UNIVERSITE IBN ZOHR
FACULTE DES SCIENCES
AGADIR

جامعة ابن زهر
كليـــــة العلـــــوم
أكاديـر

Centre des Etudes Doctorales Ibn Zohr

Formation doctorale : Sciences de la vie et ressources naturelles

THESE

Présentée par
Idriss TALIBI

Pour l'obtention du grade de
Docteur de l'Université Ibn Zohr

Spécialité : **Phytopathologie**

Recherche de moyens alternatifs aux fongicides de synthèse pour le contrôle de la pourriture amère des agrumes

Soutenue le 08 janvier 2013
Devant la commission d'examen composée de :

Mr. M.A. SERGHINI, Professeur, faculté des sciences, Agadir	**Président**
Mr. M. ACHOURI, Professeur, I.A.V Hassan II, Complexe Horticole, Agadir	**Rapporteur**
Mr. A. OUHAMMOU, Professeur, faculté des sciences Semlalia, Marrakech	**Rapporteur**
Mr. A. CHIHAB EDDINE, Professeur, faculté des sciences, Agadir	**Rapporteur**
Mme. A. AIT ALLA Professeur, faculté des sciences, Agadir	**Examinatrice**
Mr. E.H. BOUDYACH, Professeur, faculté des sciences, Agadir	**Directeur de thèse**
Mr. H. BOUBAKER, Professeur, faculté des sciences, Agadir	**Directeur de thèse**
Mr. M. NOUBAA, Directeur de la station de conditionnement de la coopérative M'BROUKA	**Membre invité**

Remerciements

J’ai eu la chance et l’honneur de travailler durant ces années de doctorat au sien de l’équipe de phytopathologie et lutte intégrée, Laboratoire des Biotechnologies et Valorisation des Ressources naturelles (LBVRN). Je tiens, en tout premier lieu à remercier chaleureusement mes directeurs de thèse, le Professeur El Hassan BOUDYACH, le Professeur Hassan BOUBAKER et le Professeur Abdellah AIT BEN AOUMAR pour leur confiance, leur patience, leur soutien ainsi que pour les conseils qu’ils m’ont prodigués tout au long de cette thèse. J’apprécie beaucoup leurs compétences à la fois professionnelles et humaines. Je tiens aussi à vous remercier de m’avoir supporté et pour l’autonomie que vous m’avez octroyée durant toutes ces années.

J’exprime ma gratitude au Professeur Fouad MSANDA et au Professeur Baha SAADI pour leur contribution dans la récolte des échantillons de plantes et pour le partage de leurs connaissances sur l’identification des plantes utilisées dans cette étude.

Je remercie également le Professeur Abdelhamid EL MOUSADIK responsable du laboratoire LBVRN pour sa disponibilité, ses remarques, ses conseils et ses encouragements.

Merci également au Professeur Fatima HAMADI et Docteur Soumaya EL ABED pour leur collaboration dans la partie analyse phytochimique des fractions de plantes et pour leur disponibilité.

Je remercie pareillement le Professeur Lalla Amina IDRISSI HASSANI, Directrice du centre des études doctorales Ibn Zohr, pour sa disposition, ses conseils et pour la qualité et la richesse des formations dont elle nous a fait profiter.

Je remercie Le Professeur Mohamed Amine SERGHINI, Professeur à la faculté des sciences d’Agadir de m’avoir fait l’honneur de présider le jury de cette thèse. Mes remerciements vont également à Monsieur Ahmed OUHAMMOU, Professeur à la faculté des sciences Semlalia Marrakech, et Monsieur Mohamed ACHOURI, Professeur à l’institut agronomique et vétérinaire (IAV) Complexe Horticole d’Agadir et Monsieur Abderrahim CHIHAB EDDINE, Professeur à la faculté des sciences Agadir et Madame Aicha AIT ALLA Professeur à la faculté des sciences d’Agadir d’avoir accepté de juger ce travail malgré leurs nombreuses sollicitations. C’est un très grand honneur d’avoir fait votre connaissance et de pouvoir vous soumettre mes travaux de recherche.

Je remercie également Monsieur Mohamed NOUBAA, Directeur de la station de conditionnement de la coopérative agricole M’brouka pour la fourniture des fruits utilisés dans ce travail.

Mes remerciements aussi à tous les professeurs que j'ai côtoyé au cours de ces années pour leur amitié et leur gentillesse.

Merci également au Professeur Naima TAQARORT qui a contribué à mon initiation au travail au laboratoire, qu’elle trouve ici toute ma reconnaissance.

Un remerciement spécial à mes amis et collègues Latifa, Aissam, Rachida, Redouan, Hicham, Fayza, Nadia, Hasna, Meryem, Naima, Fadma pour leur soutien et leur amitié ce qui a rendu cette expérience plus agréable.

Je tiens enfin à exprimer toute ma gratitude à ma famille et mes meilleurs amis dont le soutien a été essentiel tout au long de mes études, et tout particulièrement au cours de ce travail. A mes parents, qui me sont très chers.

Merci beaucoup

Je dédie cette thèse

À mes très chers parents,
À mes frères et soeurs, Naima, Touda, Ismail, Rachida, Rachid, Essaid, Leila et Aissa
À tous mes amis

À la mémoire de Sana HADDI

AVANT PROPOS

Ce travail de thèse a été réalisé, dans le cadre des formations doctorales organisées par le centre des études doctorales Ibn Zohr, entre mars 2009 et mars 2012, dans le laboratoire des biotechnologies et valorisation des ressources naturelles (LBVRN), faculté des sciences, Université Ibn Zohr, sous la direction de Messieurs les Professeurs El Hassan BOUDYACH et Hassan BOUBAKER. Il fait parti des recherches menées par l'équipe de phytopathologie et lutte intégrée (EPLI), et qui ont pour point commun le développement de méthodes biologiques de lutte, alternatives à la lutte chimique, permettant de mieux contrecarrer le développement des maladies des plantes tout en réduisant impact sur l'environnement. Ces recherches tournent principalement autour de deux thématiques qui sont le fer de lance de notre laboratoire (LBVRN), et se rapportent à deux cultures très importantes dans la région du Souss-Massa-Draa (SMD), en particulier, et dans le Maroc en général, à savoir : les agrumes et la tomate.

Les chapitres 1, 2, 3, 4 et 5 ont été rédigés en anglais et présentés sous forme d'articles. Les chapitres 2, 3 et 5 ont été publiés, respectivement, dans les revues *Crop Protection*, *Letters in Applied Microbiology* et *Plant Pathology Journal*. Pour sa part, le chapitre 1 a été soumis pour publication dans la revue *Plant Pathology Journal*. Le chapitre 4 sera soumis pour publication une fois les analyses terminées. Certains aspects de la présente recherche ont été, aussi, présentés dans des congrès internationaux sous forme de communications orales ou affichées.

Publications dans des revues internationales :

Talibi, I., Askarne, L., Boubaker, H., Boudyach E.H., Ait Ben Aoumar A. **2011**. *In vitro* and *in vivo* Screening of organic and inorganic salts to control postharvest citrus sour rot caused by *Geotrichum candidum*. ***Plant Pathology journal*** 10, 138-145.

Talibi, I., Askarne, L., Boubaker, H., Boudyach, E., Msanda, F., Saadi, B., Ait Ben Aoumar, A. **2012**. Antifungal activity of some Moroccan plants against *Geotrichum candidum*, the causal agent of postharvest citrus sour rot. ***Crop Protection*** 35, 41-46.

Talibi, I., Askarne, L., Boubaker, H., Boudyach, E.H., Msanda, F., Saadi, B., Ait Ben Aoumar, A. **2012**. Antifungal activity of Moroccan medicinal plants against citrus sour rot agent *Geotrichum candidum*. ***Letters in Applied Microbiology*** 55, 155-161.

Talibi, I., Boubaker, H., Boudyach, Ait Ben Aoumar, A. Alternative methods for the control of postharvest citrus diseases. Plant Pathology Journal (2013) submitted.

Communications dans des congrés :

Talibi I., Askarne L., Boubaker H., Boudyach E. H., and Ait Ben Oumar, A., *In vitro* and *in vivo* antifungal activity of eight medicinal plants against citrus sour rot agent *Geotrichum candidum.* The international Citrus Congress (ICC 2012), Valence, Espagne. (Communication affichée).

Talibi I., Askarne L., Boubaker H., Boudyach E. H., and Ait Ben Oumar, A., Effect of organic and inorganic salts as alternative strategie for the control of postharvest citrus sour rot agent *Geotrichum candidum.* The international Citrus Congress (ICC 2012), Valence, Espagne. (Communication affichée).

