; Verslues and Sharma, 2010; Ben Rejeb et al., 2014; Mansour and Ali, 2017). Consistent with this line of reasoning is evidence that PDH is stimulated in *P. vulgaris* under nitrogen deficiency suggesting that proline may be used as a nitrogen source for growth (Hayat et al., 2012). Similarly, exogenous proline was also used as a source of nitrogen by *Vigna radiata* L. seedlings under stress conditions (Posmyk and Janas, 2007).

The above studies provide preliminary evidence that exogenous proline alleviates the negative effects of salt by

TABLE 1 | Effects of exogenous proline on seed germination, plant growth, photosynthesis, nutrient acquisition, water uptake, ionic toxicity, proline metabolism, gene expression, antioxidant activities, and biological nitrogen fixation in different plant species under salt stress.

Plant name	Salt concentration	Exogenous proline concentration	Application method	Variable	Effect of exogenous proline		
					Without stress	With stress	References
Cucumis melo	150 mM	10 mM	Foliar spray	Growth		+	Kaya et al. (2007)
				Chlorophyll content		+	
				Electrolyte leakage		+	
				Proline content	Not shown	+	
				Relative water content		+	
				Stomatal density		+	
				Nutrient acquisition and Na/K ratio		+	
Cucumis sativus	100 mM	10 mM	Foliar spray	Growth	–	+	Huang et al. (2009)
				Proline content	+	+	
				Relative water content	–	+	
				MDA	+	+	
				Antioxidant enzyme activities	+	+	
				Na^+, Cl^- and K^+ content	–	–	
Eurya emarginata	200 mM	10 mM	Nutrient solution	Growth	–	–	Zheng et al. (2015)
				MDA	–	+	
				Na^+/K^+ ratio	–	+	
				Antioxidant enzyme activities	–	+	
				P5CS activity	–	+	
				PDH activity	+	–	
Glycine	15 mM	25 mM	Not shown	Number of nodules	–	+	Sabagh et al. (2017)
				Biological nitrogen fixation	–	+	
				Nitrogenase activity	+	+	
Helianthus annus	60 mM 120 mM	30 mM 60 mM	Foliar spray	Growth	+	+	Khan et al. (2014)
				Chlorophyll content	+	+	
				Na+ and K+ content	+	+	
				Nitrate reductase activity	-	+	
				Protein content	+	+	
				Total amino acids	+	+	
				Total sugars	–	-	
Mung bean	300 mM	15 mM	Nutrient solution	Glutathione	Not shown		Hossain and Fujita (2010)
				Antioxidant enzyme activities			
				MDA and H2O2			
Nicotiana tabacum	200 mM	20 mM	Medium solution	Non enzymatic antioxidant activities	Not shown	+	Hoque et al. (2008)
				Antioxidant enzyme activities		+	
				Carbonyl content		+	
Olea europaea	100 mM 200 mM	25 mM 50 mM	Nutrient solution	Relative water content and leaf water potential	Not shown	+ +	Ben Ahmed et al. (2011)
				Gas exchange		+	
				Photosynthetic pigment		+	
				Compatible solute		-	
				Mineral ion contents		+	
				Na^+/K^+ and $Na^+/Ca2^+$ ratio			
Onobrychi sviciaefolia	25 mM 100 mM	2.5 mM	Nutrient solution	Growth	-	+	Wu et al. (2017)
				Water content	-	+	
				MDA	-	-	
				Na^+/K^+ ratio	-	+	
				Proline content	+	+	
Oryza sativa	100 mM 200 mM 300 mM 400 mM	1 mM 5 mM 10 mM	Seed pretreatment	Seed germination	-	+	Deivanai et al. (2011)
				Growth	-	+	
				Chlorophyll content	+	+	
				Proline content	+	-	
				Protein content	+	+	
	100 mM	10 mM	Nutrient solution	Growth	-	-	Nounjan et al. (2012)
				Na^+/K^+ ratio	+	+	
				Proline content	+	+	
				H2O2 content	+	+	
				Antioxidant enzyme activities	+	+	
				P5CS gene expression	+	+	

(Continued)

TABLE 1 | Continued

Plant name	Salt concentration	Exogenous proline concentration	Application method	Variable	Effect of exogenous proline		
					Without stress	With stress	References
				P5CR gene expression	+	+	
				Antioxidant enzyme gene expression	+	+	
	150 mM	5 mM 10 mM	Growth medium	Growth	+	+	Teh et al. (2016)
				Nitrogen-metabolism enzyme activities	-	+	
				Nitrogen content	-	+	
Pisum sativum	100 mM	60 mM	Foliar spray	Growth	+	+	Shahid et al. (2014)
				Gas exchange	+	+	
				Chlorophyll content	+	+	
				Relative water content	+	+	
				Compatible solute	+	+	
				H2O2, MDA and electrolyte leakage	-	+	
Sorghum bicolor	75 mM	30 mM	Foliar spray	Growth	-	+	de Freitas et al. (2019)
				Membrane damage	-	+	
				Relative water content	-	+	
				Gas exchange	-	+	
				Nutrient uptake	-	+	
				K^+/Na^+ ratio	-	+	
				Amino acids	-	+	
				Proline content	+	+	
				P5CS activity	-	+	
				OAT activity	-	-	
				ProDH activity	+	+	
				P5CS gene expression	-	+	
				OAT gene expression	+	+	
				ProDH gene expression	+	+	
Triticum durum	120 mM	12 mM	Seed pretreatment	Growth	-	+	Rady et al. (2019)
				Photosynthetic activities	-	+	
				K^+/Na^+ ratio	-	+	
				Proline content	-	+	
				Proline metabolism enzyme activities	-	+	
				MDA and H2O2 content	-	+	
				Antioxidant enzyme activities	-	+	
				Non-enzymatic antioxidant activities	-	+	
Zea mays	25 mM 50 mM	25 mM 50 mM 100 mM	Foliar spray	Growth	Not shown	+	Alam et al. (2016)
				Grain yield		+	
				Chlorophyll		+	
				Nutrient uptake		+	
				K^+/Na^+ ratio		+	
	80 mM	30 mM	Foliar spray	Growth	-	+	de Freitas et al. (2018)
				Ion content	-	-	
				K^+/Na^+ ratio	-	-	
				Proline content	+	+	
				P5CS activity	-	+	
				ProDH activity	+	+	
				Antioxidant enzyme activities	-	+	
				Non enzymatic antioxidant activities	-	-	
				MDA and H2O2 content	-	+	

+ and - indicate positive and negative effects, respectively.

improving uptake of some nutrients as well as stimulating the activity of some enzymes involved in nutrient assimilation. However, research into the effect of exogenous proline on the translocation of micronutrients is limited.

Proline Treatment Mediates Reduction in Ion Toxicity Due to Salt Stress

High salt concentrations increase Na^+ and Cl^- contents in plants and decrease the abundance of other cations such as K^+ and Ca^{2+}, which leads to mineral nutrient imbalance (Zhu and Gong, 2014). Indeed, under salty conditions, sustaining ion homeostasis is one of the adaptive strategies that tolerant plants use to cope with salt stress. These strategies may help the plant to prevent potentially toxic effects of the build-up of ions like Na^+ and Cl^- that cause various types of damage to lipids, proteins and nucleic acids (Zhu and Gong, 2014; Bargaz et al., 2015; Rizwan et al., 2015). Application of 5 mM proline in a foliar spray decreased Na^+ content and increased K^+/Na^+ ratio in *P. vulgaris*

(Abdelhamid et al., 2013). More recently, de Freitas et al. (2018) reported that external application of proline decreased both Na^+ and Cl^- contents, but increased the K^+ content and the K^+/Na^+ ratio in salt-stressed *Z. mays*. Similar results have been reported in *S. bicolor* (de Freitas et al., 2019). Khan et al. (2014) demonstrated that exogenous proline alleviated the negative effect of 120 mM salt, and enhanced K^+ content, and reduced Na^+ concentration in *H. annuus*. In salt-stressed *O. europaea*, exogenous proline improved salt tolerance through maintaining a low Na^+ content, a high K^+ content and lowered Na^+/K^+ and Na^+/Ca^{2+} ratios in both young and old leaves (Ben Ahmed et al., 2011). Compared to salt-stressed plants, exogenous proline application increased the K^+/Na^+ ratio in *O. sativa* under 100 mM NaCl (Sobahan et al., 2012) and in *Z. mays* under 50 mM NaCl (Alam et al., 2016). Recently, Wu et al. (2017) reported that 2.5 mM exogenous proline decreased the Na^+/K^+ ratio in *Onobrychis viciifolia* Scop under 100 mM NaCl.

Removing Na^+ from the cytosol and compartmentalizing it in the vacuole are important strategies to maintain a low Na^+ concentration (Bargaz et al., 2015). Transgenic *Saccharum officinarum* overexpressing the *P5CS1* gene had a low Na^+ content compared to wild type (Guerzoni et al., 2014). Ben Ahmed et al. (2011) had previously suggested that the lower accumulation of Na^+ in proline-treated *O. europaea* under salt stress may be due to the effect of exogenous proline on the ability of root to exclude the salt ions Na^+ and Cl^- from the xylem to the shoot. The activity of some transporters, like a plasma membrane Na^+/H^+ antiporter encoded by the *SALT overly sensitive* (*SOS*) gene, facilitates the export of Na^+ from the cytosol to the leaves, protecting the plant from its toxicity (Zhu, 2003; Bargaz et al., 2015). Proline does not always act in this way to induce salt tolerance. Indeed, in *C. sativus*, exogenous proline has no significant effect on Na^+ and K^+ concentrations in leaves but improves leaf water content under 100 mM NaCl (Huang et al., 2009). This higher water content due to the exogenous application of proline may dilute the salt and therefore limit salt toxicity leading to better plant growth. This was confirmed by Ben Ahmed et al. (2011) who reported that the large reduction in Na^+ accumulation in leaves and roots in response to exogenous proline application was due to its interference in osmotic adjustment and/or its dilution. Additional studies on the effect of exogenous proline on membrane transporters, such as Na^+/H^+ antiporters and K^+/H^+ symporters, are needed to investigate the mechanism by which exogenous proline reduces salt ion toxicity.

Exogenous Proline Improves Photosynthesis Under Salt Stress

Abiotic stresses, including salt stress, cause stomata to close and chlorophyll synthesis to slow down (Hayat et al., 2012), while activating chlorophyllase activities (Jamil et al., 2007), damaging chloroplast structure and destabilizing pigment protein complexes (Singh and Dubey, 1995). These effects lead to a reduction in photosynthesis and, as a result, plant growth inhibition (Farissi et al., 2018). The beneficial effect of exogenous proline on plant growth under salt stress has often been associated with a change in photosynthesis parameters (**Table 1**) (Hayat et al., 2012; Mansour and Ali, 2017). Ben Ahmed et al. (2011) found that proline supplements to two-year-old *O. europaea* exposed to 100 or 200 mM NaCl resulted in higher levels of net photosynthesis, chlorophyll *a* and *b* and carotenoid contents as compared to salt-stressed plants without supplements. In a similar study, Wani et al. (2016) reported that exogenous proline increased various photosynthetic attributes including net photosynthesis, leaf area, stomatal conductance, intercellular CO_2, transpiration rate, and quantum efficiency of photosystem II (Fv/Fm) in two salt-stressed *B. juncea* cultivars. Similar results were obtained in *Solanum melongena* (Shahbaz et al., 2013) and in *Pisum sativum* (Shahid et al., 2014). Nawaz et al. (2010) also reported a positive effect of exogenous proline on chlorophyll *a* and total chlorophyll contents in salt-stressed *S. bicolor*. However there was no equivalent significant difference in chlorophyll b content under 50 and 100 mM of NaCl. These findings strongly suggest that exogenous proline influences plant growth under salt stress by enhancing photosynthetic processes.

Exogenous Proline Application Reduces Oxidative Stress in Salt-Stressed Plants

ROS are continuously generated in stressed plants due to the incomplete reduction of oxygen. Some of them can play a role as second messengers to trigger tolerance to abiotic stresses (Ben Rejeb et al., 2014). Proline has been considered to be a molecular chaperone due to its capacity to scavenge ROS, to stabilize protein and other macromolecular complexes, and to provide cellular redox potential (Szabados and Savouré, 2010; Ben Rejeb et al., 2014). Furthermore, under salt stress, exogenous proline increases enzymatic and non-enzymatic antioxidant activities, which improves plant tolerance. Indeed, Hossain and Fujita (2010) reported that exogenous application of 15 mM proline to the growth medium of mung bean exposed to 300 mM NaCl significantly decreased malondialdehyde (MDA) and H_2O_2 contents, and this decrease correlated significantly with an increase in glutathione content and glutathione peroxidase, glutathione-S-transferase and glutathione reductase activities. In the previously cited study by Wani et al. (2016), 20 mM proline sprayed on two *B. juncea* cultivars growing under three different concentrations of salt (2.8, 4.2, and 5.6 $dS.m^{-1}$), reduced electrolyte leakage and increased the activities of some antioxidant enzymes like CAT, SOD and POX. At the same time, proline itself can contribute to ROS scavenging and hence to plant salt tolerance, including when it is supplied exogenously (Ben Rejeb et al., 2014). However, Nounjan et al. (2012) showed that exogenous application of 10 mM proline to salt stressed *O. sativa* seedlings decreased the activity of SOD, POX, and CAT and increased H_2O_2 content. In agreement with those results, Huang et al. (2009) reported that foliar spray of proline lowered the MDA content and the SOD activity in a salt-sensitive *C. sativus* cultivar under 100 mM NaCl. An increase in POX activity in response to exogenous proline was also measured in the salt-stressed cucumber. Mansour and Ali (2017) suggested that the decrease in antioxidant activities under salt stress in response to exogenous proline may be involved in the improvement

of salt tolerance through ROS signaling. Species-specific differences may explain these contradictory results on proline effects.

Symbiotic Nitrogen Fixation Is Enhanced by Proline Treatment Under Salt Stress Conditions

Soil inorganic nitrogen deficiency is one of the most limiting factors for plant growth. However, the biological reduction of atmospheric nitrogen to ammonium by rhizobia-legume symbiosis can provide enough nitrogen to maximize growth and yield (Zahran, 1999; Kraiser et al., 2011). Encouraging rhizobia–legume symbiosis is a sustainable approach to increasing crop production, while decreasing dependency on chemical nitrogen fertilizer in traditional agriculture, which causes widespread environmental pollution (Ferguson et al., 2019). Salt stress limits the distribution, survival, and infectivity of rhizobia by decreasing the number and the biomass of nodules, and diminishing leghemoglobin synthesis and nodule respiration leading to a decrease in nitrogenase activity and nitrogen fixation rate (Zahran, 1999; Faghire et al., 2011; Monica et al., 2013). Improving BNF under salt stress is considered to be a major goal for crop scientists. Several strategies have been adopted to improve BNF under high-salt conditions including the selection of the most tolerant rhizobium–legume combinations, use of arbuscular mycorrhizal fungi, improvement of agricultural practice, genetic breeding and plant genetic modification, seed priming and exogenous application of compounds like hormones and osmoprotectants (Faghire et al., 2011; Faghire et al., 2013; Sabagh et al., 2017; Farooq et al., 2017).

Although positive correlations between endogenous proline and BNF under salt stress have been reported in many studies (Tejera et al., 2005; Verdoy et al., 2006; Fahmi et al., 2011; Kim and Nam, 2013; Bargaz et al., 2015), very few studies have focused on the effect of exogenous proline. Sabagh et al. (2017) studied salt-stressed *Glycine max* induced by 15 mM NaCl, and supplied 25 mM proline in the growing medium. The result was an increase in nodule number and biomass. Furthermore, the loss in nitrogenase activity caused by salinity was overcome when proline was applied (Sabagh et al., 2017). Similar results were observed in *Cicer arietinum* growing under conditions of cadmium toxicity, where 20 mM exogenous proline alleviated the negative effect of cadmium (25 mg/kg) and increased the number of nodules, the leghemoglobin content and the nitrogenase activity (Alyemeni et al., 2016). Moreover, the positive effect of exogenous proline on nitrogenase activity under salt stress has been reported not only in plants but also in some bacterial strains like *Klebsiella pneumonia* (Le Rudulier et al., 1982). Investigating the relationship between proline metabolism and BNF, Ren et al. (2018) demonstrated that overexpression of *StP5CS* enhanced the relative expression of two nodulation-related genes and one leghemoglobin gene. This was reflected by an increase in nodulation and nitrogen fixation under salt stress. Furthermore, overexpression of *P5CS* from *Vigna aconitifolia* in *M. truncatula* enhanced tolerance to salt stress and improved nitrogenase activity (Verdoy et al., 2006). In addition, Kim and Nam (2013) demonstrated that *P5CS3* regulated *M. truncatula* nodule number under salt stress. The above studies show that exogenous proline may improve BNF under salt stress, but the detailed mechanisms behind this relationship are still not clear as well as its relevance to field conditions.

Proline Toxicity in Salt-Stressed Plants

Despite the protective roles of exogenous proline on salt-stressed plants, several papers reported that its positive effect is concentration-dependent, high concentration could cause a toxic effect in plants (Hellmann et al., 2000; Maggio et al., 2002). For example, while low concentrations (20–33 mM) alleviated the deleterious effect of salt stress, external supplementation of high proline concentration (50 and 100 mM) was found to be toxic for both salt-stressed and unstressed callus culture of mung bean (Kumar and Sharma, 1989). In agreement with that, Rodriguez and Heyser (1988) demonstrated that 10 mM of exogenous proline seriously inhibited the normal growth of Distichlis suspension cultures under 260 mM of salt stress. Similarly, in salt stressed *Oryza sativa*, while low concentrations (20–30 mM) of proline were effective in mitigating the adverse effect of 100 mM NaCl on growth, higher concentrations (40 to 50 mM) resulted in growth reduction (Roy et al., 1993). In addition, in contrast to 1 mM, the external supplementation of 10 mM of proline to salt stressed *Solanum lycopersicum* decreased leaf and root fresh weights, even leading to plant death if proline is added in high concentration (Heuer, 2003). Furthermore, Rajendrakumar et al. (1997) showed that proline at high concentration could destabilize the DNA helix, lower the DNA melting point, increase susceptibility to S1 nuclease and insensitivity to DNAase1. Interestingly *p5cdh* and *prodh* mutants were shown to be more sensitive to proline treatments (Mani et al., 2002; Nanjo et al., 2003; Deuschle et al., 2004; Cabassa-Hourton et al., 2016), indicating the importance of proline catabolism in the regulation proline level for plants. However, the underlying mechanism of proline toxicity remains elusive.

CONCLUSIONS AND PROSPECTS

Exogenous proline application can improve salt tolerance by regulating physiological, biochemical and enzymatic processes and have a positive effect on plant growth, development and productivity under salt stress conditions. To focus on where potential solutions will be found in future crop research, the proposed beneficial effects of exogenous proline on salt stress tolerance in developing plants are summarized in **Figure 6**.

Exogenous proline reduces Na^+ and Cl^- content and increases K^+/Na^+ ratio in many plant species (**Table 1**) (*e.g.* Abdelhamid et al., 2013; de Freitas et al., 2018; de Freitas et al., 2019). Na^+/H^+ is an antiporter plasma membrane transporter, encoded by an

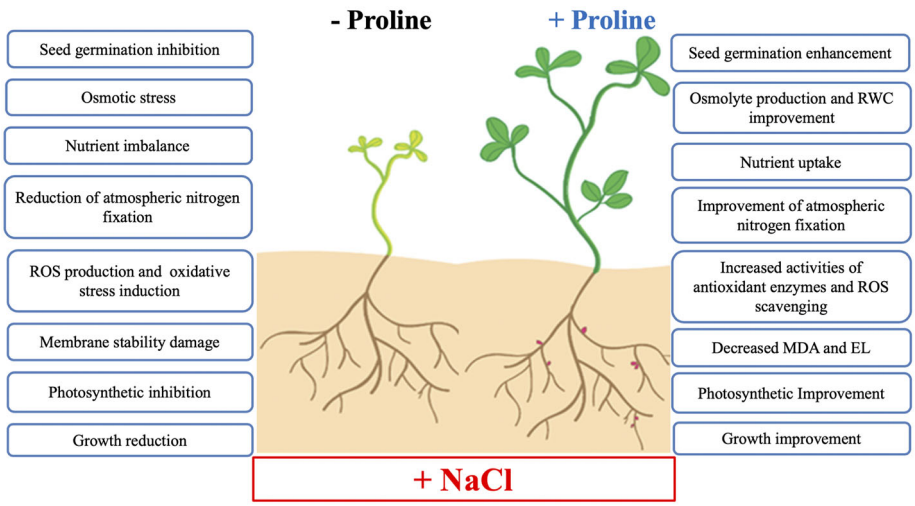

FIGURE 6 | Summary of the main effects of exogenous proline in plant salt tolerance. Abbreviations: RWC, relative water content; ROS, reactive oxygen species; MDA, malondialdehyde; EL, electrolyte leakage.

SOS1 gene, that pumps Na^+ from root cells to leaves, boosting salt stress tolerance (Zhu and Gong, 2014; Bargaz et al., 2015). High-affinity K^+ transporter (HKT) is another transporter that mediates salt tolerance in various plant species through regulation of the transport of salt ions from root to shoot (Kaundal et al., 2019; Thouin et al., 2019). In view of the important roles of these two transporters in plant salt tolerance, it would be interesting to investigate how exogenous proline can regulate the *SOS1* and *HKT* gene expression under salt stress and their relationship with salt tolerance.

Water restriction is one of the main effects of salt stress in plants (Farissi et al., 2014). Exogenous proline was widely reported to increase plant water content under salt stress (**Table 1**), and this may contribute to salt dilution and as a result plant growth improvement (Huang et al., 2009; Zheng et al., 2015). Aquaporins are a group of transporters that facilitate absorption of water by plant from soil. Under salt stress, there is a positive correlation between the expression of aquaporin genes and salt tolerance of *Eutrema salsugineum* (Qin et al., 2019). To better understand the mechanism by which exogenous proline improves plant water relations under salt stress, the effect of this osmoprotectant on the expression of aquaporin genes under salt stress will be interesting to investigate.

BNF is an important process that improves soil fertility but it is very sensitive to salt stress from the establishment of the symbiosis to nitrogen fixation (Zahran, 1999; Monica et al., 2013). The ability of exogenous proline to improve nitrogen acquisition under salt conditions was reported in several species (Kaya et al., 2007; Abdelhamid et al., 2013; Alam et al., 2016). The beneficial effect of this molecule in nitrogen nutrition of legumes through nitrogenase activity, however, is poorly understood and very few studies have been done. It will be important to focus on the effect of exogenous proline on nitrogenase gene expression under salt stress to better understand the effect of this multifunctional amino acid on BNF.

The effect of exogenous proline in alleviating the negative impact of salt stress appears to be both dose- and species-dependent. It is still not clear how proline works in reducing the detrimental effect of salt stress and further research is needed. Omics approaches can provide a more holistic molecular perspective of biological systems compared to traditional approaches. Transcriptome analysis has been widely applied to explore genes that are differentially expressed in response to abiotic stresses. These data are essential to identify and potentially manipulate genes that impact stress tolerance under diverse environmental conditions. Increasing amounts of data suggest that proline has certain regulatory functions. Using transcript profiling, Oono et al. (2003) showed that proline can also trigger expression of one third of rehydration-inducible plant genes. Most of the known proline-responsive genes have the conserved PRE cis-acting element in their promoter regions, which is a target of specific bZIP-type transcriptional activators (Oono et al., 2003; Satoh et al., 2004; Weltmeier et al., 2006). From this starting point, the proline-related signaling pathway requires further elucidation using multiomics technologies that dissect the multiple corresponding genes or metabolites. Therefore, further large-scale analyses of transcript, protein and metabolite responses are required to understand how plants respond to proline and the adaptive value of proline in plant stress adaptation.

AUTHOR CONTRIBUTIONS

AEM proposed and wrote the review. CC-H and MF commented on the content of the review and revised the text. AS revised the text at different stages of the writing process and contributed to

the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapitre II (Article 4):

Effet du Si sur la viabilité des embryons, la mobilisation des réserves et la stabilité membranaires des graines en germination de *Medicago sativa* sous contrainte saline.

I Introduction

La germination des graines est l'un des stades critiques dans le cycle de vie des plantes (Hubbard et al. 2012). Cependant, ce stade est très sensible aux contraintes abiotiques en particulier la contrainte saline, qui induit à la fois une contrainte osmotique et une toxicité ionique (Debez et al. 2001; Ghoulam et Fares 2001). Des études récentes ont montré que le Si pourrait améliorer la germination des graines sous contrainte saline (Naguib et Abdalla 2019; Wang et al. 2010; Zhang et al. 2015).

II Objectifs

L'objectif de ce chapitre est de caractériser l'effet du Si ($CaSiO_3$) sur la germination des graines de deux variétés marocaines de *M. sativa*, *Oued LMalah* (*OL*) (variété tolérante) et *Demnate 201* (*Dm*) (variété sensible), et une variété européenne sensible *NS Mediana ZMS V* (*NS Med*), qui présentent des réponses contrastées sous contraintes salines. Nous visons à comprendre les mécanismes induits par le Si pour améliorer la germination des graines à travers la mobilisation des réserves de l'embryon et sa viabilité et la régulation du métabolisme oxydatif.

III Méthodologie

Les graines de deux variétés marocaines *OL* et *Dm* et une variété européenne *NS Med* ont été désinfectées et mises à germer dans des boites de Pétri contenant deux couches de papier filtre stérile à raison de 50 graines par boite. Les graines ont été par la suite imbibées avec 5 mL d'une solution de NaCl (0 ou 200 mM) en présence et en absence de 3 mM $CaSiO_3$. La germination a été suivie dans une étuve à l'obscurité et à 25 ± 1 °C pendant 8 jours avec un comptage journalier des graines germées. Des paramètres de germination, physiologiques et biochimiques, comprenant la mobilisation des réserves glucidiques et protéiques, la viabilité de l'embryon, la régulation de métabolisme oxydatif et l'équilibre nutritionnelle ont été étudiés. Des résultats préliminaires ont montré que 3 mM Si était la concentration la plus bénéfique pour améliorer la germination des graines de luzerne. Par conséquent, le présent chapitre analyse l'effet de 3 mM de Si sur la germination des graines de *M. sativa* exposées au stress salin.

IV Résultats

L'exposition des graines de *M. sativa* au stress salin a réduit de façon significative la germination des graines des trois variétés, en particulier la variété européenne *NS Med*. Ceci est dû à une réduction de la viabilité de l'embryon et sa capacité à mobiliser les réserves glucidiques et protéiques ainsi que l'induction du stress oxydant. L'ajout de 3 mM Si dans le milieu

améliore la viabilité de l'embryon, stimule la mobilisation des réserves, induit l'activité des enzymes antioxydantes entrainant une réduction du stress oxydant.

V Conclusion

Les résultats de ce travail ont montré que le Si réduit l'effet délétère de la salinité sur la germination des graines de *M. sativa* et améliore la viabilité de l'embryon, la mobilisation des réserves et la stabilité des membranes cellulaires.

Ces données font l'objet d'un article soumis.

Silicon improves seed germination and seedling growth and alleviates salt stress in *Medicago sativa* L. by regulating seed reserve mobilization and antioxidant system defense.

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Abstract

Exogenous application of silicon (Si) provides benefits to various plant species including alfalfa. However, knowledge about the effects of Si on seed germination in alfalfa under salt stress still scarce. Therefore, this study aimed at exploring the effect of Si in *Medicago sativa* L. seed germination and seedling growth under salinity. Seeds of two Moroccan alfalfa varieties, *Ouad Lmaleh* (*OL*) and *Demnate 201* (*Dm*), and the European variety *NS Mediana ZMS V* (*NS Med*) were germinated at 25 °C in Petri dishes containing sterilized filter paper moistened with 200 mM NaCl solution with or without 3 mM Si. Exogenous Si significantly improved seed germination by enhancing reserve mobilization and embryo viability. Si alleviated salt-mediated oxidative stress in alfalfa by reducing hydrogen peroxide (H_2O_2), electrolyte leakage and malondialdehyde contents and by increasing proline accumulation. Besides, histochemical staining of H_2O_2 revealed that Si reduces H_2O_2 accumulation induced by salt stress. Enzyme activity analyses revealed that Si triggered the activity of some critical enzymes related to oxidative stress alleviation, including superoxide dismutase (SOD) and catalase, together with an accumulation of SOD isoforms, as visualized by native gel PAGE. Furthermore, inductively coupled plasma-optical emission spectroscopy also showed a key role of Si in alleviating the devastating impact of salt by reducing Na^+ content and increasing K^+ content, leading to higher K^+/Na^+ ratio. Our findings suggest that 3 mM Si application can constitute a promising way to improve alfalfa seed germination and seedlings growth under salt stress.

Keywords: Silicon; *Medicago sativa;* Salinity; Germination; Embryo viability; Reserve mobilization.

I Introduction

As a perennial legume, alfalfa (*Medicago sativa* L.) is one of the most cultivated forage crop in the Mediterranean area (Bouizgaren 2007), thanks to its high ability to fix atmospheric nitrogen when associated with rhizobia, high protein content and highly digestible fibers (Li et al. 2010). In Morocco, *M. sativa* occupies about 25% of the total area of forage crops with 455 000 ha (Mouradi et al. 2018). Its pivoting root system allows water absorption by up to 5 m depth (Hamidi and Safarnejad 2010). However, germination and early seedling development are often frequently exposed to abiotic stresses such as salinity and drought stress (Vicente et al. 2020).

Seed germination, the first stage in plant life cycle, is a key process in plant development. Successful seed germination and seedling establishment are determining futures for the propagation of flowering plants, which reproduce by sexual breeding (Hubbard et al. 2012). However, several glycophyte plant species are sensitive to increasing salinity levels particularly during germination and seedling growth (Debez et al. 2001; Ghoulam and Fares 2001). Indeed, salt stress either delays or entirely prevents seed germination (Haghighi et al. 2012). Salinity can inhibit germination, seedling emergence, and seedling establishment by primary stresses osmotic stress and toxicity associated with excess Na^+ and Cl^- uptake (Arzani 2008; Munns and Tester 2008). Secondary stresses include nutrient imbalance and oxidative stress. The latter is induced by reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which disturb cellular metabolism due to the peroxidation of proteins, lipids and nucleic acids (Wang et al. 2010). Likewise, reports also showed that salt stress induced the modification of the structural organization of proteins, reduction of activities of hydrolytic enzymes leading to the lack of the mobilization of seed reserve (Farissi et al. 2011; Farooq et al. 2017; Ibrahim 2016; Promila and Kumar 2000). According to Song et al. (2005), the response of seeds to their environmental conditions strongly determined the ecological distribution of plants.

Salt-affected soils represent about 23% of the arable lands in the world, that is to say about 20% of the total irrigated lands (Assouline et al. 2015; Jouyban 2012). Therefore, the utilization of salt tolerant plants or the enhancement of seed germination and seedling growth by using alternative approaches may be of great interest to exploit salt-affected lands. Exogenously applied compounds to get fast and uniform germination under salt stress are of great interest. Hu et al. (2012) reported that the use of glucose as an exogenous compound has successfully improved *Triticum aestivum* seed germination under salt stress. However, the use of this technique under field conditions is limited and present high cost-to-benefit ratio. Furthermore,

the germination of *Crithmum maritimum* seeds was improved under salt stress by using nitrate as an exogenous compound (Atia et al. 2009). However, nitrate is documented to be also expensive and to have a harmful environmental impact (Farissi et al. 2014). Therefore, the use of silicon (Si), which is easily available and does not affect the environment, could be a sustainable approach to overcome the negative effects of salt stress on seed germination, seedlings growth and plant productivity.

Si is the second most abundant element after oxygen in the earth's crust. Although it is considered to be non-essential for plant growth and development, its application has been considered to be one of the most effective strategies in improving plant tolerance to different abiotic stresses including heavy metals, drought and salinity (Abu-muriefah 2015; Hameed et al. 2013; Shi et al. 2014; Wang et al. 2010). However, only little information is available on the mechanisms by which Si mediated stress tolerance during germination and early seedling growth in particular in legumes, which are considered as Si rejective (El Moukhtari et al. 2021; Zhang et al. 2017). Indeed, in *Lens culinaris*, Biju et al. (2017) demonstrated the beneficial effect of Si on seed germination under drought through improvement of hydrolytic and antioxidant enzyme activities and osmolyte accumulation. This effect was Si concentration dependent. Similarly, according to Zhang et al. (2015), the enhancement of *Glycyrrhiza uralensis* seed germination under salt stress is related to the ability of Si, at a suitable concentration, to improve antioxidant enzyme activities. Furthermore, Al-Huqail et al. (2019) recently reported that Si application decreased lipid peroxidation and increased the activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) in salt-stressed *Acacia gerrardii*. The effect of exogenous Si application on seed germination and seedlings growth in *M. sativa* under salt stress remains to be investigated. Therefore, the present study aims (i) to assess the effect of Si on *M. sativa* seed germination and seedling growth under salt conditions and (ii) to elucidate the role of exogenous Si in stimulating seed germination by evaluating seed reserve mobilization, embryo viability, the activity of key antioxidant enzymes, the degree of cell membrane integrity and nutrient homeostasis of *M. sativa* seedlings.

II Materials and methods

II-1 Plant materials

Seeds of two Moroccan *M. sativa* varieties, *Ouad Lmaleh* (*OL*) and *Demnate 201* (*Dm*) and of the European *NS Mediana ZMS V (NS Med*) were used as plant materials. *OL* and *Dm* are ones of the most cultivated varieties by Moroccan farmers in their traditional agroecosystems. These varieties display good adaptation to local habitats due to natural and human selection. In a preliminary experiment, the two varieties *OL* and *Dm* have been characterized as salt tolerant, whereas *NS Med* was salt sensitive according to seed germination test and seedlings growth (data not shown). Seeds were supplied by the National Institute of Agronomic Research (INRA-Marrakesh).

II-2 Si treatment and germination conditions

Calcium silicate ($CaSiO_3$; Merck) was used in this study as a source of Si to study the impact of Si on *M. sativa* salt tolerance during germination and early seedling growth. For each variety, three replicates of fifty homogenous seeds were considered for each treatment. Seeds were surface sterilized for 5 min in 6% sodium hypochlorite, rinsed thoroughly with sterilized distilled water and placed on two layers of sterilized filter paper in a Petri dish. Seeds were then soaked with 5 mL of Si with or without 200 mM NaCl. Likewise, seeds of each variety were soaked with 5 mL of sterilized distilled water or 200 mM NaCl and used either for control or stress conditions, respectively. Preliminary experiments showed that 3 mM of Si was the optimal concentration for alleviating the negative effect of salt stress on *M. sativa* seed germination and seedlings growth. Therefore, in the present work 3 mM of Si was used.

II-3 Germination parameters assessed

Germination was conducted for 8 days in the dark with a mean temperature of 25±1 °C. The number of germinated seeds with a radicle above 1 mm was recorded every 24h. Final germination percentage (FGP), velocity index (VI) and time to reach 50% of germination (T_{50}) were assessed as described previously (Farissi et al. 2011; Farooq et al. 2005; Khan and Ungar 1984).

At the end of the experiment, seedling fresh weight (SFW) and total seedling length (TSL) were determined. The means of five random seedlings per Petri dish were calculated and grouped in three replicates per treatment per variety.

II-4 Effect of Si treatment on seed reserve mobilization: soluble sugar and protein contents

Seeds were allocated to germinate in the dark under 200 mM NaCl with or without 3 mM of Si treatment. Representative samples (100 mg) of germinated and non-germinated seeds were harvested at various germination times (0h, 6h, 2d, 4d, and 6d after the beginning of the test) and used for sugars (Dubois et al. 1956) and proteins (Bradford 1976) analyses.

II-5 Effect of Si treatment on embryo viability

Embryo viability of the non-germinated seeds was assessed by cytochemical method using 2,3,5 triphenyltetrazolium chloride (TTC) (Merck) as described previously (Lamsaadi et al. 2022). TTC assay is based on the fact that viable embryos turn red due to the reduction of TTC by cell respiratory activity (Nachlas et al. 1960). Three replicates of 10 non-germinated seeds from each treatment were taken after 6h and 24h of imbibition and incubated for 24h in 10 mg mL^{-1} TTC in distilled water at 30 °C in the dark. Embryos were then isolated under a binocular magnifying glass with 10x magnifications and photographed.

II-6 Malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and proline contents and electrolyte leakage (EL) determination.

MDA content was determined according to Dhindsa et al. (1981). Fresh samples (100 mg) of 8-day old seedlings were homogenized in 1.5 mL of 0.1% thiobarbituric acid (TBA) followed by centrifugation for 10 min at 10 000 ×*g* at 4 °C. To 1 mL of the resulted supernatant, 1 mL of trichloroacetic acid (TCA) (20%) containing 0.5% TBA was added and the mixture was heated at 95 °C for 30 min. After cooling down, the absorbance was measured at 532 nm and 600 nm. The MDA concentration was estimated using its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as ηmol g^{-1} FW. Three replicates per treatment were performed.

H_2O_2 content in the 8-day old seedlings was determined as described previously by Velikova et al. (2000). 100 mg of fresh samples were homogenized with 5 mL of TCA (0.1%) in an ice bath. Then, the homogenate was centrifuged at 12 000 ×*g* for 10 min at 4 °C and to 0.5 mL of supernatant, 0.5 mL of potassium buffer (pH 7.0) and 1 mL of 1 M potassium iodide were added. The absorbance of the mixture was read at 390 nm. The H_2O_2 content was calculated by using a standard curve of known H_2O_2 concentrations and expressed as µmol H_2O_2 g^{-1} FW. The results are expressed as a mean of three replicates per treatment.

The histochemical staining of H_2O_2 was performed by cytochemical method using 3,3'diaminobenzidine (DAB) (Kumar et al. 2014). 8-day old seedlings from different treatments were immersed in DAB solution (1 mg mL^{-1}) prepared in water (pH 3.8) and shaken at 200 rpm for an overnight. For proper visualization of the stain, seedlings were immersed in absolute ethanol and heated for 10 min to remove the chlorophyll. Samples were then photographed using a digital camera.

According to Ghoulam et al. (2002), EL was determined in the 8-day old seedlings. 50 mg of fresh seedling were washed with dionized water, cut into small pieces and placed in a flask containing 10 mL of deionized water. The flasks were incubated under agitation (100 tour. min^{-1}) in a water bath for 24 hours at 25 °C. The initial electrical conductivity (EC_1) was measured by using a conductivity meter (DDS-12DW, Benchtop Conductivity Meter). The final electrical conductivity (EC_2) was determined after autoclaving samples for 20 min at 121 °C. For each variety, three replicates per treatment were considered. EL was measured by using the formula below:

$$\text{EL (\%)} = \frac{\text{EC1}}{\text{EC2}} \times 100$$

Free proline was determined in 8-day old seedlings as described by Bates et al. (1973). Around 30 mg of frozen seedlings were ground to powder in liquid nitrogen and resuspended in 1 mL of aqueous sulfosalicilic acid (3% w/v). Samples were centrifuged at 18 000 *xg* for 10 min at 4 °C and to 400 µL of the obtained supernatant, equal volume of ninhydrin (3%) and glacial acetic acid were added. The mixture was heated for 1 h at 100 °C then placed to cool down in an ice bath. Toluene (800 µL) was added to extract the pink phase. Proline content was determined by measuring the OD at 520 nm using NP80 Nanophotometer® (Implen) using calibration curves and expressed as µmol proline g^{-1} FW.

II-7 Effect of Si and salt treatments on the enzymatic antioxidant activities

For enzymatic antioxidant activities, fresh seedlings (0.5 g) were ground in liquid nitrogen and resuspended at 4 °C in 5 mL of phosphate buffer (50 mM, pH 7.5) containing 1% of PVP and 0.1 mM of EDTA. Homogenates were centrifuged at 12 500 ×*g* for 20 min at 4 °C and the supernatant was used for superoxide dismutase (SOD) and catalase (CAT) assay.

SOD (EC 1.15.1.1) activity was performed according to the method described previously by Beyer and Fridovich (1987). For this purpose, 30 µL of enzyme extract was added to 1 mL of 50 mM phosphate buffer (pH 7.8) containing nitroblue tetrazolium (NBT) (65 µM), L-methionine (13.3 µM) and riboflavin (1.33 µM). The photoreduction of NBT was done by

exposing the reaction mixture to a fluorescent lamp for 5 min, and the ability of the enzyme to inhibit the reduction of NBT was measured at 560 nm. One enzymatic unit (EU) of SOD was defined as the amount required for the inhibition of 50% NBT. The activity was expressed as EU min^{-1} mg^{-1} protein.

For CAT (EC.1.11.1.6) activity, the method of Hwang et al. (1999) was adopted. The reaction mixture was obtained in a final volume of 1 mL by mixing 50 mM potassium buffer (pH 7.0) containing 20 mM of H_2O_2 and 10 µL of protein extract. The decrease of H_2O_2 was followed spectrophotometrically using Jasco V-730 spectrophotometer at 240 nm and the activity of CAT was determined by using a value of 39.4 M^{-1} cm^{-1} for the extinction coefficient of H_2O_2 and expressed as ηmol H_2O_2 min^{-1} mg^{-1} protein.

II-8 In gel SOD activity assay

SOD isoformes in 8-day old *M. sativa* seedlings were visualized in 12% non-denaturating acrylamide gel following Eyidogan's procedure (Eyidogan and Öz 2007). Equal amount of protein (11.5 µg) from the different treatments were subjected to discontinuous PAGE under non-denaturing conditions. After 1h of migration, SOD activity was detected by determining its ability to inhibit the photochemical reduction of NBT as described previously (Beauchamp and Fridovich 1971). The gel was incubated with revelation buffer and exposed to light until it becomes uniformly blue except at positions containing superoxide dismutase.

II-9 Analysis of Si, K^+, Na^+ and Ca^{2+}

For Si, K^+, Na^+ and Ca^{2+} analyses, samples were prepared according to Lamsaadi et al. (2022) which was adopted from Liu et al. (2013). 8-day old *M. sativa* seedlings were incinerated at 600 °C for 6 h in a Protherm Furnaces (PLF 120/12). Ashes were recovered in a mixture of concentrated hydrochloric acid (HCl) and nitric oxide (HNO_3) and heated for one hour at 200 °C. The mixture was adjusted to 100 mL using deionized water and filtered. The resulting solution was used for nutrient analyses using inductively coupled plasma optical emission spectroscopy (Optima 8000 ICP-OES). Blank was prepared by applying the same procedure and reagent solutions without plant sample.

II-10 Effect of salt and Si on K^+/Na^+ ratio and the morphology of *M. sativa* seedlings surface area

This was done using the scanning electron microscopy (SEM)-JEOL JSM IT100 system. 8-day old *M. sativa* seedling samples were placed on aluminum stubs coated with conductive carbon

tape. The samples were then coated with 2 nanometer platinum layer and imaged. K^+/Na^+ ratio was calculated based on the weight %.

II-11 Data analysis

Data were analyzed using SPSS version 22. A three-way analysis of variance (ANOVA III) was conducted for the FGP, VI, T_{50}, TSL, SFW, MDA, H_2O_2, EL, proline, SOD, CAT, Si, Na^+, K^+ and Ca^{2+}. Data from the sugar and protein contents were statistically analyzed using a four-way analysis of variance (ANOVA IV), where varieties, salinity, silicon and time were the independent variables. Tukey's test was used to compare the means. Correlations among the measured parameters were done using Pearson's correlation coefficient.

III Results

III-1 Effect of salt and Si on *M. sativa* seed germination and seedlings growth

The influence of NaCl and Si on the final germination percentage (FGP), velocity index (VI) and time to 50% of germination (T_{50}) of *M. sativa* seed are shown in Table 1. FGP and VI were significantly reduced upon salt stress, especially in *NS Med* where they decreased by 55% and 72%, respectively. T_{50} also significantly increased in the three *M. sativa* varieties with the highest increment rate of 582% for *NS Med*. However, Si supply significantly improved seed germination under salt stress, as indicated by higher FGP and VI, and lower T_{50}. In the absence of salt stress, addition of 3 mM Si did not have any effect on all of the above-mentioned parameters. Under normal conditions, all three studied *M. sativa* varieties exhibited 100% FGP. Total seedling length (TSL) and seedling fresh weight (SFW) of *M. sativa* were considerably influenced with the supplementation of NaCl and Si to the medium (Table 1). Our results unveiled the fact that the addition of NaCl to the medium severally reduced both TSL and SFW in the three *M. sativa* varieties studied. Interestingly, the comparison among the three studied *M. sativa* varieties showed significant differences, with the *NS Med* variety being the most affected with reduction rates of 62% and 69% for TSL and SFW, respectively. However, this negative effect was relieved by Si treatment, with an effect more pronounced with the salt sensitive *NS Med* variety as compared with the other varieties. Indeed, TSL of Si-treated salt-stressed *NS Med* was increased by 105% compared to salt-stressed *NS Med* seedlings. Similarly, relative to salt-stressed seedlings, Si incorporation to the medium resulted in a higher fresh weight of *NS Med* seedlings by 80% under salt stress.

Table 1 Effect of exogenous silicon (Si) on the final germination percentage (FGP), velocity index (VI), time to reach 50% of germination (T_{50}), total seedlings length (TSL) and seedlings fresh weight (SFW) of three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under control (-NaCl) and stress (+ NaCl) conditions, in the absence (-Si) or presence (+Si) of externally added 3 mM Si. Values represent means of three replicates ± standard errors

Varieties	Salt stress	FGP (%)		VI		T_{50}		TSL (cm)		SFW (g)	
		-Si	+Si	-Si	+Si	-Si	+Si	-Si	+Si	-Si	+Si
OL	-NaCl	100.00±0.00	100.00±0.00	50.00±0.00	49.90±0.11	0.53±0.01	0.52±0.01	7.60±0.28	7.60±0.27	1.70±0.08	1.70±0.03
	+NaCl	92.00±0.66	100.00±0.00	30.00±1.00	45.50±0.60	3.06±0.16	1.70±0.04	4.20±0.04	5.07±0.50	1.05±0.07	1.28±0.06
Dm	-NaCl	100.00±0.00	100.00±0.00	46.60±0.20	46.40±0.90	0.85±0.05	0.77±0.05	6.05±0.12	6.50±0.35	1.76±0.06	1.67±0.01
	+NaCl	51.30±6.20	85.30±4.80	16.90±2.10	32.20±2.50	3.34±0.08	2.24±0.16	2.64±0.20	3.70±0.25	0.58±0.11	0.89±0.09
NS Med	-NaCl	100.00±0.00	100.00±0.00	49.80±0.20	49.75±0.16	0.51±0.00	0.51±0.01	7.30±0.32	8.44±0.29	1.40±0.04	1.56±0.08
	+NaCl	45.30±6.20	78.70±3.11	14.10±2.10	25.90±0.60	3.48±0.27	2.59±0.07	2.75±0.37	5.65±0.68	0.44±0.09	0.79±0.14

III-2 Seed reserve mobilization: soluble sugar and protein contents

The effect of salt stress and exogenous Si on soluble sugar and protein contents in germinating seeds of *M. sativa* is presented in Fig. 1. Under normal conditions, content of soluble sugars gradually decreased over time in the three tested *M. sativa* varieties. However, in the presence of 200 mM NaCl, soluble sugar content varies depending on the varieties. In *OL* and *Dm* varieties, soluble sugars decreased at two days of germination, no significant change was noted for *NS Med* until after the fourth day. However, the addition of Si under salt stress caused a gradual decrease in soluble sugar content in the three studied varieties. Under unstressed conditions, there was no significant difference between Si-treated and untreated seeds for the three tested *M. sativa* varieties. ANOVA IV showed that the interaction variety-salinity, variety-Si, variety-time, salinity-Si, salinity-time and Si-time were significant (Table S1).

Decrease in soluble protein content was observed at day 2 and thereafter lowers over time in control condition in all of the tested varieties (Fig. 1). Over the six days period, soluble protein content varies from 6.9 mg g^{-1} FW to 0.6 mg g^{-1} FW, from 6.6 mg g^{-1} FW to 0.4 mg g^{-1} FW and from 7 mg g^{-1} FW to 0.6 mg g^{-1} FW for *OL*, *Dm* and *NS Med,* respectively. However, under stress, soluble protein content remained unchanged during the first two days of the experiment. After two days soluble protein content decreased. In the *OL* variety, the lowest value was noted on day 6 (1.9 mg g^{-1} FW). However, for *NS Med*, seed soluble protein content decreased only slightly from 6.6 mg g^{-1} FW on day 2 to 5.2 mg g^{-1} FW on day 4 and remained unchanged afterwards. Under salt stress, Si application favored soluble protein decrease, especially after the fourth day of the experiment, with the lowest content found at the sixth days in all tested *M. sativa* varieties.

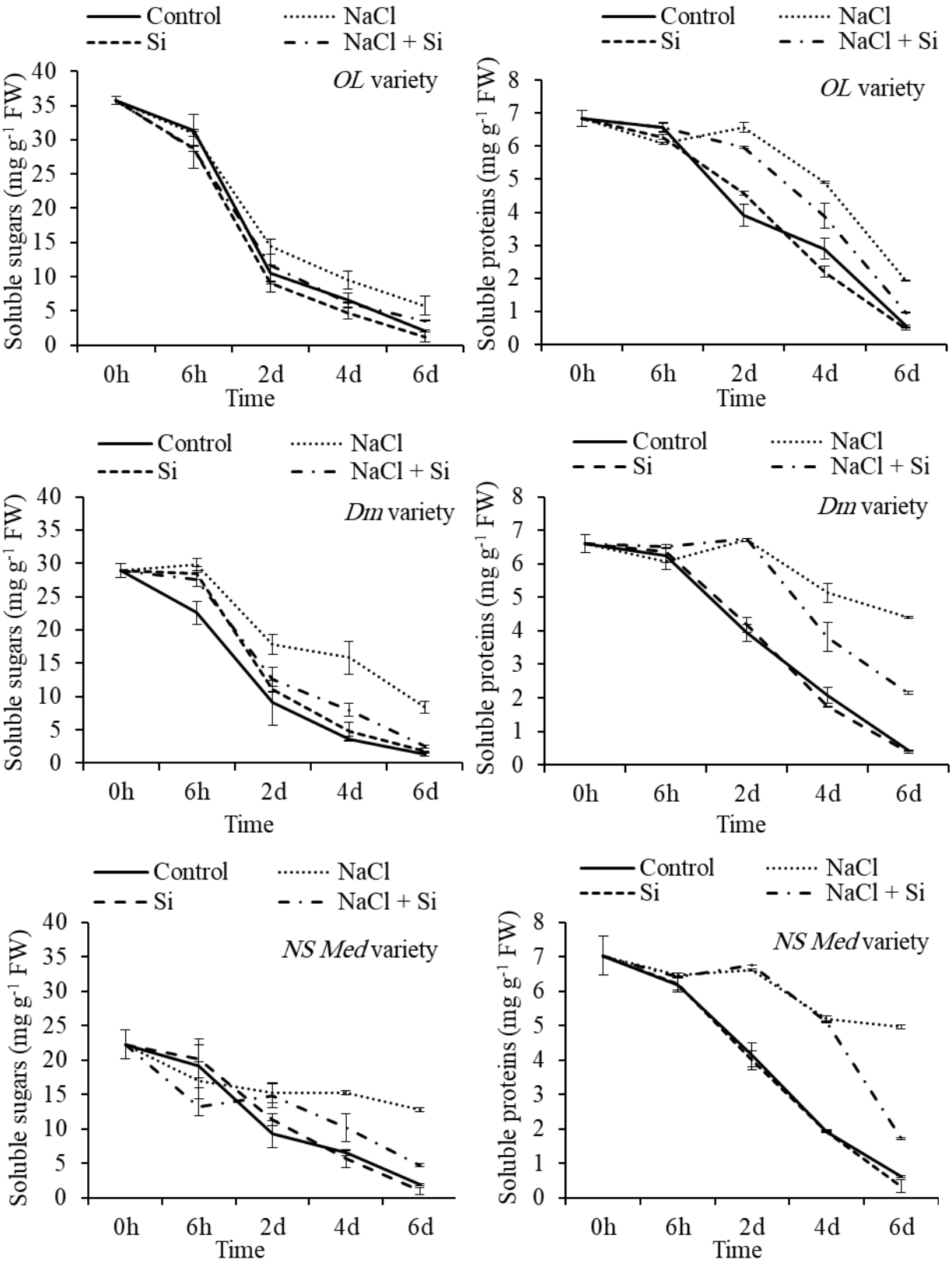

Fig. 1 Effect of 3 mM of silicon (Si) on seed reserve mobilization in three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under control (0 mM NaCl) and stress (200 mM NaCl). Data presented as a means of three replicates ± standard errors. Solid line, dashed line, dotted line and dash-

dotted line represent the control, the Si treatment, the NaCl treatment and the NaCl-Si combination, respectively

III-3 Effect of salt and/or Si on *M. sativa* embryo viability

The effect of Si and NaCl on the embryo viability of *M. sativa* is shown in Fig. 2. Depending to the variety, treatments as well as the post germinative stage different staining patterns intensity were observed, ranging from white, pale red to bright red. Under control or Si-treatment, embryos of the three varieties were bright red at the different time of the experiment, indicative of a normal cell respiration and viability. However, under 200 mM NaCl treatment, we noticed that the embryo showed very little color either after 6h or 24h, indicative of a lower respiration and lower viability. However, when Si was applied to the seeds with 200 mM NaCl, an intense staining was restored.

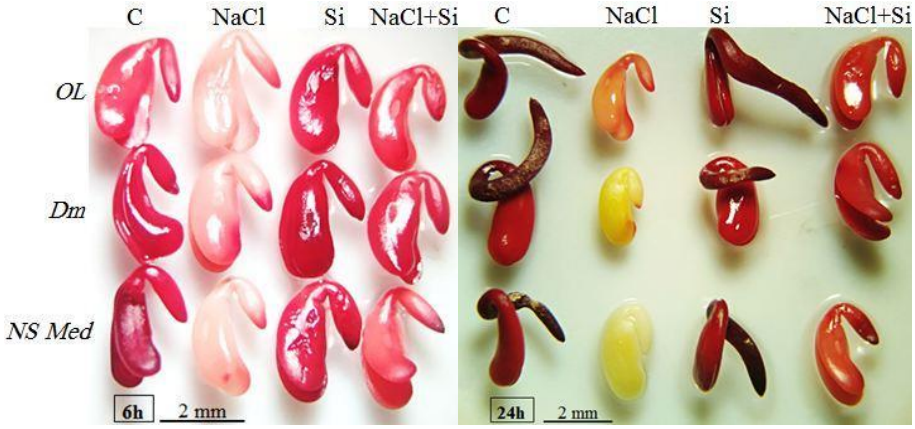

Fig. 2 Photographs showing the embryo viability of three *M. sativa* varieties (*OL, Dm* and *NS Med*) by 2,3,5 triphenyltetrazolium chloride (TTC) from the control (0 mM $CaSiO_3$ + 0mM NaCl) (C) and treated (200 mM NaCl (NaCl), 3 mM Si (Si) and 3 mM Si+200 mM NaCl (NaCl+Si)) seeds taken at different germination times (6h and 24h). Photographs were taken under binocular magnifying glass with 10x magnifications. Embryo viability was indicated by red color due to the reduction of TTC by respiratory activity in the cells. Embryos and seedlings shown are representative of embryos observed in three independent experiments

III-4 Effect of salt and Si on MDA, H_2O_2 and EL

Salt stress caused a significant increase in MDA content in the three *M. sativa* varieties compared to controls (Table 2). Comparison among the varieties showed that the highest MDA content was recorded for *NS Med* (7.14 ηmol g^{-1} FW). However, application of Si along with NaCl significantly decreased MDA content in all of the studied varieties, especially in *NS Med* where a reduction rate of 58% for MDA was found compared to Si-untreated salt-stressed

seedlings. Under non-stressed conditions, Si decreased MDA in *OL* variety. Results related to statistical analysis showed that variety, salinity and salinity-Si were significant (Table S2).

H_2O_2 content significantly increased upon salt stress in all of the three *M. sativa* varieties with a significant difference between them (Table 2). In salt-stressed *OL*, *Dm*, and *NS Med* seedlings, H_2O_2 content was 1.66, 1.99, and 3.41-fold higher, respectively, compared to their respective untreated controls. However, under the combined effect of Si and NaCl, the H_2O_2 content was much lower (0.97, 1.12 and 1.21-fold in *OL*, *NS Med* and *Dm*, respectively), relative to untreated control. ANOVA III indicated that all factors were significant (Table S2).

Eight days after imbibition, histochemical staining showed a high H_2O_2 accumulation in seedlings of the three *M. sativa* varieties exposed to salt stress, with a higher accumulation in *NS Med* (Fig. 3). However, exogenously applied Si at 3 mM to salt-stressed seedlings alleviated this effect in *OL* and *Dm* varieties. No difference was noted between Si-treated and -untreated controls.

As a consequence of lipid peroxidation, as shown in Table 2, the imposition of salt stress significantly and differently increased electrolyte leakage (EL) among the studied varieties. The highest EL values of 86% and 80% were found in salt stressed *Dm* and *NS Med*, respectively; while the lowest value was observed in *OL* seedlings (51%). However, Si supply predominantly alleviated NaCl deleterious effects by reducing EL. Indeed, EL in Si-treated salt-stressed *M. sativa* seedlings were only 55%, 46% and 39%, respectively for *Dm*, *NS Med* and *OL* variety. Under non-stressed conditions, there was no significant difference between controls and Si-treated seedlings (Table 2). According to ANOVA III, the interaction variety-Si and variety-salinity-Si were not significant (Table S2).

Table 2 Effect of 3 mM of exogenous silicon (+Si) on malondialdehyde (MDA), hydrogen peroxide (H_2O_2) contents and electrolyte leakages (EL) of 8-day-old seedlings of the three tested *M. sativa* varieties (*OL*, *Dm* and *NS Med*) without salt stress (-NaCl) and under stressed (+NaCl) conditions. Values represent means of three replicates ± standard errors

Varieties	Salt stress	MDA (ηmol g^{-1} FW)		H_2O_2 (µmol g^{-1} FW)		EL (%)	
		-Si	+Si	-Si	+Si	-Si	+Si
OL	-NaCl	1.61±0.38	1.44±0.24	0.178±0.01	0.158±0.01	17.80±2.82	22.27±2.80
	+NaCl	4.71±0.30	3.85±0.10	0.296±0.01	0.174±0.00	51.26±2.74	38.66±2.04
Dm	-NaCl	0.41±0.15	1.87±0.19	0.105±0.01	0.093±0.00	18.18±5.70	19.59±30.00
	+NaCl	3.89±0.50	3.72±0.14	0.209±0.01	0.128±0.01	85.68±5.99	55.30±4.73
NS Med	-NaCl	1.85±0.23	2.86±1.06	0.202±0.01	0.157±0.00	6.53±2.32	7.06±0.55
	+NaCl	7.14±0.93	2.97±0.09	0.690±0.01	0.227±0.00	80.29±3.53	45.94±4.61

Fig. 3 Histochemical staining of hydrogen peroxide (H_2O_2) using 3,3'diaminobenzidine (DAB) in 8-day old seedlings of three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under control (C), 3 mM Si (Si), 200 mM NaCl (NaCl) and 200 mM NaCl+3 mM Si (NaCl+Si) treatments. Browning indicated the presence of H_2O_2 due to the polymerization of DAB. Photographs were taken under binocular magnifying glass with 10x magnifications. Arrows indicate the site of the accumulation of H_2O_2

III-5 Effect of NaCl and Si on proline accumulation

Because proline content increases under stress in plants, proline content was analyzed in 8-day old *M. sativa* seedlings as shown in Fig. 4. The recorded data showed that proline content increased significantly ($P \leq 0.001$) upon salt treatment in the three *M. sativa* varieties studied. *NS Med* showed the highest proline content (8.59 µmol g FW^{-1}). Moreover, the exposure of Si together with 200 mM NaCl enhanced proline accumulation. Indeed, under the combined NaCl and Si treatments, proline content was increased by 40%, 33% and 31%, respectively in *OL*, *Dm* and *NS Med* compared to salt-stressed condition alone. When applied to untreated controls, Si has no significant effect on proline content in both *Dm* and *NS Med* varieties.

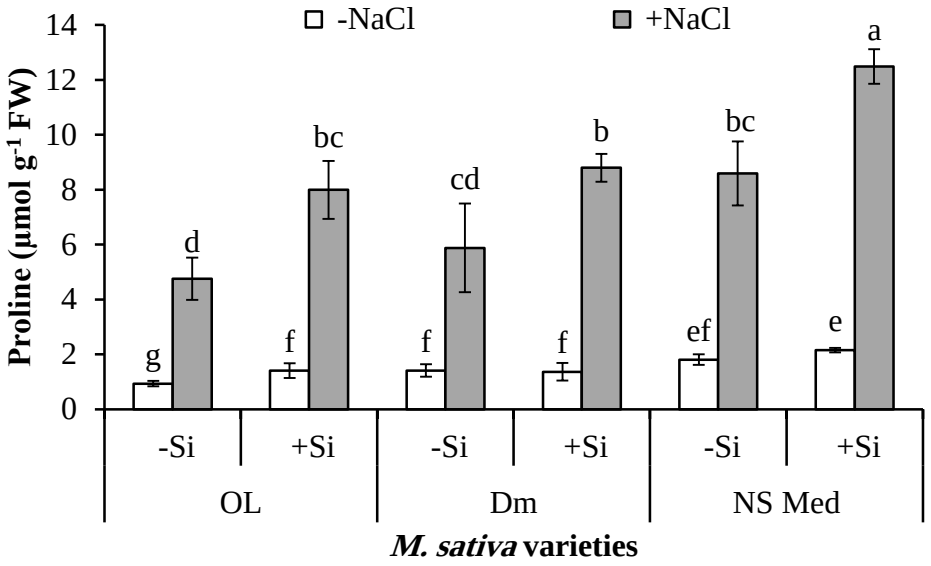

Fig. 4 Effect of 200 mM NaCl and 3 mM Si on proline accumulation in 8-day old seedlings of three *M. sativa* (*OL*, *Dm* and *NS Med*) varieties. Bars represent means of three biological replicates ± standard errors and the letters above indicate the statistical significance (LSD) between individual treatment at $P \leq 0.05$

III-6 Effect of NaCl and Si on SOD and CAT activities and SOD isoforms

The influence of 3 mM Si and 200 mM NaCl on SOD activity in the three *M. sativa* varieties studied is shown in Fig. 5a. Our findings indicated that exposure of *M. sativa* seedlings to salt stress triggered the activity of SOD in the three varieties studied. In all three varieties, together with NaCl, Si further increased SOD activity (Fig. 5a). When Si was applied alone, SOD activity decreased significantly ($P < 0.001$) only in *OL* and *Dm*, not in *NS Med*. According to ANOVA III, the interaction variety-salinity was not significant (Table S2). Moreover, in gel SOD activity revealed SOD isoforms (MnSOD, FeSOD and Cu/ZnSOD) in control and Si-treated seedlings (Fig. S1). However, under 200 mM NaCl alone or combined with 3 mM Si, MnSOD disappeared (Fig. S1).

Similarly, seedlings of the three *M. sativa* varieties exposed to salt stress had an enhanced CAT activity (Fig. 5b). *OL* and *NS Med* varieties showed the highest CAT activity, while the lowest activity was recorded in *Dm* variety. Likewise, Si application in combination to salt stress induced further CAT activity with a maximum increase in *OL*. Under non-stressed conditions, applying Si increased the activity of CAT in the three *M. sativa* varieties studied. ANOVA III showed that all factors and their interactions were significant for CAT activity (Table S2).

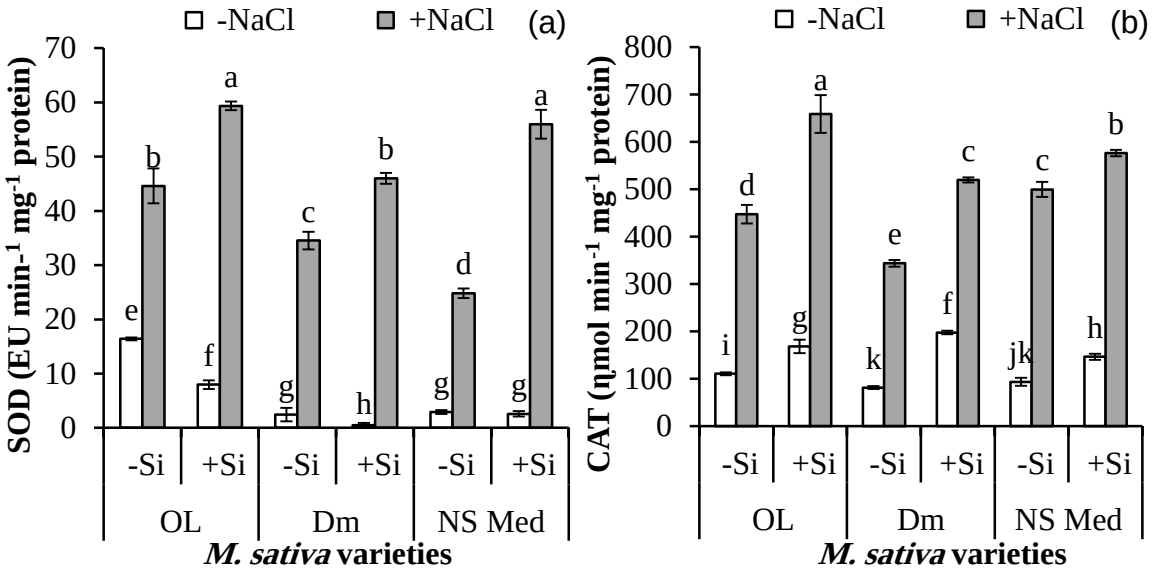

Fig. 5 Effect of 3 mM of silicon (+Si) on superoxide dismutase (SOD) (a) and catalase (CAT) (b) activities of 8-day-old seedlings in three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under 200 mM salt stress (+NaCl) and unstressed (-NaCl) conditions. Bars represent means of three replicates ± standard errors and the letters above indicate the statistical significance (LSD) between individual treatments at $P \leq 0.05$

III-7 Na^+, K^+, Ca^{2+} and Si content analyses

Si content in 8-day old *M. sativa* seedlings decreased in response to 200 mM NaCl stress but increased significantly in response to Si treatment alone as compared to Si-untreated controls (See Table 3). However, under combination of salt stress and Si, Si content reduced by 68% and 29% in *OL* and *Dm*, respectively, whereas in *NS Med* it increased by 454% relative to unstressed Si-treated seedlings. Statistical analysis revealed that all factors and their interactions were significant for Si content (Table S2).

Results in Table 3 indicated that salt stress imposition elevated Na^+ content in *OL*, *Dm* and *NS Med* by 266%, 266% and 268% respectively, while it significantly reduced K^+ by 48%, 26% and 66% in the respective salt-stressed varieties. Moreover, the increase in Na^+ and the decrease in K^+ reduced K^+/Na^+ ratio from 6.9 to 0.9 for *OL*, from 5.2 to 0.8 for *Dm* and from 7.4 to 0.7 for *NS Med* (Fig. 6). However, treatment with Si significantly increased K^+ concentration, while it significantly decreased Na^+ content leading to a higher K^+/Na^+ ratio with 1, 1.2 and 0.9 ratio for *OL*, *Dm* and *NS Med*, respectively in comparison to untreated controls (Table 3; Fig. 6). According to statistical analysis, all factors and their interactions were significant (Table S2).

Salt stress significantly increased Ca^{2+} content (Table 3) in the three *M. sativa*, with the highest increment rate of 244% recorded for *Dm* variety followed by *OL* (183%). However, unlike *NS Med* variety, applying 3 mM Si to salt-stressed seedlings decreased Ca^{2+} concentrations from 60.8 to 48.3 mg g^{-1} DW for *OL* and from 68.1 to 63.1 mg g^{-1} DW for *Dm* reflected 21% and 8% of reduction rate respectively for *OL* and *Dm*.

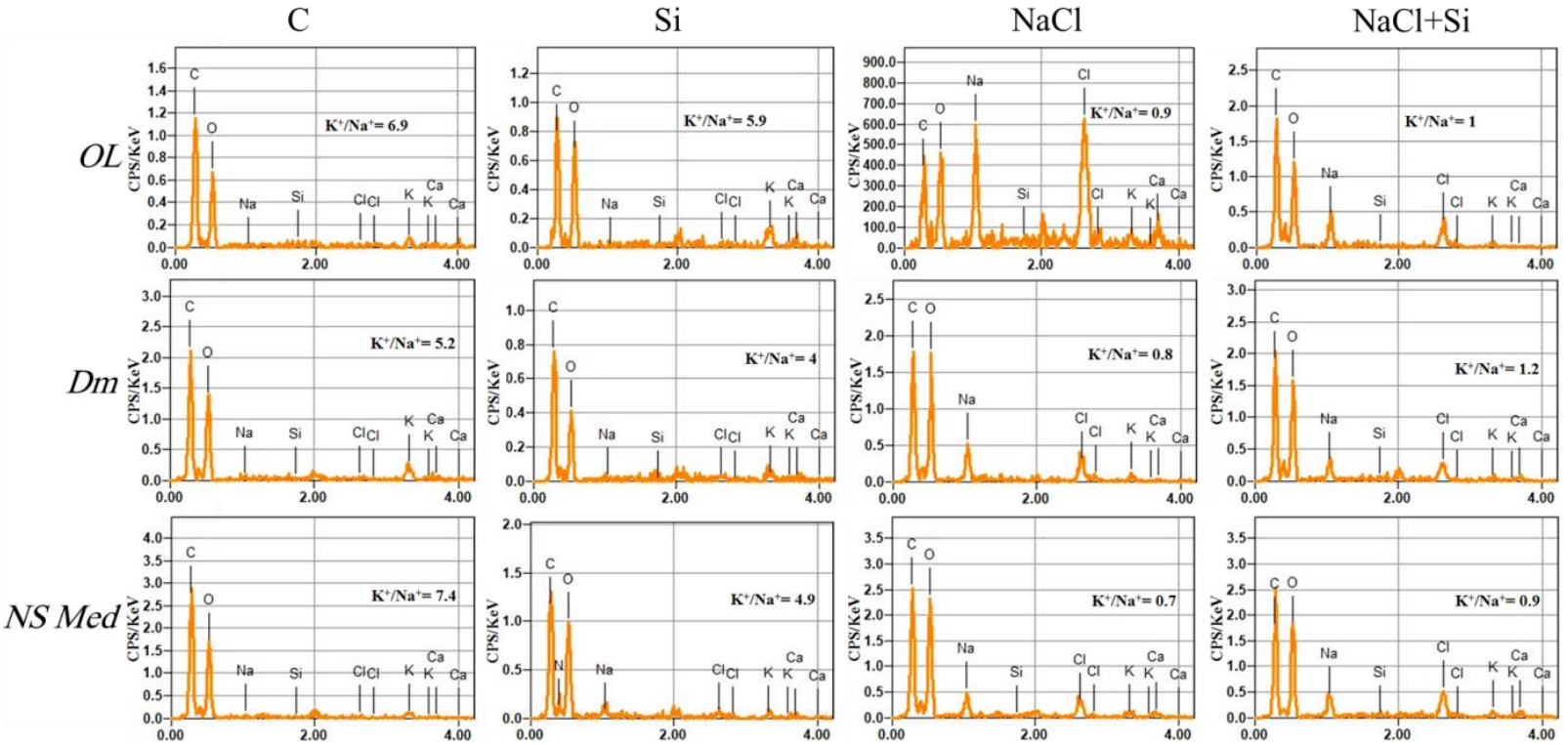

Fig. 6 Effect of NaCl (200 mM) and $CaSiO_3$ (3 mM) treatments on the K^+/Na^+ ratio of 8-day old seedlings of the three studied *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under control (C), 3 mM Si (Si), 200 mM NaCl (NaCl) or the combination of Si and NaCl (NaCl+Si)

Table 3 Effect of salt (200 mM NaCl) and Si (3 mM $CaSiO_3$) on Si, Na^+, K^+ and Ca^{2+} content of three *M. sativa* varieties (*OL*, *Dm* and *NS Med*). C, control; NaCl, salt stress; Si, $CaSiO_3$ treatment; NaCl+Si, combination of salt stress and $CaSiO_3$ treatment. Values are the mean of three replicates and the letters indicate statistically significant values

	OL				***Dm***				***NS Med***			
	C	**Si**	**NaCl**	**NaCl+Si**	**C**	**Si**	**NaCl**	**NaCl+Si**	**C**	**Si**	**NaCl**	**NaCl+Si**
Si (mg kg^{-1})	7.90g	2061.00a	4.80i	659.60c	5.60h	679.80b	4.20i	479.40d	8.10g	75.20f	2.78j	416.90e
Na^+ (mg g^{-1})	13.00f	17.30d	47.60a	37.10c	13.00f	17.30d	47.60a	37.10c	12.20f	14.70e	44.90b	46.10a
K^+ (mg g^{-1})	89.60b	101.70a	46.70g	37.50i	60.60d	59.10e	44.80h	53.40f	89.70b	71.10c	30.80j	43.30h
Ca^{2+} (mg g^{-1})	21.50h	28.40f	60.80c	48.30e	19.80h	24.80g	68.10a	63.10b	23.00g	24.60g	54.90d	61.10c

III-8 Effect of NaCl and Si on K^+/Na^+ ratio in *M. sativa* seedlings

Analyzing ion contents particularly Na^+ and K^+ using scanning electron microscopy (SEM) showed an improved K^+/Na^+ ratio in the three *M. sativa* varieties after Si treatment (Fig. 6). Na^+ concentration in seedlings treated with 200 mM NaCl was increased, whereas K^+ concentration was decreased resulted in lower K^+/Na^+ ratio of 0.9, 0.8 and 0.7 respectively for *OL*, *Dm* and *NS Med* as compared to controls (6.9, 5.2 and 7.4 observed respectively for *OL*, *Dm* and *NS Med*) (Fig. 6). 3 mM Si supplementation decreased Na^+ content of the three salt-stressed *M. sativa* varieties resulted in a high K^+/Na^+ ratio. In addition, the SEM images showed significant changes in the morphology of the investigated surface area in the three *M. sativa* varieties (Fig. S2). Under 200 mM NaCl treatment, the investigated surface area revealed shrinkage, whereas the supplementation of Si to salt-stressed *M. sativa* seedlings showed a surface area with less deformation, for the three studied *M. sativa* varieties (Fig. S2). *M. sativa* seedlings treated with Si only showed a smooth surface area as compared to the untreated *M. sativa* control seedlings (Fig. S2).

III-9 Data analysis

Principal component analysis (PCA) of all the parameters is presented in Fig. 7a. PCA obtained from the three *M. sativa* varieties under control, salt stress, salt stress supplemented with Si and Si alone including germination traits (FGP, VI and T_{50}), TSL, SFW, seed reserve mobilization (Soluble sugar and soluble protein), antioxidant enzymes (SOD and CAT), proline content, oxidative stress indicator (MDA, H_2O_2 and EL) and nutrient analyses (Si, Na^+, K^+ and Ca^{2+}) explained a total of 77.61% (PC1 = 67.62%; PC2 = 9.99%) of variability in the data (Fig. 7a). Our results demonstrated that applied NaCl decreased seed germination by inducing an ionic toxicity since the close significant negative correlation observed between Na^+ content and the germination traits such as FGP (r= -0.705) and VI (r= -0.881) (Figs. 7a, b). However, high significant positive correlation (r= 0.9) was observed between Na^+ content and T_{50} indicating that NaCl affect seeds by delaying or totally prevent their germination (Figs. 7a, b). In addition, a significant positive correlation was observed between T_{50} and oxidative stress indicators (MDA (r= 0.723), H_2O_2 (r= 0.622) and EL (r= 0.903)) (Fig. 7b) indicating that seedlings were facing an oxidative stress. Likewise, PCA test (Fig. 7a) showed a positive correlation between Si addition and seed germination traits (FGP and VI) as well as the length of seedlings and their fresh weight which explain the positive role of Si on seed germination and early seedlings growth.

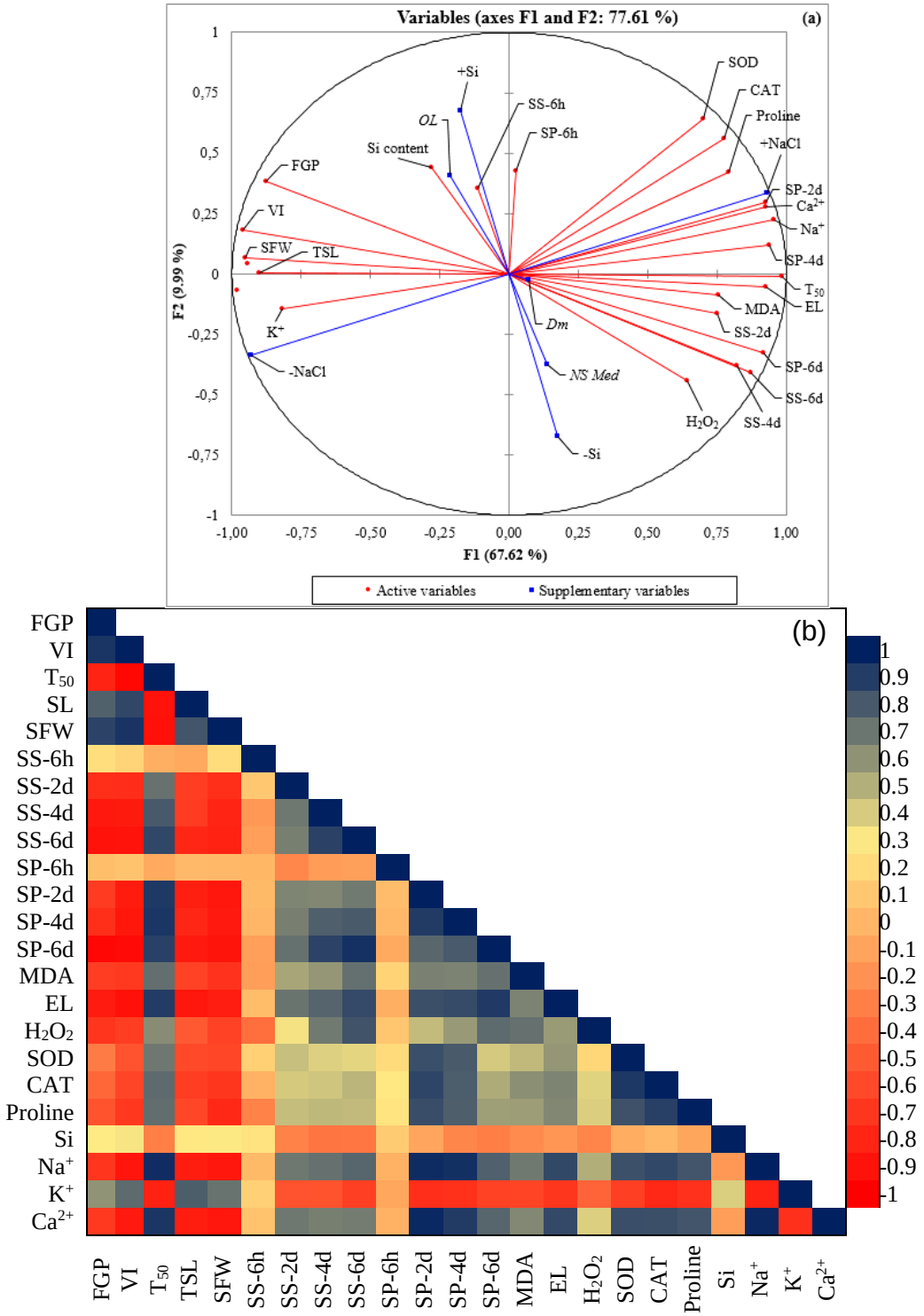

Fig. 7 Principal component analysis (PCA) (a) and Pearson's correlation matrix (b) of studied parameters in *M. sativa* varieties in response to silicon as well as salt in the medium. For PCA, the most variables (arrows), silicon concentration, NaCl concentration as well as the three different varieties are projected onto the F1-F2 principal factorial plane that explains 78.98 % of the variation. For the Pearson's correlation matrix, correlations are displayed in blue (positive) and in red (negative); color intensity is proportional to correlation coefficient. FGP: finale germination percentage; VI: velocity index; T_{50}: time to reach 50% of germination; TSL: total seedlings length; SFW: seedling fresh weight; SS 6h, 2d, 4d, 6d: soluble sugars at 6h, 2d, 4d and 6d; SP 6h, 2d, 4d, 6d: soluble protein at 6h, 2d, 4d and 6d; MDA: malondialdehyde; H_2O_2: hydrogen peroxide; EL: electrolyte leakage; SOD: superoxide dismutase; CAT: catalase; Ca^{2+}: calcium; Na^+: sodium; K^+: potassium; Si: silicon

IV Discussion

Water is essential for seed imbibition as well as for reserve mobilization leading to seed germination. However, salt-contaminated soil causing an osmotic stress limiting water uptake availability, leading to the inhibition of germination (Farissi et al. 2011). Here, we investigated the ability of exogenous Si to alleviate salt stress tolerance and improve seed germination in three *M. sativa* varieties previously investigated at the plant vegetative state (El Moukhtari et al. 2021). Salt stress reduced FGP and delayed germination differently according to the investigated *M. sativa* variety. Indeed, salt-stressed *NS Med* variety exhibited higher reduction rates for FGP and longer T_{50} than the salt stressed-*OL* variety. The decrease in FGP was confirmed by VI, which decreased from 762 to 390 for *OL*, from 606 to 182 for *Dm* and from 733 to 127 for *NS Med* after 8 days of germination under 200 mM NaCl. Previous studies have also reported that salt stress can affect seed germination percentage, VI and T_{50} in *M. sativa* (Ma et al. 2017), in *Phaseolus sp.* (Bayuelo-Jiménez et al. 2002), in *Chenopodium quinoa*, in *Amaranthus caudatus* (Moreno et al. 2018) and in *Cakile maritima* (Debez et al. 2018). The application of Si was able to alleviate the detrimental effect of salt stress in *M. sativa* seed germination. When 3 mM Si was supplied exogenously in combination with salt stress, Si increased FGP and VI particularly in *NS Med* with high improvement rates by 74% and 84%, respectively in comparison to NaCl-treated seedlings. Furthermore, Si decreased T_{50} from 3.05 to 1.71 for *OL*, from 3.34 to 2.32 for *Dm* and from 3.48 to 2.59 for *NS Med* indicating reductions rates of 44%, 33% and 26%, respectively. Our results are in agreement with the findings of Zhang et al. (2015) who showed the beneficial effects of exogenous Si on *G. uralensis* seed germination under salt stress. Similar findings were also published by Wang et al. (2010) on *Momordica charantia* seed germination under salt stress. Biju et al. (2017) demonstrated that Si may improve FGP, germination index and seedlings vigor index of lentil seeds subjected to drought stress and the effect was more pronounced in drought sensitive-genotypes than in drought tolerant ones, which also support our findings. The positive effect of Si on seed germination under osmotic stress was also reported in other plant species like tomato (Haghighi et al. 2012), wheat (Bybordi 2014) and maize (Zargar and Agnihotri 2013). According to the study conducted by Farissi et al. (2011) in some Moroccan *M. sativa* populations, the effect of salt stress on seed germination could be explained by the toxic effect of accumulated Na^+ and Cl^- on some metabolic activities during germination process. This supports our findings on an increased Na^+ content and the decreased of K^+ content in salt-stressed *M. sativa* varieties.

However, Si addition resulted in increasing Si content and K^+/Na^+ ratio under either stressed or unstressed conditions.

The inhibition of *L. culinaris* seed germination under salt stress was related to the inhibition of some hydrolytic enzyme activities, such as α-amylase, β-amylase and α-glucosidase (Sidari et al. 2008). Interestingly, similar results were obtained in the same plant species under drought stress (Biju et al. 2017). Exogenous Si addition was found effective to improve *L. culinaris* seed germination, and the improvement was partially related to the ability of Si to enhance the activity of hydrolytic enzymes (Biju et al. 2017; Sidari et al. 2008). This is in agreement with our findings concerning salinity and Si effects on seed reserve mobilization. Indeed, Si application lowered soluble sugar and protein contents in germinating seeds. Thus, a highly significant negative correlation was observed between soluble sugars and protein seed contents and germination parameters (FGP and VI). T_{50} showed a highly positive correlation with soluble sugar and protein seed contents. These findings suggest that improvement of seed germination by Si treatment under salt stress is mediated through a better seed reserve mobilization. Our results also showed that depending to the varieties, salinity delayed or completely inhibited seed germination and that Si improved embryo viability under salt stress in the three *M. sativa* varieties. Altogether these data support the important role of Si on *M. sativa* seed germination under salt stress.

Seedling growth following seed germination is also sensitive to abiotic stress, particularly salinity (Debez et al. 2001; Ghoulam and Fares 2001). Our results showed a significant reduction in seedling growth for the three studied *M. sativa* varieties under 200 mM NaCl, as evaluated by TSL and SFW. *NS Med* variety appeared to be the most affected by salt stress with a decrease of SFW and TSL by 68% and 62%, respectively, while *OL* was the less affected with a decrease of 38% and 45%, respectively. However, when Si was applied together with salt-stress, the negative effect of NaCl on seedling growth was significantly reduced in all of the three studied varieties. A less pronounced decrease of 44% and 23% in *NS Med* and 25% and 33% in *OL,* respectively was observed for FW and TSL. The effect of Si on seedling biomass could be attributed to Si-mediated water uptake through the upregulation of aquaporin genes (Zhu et al. 2015). Our results agree with previous data in some legumes like *L. culinaris* (Biju et al. 2017). Authors showed that Si was able to improve seedlings growth under osmotic stress as reflected by higher fresh and dry weights. The effect of exogenous Si on seed germination and seedlings growth was also observed in *G. uralensis* and *M. charantia* under salt stress

(Wang et al. 2010; Zhang et al. 2015) and in *Solanum lycopersicum* under water stress (Shi et al. 2014).

Salinity is known to induce secondary stress such as oxidative stress mediated by ROS accumulation (Ben Othman et al. 2017; Kiani et al. 2021). ROS disturb the normal cellular metabolism by triggering peroxidation of lipids, proteins and nucleic acids (Farissi et al. 2014, 2018). According to Gong et al. (2008), MDA content, resulting from lipid peroxidation, could be used as an indicator of oxidative stress in plants under abiotic stress. Our results showed a significant increase in MDA content in *M. sativa* seedlings under 200 mM NaCl together with higher EL and higher H_2O_2. This oxidative stress was especially pronounced in *NS Med* variety where the highest values of 7.14 ηmol g^{-1} FW, 0.69 µmol g^{-1} FW and 80.29% were observed for MDA, H_2O_2 and EL, respectively. The *in situ* visualization of H_2O_2 also showed strong accumulation of H_2O_2 in *M. sativa* seedlings of the three varieties upon salt stress. However, the oxidative stress appeared to be significantly alleviated in Si-treated seedlings, which displayed a reduced MDA and H_2O_2 content as well as EL. According to Lee et al. (2001), SOD which converted superoxide to O_2 and H_2O_2, is considered to be a primary ROS scavenger in salt-stressed plants. H_2O_2 will be then converted to H_2O by the action of CAT (Farissi et al. 2018). Our present work showed that 200 mM NaCl increased the activity of both SOD and CAT and their activities were even more pronounced when Si was added to salt stress. The positive effect of Si on oxidative stress has been attributed to the ability of Si to induce antioxidant enzyme activities (Coskun et al. 2016; Rizwan et al. 2015; Zhu and Gong 2014). Furthermore, Zhang et al. (2015) demonstrated that Si could improve seedlings growth under salt stress through an increase of antioxidant enzyme levels. Our results corroborate with previous findings by Zhang et al. (2015) in *G. uralensis*, by Wang et al. (2010) in *M. charantia* under salt stress, by Biju et al. (2017) in *L. culinaris* and by Shi et al. (2014) in *S. lycopersicum* under drought stress. All these works conclude to a protective role of Si in response to oxidative stress. Moreover, beside osmoregulation, proline has been reported as a non-enzymatic ROS-scavenger (El Moukhtari et al. 2020; Szabados and Savouré 2010). In the present study, Si supplementation alleviated the oxidative stress induced by salinity stress through proline accumulation, since the close positive correlation observed between MDA and EL and proline (Figs. 7a, b). Previous investigations showed that proline accumulation upon Si treatment improved oxidative stress induced under salinity (Abbas et al. 2015; Ahmad et al. 2018). Likewise, PCA test showed a highly significant positive correlations between the activities of SOD, CAT and proline with the contents of MDA, H_2O_2 and EL. Similar results were observed

in *M. sativa* (Wang et al. 2011) and in diverse legume plants, including *A. gerrardii* (Al-Huqail et al. 2019) and *G. uralensis* (Li et al. 2016).

V Conclusions

In this study, *M. sativa* seed exposure to salt stress resulted in seed germination inhibition by affecting reserve mobilization of the embryo and by inducing oxidative stress and ionic toxicity. However, supplementation of 3 mM Si was found to be effective to improve *M. sativa* seed germination and seedling growth. Si treatment improved reserve mobilization to the embryo and its viability. Si was also found effective in reducing the oxidative stress and ionic toxicity by increasing antioxidant enzyme activities, proline content and K^+/Na^+ ratio. Our study not only highlights the impact of NaCl on seed germination but also provides the first step for more detailed investigations of the impact of Si on *M. sativa* seed germination and seedlings growth under salt stress.

Statements and Declarations:

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable

Code availability

Not applicable

Authors' contributions

AEM, AS and MF conceptualized the work; AEM and NL carried out most of the experimental work; SL, MM, CC, PC, AS and MF brought their expertise to the work; AEM carried out the statistical analysis; AEM delivered a first draft of the manuscript; MF, SL, MM, CC, PC and AS revised the manuscript; MF and AS supervised the work. All authors read and approved the final version of the manuscript.

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Supplementary data

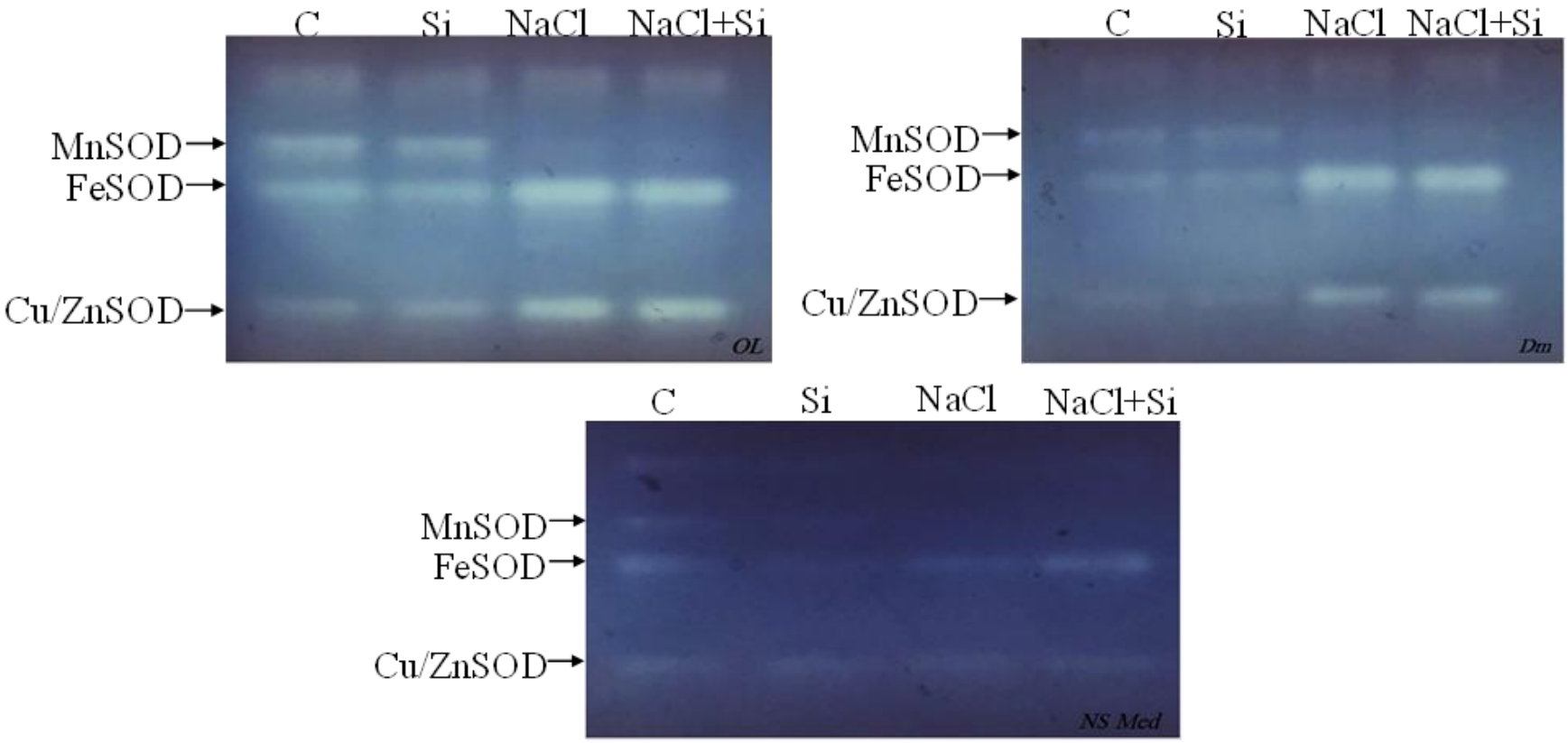

Fig. S1 Effect of NaCl (200 mM) and $CaSiO_3$ (3 mM) treatments on superoxide dismutase (SOD) isoforms of 8-day old seedlings of three *M. sativa* varieties. Proteins (11.5 µg protein per well) were separated by native-PAGE (12% polyacrylamide gels) and stained for SOD activity as described in Materials and methods. C: control, Si: 3 mM $CaSiO_3$, NaCl: 200 mM NaCl, NaCl-Si: 200 mM NaCl+3 mM $CaSiO_3$

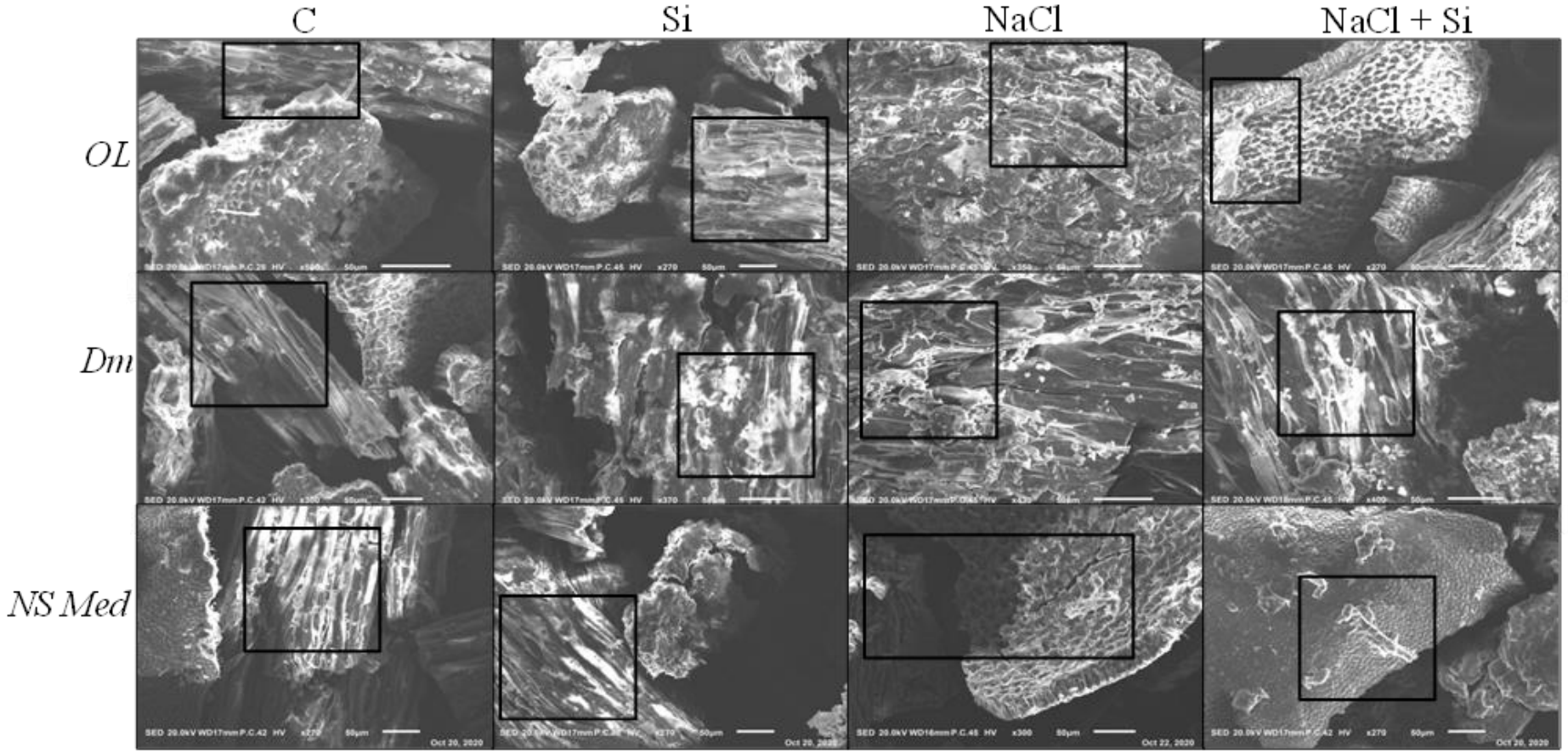

Fig. S2 Scanning electron microscopy (SEM) images of the effect of NaCl and Si on the surface of mapped elements of 8-day old seedlings of the three studied *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under control (C), 3 mM Si (Si), 200 mM NaCl (NaCl) or the combination of Si and NaCl (NaCl+Si). Scale bar = 50 µm

Table S1 Result of four-way ANOVA test for independent variables including *M. sativa* varieties (*OL*, *Dm* and *NS Med*), salinity treatment (0 and 200 mM NaCl) and silicon treatment (0 and 3 mM $CaSiO_3$) and their interaction on seed reserve mobilization

	Varieties (V)	Salinity (S)	Silicon (Si)	Time (T)	V-S	V-Si	V-T	S-Si	S-T	Si-T	V-S-Si	V-S-T	V-Si-T	S-Si-T	V-S-Si-T
Sugars	7.56^{***}	139.52^{***}	41.39^{***}	671.42^{***}	7.53^{***}	0.65^{ns}	32.97^{***}	55.42^{***}	14.39^{***}	7.67^{***}	12.73^{***}	2.56^{*}	2.74^{*}	2.10^{ns}	0.66^{ns}
Proteins	16.75^{***}	2299.51^{***}	104.74^{***}	3223.79^{***}	72.66^{***}	1.27^{ns}	18.49^{***}	69.15^{***}	247.23^{***}	62.30^{***}	1.88^{ns}	12.46^{***}	11.79^{***}	43.21^{***}	10.05^{***}

ns = $P \geq 0.05$; * = $P \leq 0.05$; ** $P \leq 0.01$; *** = $P \leq 0.001$

Table S2 Result of three-way ANOVA test for independent variables including *M. sativa* varieties (*OL*, *Dm* and *NS Med*), salinity treatment (0 and 200 mM NaCl) and silicon treatment (0 and 3 mM $CaSiO_3$) and their interactions on different tested parameters

	Varieties (V)	Salinity (S)	Silicon (Si)	V-S	V-Si	S-Si	V-S-Si
FGP	66.32^{***}	303.25^{***}	8.25^{***}	65.98^{***}	1.57^{ns}	8.14^{***}	1.63^{ns}
VI	293.62^{***}	2422.43^{***}	28.10^{***}	263.68^{***}	2.30^{*}	29.13^{***}	2.03^{*}
T50	58.76^{***}	2720.72^{***}	28.52^{***}	51.01^{***}	1.39^{ns}	27.32^{***}	1.36^{ns}
TSL	31.15^{***}	585.64^{***}	6.00^{***}	0.64^{ns}	1.85^{ns}	3.92^{**}	0.52^{ns}
SFW	49.84^{***}	668.22^{***}	2.13^{ns}	23.82^{***}	0.68^{ns}	1.98^{ns}	0.52^{ns}
MDA	3.31^{*}	46.68^{***}	1.49^{ns}	0.01^{ns}	2.63^{ns}	9.97^{*}	2.98^{ns}
H_2O_2	751.66^{***}	1234.20^{***}	985.01^{***}	317.55^{***}	276.34^{***}	622.44^{***}	197.37^{***}
EL	6.24^{**}	221.12^{***}	15.75^{***}	10.77^{***}	1.75^{ns}	21.95^{***}	0.85^{ns}
SOD	115.80^{***}	3303.38^{***}	132.90^{***}	0.85^{ns}	32.62^{***}	283.47^{***}	15.05^{***}
CAT	25.19^{***}	2703.28^{***}	255.57^{***}	32.68^{***}	12.44^{***}	30.30^{***}	7.24^{*}
Si	89.33^{***}	82.64^{***}	515.73^{***}	111.74***	88.93***	80.62***	112.00***
Na^+	19783.69^{***}	74865281.90^{***}	196089.26^{***}	236963.47^{***}	243323.01^{***}	2397605.23^{***}	451553.28^{***}
K^+	6569811.90^{***}	115851520.20^{***}	39366.85^{***}	14973030.15^{***}	351160.28^{***}	990032.73^{***}	5219834.39^{***}
Ca^{2+}	561727.55^{***}	114809147.60^{***}	14273.88^{***}	1462998.39^{***}	335407.70^{***}	1543590.13^{***}	1099035.57^{***}

FGP, Final germination percentage; VI, velocity index; T50, time to reach 50% of germination; TSL, total seedlings length; SFW, seedlings fresh weight; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; EL, electrolyte leakage; SOD, superoxide dismutase; CAT, catalase; Si, silicon; Na^+, sodium; K^+, potassium; Ca^{2+}, calcium; ns = $P \geq 0.05$; * = $P \leq 0.05$; ** $P \leq 0.01$; *** = $P \leq 0.001$

Chapitre III (Article 5)

L'effet de silicium exogène sur la photosynthèse, l'ajustement osmotique, le métabolisme oxydatif et la nutrition minérale de la luzerne (*Medicago sativa* L.) sous contrainte saline

I Introduction

La luzerne (*Medicago sativa* L.) est l'une des espèces fourragères les plus cultivées dans le bassin méditerranéen grâce à ses effets bénéfiques sur la fertilité des sols et sa grande adaptation aux climats locaux et aux conditions du sol (Mouradi et al. 2018). Cependant, une réduction significative de la croissance de la luzerne et du rendement fourrager a été enregistrée en raison de diverses contraintes abiotiques, notamment la sécheresse et la salinité (Elgharably et Benes 2021; Saidi et al. 2021). Il a été démontré que la présence du Si dans le milieu a plusieurs effets bénéfiques sur les plantes vis-à-vis de contraintes abiotiques telles que la salinité (Debona et al. 2017). L'application exogène de Si s'est avérée efficace pour améliorer la croissance, la photosynthèse, la nutrition hydrique et la fixation biologique de l'azote (FBN) des Fabacées exposées au stress salin (Meng et al. 2020; Etesami et Adl 2020; Kurdali et al. 2019).

II Objectif

L'objectif principal de ce chapitre est de comprendre l'effet du Si sur l'amélioration de la tolérance de la symbiose *M. sativa-Ensifer meliloti* au stress salin à travers l'étude de l'ajustement osmotique, l'amélioration de la photosynthèse et de la nutrition minéral ainsi que la régulation du métabolisme oxydatif.

III Méthodologie

Les graines de *M. sativa* (*OL*, *Dm* et *NS Med*) ont été mises à germer directement dans des pots contenant de la perlite dans une chambre de croissance avec une température de 25 ± 1 °C, une humidité relative de 80% et une photopériode de 16h. Après la levée, le nombre de jeunes plantules a été ajusté à 5 par pot. 15 jours après le semis, les jeunes plantules ont été inoculées par une souche *E. meliloti* Rm41 et arrosées par une solution nutritive dépourvue d'azote. Les plantes ont été divisées en deux lots. Le premier lot arrosé par la solution nutritive contenant 0 mM NaCl (témoin) et le deuxième lot arrosé par la solution nutritive additionnée de 120 mM NaCl (Stressé). En parallèle la moitié de chaque lot a été traitée avec 3 mM Si une fois par semaine, tandis que l'autre moitié n'a pas été traitée avec du Si. Le Si a été appliqué dans la solution nutritive. Après un mois de stress, les plantes ont été récoltées et soumises à un ensemble d'analyses agro-physiologiques, enzymatiques et nutritionnelles. L'effet du Si sur la symbiose *M. sativa-E. meliloti* a été évalué à travers le nombre de nodosités et la teneur des plantes en azote.

IV Résultats

Les résultats obtenus indiquent que la contrainte saline a significativement réduit la croissance des plantes des trois variétés de luzerne en réduisant la biomasse fraiche et sèche, le nombre de nodule, le nombre de feuilles et la hauteur des plantes. Ceci est accompagné avec une réduction de la teneur des plantes en azote (N) et de la teneur relative en eau (TRE). D'autre part, la contrainte saline réduit la teneur des plantes en potassium (K^+) et augmente celle du sodium (Na^+). Les teneurs des feuilles en chlorophylle, la fluorescence chlorophyllienne et la conductance stomatique sont aussi diminuées. La comparaison de ces variétés a montré que la variété européenne est la plus affectée par la contrainte saline. Cependant, l'ajout de 3 mM Si dans la solution nutritive des plantes stressées a induit une accumulation significative des solutés compatibles qui améliore la nutrition hydrique des plantes reflétée par une TRE plus élevée. De même, l'ajout de Si dans le milieu augmente le rapport K^+/Na^+ et améliore la capacité antioxydante des plantes, en particulier celles traitées par 120 mM NaCl. L'accumulation des solutés compatibles et la régulation du métabolisme oxydatif chez les plantes stressées en réponse à l'ajout du Si a rétabli la croissance des plantes de luzerne en termes de biomasse, nombre de feuilles et hauteur des plantes, l'établissement de la symbiose en termes de nombre de nodosités, teneur en N, et la photosynthèse en termes de pigments photosynthétiques, fluorescence chlorophyllienne et conductance stomatique.