Talibi, I., Askarne, L., Boubaker, H., Boudyach, E.H., Msanda, F., Saadi, B., Ait Ben Aoumar, A., Antifungal activity of solvent extracts of some Moroccan aromatic and medicinal plants against *Geotrichum candidum*, the causal agent of citrus sour rot. Rabat, 22-23 Juin 2012, Journées Internationales sur les Substances Naturelles et Développement Durable (Communication orale)

Talibi, I., Askarne, L., Boubaker, H., Boudyach, E.H., Msanda, F., Saadi, B., Ait Ben Aoumar, A., Biological control of postharvest citrus sour rot by solvent extracts of some Moroccan aromatic and medicinal plants. The 3rd International Workshop on Industrial Biotechnology, 23-24 Avril 2012, Sfax-Tunisie (Communication orale)

Talibi, I., Askarne, L., Boubaker, H., Msanda, F., Ait Ben Aoumar, A., et Boudyach, E.H., Phytochemical analysis and antifungal activity of extracts from selected south Moroccan plants against *Geotrichum candidum*, 12-13 Mai 2011, Mohammedia, 4ème édition du Symposium international sur les plantes aromatiques et médicinales (Communication affichée).

Talibi, I., Askarne, L., Boubaker, H., Boudyach, E.H., Msanda, F., Saadi, B., Ait Ben Aoumar, A., Etude de l'activité antifongique de certaines plantes médicinales et aromatiques du sud marocain sur l'agent de la pourriture amère des agrumes en post-récolte. 14-16 Octobre 2010, Congrès international sur les plantes aromatiques et médicinales, Fès (Communication orale)

Talibi, I., Askarne, L., Boubaker, H., Boudyach, E.H., Msanda, F., Ait Ben Aoumar, A., Evaluation de l'activité antifongique des extraits aqueux de certaines plantes du sud Marocain contre *Geotrichum candidum*, agent de la pourriture amère des agrumes en post-récolte. 25-26 Décembre 2009, Workshop de biotechnologies au service du développement durable, Béni Mellal (Communication affichée).

CONTENTS

Chapter two : Screening of the antifungal activity of forty three plant extracts for the control of *Geotrichum candidum*

Chapter Three : Evaluation of the effectiveness of organics fractions of the most active plants species, against *G. candidum*

Chapter four : Bioguided fractionation

Chapter five : Screening of organic and inorganic salts against citrus sour rot agent

List of abreviations

%	Percentage
°C	Degree Celsius
CHL	Chloroform
DCM	Dichloromethan
Et. Ac	Ethyle Acetat
EDTA	Ethylenediaminetetraacetic Acid
µg	Microgram
FAO	Food And Agriculture Organization
µL	Microliter
µm	Micrometer
g	Gram
GI	Germination Inhibition
GAE	Gallic Acid Equivalent
GRAS	Generally Recognized As Safe
HEX	Hexane
h	Hour
ha	Hectare
l	Liter
MGI	Mycelial Growth Inhibition
MeOH	Methanol
M	Molar
MIC	Minimal Inhibitory Concentration
MFC	Minimum Fungicidal Concentration
mg	Milligram
mL	Millilitre
mm	Millimeter
mM	Millimolar
nm	Nanometre
NYDA	Nutrient Yeast Dextrose Agar
NYDB	Nutrient Yeast Dextrose Broth
PDA	Potato Dextrose Agar
RH	Relative Humidity
SMD	Souss Massa Draa
spp.	Several Species
t	Ton
TLC	Thin Layer Chromatography
TPC	Total Phenolic Content
UV	Ultra-Violet
v/v	Volume Per Volume
w/v	Weight Per Volume

List of figures

List of tables

Abstract

The objective of this thesis was to evaluate the antifungal potential of 43 plant extracts and 34 organic and inorganic salts in order to control *Geotrichum candidum*, the causal agent of citrus sour rot.

The plant species tested are harvested in different regions in the Souss-Massa-Draa Region, southern Morocco between 2008 and 2009. Aqueous extracts of these plants are screened both *in vitro* and *in vivo* for their antifungal activity against *G. candidum*. Results showed that among 43 plant species tested, *Cistus villosus, Halimium antiatlanticum, H. umbellatum, Inula viscosa, Anvillea radiata, Pistacia atlantica, Rubus ulmifolius* and *Ceratonia siliqua* were the most active against *G. candidum*. The eight selected plants are then subjected to successive fractionation with organic solvents of increasing polarity (hexane, chloroform, ethyl acetate and methanol). Results showed that methanol extract of *C. villosus* has completely controlled sour rot development. Moreover, the disease incidence was lowered to 3.33 and 11.66% when fruit were treated with methanol extracts of *C. siliqua* and *H. umbellatum*, respectively. Methanol extracts of *C. villosus* and *H. umbellatum* are then subjected to further fractionation using the bioguided fractionation method. The subfractions F.7 from *C. villosus* and F.6 from *H. umbellatum* are the most active against *G. candidum* by inhibiting the mycelial growth of the pathogen with 40.33 and 27 mm diameter.

The assessment of the antifungal potential of 34 salts for the control of sour rot showed that ammonium carbonate and EDTA recorded the lowest minimum inhibitory concentration of 0.1% (w/v) followed by boric acid, sodium carbonate and sodium metabisulfite with MIC of 0.25%. Also of interest, EDTA, boric acid, sodium metabisulfite, sodium carbonate, sodium sulfate and sodium thiosulfate have completely inhibited the arthrospores germination of *G. candidum* at 75 mM. On citrus fruit, sodium salicylate, boric acid and EDTA were the most active by reducing the incidence of sour to 25.93% and 38.89%. These findings suggest that *C. villosus*, *C. siliqua* and *H. umbellatum* plants as well as sodium salicylate, boric acid and EDTA may be useful and effective agents for control of citrus sour rot. Such products therefore represent a promising alternative to the use of synthetic fungicides.

Key words : *Geotrichum candidum*, Sour rot, Plant extracts, inorganic and organic Salts, Citrus fruit, Postharvest

Résumé

Le but de cette thèse était d'évaluer le potentiel antifongique de 43 plantes et 34 sels organiques et inorganiques pour contrôler la pourriture amère des agrumes, causée par le champignon *Geotrichum candidum*. Les plantes testées ont été récoltées dans différentes régions de la région du Souss-Massa-Draa, au sud du Maroc entre 2008 et 2009. Les extraits aqueux de ces plantes ont été évalués, à la fois *in vitro* et *in vivo,* pour leur activité antifongique contre *G. candidum*. Les résultats ont montré que, parmi les 43 espèces végétales testées, *Cistus villosus*, *Halimium antiatlanticum*, *H. umbellatum*, *Inula viscosa*, *Anvillea radiata*, *Pistacia atlantica*, *Rubus ulmifolius* et *Ceratonia siliqua* étaient les plus actives contre *G. candidum*. Les huit plantes sélectionnées ont été ensuite soumises à un fractionnement successif avec des solvants organiques de polarité croissante (l'hexane, le chloroforme, l'acétate d'éthyle et le méthanol). Les résultats ont montré que l'extrait méthanolique de *C. villosus* a complètement contrôlé le développement de la pourriture amère. En outre, l'incidence de la maladie a été abaissé à 3,33 et 11,66% lorsque les fruits ont été traités par les extraits méthanoliques de *C. siliqua* et *H. umbellatum*, respectivement. Les meilleures extraits ont, par la suite, fait l'objet d'une purification par la méthode du fractionnement bio-guidé. La fraction F.7 de l'extrait méthanolique de *C. villosus* est la plus active *in vitro* contre *G. candidum* en inhibant la croissance mycélienne du champignon avec un diamètre de la zone d'inhibition de 40,33 mm. Concernant *H. umbellatum*, le meilleur degré d'inhibition est obtenu avec la fraction F.6 avec un diamètre de 27 mm. De même, l'évaluation du potentiel antifongique de 34 sels contre *G. candidum*, a révèlé que le carbonate d'ammonium et l'EDTA ont enregistré la plus faible concentration minimale inhibitrice (CMI) qui est de 0,1% (v/v) suivi par le carbonate de sodium et le métabisulfite de sodium avec une CMI de 0,25%. L'effet des meilleurs sels sur la germination des arthrospores a montré que l'EDTA, l'acide borique, le métabisulfite de sodium, le carbonate de sodium, le sulfate de sodium et le thiosulfate de sodium ont complètement inhibé la germination des arthrospores, à la fois à 100 et à 75 mM. Appliqués sur les fruits, le salicylate de sodium, l'acide borique et l'EDTA ont permis de réduire l'incidence de la pourriture amère à 25,93 % et 38,89% contre 100% chez le témoin. Cette étude démontre que les extraits de *C. villosus*, *C. siliqua* et *H. umbellatum* ainsi que le salicylate de sodium, l'acide borique et l'EDTA pourraient être mis à profit pour lutter contre la pourriture amère des agrumes en post-récolte et représentent donc une alternative prometteuse à l'utilisation des fongicides de synthèse.

Mots clés : *Geotrichum candidum*, pourriture amère, Extraits de plantes, Sels, agrumes, Post-récolte.