V Conclusion

Nos résultats démontrent que l'ajout du Si représente une méthode efficace pour améliorer la tolérance des plantes de luzerne aux contraintes salines à travers l'amélioration de la photosynthèse, l'induction de l'accumulation des solutés compatibles et la régulation du métabolisme oxydatif. Nos résultats montrent également que le Si exogène a un effet bénéfique sur la fixation biologique de N_2.

Silicon improves physiological, biochemical, and morphological adaptations of alfalfa (*Medicago sativa* L.) during salinity stress

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Abstract
Silicon (Si) application to crops is a promising way for the deployment of sustainable agriculture. Here, the effects of Si on salt stress tolerance were investigated in alfalfa (*Medicago sativa* L.)-rhizobia symbioses. Two Moroccan, *Ouad-Lmaleh* (*OL*) and *Demnate-201* (*Dm*), and one European, *NS-Mediana-ZMS-V* (*NS-Med*), alfalfa varieties were associated to *Ensifer meliloti* Rm41 rhizobial strain. One-month-old alfalfa plants were exposed to 120 mM NaCl for five weeks with or without 3 mM of Si. The plants subjected to salt stress showed reduced biomass, chlorophyll (Chl) contents and relative water content (RWC) in comparison to the controls. The alfalfa-rhizobia symbiosis was also impaired under stress as reflected by less root nodulation and lower nitrogen (N) content and nitrogen content index (NCI). Added Si significantly increased plant biomass, nodules number, N content, NCI, Chl contents and RWC under salt stress. Results showed that salt-stressed alfalfa increased malonyldialdehyde (MDA), hydrogen peroxide (H_2O_2) and electrolyte leakage (EL). However, Si incorporation in the cultured media reduced oxidative damages under salt-stress particularly in *NS-Med* variety by 26%, 70% and 70% for MDA, H_2O_2 and EL respectively. The lower amount of MDA, H_2O_2 and EL in the Si-treated plants seems to be related to its capacity to modulate superoxide dismutase and polyphenol oxidase activities and increase total polyphenol, flavonoid and carotenoid contents. Besides, compatible osmolytes, such as proline, glycine betaine and soluble sugars were found increased particularly in Si-treated *OL* plants by 46%, 33% and 26% respectively in comparison to Si-untreated plants. Alfalfa varieties reacted differently to Si treatment. Sodium concentration in alfalfa plants increased under salinity and reduced by Si treatment with an increase in the potassium content. Our findings showed that exogenous Si application could be a promising way to mitigate the toxic effect of salt and could improve alfalfa growth and its rhizobial symbiosis when grown in salt-affected soils.

Keywords *Medicago sativa* L. · Salt stress · Silicon · Nodulation · Nitrogen fixation · Oxidative stress

1 Introduction

Due to its high advantageous effects on soil fertility and its high adaptation to local climates and soil conditions, the perennial forage legume alfalfa (*Medicago sativa* L.) is one of the most cultivated forage species in the Mediterranean basin (Mouradi et al. 2018). In Morocco, alfalfa culture occupies about 455 000 ha, 25% of the total area dedicated to forage crop (Mouradi et al. 2018). However, a significant decline in alfalfa growth and forage yield was recorded due to various abiotic constraints including heat, nutrient deficiency, drought and salinity (Wassie et al. 2020; Gao et al. 2020; He et al. 2021; Qiu et al. 2021). Salinity is one of the most devastating environmental constraints that alfalfa encounters (Elgharably and Benes 2021; Saidi et al. 2021). It is estimated that more than 800 million hectares of irrigated land is considered as salt affected, which is expected to increase further due to the current irrigation practices and global climate change (Roy et al. 2014; Van Zelm et al. 2020). Salt stress reduces plant growth through osmotic stress and accumulation of either sodium (Na^+) and/or chloride (Cl^-) resulting in ionic toxicity (Tester and Davenport 2003; Singh and Jha 2016; Sandhu et al. 2017). Additionally, salt stress impaired photosynthesis activity in alfalfa by reducing chlorophyll (Chl) content and lowering carbon

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assimilation (López et al. 2008; Farissi et al. 2018). Furthermore, salinity also triggers an oxidative stress through the overproduction of reactive oxygen species (ROS), devastating radicals for the normal cell metabolism (Arora et al. 2020). To cope with the harmful effects of salinity stress, plants have developed various physiological and biochemical responses to avoid and/or tolerate this constraint (Munns and Tester 2008; Gupta and Huang 2014). Some common strategies include the accumulation of compatible solutes (Al-Farsi et al. 2020). One of the most widespread accumulated osmolytes is the amino acid proline (Szabados and Savouré, 2010). Transcriptional regulation of proline biosynthesis as well as proline accumulation was observed in alfalfa upon addition of salt (Armengaud et al. 2004). Furthermore, the osmoprotectant betaine including proline betaine and glycine betaine have been identified as the main betaine accumulated in alfalfa particularly under salt stress (Pocard et al. 1991; Trinchant et al. 2004). Furthermore, the antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), polyphenol oxidase (PPO), total polyphenol, flavonoids and carotenoids, have been reported to act concertedly to allow plants to tolerate at high salinity level (Singh and Jha 2017; Arora et al. 2020).

Conventional breeding has been used to improve alfalfa varieties tolerance to salt (Bhattarai et al. 2020). However, this is time consuming and had limited success (Fita et al. 2015). The application of exogenous compound such as glucose or nitrate to improve salt tolerance (Atia et al. 2009; Hu et al. 2012) had been proven inappropriate due to a high cost-to-benefit ratio and harmful environmental impact (Farissi et al. 2014). Developing innovative methods, both ecofriendly and cost effective like supplementary silicon (Si) is mandatory to help mitigating salinity effects on alfalfa plants.

Si is the second most abundant element in the Earth's crust (Sutton et al. 2018). It has been reported that its presence has several beneficial effects on plants against a variety of abiotic stresses including salinity (Debona et al. 2017). One of its most fascinating roles lies is its deposition in plant cell walls and modification of cell turgor in association with decreasing stomatal conductance (*gs*) under stress (Coskun et al. 2019). Moreover, several benefits of Si have been described by Meng et al. (2020), where Si application alleviated the negative impact of salt, and increased plant biomass, photosynthesis and water use efficiency of alfalfa. Furthermore, exogenous Si application was found to enhance biological nitrogen fixation (BNF) under several abiotic stressors, and that was demonstrated by the improved nodule number, nitrogenase activity, leghemoglobin content resulting in a higher nitrogen (N) content (Garg and Singh 2018; Kurdali et al. 2019; Etesami and Adl 2020). Additionally, salt-mediated oxidative stress in alfalfa was found alleviated by exogenous Si as reflected by low malonyldialdehyde (MDA), hydrogen peroxide (H_2O_2) and peroxide (O_2^-) content (Meng et al. 2020). The reduction in oxidative stress upon Si treatment has been reported by several authors to be mediated by the induction of the antioxidant activities (Kim et al. 2017). This includes the activity of related enzymes (ascorbate peroxidase (APX), SOD, CAT, etc.) and the contents of the non-enzymatic antioxidants (Ascorbic acid, glutathione, etc.) (Eraslan et al. 2008; Hasanuzzaman et al. 2018; Meng et al. 2020). Besides, Si was demonstrated effective in reducing Na^+ and Cl^- uptake into plant under salt stress (Tuna et al. 2008). Furthermore, Si has also been reported to reduce salt ion translocation to the aerial parts preventing the deleterious effect of Na^+ on photosynthetic machinery (Abbas et al. 2015). These positive effects have been reported mostly in monocot, which are considered high Si accumulator, but also in some dicots including legumes (Al Murad et al. 2020).

Although the protective role of exogenous Si under salinity is documented on several plant species including alfalfa and other legumes (Etesami and Adl 2020; Meng et al. 2020; Wang and Han 2007), the role of Si in stress tolerance in relation with symbiosis establishment and N fixation remains to be established. Therefore, the main objective of this study was to evaluate whether Si application alleviates salt stress in alfalfa-rhizobia symbioses in terms of nodulation and N fixation involving three alfalfa varieties and an *Ensifer meliloti* Rm41 strain, with contrasting sensitivity to salt stress with the hypothesis that Si can improve the N_2 fixing symbiosis performance under this constraint. The study also aimed (i) to assess the protective roles of Si on NaCl damaging effects in terms of plant biomass, RWC, and photosynthesis in terms of Chl content, *gs* and quantum yield of photosystem II (PS II) (ii) and to explore the ability of Si to protect alfalfa plants from salt toxicity by evaluating membrane integrity in terms of MDA content and electrolyte leakage (EL), ROS detoxification in terms of H_2O_2 and O_2^-, compatible osmolytes, antioxidative defense systems and nutrient homeostasis in terms of Na^+, K^+, calcium (Ca^{2+}) and Si contents.

2 Materials and methods

2.1 Plant material

This study was carried out using two Moroccan alfalfa (*Medicago sativa* L.) varieties, *Oued Lmaleh* (*OL*) from Oasis and *Demnate 201 (Dm*) from High Alas Mountains, and a European variety *NS Mediana ZMS V (NS Med).* In a previous study, the two varieties *OL* and *Dm* have been characterized as salt tolerant, whereas *NS Med* as sensitive according to seed germination test and seedlings growth (unpublished data). The two varieties *OL* and *Dm* are among the most

cultivated varieties in traditional Moroccan agroecosystems. These varieties showed their good adaptation to the local habitats due to natural and human selection. The seeds were supplied by the National Institute of Agronomic Research (INRA-Marrakesh).

2.2 Rhizobial strain isolation, characterization and identification

Ensifer meliloti strain Rm41 was isolated from the nodules of *M. sativa* grown in the Beni-Mellal region in Morocco. The isolate was plated on solid Yeast Extract Mannitol (YEM) medium supplemented with 25 µg mL^{-1} Congo Red according to Vincent (1970) for 48 h at 28 °C. Bacterial culture was repeated several times by single colony streaking on YEM medium until pure and easy to characterize cultures were obtained. The isolate was subjected to infectivity test with alfalfa seedlings and selected for its ability to grow under 1.02 M NaCl and 20% polyethylene glycol (PEG-6000) and its ability to solubilize tricalcium phosphate (Fahsi et al. 2021), exopolysaccharides and indole acetic acid production (Dueñas et al. 2003; Elhaissoufi et al. 2020) and to promote N_2 fixing (Geetha et al. 2014) (Supplemental Table 1). Alfalfa seeds germination percentage has been improved by 12% under 200 mM NaCl in the presence of the rhizobia isolate (unpublished data). The rhizobia isolate was identified as *E. meliloti* strain Rm41 (accession number: CP021808.1) using the housekeeping gene *gyrB* (Farssi et al. 2021).

2.3 Plant growth conditions and treatments

The present study was conducted in a growth chamber at the Polydisciplinary Faculty (Sultan Moulay Slimane University, Morocco) with the following conditions, 25 ± 1 °C, 60%—80% relative humidity, 16 h photoperiod (74 µmol photons s^{-1} m^{-2}). Seeds were surface-sterilized with sodium hypochlorite (6%) for 5 min and then rinsed five times with sterile deionized water. Germination of 10 seeds was carried out in 10 cm diameter, 15 cm tall plastic pot containing sterilized perlites (50 g per pot) with the following characteristics: granule size, 0–6 mm; pH 6.5–8.0; EC 0.0–0.1 µS cm^{-1}. Two weeks after sowing, five homogenous seedlings were selected per pot and inoculated three times with the rhizobial strain *E. meliloti* Rm41. The inoculum was prepared by growing the Rm41 strain in liquid YEM medium for 3 days at 28 °C to an approximate cell density of 10^8 bacteria per mL. Seedlings were irrigated weekly with a nitrogen (N) free nutrient solution (Hoagland and Arnon 1950) [KH_2PO_4 (250 µmol), $MgSO_4$ (1000 µmol), K_2SO_4 (750 µmol), $CaCl_2$ (1650 µmol), Fe-EDTA (16 µmol), $MnSO_4$ (6 µmol), H_3BO_3 (4 µmol), $ZnSO_4$ (1 µmol), $NaMoO_4$ (0.1 µmol), and $CuSO_4$ (1 µmol)]. Urea (2 mM) was supplied the initial week of growth to avoid N deficiency during nodule development. Thereafter, the plants were irrigated by N-free nutrient solution with NaCl concentration increased gradually by 40 mM every two days up to 120 mM. Preliminary experiments having shown that 3 mM of Si is the best concentration for improving alfalfa plant growth. Therefore, the present work was designed with 3 mM of Si. After five weeks of stress, parameters related to growth, nodulation, photosynthesis, osmotic adjustment, oxidative stress metabolism and nutrient element were assessed. Each pot was planted with five plants, and each treatment was represented by four replicates, resulting in a total of 48 pots and 240 plants.

2.4 Plants growth, nodulation and physiological parameters

To assess the effect of Si on plant growth under salt stress, plant height and leaf number were determined. The effect of Si on nodule formation under salt stress was also assessed by counting the number of nodules immediately after the harvest in three plants of each treatment. Fresh weight (FW) was determined. Shoots and roots were dried at 80 °C for 48 h and dry weight (DW) was determined in five random plants per pot and grouped as three replicates.

Salt tolerance index (STI) was calculated in three replicates as described by Bağci et al. (2003) using the formula underneath

$$\text{STI} : \frac{\text{TDW at Sx}}{\text{TDW at S}^1} \times 100$$

With TDW is the total dry weight, S_x x treatment (NaCl or NaCl × Si) and S_1 control treatment.

Leaf relative water content (RWC) was determined in well-developed leaves as previously described (Ghoulam et al. 2002). Fresh leaf discs of 10 mm diameter were weighted to determine their FW. Then, they were immersed in distilled water for 6 h to reach full turgidity, and their turgid weight (TW) was determined after wiping excess water from the surface of the leaf discs. Then, the samples were dried for 24 h at 70 °C and their DW was determined. RWC was defined as follow:

$$RWC(\%) = \frac{\text{FW} - DW}{\text{TW} - DW} \times 100$$

Photosynthetic pigments were extracted according to Arnon (1949). Fresh leaf materiel (50 mg) was homogenized in a mortar using diluted acetone (80%) against 80% of acetone as a blank, centrifuged for 10 min at 10 000 x*g* and absorbance of supernatant was read at 480, 645 and 663 nm using a SP-UV1000 Spectrophotometer. The concentration of Chlorophyll a (Chl a), Chlorophyll b (Chl b),

Total Chl and carotenoids were calculated following formulas described in D'Souza and Devaraj (2013).

Chl stability index (CSI) was calculated in three replicates per treatment following the formula described in Sairam et al. (1997): $\text{CSI} = \frac{\text{Total Chl at } X \text{ treatment}}{\text{Total Chl under control}} \times 100$.

Chl fluorescence was measured after one month of salt stress in the third youngest leaf after 20 min of dark adaptation using a portable Chl fluorescence meter (Handy PEA, Hansatech, England). Six plants per treatment were considered and grouped as three replicates.

Using a porometer system (Leaf Porometer LP1989, Decagon Device, Inc., Washington, USA) under a temperature of 25 ± 1 °C and a relative humidity of $55 \pm 5\%$, stomatal conductance (g_s) was measured between 9 and 12 h a.m. on the second youngest leaf. Six plants per treatment were considered and grouped as three replicates.

For leaf area (LA), it was determined at the end of the stress period in three random leavs of three random plants from each treatment as described by Farssi et al. (2021). This parameter was assessed by image analysis using Mesurum softwar version 3.4.4.0. The leaves were cut and laid out on a white sheet containing a scale, and then they were scanned using a digital scanner.

2.5 Biochemical analyses

Malonyldialdehyde (MDA) was determined using the thiobarbituric acid (TBA) method (Heath and Packer 1968). One hundred milligram of fresh leaf was homogenized in 1 mL of TBA (0.5%) prepared in trichloroacetic acid (TCA) (20%). The homogenate was heated for 30 min at 95 °C, and then rapidly cooled in ice bath. Samples were centrifuged at 10 000 × *g* for 10 min at 4 °C. Supernatant was used to read the OD at 532 nm. The nonspecific reaction was subtracted by measuring the OD at 600 nm. MDA concentration was determined using its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as µmol MDA g^{-1} FW. Three replicates per treatment were considered for MDA determination.

Hydrogen peroxide (H_2O_2) content in leaves was determined as described previously by Patterson et al. (1984). Around 100 mg of fresh leaves were homogenized in 2 mL of cold acetone (-20 °C) using mortar and pestle. Titanium ($TiCl_2$) reagent (20% in concentrated hydrochloric acid (HCl)) was added to known volume of supernatant to get 2% of $TiCl_2$. Then, the Ti-H_2O_2 complex was precipitated by adding 0.2 mL of ammonia to each 1 mL of supernatant. Samples were centrifuged at 10 000 × g for 10 min at 4 °C and the precipitate was washed five times with cold acetone. The precipitate was then dissolved in 3 mL of 2 N sulfuric acid (H_2SO_4) and the OD was read at 410 nm. H_2O_2 content was determined using standard curves established with known H_2O_2 concentrations. Three replicates for each treatment were established.

For the electrolyte leakage (EL), fresh leaf samples were rinsed three times with deionized water to remove surface minerals. Samples were then placed in tubes containing 10 mL of deionized water and shaken for 24 h at 25 °C as described by Ghoulam et al. (2002). Then, the initial electrical conductivity (EC_1) was determined at 25 °C using a conductivity meter (DDS-12DW, Benchtop Conductivity Meter). Samples were then autoclaved for 20 min at 120 °C. Samples were left to cool, and the final electrical conductivity (EC_2) was carried out at 25 °C. The EL was determined in three replicates per treatment using below formula:

$$EL(\%) = \frac{EC_1}{EC_2} \times 100$$

H_2O_2 staining was determined in leaves using 3,3'-diaminobenzidine (DAB) method as described previously (Kumar et al. 2014). Leaves from different treatments were immersed in DAB solution (1 mg mL^{-1}) prepared in water (pH 3.8) and shaken at 200 rpm for an overnight. After draining off the staining solution, leaves were immersed in absolute ethanol and heated to remove the Chl. Samples were transferred on a paper towel saturated with 60% glycerol and photographed using a digital camera.

Superoxide (O_2^-) in leaves was detected by nitro blue tetrazolium (NBT) staining method (Kumar et al. 2014). Leaf samples were immersed for an overnight in 50 mM sodium phosphate buffer (pH 7.5) containing 0.2% (W/V) NBT. For proper visualization of the stain, Chl was removed by immersing the leaves in absolute ethanol and heating for 10 min. Leaves were transferred on a paper saturated with 60% glycerol and photographed using a digital camera.

Glycine betaine content was estimated according to Grieve and Grattan (1983). Dried plant material (0.25 g) was finely powdered and homogenized with 10 mL of deionized water for 48 h at 25 °C. Extracts were diluted with one volume of H_2SO_4 (2 N). Then, 0.5 mL was collected and cooled on ice for 1 h, before adding 200 µL of cold KI-I_2 reagent. Samples were stored at 4 °C for 16 h and then centrifuged at 10 000 rpm for 15 min at 4 °C. The supernatant was removed and periodite crystals were dissolved in 4 mL of 1,2-dichloroethane. The absorbance was read at 365 nm after 2 h. The results were expressed as mmol glycine betaine g^{-1} DW. Three replicates per treatment were performed.

Free proline was determined in 100 mg of fresh leaf after one month of stress according to the method described by Bates et al. (1973). Fresh plant material was homogenized in 3 mL of aqueous sulfosalicylic acid (3% w/v). The extract was centrifuged at 14 000 × *g* for 10 min at 4 °C. Equal volume of ninhydrin (3%) and glacial acetic acid were added to the supernatant. The mixture was incubated in a boiling

water bath for one hour. Samples were left to cool in an ice bath and the pink coloration was extracted with 2 mL of toluene. The optical density was measured at 520 nm. Proline content was determined using calibration curves and expressed as µmol g^{-1} FW. The results were expressed as a mean of three replicates per treatment.

Soluble sugar content was determined according to the method described previously (Dubois et al. 1956). Samples of 100 mg of fresh materiel were homogenized in 4 mL of ethanol (80%) with a mortar and pestle. After 10 min of centrifugation at 2 800 ×*g*, 1 mL of phenol (5%) and 5 mL of concentrated H_2SO_4 was added to 1 mL of the supernatant and the mixture was left to cool for 30 min. The optical density was measured at 485 nm and the levels of soluble sugars were determined using calibration curves established with reference glucose solutions and expressed as mg g^{-1} FW. The results were expressed as a mean of three replicates per treatment.

Total polyphenols were extracted using Folin-Ciocalteu (FC) assay (Skotti et al. 2014). One hundred milligram of fresh leaves was homogenized in 1 mL of methanol (80%) at 4 °C. Homogenate was centrifuged at 12 000 ×*g* for 20 min at 4 °C. Then, to 50 µL of supernatant, 250 µL of FC reagent and 4.7 mL of distilled water were added. After 3 min of incubation at room temperature, the volume was adjusted to 6.5 mL with Na_2CO_3 (20%). The mixture was incubated in the dark for 1 h then the OD was read at 725 nm. Gallic acid was used to make the calibration curve and the results were expressed as milligrams of gallic acid equivalent per g^{-1} FW. For each treatment, three replicates were considered.

The aluminum chloride ($AlCl_3$) assay was used to determine the content of flavonoids in 100 mg of fresh material (Chang et al. 2002). Leaves were homogenized in 1 mL of methanol (80%) at 4 °C. The extract was centrifuged at 12 000 ×*g* for 20 min. 100 µL of the result supernatant was diluted in 300 µL of methanol (95%) and then 20 µL of $AlCl_3$ (10%) and 20 µL of potassium acetate (1 M) were added. The volume was adjusted to 1 mL with distilled water and the mixture was incubated for 30 min at room temperature. OD was read at 415 nm, and the flavonoids content was determined using calibration curve established with known concentration of quercetin. Results were expressed as mean of three replicates.

For anthocyanins, 20 mg of fresh leaf was incubated in 0.5 mL of methanol/HCl (99/1, v/v) at 4 °C for 24 h. After 10 min of centrifugation at 6 000 x*g*, the optical density of supernatant was measured at 530 and 657 nm. The anthocyanin content was calculated using the following equation established by Panuccio et al. (2016):

$$\text{Anthocyanin } (\mu g\, g^{-1} FW) = \frac{[\text{A530 nm} - (0.025 \times \text{A657 nm}) \times \text{mL of extract}]}{\text{g fresh weight}}$$

2.6 Enzymatic antioxidants activities determination

Fresh leaves (0.5 g) were ground at 4 °C in 5 mL of 50 mM phosphate buffer (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged for 20 min at 20 000 ×*g* at 4 °C and the supernatant was used for superoxide dismutase (SOD) (EC 1.15.1.1) assay. SOD activity was assayed according to the method described by Beyer and Fridovich (1987). 30 µL of enzyme extract was added to 1 mL of 50 mM phosphate buffer (pH 7.8) containing NBT (65 µM), L-methionine (13.3 µM) and riboflavin (1.33 µM). The mixture was exposed to a fluorescence lamp for 5 min and then the OD was measured at 560 nm. One enzymatic unit (EU) of SOD was defined as the amount required for the inhibition of 50% NBT. The activity was expressed as EU min^{-1} mg^{-1} protein.

Polyphenol oxidase (PPO) (EC 1.14.18.1) was assayed by following the oxidation of catechol at 410 nm (Hori et al. 1997). 100 mg of fresh material was homogenized at 4 °C in 1 mL of 50 mM phosphate buffer (pH 6) with 5% PVPP. Supernatant was collected after 20 min of centrifugation at 12 500 ×*g* at 4 °C and used for PPO assay. Enzyme extract was added to 1 mL of 0.1 M phosphate buffer (pH 6) contained 10 mM catechol and the increase in OD at 410 nm was followed for 3 min.

2.7 Nutrients analysis

Plant nitrogen (N) concentration was determined according to the Kjeldahl method. Dry material (0.5 g) was put in matrass tubes and added by 5 mL of concentrated H_2SO_4 and 2 g catalyst and then digested for 4 h at 420 °C. The distillation was done through 10 mL of boric acid and 20 mL of NaOH. The total N concentration was determined by titration of 5 mL of the distillate by H_2SO_4 (0.01 mol L^{-1}) using bromocresol green and methyl red as color indicators. Nitrogen content index (NCI) was calculated for the treated alfalfa plants relative to controls using the following formula

$$NCI(\%) = \frac{\text{Total N content at x treatment}}{\text{Total N content under control}} x100$$

For Si, potassium (K^+), sodium (Na^+) and calcium (Ca^{2+}), samples were prepared according to Liu et al. (2013) with slight modifications. Plant ashes were dissolved in a mixture of concentrated HCl/nitric oxide (2/1, v/v) and heated for one hour at 200 °C. Then the mixture was adjusted to 100 mL using deionized water and filtrated using filter paper. The resulting solution was used for nutrient analyses using inductively coupled plasma

optical emission spectroscopy (Optima 8000 ICP-OES). Blank was prepared by applying the same procedure and reagent solutions without sample.

2.8 Data analysis

Statistical analysis was performed using SPSS version 22. It concerned a three-way analysis of variance (ANOVA III), where varieties, salinity and silicon are the independent variables. Means were compared using Tukey's test.

3 Results

3.1 Effects of salt and Si on growth and nodulation of alfalfa plants

Salt stress caused a significant decrease in fresh weight (FW) in both shoots and roots relative to untreated control alfalfa plants (Table 1) (Supplemental Table 2). As compared to the corresponding controls, *NS Med* variety was the most affected in both shoots and roots with a reduction of FW of 80% and 83% respectively, whereas the reduction of FW for *OL* variety was only 44% for the shoots and 38% for the roots. However, Si supplementation to salt-stressed alfalfa plants resulted in a higher FW compared to salt stressed plants in the three tested varieties. A maximum effect of Si supplementation was observed in *NS Med* variety shoots FW (plus 265% FW) and in *Dm* variety roots FW (plus 472%) compared to salt-stressed plants. When applied to unstressed alfalfa plants, Si impaired SFW in the three alfalfa varieties and RDW in both *Dm* and *NS Med*. Statistical analysis (Supplemental Table 2) indicated that all factors (variety, salinity and Si) and their interactions were significant.

Dry biomass (DW) production was severely decreased under salinity treatment in both shoots and roots parts and for the three studied alfalfa varieties. Indeed, DW of *NS Med* variety seemed the most affected by salt stress with reduction of 80% and 88% respectively for shoots and roots, whereas DW of *Dm* and *OL* varieties were the less affected by salt stress in shoots and roots (Table 1). DW in Si-treated salt-stressed plants was higher than in their salt-stress counterparts, especially in *NS Med* shoots (plus 211%) and in *Dm* roots (plus 515%). However, under normal condition, Si incorporation to the growth medium significantly decreased DW of both shoot and root in alfalfa plants. When DW was measured, a significant interaction ($P < 0.001$) was also observed for salinity × variety, salinity × Si and salinity × variety × Si. However, in DW, contrarily to FW, variety × Si interaction was not significant (Supplemental Table 2).

The impact of NaCl and Si on the number of nodules of alfalfa plants is shown in Fig. 1. Our results unveiled

Table 1 Effect of exogenous Si (3 mM) application on shoot (SFW) and root (RFW) fresh weights, shoot (SDW) and root (DRW) dry weights in three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under either salt stress (120 mM NaCl) or non-stress conditions

		SFW (mg plant^{-1})		RFW (mg plant^{-1})		SDW (mg plant^{-1})		RDW (mg plant^{-1})	
		-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl
OL	-Si	38.80 ± 1.00^{b}	21.70 ± 0.70^{d}	39.50 ± 1.30^{c}	24.60 ± 1.60^{d}	12.40 ± 0.60^{b}	5.40 ± 1.20^{c}	5.00 ± 1.20^{bc}	4.30 ± 0.60^{c}
	+Si	26.10 ± 1.40^{c}	63.20 ± 2.10^{a}	88.00 ± 4.00^{b}	128.50 ± 15.50^{a}	7.30 ± 1.30^{c}	15.60 ± 0.80^{a}	7.50 ± 1.10^{ab}	9.00 ± 1.80^{a}
Dm	-Si	82.70 ± 4.60^{a}	18.10 ± 1.40^{d}	144.20 ± 5.80^{a}	24.20 ± 0.80^{c}	17.80 ± 1.70^{a}	8.60 ± 0.90^{b}	10.00 ± 1.10^{ab}	2.00 ± 0.20^{c}
	+Si	35.70 ± 1.00^{b}	26.30 ± 2.00^{c}	103.90 ± 8.10^{b}	138.60 ± 1.20^{a}	9.80 ± 0.40^{b}	8.40 ± 2.00^{b}	8.40 ± 1.20^{b}	12.30 ± 0.50^{a}
NS Med	-Si	94.70 ± 1.40^{a}	19.40 ± 0.50^{d}	151.10 ± 1.40^{a}	26.00 ± 0.70^{d}	17.30 ± 1.40^{a}	3.40 ± 0.40^{c}	21.60 ± 0.10^{a}	2.70 ± 0.10^{c}
	+Si	34.40 ± 1.00^{c}	70.90 ± 7.00^{b}	119.90 ± 4.60^{b}	107.80 ± 0.50^{c}	8.80 ± 1.40^{b}	10.60 ± 2.70^{b}	7.50 ± 1.20^{b}	7.10 ± 2.00^{b}

-NaCl, 0 mM NaCl treatment; +NaCl, 120 mM NaCl treatment; -Si, 0 mM $CaSiO_3$ treatment; +Si, 3 mM $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation and the letters indicate statistically significant values

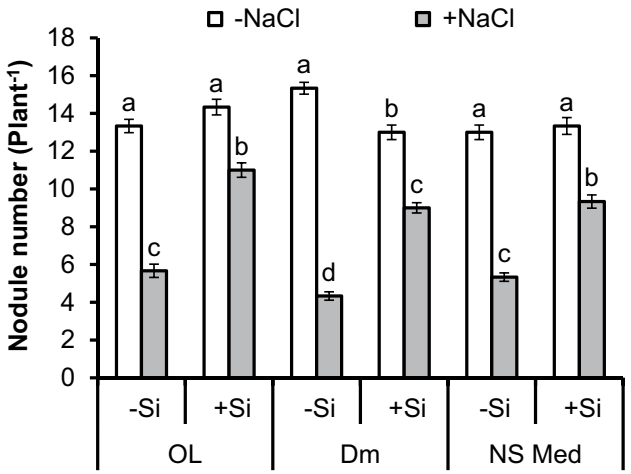

Fig. 1 Effect of exogenous Si (3 mM) application on nodule number of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*) under either salt stress (120 mM NaCl) or non-stress conditions. -NaCl, 0 mM NaCl treatment; + NaCl, 120 mM NaCl treatment; -Si, 0 mM $CaSiO_3$ treatment; + Si, 3 mM $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

the fact that the addition of NaCl to the medium severally reduced the number of nodules in the three alfalfa varieties. The reduction rate was significantly higher in *Dm* (72%) than in both *OL* (57%) and *NS Med* (59%). However, 3 mM Si supplementation significantly increased the number of nodules of alfalfa under salt stress (Supplemental Fig. S1). This increase was most spectacular for *Dm* variety, which had the highest increament rate of 108% relative to Si-untreated salt stressed plants. Under normal conditions Si increased the number of nodules, except for the *Dm* variety. The interactive effect of salinity and silicon was statistically significant (Supplemental Table 2).

Plant height of alfalfa plants was considerably lower under salt stress than without salt (Fig. 2a). *NS Med* showed the most important diminution, minus 37% compared to the untreated control. However, Si supplementation alleviated the adverse effect of NaCl and significantly improved plant height in all of the three studied varieties ($P \leq 0.001$; Supplemental Table 2). Applied Si increased plant height by 43%, 22% and 24% in *OL*, *Dm* and *NS Med* respectively compared to plants treated by NaCl alone. Si supply to the growth medium of the unstressed alfalfa plants increased plant height only in *OL*. Significant interactions were observed for NaCl × Si, NaCl × variety, Si × variety and NaCl × Si × variety ($P \leq 0.001$; Supplemental Table 2).

Salt stress significantly reduced the number of leaves of alfalfa plants (Fig. 2b) ($P \leq 0.001$; Supplemental Table 2). Compared to untreated controls, leaf number was reduced under salt stress by 52% in *NS Med*, whereas in *OL* the reduction did not exceed 35%. Applied Si to salt-stressed plant significantly improved the number of leaves in the three studied varieties with a maximum increase of 43% in *OL*. However, in non-stressed plants, Si showed a negative effect on the number of leaves particularly in *OL* where the reduction reached 25% relative to the untreated control. Significant interactions ($P \leq 0.001$; Supplemental Table 2) were observed for NaCl × variety and NaCl × Si.

Salinity and Si treatments affected the leaf area (LA). Under salt stress, alfalfa LA decreased significantly in the three varieties with the highest reduction rate of 37% observed for *OL* variety relative to the untreated control (Fig. 3; Supplemental Fig. S2). However, the application of Si improved the LA of *OL*, *Dm* and *NS Med* by 9%, 32% and 13% respectively, relative to Si-untreated salt stressed plants. Likewise, under normal condition, Si addition improved LA of *Dm* alfalfa plants by 38% relative to the untreated control. However, Si application has a negative impact on LA of *OL* (19%) and *NS Med* (17%) compared to the control without Si (Fig. 3). Significant interaction ($P \leq 0.001$; Supplemental Table 2) of variety × silicon was observed for LA.

Results related to statistical analysis (See supplemental Table 2) revealed that NaCl × variety, NaCl × Si and variety × Si interactions were significant ($P \leq 0.001$) for the salt

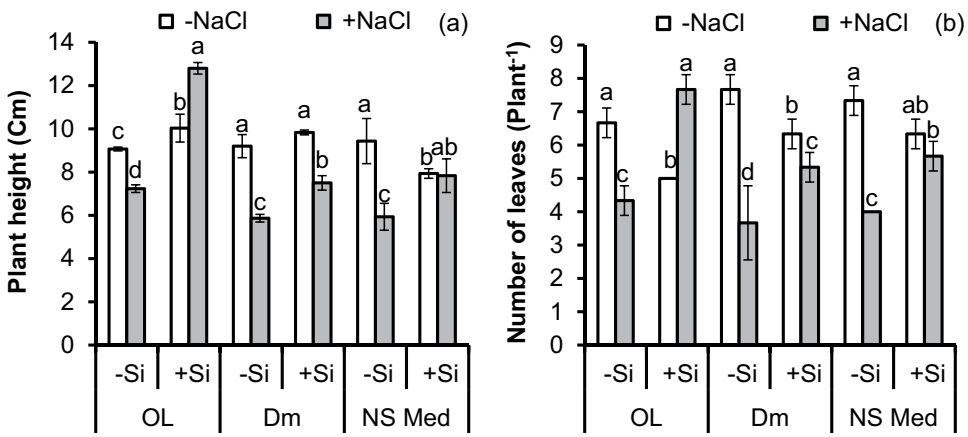

Fig. 2 Effect of salinity (120 mM NaCl) and exogenous Si (3 mM) on plant height (**a**) and number of leaves (**b**) of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*). -NaCl, 0 mM NaCl treatment; + NaCl, 120 mM NaCl treatment; -Si, 0 mM $CaSiO_3$ treatment; + Si, 3 mM $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

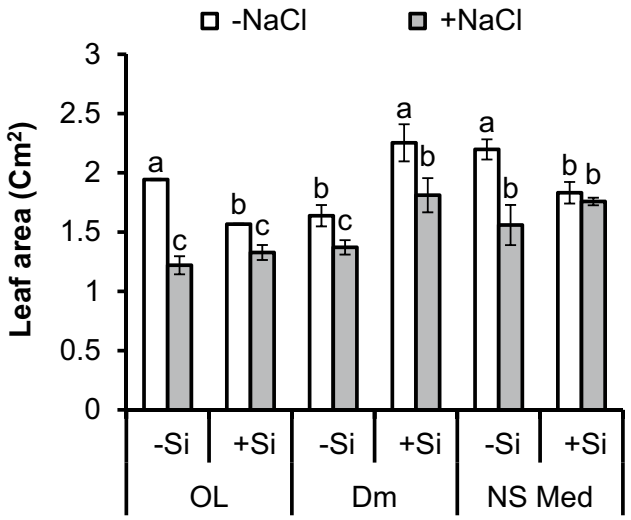

Fig. 3 Effect of exogenous Si (3 mM) application on leaf area (LA) of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*) under either salt stress (120 mM NaCl) or non-stress conditions. -NaCl, 0 mM NaCl treatment; + NaCl, 120 mM NaCl treatment; -Si, 0 mM $CaSiO_3$ treatment; + Si, 3 mM $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

tolerance index (STI), while the interaction NaCl × variety × Si was not significant ($P \geq 0.05$). Results in Fig. 4 indicated that Si-untreated salt-stressed alfalfa plants exhibited low value of STI, whereas a significant ($P \leq 0.001$) increase of STI was recorded in salt-stressed plant supplied with Si.

3.2 Effect of salt and Si on physiological performance of alfalfa plants

Leaf RWC significantly decreased under salt stress (Fig. 5). RWC were lower after salt stress, from 58.30 to 52.57 for *OL*, from 63.40 to 55.98 for *Dm* and from 72.43 to 59.06 for *NS Med*. Si supplementation improved RWC in alfalfa varieties as compared to Si-untreated plants. The strongest effect of Si treatment on RWC was found in *Dm* by 15% improvement, followed by 12% for *OL* and *NS Med* relative to Si-untreated salt stressed plants. Interestingly, Si incorporation to the growth medium of alfalfa plants having 0 mM NaCl elevated remarkably RWC in *OL* and *Dm* alfalfa varieties (Fig. 5).

The effect of Si and salinity on photosynthetic pigments of alfalfa plants is shown in Table 2. The results of the present work indicated that photosynthetic pigments (Chl a, Chl b, total Chl and carotenoids) were significantly reduced in salt-stressed alfalfa plants ($P \leq 0.001$; Supplemental Table 2). Compared to the other varieties, *NS Med* was the most affected with reduction rates of 30%, 46%, 37% and 58% for Chl a, Chl b, total Chl and carotenoids respectively, relative to untreated controls. Conversely, addition of Si along with NaCl significantly improved all the photosynthetic pigment contents. When supplemented solely with Si in controls, no significant difference was noted as compared to the corresponding untreated controls. ANOVA three-way (Supplemental Table 2) indicated a significant interaction for variety × NaCl, NaCl × Si and variety × NaCl × Si, whereas the interaction variety × silicon was not significant.

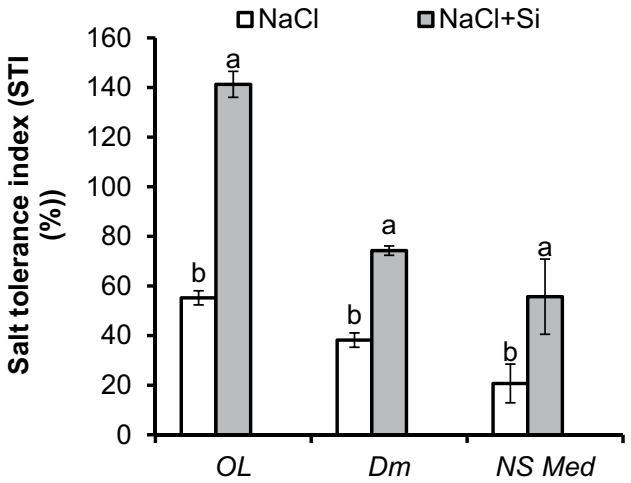

Fig. 4 Salt tolerance index of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*) grown under 120 mM NaCl combined or not with 3 mM of $CaSiO_3$. NaCl, NaCl treatment; NaCl + Si, combination of NaCl and Si treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

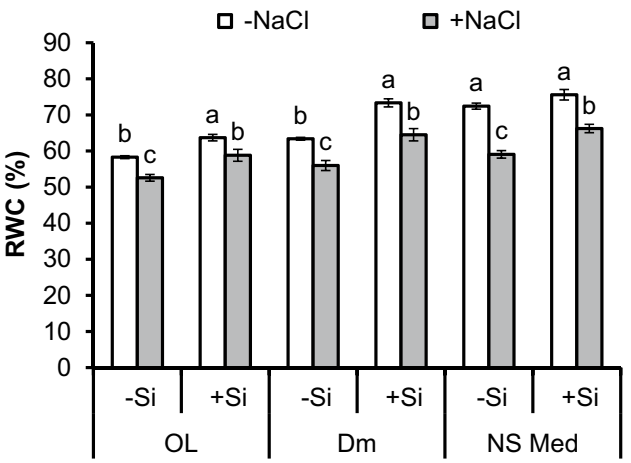

Fig. 5 Effect of exogenous Si (3 mM) application on leaf relative water content (RWC) of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*) under either salt stress (120 mM NaCl) or non-stress conditions. -NaCl, 0 mM NaCl treatment; + NaCl, 120 mM NaCl treatment; -Si, 0 mM $CaSiO_3$ treatment; + Si, 3 mM $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

Table 2 Effect of salinity (120 mM NaCl) and exogenous Si (3 mM) on chlorophyll (Chl) a, Chl b, total Chl and carotenoids (Car) in three *M. sativa* varieties (*OL*, *Dm* and *NS Med*)

		Chl a (mg g^{-1} FW)		Chl b (mg g^{-1} FW)		Total Chl (mg g^{-1} FW)		Car (mg g^{-1} FW)	
		-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl
OL	-Si	8.23 ± 0.31^{b}	6.73 ± 0.08^{c}	7.31 ± 0.07^{a}	5.45 ± 0.25^{b}	15.54 ± 0.36^{b}	12.18 ± 0.33^{c}	30.50 ± 0.94^{b}	26.06 ± 1.52^{c}
	+Si	9.77 ± 0.63^{a}	8.24 ± 0.23^{b}	7.15 ± 0.34^{a}	7.50 ± 0.46^{a}	16.92 ± 0.97^{a}	15.74 ± 0.41^{ab}	32.92 ± 0.79^{a}	29.31 ± 0.71^{b}
Dm	-Si	7.23 ± 0.09^{a}	5.69 ± 0.22^{b}	7.05 ± 0.34^{a}	4.80 ± 0.44^{c}	14.29 ± 0.43^{a}	10.49 ± 0.66^{c}	31.83 ± 1.41^{a}	16.61 ± 1.23^{d}
	+Si	7.46 ± 0.09^{a}	7.01 ± 0.33^{a}	7.05 ± 0.50^{ab}	6.41 ± 0.24^{b}	14.52 ± 0.51^{a}	13.42 ± 0.42^{ab}	29.40 ± 0.90^{b}	24.48 ± 0.95^{c}
NS Med	-Si	7.25 ± 0.11^{a}	5.11 ± 0.13^{b}	6.94 ± 0.47^{a}	3.78 ± 0.23^{b}	14.19 ± 0.55^{a}	8.88 ± 0.23^{b}	30.91 ± 0.92^{b}	13.01 ± 1.04^{d}
	+Si	7.29 ± 0.29^{a}	7.48 ± 0.24^{a}	6.42 ± 0.38^{a}	6.49 ± 0.23^{a}	13.71 ± 0.08^{a}	13.97 ± 0.46^{a}	33.75 ± 0.59^{a}	19.88 ± 1.40^{c}

-NaCl, 0 mM NaCl treatment; +NaCl, 120 mM NaCl treatment; -Si, 0 mM $CaSiO_3$ treatment; +Si, 3 mM $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation and the letters indicate statistically significant values

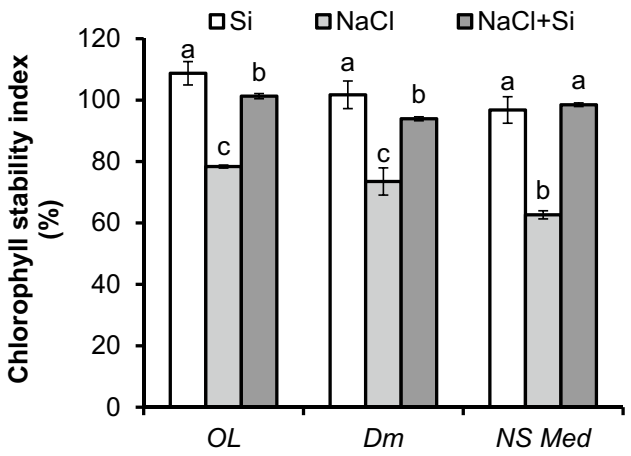

Fig. 6 Effect of salinity (120 mM NaCl) and exogenous Si (3 mM) on chlorophyll stability index of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*). Si, 3 mM $CaSiO_3$ treatment; NaCl, 120 mM NaCl treatment; NaCl + Si, combination of NaCl and $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

Results presented in Fig. 6 showed the effect of the applied treatments (Si and NaCl) on Chl stability index (CSI) of alfalfa plants. Our present finding indicated that CSI reduced significantly ($P \leq 0.01$) in the three alfalfa varieties upon salt stress. However, application of Si along with NaCl significantly improved this parameter in alfalfa plants. Furthermore, Si applied solely to control plants induced a significant increase of this parameter in all of the three used varieties.

Significant decrease ($P \leq 0.001$) of photosynthetic parameter F_v/F_m was measured in the three studied alfalfa varieties under salt stress as shown in Fig. 7a. Indeed, under salt stress, the comparison between different studied alfalfa varieties revealed that the lowest F_v/F_m values were recorded in *Dm* and *NS Med* (0.75 and 0.76 respectively), while the *OL* variety was less affected by salt stress with an F_v/F_m value of 0.78. Furthermore, we have observed that when plants were treated with Si along with salt stress, they displayed higher values of F_v/F_m than in Si-untreated salt-stressed plants. Indeed, under the combined effect of salt and Si, the

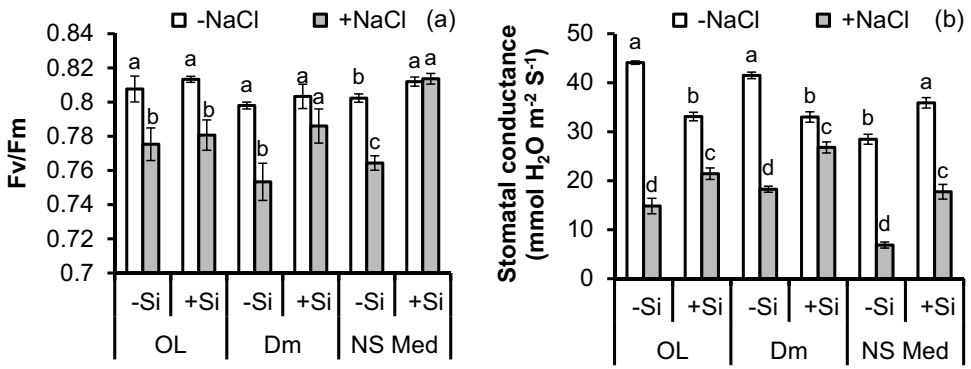

Fig. 7 Effect of salinity (120 mM NaCl) and exogenous Si (3 mM) on Maximum quantum yield of PS II (F_v/F_m) (**a**) and stomatal conductance (*gs*) (**b**) of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*). –NaCl, 0 mM NaCl treatment; +NaCl, 120 mM NaCl treatment; -Si, 0 mM $CaSiO_3$ treatment; +Si, 3 mM $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

F_v/F_m values were 0.79, 0.81 and 0.78 respectively in *Dm*, *NS Med* and *OL*. In addition, applied Si solely to untreated alfalfa plants showed similar F_v/F_m values to that of controls.

Salinity induced a marked decline in stomatal conductance (g_s) in alfalfa plants, with the lowest g_s noted for *NS Med* variety compared to untreated controls (Fig. 7b). On the other hand, Si addition increased g_s in the three studied varieties. Compared to salt-stressed alfalfa plants, 3 mM Si application in combination with NaCl treatment significantly increased g_s in *OL*, *Dm* and *NS Med* varieties by 31%, 32% and 61% respectively. However, Si supplied to the unstressed alfalfa plants increased g_s only in *NS Med*. For this parameter, there were significant interactions between NaCl × Si, NaCl × variety, Si × variety and NaCl × Si × variety ($P \leq 0.001$; Supplemental Table 2).

3.3 Effects of salt and Si on the biochemical parameters of alfalfa

Table 3 indicated that when alfalfa plants were subjected to 120 mM NaCl, MDA increased remarkably in the three varieties but decrease in concentrations was observed in Si treated plants. In the treatment with 120 mM NaCl, MDA content decreased in Si-treated *OL* (plus 16%), *Dm* (plus 31%) and *NS Med* (plus 26%) relative to Si-untreated plants. For *OL* and *NS Med* varieties, there was no significant difference between Si-treated and untreated controls. Statistical analysis showed that the interaction salinity × Si was significant ($P < 0.01$; Supplemental Table 2).

H_2O_2 content significantly increased in alfalfa plants grown under salt stress as compared to those grown under normal condition (see Table 3). In *NS Med* and *Dm*, H_2O_2 content elevated 4.02-fold under salt stress as compared to control, while in *OL* it was only 1.61-fold higher. Alfalfa plants treated exogenously with Si showed less H_2O_2 concentration compared to Si-untreated salt stressed plants. Indeed, when Si along with NaCl, H_2O_2 content decreased by 57%, 33% and 70% respectively in *OL*, *Dm* and *NS Med*. No significant difference was noted between Si-treated and untreated control. Statistical analysis indicated that salinity effect was significant (Supplemental Table 2).

Results in Table 3 showed that salinity significantly increased EL in the three studied alfalfa varieties, with a particular high EL value observed in *NS Med* (40.33%). However, this effect was alleviated by Si application. As compared to salt stressed plants, EL was decreased by 27%, 24% and 27% in *OL*, *Dm* and *NS Med* respectively. No significant difference was noted between Si-treated and untreated *OL* control. Significant ($P \leq 0.001$; Supplemental Table 2) interactions were observed for variety × salinity and salinity × Si.

The visual distribution of H_2O_2 in alfalfa leaves is shown in Fig. 8a. H_2O_2 accumulated in leaves of the three varieties in response to salt stress. Although, the staining intensity was not quantified, H_2O_2 accumulation under salt stress appeared higher in *OL* and *Dm* varieties than in *NS Med*. However, this effect seems to be alleviated by Si supplementation as the brown coloring indicative of H_2O_2 are not visible in the leaves of salt-stressed alfalfa treated with Si. Under unstressed conditions, plants of the three varieties did not stain brown with DAB.

Leaves of the three alfalfa varieties grown either under control or Si treatment did not stain blue with NBT. However, under salt treatment, leaves were stained blue with NBT, indicative of the presence of superoxide, particularly in those *OL* and *NS Med* (Fig. 8b). In contrast, although the presence of salt, the intensity of the staining was decreased in the three alfalfa varieties in response to Si supplementation.

Glycine betaine content increased significantly ($P \leq 0.001$; Supplemental Table 2) upon salt stress in all three alfalfa varieties relative to untreated controls with highest accumulation noted in *NS Med* variety (7.66 mmol g^{-1} DW) (Table 4). In addition, in *OL* variety, application of Si along with NaCl induced a further increase in glycine betaine content, whereas in *Dm* and *NS Med* there were no significant difference between plants treated with salt stress alone and those treated with both Si and NaCl. Furthermore,

Table 3 Effect of exogenous Si (3 mM) application on malonyldialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents and electrolyte leakage (EL) in three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under either salt stress (120 mM NaCl) or non-stress conditions

		MDA (ηmol g^{-1} FW)		H_2O_2 (ηmol g^{-1} FW)		EL (%)	
		-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl
OL	-Si	34.76 ± 0.89^{c}	47.44 ± 0.83^{a}	0.23 ± 0.06^{c}	0.37 ± 0.05^{ab}	11.80 ± 1.10^{c}	27.65 ± 1.86^{a}
	+Si	36.17 ± 1.78^{bc}	39.48 ± 2.58^{b}	0.21 ± 0.09^{bc}	0.16 ± 0.03^{c}	11.74 ± 0.46^{c}	20.21 ± 1.03^{b}
Dm	-Si	22.57 ± 0.89^{c}	42.66 ± 0.75^{a}	0.29 ± 0.01^{c}	1.17 ± 0.01^{a}	8.67 ± 0.17^{d}	34.33 ± 0.28^{a}
	+Si	32.59 ± 2.66^{b}	29.34 ± 0.81^{b}	0.32 ± 0.08^{c}	0.78 ± 0.07^{b}	12.73 ± 0.55^{c}	26.20 ± 0.34^{b}
NS Med	-Si	29.71 ± 9.41^{bc}	47.73 ± 2.58^{a}	0.28 ± 0.10^{b}	1.13 ± 0.06^{a}	9.82 ± 0.94^{d}	40.33 ± 0.23^{a}
	+Si	27.44 ± 3.08^{c}	35.13 ± 1.13^{b}	0.43 ± 0.10^{b}	0.34 ± 0.11^{b}	16.74 ± 2.95^{c}	29.51 ± 4.90^{b}

-NaCl, 0 mM NaCl treatment; +NaCl, 120 mM NaCl treatment; -Si, 0 mM $CaSiO_3$ treatment; +Si, 3 mM $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation and the letters indicate statistically significant values

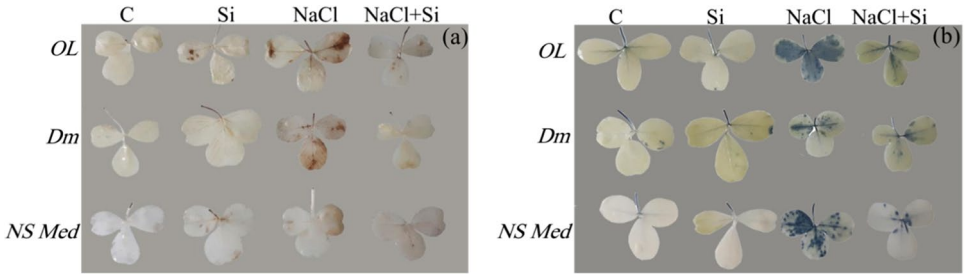

Fig. 8 Histochemical detection of hydrogen peroxide (H_2O_2) (**a**) and superoxide (O_2^-) (**b**) of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*) grown for one month under 120 mM NaCl and treated with 3 mM Si. C, control; NaCl, salt stress; Si, $CaSiO_3$ treatment; NaCl + Si, combination of salt stress and $CaSiO_3$ treatment. Brown and blue residue from diaminobenzidine and NBT staining indicates sites of H_2O_2 and O_2^- accumulation respectively

Table 4 Effect of salinity (120 mM NaCl) and exogenous Si (3 mM) on glycine betaine, proline and soluble sugars accumulation in three *M. sativa* varieties (*OL*, *Dm* and *NS Med*)

		Glycine betaine (mmol g^{-1} FW)		Proline (µmol g^{-1} FW)		Soluble sugars (mg g^{-1} FW)	
		-NaCl	+ NaCl	-NaCl	+ NaCl	-NaCl	+ NaCl
OL	-Si	2.13 ± 0.36^{c}	5.80 ± 0.63^{b}	0.79 ± 0.06^{c}	2.01 ± 0.12^{b}	37.08 ± 4.01^{d}	78.17 ± 5.06^{b}
	+ Si	2.31 ± 0.26^{c}	7.74 ± 0.45^{a}	0.54 ± 0.05^{d}	2.93 ± 0.09^{a}	63.45 ± 1.74^{c}	98.12 ± 2.60^{a}
Dm	-Si	2.24 ± 0.31^{b}	5.37 ± 0.16^{a}	0.63 ± 0.09^{b}	2.92 ± 0.14^{a}	37.53 ± 1.94^{d}	71.35 ± 1.15^{c}
	+ Si	2.00 ± 0.55^{b}	5.51 ± 0.08^{a}	0.64 ± 0.08^{b}	2.89 ± 0.17^{a}	79.50 ± 4.60^{b}	98.12 ± 4.90^{a}
NS Med	-Si	2.02 ± 0.35^{b}	7.66 ± 0.45^{a}	0.55 ± 0.08^{d}	2.99 ± 0.12^{b}	42.81 ± 2.17^{d}	100.83 ± 1.41^{b}
	+ Si	1.83 ± 0.45^{b}	7.94 ± 0.20^{a}	0.95 ± 0.05^{c}	3.38 ± 0.08^{a}	82.66 ± 2.37^{c}	114.22 ± 3.25^{a}

-NaCl, 0 mM NaCl treatment; + NaCl, 120 mM NaCl treatment; -Si, 0 mM $CaSiO_3$ treatment; + Si, 3 mM $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation and the letters indicate statistically significant values

application of Si solely in non-treated alfalfa plants showed similar glycine betaine content to that of untreated controls.

Under salt stress, proline content increased in three studied alfalfa varieties in comparison to untreated controls as indicated in Table 4. Proline highest accumulations were observed in *NS Med* and *Dm* (2.99 and 2.92 µmol g^{-1} FW respectively). Furthermore, when the exogenous Si was added to salt, proline content increased further, particularly in *NS Med* (3.38 µmol g^{-1} FW) and *OL* (2.93 µmol g^{-1} FW) compared to plants exposed to salt stress alone, whereas no significant difference was noted between *Dm* plants treated with NaCl and those treated with NaCl combined with Si. Addition of Si to non-stressed plant growing medium caused a slight reduction of proline content in *OL* comparing to the control. Under the same conditions, a slight increase in proline content was observed in *NS Med* variety.

Soluble sugars content also increased significantly in all of the three studied alfalfa varieties and that contents were more pronounced in Si-supplemented salt-stressed plants (Table 4) ($P \leq 0.001$; Supplemental Table 2). Highest soluble sugar contents were recorded in *NS Med*, with 101 and 114 mg glucose g^{-1} FW under salt and salt combined with Si respectively.

Table 5 showed that salt stress application resulted in an increase in the amount of total polyphenol with a significant difference between the varieties. The highest total polyphenol content of 101.11 mg gallic acid g^{-1} FW was shown in *Dm* variety compared to 75.33 mg gallic acid g^{-1} FW observed under control condition reflected an increment rate of 34%. Furthermore, when Si was applied to salt-stressed plants, total polyphenol content was further increased in the three alfalfa varieties. Indeed, under this condition, total polyphenol was increased by 16%, 11% and 18% respectively in *OL*, *Dm* and *NS Med* as compared to Si-untreated salt stressed plants. When applied to unstressed control plants, Si caused a significant decrease of total polyphenol in *OL* and *Dm*, while in *NS Med* significant increase was shown. Results of ANOVA (Supplemental Table 2) indicated that all factors (Variety, Si and salinity) and their interaction were significant ($P < 0.001$).

Salt stress significantly enhanced the flavonoids contents of the three alfalfa varieties as compared to unstressed plants (Table 5). In addition, the flavonoids content under salt stress was ranged differently among the varieties with the highest content of 12.08 mg quercetin g^{-1} FW observed for *Dm*. Furthermore, while the content of flavonoids did not change significantly in *Dm* under the

Table 5 Effect of exogenous Si (3 mM) application on total polyphenol, flavonoids and anthocyanin content in three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under either salt stress (120 mM NaCl) or non-stress conditions

		Total polyphenol (mg GA g^{-1} FW)		Flavonoids (mg quercitin g^{-1} FW)		Anthocyanin (µg g^{-1} FW)	
		-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl
OL	-Si	60.01 ± 0.99^{c}	68.91 ± 0.69^{b}	6.39 ± 0.09^{c}	7.66 ± 0.35^{b}	6.66 ± 0.18^{b}	3.73 ± 0.08^{d}
	+Si	28.84 ± 2.24^{d}	80.24 ± 0.37^{a}	4.67 ± 0.10^{d}	10.00 ± 0.16^{a}	8.07 ± 0.88^{a}	5.77 ± 0.55^{c}
Dm	-Si	75.33 ± 1.85^{c}	101.11 ± 0.80^{b}	7.60 ± 0.06^{b}	12.08 ± 0.28^{a}	7.85 ± 0.56^{a}	2.28 ± 0.45^{c}
	+Si	56.28 ± 1.89^{d}	111.77 ± 4.42^{a}	4.61 ± 0.16^{c}	12.01 ± 1.19^{a}	6.99 ± 1.21^{ab}	6.21 ± 0.66^{b}
NS Med	-Si	64.13 ± 1.24^{c}	75.85 ± 0.88^{b}	6.84 ± 0.07^{d}	9.91 ± 0.17^{b}	7.50 ± 0.18^{a}	2.01 ± 0.34^{c}
	+Si	75.51 ± 0.44^{b}	89.82 ± 2.36^{a}	8.29 ± 0.23^{c}	11.67 ± 0.04^{a}	6.72 ± 0.11^{b}	6.30 ± 0.46^{b}

GA, gallic acid; -NaCl, 0 mM NaCl treatment; +NaCl, 120 mM NaCl treatment; -Si, 0 mM $CaSiO_3$ treatment; +Si, 3 mM $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation and the letters indicate statistically significant values

combined effect of salt and Si, in *OL* and *NS Med* a further increase of 31% and 18% were observed respectively relative to Si-untreated salt stressed plants. Under unstressed conditions, unlike *NS Med* variety, Si significantly reduced flavonoids content in *OL* and *Dm*. Statistical analysis indicated that, except for Si, all factors and their interaction were significant ($P < 0.001$; Supplemental Table 2) for flavonoids.

Anthocyanin content was markedly reduced in salt-stressed alfalfa plants ($P \leq 0.001$; Supplemental Table 2). Compared to the other varieties, *NS Med* was the most affected variety by salt stress, as reduction rate reached 73% relative to untreated controls (Table 5). Conversely, supplying Si along with NaCl significantly improved anthocyanin content in the three alfalfa varieties plants with an obvious particular high improvement rate of 213% noted for *NS Med* relative to Si-untreated salt stressed plants. When supplemented solely with Si on controls, no significant difference was noted as compared to the corresponding untreated controls.

3.4 Effect of salt and exogenous Si on antioxidant enzymes of alfalfa

In the presence of salt, the activity of SOD increased significantly in the three alfalfa varieties (Fig. 9a). Indeed, as compared to controls, 120 mM NaCl treatment resulted in 4.88, 3.62 and 1.54-fold increases in the activity of SOD in *OL, Dm* and *NS Med* varieties respectively. Moreover, the activity of SOD in the three salt-stressed alfalfa varieties was further elevated when they were treated with 3 mM Si with 79, 86 and 36 EU mg $protein^{-1}$ for *OL*, *Dm* and *NS Med* respectively. Statistical analysis indicated a significant interaction for variety × salinity (Supplemental Table 2).

PPO activity was similar under normal conditions in the three alfalfa varieties (Fig. 9b). However, salt stress significantly reduced PPO activity in the three alfalfa varieties with *OL* and *NS Med* presented the most reduction rates of 34% and 33% respectively. Additionally, when salt stressed plants were treated with 3 mM Si, the activity was further decreased in *OL* and *Dm* varieties with no significant difference between Si-treated and untreated salt stressed *NS*

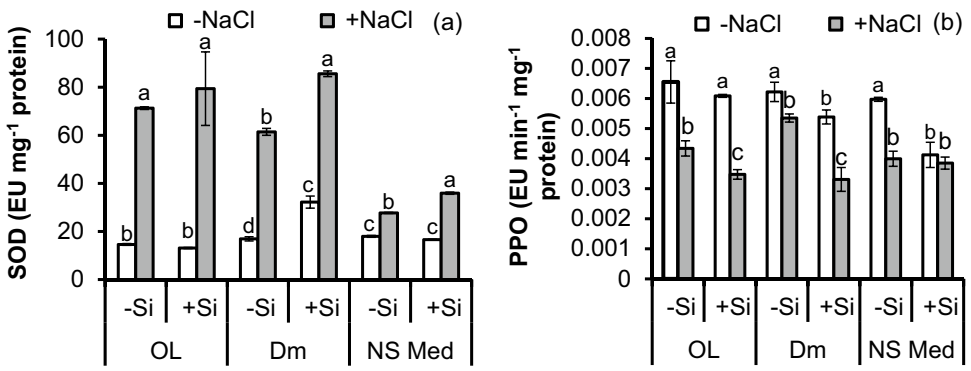

Fig. 9 Effect of exogenous Si (3 mM) application on superoxyde dismutase (SOD) (**a**) and polyphenol oxidase (PPO) (**b**) activities of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*) under either salt stress (120 mM NaCl) or non-stress conditions. –NaCl, 0 mM NaCl treatment; +NaCl, 120 mM NaCl treatment; -Si, 0 mM $CaSiO_3$ treatment; +Si, 3 mM $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

Med plants. Si supplementation to control plants reduced significantly PPO activity in *OL* and *Dm*. Statistical analysis showed that NaCl and Si were significant ($P \leq 0.001$; Supplemental Table 2).

3.5 Effect of salt and exogenous Si treatments on nutrient elements

Imposition of 120 mM salt stress significantly reduced N content in the three alfalfa varieties with *NS Med* being the most affected with a 31% reduction (Table 6). However, all three Si-treated salt-stressed alfalfa varieties had greater N content than their Si-untreated salt-stressed counterparts. Indeed, N content in Si-treated salt-stressed *OL*, *Dm* and *NS Med* was 1.34, 1.69 and 1.48-fold higher respectively compared to their respective salt-stressed controls. Under unstressed conditions, N content increased in Si-treated *OL* and *NS Med*. Statistical analysis indicated that all factors and their interaction were significant for N content (Supplemental Table 2).

Nitrogen content index (NCI) of alfalfa plants was considerably influenced by the addition of NaCl and Si to the medium as shown in Fig. 10. The addition of NaCl to the medium severally reduced NCI in all three investigated alfalfa varieties. However, Si incorporation to the growth medium of alfalfa plants having 120 mM NaCl increased NCI by 14%, 70% and 18% in *OL*, *Dm* and *NS Med* respectively as compared to Si-untreated salt-stressed control. Si alone also increased NCI in *OL* and *NS Med* (Fig. 10).

Statistical analysis (Supplemental Table 2) showed that all factors and their interaction were significant ($P \leq 0.001$) for Si contents. Si content in all alfalfa varieties increased due to Si supplementation (Table 6). In addition, Si application along with salt stress significantly increased Si content in *Dm* and *NS Med* varieties as compared to Si-treated control plants. When no Si was added to the watering solution, salt stress alone induced a slight increase in Si content in the three alfalfa varieties.

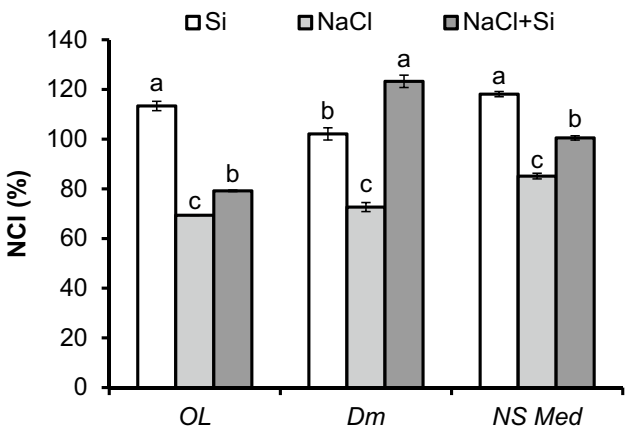

Fig. 10 Effect of salinity (120 mM NaCl) and exogenous Si (3 mM) on nitrogen content index (NCI) of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*). Si, 3 mM $CaSiO_3$ treatment; NaCl, 120 mM NaCl treatment; NaCl + Si, combination of NaCl and $CaSiO_3$ treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

Accumulation of Na^+ and Ca^{2+} in all alfalfa varieties increased significantly due to salt imposition, whereas accumulation of K^+ decreased significantly (Table 6). However, Si supplementation counteracted the negative impact of salt stress in increasing K^+ and reducing Na^+ with a significant difference between the varieties. In *NS Med*, supplemented Si to salt-stressed plants reduced Na^+ by 7% while K^+ was increased by 23% relative to Si-untreated salt stressed alfalfa plants. Results related to statistical analysis (Supplemental Table 2) indicated that all factors and their interaction were significant for K^+ and Ca^{2+} content while for Na^+ the interaction NaCl × Si was not significant.

Table 6 Effect of salt (120 mM NaCl) and Si (3 mM $CaSiO_3$) on nitrogen (N), Si, sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) content in three *M. sativa* varieties (*OL*, *Dm* and *NS Med*)

	OL				*Dm*				*NS Med*			
	C	Si	NaCl	NaCl + Si	C	Si	NaCl	NaCl + Si	C	Si	NaCl	NaCl + Si
N (%)	1.35^{b}	1.50^{a}	0.94^{d}	1.07^{c}	1.10^{b}	1.15^{b}	0.82^{c}	1.39^{a}	1.15^{b}	1.38^{a}	0.79^{c}	1.17^{b}
Si (mg Kg^{-1} DW)	5.99^{d}	4337.00^{a}	27.32^{c}	3814.00^{b}	5.82^{c}	4803.00^{b}	7.03^{c}	7310.00^{a}	8.76^{d}	6674.00^{b}	24.17^{c}	7450.00^{a}
Na^+ (mg g^{-1} DW)	32.40^{c}	28.00^{c}	242.80^{b}	299.10^{a}	42.00^{d}	48.80^{c}	73.50^{a}	63.30^{b}	45.70^{c}	27.20^{d}	60.20^{a}	55.90^{b}
K^+ (mg g^{-1} DW)	79.60^{c}	75.30^{c}	246.00^{b}	257.30^{a}	68.10^{b}	108.30^{a}	65.90^{c}	54.40^{d}	86.80^{a}	68.20^{b}	55.80^{c}	68.90^{b}
Ca^{2+} (mg g^{-1} DW)	60.20^{d}	55.10^{d}	323.90^{b}	402.50^{a}	71.20^{c}	89.40^{b}	95.30^{a}	58.90^{d}	76.50^{b}	51.80^{c}	80.40^{a}	77.00^{b}

C, control; Si, $CaSiO_3$ treatment; NaCl, salt stress; NaCl + Si, combination of salt stress and $CaSiO_3$ treatment. Values are the mean of three replicates and the letters indicate statistically significant values

4 Discussion

Soil salinity is the most severe abiotic stress that restricts plant growth through cellular ionic toxicity and osmotic stress (Ahmad et al. 2019). This work revealed that salinity stress significantly reduced alfalfa plants growth by reducing fresh and dry biomass, plant height, leaf number and leaf area. Salt stress has also impaired alfalfa-rhizobia symbiosis by reducing number of nodules of the three alfalfa varieties. The adverse effect of salt on plant growth was higher in the European variety *NS Med* as compared to the two other varieties. However, Si incorporation to growth medium of salt-stressed alfalfa plants increased significantly all the above-mentioned growth parameters. The results were in accordance with those of Lee et al. (2010), who stated that Si incorporation to the growth medium of salt-stressed soybean plants significantly increased fresh and dry weight as well as plant height. Similar results were found in alfalfa (Meng et al. 2020), maize (Ali et al. 2021) and tomato (Costan et al. 2020). In contrast, under unstressed condition, 3 mM Si-treated alfalfa plants had restricted growth and a noticeable decline in SFW, RFW, SDW, RDW and number of leaves. Similar results have been found by Kabir et al. (2016) on alfalfa, where 1 mM Si applied in the form of potassium silicate (K_2SiO_3) impaired SDW, RDW, shoot height and root length under normal condition. Furthermore, as legume-rhizobia symbiosis establishment is triggered by flavonoids, previous investigations showed that Si modulates flavonoids metabolism (Zhang et al. 2013) which partially explain in our study the increase in nodule number under salt stress in response to Si supplementation. In the same line, Si supplementation has been reported to increase nodule number in alfalfa under elevated temperature (Johnson et al. 2018). Likewise in soybean, Steiner et al. (2018) demonstrated that Si supplementation triggered symbiosis establishment as shown by numerous and bigger nodules.

Apparently, salt stress drastically reduced N content and nitrogen content index in the three alfalfa varieties. The decreased of N content under salt stress could be the result of the decreased nodule number, since the significant positive correlation (r = 0.7; Fig. 11; Supplemental Fig. S3) noted between N content and nodule number. However, Si application counteracted the negative impact of salt and positively improved the amount of nitrogen, as estimated by N content. Moreover, Si incorporation to the growth medium of alfalfa plants without salt significantly improved N content and nitrogen content index. This higher N content in plants, either stressed or not, subjected to Si supplied to the growth solution is in agreement with the previous works in soybean (Steiner et al. 2018). In *Sesbania aculeate*, Si was also found effective in mediating nodule formation and development and, as a result, biological N_2 fixation (Kurdali et al. 2019). Similarly, the positive impact of Si in nodule number, nitrogenase activity and N content was reported in *Cajanus cajan* under cadmium and zinc toxicity (Garg and Singh 2018). Here, a positive correlation was found between N and plant growth, indicating the important role of Si in legumes plants growth particularly when their growth is depending on biological nitrogen fixation.

We found that salt stress imposition not only disrupts alfalfa plant cell physiology but also affects photosynthesis. Photosynthetic pigment content (Chl and carotenoids) was vastly reduced under salt stress, which in turn impaired plant growth and nodule development. This reduction in pigment content was more prominent in the European variety (*NS Med*) as compared to the Moroccan varieties (*OL* and *Dm*). The reduction in Chl content in our study is partially explained by the toxic effect of Na^+ because of the close negative relationship found between Na^+ accumulation and photosynthetic pigments content (Zhu and Gong 2014; Rizwan et al. 2015). However, this was alleviated by the addition of Si. According to Alamri et al. (2020), Si could increase Chl content under salt stress by increasing the activity of some Chl synthesis enzymes including δ-aminolevulinic acid dehydratase and porphobilinogen deaminase and inhibiting those responsible for Chl degradation including chlorophyllase, Chl-degrading peroxidase and pheophytinase. Recently, previous studies indicated that exogenous Si treatment considerably improved photosynthetic traits (Hussain et al. 2021), which might be the reason of increased plant growth reflected by higher plant biomass, plant height, leaf number and area. This was evident in our present work since the close positive correlation recorded between Chl b and plant growth parameters (shoot (r = 0.52) and root (r = 0.62) fresh weight, shoot dry weight (r = 0.61), plant high (r = 0.74) and leaf number (r = 0.7)) (Fig. 11; Supplemental Fig. S3). Another consequence of salt stress on photosynthesis is the decrease of Chl fluorescence, especially the photosystem II parameter F_v/F_m ratio (Farissi et al. 2018). This might be a consequence of Chl reduction in response to salt (Ganieva et al. 1998). Our results indicated that salt stress decreased F_v/F_m ratio in all of the three studied varieties and this negative effect was reversed by Si treatment. In addition, a significant positive correlations (up to r = 0.7) between F_v/F_m ratio and Chl content was shown (Fig. 11), which agrees with previous studies (Ganieva et al. 1998). Si content was positively correlated with the increase of all photosynthetic parameters in this study and also in other plants species such as sweet pepper (Abdelaal et al. 2020), lettuce (Lemos Neto et al. 2020), sea barley (Laifa et al. 2020) and sugarcane (Verma et al. 2021a and b). On the one hand, these positive effects can be attributed to the involvement of Si in reducing Na^+ and Cl^- uptake by plants

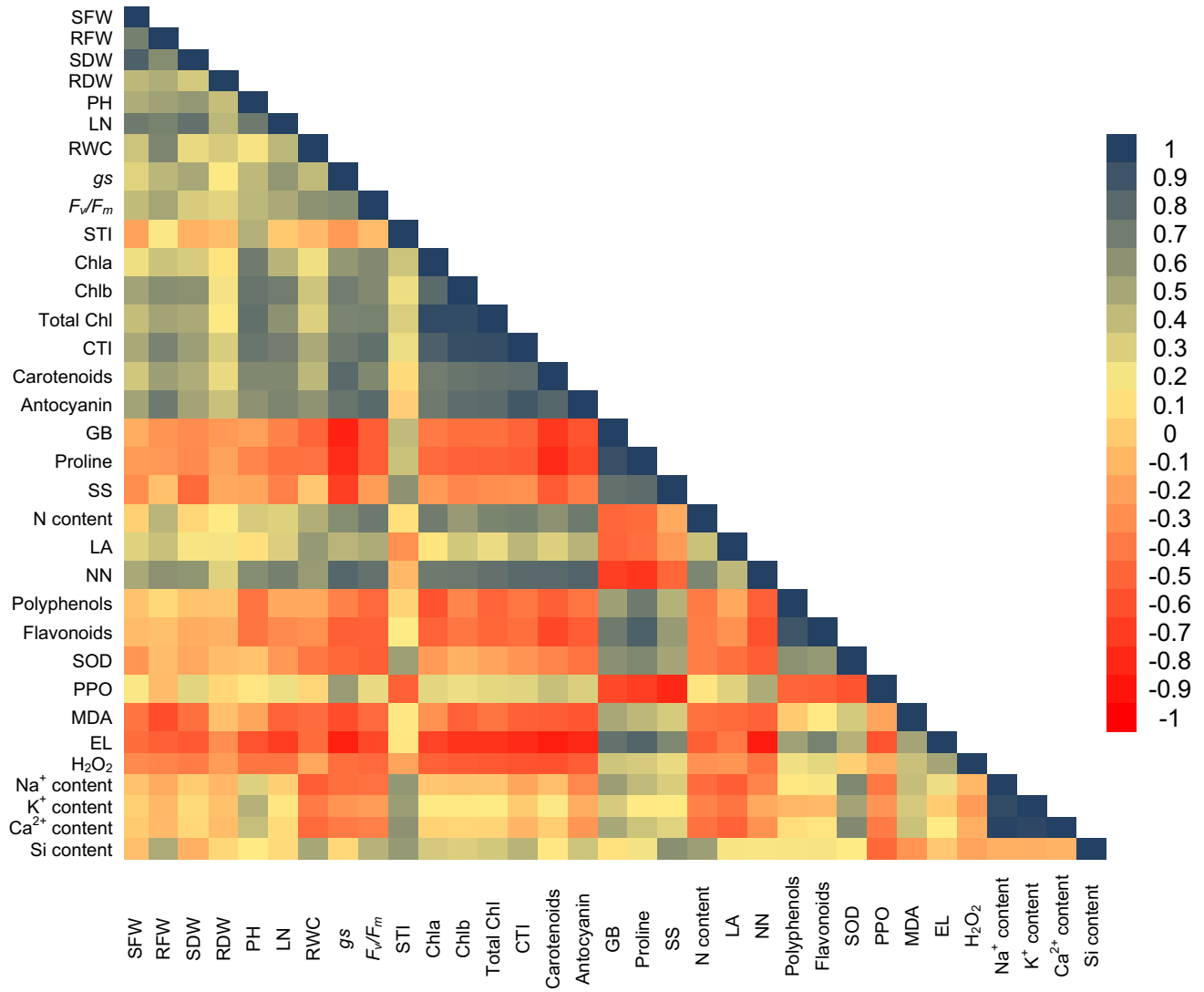

Fig. 11 Pearson's correlation matrix between plant growth, photosynthesis traits, compatible osmolyte, membrane stability, enzymatic and non-enzymatic antioxidants and nutrient element in three alfalfa (*Medicago sativa* L.) varieties (*OL*, *Dm* and *NS Med*) treated with 0 or 120 mM NaCl with or without 3 mM $CaSiO_3$. Correlations are displayed in blue (positive) and red (negative); color intensity is proportional to the correlation coefficient. EL: electrolyte leakage, Ca^{2+} content: calcium content, Chl a: chlorophyll a, Chl b: chlorophyll b, CTI: chlorophyll tolerance index, F_v/F_m: photochemical efficiency of photosystem II, GB: glycine betaine; g_s: stomatal conductance, H_2O_2: hydrogen peroxide, K^+ content: potassium content, LA: leaf area, LN: leaf number, MDA: malonyldialdehyde, N content: nitrogen content, Na^+ content: sodium content, NN: nodule number, PH: plant height, RDW: root dry weight, RFW: root fresh weight, RWC: relative water content, SDW: shoot dry weight, SFW: shoot fresh weight, SS: soluble sugars, STI: salt tolerance index, SOD: superoxyde dismutase, Total Chl: total chlorophyll, PPO: polyphenol oxidase, Si content: silicon content

(Zhu and Gong 2014; Rizwan et al. 2015) since the close negative correlation found between Na^+ content and photosynthetic pigment. On the other hand, Si could be involved in the regulation of some aquaporin related-genes expression, and as a result, maintain of plant water content under salt stress (Liu et al. 2015).

Under limiting water conditions, plants close their stomatal pores to limit water loss by transpiration and consequently lead to reduction of CO_2 assimilation and to perturbation of photosynthetic activities (Antolín et al. 2010). Furthermore, Xu et al. (2018) demonstrated that Si supplementation resulted in improved water use efficiency, which in return improved transpiration rate, *gs* and net photosynthetic rate. Similarly, when applied to the nutrient solution, exogenous Si was found to improve transpiration rate and stomatal conductance and this is closely related to its ability to alleviate osmotic stress by reducing leaf water potential and leaf osmotic potential and increasing leaf turgor potential (Romero-Aranda et al. 2006). In this study, salt stress induced a significant decrease of RWC in the three studied

alfalfa varieties. The variety *OL* was able to maintain adequate relative water content under stress as compared to control, while the other *Dm* and *NS Med* varieties were the most affected by salt stress. However, as previously reported by Mahmood et al. (2016) and Tuna et al. (2008), exogenous Si application significantly elevated this parameter allowing the plant to grow normally in the presence of salinity. Significant improvement of relative water content under salt stress in response to Si treatment has been reported in several plant species including *Onobrychis viciaefolia* (Wu et al. 2017), wheat (Tuna et al. 2008) and *Vigna radiata* (Ahmad et al. 2018). As well as for RWC, salinity caused a significant decrease in *gs* particularly in *NS Med* and as reported by Abbas et al. (2015) in *Abelmoschus esculentus*, Si addition alleviated this negative effect by improving *gs* in all of the three alfalfa varieties.

Compatible osmolytes are small molecules that can act as osmoprotectant, alleviating salt stress by regulating cellular osmotic pressure (Al Murad et al. 2020). Furthermore, ability of stressed plant to accumulate compatible osmolytes may define their tolerance capability (Kavi Kishor and Sreenivasulu 2014; Slama et al. 2015). In our work, salt stress raised significantly the content of compatible solutes, including glycine betaine, proline and soluble sugars. Interestingly, the accumulation of compatible solutes was further elevated in salt-stressed plants with Si supplied to the growing medium, indicating the contribution role of Si in maintaining cell turgor pressure. Furthermore, K^+ was found higher in salt-stressed alfalfa plants particularly those threated with Si. The greater accumulation of these, organic and inorganic, solutes in response to Si favors the role of exogenous Si in osmotic adjustment under saline conditions. Previous research in *Glycyrrhiza uralensis* indicated that Si increased salt tolerance by regulating osmolytes accumulation allowing osmotic potential adjustment (Zhang et al. 2017). Similarly, in a study conducted in cucumber by Zhu et al. (2020), Si incorporation in cultured media with salinity resulted in a significant increase in the activity of pyrroline-5-carboxylase synthase and inhibiting the activity of proline dehydrogenase resulting in a high proline content.

The effect of salt stress could also be seen in the form of oxidative stress. MDA is usually used as a membrane stability indicator (Gong et al. 2008) and its increase is often accompanied with high EL and ROS production (Shanker et al. 2004; Mandhania et al. 2006). In our study, salt stress induced ROS (H_2O_2 and O_2^-) accumulation in the three alfalfa varieties. This is correlated with membrane alteration (MDA, $r = 0.57$) and leakage (EL, $r = 0.64$) which indicates membrane damage. Furthermore, leaf staining revealed H_2O_2 and O_2^- accumulation noticeably when plants were grown under salt stress without Si. Similar to the present study, previous investigations showed that ROS content was considerably increased upon salt stress, which consequently impaired membrane integrity as reflected by higher MDA content and higher EL (Khan et al. 2020; Sheikhalipour et al. 2021). Si application, however, significantly alleviated the deleterious effect of salt on cell membrane, which was evident from the considerably decreased MDA, EL, H_2O_2 and O_2^- in all of tested alfalfa varieties. Several reports have indicated the decrease in ROS production, MDA contents and EL level under stress in response to Si application (Coskun et al. 2016). In alfalfa, Si application was able to reduce H_2O_2, O_2^- and MDA contents under salt stress (Meng et al. 2020). The decrease in oxidative stress in response to Si supplementation was associated with an increase in the activity of some antioxidant enzymes including CAT, SOD and peroxidase (POD) (Liang et al. 2003; Coskun et al. 2016; Meng et al. 2020). Non-enzymatic antioxidant polyphenols, flavonoids and anthocyanins were also indicative of a positive effect of Si application in response to salt stress. Furthermore, beside osmoregulation, proline and glycine betaine have been reported having a non-enzymatic protective role under salt stress (Szabados and Savouré 2010; Hussain Wani et al. 2013; El Moukhtari et al. 2020). In this study, Si treatment alleviates oxidative stress through proline and glycine betaine accumulation, since the close positive correlation noted between MDA, proline and glycine betaine. This suggests that Si application reduced salt-mediated oxidative stress not only by inducing the activity of antioxidant enzymes but also by the increase in the content of non-enzymatic antioxidant compounds.

5 Conclusions

Salt stress markedly affected plant growth and caused an oxidative stress reflected by high MDA and ROS production. Applying Si improves alfalfa plant growth and membrane stability by improving nodulation, photosynthesis and nitrogen fixation and stimulating the activity of both enzymatic and non-enzymatic antioxidants thereby preventing ROS induced oxidative damage. Likewise, Si supplementation reduced Na^+ content and induced further accumulation of the organic solutes proline, glycine betaine and soluble sugars as well as the inorganic solute K^+, which allows an increase of leaf relative water content in salt-stressed alfalfa plants. Our findings strongly suggest and recommend that 3 mM Si application could be a promising way to improve alfalfa production in case of salt stress. Then, the development of fertilizers based on Si is a very interesting prospect for the mitigation of the deleterious effects of salt stress.

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Declarations

Conflicts of interest The authors declare no conflict of interest.

Data availability Not applicable.

Code availability Not applicable.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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Supplementary data

Supplemental Table 1 Plant growth-promoting activities of *Ensifer meliloti* Rm41 strain

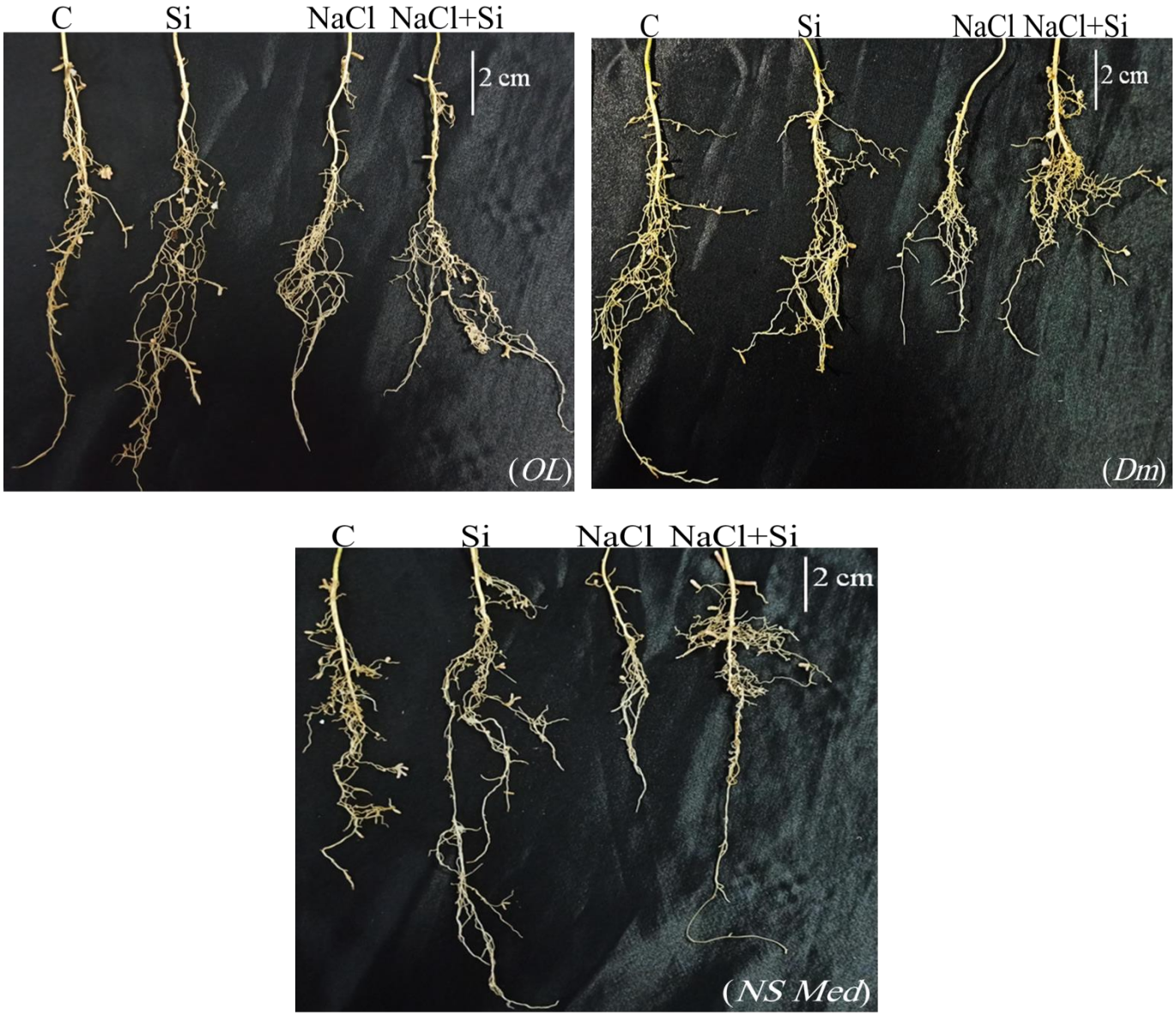
Phosphate Solubilization ($\mu g\ mL^{-1}$)	Production of Exopolysaccharide ($\mu g\ mL^{-1}$)	Production of IAA ($\mu g\ mL^{-1}$)	Nitrogen Fixation
85.9 5± 6.38	163.65 ± 15.33	0.294 ± 0.07	+

+ indicated the ability of the Rm41 strain to grow on N-free solid medium.

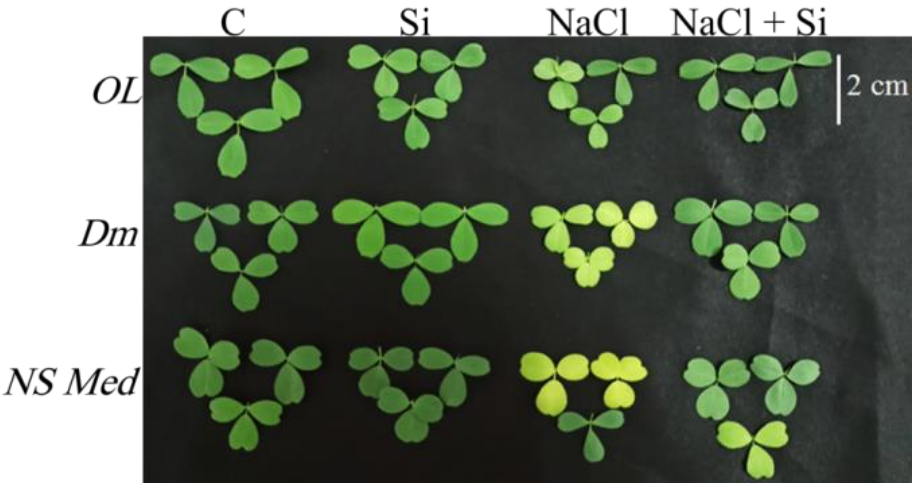
Supplemental Table 2 Result of three way ANOVA test for independent variables including alfalfa (*M. sativa* L.) varieties, salinity treatment and silicon treatment and their interaction

	Variety (V)			Salinity (S)			Silicon (Si)			V×S		V×Si		S×Si		V×S×Si	
	df	F	CV(%)	df	F	CV(%)	df	F	CV(%)	df	F	df	F	df	F	df	F
Shoot FW	2	78.49***	73.6	1	163.567***	110.1	1	6.685*	57.6	2	129.09***	2	65.56***	1	932.57***	2	61.83***
Root FW	2	58.26***	78.1	1	139.332***	80.7	1	275.5***	18	2	74.529***	2	30.59***	1	375.08***	2	26.83***
Shoot DW	2	1.239ns	84.3	1	29.430***	61	1	1.287ns	35.5	2	10.568**	2	8.635**	1	98.298***	2	3.897*
Root DW	2	0.454ns	85.3	1	1.805ns	79.2	1	0.131ns	27	2	1.143ns	2	1.071ns	1	3.721ns	2	0.703ns
Nodule number	2	0.913ns	85.8	1	138.793***	36.4	1	16.533***	24.4	2	1.348ns	2	1.174ns	1	22.011***	2	0.913ns
Plant height	2	29.25***	88.4	1	36.614***	35.3	1	44.63***	22	2	18.027***	2	15.63***	1	42.707***	2	5.315**
Number of Leaves	2	0.188ns	68.7	1	42.250***	30.7	1	4*	16.7	2	13.563***	2	0.813ns	1	64***	2	2.688ns
Leaf area	2	6.591**	52.2	1	27.094***	21.6	1	1.826ns	20.4	2	0.303ns	2	7.776**	1	3.638ns	2	2.358ns
RWC	2	41.806***	52.4	1	84.880***	9.2	1	56.396***	9.8	2	3.809*	2	1.979ns	1	0.405ns	2	0.774ns
STI	2	41.32***	208.7	1	76.372***	73.2	1	290.59***	50.8	2	13.917***	2	13.62***	1	4.437*	2	1.636ns
Chl a	2	53.043***	81.8	1	79.812***	16.4	1	80.043***	14.5	2	1.866ns	2	2.798ns	1	18.711***	2	6.830**
Chl b	2	11.490***	70.5	1	59.404***	22.4	1	34.439***	9.4	2	2.411ns	2	0.271ns	1	53.240***	2	2.204ns
Total Chl	2	40.556***	74.8	1	109.435***	18.8	1	84.27***	10.9	2	0.104ns	2	1.415ns	1	57.198***	2	5.257**
Carotenoids	2	41.998***	77.7	1	404.618***	26.8	1	48.76***	17.7	2	47.490***	2	1.940ns	1	25.834***	2	7.818**
CSI	2	14.318***	67.3	1	197.307***	17.2	1	150.405***	6	2	0.724ns	2	2.021ns	1	104.091***	2	10.588***
g_s	2	90.80***	86	1	1400.78***	35.8	1	22.48***	24.2	2	13.867***	2	50.25***	1	167.55***	2	22.24***
F_v/F_m	2	6.401**	61.6	1	80.841***	2.9	1	35.345***	2.1	2	2.257ns	2	5.264**	1	13.468***	2	3.814*
MDA	2	3.244*	91.7	1	15.599***	18.4	1	2.783ns	18.1	2	0.396ns	2	0.487ns	1	8.435**	2	0.931ns
EL	2	6.280**	56	1	151.421***	30.5	1	3.183ns	46.7	2	3.959*	2	0.166ns	1	18.5***	2	1.072ns
H_2O_2	2	2.109ns	173.8	1	5.036*	249.8	1	2.832ns	82.8	2	1.375ns	2	1.354ns	1	3.509ns	2	1.786ns
GB	2	12.866***	80.8	1	666.34***	18.2	1	3.920*	69.2	2	17.347***	2	4,022*	1	5,892*	2	1.550ns
Proline	2	21.665***	88.8	1	1881.76***	15.6	1	22.853***	67.6	2	13.794***	2	6.352**	1	14.474***	2	15.969***
Soluble sugars	2	46.120***	68.4	1	619.391***	16.6	1	369.9***	19.7	2	13.756***	2	5.187**	1	30.138***	2	3.957*
SOD	2	19.638***	211.7	1	150.165***	40.4	1	6.728*	93.2	2	17.053***	2	2.576ns	1	1.893ns	2	0.001ns
PPO	2	2.692ns	69.7	1	46.209***	23.9	1	17.704***	27.9	2	2.441ns	2	0.832ns	1	0.004ns	2	3.126ns
Polyphenols	2	117.045***	59.5	1	377.782***	20.2	1	0.112ns	35.4	2	31.374***	2	22.285***	1	75.294***	2	16.749***
Flavonoids	2	19.186***	59.1	1	195.925***	18.5	1	0.188ns	36.7	2	9.064***	2	9.388***	1	16.840***	2	3.511*
Anthocyanin	2	0.921ns	68	1	130.254***	43.8	1	42.84***	16.8	2	0.398ns	2	0.070ns	1	46.950***	2	7.897***
N	2	45.526***	73.4	1	675.494***	18.5	1	645.09***	13.2	2	189.62***	2	24.192***	1	85.197***	2	133.54***
Si	2	13832080.2***	156.3	1	3913920,2***	188.3	1	58849808***	26.2	2	3419396.4***	2	13874997.9***	1	3704438.4***	2	3512634.9***
Na^+	2	263.346***	376.1	1	569.484***	167.9	1	0.018ns	183.6	2	326.461***	2	3.775*	1	2.186ns	2	4.256*
K^+	2	34291.028***	276.5	1	17162.016***	162.9	1	228.769***	110.5	2	38476.798***	2	223.850***	1	4.765*	2	1463.767***
Ca^{2+}	2	70256.174***	305.6	1	109201.921***	180.8	1	733.747***	166.9	2	85928.737***	2	1985.810***	1	1494.865***	2	2333.031***

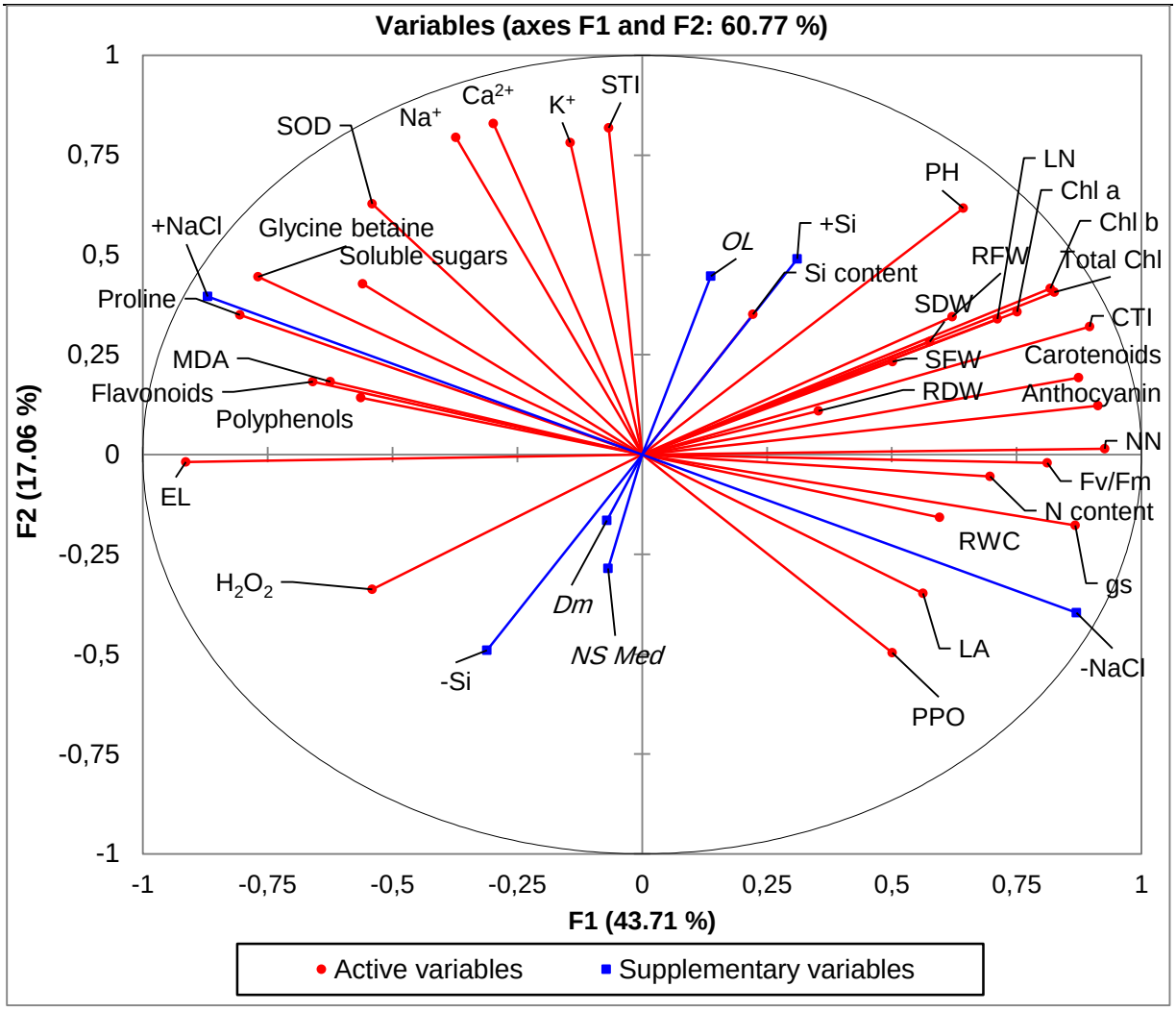
df, degree of freedom; F, ficher; CV, coefficint of variation; ns non-significant; * significant at p<0.05; ** significant at p<0.01; *** significant at p<0.001



Supplemental Fig. S1 Effect of exogenous Si (3 mM) application on root length and nodule number of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*) under either salt stress (120 mM NaCl) or non-stress conditions. C, control; NaCl, salt stress; Si, $CaSiO_3$ treatment; NaCl+Si, combination of salt stress and $CaSiO_3$ treatment.



Supplemental Fig. S2 Effect of exogenous Si (3 mM) application on leaf phenotype of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*) at the 60th day after germination under either salt stress (120 mM NaCl) or non-stress conditions. C, control; NaCl, salt stress; Si, $CaSiO_3$ treatment; NaCl+Si, combination of salt stress and $CaSiO_3$ treatment.



Supplemental Fig. S3 Principal component analysis of studied parameters for the three studied alfalfa (*M. sativa* L.) varieties in response to silicon as well as salt in the medium. The most variables (arrows), silicon concentration, NaCl concentration as well as the three different varieties are projected onto the F1-F2 principal factorial plane that explains 60.77% of the variation. EL: electrolyte leakage, Ca^{2+}: calcium content, Chl a: chlorophyll a, Chl b: chlorophyll b, CTI: chlorophyll tolerance index, F_v/F_m: photochemical efficiency of photosystem II, *gs*: stomatal conductance, H_2O_2: hydrogen peroxide, K^+: potassium content, LA: leaf area, LN: leaf number, MDA: malonyldialdehyde, N content: nitrogen content, Na^+: sodium content, NN: nodule number, PH: plant height, RDW: root dry weight, RFW: root fresh weight, RWC: relative water content, SDW: shoot dry weight, SFW: shoot fresh weight, STI: salt tolerance index, SOD: superoxyde dismutase, Total Chl: total chlorophyll, PPO: polyphenol oxidase, Si content: silicon content.

Chapitre IV

L'apport exogène de la proline améliore les pigments photosynthétiques et le rapport K^+/Na^+ et réduit le stress oxydant chez *Medicago sativa* sous contrainte saline

I Introduction

La proline exogène est décrite comme molécule à effet biostimulant pour palier le problème de la salinité (Meena et al. 2019; Heuer 2010). La stabilisation des protéines et des complexes macromoléculaires, le piégeage des radicaux libres et la régulation du potentiel redox cellulaire sont des effets bénéfiques de la proline décrits pour des plantes sous contraintes salines (Ben Rejeb et al. 2014). Chez *Sorghum bicolor*, l'apport exogène de la proline réduit l'effet délétère de 75 mM NaCl et améliore la biomasse des plantes, la surface foliaire, la nutrition hydrique et les échanges gazeux (de Freitas et al. 2019). De même, chez *Zea mays*, de Freitas et al. (2018) ont démontré que l'apport exogène de la proline réduit la toxicité ionique et la peroxydation lipidique et induit une forte activité des enzymes antioxydantes sous stress salin. Cependant, à notre connaissance, aucun travail n'a été publié sur l'effet de la proline dans la tolérance de la luzerne au stress salin.

II Objectif

Dans ce chapitre, nous avons étudié l'effet de la proline (20 mM) sur l'amélioration de la croissance de trois variétés de *M. sativa* sous contrainte saline sévère (200 mM de NaCl).

III Méthodologies

Des graines de *M. sativa* (*OL*, *Dm* et *NS Med*) ont été mises à germer comme décrit précédemment dans le chapitre III. Par la suite, le nombre de plantes par pot a été ajusté à 3. Durant toute la période de l'expérimentation, la culture a été arrosée deux fois par semaine avec une solution nutritive dépourvue d'azote (Hoagland). Au stade un mois, le traitement salin a été appliqué par irrigation des plantes par deux solutions de NaCl : 0 mM (témoin) et 200 mM NaCl (Stressé). Les plantes de chaque traitement (témoin et stressé) ont été par la suite divisées en deux lots : un lot correspondant aux plantes non traitées avec de la proline (0 mM proline) et un autre lot correspondant aux plantes traitées une fois par semaine avec 20 mM proline. La proline a été ajoutée à la solution nutritive utilisée pour arroser les plantes. Le sel a été appliqué pendant un mois puis l'effet de la proline a été évalué à travers des mesures de croissance, des paramètres physiologiques, biochimiques, enzymatiques et nutritionnelles.

IV Résultats

Les résultats montrent que la contrainte saline affecte négativement la croissance des plantes en réduisant la biomasse, la hauteur et le nombre de feuilles des plantes. La contrainte saline

réduit également la surface des feuilles et leur teneur en pigments photosynthétiques. Cependant, le traitement des plantes exposées à la contrainte saline avec 20 mM de proline induit une forte accumulation de proline *in planta*. La biomasse, le nombre de feuilles et la hauteur des plantes, la surface foliaire et la teneur en pigments photosynthétiques sont supérieures dans ces conditions. La proline exogène réduit également l'effet négatif de la contrainte saline sur la stabilité membranaire en réduisant la teneur des feuilles en MDA. Cela est bien corrélé avec un rapport K^+/Na^+ très élevé et des activités très fortes de la SOD, la CAT, l'ascorbate peroxydase (APX) et la glutathionne peroxydase (GR).