ملخص:

في إطار البحث عن البدائل الصحية والطبيعية للمبيدات الفطرية الاصطناعية، تمت دراسة تأثير 43 نوعا من النباتات الطبية والعطرية، و 34 نوعا من الأملاح العضوية وغير العضوية على الفطر المسبب للعفن المر عند الحوامض الذي يسببه الفطر المعروف باسم *Geotrichum candidum*. تم جلب النباتات المختبرة في هذه الدراسة من مناطق مختلفة من الجنوب المغربي بين عامي 2008 و 2009، وتم تقيم المستخلصات المائية لهذه النباتات ضد *G.candidum* سواء بالتجارب المختبرية أو الحية. أظهرت النتائج أن من بين 43 نبتة التي تم اختبارها *Cistus : villosus, Halimium antiatlanticum, H. umbellatum, Inula viscosa, Anvillea radiata, Pistacia atlantica, Rubus ulmifolius, Ceratonia siliqua* هي الأكثر نشاطا ضد هذا الفطر. هذه النباتات تم اختبار تأثير مستخلصاتها العضوية على الفطر المسبب للعفن باستعمال مذيبات عضوية (Hexane, Chloroforme, Acétate d'éthyle, Méthanole)، أوضحت النتائج أن مستخلص الميثانول لنبتة *C.villosus* سيطر تمام على تطور العفن، بالإضافة إلى ذلك تم تخفيض معدل الإصابة بالمرض إلى 3.33% و 11.66% عندما تمت معالجة الفاكهة بمستخلصات الميثانول ل *C.siliqua* و *H.umbellatum* على التوالي. و بالمثل أظهرت دراسة تأثير الأملاح العضوية وغير العضوية المختبرة أن Salicylate sodium ; Acide borique ;EDTA حفظوا من حالات العفن بنسبتي 25.93% و 38.89%. أثبتت هذه الدراسة أنه يمكن تسخير مستخلصات *C. villosus* و *C. siliqua* و *H. umbellatum* بالإضافة إلى EDTA; Acide borique للحد من العفن المر للحوامض، هذه الوسائل تعتبر بدائل واعدة لاستخدام المبيدات الفطرية الاصطناعية.

كلمات مفتاح : حوامض امالح، ، مستخلصات النباتات ، عفن مر , *Geotrichum candidum*

INTRODUCTION GÉNÉRALE

Les agrumes comptent parmi les principales cultures fruitières, dont le fruit est largement consommé aussi bien frais que sous forme de jus ou autre formes (conserve, confiture…). L'importance des fruits d'agrumes est attribuée à leur richesse en vitamine C (acide ascorbique), en fibres alimentaires, en composés phénoliques, en oligo-éléments, et pour leur potentiel antioxydant (Gorinstein *et al.*, 2001). Les agrumes sont cultivés dans plus de 137 pays à travers le monde (Ismail and Zhang, 2004). À l'échelle mondiale, la production annuelle en fruit frais est d'environ 120 millions de tonnes. Au Maroc, la culture des agrumes couvre une superficie de 76500 hectares assurant une production annuelle d'environ 1,5 millions de tonnes. Ce tonnage couvre à la fois les besoins du marché national en fruit frais, assure l'exportation d'environ 570.000 t/an et alimente les unités de transformation. Ce secteur génére des effets importants sur l'emploi à travers la création de près de 21 millions de journnées de travaille par an. De ce fait, le secteur des agrumes est classé parmi les plus importants de l'économie nationale. La région du Souss-Massa-Draa vient au premier rang à l'échelle nationale, en assurant environ 50 % de la production et de l'exportation nationale (Anonyme, 2011).

En raison de leur teneur élevée en eau et de leur richesse en élément nutritifs, les fruits d'agrumes sont très susceptibles aux attaques des champignons pathogènes (Eckert and Ogawa, 1985; Tripathi and Dubey, 2004). Au Maroc, comme dans les pays en développement, les pertes pendant la récolte, le transport et le stockage des fruits sont élevées. Elles peuvent aller jusqu'à 50%, ou plus, de la récolte (Eckert and Ogawa, 1985; Wisniewski and Wilson, 1992). Les agrumes, comme toutes les autres cultures, sont affectés par plusieurs maladies cryptogamiques. Ces maladies sont dues à des infections initiées soient avant la récolte (e.g. *Alternaria citri, Colletotrichum gloesporioides, Lasiodiplodia theobromae, Trichoderma viride*) ou après la récolte des fruits (e.g. *Aspergillus niger, Geotrichum candidum, Penicillium spp., Rhizopus stolonifer, Trichoderma viride*) (Sommer, 1982; Eckert and Brown, 1986; Holmes *et al.*, 1994). Cependant, la quasi-totalité des pertes d'origine parasitaire, en post-récolte, sont dues aux champignons suivants : *Geotrichum candidum* (agent de la pourriture amère), *Penicillium digitatum* (agent de la pourriture verte) et *P. italicum* (agent de la pourriture bleue). En effet, plus de 90% des dégâts sur fruits d'agrumes en post-récolte sont dus aux champignons précités (Boubaker, 1993; Holmes *et al.*, 1994).

La pourriture amère, causée par le champignon *Geotrichum candidum* Link ex Pers., est l'une des plus redoutables maladies d'agrumes en post-récolte, notamment pendant la saison des pluies et le déverdissage des fruits. Elle a été rapportée dans la majorité des régions agrumicoles et cause des pertes économiques importantes (Eckert, 1978; Rippon and Morris, 1981; Brown, 1988; Hershenhorn *et al.,* 1992;). Elle atteint toute les variétés d'agrumes à différents degrés d'importance (Boubaker, 1993). L'agent causal de la maladie est un parasite de blessure qui pénètre dans les fruits par les blessures profondes qui peuvent se produire pendant la récolte, le conditionnement ou lors du transport (Brown, 1979; Palou *et al.,* 2008). Le champignon dégrade le fruit en secrétant des enzymes pectinolytiques qui transforment le fruit en une masse molle. Cet aspect favorise la propagation de la maladie par contact entre fruit saint et fruit infecté. L'infection par *G. candidum* dépend de plusieurs facteurs tels que la présence de blessures fraîches et profondes, la turgescence de la peau des fruits, le stade de maturité des fruits et les conditions de l'environnement avec une température optimale qui se situe entre 25 et 30°C et une humidité relative élevée (Brown, 1979; Baudoin and Eckert, 1985; Eckert and Brown, 1988; Cohen *et al.,* 1991; Suprapta *et al.,* 1995).

Etant donné qu'il est plus facile d'augmenter le volume des exportations en protégeant mieux les fruits après leur récolte que de vouloir augmenter la production, cette démarche s'avère moins coûteuse et plus simple à atteindre dans un délai assez court. Dans ce contexte, des traitements chimiques (utilisation des fongicides) et physique (stockage au froid) sont pratiqués dans les stations de conditionnement afin de limiter les pertes dues aux pathogènes de post-récolte (Palou *et al.,* 2008). Néanmoins, ces traitements ont montré peu d'efficacité contre la pourriture amère des agrumes en post-récolte. En effet, le stockage au froid n'a qu'un effet fongistatique ; la maladie se développe d'une manière explosive lorsque le fruit est transféré à la température ambiante. En revanche, les fongicides homologués pour contrôler les maladies d'agrumes en post-récolte (Imazalil, Thiabendazole) n'ont aucun effet sur *G. candidum* (Suprapta *et al.,* 1997; Brown and Miller, 1999; Mercier and Smilanick, 2005; Liu *et al.,* 2009b). La guazatine est le seul fongicide qui peut contrôler efficacement l'agent de la pourriture amère (Brown and Eckert, 1988). Cependant, ce produit n'est pas homologué en traitement de post-récolte dans la plupart des pays agrumicoles. En outre, l'utilisation des fongicides est de plus en plus restreinte en raison de la réglementation qui devient de plus en plus sévère, de leur toxicité, de leur effet sur l'environnement et de l'inquiétude croissante du consommateur à propos des résidus de pesticides dans les fruits (Zhang and Swingle, 2003; Tripathi and Dubey, 2004; Palou *et al.,* 2008)**.** Les mesures

prophylactiques, telles que la minimisation des blessures des fruits, et les mesures d'hygiènes dans les stations de conditionnement restent donc le seul moyen pour contrôler cette maladie.