V Conclusions

Nos résultats montrent que la proline exogène a des effets très positifs sur la biomasse, la photosynthèse, l'accumulation des compatibles osmolytes et la stabilité membranaire des plantes de *M. sativa* au cours d'une contrainte saline.

Ces données font fait l'objet d'un projet d'article intitulé « Exogenous proline supply improves growth, antioxidant defense system, and nutrient homeostasis in *Medicago sativa* during salinity stress ».

Exogenous proline supply improves growth, antioxidant defense system, and nutrient homeostasis in *Medicago sativa* during salinity stress

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Abstract

Salinity stress is a severe environmental trait for crop plants. Exogenous proline has emerged as a promising way to improve plant tolerance to abiotic stresses, including salinity. A pot experiment was conducted using two Moroccan, *Ouad Lmaleh* (*OL*) and *Demnate 201* (*Dm*), and one European, *NS Mediana ZMS V* (*NS Med*), alfalfa varieties to investigate the effect of exogenous proline on physiological and biochemical responses of alfalfa (*Medicago sativa*) during salinity stress. Results indicated that salt stress reduced shoot and root dry weight, plant height and leaf number, with *NS Med* being the most affected with reductions rates of 75%, 85%, 53%, and 65%, respectively. Salinity also reduced photosynthetic pigments, potassium (K^+) and increased malonyldialdehyde (MDA) and sodium (Na^+) contents. The injury impact of salinity stress on alfalfa plant growth was alleviated by exogenous proline treatment, as evidenced by increasing plant biomass and plant height and leaf number. Proline-treated salt-stressed plants also showed higher photosynthetic pigments and K^+ and reduced Na^+ contents. Proline treatment also effectively reduced MDA content under salt stress, particularly in *NS Med* variety, by 26%. The lower amount of MDA in the proline-treated plants seemed to be related to its capacity to modulate antioxidant enzymes activities such as superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase. Furthermore, proline treatment boosted the accumulation of proline content, which positively correlated with plant growth and photosynthetic pigments. Our findings suggested that exogenous proline treatment could be promising to mitigate the effect of salinity on alfalfa plant.

Keywords: *Medicago sativa*; Proline; Salinity; Photosynthetic pigments; Antioxidant enzymes.

I Introduction

Alfalfa (*Medicago sativa* L.) is a prominent perennial forage legume because of its high biomass production, livestock nutritional value and biological nitrogen fixation (Mouradi et al. 2016; Zhang et al. 2019). Additionally, the deep root system of alfalfa allows it to utilize deep soil moisture (Huang et al. 2018). Therefore, alfalfa is considered a drought-tolerant species (Norton et al. 2021; Zhang et al. 2019). However, although alfalfa can adapt to water deficit, its growth was extremely reduced under some abiotic stresses such as salinity inducing osmotic stress and ionic toxicity (El Moukhtari et al. 2021).

Salinity is considered one of the global threats to the growth and development of crops. Today, it is estimated that about 800 million hectares of irrigated land are salt-affected, and this is continuously increasing (Roy et al. 2014; Van Zelm et al. 2020). Indeed, between 2 000 and 4 000 ha of irrigated land are to be loosed every day because of salinity (Jayakannan et al. 2015). Salinity impairs plant growth by causing osmotic stress and ionic toxicity accompanied by nutrient imbalance (Munns and Tester 2008). Salinity was also reported to increase reactive oxygen species inducing oxidative stress, harmful to cell membranes, lipids, proteins and nucleic acids (Parihar et al. 2015). Plants have developed some tolerance mechanisms to avert these negative impacts, including osmoregulation (Nadeem et al. 2019). In alfalfa, the accumulation of the osmolyte proline is one of the widespread responses to salinity stress (Farissi et al. 2013; Armengaud et al. 2004; El Moukhtari et al. 2021).

Proline is a multifunctional amino acid that provides an effective response to mitigate the harmful effects of abiotic stresses (Szabados and Savouré 2010). Its accumulation has been reported in response to several environmental constraints, particularly salt and drought stresses (Verbruggen and Hermans 2008). Under stress conditions, proline is believed to fulfill various defense mechanisms, including osmotic adjustment, free radical detoxification and membrane and protein stabilization (Meena et al. 2019; Chun et al. 2018). Proline has also been reported to provide cellular redox potential, protect RuBisCo activity and mitochondrial electron transport chain complex II, mediate chlorophyll synthesis, modulate gene expression, and improve biological nitrogen fixation in legumes (El Moukhtari et al. 2020). Furthermore, when applied exogenously, proline enhances the ROS scavenging efficiency and improves sugar beet's leaf membrane stability index under drought stress conditions, protecting them from oxidative stress (Ghaffari et al. 2019). Additionally, under salinity stress, exogenous proline improves *Z. mays* plants stress tolerance by promoting ion homeostasis and osmotic adjustment (de Freitas et al. 2018).

Exogenous proline mediated the tolerance of plants to abiotic stress has been consistently reported in *Oryza sativa* under salt stress (Teh et al. 2016), in *Z. mays* under drought stress (Hanif et al. 2021), in *Olea europaea* under lead stress (Zouari et al. 2018) and in *Cajanus cajan* under cadmium stress (Hayat et al. 2021). However, to date, no scientific study has investigated the potential role of exogenous proline in alleviating salt stress in *M. sativa*. Therefore, in this study we evaluate the effect of exogenous proline on growth in terms of biomass, plant height and leaf number, photosynthesis in terms of chlorophyll content and leaf area, osmotic adjustment in terms of proline and soluble sugars, oxidative stress alleviation in terms of malonyldialdehyde, superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase and nutrient homeostasis in terms of sodium and potassium, in salt stressed *M. sativa* plants, with the hypothesis that proline treatment might improves the tolerance to salt stress. The present study's findings will open up new perspectives for using proline to relieve salt stress in alfalfa, particularly in semi-arid regions.

II Materials and methods

II-1 Plant material

A pot experiment was conducted under greenhouse conditions at the Sorbonne University (Paris, France) to study the effect of exogenous proline on salt stress tolerance in alfalfa (*Medicago sativa* L.). Seeds of two Moroccan *M. sativa* varieties, *Oued Lmaleh* (*OL*) from Oasis and *Demnate 201* (*Dm*) from High Alas Mountains, and a European variety *NS Mediana ZMS V* (*NS Med*), supplied by the National Institute of Agronomic Research (INRA-Marrakesh, Morocco) were used as material in this work. These varieties were selected for their contrasting response to salt stress (El Moukhtari et al. 2021).

II-2 Growth conditions

Seeds with homogenous size were sterilized by immersing in 6% sodium hypochlorite for 5 min and rinsed five times with sterile deionized water. Germination of sterilized seeds was carried out in plastic pots containing sterilized perlites (granule size, 0.6-6 mm; pH 6.8; EC 0.0-0.1 µS cm^{-1}) and vermiculite (granule size, 1-4 mm; pH 7; EC 0.1-0.3 µS cm^{-1}) with a ratio of 6:1 (v/v). The growth environment conditions were as follows: 16 h photoperiod at 80-100 µmol photons s^{-1} m^{-2} at 25 ± 1 °C and 60% - 80% relative humidity. Two weeks after sowing, three uniform alfalfa seedlings were maintained per pot and inoculated with an *Ensifer meliloti* Rm41 strain. Seedlings were then irrigated with a nitrogen (N) free nutrient solution (Hoagland and Arnon 1950) [KH_2PO_4 (250 µmol), $MgSO_4$ (1000 µmol), K_2SO_4 (750 µmol), $CaCl_2$ (1650 µmol), Fe-EDTA (16 µmol), $MnSO_4$ (6 µmol), H_3BO_3 (4 µmol), $ZnSO_4$ (1 µmol), $NaMoO_4$ (0.1 µmol), and $CuSO_4$ (1 µmol)] once a week. To avoid N deficiency during nodule development, urea was supplied during the initial week of growth at the final concentration of 2 mM. After one month of growth, alfalfa plants of each variety were divided into four groups as follows: Control (C), untreated plants; proline, plants treated with 20 mM proline; salinity, plants treated with 200 mM NaCl; proline + salinity, plants treated with 200 mM NaCl and 20 mM proline. For each variety, five replicates per treatment were considered. Proline was applied in the growth solution. Proline concentration was selected according to preliminary experiments showing that 20 mM of proline is the best concentration for improving alfalfa plant growth. Stress was applied for one month, and then the two-month-old alfalfa plants were harvested to assess growth, physiological, biochemical, nutrients and antioxidant defense systems.

II-3 Plant growth and physiological parameters

Several growth indices were measured to observe the effect of exogenous proline on plant growth under salt stress. First, plant height and leaf number were determined just before the harvest. Next, the fresh weight (FW) of shoot and root was determined immediately after the harvest in three random plants of each treatment. Shoots and roots were then dried at 80 °C for 48 h, and their dry weight (DW) was determined.

According to Bağci et al. (2003), the salt tolerance index (STI) was calculated

$$\text{STI: } \frac{TDW\ at\ Sx}{TDW\ at\ S1} \times 100$$

With TDW is the total dry weight, S_x x treatment (NaCl or NaCl × P) and S_1 control treatment. The leaf area (LA) of alfalfa plants was determined in three random plants from each treatment immediately after the harvest (El Moukhtari et al. 2021). Brief, leaves were cut, laid out on a white sheet containing a scale, and scanned using a digital scanner. Images were then analyzed using Mesurum software version 3.4.4.0.

Photosynthetic pigments were determined according to the method described in Arnon (1949). Frozen leaf material (100 mg) was ground to a fine powder in liquid nitrogen and resuspended in 1 mL of 92% acetone. Aliquots were then centrifuged for 10 min at 10000 *xg* at 4 °C and the resulted supernatant was collected and adjusted to 2 mL using 80% acetone. The optical density (OD) was determined at 460, 645 and 663 nm using 80% acetone as a blank. Using the given formula, chlorophyll (Chl) a, Chl b, total Chl and carotenoids contents were determined.

Chl *a* (mg g^{-1} FW) = [12.70 × *A*663 − 2.690 × *A*645] × *V*/(1000 × *W*)

Chl *b* (mg per gram FW) = [22.90 × *A*645 − 4.680 × *A*663] × *V*/(1000 × *W*)

Total Chl (mg g^{-1} FW) = [20.2 × *A645* + 8.02 × *A663*] × *V*/(1000 × *W*)

Carotenoids (mg g^{-1} FW) = *A480* + (0.14 × *A663* – 0.638 × *A645*)

II-4 Biochemical parameters

According to the method of Heath and Packer (1968), malonyldialdehyde (MDA) was determined in fresh leaves. Around 100 mg of frozen leaves were homogenized to powder in liquid nitrogen and resuspended in 1 mL of thiobarbituric acid (0.5%) prepared in 20% trichloroacetic acid. The mixture was then heated for 30 min at 95 °C. Ice bath was used to stop the reaction, and then samples were centrifuged at 4 °C for 10 min at 10 000 *xg*. The supernatant was recuperated and used to read the OD at 532 nm and 600 nm. MDA concentration was determined using its extinction coefficient (155 mM^{-1} cm^{-1}) and expressed as µmol MDA g^{-1} FW.

For proline content, the method of Bates et al. (1973) was adopted. Frozen fresh material leaves (30-40 mg), ground to powder in liquid nitrogen, were resuspended in 1 mL of 3% (w/v) aqueous sulfosalicilic acid solution. The extract was then centrifuged at 4 °C for 10 min at 18000 *xg* and to 400 µL of the resulted supernatant, equal volume of ninhydrin (3%) and glacial acetic acid were added. The mixture was heated for 1h at 95 °C. After cooling down, 800 µL of toluene was added, and the OD of the pink phase was determined at 520 nm using NP80 Nanophotometer® (Implen). Proline content was determined using calibration curves established with known proline concentrations and expressed as µmol proline g^{-1} FW.

Soluble sugars were determined following the method of Yemm and Willis (1954). Frozen fresh leaves (100 mg) were ground to powder in liquid nitrogen. Powder was resuspended in 1 mL of 80% ethanol and centrifuged at 5000 rpm for 20 min at 4 °C. Supernatant was recuperated, and to 0.25 mL, 0.25 mL of distilled water and 1 mL of cold anthrone solution (0.2% in 80% sulfuric acid) were added. After 10 min of incubation at 100 °C, the OD was read at 630 nm, and the soluble sugars concentrations were estimated using calibration curves established with known glucose concentrations.

II-5 Enzymatic parameters

Frozen fresh leaves material (0.1 g) was ground to fine powder in liquid nitrogen and resuspended in 3.5 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediaminatetraecetate (EDTA), 1% polyvinylpyrrolidone, 0.1% protease inhibitor, 0.1 mM phenylmethylsulfonyl fluoride and 2 mM dithiothreitol (Araújo et al., 2016). The extract was centrifuged at 14, 000 ×*g* for 30 min at 4 °C. 2.5 mL of the resulted supernatant was recovered and transferred to PD10 desalting columns (GE Healthcare) previously equilibrated with 100 mM potassium phosphate buffer containing 0.1 mM EDTA. Extracts were then eluted with the same buffer. The resulted supernatant was recovered and used for the determination of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities.

SOD activity was determined following Beyer and Fridovich (1987) method. The reaction mixture contained 30 µL enzyme extract, 50 mM phosphate buffer (pH 7.8), 65 µM NBT, 13.3 µM L-methionine and 1.33 µM riboflavin in a final volume of 1.03 mL. The reaction was started by exposing tubes to a fluorescence lamp for 5 min, and then the OD has measured at 560 nm in a non-irradiated reaction mixture as a blank. One enzymatic unit (EU) of SOD was defined as the amount required to inhibit 50% NBT. The activity was expressed as EU min^{-1} mg^{-1} protein.

CAT activity was determined as described previously (Aebi 1984). The 3 mL reaction mixture consisted of 50 mM potassium buffer (pH 7.0), 10 mM H_2O_2 and enzyme extract. After the addition of H_2O_2, the decrease in OD at 240 nm was followed at intervals of 20 s for 3 min. The activity of CAT was determined using the extinction coefficient of H_2O_2 and expressed as ηmol min^{-1} mg^{-1} protein.

APX activity was assayed by recording ascorbate oxidation, as described by Nakano and Asada (1981). The assay was performed 3 mL reaction mixture containing 50 mM phosphate buffer (pH 7), 0.1 mM EDTA, H_2O, 0.25 mM H_2O_2, 0.25 mM ascorbate and enzyme extract. The decrease in absorbance at 290 nm was followed spectrophotometrically for 10 min. APX activity was determined using the extinction coefficient of ascorbate and expressed as ηmol min^{-1} mg^{-1} protein.

GR activity was determined spectrophotometrically by Carlberg and Mannervik (1985). The reaction mixture in a final volume of 1 mL consisted of 100 mM phosphate buffer (pH 7.8), 3 mM magnesium chloride, 10 mM glutathione disulfide, 0.2 mM NADPH and enzyme extract. The NADPH was added last to initiate the reaction, and the decrease in OD was followed at 340 nm. GR activity was calculated using the extinction coefficient of NADPH of 6.22 mM^{-1} Cm^{-1} and expressed as ηmol min^{-1} mg^{-1} protein.

The concentration of soluble proteins for all enzymes was quantified using the Bradford method (Bradford 1976).

II-6 Nutrient analyses

For Na^+ and K^+, the method of Oukaltouma et al. (2021) was adopted. Briefly, dried samples of shoots and roots (0.5 g) were incinerated for 6h at 550 °C. Ashes formed were recovered in 3 mL of 10N hydrochloric acid. Samples were filtrated using filter paper, and the volume was adjusted to 50 mL with distilled water. Na^+ and K^+ concentrations were determined using a flame spectrophotometer (Model 410).

II-7 Statistical analysis

Statistical analysis was performed using SPSS version 22. It concerned a three-way analysis of variance (ANOVA III) with alfalfa varieties, salinity and proline were the independent variables. Means were compared using Tukey's test.

III Results

III-1 Growth parameters

Salinity stress remarkably decreased the dry weight of shoots and roots of the three alfalfa varieties (Table 1). Additionally, among the three alfalfa varieties, *NS Med* was the most affected, with reductions rates of 75% and 85% observed for the shoot and root dry weight, respectively, compared to control. However, external supplementation of 20 mM proline to the growth medium of salt-stressed alfalfa plants reduced the impact of salt stress on both shoot and root dry weight. Indeed, under the combined effect of proline and NaCl, the reduction rates were only 72% and 13% for *OL*, 36% and 61% for *Dm* and 52% and 71% for *NS Med*, respectively, for the shoot and root dry weight (Table 1). Under normal conditions, exogenous proline supplementation has no significant effect on the shoot dry weight of the three alfalfa varieties. In contrast, for root dry weight, a significant increase was observed for *OL* variety. Salt stressed alfalfa plants showed a lower salt tolerance index, particularly the European *NS Med* variety, where the lowest (19%) value was recorded (Table 1). In contrast, salt-stressed alfalfa plants treated with 20 mM proline revealed improved STI of 46%, 47% and 37% for *OL*, *Dm* and *NS Med*, respectively (Table 1).

III-2 Plant height, leaf number and area

Exposure to salinity stress caused significant decreases in alfalfa plant growth which were indicated by the reduction in plant height, leaf number and area (Table 1). Compared to the other varieties, *NS Med* showed the highest reduction rates of 53% and 65% for plant height and leaf number. In contrast, the highest reductions rates (37%) for leaf area were observed in *OL* relative to the unstressed control. However, values of these growth parameters in salt-stressed plants were remarkably increased upon proline treatment. Indeed, 20 mM proline treatment increased plant height, leaf number and area by 15%, 40% and 9% in *OL*, by 35%, 49% and 32% in *Dm* and by 38%, 87% and 13% in *NS Med*, respectively relative to the untreated stressed alfalfa plants (Table 1). Furthermore, when applied to the unstressed alfalfa plants, proline increased leaf area in the Moroccan mountainous *Dm* variety and leaf number in both *OL* and *NS Med*.

Table 1. Effect of exogenous proline (20 mM) application on SDW, RDW, STI, PH, LN, and LA in three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under either salt stress (200 mM NaCl) or non-stress conditions.

		SDW	RDW	STI	PH	LN	LA
OL	Control	0.37 ± 0.003^{a}	0.17 ± 0.01^{b}		31.77 ± 0.76^{a}	62.33 ± 0.90^{b}	1.94 ± 0.01^{a}
	Proline	0.33 ± 0.03^{a}	0.41 ± 0.02^{a}		30.33 ± 0.64^{a}	64.33 ± 0.51^{a}	1.57 ± 0.004^{b}
	NaCl	0.10 ± 0.002^{b}	0.07 ± 0.001^{c}	30.73 ± 1.36^{b}	22.53 ± 0.45^{c}	34.66 ± 0.89^{d}	1.22 ± 0.08^{c}
	NaCl+Proline	0.10 ± 0.006^{b}	0.15 ± 0.01^{b}	46.38 ± 1.15^{a}	25.93 ± 0.21^{b}	48.33 ± 1.03^{c}	1.33 ± 0.06^{c}
Dm	Control	0.29 ± 0.01^{a}	0.71 ± 0.06^{a}		31.63 ± 0.32^{a}	67.33 ± 0.64^{a}	1.64 ± 0.09^{b}
	Proline	0.28 ± 0.01^{a}	0.78 ± 0.02^{a}		31.00 ± 0.19^{a}	56.66 ± 0.64^{b}	2.25 ± 0.16^{a}
	NaCl	0.14 ± 0.01^{c}	0.12 ± 0.02^{c}	26.74 ± 3.41^{b}	16.63 ± 0.32^{c}	30.33 ± 0.89^{d}	1.37 ± 0.06^{c}
	NaCl+Proline	0.19 ± 0.006^{b}	0.27 ± 0.01^{b}	46.79 ± 1.91^{a}	22.46 ± 0.55^{b}	45.33 ± 0.64^{c}	1.81 ± 0.14^{b}
NS Med	Control	0.38 ± 0.02^{a}	0.57 ± 0.01^{a}		29.66 ± 1.02^{a}	44.00 ± 0.38^{b}	2.19 ± 0.09^{a}
	Proline	0.39 ± 0.02^{a}	0.31 ± 0.003^{b}		30.23 ± 0.47^{a}	46.66 ± 1.02^{a}	1.83 ± 0.09^{b}
	NaCl	0.09 ± 0.01^{c}	0.08 ± 0.01^{d}	19.15 ± 1.26^{b}	13.96 ± 0.39^{c}	15.33 ± 0.64^{d}	1.56 ± 0.17^{b}
	NaCl+Proline	0.18 ± 0.01^{b}	0.16 ± 0.01^{c}	36.51 ± 0.94^{a}	19.23 ± 0.45^{b}	28.66 ± 0.64^{c}	1.76 ± 0.03^{b}

SDW, shoot dry weight; RDW, root dry weight; STI, salt tolerance index; PH, plant height; leaf number, LN; LA, leaf area. NaCl, 200 mM NaCl treatment; Proline, 20 mM proline treatment; NaCl+Proline, combination of NaCl and proline treatment. Values are the mean of three replicates ± standard deviation and the letters indicate statistically significant values.

III-3 Photosynthetic pigments

Alfalfa plants exposed to 200 mM NaCl stress showed reduced photosynthetic pigments (Table 2). Indeed, compared to control, Chl a, Chl b, total Chl and carotenoids were decreased, respectively by 76%, 86%, 79% and 73% in *OL*, by 78%, 79%, 78% and 76% in *Dm* and by 86%, 93%, 89% and 70% in *NS Med*. However, in proline-treated alfalfa plants, the reduction rates were only 64%, 79%, 69% and 63% in *OL*, 55%, 73%, 61% and 49% in *Dm* and 43%, 77%, 57% and 59% in *NS Med*, respectively for Chl a, Chl b, total Chl and carotenoids (Table 2). Under unstressed conditions, proline supplementation increased significantly Chl a and carotenoids contents in *OL*.

Table 2. Effect of salinity (200 mM NaCl) and exogenous proline (20 mM) on Chl a, Chl b, total Chl and carotenoids in three *M. sativa* varieties (*OL*, *Dm* and *NS Med*).

		Chl a (mg g^{-1} FW)		Chl b (mg g^{-1} FW)		Total Chl (mg g^{-1} FW)		Car (mg g^{-1} FW)	
		-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl
OL	-Proline	0.79 ± 0.02^{b}	0.19 ± 0.008^{d}	0.44 ± 0.02^{a}	0.06 ± 0.004^{d}	1.23 ± 0.02^{a}	0.25 ± 0.01^{c}	0.36 ± 0.002^{b}	0.10 ± 0.004^{d}
	+Proline	0.93 ± 0.007^{a}	0.28 ± 0.003^{c}	0.29 ± 0.01^{b}	0.09 ± 0.0003^{c}	1.23 ± 0.02^{a}	0.38 ± 0.003^{b}	0.45 ± 0.007^{a}	0.13 ± 0.002^{c}
Dm	-Proline	0.72 ± 0.003^{a}	0.16 ± 0.01^{c}	0.33 ± 0.02^{a}	0.07 ± 0.002^{d}	1.05 ± 0.02^{a}	0.23 ± 0.008^{d}	0.34 ± 0.013^{a}	0.08 ± 0.001^{c}
	+Proline	0.70 ± 0.02^{a}	0.32 ± 0.009^{b}	0.22 ± 0.005^{b}	0.09 ± 0.002^{c}	0.92 ± 0.02^{b}	0.41 ± 0.008^{c}	0.34 ± 0.003^{a}	0.17 ± 0.01^{b}
NS Med	-Proline	0.71 ± 0.03^{a}	0.10 ± 0.002^{c}	0.47 ± 0.04^{a}	0.03 ± 0.008^{d}	1.18 ± 0.07^{a}	0.13 ± 0.009^{d}	0.34 ± 0.003^{b}	0.10 ± 0.001^{d}
	+Proline	0.69 ± 0.02^{a}	0.41 ± 0.008^{b}	0.27 ± 0.01^{b}	0.11 ± 0.005^{c}	0.96 ± 0.006^{b}	0.51 ± 0.004^{c}	0.36 ± 0.004^{a}	0.14 ± 0.006^{c}

Chl, chlorophyll. -NaCl, 0 mM NaCl treatment; +NaCl, 200 mM NaCl treatment; -Proline, 0 mM proline treatment; +Proline, 20 mM proline treatment. Values are the mean of three replicates ± standard deviation and the letters indicate statistically significant values.

III-4 Proline and soluble sugars contents

Alfalfa plants treated with either proline or NaCl showed increased proline content, with the highest value observed for *NS Med* under either proline (5.50 µmol proline g^{-1} FW) or NaCl (14.39 µmol proline g^{-1} FW) treatments (Figure 1). Moreover, the proline content was further increased when alfalfa plants were treated with both proline and NaCl. Indeed, under the combined effect, proline content reached 28.85, 24.66 and 31.97 µmol proline g^{-1} FW in *OL*, *Dm* and *NS Med*, respectively.

Under salt stress conditions, soluble sugars concentration increased from 8.01 to 10.58 mg glucose g^{-1} FW in *OL*, from 7.81 to 10.95 mg glucose g^{-1} FW in *Dm* and from 5.76 to 10.28 mg glucose g^{-1} FW in *NS Med* (Figure 1). In contrast, proline application to plants submitted to salt stress remarkably reduced soluble sugars, particularly in *OL* and *Dm*, where the reduction rates reached 36% and 32%, respectively, relative to proline-untreated salt-stressed controls (Figure 1). Under normal conditions, proline induced a slight increase in soluble sugars in *OL* and *NS Med* compared to untreated controls.

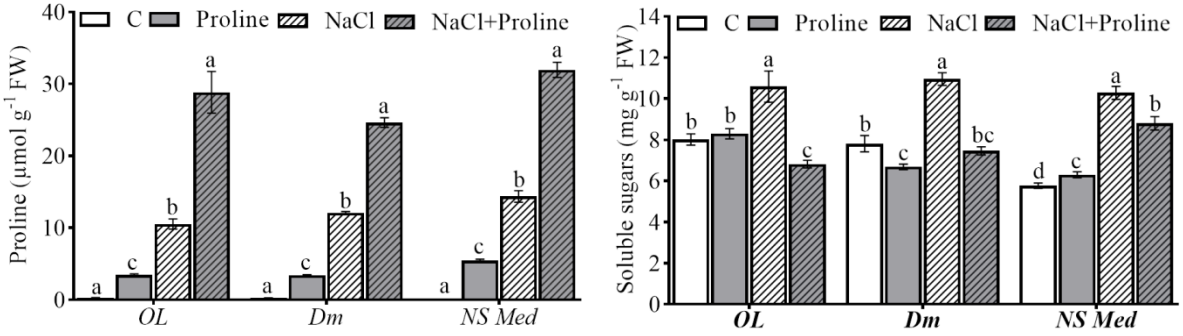

Figure 1. Effect of salinity (200 mM NaCl) and exogenous proline (20 mM) on proline and soluble sugars contents of three *M. sativa* L. varieties (*OL*, *Dm* and *NS Med*). C, control; Proline, 20 mM proline treatment; NaCl, 200 mM NaCl treatment; NaCl+Proline, combination of NaCl and proline treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values.

III-5 MDA content

Salt stress resulted in a significant increase in MDA content in the three alfalfa varieties, with a significant difference (Figure 2). Relative to the unstressed controls, *NS Med* showed the highest increment rate of 54%, followed by *OL* and *Dm* with increments rates of 44% and 32%, respectively. Proline treatment lowered the level of MDA in alfalfa plants subjected to salinity stress (Figure 2). A significant decrease of 5%, 18% and 26% were observed, respectively, in *OL*, *Dm* and *NS Med* treated with both NaCl and proline compared to those treated with NaCl only. Under normal conditions, proline treatment reduced MDA in *Dm* only.

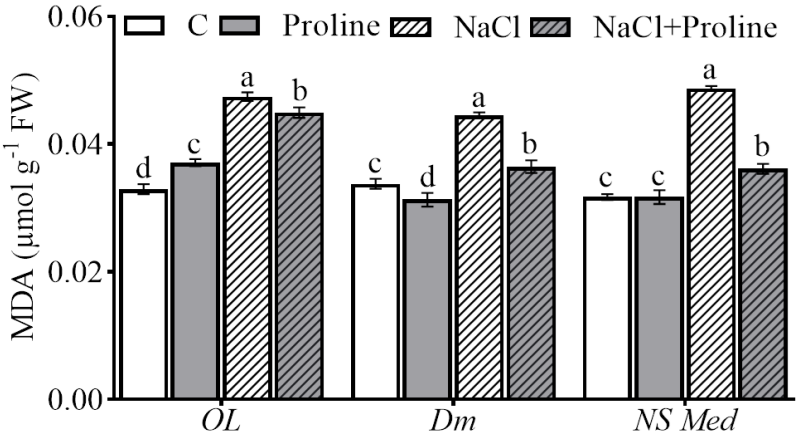

Figure 2. Effect of salinity (200 mM NaCl) and exogenous proline (20 mM) on malonyldialdehyde (MDA) content of three *M. sativa* L. varieties (*OL*, *Dm* and *NS Med*). C, control; Proline, 20 mM proline treatment; NaCl, 200 mM NaCl treatment; NaCl+Proline, combination of NaCl and proline treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values.

III-6 Enzymatic antioxidant

The activities of SOD, CAT, APX and GR were improved upon salt stress in the three alfalfa varieties relative to control (Figure 3). Indeed, SOD, CAT, APX and GR were 17.16, 1.87, 12 and 32.43-fold higher in *OL*, 17.30, 2.05, 14.14 and 30-fold higher in *Dm* and 21.39, 1.35, 16.36 and 58.59-fold higher in *NS Med*, respectively relative to their corresponding controls. Moreover, the exogenous addition of 20 mM proline furthered the increase of CAT activity in the three salt-stressed alfalfa varieties and SOD activity in the salt-stressed *Dm* variety (Figure 3). However, APX and GR in salt-stressed alfalfa plants were decreased significantly in response to proline addition. Moreover, under unstressed conditions, proline treatment significantly increased SOD, APX and GR in the three alfalfa varieties and CAT in *NS Med* variety as compared to control (Figure 3).

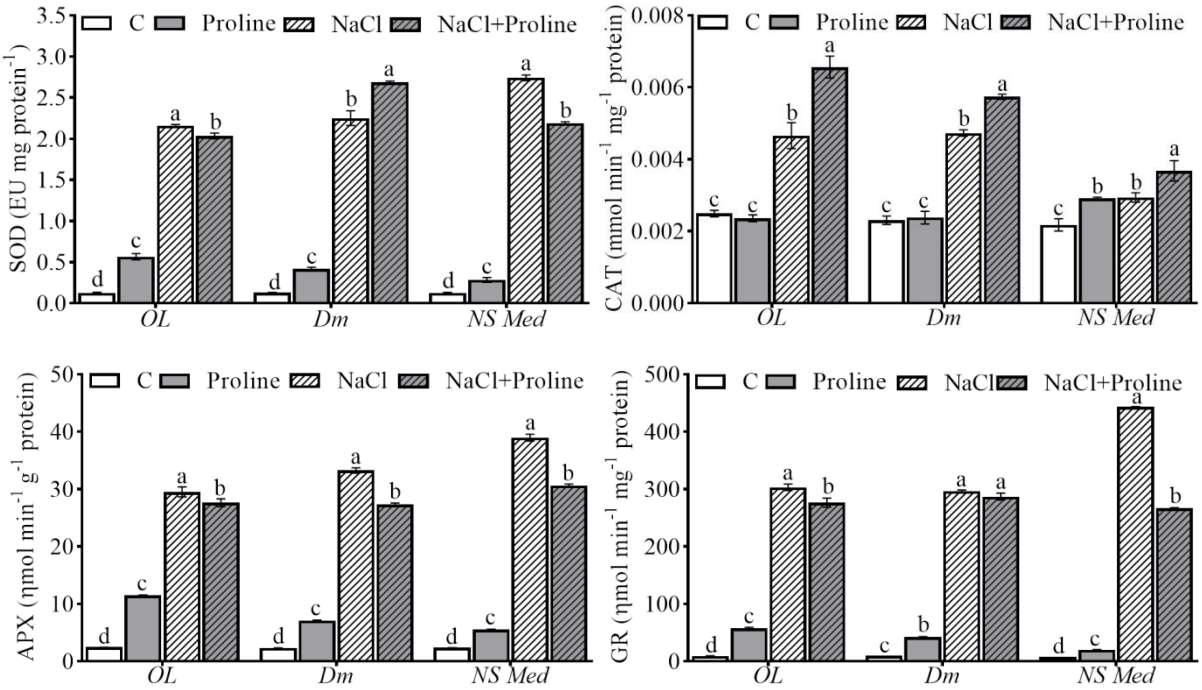

Figure 3. Effect of salinity (200 mM NaCl) and exogenous proline (20 mM) on superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activities of three *M. sativa* L. varieties (*OL*, *Dm* and *NS Med*). C, control; Proline, 20 mM proline treatment; NaCl, 200 mM NaCl treatment; NaCl+Proline, combination of NaCl and proline treatment. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values.

III-7 Nutrient analysis

Salt-stressed alfalfa plants displayed increased content of Na^+ in the shoot (21.36, 9.99, and 21.89-fold higher in *OL*, *Dm*, and *NS Med*, respectively) and root (4.80, 3.69, and 6.82-fold higher in *OL*, *Dm*, and *NS Med*, respectively), and reduced content of K^+ in the shoot (1.02, 1.29, and 1.16-fold lower in *OL*, *Dm*, and *NS Med*, respectively) and root (6.13, 1.45, and 1.88-fold lower in *OL*, *Dm*, and *NS Med*, respectively) compared to the unstressed controls (Table 3). However, when salt-stressed alfalfa plants were treated with 20 mM proline, Na^+ was reduced, whereas the amount of K^+ was increased, resulting in a higher K^+/Na^+ ratio. Indeed, K^+/Na^+ ratio increased from 0.33 to 0.35, from 0.24 to 0.37, and from 0.28 to 0.32 in the shoot of *OL*, *Dm*, and *NS Med*, respectively and from 0.13 to 0.28, and from 0.19 to 0.25 in the root of *OL* and *NS Med*, respectively (Table 3). However, in the root of *Dm*, K^+/Na^+ ratio was decreased from 0.18 under salt stress to 0.16 under the combined treatment of proline and NaCl. Under normal conditions, proline treatment reduced K^+/Na^+ ration in the shoot and root of the three alfalfa varieties.

Table 3. Effect of 20 mM proline on Na^+ and K^+ contents and K^+/Na^+ ratio in shoot and root of three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) grown under either 0 mM or 200 mM NaCl stress.

Treatment		Shoot			Root		
		Na^+ (mg g^{-1} DW)	K^+ (mg g^{-1} DW)	K^+/Na^+	Na^+ (mg g^{-1} DW)	K^+ (mg g^{-1} DW)	K^+/Na^+
OL	Control	1.75 ± 0.10^{d}	12.53 ± 0.17^{b}	7.21 ± 0.34^{a}	2.55 ± 0.12^{d}	9.54 ± 0.10^{a}	3.77 ± 0.15^{a}
	Proline	4.16 ± 0.23^{c}	14.39 ± 0.14^{a}	3.49 ± 0.17^{b}	2.80 ± 0.05^{c}	3.55 ± 0.11^{b}	1.27 ± 0.02^{b}
	NaCl	37.48 ± 0.51^{a}	12.21 ± 0.18^{c}	0.33 ± 0.001^{d}	12.25 ± 0.10^{a}	1.56 ± 0.04^{d}	0.13 ± 0.002^{d}
	NaCl+Proline	35.70 ± 0.11^{b}	12.51 ± 0.03^{b}	0.35 ± 0.002^{c}	11.47 ± 0.24^{b}	3.22 ± 0.08^{c}	0.28 ± 0.003^{c}
Dm	Control	3.79 ± 0.11^{d}	11.61 ± 0.06^{c}	3.08 ± 0.10^{a}	3.29 ± 0.05^{d}	3.24 ± 0.03^{a}	0.99 ± 0.01^{a}
	Proline	5.96 ± 0.08^{c}	14.43 ± 0.02^{a}	2.42 ± 0.03^{b}	3.63 ± 0.05^{c}	2.37 ± 0.03^{b}	0.65 ± 0.02^{b}
	NaCl	37.90 ± 0.05^{a}	8.96 ± 0.18^{d}	0.24 ± 0.005^{d}	12.13 ± 0.08^{a}	2.22 ± 0.06^{c}	0.18 ± 0.01^{c}
	NaCl+Proline	35.98 ± 0.27^{b}	13.25 ± 0.26^{b}	0.37 ± 0.004^{c}	9.43 ± 0.03^{b}	1.48 ± 0.09^{d}	0.16 ± 0.01^{c}
NS Med	Control	2.34 ± 0.07^{d}	16.68 ± 0.41^{a}	7.16 ± 0.38^{a}	2.33 ± 0.09^{d}	5.66 ± 0.11^{a}	2.43 ± 0.05^{a}
	Proline	4.77 ± 0.09^{c}	16.67 ± 0.21^{a}	3.50 ± 0.11^{b}	3.92 ± 0.12^{c}	4.44 ± 0.11^{b}	1.13 ± 0.02^{b}
	NaCl	51.34 ± 0.73^{a}	14.37 ± 0.11^{b}	0.28 ± 0.005^{d}	15.93 ± 0.08^{a}	3.00 ± 0.03^{d}	0.19 ± 0.003^{d}
	NaCl+Proline	39.31 ± 0.10^{b}	12.64 ± 0.17^{c}	0.32 ± 0.01^{c}	13.03 ± 0.07^{b}	3.31 ± 0.13^{c}	0.25 ± 0.01^{c}

K^+, potassium; Na^+, potassium. Proline, 20 mM proline treatment; NaCl, 200 mM NaCl treatment; NaCl+Proline, combination of NaCl and proline treatment. Values are the mean of three replicates ± standard deviation, and the letters indicate statistically significant values.

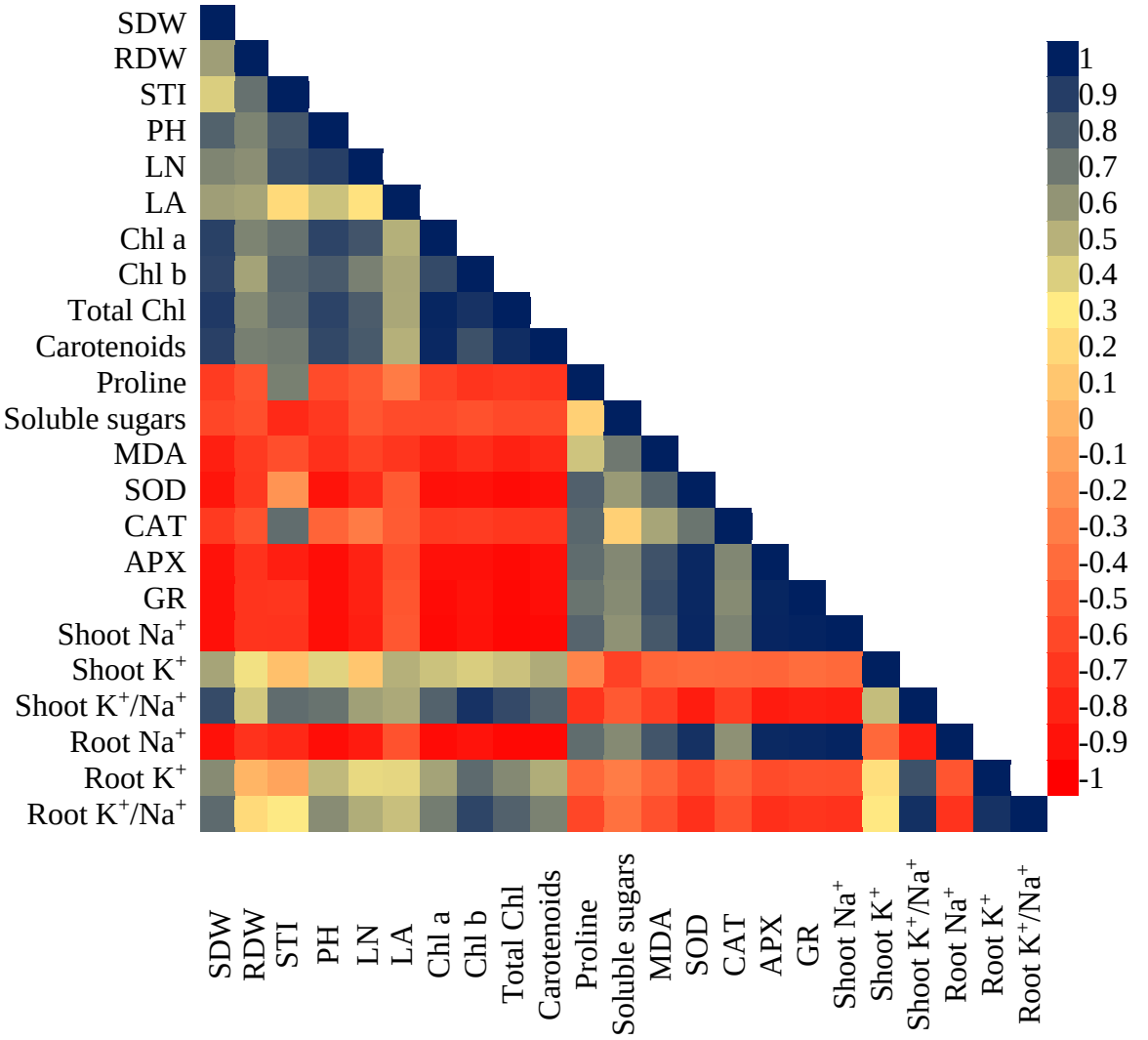

Figure 4. Pearson's correlation matrix between plant growth, photosynthetic pigments, compatible osmolyte, lipid peroxidation, enzymatic antioxidants and nutrient element in three alfalfa (*Medicago sativa* L.) varieties (*OL*, *Dm* and *NS Med*) treated with 0 or 200 mM NaCl with or without 20 mM proline. Correlations are displayed in blue (positive) and red (negative); color intensity is proportional to the correlation coefficient. SDW, shoot dry weight; RDW, root dry weight; STI, salt tolerance index; PH, plant height; LN, leaf number; LA, leaf area; MDA, malonyldialdehyde; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase.

IV Discussion

Salinity is one of the key restrictive factors limiting crop plant development (Van Zelm et al. 2020). It reduces plant growth by inducing photosynthesis reduction, oxidative stress, membrane damage and nutrient imbalance (Arif et al. 2020). Using exogenous amino acids such as proline can serve as a good strategy to mitigate salinity-reduced plant growth and productivity (El Moukhtari et al. 2020). These effects have been reported in several plant species (de Freitas et al. 2018; Sobahan et al. 2012; Hoque et al. 2007). However, in alfalfa, the effect is poorly understood. This study provides novel knowledge on the effect of exogenous proline on salt stress tolerance in alfalfa.

In our study, 200 mM NaCl stress greatly inhibited plant growth of alfalfa as manifested by lower biomass, fewer and small leaves and short plant. However, treatment with 20 mM proline mitigated the negative impact of salt stress and significantly improved shoot and root dry weight, plant height, leaf number and area. Several previous investigations have reported the positive role of exogenous proline in ameliorating the deleterious effects of salinity on plant growth of numerous plant species (Hayat et al. 2012; Heuer 2003; Hoque et al. 2007). For example, foliar spray with 30 mM proline improved both shoot and root dry weight (de Freitas et al. 2018). Similar results have been found in sorghum plants (de Freitas et al. 2019). In addition, the authors demonstrated that 30 mM proline foliar spray alleviated the negative impact of salt stress and significantly improved fresh and dry biomasses and leaf area. The positive impact of exogenous proline on plants growth was also reported in *Triticum aestivum* L. and *Lens culinaris* under drought (Bekka et al. 2018), in tobacco under cadmium stress (Islam et al. 2009), and in *Abelmoschus esculentus* under heat stress (Hussain et al. 2021).

Under salt stress, K^+ was reported to be substituted by Na^+ because of their similarity led to a lower K^+/Na^+ ratio (Benito et al. 2014). Thus, salt stress initially provokes ionic toxicity in higher plants, which eventually disturbs physiological attributes limiting plants' growth and development (Flowers et al. 2015). In our study, alfalfa plants exposed to 200 mM NaCl displayed increased Na^+ content and reduced K^+ content in both the shoot and root of the three alfalfa varieties. Moreover, the increased Na^+ content, particularly in the shoot part, was negatively correlated (Figure 4) with the photosynthetic pigment contents, including Chl a (r=-0.5; p=0.001), Chl b (r=-0.9; p=0.001), total Chl (r= -0.9; p=0.001) and carotenoids (r= 0.9; p=0.001). This partially explained the reduced plant growth under salt stress. However, proline-treated salt-stressed plants tend to maintain higher K^+/Na^+ ratio and photosynthetic pigments. Importantly, the increased K^+/Na^+ ratio and photosynthetic pigments were positively correlated

(Figure 4) with plant growth parameters, corroborating de Freitas's findings (de Freitas et al. 2019). Under salt stress conditions, the modulation of the activity of some transporters such as Na^+/H^+ antiporters and K^+/H^+ symporters is one of the proline's lies (El Moukhtari et al. 2020), which explains a part of the decreased Na^+ and increases K^+ contents in our study. Moreover, as Na^+ was reported to increase Chl-degrading enzymes and inhibit those responsible for Chl synthesis (Alamri et al. 2020), exogenous proline may positively affect the activity of these enzymes.

Salinity could also induce osmotic stress (Van Zelm et al. 2020). To overcome salinity-mediated osmotic stress, tolerant plants adjust their osmotic potential by accumulating compatible osmolytes, including proline (Munns and Tester 2008). In the current work, the exposition of alfalfa plants to 200 mM NaCl stress resulted in a spectacular increase in the content of proline and soluble sugars in the three alfalfa varieties. Furthermore, the treatment of salt-stressed alfalfa plants with 20 mM proline furthered the content of the three alfalfa plants on proline. In contrast, soluble sugars content was significantly decreased compared to proline untreated salt-stressed alfalfa plants. Similar results have been found by Ben Ahmed et al. (2010, 2011) on *Olea europaea*. Authors demonstrated that while the content of both proline and soluble sugars was increased under salinity, exogenous proline treatment further increased proline but decreased soluble sugars. The greater accumulation of proline in salt-stressed plants treated with proline could result from increased endogenous production, as exogenous proline was shown to upregulate genes encoding proline synthesis enzymes, including *Pyrroline-5-Carboxylate Synthetase* (*P5CS*) and *P5C Reductase* (*P5CR*) genes (Nounjan et al. 2012). Another explanation for the higher amount of proline in proline-treated alfalfa plants is the assimilation of proline applied exogenously to the soil solution. On the other hand, the decreased level of soluble sugars in salinized plants supplied with 20 mM proline was explained by Ahmed et al. (2010) by the fact that proline could be able to adjust the osmotic potential of the plant which limits the need of salt-stressed plants for soluble sugars synthesis and, thus, relaxing the pressure on the photosynthetic chain.

The effect of salinity could also be seen in oxidative stress (Arora et al. 2020). In the present study, salinity-induced membrane damage was reflected by higher MDA content. However, 20 mM proline treatment reduced the accumulation of MDA, supporting the beneficial effect of proline in alleviating oxidative stress caused by salt stress (El Moukhtari et al. 2020). Our findings are consistent with the investigations in *O. sativa*, *Cucumis sativus* and *Onobrychis viciaefolia*, in which proline treatment decreased lipid peroxidation of salt-stressed plants

(Sobahan et al. 2016; Wu et al. 2017; Huang et al. 2009). The low lipid peroxidation under proline treatment seemed to result from the activation of antioxidant enzymes (Hayat et al. 2012). In our study, 200 mM NaCl treatment increased the activities of SOD, CAT, APX, and GR. However, the application of both proline and NaCl further increased the activity of CAT in the three alfalfa varieties and SOD in *Dm* only. These findings are consistent with those of de Freitas et al. (2018), Nounjan and Theerakulpisutand (2012), and Hossain and Fujita (2010). They stated that antioxidant enzymes, including SOD, CAT, APX, and GR, increased under either salinity or salinity plus proline treatment.

V Conclusions

In conclusion, salt stress considerably reduced the plant's growth and caused lipid peroxidation and ionic toxicity reflected by high MDA content and low K^+/Na^+ ratio. However, proline treatment effectively alleviated the negative impact of salinity on alfalfa plants. Indeed, proline treatment reduced MDA and Na^+ contents and increased plant biomass and height, leaf number and area, photosynthetic pigments, proline and K^+ contents, and antioxidant enzymes activities. Our findings suggested that proline treatment might be a successful strategy for increasing alfalfa production under salinity stress.

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Chapitre V (article 6)

L'apport séparé de proline et de Si améliore la tolérance de la luzerne (*Medicago sativa*) aux contraintes salines plus que l'apport combiné des deux molécules.

I Introduction

L'apport exogène de silicium (Si) ou de la proline a été décrit pour leur effet bénéfique sur la tolérance des plantes aux contraintes abiotiques notamment la contrainte saline (Souri et al. 2020; Ben Rejeb et al. 2012). Les études sur l'effet combiné de Si et de proline sur la tolérance des plantes aux contraintes environnementales sont rares, et des résultats contrastés ont été décrits dans ce sens. Par exemple, chez *Phaseolus vulgaris*, Rady et al. (2019) ont démontré que l'apport exogène de la proline ou de Si peut améliorer la tolérance des plantes au stress salin et au stress métallique. De même, sous contrainte hydrique, l'effet de la proline et de Si sur la croissance de *Beta vulgaris* est plus marqué lorsqu'elles sont appliquées simultanément (AlKahtani et al. 2021). Cependant, chez *Vigna radiata*, dos Santos et al. (2022) ont démontré que l'apport séparé de la proline et de Si ont des effets très bénéfiques sur la tolérance des plantes au déficit hydrique, plus important que l'apport combiné des deux molécules. A notre connaissance, aucun travail n'a été publié sur l'effet combiné de ces deux molécules chez la luzerne.

II Méthodologies

Après avoir déterminé les concentrations bénéfiques de proline (20 mM) et du Si (3 mM) pour améliorer la tolérance de la luzerne à la contrainte saline, nous avons cherché à étudier sur leur effet combiné sur l'amélioration de la croissance des trois variétés de *M. sativa* sous contrainte saline sévère (200 mM de NaCl).

III Matériels et méthodes

Pour étudier l'effet de l'apport combiné ou séparé de la proline et de Si, les graines des trois variétés ont été cultivées sous serre et inoculées avec la même souche d'*E. meliloti* comme mentionné dans le chapitre III. Après un mois de croissance, les plantes ont ensuite été traitées avec du Si (3 mM) et/ou de la proline (20 mM) en présence et en absence de NaCl (200 mM). Au stade 47 jours (17 jours de stress), les plantes ont été récoltées et l'effet de la proline et/ou de Si a été évalué à travers le dosage de la chlorophylle, de MDA, des sucres solubles, de la proline ainsi que le dosage de certains éléments minéraux (Na^+, K^+ et P). L'effet de ces deux molécules exogènes sur le renforcement de la tolérance de *M. sativa* au stress oxydant induit par la salinité a été évalué à travers le dosage de l'activité de certains enzymes antioxydantes notamment la CAT et la SOD.

IV Résultats

Les résultats ont montré que l'application séparée de Si et de la proline entraine une augmentation de la teneur des plantes en pigments photosynthétiques, en proline, en K^+ et en phosphore et réduit la teneur des plantes en Na^+. Cependant, considérant ces paramètres, l'application combinée de Si et de la proline n'a montré aucun effet en comparaison avec les plantes traitées uniquement avec du NaCl. En revanche, l'application séparée de Si et de proline réduit la teneur en sucres solubles et en MDA avec une augmentation dans l'activité de la CAT et de la SOD chez les trois variétés étudiées.

V Conclusions

En conclusion, l'application combinée de proline et de Si n'a pas montré d'effets bénéfiques ou synergiques sur des plantes sous contraintes salines par rapport à des plantes traités avec soit de la proline soit du Si.

Ces données font l'objet d'un manuscrit intitulé ''Salt stress is alleviated by either proline or silicon but not by their combination, in three alfalfa (*Medicago sativa* L.) varieties'' accepté sous réserve de modifications pour publication.

Salt stress is alleviated by either proline or silicon but not by their combination, in three alfalfa (*Medicago sativa* L.) varieties

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Abstract

Salinity is one of the most deleterious abiotic stress limiting plant growth and productivity. In the present study, we aimed to assess the interplaying roles of 20 mM proline and 3 mM silicon (Si) in the mitigation of salt stress (200 mM NaCl) effects on three alfalfa (*Medicago sativa* L.) varieties: two Moroccan, *Ouad Lmaleh* (*OL*) and *Demnate 201* (*Dm*), and one European, *NS Mediana ZMS V* (*NS Med*). Salt stress strongly reduced plant biomass, with *NS Med* being the most affected. A beneficial effect of independently supplied proline or Si in salt-stressed alfalfa plants was observed on dry biomass and shoot-to-root ratio, on photosynthetic pigments, nutrient contents and K^+/Na^+ ratio. Moreover, stress indicators such as Na^+, malonyldialdehyde and hydrogen peroxide were significantly reduced. Proline or Si treatment also led to higher activities of both superoxide dismutase and catalase. However, when applied together, proline and Si beneficial effects were less efficient in comparison with their single application for almost all the investigated parameters. We conclude that application of either proline or Si provides beneficial effects in mitigating the toxic effect of salt on alfalfa and could be a promising way to improve its growth in salt-affected soils. However, a combined Si and proline treatment is unadvisable.

Keywords: *Medicago sativa*; Salinity; Silicon; Proline; Plant development; Oxidative stress.

I Introduction

Alfalfa (*Medicago sativa* L.) is an important forage legume, one of the most cultivated feed worldwide (Annicchiarico 2015; Al-Farsi et al. 2020; Acharya et al. 2021) as a supply of proteins for livestock (Radović et al. 2009). Alfalfa has also an important ecosystem role especially in the nitrogen cycle due to its ability to fix biological nitrogen when associated with rhizobia (Rocci et al. 2019). However, alfalfa crop production is strongly affected by various abiotic constraints including drought, salinity, elevated temperature, heavy metals and nutrient deficiency (Wassie et al. 2020; Gao et al. 2020; He et al. 2021; Qiu et al. 2021). Salinity is one of the most critical abiotic stresses limiting plant growth and development (Elgharably and Benes 2021). In addition, this constraint becomes an increasingly important limiting factor for crops due to climate change (Roy et al. 2014; Van Zelm et al. 2020). In fact, salt stress triggers ionic imbalance, specific ion toxicity and osmotic stress. As a consequence, restricted photosynthesis, increased reactive oxygen species (ROS) production, ROS mediated oxidative damage, hormonal imbalance, contribute to retardation of plant development in saline soils (Munns and Tester 2008). To improve plant salinity resistance, various approaches have been adopted (Johnson and Puthur 2021). Some of them, such as conventional breeding, are time-consuming. Others, using genetic modifications, are not accepted in many countries (Savvides et al. 2016). The use of exogenous compounds, such as silicon (Debona et al. 2017; Lesharadevi et al. 2021) or proline (El Moukhtari et al. 2020), is a sustainable approach to overcoming the negative effects of salt stress on plant growth, and productivity.

Proline is one of the most common accumulated osmolytes in plants in response to various stress conditions including water stress and salinity (Forlani et al. 2019; Meena et al. 2019). It is presented as a multifunctional amino acid that is important in the regulation of plant growth and development particularly under abiotic stresses (Szabados and Savouré 2010; Alvarez et al. 2021). When applied exogenously, proline has been reported to confer a wide range of protective roles to various plants (Meena et al. 2019; El Moukhtari et al. 2020). Under salt stress, exogenous proline may regulate ion uptake and transport, photosynthesis, reduction of lipid membrane oxidation, both enzymatic and non-enzymatic antioxidants and stabilizes subcellular structures (Hoque et al. 2008; Shahbaz et al. 2013; de Freitas et al. 2018). Proline can also play an important role in osmotic adjustment to protect plants from salt-induced osmotic stress (Zheng et al. 2015). Furthermore, this key amino acid has also been linked to cascade signaling pathways that help plant growth under salinity-stressed conditions (Yang et

al. 2009; Singh et al. 2014). In *Cicer arietinum*, proline supply has also been proven efficient in improving the efficiency of nitrogen fixation under cadmium stress (Alyemeni et al. 2016).

Silicon (Si) is the second most important element after oxygen in term of abundance, representing up to 28.8% of the Earth's crust (de Tombeur et al. 2021). Although Si is not characterized as an essential nutrient element for plant growth and development (El Moukhtari et al. 2021b; Coskun et al. 2019), its exogenous application has been proven to be efficient against abiotic stresses (Debona et al. 2017; Lesharadevi et al. 2021). Regarding salinity, Si treatment has been shown to preserve nutritional balance, optimal K^+/Na^+ ratio, modulate scavenging enzyme activities, and to limit ROS production (Tuna et al. 2008; Al Murad et al. 2020; Meng et al. 2020). Si has also been found to improve photosynthesis, water nutrition, nitrogen fixation and osmolyte accumulation, which all are essential for plant growth and biomass production (Kurdali et al. 2019; Al Murad et al. 2020; El Moukhtari et al. 2021a).

Interactive effects between proline and Si have been reported on *Phaseolis vulgaris* (Rady et al. 2019) and *Olea europea* L. (Radhi et al. 2021) under salt stress and on *Beta vulgaris* L. (Alkahtani et al. 2021) and recently in *Vigna radiata* L (dos Santos et al. 2022) under drought stress. However, the interactive effect between proline and Si in alfalfa grown under salinity was not investigated yet. Therefore, the aim of this work was to investigate the individual and combined impacts of proline and Si on alfalfa growth through the investigation of some plant development parameters. Membrane stability in terms of malonyldialdehyde (MDA), oxidative stress in terms of hydrogen peroxide (H_2O_2), superoxide dismutase (SOD) and catalase (CAT) as well as nutrient homeostasis in terms of phosphorus (P), sodium (Na^+), potassium (K^+) and Si contents were also assessed.

II Materials and methods

II-1 Plant material

Seeds of two Moroccan *Medicago sativa* L. varieties, *Oued Lmaleh* (*OL*) from Oasis and *Demnate 201* (*Dm*) from High Alas Mountains, and a European variety *NS Mediana ZMS V* (*NS Med*) were supplied by the National Institute of Agronomic Research (INRA-Marrakesh, Morocco) and used as material in this work. In a previous study, the two varieties *OL* and *Dm* were characterized as salt tolerant, whereas *NS Med* was described as sensitive (El Moukhtari et al. 2021a). The two varieties *OL* and *Dm* are among the most cultivated varieties in traditional Moroccan agroecosystems, showing a good adaptation to local habitats.

II-2 Plant growth conditions and treatments

Seeds were surface-sterilized by immersion in sodium hypochlorite (6%) for 5 min, rinsed five times with sterilized distilled water and sowed in plastic pots (6 cm in diameter, 6 cm depth) containing sterilized perlites (granule size, 0.6-6 mm; pH 6.8; EC 0.0-0.1 µS cm^{-1}) and vermiculite (granule size, 1-4 mm; pH 7; EC 0.1-0.3 µS cm^{-1}) with a ratio of 6:1 (v/v). Plants were grown in a greenhouse with 16/8 h light/dark cycle at 80-100 µmol photons s^{-1} m^{-2} at 25 ± 1 °C and 60% - 80% relative humidity. After shoot emergence, three seedlings of same size were left in each pot. Fifteen-day-old-seedlings were inoculated three times with the rhizobial strain *Ensifer meliloti Rm41* as described previously (El Moukhtari et al. 2021a). Seedlings were irrigated weekly with nitrogen (N) free nutrient solution (Hoagland and Arnon 1950) [KH_2PO4 (250 µmol), $MgSO_4$ (1000 µmol), K_2SO_4 (750 µmol), $CaCl_2$ (1650 µmol), Fe-EDTA (16 µmol), $MnSO_4$ (6 µmol), H_3BO_3 (4 µmol), $ZnSO_4$ (1 µmol), $NaMoO_4$ (0.1 µmol), and $CuSO_4$ (1 µmol)]. The pH of the nutrient solution was adjusted to 6.8-7 before use. Urea (2 mM) was supplied the first week to avoid any N deficiency during nodule development. One month after sowing, pots were sorted in control (0 mM NaCl) and stress (200 mM NaCl). Four treatments were applied: i) control, ii) 20 mM proline supplementation, iii) 3 mM Si supplementation (in $CaSiO_3$), and iv) 20 mM proline and 3 mM Si in combination. Each treatment was replicated 5 times. Salt was added gradually to the growth medium to avoid osmotic chock, incrementing NaCl by 40 mM every two days up to 200 mM. Seventeen days after the beginning of stress treatment, plants were harvested. All the biochemical and enzymatic parameters were measured using the youngest (first to third) fully expanded leaves of alfalfa plants.

II-3 Plant dry weight and shoot to root ratio measurements

Seventeen days after stress application, three plants from each treatment were selected randomly and used for biomass determination. For this purpose, the shoots were cut off from the root and subsequently dried at 80 °C. Dry weight (DW) was measured after 48 h. Roots were cleaned by hand, washed thoroughly with deionized water and dried afterwards at 80 °C for 48h. The shoot to root ratio was calculated by taking the shoot DW of the plants and dividing it by the DW of the root.

II-4 Salt tolerance index (STI)

STI was calculated as described by Bağci et al. (2003) using below formula

$$STI\ (\%) = \frac{TDW_{at\ Sx}}{TDW_{at\ S1}}\ x\ 100$$

With TDW is the total dry weight, Sx treatment (NaCl, NaCl × Si, NaCl×Proline or NaCl×Si×Proline), S1 control treatment.

II-5 Estimation of photosynthetic pigment contents

Photosynthetic pigments were extracted according to Launay et al. (2019), which was adapted from the method developed by Arnon (1949). Around 100 mg of frozen leaf materiel were ground to fine powder in liquid nitrogen and resuspended in 1 mL of 92% acetone (V/V) and then centrifuged at 10000 *xg* for 10 min at 4 °C. The resulted supernatant was collected and adjusted to 2 mL with 80% acetone (V/V). The absorbance was read at 480, 645 and 663 nm using 80% acetone as a blank. The concentration of chlorophyll (Chl) a, Chl b and carotenoids was determined according to D'souza and Devaraj (2013).

II-6 Proline and soluble sugar content determination

Bates et al. (1973) method was followed for proline determination with slight modification. Around 50 mg of clean, washed to eliminate any traces of exogenous proline, frozen leaves were ground to powder in liquid nitrogen and resuspended in 1 mL of aqueous sulfosalicilic acid (3% w/v). Samples were centrifuged at 18000 *xg* for 10 min at 4 °C. To 400 µL of the obtained supernatant, equal volume of 3% ninhydrin (W/V) and glacial acetic acid were added. The mixture was heated at 100 °C for 1 h then placed to cool down in an ice bath. Toluene (800 µL) was added to extract the colored pink phase. Proline content was determined by measuring

the optical density (OD) at 520 nm using NP80 Nanophotometer® (Implen) using calibration curves and expressed as µmol proline g^{-1} FW.

For soluble sugars quantification, 50 mg of frozen leaves were ground to powder in liquid nitrogen and resuspended in 1 mL of 80% ethanol. After 20 min of centrifugation at 2.700 x*g* at 4 °C, supernatant was collected and used to measure soluble sugars (Yemm and Willis, 1954). Thus, to 0.25 mL of supernatant, 0.25 mL of distilled water and 1 mL of cold anthrone solution (0.2%, W/V, in 80% sulfuric acid, V/V) were added. The mixture was heated at 100 °C for 10 min and the OD was read at 630 nm. Soluble sugars concentrations were estimated using calibration curves established with known concentration of glucose.

II-7 Oxidative stress markers

Malonyldialdehyde (MDA) was determined in fresh leaves using the thiobarbituric acid (TBA) method (Heath and Packer 1968). 100 mg of frozen leaves were homogenized in 1 mL of TBA (0.5%) prepared in 20% trichloroacetic acid (TCA) and heated at 95 °C for 30 min. Samples were then rapidly cooled in an ice bath and centrifuged at 10 000 ×*g* for 10 min at 4 °C. Supernatant was used to read the OD at 532 nm and 600 nm. MDA concentration was determined using its extinction coefficient of 155 mM^{-1} cm^{-1} of 532 nm and expressed as µmol MDA g^{-1} FW.

Hydrogen peroxide (H_2O_2) content in *M. sativa* leaves was determined as described by Velikova et al. (2000). 100 mg of frozen leaf samples were ground in powder in liquid nitrogen and resuspended in 5 mL of TCA (0.1%) in an ice bath. The homogenate was centrifuged at 12000 ×*g* for 10 min at 4 °C and to 0.5 mL of supernatant, 0.5 mL of potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide were added. The absorbance of the mixture was read at 390 nm and the content of $H_2O_2$was calculated and expressed as µmol H_2O_2 g^{-1} FW.

H_2O_2 staining in leaves was determined as described by Ben Othman et al. (2017) using 3,3'-diaminobenzidine (DAB) method. Leaves from different treatments were immersed in DAB solution (1 mg mL^{-1}) prepared in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (10 mM, pH 5.8) and shaken at 200 rpm for an overnight. After draining off the staining solution, leaves were immersed in absolute ethanol and heated to remove the Chl. Samples were photographed using a digital camera on top of a sheet of paper saturated with 60% glycerol (V/V).

II-8 Estimation of antioxidant enzyme activity

Fresh leave samples (0.5 g) were ground in liquid nitrogen and resuspended at 4 °C in 5 mL of 50 mM phosphate buffer (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid and 1%

polyvinylpolypyrrolidone. The homogenate was centrifuged for 20 min at 20000 ×*g* at 4 °C and the supernatant was used for superoxide dismutase (SOD) and catalase (CAT) assay.

SOD activity was assayed as described by Beyer and Fridovich (1987). For this purpose, 30 µL of enzyme extract was added to 1 mL of 50 mM phosphate buffer (pH 7.8) containing nitroblue tetrazolium (65 µM), L-methionine (13.3 µM) and riboflavin (1.33 µM). The mixture was exposed to a fluorescence lamp for 5 min and then the OD was measured at 560 nm using a NP80 Nanophotometer® (Implen). One enzymatic unit (EU) of SOD was defined as the amount required for the inhibition of 50% NBT. The activity was expressed as EU min^{-1} mg^{-1} protein.

CAT activity was estimated according to Hwang et al. (1999) by following the decomposition of H_2O_2 for 2 min. The reaction mixture was obtained in a final volume of 1 mL by mixing 50 mM potassium buffer (pH 7.0) containing 20 mM of H_2O_2 and 10 µL of protein extract. The decrease of H_2O_2 was followed spectrophotometrically using Jasco V-730 spectrophotometer at 240 nm. The activity of CAT was determined by using a value of 39.4 M^{-1} cm^{-1} for the extinction coefficient of H_2O_2 and expressed as ηmol H_2O_2 min^{-1} mg^{-1} protein.

II-9 Estimation of Si content

Si content was determined by the colorimetric molybdenum blue method following the method described in Dai et al. (2005) with modifications. Dry samples were ground to powder in plastic tubes in order to avoid Si contamination from mortar and pestle. 300 µL of 50% (W/V) sodium hydroxide (NaOH) was added to 10 mg of powder in polyethylene tubes. Tubes were then autoclaved at 121 °C for 21 min. After cooling down, samples were brought to 500 µL with deionized water and centrifuged at 29,750 *xg* for 15 min. 0.16 mL of supernatant was added to 2 mL tubes containing 1.2 mL of 20% acetic acid and gently mixed by vortex. Then, 400 µL of ammonium molybdate (43.7 mM, pH 7) was added and the mixture was gently mixed by vortex and incubated at room temperature. After 5 min, 200 µL of tartaric acid (20%) and 40 µL of reducing solution containing 8 g L^{-1} sodium sulphite (Na_2SO_3), 1.6 g L^{-1} 1-amino-2-naphthol-4-sulfonic acid and 100 g L^{-1} sodium bisulphite ($NaHSO_3$) were added and the mixture was then mixed by vortexing and incubated for 30 min at room temperature. The OD was then measured at 650 nm with a NP80 Nanophotometer® (Implen) and Si concentrations were calculated using standard curve prepared from SiO_2 standard solutions.

II-10 Determination of phosphorus (P), sodium (Na^+) and potassium (K^+) contents

Dry samples (0.5 g) from shoot and root alfalfa plants were incinerated for 6 h at 600 °C. Ash formed was recovered by adding 3 mL of HCl (10 N). After filtration, the solution was adjusted to 50 mL using distilled water.

For the P determination, to 1 mL of the obtained solution, 4 mL of distilled water and 5 mL of a mixture of 2.5% (w/v) sodium molybdate and 0.15% (w/v) hydrazine sulfate were added and the mixture was heated in a water bath (95 °C) for 10 min. After cooling down, the OD was determined at 825 nm and P concentration was calculated based on a standard curve established with KH_2PO_4 solutions.

According to Oukaltouma et al. (2021), Na^+ and K^+ were determined in the obtained solution using flame emission photometer (FP6450).

II-11 Statistical analysis

Data were analyzed using SPSS version 22. It concerned a four-way analysis of variance (ANOVA IV), where varieties, salinity, proline and silicon were the independent variables. Means were compared using Tukey's test. The difference was considered significant at $P \leq 0.05$ and indicated by different letters. The degree of correlation between the different studied parameters was also investigated based on Pearson's correlation matrix.

III Results

III-1 Effect of proline and Si on plant growth and salt tolerance index (STI) in salt-stressed plants

Exposure of alfalfa plants to 200 mM NaCl stress significantly decreased both shoot and root dry weight (DW), with *NS Med* being the most affected in both shoot (64%) and root (47%) (Table 1). Salinity also drastically reduced shoot to root ratio with the highest reduction rate of 34% recorded in *NS Med*. Supply of either proline or Si to salt-stressed alfalfa plants resulted in a higher DW and shoot to root ratio. Proline-treated stressed plants showed most growth improvement with a 45%, 68% and 122% increase for SDW, 17%, 21% and 33% increase for RDW and 24%, 40% and 66% increase for shoot to root ratio for *OL*, *Dm* and *NS Med*, respectively. In contrast, when proline was applied together with Si to salt-stressed alfalfa plants, shoot and root DW were lower (Table 1). Under normal growth conditions, the individual application of proline or Si increased SDW and shoot to root ratio and importantly this increase was more pronounced when proline was applied together with Si (Table 1).

Results illustrated in table 1 revealed that salt-stressed alfalfa plants exhibited low value of STI with *NS Med* showing the lowest STI value. Whereas individual application caused a significant increase of STI as compared to salt-stressed plant ($P \leq 0.001$), the combined application of proline and Si has no significant effect on STI (Table 1).

Table 1 Effect of 20 mM proline (Pro) and 3 mM $CaSiO_3$ (Si) on shoot dry weight (SDW), root dry weight (RDW), shoot to root (S to R) ratio and salt tolerance index (STI) of three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under either salt stress (200 mM NaCl) or non-stress conditions. Values are the mean of three replicates ± standard deviation. The letters indicate statistically significant values

	Varieties	Control	Pro	Si	Pro-Si	NaCl	NaCl-Pro	NaCl-Si	NaCl-Pro-Si
SDW (mg plant^{-1})	*OL*	25.5±1.7^{c}	42.7±1.8^{b}	27.6±2.2^{c}	43.9±2.5^{b}	12.3±1.8^{f}	17.8±1.3^{e}	16.6±1.6^{e}	11.1±1.9^{f}
	Dm	25.3±4.7^{cd}	71.3±14.0^{a}	32.1±3.0^{c}	71.3±13.9^{a}	11.7±2.4^{f}	19.6±2.2^{de}	16.6±1.4^{e}	8.1±2.6f
	NS Med	26.1±1.2^{c}	60.5±2.7^{a}	26.4±1.5^{c}	76.6±19.8^{a}	9.3±1.5^{f}	20.6±2.1^{de}	17.7±2.5^{de}	9.0±2.0^{f}
RDW (mg plant^{-1})	*OL*	25.0±0.9^{a}	25.4±0.9^{a}	22.3±1.4^{b}	22.8±2.5^{ab}	14.5±0.4^{e}	17±0.6^{c}	16.2±0.8^{cd}	14.1±1.4d^{ef}
	Dm	25.7±1.1^{a}	22.4±1.1^{b}	21.8±0.4^{b}	24.0±1.9^{ab}	13.6±1.7d^{ef}	16.5±1.1^{cd}	17.0±0.8^{c}	13.1±1.8^{ef}
	NS Med	22.6±1.8^{ab}	23.7±1.8^{ab}	20.3±0.9^{b}	21.2±0.6^{b}	12.0±0.7^{f}	16.0±1.2^{cde}	13.3±0.9^{ef}	10.4±1.4^{f}
S to R ratio (Plant^{-1})	*OL*	1.02±0.02^{f}	1.67±0.01^{c}	1.23±0.01^{e}	1.94±0.09^{c}	0.84±0.05^{g}	1.04±0.02^{f}	1.02±0.02^{f}	0.78±0.03^{g}
	Dm	0.97±0.04^{f}	3.15±0.26^{a}	1.46±0.06^{d}	2.94±0.21^{ab}	0.84±0.05^{g}	1.18±0.03^{e}	0.97±0.03^{f}	0.59±0.07^{h}
	NS Med	1.16±0.06^{e}	2.56±0.07^{b}	1.30±0.04^{e}	3.65±0.58^{a}	0.77±0.05^{g}	1.28±0.02^{e}	1.35±0.15^{de}	0.88±0.14^{fg}
STI (%)	*OL*	---	---	---	---	53.07±3.56^{b}	68.84±1.76^{a}	64.89±3.52^{a}	49.90±5.01^{bc}
	Dm	---	---	---	---	49.60±5.62^{bc}	70.78±6.40^{a}	65.81±3.33^{a}	41.43±8.71^{bc}
	NS Med	---	---	---	---	43.80±4.38^{bc}	75.41±6.62^{a}	63.82±4.57^{a}	39.96±4.43^{c}

III-2 Effect of proline and Si on photosynthetic pigments in salt-stressed plants

Contents of photosynthetic pigments (Chl a, Chl b and carotenoids) were significantly reduced in salt-stressed plants in the three alfalfa varieties (Fig. 1). Comparatively, *OL* was the most affected for Chl a and Chl b with reductions rates of 56% and 59%, respectively, whereas for carotenoids the highest reduction rate of 51% was recorded in *NS Med*. Exogenously supplied proline and Si individually led to a significant increase ($P \leq 0.001$) in photosynthetic pigments with proline treatment conferring the highest values. In contrast, under their combined application, a significant reduction in the contents of all photosynthetic pigments was observed in the three salt-stressed alfalfa varieties in comparison to individual proline or Si treatment. Under normal conditions, and compared to other treatments, proline supply to the growth medium of alfalfa plants increased significantly ($P \leq 0.001$) all contents of Chl a, Chl b and carotenoids in the three alfalfa varieties as compared to untreated control.

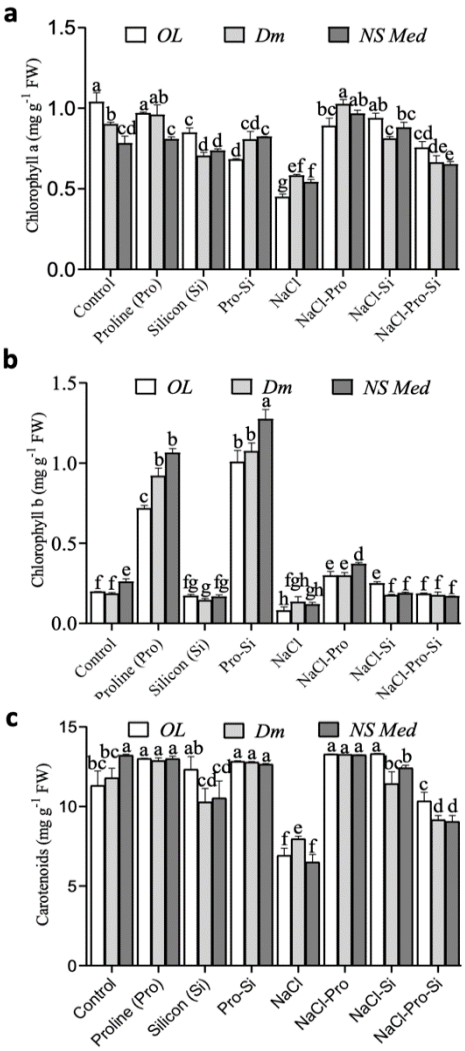

Fig. 1 Effect of 20 mM proline and 3 mM $CaSiO_3$ (Si) on chlorophyll a (a), chlorophyll b (b) and carotenoids (c) contents of three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under either salt

stress (200 mM NaCl) or non-stress conditions. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

III-3 Effect of proline and Si on solute content accumulation in response to salt stress

Proline content was significantly higher ($P \leq 0.001$) upon salt stress particularly in *OL* where the rate of increase reached around 690% relative to control (Fig. 2). Additionally, under salt stress, either proline or Si caused a remarkable increase in proline content although a combined application of both proline and Si further sustained the increase (Fig. 2). Under the combined application of both Si and proline, proline content increased from 0.53 to 11.69 µmol proline g^{-1} FW for *OL*, from 0.29 to 12.68 µmol proline g^{-1} FW for *Dm* and from 0.87 to 14.45 µmol proline g^{-1} FW for *NS Med*. Under normal conditions, proline alone or combined with Si caused significant ($P \leq 0.001$) increase in proline contents in the three alfalfa varieties, with the highest increases observed in plants treated with proline alone.

Soluble sugars accumulate in response to salt stress in alfalfa plants (Fig. 2b). Soluble sugar content further increased when salt-stressed plants were treated with both proline and Si. Indeed, relative to untreated-salt stressed plants, the simultaneous application of proline and Si increased soluble sugars by 4%, 19% and 8% in salt stressed-*OL*, *Dm* and *NS Med*, respectively. Stress-induced sugar accumulation was significantly prevented by the sole application of either 20 mM proline or 3 mM Si to salt-stressed alfalfa plants in the three varieties but not with the combination of both. Under normal growth conditions, only *OL* variety responded to the supplementation of proline alone or with Si by a significant increase of soluble sugars (Fig. 2b).

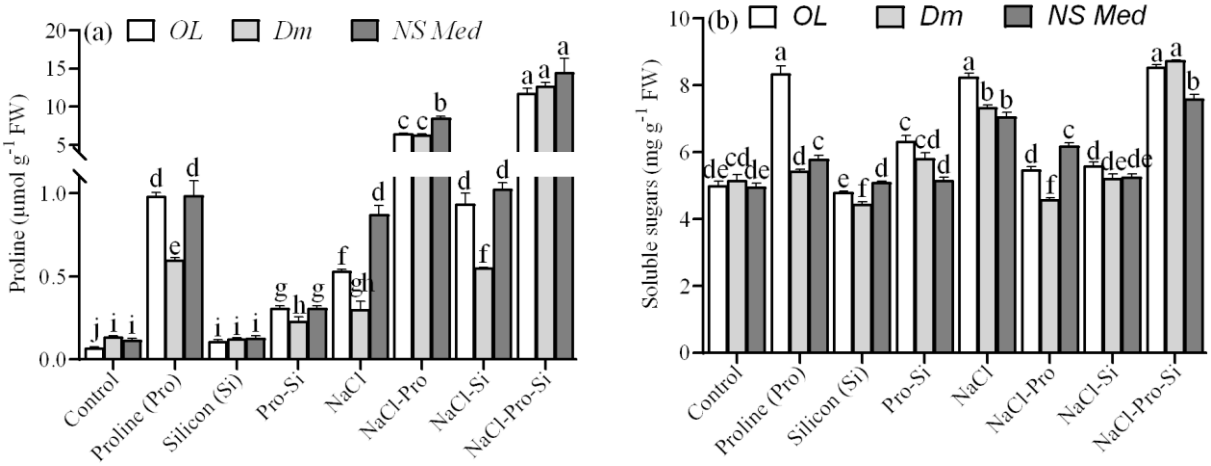

Fig. 2 Effect of 20 mM proline and 3 mM $CaSiO_3$ (Si) on proline (a) and soluble sugars (b) contents of three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under either salt stress (200 mM NaCl) or non-stress conditions. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

III-4 Effect of proline and Si on oxidative stress markers in salt-stressed plants

The MDA, a product of lipid peroxidation and a marker of oxidative stress, accumulated in the three alfalfa varieties in response to salt stress (Fig. 3a). The highest MDA level being found in salt-stressed *OL* (0.054 µmol MDA g^{-1} FW), corresponding to a three-fold increase compared to the control (0.016 µmol MDA g^{-1} FW) (Fig. 3a). Either proline or Si were efficient in reducing MDA content in salt-stressed plants. Salt-stressed *OL*, *Dm* and *NS Med* exhibited 39%, 34% and 31% less MDA contents when treated with proline and 44%, 40% and 43% when treated with Si, respectively. However, no reduction in MDA contents was observed in salt-stressed plants treated simultaneously with proline and Si (Fig. 3a).

H_2O_2 also increased dramatically in salt-stressed alfalfa plants (Fig. 3b) by 4.89, 4.93 and 6.19-fold in *OL*, *Dm* and *NS Med* varieties, respectively (Fig. 3b). Either proline or Si significantly reduced H_2O_2 content ($P \leq 0.001$) in salinity exposed alfalfa. Indeed, Si-treated salt-stressed alfalfa plants showed reduction of 51%, 64% and 60%, respectively for *OL*, *Dm* and *NS Med* relative to Si-untreated salt-stressed plants. Under the combined application of proline and Si, there were no significant differences between treated and untreated salt-stressed plants. Under normal conditions, proline and Si has no effect on H_2O_2 content (Fig. 3b).

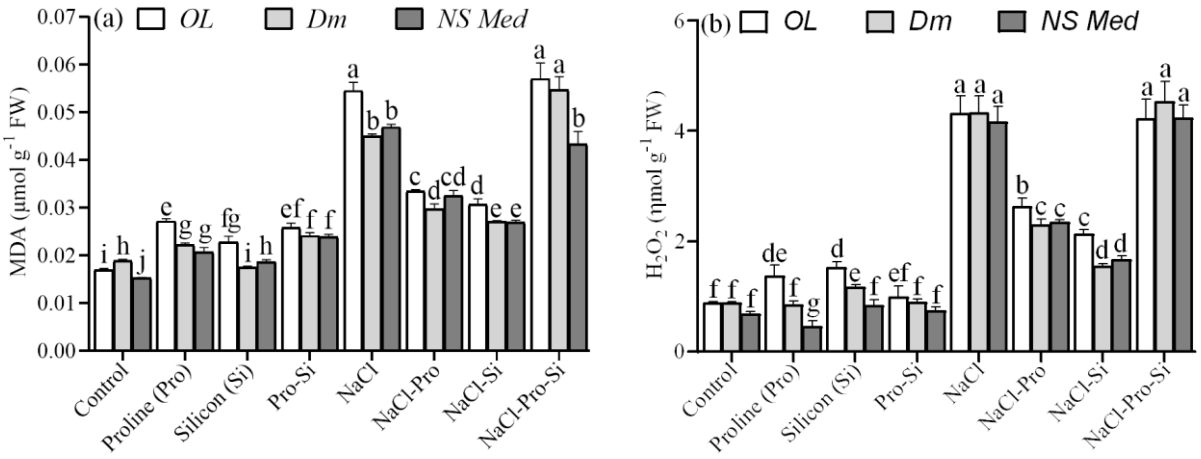

Fig. 3 Effect of 20 mM proline and 3 mM $CaSiO_3$ (Si) on malonyldialdehyde (MDA) (a) and hydrogen peroxide (H_2O_2) (b) contents of three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) under either salt stress (200 mM NaCl) or non-stress conditions. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

A histochemical study (Fig. 4) revealed DAB-stained leaves in salt-stressed alfalfa, indicating an accumulation of H_2O_2 in response to salt stress. Except for proline-treated *OL* plants, leaves of salt-stressed alfalfa treated with either proline or Si were less stained by DAB (Fig. 4).

However, under the combined application of proline and Si, the DAB staining remained in *OL* and *NS Med*, but decreased in *Dm*.

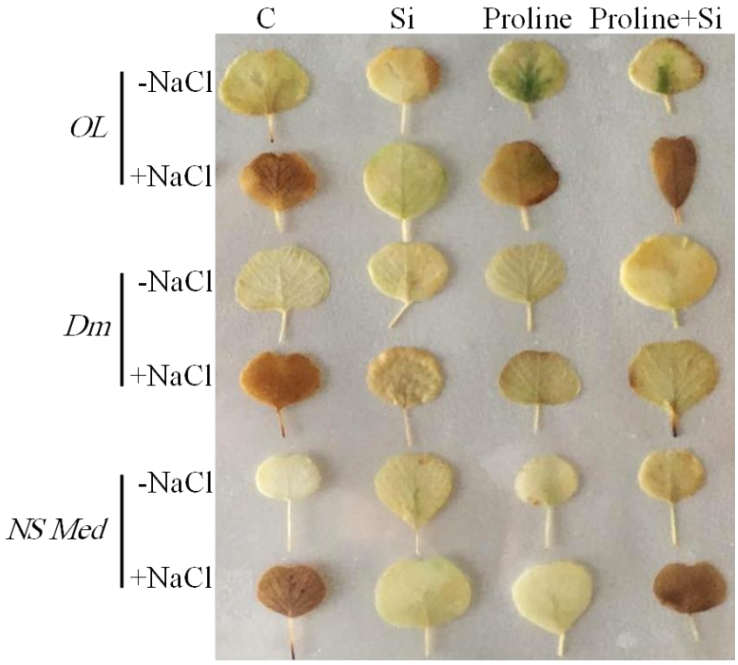

Fig. 4 Histochemical detection of hydrogen peroxide (H_2O_2) of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*) grown for seventeen days under 200 mM NaCl and treated with 20 mM proline and/or 3 mM $CaSiO_3$ (Si)

III-5 Effect of proline and Si on antioxidant enzymes activities in salt-stressed plants

The impact of NaCl, proline and Si on SOD activity is shown in Fig. 5a. SOD activity increased significantly in the three alfalfa varieties in response to NaCl treatment. SOD activity was especially higher in *NS Med* and *Dm* (67.45 and 4.16-fold higher, respectively when compared to their respective control). Furthermore, under salt stress, SOD activity was further increased in *OL* and *NS Med* in response to proline supply and in the three alfalfa varieties in response to Si supply with Si treatment showing the highest increase. Indeed, relative to salt-stressed plants, SOD activity in Si-treated salt-stressed *OL*, *Dm* and *NS Med* increased by 74%, 24% and 118%, respectively. However, *Dm* plants receiving both proline and Si treatments had lower SOD activity with no significant effect in the two other varieties.

CAT activity in leaves of alfalfa plants was notably influenced by NaCl, proline and Si as indicated in Fig. 5b. CAT activity was respectively 6.66, 21.41 and 7.75-fold higher in salt-stressed *OL*, *Dm* and *NS Med* than control plants. Moreover, supplementation of salt-stressed alfalfa plants with proline or Si led to a higher CAT activity. Salt-stressed *OL* revealed a higher CAT activity (10.65 ηmol H_2O_2 min^{-1} mg^{-1} protein) upon proline treatment, while in *Dm* and

NS Med the highest CAT activity was observed under Si treatment (11.69 and 11.46 ηmol H_2O_2 min^{-1} mg^{-1} protein for *Dm* and *NS Med*, respectively), as compared to untreated salt-stressed plants (0.68, 0.32 and 0.81 ηmol H_2O_2 min^{-1} mg^{-1} protein for *OL*, *Dm* and *NS Med*, respectively). The combined application of proline and Si caused a slight increase in CAT activity in salt-stressed *OL* only. Under normal conditions, single or combined supply of proline and Si increased significantly CAT activity in *Dm* ($P \leq 0.001$).

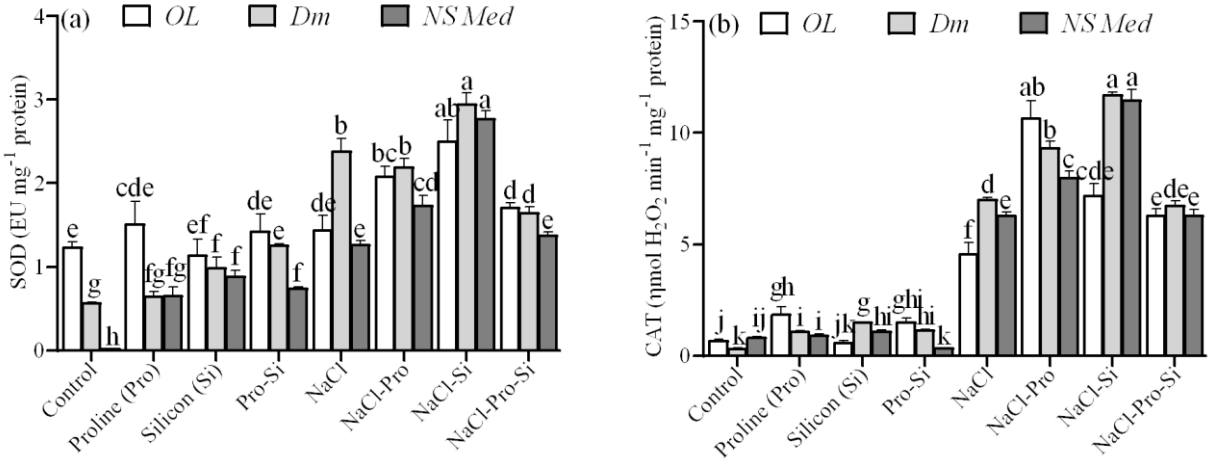

Fig. 5 Effect of 20 mM proline and 3 mM $CaSiO_3$ (Si) on superoxyde dismutase (SOD) (a) and catalase (CAT) (b) activities of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*) under either salt stress (200 mM NaCl) or non-stress conditions. Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

III-6 Effect of proline and Si on Si concentrations in shoot and root of salt-stressed alfalfa plants

Under control growth conditions, Si content is higher in roots than in shoots in the three alfalfa varieties (Fig. 6). Si incorporation to growth medium of the unstressed-alfalfa plants significantly increased the Si contents ($P \leq 0.001$), especially in roots (Fig. 6). When supplied with both Si and proline, Si content increased in shoots, while it remains low in roots (Fig. 6a, b). Salt stress led to an increased root Si content of *OL*, *Dm* and *NS Med* by up 135%, 104% and 68%, whereas in shoot, Si contents were decreased by 12%, 48% and 34%, respectively (Fig. 6a, b). Furthermore, when Si was supplied to salt-stressed alfalfa plants, Si contents were 1.74, 2.44 and 2.67-fold higher in the root of *OL*, *Dm* and *NS Med*, respectively relative to their respective Si-untreated salt stressed controls. In shoots of salt-stressed plants, proline application alone led to an increase of Si content. Under salt stress, the combined application of Si and proline induced a slight increase in Si contents in the shoots of *Dm* and *NS Med* and the roots of *NS Med* as compared to alfalfa plants treated with NaCl alone.

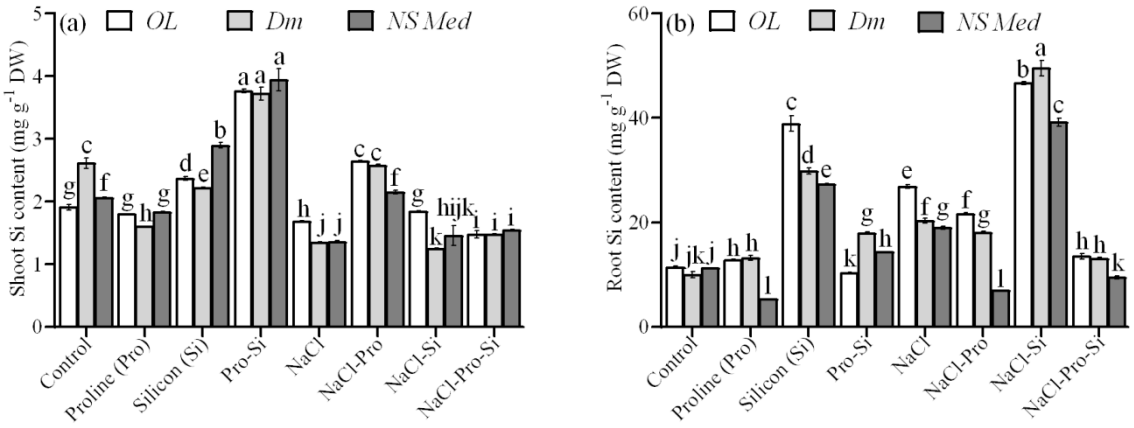

Fig. 6 Effect of proline, $CaSiO_3$ and NaCl on Si contents in shoots (a) and roots (b) of three alfalfa (*M. sativa* L.) varieties (*OL*, *Dm* and *NS Med*). Values are the mean of three replicates ± standard deviation. The letters above bars indicate statistically significant values

III-7 Effect of proline and Si on nutrient elements of salt-stressed plants

Data shown in table 2 indicated that P content was drastically reduced under 200 mM NaCl treatment as compared to control. P content was decreased by 37%, 27% and 43% in shoots and by 41%, 54% and 64% in roots, respectively in *OL*, *Dm* and *NS Med*. However, single or combination of proline and Si remarkably mitigated NaCl effect as reflected by a significant increase of P contents. In shoot, Si supply showed the highest P contents, whereas in the root the combination of both proline and Si treatment is the most beneficial for P content. P content in salt-stressed *OL*, *Dm* and *NS Med* was 2.04, 2.75 and 5.41-fold higher in alfalfa plants treated with both proline and Si as compared to the untreated salt-stressed plants. Under normal conditions, Si supplied alone or in combination with proline caused a significant increase in P content.

Under 200 mM NaCl stress, Na^+ content in both shoot and root of the three alfalfa varieties showed significant increases. K^+ content decreased compared to control. The effect was more pronounced in the root than in the shoot for both Na^+ (144%, 71% and 66% increases for *OL*, *Dm* and *NS Med*, respectively, Table 2) and K^+ (89%, 91% and 94% decreases for *OL*, *Dm* and *NS Med*, respectively, Table 2). Consequently, K^+/Na^+ ratio in roots markedly decreased under salt stress from 2.97 to 0.13 for *OL*, from 1.48 to 0.07 for *Dm* and from 1.52 to 0.05 for *NS Med*. Individual supply of proline and Si to salt-stressed alfalfa plants significantly reduced Na^+ content and increased K^+ content, resulted in a higher K^+/Na^+ ratio and the positive effect was more spectacular for the shoot than the root, with an obvious effect observed for Si (Table 2). Indeed, K^+/Na^+ ratio was 2.60, 1.93, and 1.55-fold higher in the shoots of Si-treated salt-stressed *OL*, *Dm* and *NS Med*, respectively as compared to their respective Si-untreated salt-stressed

controls (Table 2). The simultaneous supplementation of proline and Si increased significantly K^+/Na^+ ratio only in *Dm* variety.

Table 2 Effect of 20 mM proline (Pro) and 3 mM $CaSiO_3$ (Si) on phosphorus (P), sodium (Na^+), potassium (K^+), K^+/Na^+ ratio content in three *M. sativa* varieties (*OL*, *Dm* and *NS Med*) grown under either 0 mM (-NaCl) or 200 mM (+NaCl) NaCl stress. Values are the mean of three replicates ± standard deviation

	Salt stress	Treatments	*OL*		*Dm*		*NS Med*	
			Shoot	Root	Shoot	Root	Shoot	Root
P (mg g^{-1} DW)	-NaCl	Control	2.77±0.21	3.41±0.12	2.39±0.06	2.84±0.18	1.71±0.02	2.12±0.08
		Pro	2.78±0.08	2.60±0.16	2.40±0.20	2.77±0.09	2.28±0.03	2.20±0.06
		Si	3.59±0.04	3.19±0.10	2.67±0.13	3.03±0.18	2.10±0.10	2.60±0.04
		Pro-Si	3.32±0.09	2.71±0.02	2.50±0.14	3.42±0.34	2.15±0.04	2.04±0.02
	+NaCl	Control	1.74±0.05	2.00±0.02	1.74±0.04	1.30±0.04	0.97±0.13	0.76±0.04
		Pro	2.62±0.03	2.83±0.26	2.08±0.04	2.43±0.06	2.75±0.01	3.37±0.17
		Si	2.91±0.04	3.88±0.05	2.75±0.13	3.55±0.01	2.79±0.01	3.68±0.05
		Pro-Si	2.08±0.09	4.07±0.01	2.11±0.03	3.58±0.06	1.06±0.06	4.11±0.16
Na^+ (ppm)	-NaCl	Control	39.27±0.58	16.84±0.12	40.20±0.20	28.55±0.11	41.05±0.29	26.94±0.59
		Pro	28.66±0.08	11.46±0.05	42.13±0.19	18.76±0.31	40.40±0.40	18.85±0.11
		Si	36.03±0.28	21.71±0.08	41.80±0.19	17.72±0.09	38.86±0.59	19.62±0.17
		Pro-Si	31.86±0.16	21.14±0.18	24.50±0.43	24.58±0.29	30.89±0.14	23.50±0.50
	+NaCl	Control	45.63±0.31	41.10±0.16	59.40±0.37	48.73±0.07	51.13±0.83	44.75±0.34
		Pro	30.60±0.23	44.19±1.28	44.61±0.32	44.27±0.13	23.12±13.19	48.54±0.43
		Si	33.16±0.14	43.23±1.08	44.61±0.32	40.74±0.33	46.44±0.16	38.87±0.34
		Pro-Si	59.90±0.55	49.64±0.05	44.27±0.21	44.43±0.67	54.53±0.41	42.83±0.38
K^+ (ppm)	-NaCl	Control	34.59±0.56	50.11±0.30	34.14±0.51	42.47±0.11	34.02±0.38	41.00±0.11
		Pro	35.91±1.51	45.56±0.48	35.28±1.53	36.60±1.73	35.62±0.66	35.53±0.78
		Si	35.47±0.54	40.23±0.70	35.82±0.17	23.96±0.28	34.10±1.12	37.09±0.81
		Pro-Si	36.46±0.29	48.34±0.77	36.17±0.78	40.90±0.41	36.10±0.17	42.29±0.68
	+NaCl	Control	27.48±0.90	5.64±0.08	17.36±0.42	3.96±0.06	14.04±0.38	2.39±0.07
		Pro	43.73±0.31	6.27±0.15	25.14±0.54	4.82±0.06	19.03±0.24	5.64±0.73
		Si	51.98±1.04	20.14±0.27	25.14±0.54	18.07±0.06	19.94±0.71	30.41±0.32
		Pro-Si	28.81±1.59	6.27±0.24	25.30±1.01	4.32±0.33	12.92±0.48	2.02±0.06
K^+/Na^+	-NaCl	Control	0.88±0.02	2.97±0.04	0.85±0.01	1.48±0.01	0.82±0.01	1.52±0.02
		Pro	1.25±0.05	3.97±0.03	0.83±0.03	1.95±0.09	0.88±0.01	1.88±0.04
		Si	0.98±0.01	1.85±0.03	0.85±0.01	1.35±0.02	0.87±0.03	1.89±0.02
		Pro-Si	1.14±0.01	2.28±0.02	1.47±0.05	1.66±0.01	1.16±0.01	1.80±0.04
	+NaCl	Control	0.60±0.02	0.13±0.00	0.29±0.01	0.07±0.00	0.27±0.01	0.05±0.01
		Pro	1.42±0.01	0.14±0.01	0.56±0.01	0.10±0.00	2.30±2.31	0.11±0.01
		Si	1.56±0.03	0.46±0.01	0.56±0.01	0.44±0.01	0.42±0.01	0.78±0.01
		Pro-Si	0.48±0.02	0.12±0.01	0.57±0.02	0.09±0.01	0.23±0.01	0.04±0.00

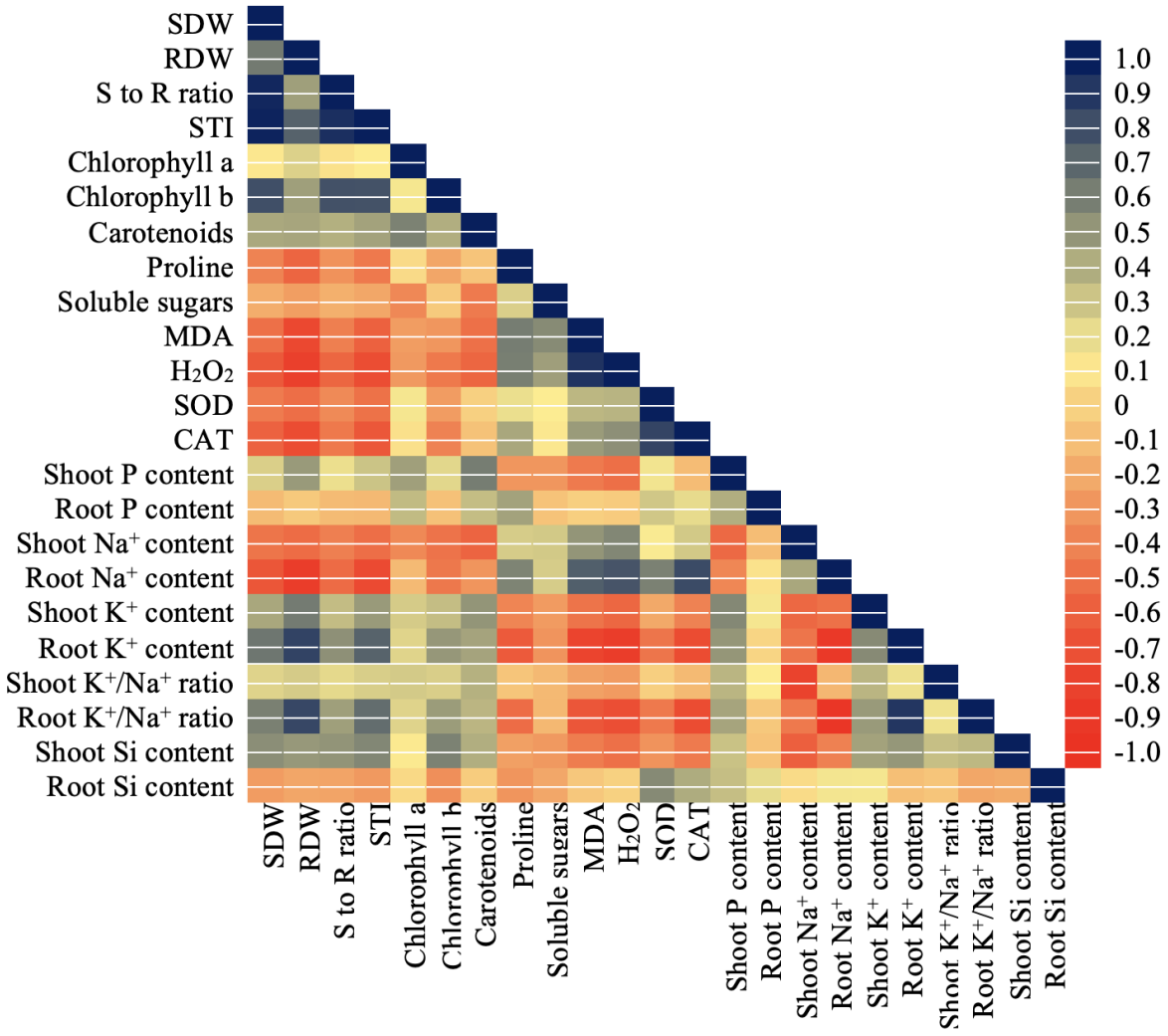

Fig. 7 Pearson's correlation matrix between plant growth, photosynthesis traits, compatible osmolyte, membrane stability, enzymatic antioxidants and nutrient element in three alfalfa (*Medicago sativa* L.) varieties (*OL*, *Dm* and *NS Med*) treated with 20 mM proline and/or 3 mM $CaSiO_3$ with or without 200 mM NaCl. Correlations are displayed in blue (positive) and red (negative); color intensity is proportional to the correlation coefficient. SDW: shoot dry weight; RDW: root dry weight; STI: salt tolerance index; MDA: malonyldialdehyde; H_2O_2: hydrogen peroxide; SOD: superoxyde dismutase; CAT: catalase

IV Discussion

In this study, the effects of individual and combined supply of 20 mM proline and 3 mM Si were investigated in alfalfa plants upon salt stress. Physiological, biochemical, enzymatic and nutritional parameters chosen were affected by salt stress and reversed when salt-stressed plants were treated with either proline or Si, but not when proline and Si were applied simultaneously.

IV-1 Salt stress affected alfalfa growth and physiological parameters

Our results indicate that salt stress significantly reduced shoot and root DW and shoot-to-root ratio in all the three alfalfa varieties. Biomass reduction under salt stress could be a consequence of a reduction of photosynthesis capacity, water uptake and/or oxidative stress (Gama et al. 2009). Comparison of the alfalfa varieties showed that the European *NS Med* variety was the most affected by salt stress, reflecting a relative diversity in salt-stress tolerance traits. This may result of specific breeding of Moroccan local landraces adapted to harsher environmental growth conditions (El Moukhtari et al. 2021a).

Alfalfa plants stressed by salinity had reduced photosynthetic pigment contents such Chl a, Chl b and carotenoids as compared to control. This effect is often attributed to a toxic effect of Na^+ and Cl^- on Chl synthesis machinery (Tavakkoli et al. 2010). Negative correlations were found between shoot Na^+ and Chl b (r=0.6; $P \leq 0.001$; Fig. 7) content as well as Na^+ and carotenoid content (r=0.7; $P \leq 0.001$; Fig. 7). Another explanation for the decrease of Chl contents under salt stress is the increase of Chl degradation enzyme activities, such as chlorophyllase, Chl-degrading peroxidase and pheophytinase (Alamri et al. 2020). Yang et al. (2020) reported that photosynthesis inhibition is one of the most plant growth-limiting factors under salt stress. This was in agreement with our observations, which revealed a highly significant correlation between shoot DW and Chl b (r=0.8; $P \leq 0.001$; Fig. 7) and carotenoid contents (r=0.6; $P \leq 0.001$; Fig. 7).