Il y a donc un besoin évident et croissant de trouver et de mettre en œuvre des méthodes de lutte alternatives à la lutte chimique pour le contrôle de la pourriture amère. L'objectif est d'accomplir un contrôle satisfaisant des pourritures de post-récolte en adoptant des programmes de gestion intégrée des maladies. Au cours des deux dernières décennies, la lutte biologique, par ses méthodes respectueuses de l'environnement et de la santé du consommateur, est apparue comme une stratégie efficace pour lutter contre les maladies d'agrumes en post-récolte. Parmi les différentes approches biologiques, l'utilisation des antagonistes microbiens et des substances naturelles, d'origine végétale ou animale, est très prometteuse et gagne de plus en plus la confiance des consommateurs. En effet, plusieurs travaux ont porté sur la recherche de microorganismes antagonistes des champignons pathogènes des agrumes en post-récolte (Eckert and Ogawa, 1988; Wisniewski and Wilson, 1992 ;Arras *et al.*, 1998; Bull *et al.,* 1998; Droby *et al.,* 2002; Mercier and Smilanick, 2005; Cañamás *et al.,* 2008; Taqarort *et al.,* 2008). Cependant seuls quelques uns de ces antagonistes (*Candida oleophila; Pseudomonas syringae; Cryptococcus albidus)* sont utilisés à l'échelle commerciale (Droby *et al.,* 1998; Palou *et al.,* 2008). Une autre approche de la lutte contre les maladies d'agrumes en post-récolte est l'utilisation de substances naturelles extraites à partir de plantes. Les plantes ont une capacité presque illimitée de synthétiser des substances aromatiques. La plupart sont des métabolites secondaires, dont plus de 10.000 ont été isolés, identifiés et définis pour leurs propriétés antimicrobiennes. Par ailleurs, ces métabolites sont connus par leur caractère systémique, non phytotoxique et biodégradable (Tripathi and Dubey, 2004; Kosalec *et al.,* 2005). En raison de ces propriétés, les extraits de plantes sont très attractifs comme moyen de lutte contre les pathogènes de post-récolte.

Une autre alternative prometteuse est l'utilisation des sels et additifs alimentaires qui sont des composés généralement reconnus comme sains (GRAS). Certains de ces sels sont présents naturellement dans de nombreux fruits et légumes (Foegeding and Busta, 1991; El-Mougy *et al.,* 2008;) et sont, le plus souvent, utilisés dans l'industrie alimentaire comme désinfectants ou pour contrôler le pH, le goût et la texture (Smilanick *et al.,* 1999; Hervieux *et al.*, 2002; Arslan *et al.,* 2009). En outre, ces composés ont un large spectre antimicrobien, une faible toxicité et un coût relativement faible (Corral *et al.*, 1988; Olivier *et al.,* 1998; Hervieux *et al.,* 2002; Deliopoulos *et al.,* 2010).

La plupart des travaux de recherches sur le contrôle des maladies de post-récolte sont relatifs à la lutte contre les pourritures à *Penicillium* spp. En effet, très peu de travaux ont été publiés sur la lutte contre la pourriture amère. L'objectif de cette étude est d'évaluer l'efficacité de deux stratégies alternatives au fongicides de synthése, à savoir : l'utilisation des extraits de plantes et l'utilisation des sels organiques et inorganiques pour contrecarrer le développement de la pourriture amère des agrumes en post-récolte. Pour atteindre cet objectif les points suivants ont été traités :

1) Screening *in vitro* et *in vivo* de l'activité antifongique des extraits aqueux de 43 plantes, récoltées dans différentes régions du sud du Maroc, contre *G. candidum*, l'agent de la pourriture amère des agrumes.
2) Evaluation de l'activité antifongique des fractions organiques des plantes les plus efficaces contre la pourriture amère et étude du mode d'action des meilleures fractions organiques sélectionnées.
3) Evaluation de l'activité antifongique d'une large gamme de sels organiques et inorganiques, aussi bien *in vitro* qu'*in vivo,* contre *G. candidum.*

CHAPTER ONE

Literature review:
Alternative methods for the control of postharvest citrus diseases

Submitted to Plant Pathology Journal (2013) as :

Idriss Talibi, Hassan Boubaker, Abdellah Ait Ben Aoumar, and El Hassane Boudyach.
Healthy alternatives for the control of postharvest diseases of citrus fruit.

Résumé

Les maladies de post-récolte sont à l'origine d'énormes pertes des fruits d'agrumes pendant le stockage et le transport des fruits. Ces maladies sont contrôlées principalement par l'utilisation excessive de fongicides de synthèse. Cependant, l'efficacité variable de ces fongicides, la prolifération des souches résistantes et la préoccupation croissante de la population envers les dangers liés à l'utilisation de ces produits, tels que leurs effets néfastes sur la santé et l'environnement, a nécessité le développement de stratégies alternatives pour le contrôle des maladies d'agrumes en post-récolte. Par conséquent, la lutte biologique, par ses méthodes respectueuses de l'environnement et de la santé du consommateur, constitue une alternative prometteuse à la lutte chimique. L'utilisation des micro-organismes antagonistes, des substances naturelles ou des sels organiques et inorganiques à effet antimicrobien sont les moyens alternatifs les plus appropriés pour remplacer les fongicides de synthèse qui sont soit interdits ou recommandé seulement pour une utilisation limitée. Cependant, l'application de ces alternatives seules ne peuvent pas assurer, toujours, des niveaux de contrôle commercialement acceptable des maladies d'agrumes en post-récolte en comparaison avec ceux obtenus par les fongicides de synthèse. A cet effet, la protection des fruits d'agrumes par la combinaison de différentes méthodes de lutte qui satisfait, à la fois, les exigences écologique et économiques est une bonne solution pour améliorer l'efficacité de la lutte biologique et confronter ces limites. Malgré les caractéristiques distinctives de ces méthodes alternatives, plusieurs raisons empêchent l'utilisation commerciale de ces traitements. Par conséquent, les travaux de recherche doivent prêter plus d'importance à s'assurer que les moyens de lutte alternatives efficaces répondent aux exigences commerciales.

Mots clés : Lutte biologique, Post-récote, Agrumes, Antagonistes, Extraits de plantes, sels, Additifs.

Abstract

The postharvest diseases of citrus fruit cause considerable losses during storage and transportation. These diseases are managed principally by the excessive application of synthetic fungicides. However, the increasing concern for health hazards and environmental pollution due to chemical use has necessitated the development of alternative strategies for the control of postharvest citrus diseases. Management of postharvest diseases by employing microbial antagonists, natural plant-derived products and Generally Recognized As Safe (GRAS) compounds has been demonstrated to be most suitable to replace the synthetic fungicides which are either being banned or recommended for limited use. However, application of these alternatives by itself may not always provide commercially acceptable level of control of postharvest citrus diseases comparable to that obtained with synthetic fungicides. To provide more effective disease control, than that possible with a single approach, a multifaceted approach based on the combination of different postharvest treatments has been adopted. Actually, despite the distinctive features of these alternative methods, several reasons hinder the commercial use of such treatments. Consequently, research should lend more importance to ensure appropriate tools to effective application of these alternatives to commercial requirements.

Key words : Biological control, Post-harvest, Citrus, Microbial antagonists, Plant extracts, Salt additives.

I. Introduction

Citrus is one of the most widely produced fruit. It is grown commercially in more than 137 countries around the world (Ismail and Zhang, 2004). The production of citrus fruit remains amongst the largest crops produced worldwide with almost 115.65 million tons (FAO, 2011). The contribution of the citrus industry to the world economy is enormous and it provides jobs to millions of people around the world in harvesting, handling, transportation, storage, and marketing operations. The importance of citrus fruit is attributed to its diversified use, which is widely consumed either as fresh fruit or as juice. In the world, Brazil is the major citrus fruit producing country followed by the United States of America and China. Citrus production in Morocco is 1.5 million tons from 76 500 ha under this crop (Anonymous, 2011). About 570 000 tons of fresh citrus fruit were exported (Anonymous, 2011).

Due to their higher water content and nutrient composition, citrus fruit are very susceptible to infection by microbial pathogens during the period between harvest and consumption (Eckert and Ogawa, 1985; Tripathi and Dubey, 2003). As citrus fruits have a pH lower than 4, so fungi are often the predominant microorganisms in fruit and fruit products. No bacterial postharvest disease of commercial importance is reported on citrus fruit. Contamination and infection by pathogenic fungi occur at different stages in the field and after harvest during marketing, and usually follows mechanical injury or physiological breakdown of the fruit, which allow entry to these microorganisms. In a few cases, pathogens can infect healthy tissues (Huang *et al.,* 1992). Postharvest decays of fruit can also originate from latent infections occurring in the orchard (Snowdon, 1990).

Losses, during transportation and storage of fresh citrus fruit, are estimated at around 5 and 10% in most developed countries, and from 25 to 30% or more in developing and underdeveloped countries because of the lack of adequate protection measures and storage facilities (Eckert and Ogawa, 1985; Wisniewski and Wilson, 1992). Citrus fruit are susceptible to a number of postharvest diseases that cause significant losses during the postharvest phase**.** Nevertheless, the most common and serious diseases that affect citrus fruit are green and blue molds caused, respectively, by *Penicillium digitatum* Sacc. and *Penicillium italicum* Wehmer, followed by sour rot caused by *Geotrichum candidum* Link ex Pers (Caccioni *et al.,* 1998**;** Palou *et al.,* 2002; Zheng *et al.,* 2005). These pathogens are strict wound pathogens that can infect the fruit in the grove, in the packinghouse, or during subsequent handling and storage (Brown, 1979; Palou *et al.,* 2008). The fungal inoculum is

practically always present on the surface of fruit during the season and after harvest can build up high levels unless appropriate packinghouse sanitization measures are adopted (Kanetis *et al.,* 2007). Fruit infection by these fungi is enhanced during fruit degreening operation, and during wet and rainfall seasons (Eckert, 1978; Eckert and Brown, 1986; Cohen *et al.,* 1991, Liu *et al.,* 2009b). The decays are also more prevalent as fruit increases in maturity, and at favorable temperatures and humidity (Brown, 1979; Baudoin and Eckert, 1985).