Previous investigation showed that the tolerance of plants to salt-induced osmotic stress condition was partially related to their capacity to regulate cellular osmotic pressure (Al Murad et al. 2020). In alfalfa, this is mediated by the accumulation of organic solutes such as proline, glycine betaine and soluble sugars (El Moukhtari et al. 2021a). In our study, we observed a strong correlation between proline and sugar accumulation and salt-stress tolerance.

Elevated Na^+ content in plant tissues, particularly in the aerial part, led to a dramatic accumulation of H_2O_2 (r=0.61; Fig. 7). In our study, the increase in H_2O_2 content was significantly correlated with MDA content (r=0.916; Fig. 7) indicating an oxidative stress. If

not metabolized, ROS such as H_2O_2 could induce membrane damages (Slimen et al. 2014). Furthermore, the increase in both H_2O_2 and MDA was accompanied by an increase of SOD and CAT activities, suggesting an oxidative stress response.

Alfalfa plants exposure to salt stress resulted in ionic unbalance reflected by elevated Na^+ content and reduced P and K^+ contents with low K^+/Na^+ ratio in both shoot and root parts. Because of the molecular similarity between Na^+ and K^+, K^+ is often replaced by Na^+ under salt stress resulting in lower K^+/Na^+ ratio (Van Zelm et al. 2020). Na^+ was also reported to have devastating effect on the activity of acid phosphatase (Faghire et al. 2013), which partially explain in our work the reduced P content under salinity. Altogether our results suggest that NaCl causes a typical ionic, osmotic and oxidative stress, with a varying sensitivity in the three studied varieties. *NS Med* being the most sensitive.

IV-2 Similar beneficial effect of proline or Si in salt-stress alfalfa tolerance

Exogenous supply of either proline or Si in solution to salt-stressed plants improved shoot and root DW as well as the shoot to root ratio. Interestingly, the most sensitive variety showed the most spectacular response to proline and Si. The positive impact of proline treatment on plant biomass is in line with some previous investigations, indicating the potential role of proline in alleviating the devastating effects of salinity on different plants species including *Viburnum lucidum*, *Callistemon citrinus*, *Pisum sativum*, and *Oryza sativa* (Shahid et al. 2014; Chui-Yao et al. 2016; Cirillo et al. 2016). Similarly, Si supplementation has been shown to improve significantly plant growth and biomass of salt-stressed alfalfa, wheat, and tomato (Tuna et al. 2008; Haghighi and Pessarakli 2013; Laifa et al. 2020; El Moukhtari et al. 2021a).

The improved DW of salt-stressed alfalfa plants by proline or Si treatment could be explained by the ability of either proline or Si to maintain photosynthetic pigments or to reduce oxidative stress and to alleviate the toxic effect of Na^+ (Yan et al. 2013; Meng et al. 2020).

In salt-stressed alfalfa plants, either proline or Si led to significant increases of Chl a, Chl b and carotenoids. Exogenously applied proline showed a relative stronger effect than Si in alleviating the toxic effect of Na^+ on Chl pigments. This exogenous proline effect on photosynthetic pigment accumulation has been reported in several plant species, including *Brassica juncea* (Wani et al. 2016), *Triticum aestivum* (Rady et al. 2018) and *O. europea* (Ben Ahmed et al. 2011). Previous studies also indicated that Si supplementation significantly improved photosynthetic pigments due to its ability to increase the activities of some Chl synthesis enzymes such as δ-aminolevulinic acid dehydratase and porphobilinogen deaminase (Alamri et

al. 2020). Another explanation is the ability of Si to modulate amino acids accumulation mainly glutamate, one of the common Chl synthesis precursor (Feng et al. 2010).

Regarding oxidative stress relief, we found that the supply of either proline or Si triggered an increase of both SOD and CAT activities in salt-stressed alfalfa plants. The increase in antioxidant enzyme activities in proline-treated salt-stressed alfalfa plants is reminiscent of that described in Abdelhamid et al. (2013). The authors demonstrated the important role of proline in ROS detoxification in *P. vulgaris* by promoting the activities of antioxidant enzymes including SOD and CAT. Interestingly, studying salt-stressed *Eurya emarginata*, Zhang et al. (2015) found that proline treatment was effective in alleviating salt-mediated osmotic stress by modulating the activities of pyrroline-5-carboxylase synthase (P5CS) and proline dehydrogenase (ProDH). Moreover, Meng et al. (2020) indicated that Si was able to reduce ROS accumulation in salt-stressed alfalfa plants by modulating antioxidant enzyme activities including SOD and CAT. Similarly, Si incorporation in cultured media of salt-stressed cucumber induced a significant increase in the activity of P5CS and inhibit the activity of ProDH resulting in higher proline content, required for cell osmotic adjustment (Zhu et al. 2020).

IV-3 Beneficial effect of combined proline and Si treatment on mineral balance

Supply of either proline or Si increased leaf contents of P, K and K^+/Na^+ ratio, while suppressed Na^+ uptake by salt-stressed alfalfa plants. The simultaneous supplementation of proline and Si also increased K^+ in the shoots of *Dm* and in the roots of both *OL* and *Dm*, while it significantly reduced Na^+ in the shoots of *Dm* and the roots of *Dm* and *NS Med*. The same has been reported by Radhi et al. (2021) in *O. europea* under salt stress and by Alkahtani et al. (2021) in *B. vulgaris* under drought. In salt-stressed *Zea mays*, Bosnic et al. (2018) reported that exogenous Si upregulates the expression of *Salt overly sensitive 1* (*ZmSOS1*) gene encoding a Na^+ efflux transporter resulting in a significant decrease in root Na^+ content. On the other hand, Si can also improve P content in plants by increasing root exudation of some organic acids like malate and citrate (Kostic et al. 2017), increasing soil P availability by increasing soil pH, lowering P sorption in soil (Owino-Gerroh and Gascho 2005) and upregulating some plant genes involved in P uptake (Kostic et al. 2017).

In *P. vulgaris*, Rady et al. (2019) showed that the supply of proline, Si or both significantly increased P and K^+ contents and K^+/Na^+ ratio, while it decreased Na^+ content under salt stress,

and the effect was more obvious under the combined application than that under the single supply of either proline or Si.

IV-4 Combined proline and Si treatments had no beneficial effect on salt-stressed alfalfa plants

When applied together, proline and Si did not show beneficial effect compared to their separate application, neither on DW of both shoot and root nor on the shoot to root ratio. Our findings are in accordance to those of Dos Santos et al. (2022) who demonstrated that the separate supplementation of proline and Si was much beneficial on leaf area and relative water content of water-stressed *V. unguiculata* as compared to the combined application. However, Rady et al. (2019) and Radhi et al. (2021) reported that the positive effect of proline and Si was much higher in *P. vulgaris* and *Olea europea* when applied together than applied separately. Soluble sugar contents accumulated under salt stress and even more when both proline and Si were provided, they decreased when Si or proline was provided separately. Also, our findings showed that the combined supplementation of proline and Si was found less effective in reducing oxidative stress as compared to their single supplementation. This is in contrast to what was found in *P. vulgaris* under salt-stress (Rady et al. 2019) and in *B. vulgaris* under drought (Alkahtani et al. 2021).

An explanation could be that most probably species react differently to the combination of proline and Si. Another explanation is the way that proline and Si supplementation was done. For example, in some previous investigations, proline and Si were applied as foliar spray (Rady et al. 2019; Alkahtani et al. 2021). Furthermore, we found that Si was less accumulated in roots in the presence of proline, suggesting a putative negative interaction. Additionally, the combined application of proline and Si resulted in a higher P content in both shoot and root of the three salt-stressed alfalfa varieties. One must point out that in our study, proline and Si were applied to the growth solution while in some other studies, it was applied by foliar spray.

IV-5 Synergy between proline and Si

In this work, NaCl-exposed alfalfa plants exhibited a dramatic accumulation of proline. Additionally, a single supply of either proline or Si to salt-treated alfalfa plants caused additional increases in proline content. Similarly, in a study conducted by Rady et al. (2019) in

P. vulgaris, proline content was much higher in salt-stressed plants treated with both proline and Si than those treated with either proline or Si.

In the absence of stress, proline-treated plants accumulated proline. However, proline content was lowered when Si added. This unexpected lower proline content in Si-treated plants compared to proline-treated plants could suggest a reduced proline uptake or an enhanced proline oxidation. It is noteworthy that under salt stress, proline oxidation by ProDH activity is down-regulated (Funck et al., 2010; Forlani et al., 2019). This correlates to an enhanced proline accumulation in proline and Si-treated stressed alfalfa plants. We observed that proline also modulates Si accumulation. In the absence of stress, under Si treatment, Si accumulated more in shoots when proline is present. Under salt stress, proline alone increases Si accumulation in shoots without any Si supplementation. Also, when both proline and Si are provided to salt-stressed plants, Si doesn't accumulate more than in the control. This apparent interplay between Si and proline accumulation during stress could provide an explanation for a relative lack of beneficial effect of the combination of both proline and Si treatment to salt-stressed plants. It is noteworthy that Si and proline were provided by watering, with the growth solution, while studies reporting a beneficial combined effect of proline and Si in salt-stress tolerance applied the mixture once by foliar spray (Alkahtani et al. 2021; Rady et al. 2019).

V Conclusions

Salt stress considerably reduced plant growth mainly due to oxidative stress reflected by the elevated MDA and H_2O_2 contents, and nutrient unbalance. The single supply of proline or Si in growth solution plays an important role in preserving plant biomass, shoot to root ratio, photosynthetic pigments and improving salt tolerance index in alfalfa plants grown under 200 mM NaCl stress. Furthermore, proline or Si treatments significantly reduced salt-induced oxidative stress damage by increasing enzymatic antioxidant response. Moreover, proline or Si supplementation reduced Na^+ content, while significantly improved P, K^+ content and K^+/Na^+ ratio. The comparison between the treatments revealed that single supply of proline or Si was more beneficial in improving salinity tolerance in alfalfa than the combination of both proline and Si. This was especially observed with the salt sensitive variety. Our findings suggest that proline or Si but not the combination of the two could be an effective way in mitigating salinity stress in alfalfa.

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Conflicts of Interest

The authors declare no conflict of interest.

Author contribution

Conceptualization: Savouré Arnould, Farissi Mohamed, El Moukhtari Ahmed; Methodology: El Moukhtari Ahmed; Formal analysis and investigation: El Moukhtari Ahmed, Crilat Emilie, Lamsaadi Nadia, Hidri Rabaa; Writing - original draft preparation: El Moukhtari Ahmed; Writing - review and editing: Savouré Arnould, Farissi Mohamed, Cabassa-Hourton Cecile, Carol Pierre; Funding acquisition: Savouré Arnould, Farissi Mohamed; Supervision: Savouré Arnould, Farissi Mohamed.

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Chapitre VI (article 7)

L'apport exogène de la proline et/ ou de silicium améliore la tolérance de la luzerne (*Medicago truncatula*) aux contraintes salines *via* la modulation du métabolisme de proline et le transport de Si.

I Introduction

La salinité est l'une des facteurs abiotiques majeurs limitant la croissance et la productivité des plantes. Pour pallier au problème de la salinité, l'apport exogène du Si et de la proline a été décrit parmi les approches effectives. Chez *Phaseolus vulgaris*, par exemple, l'apport combiné du Si et de la proline induit un ajustement osmotique, améliore par conséquent la tolérance des plantes au stress osmotique induit par la salinité (Rady et al. 2019). De même, sous contrainte hydrique, l'apport combiné du Si et de la proline améliore la croissance de *Beta vulgaris* en induisant une forte activité des enzymes antioxydantes (Alkahtani et al. 2021). Cependant, l'apport combiné de ces deux molécules sur la réponse génique des plantes traitées par le sel n'est pas encore étudié.

II Objectif

Le présent chapitre vise à identifier les mécanismes moléculaires impliqués dans la régulation du métabolisme de la proline ainsi que l'élucidation des déterminants contrôlant le transport et l'accumulation du Si et la caractérisation des gènes impliqués en réponse à l'application combiné de Si et de la proline chez la plante modèle *M. truncatula*.

III Méthodologies

L'expérience a été réalisée sous serre sous une température de 25 °C et une photopériode de 16h/8h. Les graines de la lignée A17 de *M. truncatula* ont été traitées à l'acide sulfurique concentré (36N) pendant 7 min, rincé abondamment à l'eau distillé stérile et mises à germer, sous une obscurité totale, dans des pots en plastique contenant de la perlite à raison de 12 pots par traitement. Après la levée (8 jours), le nombre de plantes a été ajusté à 3 par pot. Les plantes ont été arrosées avec une solution nutritive (Hoagland) dépourvue d'azote tout au long de l'expérience.

Après15 jours de semis, les jeunes plantules ont été inoculées avec la souche rhizobienne *E. meliloti E1021*. Après un mois de croissance, les plantes ont été soumises au stress salin (120 mM de NaCl) et traitées par 3 mM Si ($CaSiO_3$), 20 mM de proline ou la combinaison des deux. Le stress salin a été appliqué pendant 14 jours, puis les plantes ont été récoltées pour des analyses agrophysiologiques, biochimiques et moléculaires liées au métabolisme de la proline, le transport du Si et la tolérance au stress osmotique.

IV Résultats

Les résultats ont révélé que la contrainte saline induit une accumulation de transcrits codant l'Ornithine Aminotransférase (OAT) et de la Pyroline-5-Carboxylase Synthèse 2 (P5CS2) et réduit celui codant P5CS1. De plus, en comparaison avec les plantes stressées, le traitement des plantes stressées par 20 mM proline ou 3 mM Si induit une accumulation de transcrit codant l'OAT et cette accumulation a été plus accentuée chez les plantes traitées à la fois avec de la proline et du Si. De même, le traitement des plantes avec 20 mM proline ou 3 mM Si induit une forte accumulation de transcrit codant la proline déshydrogénase 1 (ProDH1) et réduit celui codant la P5C déshydrogénase (P5CDH) en absence ou en présence du sel. Les résultats de ce travail montrent également que la contrainte saline seule ou combinée avec le Si ou la proline induit une accumulation de transcrits codant Low silicon transporter 2 (Lsi2) et Dehydrine 2 (DHN2), et cette accumulation est plus forte chez les plantes traitées à la fois avec 120 mM NaCl, 20 mM proline et 3 mM Si.

V Conclusions

Les résultats obtenus dans ce chapitre montrent que le traitement avec du Si et/ou de la proline représente une méthode efficace pour améliorer la tolérance de la luzerne au stress salin. Nous résultats démontreront aussi que l'effet bénéfique de traitement avec de la proline et/ou du Si chez les plantes stressées implique une réponse génique. La comparaison entre les traitements appliqués a montré que l'apport combiné du Si et de la proline a un effet très positif sur la tolérance des plantes au stress salin que l'apport séparé des deux molécules.

Ces données ont fait l'objet d'un projet d'article intitulé "Proline and silicon mediate alleviation of salinity stress in *Medicago truncatula* by regulating proline metabolism and silicon transporter-related genes".

Proline and silicon mediate alleviation of salinity stress in *Medicago truncatula* by regulating proline metabolism and silicon transporter-related genes

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Abstract

Salt stress is a global issue threatening continuously crop production. Among sustainable and environmentally-friendly approaches used to alleviate the consequences of salt stress in crops growth and yield, the application of proline and silicon (Si) is of interest. Here, we investigated the outcome of single and combined application of 20 mM proline and 3 mM Si in *Medicago truncatula* as a model legume upon salt stress. The results showed that salt stress (120 mM NaCl) impairs plant growth indexes, including shoot dry weight, root dry weight, plant height, leaf number and area. Interestingly, proline or Si supplementation alleviates the adverse effects of NaCl on studied growth indexes. This was found especially pronounced with the combined application of proline and Si. Furthermore, salt-stressed plants exhibited enhanced proline content especially when they were treated with both proline and Si which correlates with the highest transcript levels of proline biosynthesis-related genes. We also found that the ornithine and glutamate pathways of proline biosynthesis are diversely regulated in *M. truncatula* during salt stress. Salt stress also greatly increased transcript level of Si transporter gene *Lsi2* together with proline and Si, coinciding with the highest shoot Si content. Our work suggests that proline metabolism and Si accumulation are key factors in proline and/or Si-induced salt tolerance in *M. truncatula*.

Keywords: *Medicago truncatula*; Salinity; Proline; Silicon; proline metabolism; Silicon-transporter.

I Introduction

Salinity is one of the most limiting factors for plant growth and yield. It was estimated that more than 800 million hectares of agricultural land in the world are salt-affected (Cao et al. 2020). Moreover, current climate prediction models estimate that more than half of the world's arable land will be salinized by 2050 (Hessini et al. 2021).

Salt stress causes osmotic stress preventing root-mediated water uptake and ion toxicity (Van Zelm et al. 2020). Salt also triggers secondary stress by the generation of excessive reactive oxygen species (ROS) (Munns and Tester 2008). To survive salinity stress, tolerant plants have evolved a variety of physiological and molecular mechanisms (Nadeem et al. 2019). Maintaining osmotic balance is one of the main plant responses to compensate the loss of turgor pressure in response to salinity-mediated osmotic stress (Liang et al. 2018). Among osmolytes, the amino acid proline is one of the main solutes, which is accumulated by plants under salt stress in order to adjust their osmotic potential (Slama et al. 2015; Szabados and Savouré 2010). Proline is synthesized from glutamate by the sequential action of pyrroline-5-carboxylate synthetase (P5CS) and P5C reductase (P5CR) (Szabados and Savouré 2010). Proline can also be synthesized from ornithine by ornithine-aminotransferase (δ-OAT) (Mansour and Ali 2017; Szabados and Savouré 2010). In *Medicago truncatula*, upregulation of genes encoding for proline biosynthesis, including *P5CS2*, *P5CS3* and *OAT* was observed upon salt stress (Armengaud et al. 2004; Nguyen et al. 2013). In addition, endogenous proline level is regulated by its degradation which is mediated by two enzymes, proline dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH) (Lebreton et al. 2020; Miller et al. 2005; Verdoy et al. 2006; Adamipour et al. 2020). Besides endogenous accumulation, previous investigations have shown that the exogenous application of proline is a possible alternative to mitigate the negative effects of salt stress in various plants, including *Zea mays* (de Freitas et al. 2018), *Trigonella foenum-graecum* (Ould Said et al. 2021) and *Vigna unguiculata* (dos Santos et al. 2022). Exogenous proline may also be perceived as a stress signal, triggering a transduction cascade to regulate gene expression. For example, upregulation of proline biosynthesis genes, *P5CS* and *P5CR*, was observed in salt-stressed rice upon proline treatment (Nounjan et al. 2012).

Silicon (Si), the second most abundant element in the earth's crust, is categorized as a non-essential element (Coskun et al. 2019). However, it has long been recognized as a beneficial element for the growth of many plant species, especially when they are facing stress (Debona et al. 2017; El Moukhtari et al. 2021). For example, in drought-stressed *Lens culinaris*, Si supplementation helps plants to adjust their osmotic potential through changes in the

accumulation of organic solutes, including proline (Biju et al. 2021). In salt-stressed *Medicago sativa*, Si treatment has been shown to preserve photosynthesis, biological nitrogen fixation, nutritional balance, modulate scavenging enzyme activities and to limit ROS production (Meng et al. 2020; El Moukhtari et al. 2021; Zhang et al. 2022). The assimilation of Si by plants involves some specific Si-transporters such as the low silicon transporter Lsi (Yamaji et al. 2008; Mitani et al. 2009). These transporters are generally identified in Monocots, which are known to be high Si accumulators (up to 10% dry weight) compared to dicots (≤1% dry weight) (El Moukhtari et al. 2021; Coskun et al. 2019). However, Si-transporters have been identified in some dicot plants, such as *Glycine max* (Deshmukh et al. 2013) and *Cucumis sativus* (Wang et al. 2015), although in other dicot plants, such as *Medicago truncatula*, no Si transporters have been identified so far.

M. truncatula, a model legume, is a Mediterranean species that presents various levels of salt tolerance in natural populations (Ané et al. 2008). Numerous agroeconomic and ecological benefits make this species a model forage crop in many Mediterranean agricultural systems. In addition, this species is considered to be a model legume crop for breeding programs due to its short diploid sequenced genome (500 Mb), good genetic transformation efficiency, a vast range of biological diversity and high conservation synteny with other cultivated legumes, such as *M. sativa*, *G. max* and *Phaseolus vulgaris* (Zhu et al. 2005; Ané et al. 2008; Rose 2008).

Growth and yield of alfalfa are adversely affected by salinity stress in many regions around the world. Preliminary data advocated proline and Si roles for improving alfalfa (*M. sativa*) grown under salinity stress through enhancing photosynthesis, nitrogen fixation, nutrient and water uptake, osmotic adjustment, and antioxidant metabolism. The present study further investigates the mechanisms by which proline and Si modulate proline metabolism and salt stress tolerance in *M. truncatula*.

II Materials and methods

II-1 Plant material and growth conditions

All the experiment was carried out using seeds of *M. truncatula* var. Jemalong A17 obtained from Montpellier Stock Centre (INRAE, France). *M. truncatula* seeds were scarified by immersion in concentrated sulfuric acid for 7 min, and then washed thoroughly with sterile distilled water and incubated in sterile distilled water an overnight at 4 °C. Seeds were planted into plastic pots (10 cm diameter and 15 cm tall) containing perlite: vermiculite (6:1, v/v), and watered with a nitrogen-free nutrient solution. At the two-leaf stage, seedlings were inoculated with an *Ensifer meliloti* 1021 strain. The bacterial strain was grown in a tryptone yeast extract medium, and the bacterial pellet (OD600nm = 2.5) was obtained by centrifugation at 5000 rpm for 5 min. The bacterial pellet was then resuspended in N-free nutrient solution (OD600nm = 0.05) and used for plant inoculation. Urea (2 mM) was supplied during the initial week of growth to avoid N deficiency. After one month of growth, plants were subjected to salt stress by adding 120 mM NaCl to the growth solution and treated with either 20 mM proline and/or 3 mM Si. Stress was applied for 14 days, and then plants were harvested for ecophysiological, biochemical, nutritional and molecular parameters.

II-2 Experimental design

To investigate single and combined application of exogenous proline and silicon (Si) on the tolerance of *M. truncatula* to salt stress, a completely randomized design was set up in sterilized perlite : vermiculite (6:1, v/v). Four weeks after sowing, pots were split into 2 plots: control (0 mM NaCl) and stress (120 mM NaCl). Each of those 2 plots was divided into four subplots: i) control, ii) 20 mM proline supplementation, iii) 3 mM Si supplementation (in $CaSiO_3$), and iv) 20 mM proline and 3 mM Si in combination. Each treatment was independently replicated 12 times, resulting in a total of 96 pots (8 x 12). Salt was added gradually to the growth medium to avoid osmotic shock, incrementing NaCl by 40 mM every two days up to 120 mM. Proline and Si were applied into the growth medium. The concentrations of NaCl, proline and Si were chosen according to preliminaries studies.

II-3 Growth index

Plant height, leaf number and shoot and root dry weight were determined before and after stress application in three replicates per treatment. Growth index was determined by comparing the growth traits before and after the stress application using the formula below:

$$Growth\ index = \frac{Growth\ traits\ after\ stress - Growth\ traits\ before\ stress}{Growth\ traits\ before\ stress} x100$$

Where growth traits are plant high, leaf number, shoot dry weight or root dry weight.

Leaf area was determined after stress application in three replicates per treatment as described in El Moukhtari et al. (2021).

II-4 Proline determination

Proline content in leaves was determined after 14 days of stress using the method described by Bates et al. (1973). Frozen fresh material (30 mg) was ground in liquid nitrogen to powder and resuspended in 1 mL of aqueous sulfosalicylic acid (3% w/v) and centrifuged at 18000 *xg* for 10 min at 4 °C. To 400 µL of the obtained supernatant, equal volume of ninhydrin (3%) and glacial acetic acid were added. The mixture was heated at 100 °C for 1h then placed to cool down in an ice bath. Toluene (800 µL) was added to extract the pink phase. Proline content was determined by measuring the OD at 520 nm using NP80 Nanophotometer® (Implen) using calibration curves and expressed as µmol proline g^{-1} FW.

II-5 Quantification of Si content

Si content was determined using the method of Dai et al. (2005) with modifications. Around 10 mg of dry samples from shoot and root were put in polyethylene tubes and 300 µL of 50% sodium hydroxide (NaOH) was added. After autoclaving the samples at 121 °C for 21 min, the volume was adjusted to 500 µL with deionized water. Samples were then centrifuged at 29750 *xg* for 15 min. 0.16 mL of supernatant was added to 2 mL tubes containing 1.2 mL of 20% acetic acid and gently mixed by vortex. Then, 400 µL of ammonium molybdate (43.7 mM, pH 7) was added and the mixture was incubated at room temperature. After 5 min, 200 µL of tartaric acid (20%) and 40 µL of reducing solution containing 8 g L^{-1} sodium sulfite (Na_2SO_3), 1.6 g L^{-1} 1-amino-2-naphthol-4-sulfonic acid, and 100 g L^{-1} sodium bisulfite ($NaHSO_3$) were added and the mixture was incubated at room temperature for 30 min. The OD was then measured at 650 nm with a NP80 Nanophotometer® (Implen) and Si concentrations were calculated using a calibration curve prepared from SiO_2 standard solutions.

II-6 Nutrient analyses

For potassium (K^+) and sodium (Na^+) determination, the method of Oukaltouma et al. (2020) was adopted. Briefly, 0.5 g of dry material from shoots and roots *M. truncatula* plants were incinerated at 600 °C for 6h. Ash formed was resuspended in 3 mL of HCl (10 N). After

filtration, the solution was adjusted to 50 mL using distilled water and used for Na^+ and K^+ analyses using a flame spectrophotometer (Model 410).

II-7 RNA extraction and droplet digital PCR (ddPCR) analysis

RNAs were extracted from the first and second youngest leaves (~100 mg of plant tissue) that were ground in liquid nitrogen using a RNeasy plant mini kit (QIAGEN), and their quality and concentration were determined using a NP80 Nanophotometer® (Implen). RNAs were first treated with DNaseI (ThermoFisher) to remove genomic DNA. First-strand cDNA was reverse transcribed from 2 µg of DNase treated RNAs. cDNA was diluted 10-fold and used (10 ηg μL^{-1}) as template in ddPCR reactions. ddPCR was conducted and data were analyzed using a QX200 ddPCR system (Bio-Rad) according to the manufacturer's protocol. The 20-µL reaction was conducted using 2× ddPCR supermix. The sample mix and 70 µL of droplet generation oil were added into their corresponding wells of the DG8TM cartridge in order to be emulsioned into 20 000 oil encapsulated nanodroplets. Data analysis was performed using the Quantasoft™ software (Bio-Rad) to calculate the number of copies per cDNA. The absolute expression quantitation of the *Pyrroline-5-Carboxylate Synthetase 1* (*P5CS1*), *P5CS2*, *Ornithine Aminotransferase* (*OAT*), *Proline Dehydrogenase 1* (*ProDH1*), *P5C Dehydrogenase* (*P5CDH*), *Low silicon transporter 2* (*Lsi2*) and *Dehydrin2* (*DHN2*) genes were normalized using *Actin2* and *RNA binding protein 1* (*RNAbp1*) as housekeeping genes (Kim et al. 2013; Plet et al. 2010). All the primers used for ddPCR are given in Table 1. For *Lsi2*, using the Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalw2/), the sex existing variants of *Lsi2* were aligner and the consensus regions were used to construct specific primers (Supplementary data).

Table 1: Primers used for this study

Target sequence	Forward primer sequence	Reverse primer sequence	Accession number
MtP5CS1	TCGACGAAAATCCCTGTTCT	TTCTGACCGCAAATTGACAC	AJ278818.1
MtP5CS2	AAATTGCGGTCGCATTACAA	CTCGGGTAGTAAGGGCTTTT	JN849370.1
MtOAT	TTATACCGGTTAGCGCAGTT	TGAATCTTCTCCAGCAGACC	AJ278819.1
MtProDH1	GGACGTTGGAATATGGGCTA	AAAACAAGCATGCCACGTAA	XM_013595747.2
MtP5CDH	AATTTCTGCGGAGATCAGGT	GCTCCATAACAATGCTCACC	AY556387.1
MtLsi2	GGAAGCGTAGATTGTGGAAATC	CGCTATTCCACTAGAACGATCAAC	XM_013596681.2
MtDHN1	CAACATGGAGAGAAAGGGGT	GTGTTGCTCATCACCATAGC	XM_013597564.3
MtActin2	GACATGGAGAAGATCTGGCA	CACTTGCATAGAGGGAGAGG	JQ028731.1
MtRNAbp1	AGGGGCAAGTTCCTTCATTT	GGTAGAAGTGCTGGCTCAGG	XM_013596338.4

II-8 Statistical analysis

Three-way ANOVAs were performed for the ecophysiological, biochemical and nutritional analyses, with salinity, proline and silicon being the independent variables. Tukey tests were performed for the comparisons of means and the difference was considered significant at $P \leq 0.05$ and indicated by different letters.

III Results

III-1 Effect of proline and Si on plant growth index

NaCl treatment reduced plant growth indexes, including SDW, RDW, plant height and leaf number indexes by 85%, 96%, 93% and 58%, respectively (Figure 1a-d). However, treatment of salt-stressed plants by 20 mM proline, 3 mM Si or their combination significantly improved all of the growth index parameters. Indeed, SDW, RDW, plant height and leaf number indexes were increased by 161%, 262%, 263% and 68% in proline-treated plants by 92%, 577%, 256% and 39% in Si-treated plants and by 231%, 439%, 320% and 100% in plants treated with both proline and Si (Figure 1). Under normal conditions, the single application of proline and Si increased SDW index only (Figure 1a). However, the combined supplementation of both proline and Si reduced RDW, plant height and leaf number indexes as compared to untreated control (Figure 1a-d).

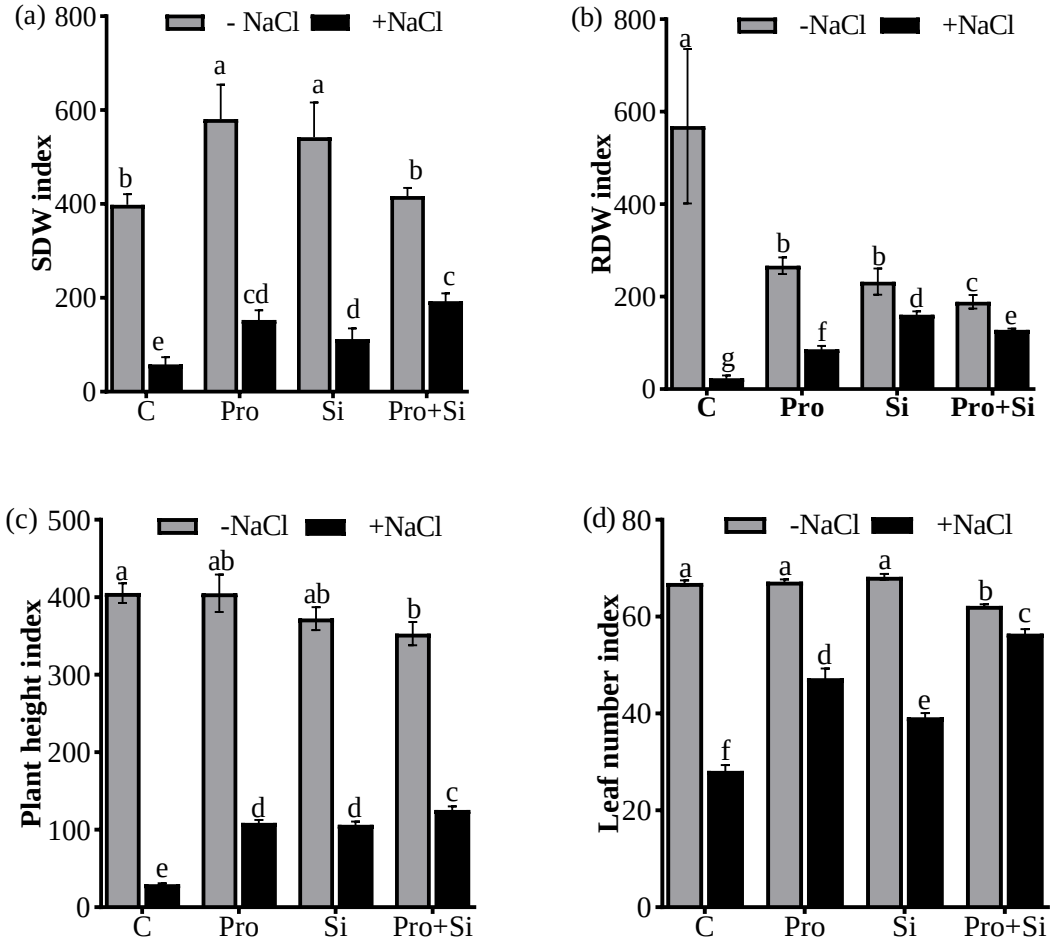

Figure 1. Effect of 20 mM proline (Pro) and 3 mM $CaSiO_3$ (Si) on shoot dry weight (SDW) (a), root dry weight (RDW) (b), plant height (c) and leaf number (d) indexes of *M. truncatula* plants

under either 0 or 120 mM NaCl stress conditions. Values are means ± standard deviation of three replicates. The letters above bars indicate statistically significant values.

III-2 Effect of proline and Si on leaf area (LA)

Data presented in Figure 2 indicated that LA was reduced by 29% upon salt stress as compared to the unstressed control plants. However, LA of salt-stressed plants was strongly enhanced by either proline (39%) or Si (24%) and (42%) when proline and Si were applied together by comparison with untreated salt-stressed plants (Figure 2). However, under normal conditions, the combined application of proline and Si reduced LA (Figure 2).

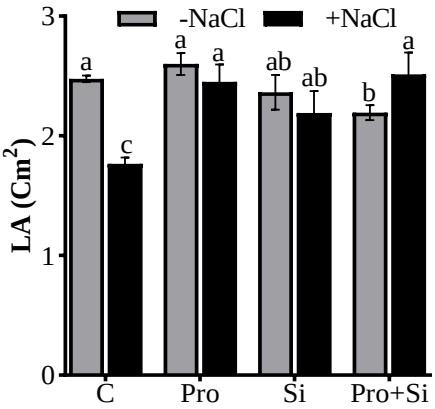

Figure 2. Effect of 20 mM proline (Pro) and 3 mM $CaSiO_3$ (Si) on leaf area (LA) of *M. truncatula* plants under either 120 mM NaCl stress (+NaCl) or non-stress (-NaCl) conditions. Values are means ± standard deviation of three replicates. The letters above bars indicate statistically significant values.

III-3 Effect of proline and Si on proline content

Under salt stress, proline content increased 33.6-fold (Figure 3). The sole application of either proline or Si caused a remarkable increase in proline content, and the combined application of both compounds further the increase. Indeed, the simultaneous supplementation of proline and Si to the growth solution of salt-stressed *M. truncatula* plants increased proline content by 88% relative to the untreated salt-stressed control (Figure 3). Under normal conditions, proline alone or combined with Si also caused a more modest (1.5-fold) yet significant ($P \leq 0.001$) increase in proline content.

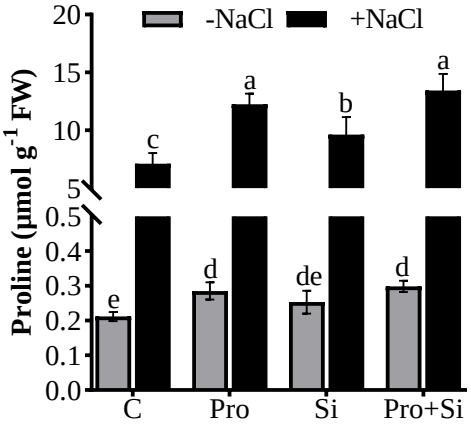

Figure 3. Effect of 20 mM proline (Pro) and 3 mM $CaSiO_3$ (Si) on proline content of *M. truncatula* plants under either 120 mM NaCl stress (+NaCl) or non-stress (-NaCl) conditions. Values are means ± standard deviation of three replicates. The letters above bars indicate statistically significant values.

III-4 Si content in *M. truncatula*

Salt stress increased shoot Si content by 100% as compared to the unstressed control (Figure 4). In addition, while separate application of proline and Si reduced Si content, their combined application furthered increased Si content in the shoot of salt-stressed *M. truncatula* plants (up to 24%) relative to untreated salt-stressed control (Figure 4a). Under normal conditions, only the combined application of proline and Si significantly ($P \leq 0.05$) increased Si content.

In roots (Figure 4b), Si content was 30% lower under salt stress than that under control. However, proline supplementation to the soil solution of salt-stressed *M. truncatula* plants improved Si content by 32% as compared to proline-untreated salt-stressed plants. In contrast, Si alone or combined with proline has no significant effects on Si content in salt-stressed plants relative to salt-stressed control (Figure 4b). Under normal conditions, proline treatment significantly increased Si content.

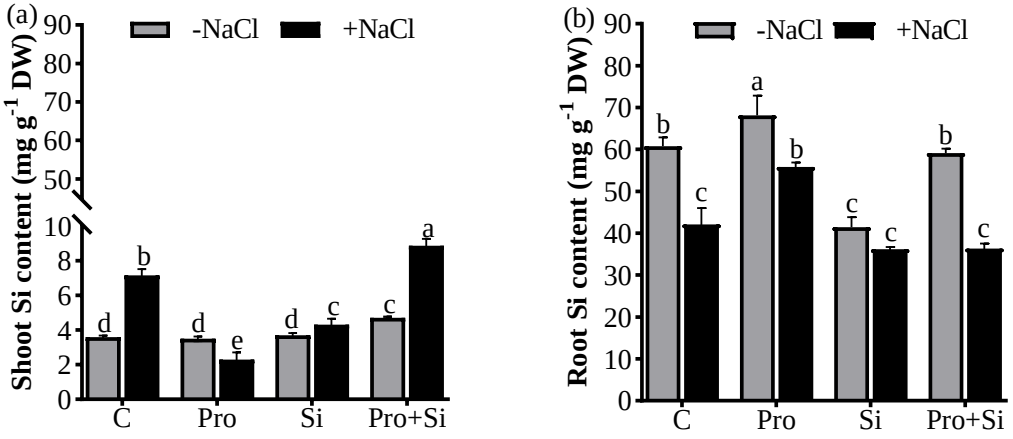

Figure 4. Effect of 20 mM proline (Pro) and 3 mM $CaSiO_3$ (Si) on shoot (a) and root (b) Si content of *M. truncatula* plants under either 120 mM NaCl stress (+NaCl) or non-stress (-NaCl) conditions. Values are means ± standard deviation of three replicates. The letters above bars indicate statistically significant values.

III-5 Effect of proline and Si on Na^+ and K^+ contents

Results presented in table 2 showed that K^+ content was significantly reduced upon salt stress, particularly in roots with a 43% decrease relative to unstressed control. Furthermore, single or combined supply of proline and Si to salt-stressed *M. truncatula* plants further reduced K^+ content in roots, whereas a slight increase was observed in response to proline treatment (proline alone or combined with Si) in shoots (Table 2). Under normal conditions, single or combined supply of proline and Si reduced K^+ in both shoots and roots.

For Na^+, results indicated that Na^+ content was 10.59 and 5.86-fold higher in shoot and root, respectively, under 120 mM NaCl treatment compared to control (Table 2). Moreover, the supplementation of proline or Si further increased Na^+ content in the shoots of salt-stressed plants with the highest increment rates of 33% observed under the combined application of proline and Si compared to the untreated salt-stressed control. However, for the roots, proline alone or combined with Si reduced significantly Na^+ content in salt-stressed plants (Table 2). Under normal conditions, single or combination of proline and Si induced a slight increase in Na^+ content in both shoots and roots.

Table 2. Effect of proline and Si on K^+ and Na^+ content in *M. truncatula* plants under normal (- NaCl) or stressed (+ NaCl) conditions.

		K^+ (mg g^{-1} DW)		Na^+ (mg g^{-1} DW)	
		- NaCl	+ NaCl	- NaCl	+ NaCl
Shoot	Control	19.11±0.31^{b}	18.51±0.16^{c}	3.60±0.09^{g}	38.11±0.28^{c}
	Proline	14.89±0.12^{e}	19.70±0.04^{a}	6.84±0.09^{f}	48.84±0.49^{a}
	Si	15.86±0.11^{d}	18.88±0.64^{bc}	7.05±0.04^{e}	41.39±0.18^{b}
	Proline+Si	17.67±0.07^{c}	19.08±0.03^{b}	8.47±0.09^{d}	50.85±0.05^{a}
Root	Control	19.78±0.32^{a}	11.24±0.06^{d}	5.01±0.32^{e}	29.34±0.17^{a}
	Proline	17.57±0.05^{b}	7.26±0.11^{e}	7.28±0.14^{d}	25.75±0.08^{b}
	Si	16.28±0.17^{c}	7.15±0.35^{e}	8.10±0.07^{c}	29.54±0.10^{a}
	Proline+Si	11.41±0.12^{d}	7.30±0.32^{e}	7.40±0.04^{d}	26.71±0.11^{b}

K^+, potassium; Na^+, sodium; -NaCl, 0 mM NaCl treatment; + NaCl, 120 mM NaCl treatment; Si, 3 mM Si ($CaSiO_3$) treatment; proline, 20 mM proline treatment; proline + Si, combination of proline and $CaSiO_3$ treatment. Values are means ± standard deviation of three replicates and the letters indicate statistically significant values.

III-6 Effect of proline and Si on the expression of *DHN2* and *Lsi2*

Transcript analyses using the highly sensitive droplet digital PCR (ddPCR) method revealed low transcript level of *DHN2* in the unstressed *M. truncatula* plants whatever the presence or not of proline and /or Si (Figure 5a). However, the addition of 120 mM NaCl induced a spectacular increase in the transcript level of *DHN2* (177-fold induction compared to control). Furthermore, single or combined supplementation of proline and Si to salt-stressed plants further increased the transcript level of *DHN2* with the highest level observed for proline-treated salt-stressed *M. truncatula* plants (2.34-fold induction compared to untreated salt-stressed *M. truncatula* plants) (Figure 5a).

For *Lsi2* (Figure 5b), the transcript level was 1.27-fold higher under salt stress than under control. Furthermore, treatment of salt-stressed *M. truncatula* plants with either proline or Si further enhanced the transcript level of *Lsi2*, and the increase was much higher under the combined application of proline and Si (1.65-fold higher compared to the untreated salt-stressed *M. truncatula* plants) (Figure 5b). Under normal conditions, treatment of *M. truncatula* plants with proline alone or combined with 3 mM Si reduced the transcript level of *Lsi2* (1.19 and 2.07-fold lower for plants treated with proline and with proline and Si, respectively).

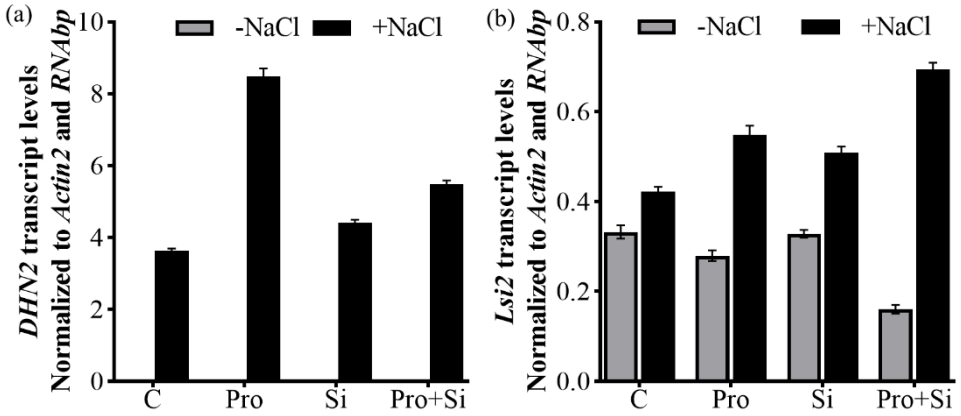

Figure 5. Effect of 20 mM proline (Pro) and 3 mM $CaSiO_3$ (Si) on *Dehydrin 2* (*DHN2*) (a) and *Low silicon transporter 2* (*Lsi2*) (b) gene expression in *M. truncatula* plants under normal (0 mM NaCl) or stressed (120 mM NaCl) conditions. *DHN2* (a) and *Lsi2* (b) expression in *M. truncatula* was quantified by droplet digital PCR after 14 d of treatment and normalized by *Actin2* and *RNAbp*.

III-7 Effect of proline and Si on the expression of proline metabolism genes

For the anabolism of proline, transcript levels of *P5CS1*, *P5CS2* and *OAT* were determined (Figure 6a-c). Transcript level of *P5CS1* was 1.88-fold lower in NaCl-treated *M. truncatula* plants than that in control plants (Figure 6a). Moreover, under salt stress, single or combined supplementation of proline and Si has no effect on the transcript level of *P5CS1*. However, under normal conditions, single or combined application of proline and Si increased *P5CS1* transcript level with the highest increment rates recorded for plants treated with Si alone (1.47-fold higher compared to untreated control).

Transcript level of *P5CS2* in salt-stressed *M. truncatula* plants was similar to control *M. truncatula* plants (Figure 6b). Exogenous proline supplementation increased *P5CS2* level by 85% and 289% under normal and stressed conditions, respectively, relative to their respective controls. In contrast, single supplementation of Si reduced *P5CS2* transcript level under salt stress as compared to control (Figure 6b).

Transcript level of *OAT* was 2.64-fold higher under salt stress than that under control (Figure 6c). In addition, as compared to NaCl-treated control, *OAT* transcript level was 4.44, 2.39 and 9.17-fold higher in salt-stressed plants treated with proline, Si and their combination, respectively. Under normal conditions, single or combined supplementation of proline and Si induced a slight increase in *OAT* transcript level (Figure 6c).

For the catabolism of proline, transcript levels of *P5CDH* and *ProDH1* were also determined using ddPCR method (Figure 6d, e). Results presented in Figure 6d indicated that salt stress lowered the transcript level of *P5CDH* by 93% relative to the unstressed control. However, as

compared to salt-stressed control, the supplementation of proline alone or combined with Si to the salt-stressed plants induced a slight increase in transcript level of *P5CDH* (Figure 6d). Furthermore, under normal conditions, the single supplementation of Si reduced the transcript level of *P5CDH*. In contrast, the transcript level of *ProDH1* was 7.21-fold higher under salt stress as compared to unstressed control (Figure 6e). Additionally, the treatment of salt-stressed *M. truncatula* plants with 3 mM Si alone or combined with 20 mM proline boosted the increase in the transcript level of *ProDH1* as compared to the untreated salt-stressed plants (79% and 115% for Si and proline plus Si, respectively) (Figure 6e). Under normal conditions, the single supplementation of proline induced a remarkable increase in the transcript level of *ProDH1*.

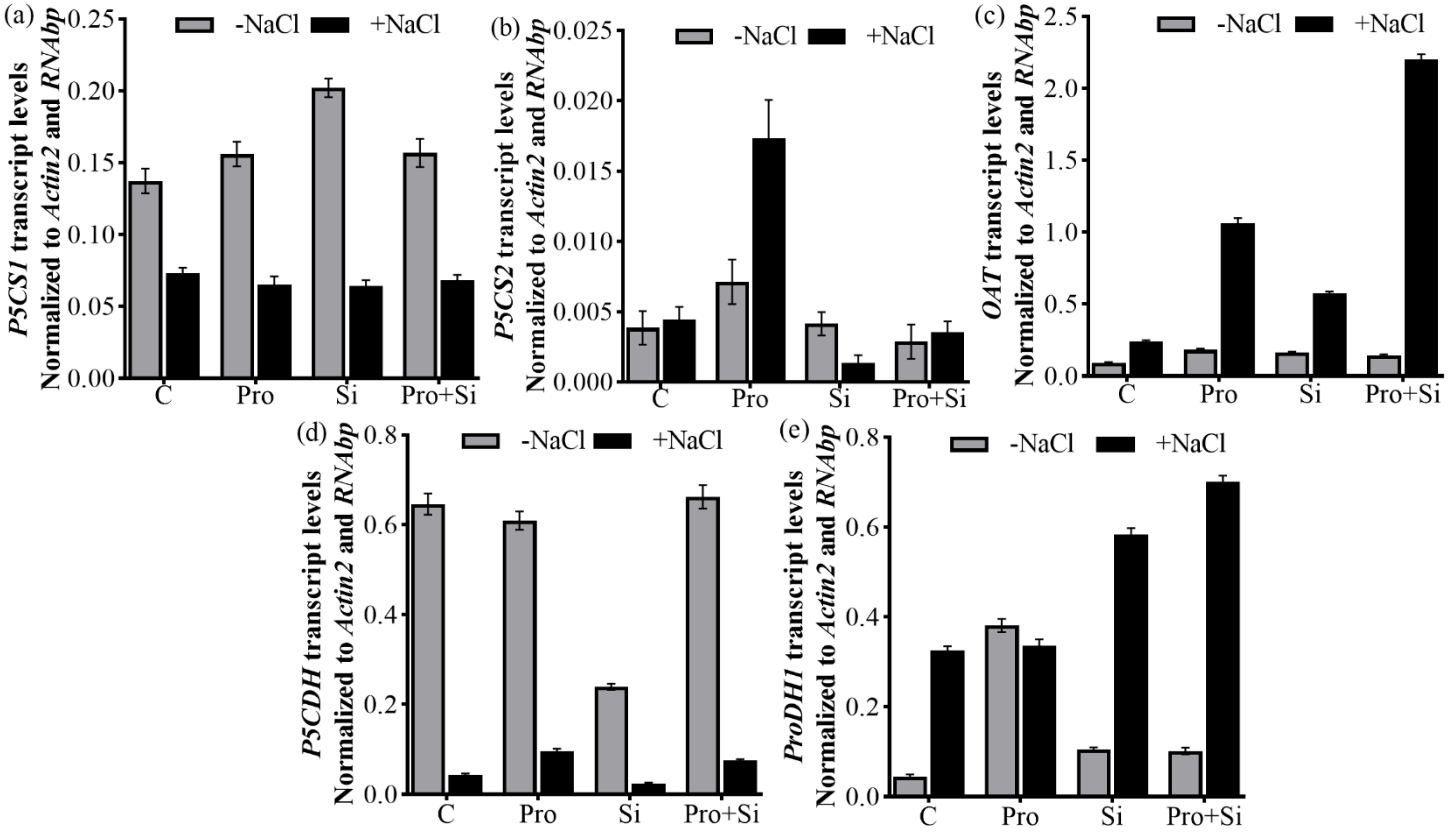

Figure 6. Effect of 20 mM proline (Pro) and 3 mM $CaSiO_3$ (Si) on the expression of proline metabolism genes of *M. truncatula* plants grown under normal (0 mM NaCl) or stressed (120 mM NaCl) conditions. *Pyrroline-5-carboxylate synthetase 1* (*P5CS1*) (a), *P5CS2* (b), *Ornithine Aminotransferase* (*OAT*) (c), *P5C Dehydrogenase* (*P5CDH*) (d) and *Proline Dehydrogenase 1* (*ProDH1*) (e) expression in *M. truncatula* was quantified by droplet digital PCR after 14 d of treatment and normalized by *Actin2* and *RNAbp*. Data are means (±SE).

IV Discussion

Salt stress is a major abiotic stress that could cause morphological, physiological and biochemical damages resulting in crop yield loss. The beneficial effects of the single application of proline and Si on plant salt tolerance have been extensively investigated (El Moukhtari et al. 2021; de Freitas et al. 2018; Nounjan et al. 2012; Deshmukh et al. 2013). However, very few studies have investigated the effect of the combined application of proline and Si (Rady et al. 2019; Radhi et al. 2021). In this study, the effects of the individual and combined supply of 20 mM proline and 3 mM Si were investigated in *M. truncatula* plants under salt stress conditions. Transcript levels of proline metabolism and Si transporters-related genes were investigated to understand the mechanistic roles induced by proline and Si and their relations to plant growth under salt stress.

IV-1 Beneficial effects of exogenous proline and/or Si on plant growth indexes of salt-stressed *M. truncatula* plants

Our results showed that all the growth indexes of salt-stressed *M. truncatula* plants were significantly ($P \leq 0.001$) higher with added proline or Si. Interestingly, with the exception of RDW, the increase in all the growth indexes was further enhanced when proline and Si were applied simultaneously as compared to their separate applications, as seen in salt-stressed common bean (*P. vulgaris*) (Rady et al. 2019) and beet (*Beta vulgaris*) under drought stress (Alkahtani et al. 2021). However, other investigations in drought-stressed cowpea (*Vigna unguiculata*) showed that separate application of either proline or Si was more beneficial compared to their combined application (dos Santos et al. 2022). The proline-mediated positive effect on biomass under salt stress could be attributed to its ability to improve osmotic adjustment mediated water uptake and gas exchange (de Freitas et al. 2019). Additionally, Si was shown to enhance photosynthesis, nitrogen fixation, nutrient status and relative water content and reduced oxidative stress in *M. sativa* grown under salt stress (El Moukhtari et al. 2021).