Currently, synthetic fungicides are the primary means of controlling post-harvest diseases of citrus fruit, especially by imazalil (IZ), thiabendazole (TBZ), sodium ortho-phenyl phenate (SOPP), fludioxonil (FLU), pyrimethanil or different mixtures of these compounds (Eckert and Ogawa, 1988**;** Holmes and Eckert, 1999; Ismail and Zhang, 2004; Smilanick *et al.,* 2005; Palou *et al.,* 2008). Continuous use of these fungicides has resulted in the appearance of isolates of fungi with multiple fungicide resistances, which further complicate the management of the decays (especially *Penicillium* rots) (Holmes and Eckert, 1999**;** Droby *et al.,* 2002; Mercier and Smilanick, 2005; Boubaker *et al.,* 2009). In addition, theses fungicides are not effective against all important pathogens. Indeed, sour rot is difficult to control with IMZ and TBZ (Eckert, 1978; Brown, 1979; Suprapta *et al.,* 1997; Mercier and Smilanick, 2005;). The synthetic fungicide guazatine is the only commercial product that can control sour rot (Brown, 1988). However, this fungicide is not authorized in several countries. Furthermore, the use of fungicides is increasingly becoming restricted owing to stringent regulation, carcinogenicity, high and acute residual toxicity, long degradation period, environmental pollution and growing public concern about chemical residues in fruit (Tripathi and Dubey, 2003; Zhang and Swingle, 2003; Palou *et al.,* 2008).

Therefore, the challenge is to develop safer and eco-friendly alternative strategies of controlling citrus postharvest diseases, which pose less risk to human health and environment. Recently, several promising biological approaches have been proposed as potential alternatives to synthetic fungicides for the control of postharvest citrus disease. These biological control strategies include : (1) use of antagonistic microorganisms; (2) application of naturally-derived bioactive compounds; and (3) induction of natural resistance. Among these biological approaches, the use of the microbial antagonists, either alone or as part of an integrated disease management policy, is quite promising and gaining popularity among consumers (Eckert and Ogawa, 1988; Wisniewski and Wilson, 1992; Droby *et al.,* 2002). Interestingly, most of the antagonistic microorganisms are isolated from fruit surfaces as epiphytic microbial population. According to Wilson *et al.* (1991), the postharvest

environments present some unique advantages for the use of biocontrol methods. Indeed, the postharvest environment provides the following advantages : (1) the partially controlled environment in storage may result in a shift in the balance of interactions between host, pathogen, and antagonistic microbe in favor of antagonist; (2) the efficacy of antagonist may be enhanced because the biocontrol product can be applied directly onto the site where needed in the harvested product; (3) the harvested commodity may be protected relatively free of potential interfering factors; (4) protection is needed for a relatively short period as compared with period of protection required for field crops; and (5) as the harvested fruits have high market value, use of a relatively high-cost biocontrol product may be justified.

The second approach to disease control is the use of natural plant-derived compounds. Indeed, these compounds have gained popularity and scientific interest for their antibacterial and antifungal activity (Tripathi and Dubey, 2003; Liu *et al.,* 2009b; du Plooy *et al.,* 2009; Gatto *et al.,* 2011; Talibi *et al.,* 2011a; Talibi *et al.,* 2012a and b). Naturally occurring plant products are very attractive as alternative or complementary control means because of their antifungal activity, non-phytotoxicity, systemicity, and biodegradability (Tripathi and Dubey, 2003; Kosalec *et al.,* 2005; Ameziane *et al.,* 2007; Gatto *et al.,* 2011; Talibi *et al.,* 2011a).

However, application of these biological control methods by itself may not always provide commercially acceptable level of control of postharvest citrus diseases. It is possible to increase the efficacy of these methods by combining with other postharvest treatments. Indeed, different disease management strategies have been integrated to provide more effective disease control than that possible with a single approach. Low-toxicity chemicals, particularly common food additives and GRAS (Generally Regarded as Safe) compounds, were evaluated for their efficacy for the control of citrus pathogens. Strictly speaking, GRAS compounds do not fall into the category of organic ingredients, but they are much less harmful than many other inorganic chemicals.

The purpose of this paper is to provide an overview of the published data on the control methods alternative to conventional chemical fungicides for the control of postharvest citrus pathogens.

II. Postharvest citrus diseases

Diseases that occur after harvest can have a significant impact on keeping quality of fresh citrus fruit. Losses from post-harvest diseases caused by various pathogens account for nearly 50 percent of the total wastage in citrus fruits (Ladaniya, 2008). Infection and contamination

occur at different stages in the field, subsequent handling and storage activities. Citrus fruits are susceptible to infection by many fungal pathogens (**Table 1.1**). These fungal pathogens can be subdivided into two categories :

- ✓ The first category includes those pathogens that enter through a natural entry point and possess a long inactive (quiescent) phase after infection, or symptoms may develop shortly after infection. The main fungi belonging to this group are: *Lasiodiplodia theobromae* (stem end rot), *Phomopsis citri* (stem end rot), *Colletotrichum gloesporioïdes* (Antracnose) and *Alternaria alternata* (Black rot) (Boubaker, 1993) (**Table 1.1**).
- ✓ The second category includes wounds pathogens that enter through injuries. In this case, the diseases appear in less than a week after the pathogen's activation upon the release of moisture and nutrients at injury sites of the fruit rind (Eckert and Brown, 1986). These include green and blue mold caused by *Penicillium spp.*, and sour rot caused by *G. candidum*. Over than 90 % of citrus fruit losses are due to the aforementioned fungi (Boubaker, 1993).

Table 1.1 : Major postharvest citrus diseases, Causal agent, Type and site of infection

Disease	**Causal agent**	**Infection site**
Anthracnose	*Colletotrichum gloeosporioides*	Intact or injured rind
Black rot	*alternaria alternata*	Natural openings
Black spot	*Guignardia citricarpa*	Intact or injured rind
Blue mould	*Penicillium italicum*	Injured rind
Brown rot	*Phytophthora spp.*	Intact rind
Greasy spot	*Mycosphaerella citri*	Intact or injured rind
Green mould	*Penicillium digitatum*	Injured rind
Lime anthracnose	*Gloeosporium limetticola*	Intact or injured rind
Sour rot	*Geotrichum candidum*	Injured rind
Stem-end rot	*Lasiodiplodia theobromae*	Button
Stem-end rot	*Phomopsis citri*	Button
Trichoderma rot	*Trichoderma viride*	Injured rind

II.1 *Penicillium* rots

Penicillium digitatum Sacc. and *Penicillium italicum* Wehmer are the two most significant and widely reported postharvest pathogens in citrus (Plaza *et al.,* 2003; Ladaniya, 2008). The rots develop only if there are injuries in the rind. The major menace of these pathogens is due to their spores, which appear as fine powder and are airborne and are

produced on the surfaces of infected fruit. Initially, the decay appears as a soft, watery, slightly discolored spot. After few days, very profuse sporulation can be seen on fruit which is completely covered by white mycelium followed by olive-green and bluish spores of *P. digitatum* and *P. italicum* respectively **(Figure 1.1).** Spores are easily dispersed if the fruits were handled or shaken or if they were exposed to air currents. Although, blue mold develops less rapidly than green mold at ambient temperatures, but it is more harmful because it spreads in the box and healthy fruits are directly attacked, regardless of injury. The development of the *Penicillium* rots is influenced by several factors such as temperature, and relative humidity. Indeed, the optimum conditions for the growth of both fungi are a temperature ranged between 20 and 25° C and high levels of relative humidity (>90%). However, *P. italicum* is more adapted to low temperatures (<10°C) than *P. digitatum* (Eckert and Ogawa, 1985).