IV-2 Effects of exogenous proline and/or Si on endogenous proline accumulation and expression of proline metabolism-related genes of salt-stressed *M. truncatula* plants

Plants maintain their physiological functions under osmotic stress by accumulating various osmoprotectants (Singh et al. 2015). Proline is the most abundant osmolyte accumulated under

salinity-mediated osmotic stress in *M. truncatula* (Armengaud et al. 2004). We found a significantly increased proline upon salt stress. Moreover, 20 mM proline treatment, 3 mM Si treatment or their combination further increased proline accumulation (Figure 3). The increase in proline content under stress conditions is the result of induction of its biosynthesis *via* P5CS and OAT and inhibition of catabolism *via* ProDH and P5CDH as well as transport process (Xue et al. 2009). We found that *OAT* transcript level was strongly induced upon salt stress while *P5CS1* was reduced, albeit with no significant effect on *P5CS2*. Furthermore, the exogenous supply of proline or Si to salt-stressed plants increased transcript level of *OAT*. This is correlated with the highest proline content when proline and Si were applied simultaneously (Figure 6c). Both *P5CS1* and *P5CS2* transcript levels were lower under salt stress in response to single or combined application of proline and Si. Previous studies showed that the biosynthesis of proline in legumes, such as *Medicago lupulina* and *Lotus corniculatus*, is mediated by the ornithine pathway catalyzed by OAT in relation to nitrogen status (AbdElgawad et al. 2015). Similarly, in *M. truncatula*, proline accumulation under salt stress is related to the expression of both *P5CS2 and OAT* genes as already described by Armengaud et al. (2004). Beside proline biosynthesis, results of the present study indicated that, under salt stress, *P5CDH* transcript level was strongly reduced (93%) while transcript level of *ProDH1* was increased (621%). Moreover, transcript level of *ProDH1* was further increased in response to proline alone or in combination with Si in the presence or not of salinity stress. In contrast, transcript level of *P5CDH* in proline-treated plants remained lower than that of the control even in the presence of salt stress. Previous investigations reported that *ProDH* is rapidly accumulated in response to proline treatment, suggesting that this response may protect plants against proline toxicity (Deuschle et al. 2001). Similarly, in *C. sativus*, Zhu et al. (2020) reported that *ProDH* transcript level was increased upon proline treatment with or without NaCl and Si, while transcript of *P5CDH* remained stable under the same conditions. The low level of *P5CDH* under salt stress could represent a tolerance mechanism, as P5C was reported to serve as a regulator of cellular stress responses (Deuschle et al. 2001).

IV-3 Effects of exogenous proline and/or Si treatments on Si assimilation and *Lsi2* expression of salt-stressed *M. truncatula* plants

Si accumulation and stress do not necessarily correlate in *M. sativa*. Recently Meng et al. (2020) showed that Si content was found to decrease with increasing salt concentration. However, other investigations found that Si content was accumulated under cadmium and salt stress

(Kabir et al. 2016; El Moukhtari et al. 2021). In the present study, Si content was reduced in both shoots and roots upon Si treatment, whatever the presence or not of NaCl. On the other hand, under salt stress, Si content was drastically reduced in roots, but was significantly increased in shoots. Interestingly, the increase in Si content under salt stress conditions was further enhanced under the combined application of proline and Si. Si accumulation was found to be modulated by Si transporters, and difference in Si uptake between treatments could be due to the involvement of Si transporters (Coskun et al. 2019; 2021). Si is acquired in the form of silicic acid by the roots through influx transporter and then, is transported up to the aerial part through efflux transporter, which are encoded by *Lsi1* and *Lsi2*, respectively (Coskun et al. 2019; El Moukhtari et al. 2021). There is no consensus on how Si supply affects *Lsi2* expression in plants (Coskun et al. 2021). In *O. sativa*, *OsLsi2* expression was found upregulated after one day of Si treatment (Kim et al. 2014) while it decreased after three days (Ma et al., 2007). In *Z. mays* and *H. vulgare*, *Lsi2* expression was downregulated with Si supply (Mitani et al. 2009), whereas in *C. sativus*, *Lsi2* was upregulated (Sun et al. 2018). In this work, we show that *Lsi2* transcript level strongly increased under salt conditions compared to control. More interestingly, treatment of salt-stressed plants with proline and/or Si further increased transcript level of *Lsi2* with highest increase observed in stressed plants treated with both proline and Si (Figure 5), which is correlated with the highest shoot Si content (Figure 4). However, under normal conditions, the expression of *Lsi2* was 2.7-fold lower under the combined application of proline and Si than of the control.

IV-4 Effects of exogenous proline and/or Si on K^+ and Na^+ uptake of salt-stressed *M. truncatula* plants

Depending on plant species, an increase in K^+ and a decrease in Na^+ is well known to contribute to osmotic adjustment and to avoid Na^+ toxicity (Gupta and Huang 2014). Accumulation of Na^+, especially in roots exposed to either drought or salinity-mediated osmotic stress, is also reported to maintain osmotic balance, especially in halophytes (Slama et al. 2015). In the present study, salt stress treatment increased Na^+ content and reduced K^+ content in both shoots and roots. However, in salt-stressed plants, Na^+ content in shoots is further increased in response to single or combined proline and Si treatment, while its content is significantly reduced in roots. Furthermore, single or combined supply of proline and Si increased significantly K^+ in the shoots of salt-stressed plants. Our results corroborate the works of Huang

et al. (2009) and Romero-Aranda et al. (2006). They found that exogenous supply of proline or Si to salt-stressed plants dramatically increased Na^+ as compared to untreated salt-stressed controls. In contrast, Rady et al. (2019) showed that the supply of proline or Si significantly increased K^+ and K^+/Na^+ ratio, while suppressed Na^+ uptake by salt-stressed *P. vulgaris* plants, the effect was more obvious under combined application than that under single supply of either proline or Si. According to Aydi et al. (2010), the accumulation of Na^+ under salt stress is one among others of the main tolerance mechanisms adopted by *M. truncatula*. Similar results have been reported by Farissi et al. (2013) on *M. sativa*, who stated that the accumulation of Na^+ may contribute to the osmotic adjustment and consequently, tolerance to drought-mediated osmotic stress.

V Conclusions

We found that single or combined proline and Si treatments alleviated the negative impact of salinity stress and significantly improved plant growth index. Proline and/or Si-mediated alleviation of salinity toxicity were mediated by modulating proline metabolism and Si transporter-related genes. We also demonstrated that *MtOAT* played a more important role than *MtP5CS1* and *MtP5CS2* for proline accumulation in leaves of *M. truncatula* under salt stress. Our findings suggest that single or combined supply of proline and Si could be an effective way in mitigating salinity stress in *M. truncatula*. These findings are also valuable from an agronomic standpoint, as they will encourage the use of proline and Si as biostimulants to maintain the production of forage crops like *M. sativa* under salinity stress.

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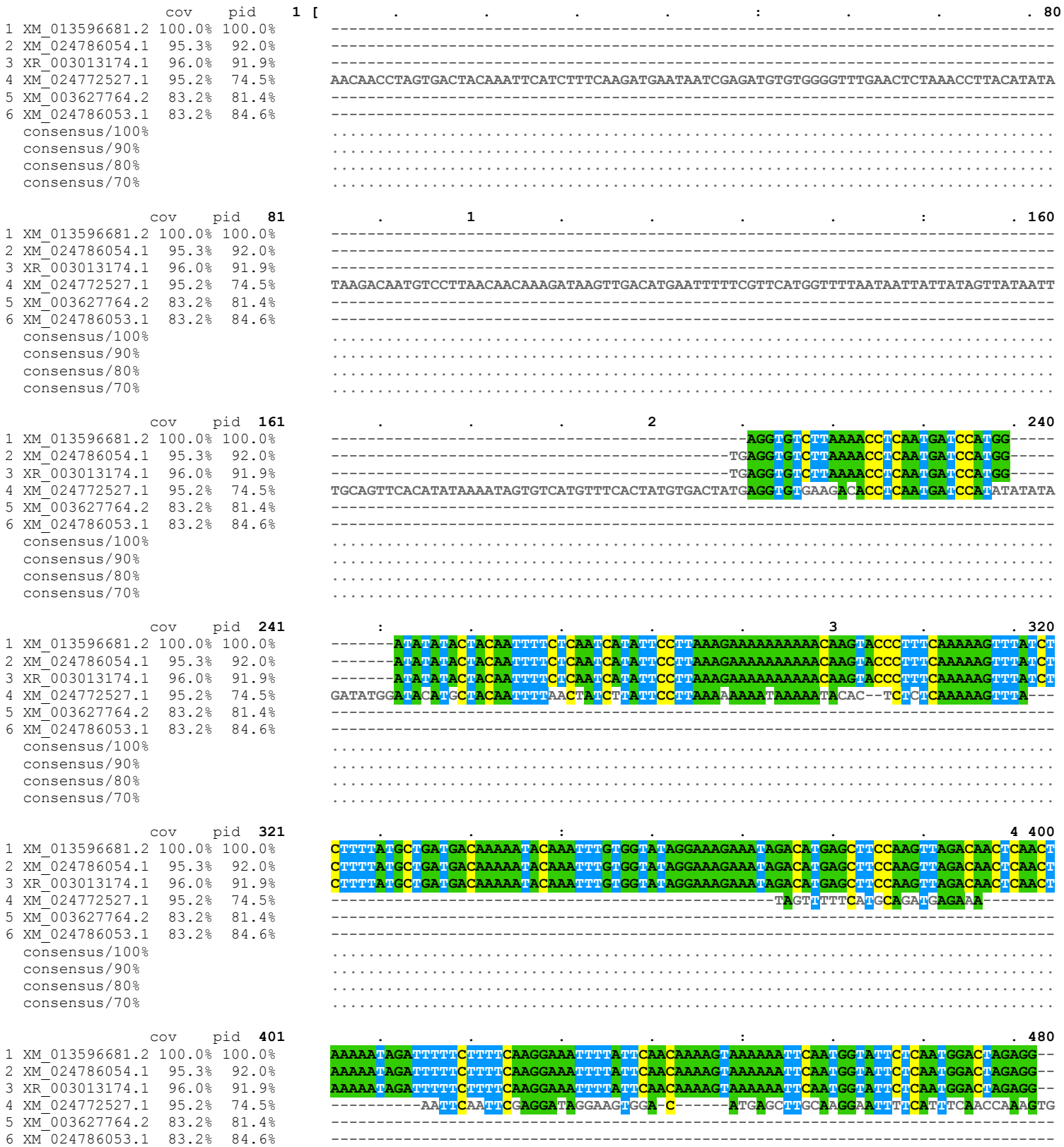
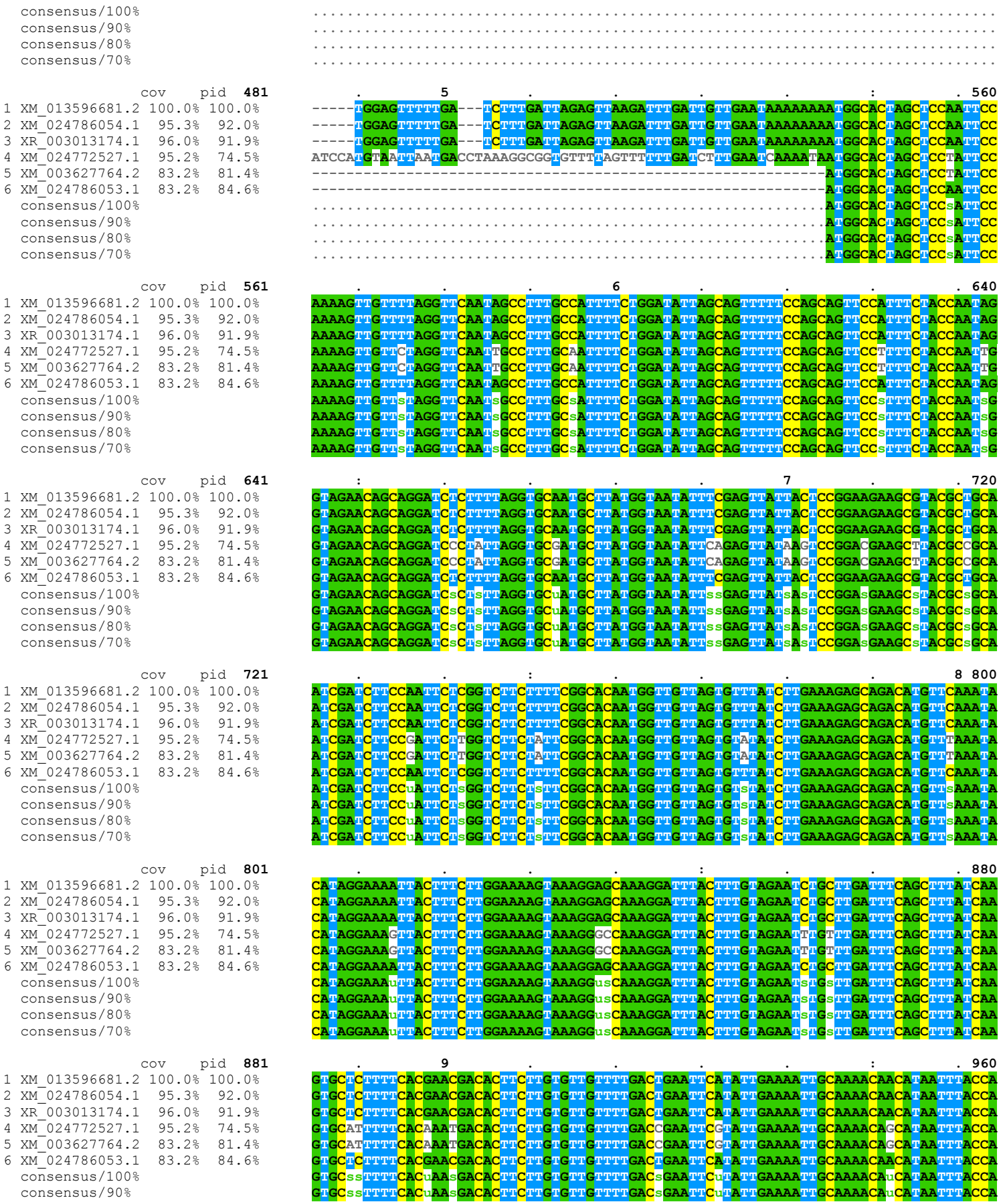
Supplementary data

Results of Lsi2 variants alignement

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consensus/90%
consensus/80%
consensus/70%
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2 XM_024786054.1 95.3% 92.0%
3 XR_003013174.1 96.0% 91.9%
4 XM_024772527.1 95.2% 74.5%
5 XM_003627764.2 83.2% 81.4%
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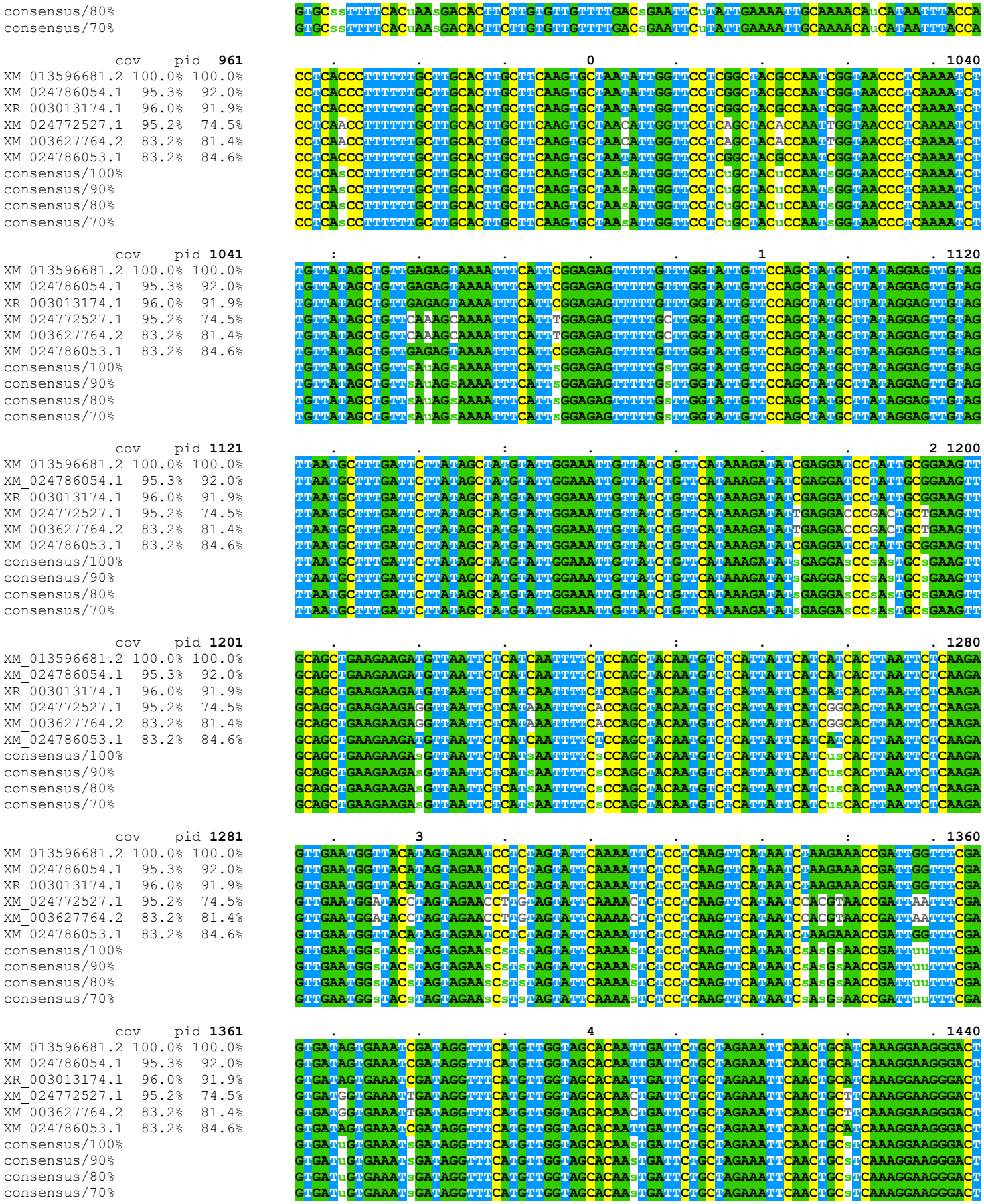
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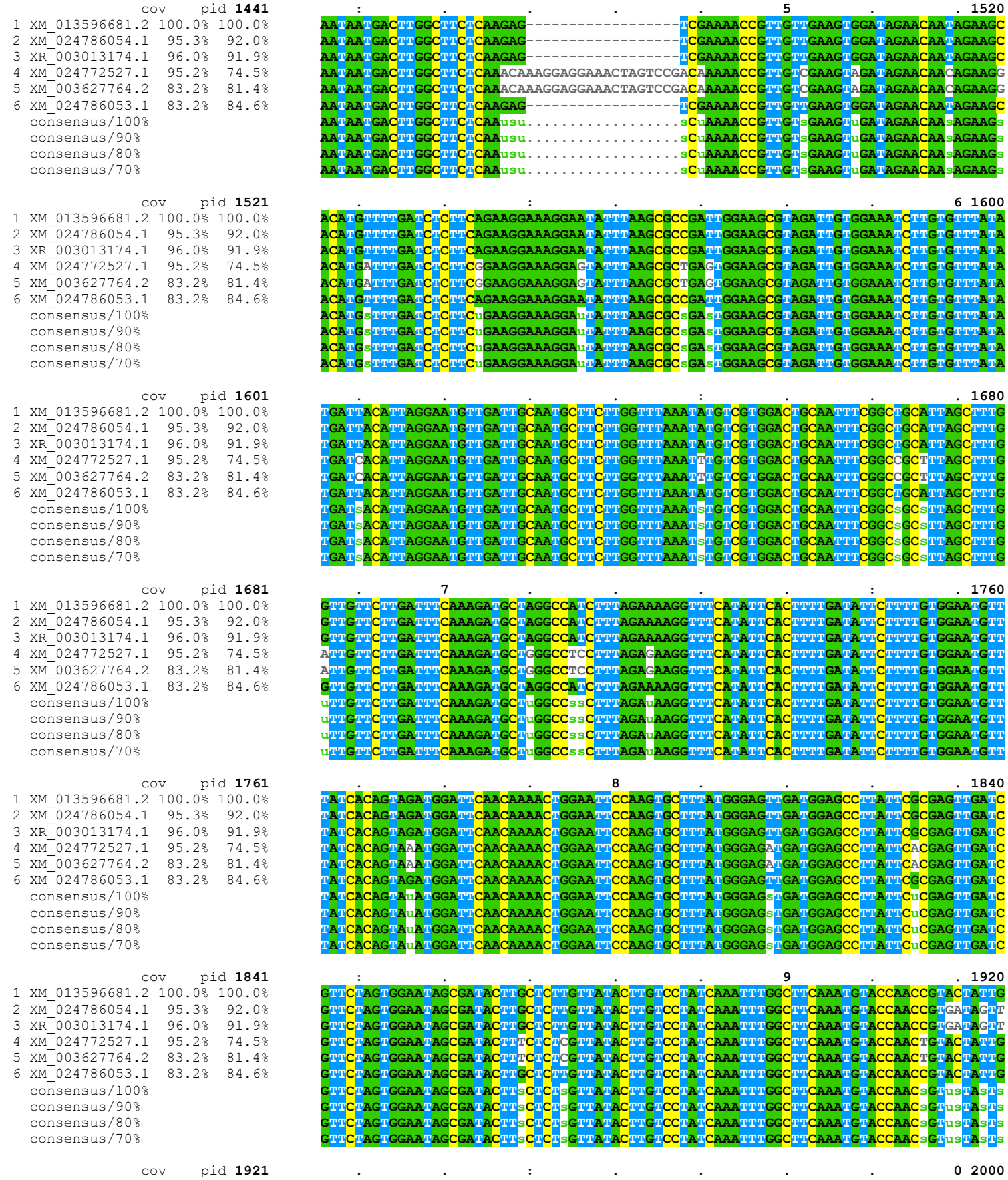
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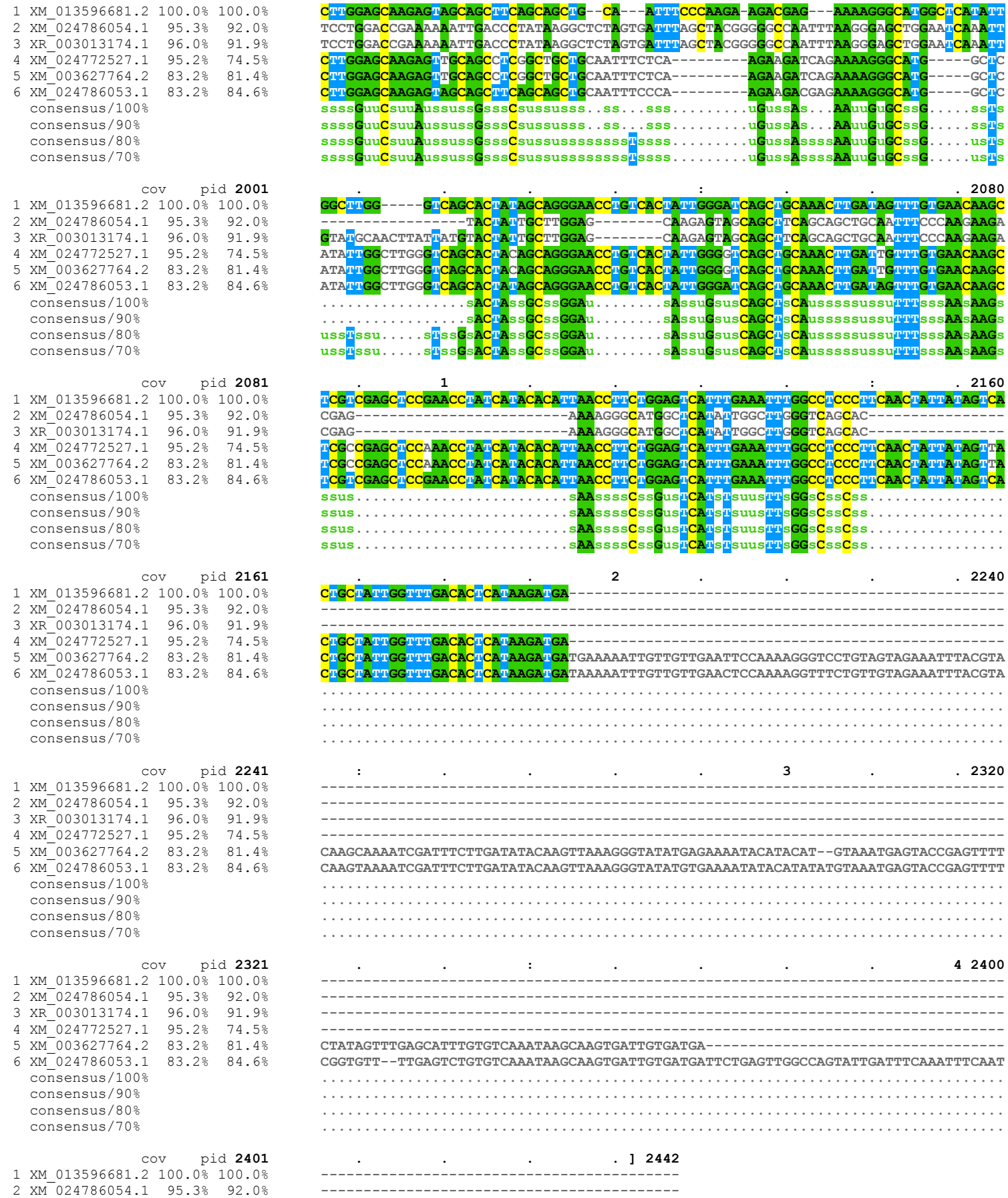
2 XM_024786054.1 95.3% 92.0%
3 XR_003013174.1 96.0% 91.9%
4 XM_024772527.1 95.2% 74.5%
5 XM_003627764.2 83.2% 81.4%
6 XM_024786053.1 83.2% 84.6%
consensus/100%
consensus/90%
consensus/80%
consensus/70%
cov pid 1521
cov pid 1601
cov pid 1681
cov pid 1761
cov pid 1841
cov pid 1921


cov pid 2001
2080
cov pid 2081
2160
cov pid 2161
2240
cov pid 2241
2320
cov pid 2321
4 2400
cov pid 2401
] 2442
1 XM_013596681.2 100.0% 100.0%
2 XM_024786054.1 95.3% 92.0%
3 XR_003013174.1 96.0% 91.9%
4 XM_024772527.1 95.2% 74.5%
5 XM_003627764.2 83.2% 81.4%
6 XM_024786053.1 83.2% 84.6%
consensus/100%
consensus/90%
consensus/80%
consensus/70%

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3 XR_003013174.1  96.0%  91.9%      ----------------------------------------
4 XM_024772527.1  95.2%  74.5%      ----------------------------------------
5 XM_003627764.2  83.2%  81.4%      ----------------------------------------
6 XM_024786053.1  83.2%  84.6%      TTAAATAGAATCTATAAGAATATGTAGAGCATGGTTCTAACA
  consensus/100%                    .......................................
  consensus/90%                     .......................................
  consensus/80%                     .......................................
  consensus/70%                     .......................................
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DISCUSSION GENERALE

L'apport exogène de proline et de silicium (Si) en réponse à des contraintes environnementales variées, notamment les contraintes hydriques et salines, sont généralement considérés comme des méthodes prometteuses permettant aux plantes d'intérêt agronomique de mieux tolérer des conditions environnementales défavorables (Hanif et al. 2021; Kaur et Asthir 2020; Debona et al. 2017; Mostofa et al. 2021). L'évaluation de l'effet de la proline et du Si sur la tolérance à la contrainte saline chez deux variétés marocaine *Ouad Lmalah* (*OL*) et *Demnate 201* (*Dm*) et une variété européenne *NS Mediana ZMS V* (*NS Med*) de *Medicago sativa* ainsi que la lignée A17 de *M. truncatula* à différents stades de développement ont été réalisées dans ce travail de thèse.

Effet du Si sur la germination des graines, la viabilité des embryons et la mobilisation des réserves chez *M. sativa* sous contrainte saline

Les résultats obtenus pour le test de germination ont montré que la contrainte saline réduit significativement le pourcentage final de germination, l'indice de vigueur et la croissance des jeunes plantules, et augmente le temps nécessaire pour la germination de 50% des graines (T_{50}). Cette contrainte réduit également la viabilité des embryons et leur capacité à mobiliser les réserves glucidiques et protéiques. La comparaison de la réponse des variétés étudiées au NaCl a montré une variation significative de leur comportement. Sur la base des résultats obtenus, la variété marocaine *OL* issue des oasis a été identifiée comme étant la plus tolérante et *Dm* celle issue des montagnes comme étant moyennement tolérante à la contrainte saline. En revanche, la variété européenne *NS Med* est considérée comme étant la plus sensible. Nos résultats concordent bien avec ceux de Bayuelo-Jiménez et al. (2002) sur *Phaseolus vulgaris*, sur *M. sativa* par Farissi et al. (2011) et sur *Amaranthus caudatus Chenopodium quinoa* par Moreno et al. (2018). Selon Debez et al. (2018), l'effet négatif du NaCl sur la germination des graines pourrait être attribué à un effet osmotique et/ou toxicité ionique. Dans la présente étude, la contrainte saline engendre une forte accumulation de Na^+ et une diminution de K^+. L'effet toxique des ions Na^+ et Cl^- sur la viabilité de l'embryon et les activités métaboliques au cours des processus de germination a donc été étudié. Le traitement exogène des graines avec le Si a été montré des effets très bénéfiques sur la germination des graines sous diverses contraintes abiotiques (Zhu et Gong 2014; Rizwan et al. 2015). Nos résultats montrent que le traitement des graines de *M. sativa* avec 3 mM Si améliore significativement la tolérance au sel des trois variétés au stade germination à travers un pourcentage de germination et un indice de vigueur plus élevés et une T_{50} plus petite. De même, l'imbibition des graines des trois variétés de *M. sativa* par 3 mM Si améliore significativement la viabilité des embryons, la mobilisation des réserves et la croissance des jeunes plantules. La tolérance des variétés à la contrainte saline est

bien associée en premier lieu à des activités antioxydantes SOD et CAT plus élevées et à une accumulation de certaines isoformes de SOD ainsi que des teneurs en proline plus élevées. Ces activités augmentent suite à l'imbibition des graines avec 3 mM Si. D'autre part, une grande stabilité membranaire reflétée par les faibles teneurs en MDA et en H_2O_2 et de faibles valeurs de perte en électrolytes sont observées chez les jeunes plantules sous contraintes salines. Nous avons également mis en évidence que le traitement des graines avec le Si peut améliorer la germination des graines de *M. sativa* sous contraintes salines, ce qui est en accord avec les résultats rapportés par Zhang et al. (2015) chez *Glycyrrhiza uralensis* et par Gou (2020) chez *Cucumis sativus.* Ces auteurs ont montré que le traitement des graines avec du Si a eu un effet antagoniste sur celui provoqué par le NaCl sur la germination des graines. Les effets positifs du traitement au Si pourraient être associés à l'induction des enzymes hydrolytiques et de l'accumulation des solutés organiques (Biju et al. 2017), la régulation du métabolisme hormonal (Arif et al. 2021), le renforcement des réponses antioxydantes (Wang et al. 2010) et la modulation de l'expression de certains gènes des graines en germination (Almutairi et al. 2016).

L'effet de la contrainte saline sur la croissance, la photosynthèse, la nutrition minérale et le métabolisme oxydatif des plantes chez *M. sativa*

Les effets inhibiteurs de la contrainte saline sur la croissance des plantes peuvent être divisés en trois catégories : effet toxique en raison de l'accumulation excessive des ions Na^+ et Cl^- et effet osmotique, auxquelles s'ajoutent des effets secondaires dus à l'accumulation excessive des espèces réactives d'oxygène (ERO) induisant un stress oxydant (Van Zelm et al. 2020). Nos résultats montrent que le traitement des plantes par le sel réduit la teneur relative en eau des plantes reflétant ainsi l'effet osmotique du NaCl. La contrainte saline induit une réduction de la teneur des plantes en K^+ et augmente celle en Na^+, réduisant par conséquence le rapport K^+/Na^+. Nos résultats ont montré également qu'en condition de salinité, une différence de rapport K^+/Na^+ est observée entre les trois variétés étudiées avec des réductions plus importantes observées chez la variété sensible. Ce résultat suggère que la sensibilité de la variété européenne *NS Med* à la salinité est due en grande partie à des perturbations de l'homéostasie K^+/Na^+. De plus, les fortes teneurs en Na^+ accumulées au cours de la contrainte saline pourraient perturber également les processus photochimiques de la photosynthèse (Farissi et al. 2018). Nos résultats montrent que les fortes teneurs en Na^+, enregistrées chez les plantes stressées, sont accompagnées avec une réduction très importante de la teneur des feuilles en pigments (Chl a, Chl b, Chl totale et caroténoïdes) et de l'efficacité photochimique maximale du PSII (F_v/F_m)

chez les plantes des deux variétés marocaines et celles de la variété européenne, avec un effet plus prononcé chez la variété européenne *NS Med*. Selon Alamri et al. (2020), l'accumulation des ions Na^+ dans les feuilles induit une forte activité de la chlorophyllase, Chl-degrading peroxidase et la pheophytinase, induisant par conséquent une réduction de la teneur en Chl. Il a été également rapporté qu'au cours d'une contrainte saline, les plantes ferment leurs stomates pour limiter la perte d'eau par transpiration, ce qui conduit à une réduction de l'assimilation du CO_2 et à une perturbation des activités photosynthétiques (Antolín et al. 2010). Dans notre étude, nous avons observé que la contrainte saline réduit la conductance stomatique (*gs*) et cette réduction est plus marquée chez la variété européenne *NS Med*. L'effet de la contrainte saline pourrait également être observé sous forme d'un stress oxydant. Le malonyldialdehyde (MDA) est généralement un indicateur de stabilité membranaire et de la peroxydation lipidique (Gong et al. 2008) et son augmentation s'accompagne souvent avec une production élevée des ERO et d'une fuite d'électrolytes (Shanker et al. 2004; Mandhania et al. 2006). Dans la présente étude, la contrainte saline induit une forte accumulation des ERO telles que le peroxyde d'hydrogène et l'ion superoxyde, ce qui suggère la présence d'un stress oxydant. Une forte peroxydation des lipides membranaires (MDA) et une fuite d'électrolytes très élevée ont été en effet observées. Les stress osmotiques et oxydants et la toxicité ionique induits chez les plantes de la luzerne sous contraintes salines ont des impacts négatifs sur l'établissement de la symbiose *M. sativa-E. meliloti* et la teneur des plantes en azote (N). Des résultats similaires ont été rapportés chez *M. sativa* (Elgharably et Benes 2021), *Cicer arietinum* (Sadji-Ait Kaci et al. 2017) et *Melilotus indicus* (Sunita et al. 2019). Selon Bargaz et al. (2013), les nodosités sont plus sensibles au stress oxydant que les autres organes (feuilles, tiges, racines, etc.). Chez *P. vulgaris*, Faghire et al. (2011) ont démontré que la contrainte saline réduit la respiration des bactéroïdes et le métabolisme carboné des nodosités, ce qui affecte négativement le fonctionnement des nodosités et par conséquent entraine une réduction de la teneur des plantes en N.

Effet de la proline et/ou du Si sur la tolérance de *M. sativa* à la contrainte saline

Le traitement exogène de plantes par de la proline ou du Si est généralement considéré comme une méthode très efficace permettant aux plantes de tolérer plusieurs contraintes abiotiques (Zhang et al. 2021; Ghouri et al. 2021; Janeeshma et al. 2021; Hayat et al. 2021; de Freitas et al. 2019). Nos résultats ont montré que l'ajout de 20 mM de proline ou 3 mM de Si dans la solution nutritive des plantes stressées atténue l'effet inhibiteur du sel sur la croissance des trois variétés de *M. sativa*, et en particulier chez la variété européenne (*NS Med*) considérée comme étant la plus sensible. En effet, le traitement de plantes stressées par de la proline ou du Si

améliore la biomasse sèche des parties aérienne et racinaire, la hauteur des plantes, le nombre de feuilles et la surface foliaire. L'apport exogène de la proline a été montré avoir des effets bénéfiques sur la croissance des plantes soumises à des contraintes salines (Ben Rejeb et al. 2012; Meena et al. 2019). Chez *Sorghum bicolor*, le traitement des plantes par 30 mM proline appliqué par voie foliaire réduit l'impact de la salinité sur la croissance des plantes et améliore significativement la biomasse fraiche et sèche des parties aérienne et racinaire ainsi que la surface foliaire (de Freitas et al. 2019). De même chez *M. sativa*, l'apport exogène de Si améliore la hauteur des plantes, la longueur des racines, le poids des tiges, des feuilles et des racines (Meng et al. 2020; Zhang et al. 2022). D'autre part, la comparaison des différents traitements appliqués révèle que l'apport séparé de la proline et de Si a des effets très bénéfiques sur la tolérance des plantes à la contrainte saline en comparaison avec un apport combiné des deux molécules. En effet, en conditions salines, aucune différence dans les paramètres éco-physiologiques mesurées n'est observée entre les plantes traitées avec de la proline et du Si de celles non traitées. Des résultats similaires ont été trouvés chez *Vigna radiata* sous déficit hydrique (dos Santos et al. 2022). Les auteurs ont démontré que l'application séparée de la proline et du Si ont des effets plus bénéfiques qu'une application combinée des deux molécules. Cependant, chez d'autres espèces telles que *P. vulgaris*, Rady et al. (2019) ont démontré que le traitement des plantes par 6 mM proline ou 6 mM Si améliore la croissance des plantes, et les effets sont plus marqués sous la combinaison des deux molécules.

L'amélioration de la croissance des plantes pourrait être le résultat de l'amélioration de la photosynthèse (Horton 2000). Nos résultats ont montré que le traitement des plantes stressées et traitées avec 20 mM proline ou 3 mM Si atténue l'effet délétère du NaCl sur des paramètres photosynthétiques telles que la teneur en chlorophylle, la conductance stomatique et l'efficacité photochimique maximale du PSII (F_v/F_m). Selon Alamri et al. (2020), le Si pourrait augmenter la teneur en chlorophylles sous contrainte saline en augmentant l'activité de certaines enzymes de synthèse de la chlorophylle, notamment l'acide δ-aminolévulinique déshydratase et la porphobilinogène désaminase, et en inhibant celles responsables de la dégradation de chlorophylle, notamment la chlorophyllase, la Chl-degrading peroxydase et la pheophytinase. De même, des études récentes ont indiqué que le traitement des plantes par le Si exogène améliore considérablement les traits photosynthétiques (Hussain et al. 2021), ce qui pourrait expliquer une meilleure croissance des plantes à travers l'augmentation de la biomasse, de la hauteur des plantes, du nombre de feuilles et de la surface foliaire. De même, plusieurs auteurs ont rapporté que l'apport exogène de la proline est capable d'améliorer la capacité photosynthétique chez plusieurs espèces végétales soumises à des contraintes

environnementales variées (Ben Rejeb et al. 2012; Meena et al. 2019). L'amélioration de la photosynthèse en conditions de contraintes en réponse à l'ajout de la proline pourrait être expliquée par la capacité de la proline à maintenir la stabilité des membranes et la conformation de protéines telles que la RuBisCo (Szabados et Savouré 2010; Zheng et al. 2021). D'autre part, la proline peut jouer le rôle d'un soluté compatible impliqué dans l'ajustement osmotique. Les plantes peuvent ainsi maintenir leur turgescence pendant la contrainte saline, en gardant leurs stomates ouverts pour l'assimilation de CO_2. Nos résultats ont aussi indiqué une accumulation significative des ERO chez les plantes traitées par NaCl, ce qui indique que ces plantes ont subi un stress oxydant mis en évidence par une forte accumulation de la MDA et des valeurs très élevées de la perte d'électrolytes. Cependant, l'apport exogène de proline ou de Si réduit la teneur en ERO, en MDA et la perte d'électrolytes. Ces résultats fournissent des arguments supplémentaires sur le rôle possible de la proline dans la protection des cellules contre les dommages oxydatifs liés aux ERO (Ben Rejeb et al. 2014 ; Szabados et Savouré 2010). Le Si a été décrit pour ses effets bénéfiques sur la réduction du stress oxydant à travers le renforcement du system antioxydant enzymatique et non-enzymatique (Al Murad et al. 2020). Nos résultats montrent que les activités enzymatiques de la SOD, CAT, APX, GR et la PPO ainsi que la teneur des molécules antioxydantes non enzymatiques telles que les polyphénols totaux, les flavonoïdes et les anthocyanines est augmentée d'une manière significative chez les plantes traitées par le NaCl et ceci est exacerbée par un apport exogène de la proline et/ou de Si.

Effet de la proline et/ou du Si sur le métabolisme de la proline et le transport de Si chez *M. truncatula* sous contraintes salines

Nos résultats montrent que l'ajout de 20 mM de proline ou 3 mM de Si dans la solution nutritive des plantes stressées améliore significativement la biomasse, la hauteur des plantes, le nombre de feuilles, et la surface foliaire, avec un effet très significatif observé chez les plantes traitées avec de la proline combinée au Si. Des résultats similaires ont été rapportés chez *Beta vulgaris* sous contrainte hydrique (AlKahtani et al. 2021). La pulvérisation foliaire de 10 mM proline ou 2 mM Si améliore la croissance des plantes et les effets sont plus marqués chez les plantes traitées à la fois par la proline et le Si. De même, chez *P. vulgaris*, la pulvérisation foliaire des plantes par ces deux molécules améliore la tolérance au stress salin et au cadmium (Rady et al. 2019).

L'amélioration de la croissance des plantes en réponse à l'ajout de proline et/ou de Si pourrait être le résultat d'une amélioration de la photosynthèse, la nutrition minérale et hydrique et la

réduction du stress oxydant (Al Mayahi et Fayadh, 2015; Meng et al. 2020; Zhang et al. 2022; de Freitas et al. 2019). L'apport exogène de la proline et/ou de Si pourrait également améliorer la croissance des plantes en conditions de stress hydrique à travers la mise en place d'un ajustement osmotique (Hayat et al. 2012 ; Rizwan et al. 2015). Chez *M. truncatula*, Armengaud et al. (2004) ont démontré que la proline est l'acide aminé le plus accumulé en condition de contrainte saline. Les résultats de ce travail indiquent que le traitement des plantes par 120 mM de NaCl induit une accumulation significative de la proline. De plus, le traitement des plantes stressées par 20 mM proline ou 3 mM Si augmente la teneur en proline et cette augmentation est accentuée chez les plantes traitées à la fois par proline et Si. Des résultats similaires ont été rapportés chez *P. vulgaris* au cours d'une contrainte saline (Rady et al. 2018). Les auteurs ont démontré que l'apport combiné de proline et de Si induit une accumulation très élevée de la proline dans les feuilles des plantes stressées en comparaison avec un apport séparé des deux molécules. Pour mieux étudier le rôle de proline et du Si dans le métabolisme de la proline, nous avons évalué l'effet de proline et/ou de Si sur les niveaux de transcripts de *MtP5CS1*, *MtP5CS2*, *MtOAT*, *MtP5CDH* et *MtProDH1* par PCR digitale (ddPCR). La contrainte saline entraine une augmentation des niveaux de transcrits des gènes codant l'OAT et cette augmentation est accentuée chez les plantes stressées et traitées par 20 mM proline et/ou 3 mM Si. De plus, la comparaison des différents traitements révèle qu'un niveau élevé de transcrits *MtOAT* est observé chez les plantes stressées et traitées par la proline combiné avec le Si. Cette accumulation élevée de transcrits *MtOAT* coïncide avec une teneur plus élevée en proline. Cependant, la salinité réduit le niveau de transcrits *MtP5CS1*, que ce soit en présence ou en absence de la proline et/ou de Si. De même, seule la proline exogène induit une augmentation des transcrits *MtP5CS2* en présence et en absence de NaCl. Nos résultats sont en accord avec ceux d'Armengaud et al. (2004). Ces auteurs ont démontré que la salinité induit une forte accumulation de transcrits *MtOAT* et *MtP5CS2* chez *M. truncatula*, et cette accumulation est corrélée positivement avec la teneur des plantes en proline. Selon AbdElgawad et al. (2015), la biosynthèse de la proline chez les légumineuses se fait principalement *via* la voie de l'ornithine, en lien avec le statut azoté des plantes. D'autres auteurs ont au contraire indiqué que la voie de l'ornithine ne dépend que du stade de développement des plantes (Schmid et al. 2005; Hayat et al. 2012).

La teneur des plantes en proline dépend aussi de sa dégradation. Les résultats de notre étude montrent que les niveaux de transcrits *MtProDH1* sont augmentés (620%) alors que ceux de *MtP5CDH* sont fortement diminués (93%) lors de la contrainte saline. De plus, les niveaux de transcrits *MtProDH1* sont plus élevés en réponse à la proline seule ou en combinaison avec le

Si, quel que soit la présence ou non de sel dans le milieu. En revanche, les niveaux de transcrits *MtP5CDH* des plantes traitées par la proline restent inférieurs à ceux des plantes témoin même en présence de sel. Des études antérieures ont montré que les transcrits *AtProDH* s'accumulent très rapidement et beaucoup plus que les transcrits *AtP5CDH*, ce qui suggère que cette réponse est impliquée dans la protection des plantes contre un niveau de proline élevé (Deuschle et al. 2001). De même, chez *C. sativus*, Zhu et al. (2020) ont rapporté que les niveaux de transcrits *ProDH* sont augmentés lors du traitement à la proline, en présence ou non du NaCl et de Si, tandis que les transcrits *P5CDH* ne sont pas affectés. Les faibles accumulations de transcrits *P5CDH* en condition de salinité pourrait représenter un mécanisme de tolérance avec P5C servant de régulateur des réponses cellulaires (Deuschle et al. 2001). Pour confirmer ces résultats, Ould Said et al. (2021) ont montré que les plantes de *Trigonella foenum-graecum* issues de graines prétraitées par 7 mM proline présentaient une teneur en P5C plus élevée et celle-ci était positivement corrélée aux activités enzymatiques antioxydantes par rapport à celles issues des graines non prétraitées. Dans la présente étude, l'apport exogène de proline et de Si module l'accumulation de Si dans les parties aériennes et racinaires des plantes. Le traitement des plantes par 3 mM Si réduit la teneur des parties aérienne et racinaire en Si en présence ou non de NaCl. D'autre part, tandis que le traitement au sel réduit considérablement la teneur en Si dans les racines, la teneur en Si est significativement augmentée dans les parties aériennes et cette augmentation est plus marquée chez les plantes sous contraintes salines lorsqu'elles sont traitées avec la proline combinée au Si. La différence de teneur en Si en fonction des traitements appliqués pourrait être due aux transporteurs du Si. Le Si est absorbé par les racines des plantes à partir de la solution de sol sous forme d'acide silicique non chargé *via* des transporteurs d'influx codés par *Lsi1* et transporté vers les parties aériennes à travers des transporteurs d'efflux codés par *Lsi2* (Debona et al. 2017; Coskun et al. 2019). Dans notre étude, les niveaux de transcrits *Lsi2* sont fortement augmentés au cours de la contrainte saline. De plus, le traitement des plantes stressées par le sel en présence de proline et/ou du Si augmente considérablement le niveau de transcrit *Lsi2* et cette augmentation est plus marquée chez les plantes stressées et traitées à la fois avec de la proline et du Si, ce qui coïncide avec une teneur des parties aériennes en Si plus élevée. Il est à mentionner, qu'à notre connaissance, aucune étude ne s'est intéressée à l'effet de la proline exogène sur l'expression de *Lsi2*. De plus, il n'y a pas de consensus sur la modulation de l'expression du gène *Lsi2* en réponse à l'ajout de Si. Des résultats contradictoires ont été rapportés même au sein d'une même espèce (Coskun et al. 2021). Chez *O. sativa* par exemple, alors que l'expression de *OsLsi2* est induite après un jour de traitement au Si (Kim et al. 2014), Ma et al. (2007) ont montré qu'une diminution de niveau

de transcrits *Lsi2* est observée après 3 jours de traitement avec le Si. Chez *Z. mays* et *H. vulgare*, un traitement au Si inhibe l'expression de *Lsi2* (Mitani et al. 2009), tandis que chez *C. sativus* les niveaux de transcrits *Lsi2* sont induits en réponse à l'ajout de Si dans le milieu (Sun et al. 2018).

CONCLUSIONS GENERALES et PERSPECTIVES

La contrainte saline engendre des effets négatifs plus ou moins marqués selon les variétés de luzerne étudiées et leur stade de développement. Au stade germination, le sel significativement réduit le taux de germination des graines, la viabilité des embryons, la mobilisation des réserves glucidiques et protéiques et la croissance des jeunes plantules. La salinité induit aussi un stress oxydant marqué par des valeurs élevées en MDA, H_2O_2 et une perte d'électrolytes chez de jeunes plantules de luzerne. En revanche, le traitement des graines avec 3 mM Si améliore la tolérance des variétés étudiées à la contrainte saline *via* l'augmentation du taux de germination des graines, la viabilité de l'embryon et la croissance des jeunes plantules. Le Si améliore aussi la stabilité membranaire, réduit les teneurs en MDA et induit les activités SOD et CAT sous contrainte saline. La comparaison entre les variétés de luzerne ciblées montre que la variété européenne *NS Med* est la plus sensible à la contrainte saline, tandis que la variété marocaine *OL* est la moins affectée.

En conditions contrôlées, la contrainte saline (120 et 200 mM NaCl) affecte la croissance, la nodulation, la photosynthèse, l'intégrité membranaire et la teneur en N chez toutes les variétés de luzerne testées. Nous avons noté que la variété marocaine *OL* maintient une croissance et une nodulation élevées sous conditions de stress par rapport à la variété européenne *NS Med*. Le traitement des plantes avec de la proline ou du Si est bénéfique pour la croissance, la nodulation, la photosynthèse, le métabolisme oxydatif et la nutrition minérale chez toutes les variétés de luzerne étudiées, en particulier sous conditions saline (120 et 200 mM NaCl). De plus, la comparaison de l'effet des différents traitements montre que l'apport séparé de proline et de Si a des effets très bénéfiques par rapport à un apport combiné.

Chez la plante modèle *M. truncatula*, la contrainte saline (120 mM NaCl) réduit tous les indices de croissance notamment la biomasse sèche des parties aérienne et racinaire, la hauteur des plantes, le nombre de feuilles et la surface foliaire. Cependant le traitement des plantes sous contraintes salines et traitées avec 20 mM proline ou 3 mM Si améliore tous les indices de croissance et l'effet améliorateur est très significatif lorsque la proline et le Si sont appliqués conjointement. Les analyses moléculaires obtenus dans cette étude démontrent que la tolérance

des luzernes à la contrainte saline est corrélée avec l'augmentation de transcrits de gènes impliqués dans le métabolisme de la proline. L'apport exogène de proline et de Si induit également l'expression des gènes impliqués dans le transport du Si et la dehydrine 2 chez les plantes traitées par NaCl.

A la lumière des résultats obtenus, nous avons identifié des aspects de recherche qui assureront la continuité de ces travaux entamés dans le cadre du projet PHC-Maghreb.

Dans le chapitre 3, nous avons démontré que l'apport exogène de Si améliore le nombre de nodosités et la teneur en azote des plantes de luzerne sous contrainte saline. Compte tenu de ces résultats et pour mieux comprendre les effets de la proline et de Si sur la fixation biologique de l'azote atmosphérique dans la symbiose *M. sativa-E. meliloti*, nous comptons continuer le travail sur les effets de ces deux molécules sur l'activité de la nitrogénase ainsi que sur la teneur des nodules en leghémoglobine au cours de contraintes salines.

Dans les résultats présentés et discutés dans le chapitre 6, nous avons observé que l'apport exogène de proline et de Si améliore la tolérance des plantes de *M. truncatula* aux contraintes salines *via* la modulation de l'expression de gènes codant des enzymes du métabolisme de la proline. Ainsi pour une meilleure compréhension des effets mécanistiques induits par la proline et le Si chez la luzerne exposée à la contrainte saline, nous envisageons de continuer les travaux de recherche concernant l'effet de ces deux molécules sur l'expression d'autres gènes liés à la tolérance à la contrainte saline notamment les gènes *Salt Overlay Sensitive 1* (*SOS1*) et *High affinity* K^+ *Transporter* (*HKT*). Etant donné que l'accumulation du Si par les plantes dépend de la présence des transporteurs spécifiques, la capacité des plantes de luzerne à accumuler le Si reste à explorer en s'intéressant à d'autres transporteurs du Si notamment Lsi1 et Lsi6.

Une approche de transcriptomique serait intéressante pour identifier de nouveaux gènes dont l'expression est modulée par le Si et ou la proline afin de mieux comprendre leur rôle dans la mise en place d'une meilleure tolérance à la contrainte chez *Medicago*.

Nous envisageons également de tester l'effet de la proline et du Si sur la tolérance de l'interaction *M. sativa-E. meliloti* aux contraintes salines dans des conditions naturelles en utilisant des sols agricoles marocains affectés par la salinité.

L'apport exogène de proline et/ou du Si présente un intérêt évident pour le développement d'une agriculture durable. Nos travaux montrent que le développement de biostimulants à base de proline ou Si est fortement recommandé pour améliorer la productivité des cultures sous contraintes environnementales.

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