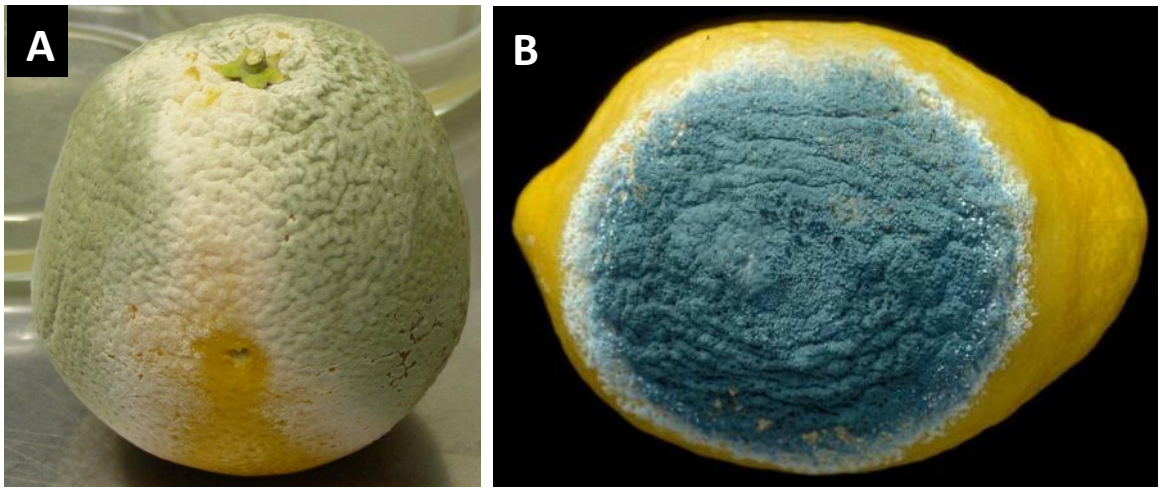


Figure 1.1 : Citrus Green (A) and Blue (B) mold in citrus fruit

II.2 Sour rot

Sour rot is an important postharvest disease of citrus fruit from most areas of the world (Eckert, 1978; Rippon and Morris, 1981; Brown, 1988; Hershenhorn *et al.*, 1992). It is the most rapidly spreading postharvest disease (El Ghaouth *et al.*, 2002). *Geotrichum candidum* Link ex Pers., the causal agent of this rot, is responsible for economically significant losses under favorable conditions for pathogen development, principally during fruit degreening and wet seasons (Eckert and Brown, 1988; Cohen *et al.*, 1991). Also, isolates of *G. candidum* have been found to be pathogenic to the fruits of tomato, orange, lemon, satsuma mandarin, grapefruit, cucumber and carrot (Suprapta *et al.*, 1995; Wells, 1997; Palou *et al.*, 2009).

II.2.1 Causal organism

Sour rot of citrus fruit was first described in 1917 and the pathogen was designated as *Oospora citri-aurantii* (Ferr.) Sacc. et Syd (Butler *et al.*, 1965; Suprapta *et al.*, 1995). Since then, several different names have been used to designate the pathogen such as *G. candidum* Link var. *citri-aurantii* (Ferr.) R. Ciferri. et F. Ciferri (Butler *et al.*, 1965) and *G. citri-aurantii* (Ferr.) Butler (asexual form) and *Galactomyces citri-aurantii* Butler (sexual form) (Butler *et al.*, 1988). However, *G. candidum* Link ex Pers is commonly the most cited to indicate the citrus sour rot pathogen (Brown, 1979; Rippon and Morris, 1981; Kitagawa and Kawada, 1984; Baudoin and Eckert, 1985; Mercier and Smilanick, 2005; Smilanick *et al.*, 2008). *G. candidum* is a filamentous fungus which grows rapidly on potato dextrose agar (PDA), producing a dull gray-white colony with chains of arthrospores. These arthrospores are produced by the fragmentation of undifferentiated hyphae. The arthroconidia, which are quite variable in size, may germinate and develop into a septate mycelium **(Figure 1.2)**.

Figure 1.2: Physiology of *Geotrichum candidum :* a) Aspect on PDA medium and b) Chains of arthrospores appearing dull gray white colony

II.2.2 Disease cycle and epidemiology

G. candidum is a common inhabitant of citrus soils and is more prevalent on lower fruit of the tree canopy and it is windborne from soil particles to surfaces of fruit (Brown, 1979; Suprapta *et al.*, 1996). The fungus can also accumulate in contaminate pallets, washer brushes, belts, conveyors, and with dirt and debris in drenchers and soak tanks. The organism is a wound pathogen requiring injury into the albedo for entry (Brown, 1979). These injuries

may be caused by insects or mechanical means, such as thorn or stem punctures, or by plugging at harvest. Fruit are more susceptible to sour rot as they become more mature, and if they contain high amounts of rind moisture (Brown and Eckert, 1988; Suprapta *et al.*, 1996). Also, the fungus develops most rapidly at ambient temperatures of 25–30ºC. Thus, the aggressiveness of the fungus increases especially during fruit degreening, wet and rainfall seasons (Eckert, 1978; Eckert and Brown, 1988; Cohen *et al.*, 1991; Liu *et al.*, 2009b). In the packing house, the sour odor, characteristic of sour rot, attracts fruit flies, which can disseminate the fungus to other injured fruit (Brown and Eckert, 1988).

II.2.3 Symptoms

Citrus sour rot infection has the most unpleasant smell of all decays known (Boubaker, 1993; Sissay, 2007). The initial symptoms of sour rot infections are similar to those of *Penicillium* rots. The lesion first appears water-soaked, light to dark yellow, and slightly raised (**Figure 1.3**). The fruit becomes a soft, stinking, semi-solid mass as the fungi secrete an active extracellular enzyme named polygalacturonase that rapidly degrade the tissue (Cohen *et al.*, 1991). Therefore, the fungus degrades the fruit thoroughly, causing it to disintegrate into a slimy and watery mass. At high relative humidity, the lesions may be covered with a yeasty, sometimes wrinkled layer of withe or cream-coloured mycelium (Baudoin and Eckert, 1985). Sour rot is often associated with green mold and is stimulated by its presence (Morris, 1982) (**Figure 1.3**).

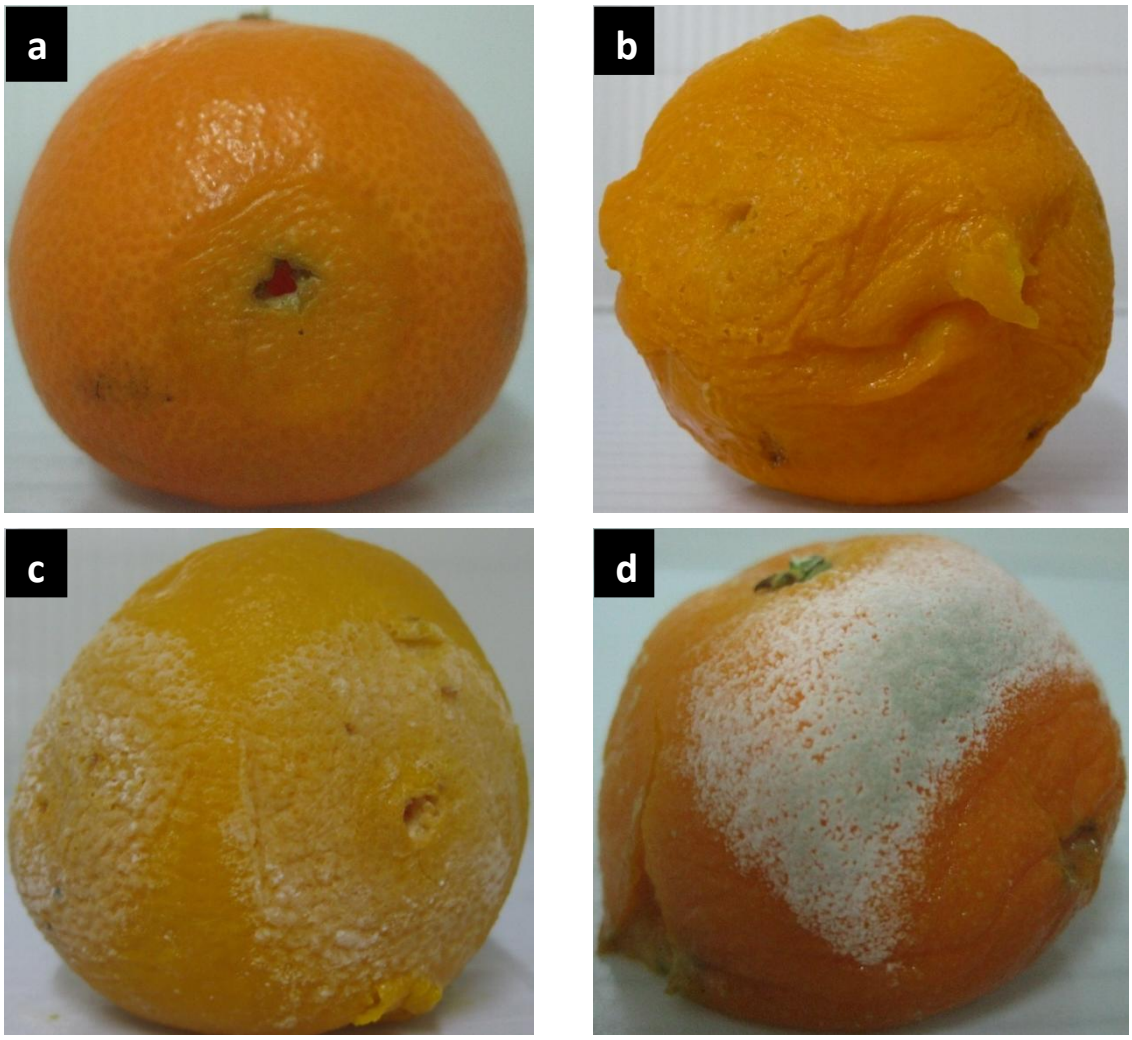


Figure 1.3 : Sour rot infection caused by *G. candidum.* a) Initial infection, b) disintegrated fruit, c) white layer of mycelium appeared at high relative humidity and d) synergism between sour rot and green mold.

III. Postharvest fungicides treatments

The use of chemicals in citrus postharvest disease management has a history going back 100 years (Ladaniya, 2008). Presently, the chemical control of various rots is the integral part of the postharvest handling system in citrus. The use of fungicides to control postharvest citrus diseases can be carried out pre or post harvestly. Field use of fungicides for the control of postharvest diseases is restricted to applications of copper or benomyl **(Table 1.2).** For the postharvest application, several fungicides are used like Sodium orthophenylphenate (SOPP), Thiabendazole, biphenyl, benomyl, thiophanate methyl, carbendazim, Imazalil and Guazatine (Eckert and Sommer, 1967; Eckert and Ogawa, 1985) **(Table 1.2)**. Some of these chemicals

are still being used (Imazalil, Thiabendazole) and others are not longer registered in many countries (Guazatine). These chemicals are used in various forms : in soak tanks or as water sprays, as fumigants or in wax, or impregnated in paper for keeping in fruit layers or as wraps. Nevertheless, the control of wounds pathogens with these fungicides depends on their prompt application at required concentrations. If the application of fungicide is delayed, the pathogen gets established, so the pathogen can escape the protective action of fungicide (Ladaniya, 2008). In addition, all fungicides are not effective against all important pathogens and there are problems related to use of these fungicides. For example, sour rot is not controlled, efficiently, by any postharvest chemical treatments except Guazatine (Brown, 1988), a chemical that is not authorized in several countries. Also, the intensive and continuous use of these fungicides has created serious problems of the emergence of resistant pathogens (El-Goorani *et al.*, 1984; Eckert, 1990; Holmes and Eckert, 1999; Kinay *et al.*, 2007; Sánchez-Torres and Tuset, 2011). Thus, all these problems require the screening of new antifungal compounds.

Table 1.2 : Majors fungicides for control of citrus postharvest diseases

Treatments	Diseases
Preharvest	
Copper	Brown rot
Benomyl	Diplodia and Phomopsis SER; Penicillium rots; Anthracnose
Postharvest	
SOPP	Green mold; Sour rot
Thiabendazol	Diplodia and Phomopsis SER; Penicillium rots; Anthracnose
Imazalil	Penicillium rots; Diplodia, Phomopsis and Alternaria SER
Diphenyl	Penicillium rots
Guazatine	Penicillium rots, Sour rot

SER : Stem end rot

IV. Integrated strategies to control postharvest citrus diseases

IV.1 Antagonistic microorganisms as biocontrol agents

It has been demonstrated that there are natural microbial antagonists on fruit surface that can suppress disease development (Wilson and Wisniewski, 1989). The use of antagonistic microorganisms for controlling the postharvest diseases of citrus fruit is based on two approaches : a) the use of natural epiphytic antagonists that already exists on fruit surface and b) the artificial introduction of selective microbial antagonists that control postharvest diseases.

IV.1.1 Naturally occurring antagonists

One of the approaches to the isolation of antagonistic microorganisms for controlling postharvest diseases is through the promotion and management of natural epiphytic microflora, already present on fruit surface. The importance of naturally occurring microbial antagonists is revealed when washed fruits develop more rot than unwashed ones (Wilson and Wisniewski, 1989). Chalutz and Wilson (1990) found that washed, dried and stored citrus fruit get infected much more rapidly than unwashed fruit. This proves that the resident epiphytic microflora on the citrus fruit is capable of controlling citrus diseases. Several antagonistic microorganisms, found to be effective to control citrus diseases in postharvest phase, are isolated from the surface of citrus fruit (Wilson and Chalutz, 1989; Borras and Aguilar, 1990; Chalutz and Wilson, 1990; Droby *et al.,* 1999a; Taqarort *et al.,* 2008; Abraham *et al.,* 2010; Osman *et al.,* 2011). According to their origin, the naturally occurring antagonists are more apt to gain public acceptance.

IV.1.2 Artificially introduced microbial antagonists

Several microbial antagonists have been identified and artificially introduced on citrus fruit to control the postharvest diseases. Artificial introduction of microbial antagonists is more effective in controlling postharvest diseases of fruits than other means of biological control (Sharma *et al.,* 2009). This success is due to the possibility of controlling the environmental conditions in the postharvest phase and to direct application of biocontrol agents toward effective sites. Currently, available microbial antagonists were developed for the control of decays originating mainly from active infection of fruit wounds and not from latent infections. Postharvest citrus diseases are controlled by a broad spectrum of microorganisms such as yeasts, bacteria and fungi **(Table 1.3)**.

IV.1.2.1 Yeasts as biocontrol agents

Treatment of citrus fruit by antagonistic yeasts is the most means used as alternative to control postharvest diseases. This importance is attributed to several positive points making yeasts recommended as potential microbial agents for controlling the postharvest diseases of fruit. First, yeasts can colonize the surface of fruit for long period even under dry conditions (Janisiewicz, 1987). Second, yeasts produce extra-cellular polysaccharides, which enhance their survivability and restrict the growth of pathogen propagules. Third, yeasts can use

nutrients rapidly and proliferate at a faster rate (Sharma *et al.,* 2009). Effective control of citrus fruit decay was observed with yeasts such as *Candida guilliermondii* (syn. : *Pichia guilliermondii*, *Debaryomyces hansenii*), *Pichia anomala*, *Candida saitoana*, *Candida famata*, *Candida oleophila*, *Candida sake*, *Aureobasidium pullulans* and *Kloeckera apiculata* (Wilson and Chalutz, 1989; Chalutz and Wilson, 1990; Arras, 1996; Arras *et al.*, 1998; El-Ghaouth *et al.,* 2000; Droby *et al.,* 2002; Lahlali *et al.,* 2004; Taqarort *et al.,* 2008) **(Table 1.3)**. Actually, at least three products based on antagonistic yeasts are commercially available under the trade names Aspire (Ecogen Inc., USA, limited to the USA), Yield Plus (Anchor Yeast, Cape Town, South Africa) and Shemer (AgroGreen Ashdod, registred in Palestine) (Droby *et al*., 2002). A few others are at different stages of commercial development and expected to reach the marketplace within future years. However, application of these biocontrol products alone did not provide commercially acceptable control of fruit diseases. The biocontrol ability of these antagonists could be enhanced by manipulation of the environment, using mixtures of beneficial organisms, physiological and genetic enhancement of the biocontrol mechanisms and integration of biocontrol with other methods such as low doses of fungicides and controlled atmosphere storage (Spotts *et al.,* 2002).

IV.1.2.2 Bacteria as biocontrol agents

Use of bacteria for postharvest diseases control of citrus has focused on their application as biofungicides. Endophytic bacteria are ubiquitous in most plant species and can be isolated from surface plant tissues, soils, roots, and the rhizosphere of various plants (Lai *et al.,* 2012). Moreover, bacteria as biocontrol agents have become of interest because of their rhizosphere competence and their ability to colonize internal tissues of plants and thus, represent an internal defense line against pathogens (Liu *et al.,* 2009a). Antagonistic bacteria are well known by their production of substances with antifungal and antibacterial properties (Smilanick and Denis-Arrue, 1992; Leelasuphakul *et al.,* 2008; Lucon *et al.,* 2010; Yanez-Mendizabal *et al.,* 2011). Significant advances in control of postharvest citrus diseases have been achieved in research by the use of bacterial antagonists such as *Pseudomonas syringae*, *P. fluorescens*, *Burkolderia* (*Pseudomonas*) *cepacia, Bacillus subtilis, B. thuringiensis*, *Pantoea agglomerans*, *Enterobacter cloacae* and *Serratia polymuthica* (Singh and Deverall, 1984; Wilson *et al.,* 1987; Smilanick and Denis-Arrue, 1992; Bull *et al.,* 1998; Meziane *et al.,* 2006; Canamas *et al.,* 2008; Lucon *et al.,* 2010) **(Table 1.3)**. However, only one species of bacteria have been mass-produced and commercialized, under the trade name BioSave 100 and 110 based on a strain of *Pseudomonas syringae,* to control postharvest citrus pathogens

Although, antibiosis might be an effective tool for controlling postharvest diseases, the importance is being given for the development of non-antibiotic producing microbial antagonists for the control of postharvest diseases of fruits (El-Ghaouth *et al.*, 2004).

IV.1.2.3 Fungi as biocontrol agents

Biological control of postharvest citrus diseases by fungal antagonists is less developed compared with yeasts and bacteria. However, the most known antagonistic fungi like *Muscodor albus* and *Homoptera parasite* are also tested on postharvest citrus diseases and have shown a reduction of fruit decay (Borras and Aguilar, 1990; Benhamou, 2004; Mercier and Smilanick, 2005) **(Table 1.3)**. Antagonistic fungi exhibit broad spectrum in terms of disease control and volatile antimicrobial compounds (Kiss, 2003; Ezra *et al.*, 2004; Mercier and Smilanick, 2005; Verma *et al.*, 2007). The fungus *Muscodor albus*, a biofumigant that produces certain low molecular weight volatiles, has been used to fumigate whole rooms of lemons to control pathogens during storage. It is reported to be effective on green mold and sour rot (Mercier and Smilanick, 2005). This fungus was reported to produce 28 organic volatile compounds that show some inhibitory effect against pathogenic fungi and bacteria (Strobel *et al.*, 2001). Numerous species of *Trichoderma* have been widely used as antagonistic fungal agents against several postharvest diseases (Okigbo and Ikediugwu, 2000; Batta, 2004). The antagonism of these fungi is the result of its richness of antimicrobial metabolites and physiological conformation (Verma *et al.*, 2007).

IV.1.3 Mode of action of antagonistic microorganisms

Understanding the mode of action of antagonists allow to develop more reliable procedures for the effective application of known antagonists and to provide a basis to select more effective and desirable antagonists or strains of antagonists (Wisniewski and Wilson, 1992). Although considerable amount of research have been reviewed on the use of the microbial antagonists, only few attempts have been made to study microbial interactions in wounds of fruit. Probably, because of the difficulty in studying the complex interactions occurring between the pathogen, antagonist, host, and possibly other microorganisms present on the fruit surface. Several mechanisms, operating alone or in concert, are involved in antagonistic interactions in the fructoplan. Nutrients and space competition, antibiosis and parasitism are the major mechanisms. Additional mechanisms such as induced resistance, interference with pathogen-related enzymes, and undoubtedly a number of still unknown mechanisms may complete the microbial arsenal (Sharma *et al.*, 2009). Often, more than one

mechanism was implicated, but in no case has a sole mechanism been found responsible for biological control (Janisiewicz and Korsten, 2002).

IV.1.3.1 Antibiosis

Antibiotic production is one of the major modes of action of antagonists. This mechanism was more found in bacteria than in yeasts and in filamentous fungi. Bull *et al.,* (1998) demonstrated that Syringomycin produced by *Pseudomonas syringae* controlled green mold on lemons and inhibited the growth of *G. candidum, P. digitatum* and *P. italicum*. Similarly, *Pseudomonas cepacia* was also found to be effective in controlling green mold in lemon by producing antibiotics (Smilanick and Denis-Arrue, 1992). Moreover, *Bacillus subtilis* and its antibiotics are considered to be potent biological control agents to suppress growth of *P. digitatum* in the postharvest protection of citrus (Leelasuphakul *et al.,* 2008). *Pseudomonas corrugate*, *Pseudomonas fluorescens*, *Bacillus thuringiensis*, *Aureobasidium pullulans* and *Pichia pastoris* were also found to control citrus postharvest diseases by production of antibiotics (Smilanick and Denis-Arrue, 1992; Liu *et al.,* 2007; Lucon *et al.,* 2010; Ren *et al.,* 2011). However, the production of these antibiotics wasn't generally detected on the fruit; raising a doubt on the role of the antibiosis in postharvest diseases control which explains the fact that antibiosis may not comprise the entire mode of action of antagonists on citrus (Bull *et al.,* 1997). Although, antibiosis might be an effective tool for controlling postharvest diseases by antagonistic bacteria, the importance is being given for the development of non-antibiotic producing microbial antagonists for the control of postharvest diseases of fruits. The main concern, related to the use of antibiotics into our food, is the adverse effect on the resistance of human's pathogens to antibiotics, because of the possible residue left in the fruit, and the possible development of resistance in fruit pathogens (El-Ghaouth *et al.,* 2004). Further search was directed to identify antagonists that did not produce antibiotics.

IV.1.3.2 Competition for nutrients and space

The nutrient sources in the peel of citrus fruit are frequently not sufficient for all microorganisms, which makes the competition between pathogen and non-pathogens for nutrient resources or site an important issue in Biocontrol. Many investigations on different biocontrol systems have concluded that the successful competition of microbial antagonists with the pathogens infecting fruits for nutrients and space may be the possible mechanism of biocontrol (Droby *et al.,* 1989; Wilson and Wisniewski, 1989; Wisniewski *et al.,* 1989; Mercier and Wilson, 1994; Arras *et al.,* 1998). Indeed, many successful microbial antagonists

that do not produce antibiotics are able to grow rapidly at the wound sites and are better adapted to extreme nutritional and environmental conditions compared with postharvest pathogens. The non-pathogenic microorganisms (especially yeasts) protect the surface of citrus fruits by rapid colonization of wounds and thereby exhausting the limited available substrates so that none are available for pathogen to grow. Results of study by Liu *et al.* (2010) revealed that *Cryptococcus laurentii* colonized citrus wounds in a short time and effectively exploit the available endogenous nutrient resources. Also *Pichia guilliermondii, Candida saitoana, Rhodotorula glutinis, Rhodosporidium paludigenum, Kloeckera apiculata, Hanseniaspora guilliermondii* and *Metschnikowia andauensis* were reported to compete citrus postharvest pathogens for nutrients and space (Arras *et al.,* 1998; El-Ghaouth *et al.,* 2000; Zheng *et al.,* 2005; Long *et al.,* 2006; Taqarort *et al.,* 2008; Liu *et al.,* 2010; Manso and Nunes, 2011) **(Table 1.3)**. The competition for nutrients and space is favored by the attachment capability of antagonistic yeasts to pathogen hyphae. The attachment may enhance nutrient competition as well as interfere with the ability of the pathogen to initiate infection (El Ghaouth *et al.,* 2002). The attachment of antagonists to pathogen hyphae has been suggested as an important factor in competition for nutrients between the antagonistic yeast *Pichia guilliermondii* and *P. italicum* on citrus fruit (Arras *et al.,* 1998). Currently, there is only fragmented data regarding the antagonist-pathogen interaction in terms of competitions for limiting nutrient essential for pathogenesis. Once more information regarding the specificity of competition between antagonistic and pathogens in fruit wounds is available and genes responses of antagonism of biocontrol agents have been characterized, it will be possible to develop antagonistic strains with a higher rate of transport and/or metabolism of limiting nutrient essential for pathogenesis (El-Ghaouth *et al.,* 2004).

IV.1.3.3 Direct parasitism

In this case, pathogen is directly attacked by a specific microbial antagonist that kills it or its propagules. Parasitism depends on close contact and recognition between antagonist and pathogen, on the secretion of lytic enzymes, and on the active growth of the parasite into the host (El-Ghatouh *et al.,* 2002). It is often referred to as hyperparasitism or mycoparasitism when interactions involve a fungus. In the literature, very little information is available on the role of direct parasitism of the microbial antagonists in controlling postharvest pathogens of citrus fruit. Arras *et al.* (1998) showed that *Pichia guilliermondii* cells had the ability to attach to the hyphae of *Penicillium italicum.* Also, the antagonistic yeast *Candida saitoana* was found to be able to attack *P. italicum* by direct parasitism (El-Ghaouth *et al.,* 2000).

IV.1.3.4 Induced resistance

Additional mode of action, such as induced resistance, has also been suggested and several microbial antagonists were shown to induce a wide range of defense responses in citrus fruit (Borras and Aguilar, 1990; Rodov *et al.,* 1994; Arras, 1996; Droby *et al.,* 2002; Benhamou, 2004; Lucon *et al.,* 2010). Droby *et al.* (2002) reported that the application of *Candida oleophila* to surface wounds of grapefruit elicited systemic resistance against *P. digitatum.* They also demonstrated that the induction of pathogen resistance required viable yeast cells. Rodov *et al.* (1994) reported that *Pichia guilliermondii* induced resistance to green mold by eliciting the production of phytoalexins (e.g. scoparone and scopoletin). Similarly, Arras (1996) showed that scoparone accumulation could be 19 times higher when the antagonist *C. famata* was inoculated 24 h prior to *P. digitatum*, and only four times higher if inoculated 24 h after the pathogen. The accumulation of the phytoalexin scoparone was correlated with increased antifungal activity in the flavedo, and resulted in enhanced resistance of the fruit to infection by *Penicillium digitatum.* Although a causal connection between the accumulation of the host defense responses and bioprotection by microbial antagonists has not yet been clearly established, the occurrence of high levels of host antifungal compounds in protected tissue suggests their implication in disease resistance (El Ghaouth *et al.,* 2004).

Table 1.3 : Microbial antagonists used for the successful control of citrus postharvest diseases and their mode of action on citrus fruits

Microbial antagonist	Pathogen	Mode of action	Reference
Bacteria :			
Pseudomonas syringae	*P. digitatum*	Antibiosis, Competition for nutrient and space	Bull *et al.,* 1998 Wilson and Chalutz ,1989
Pseudomonas cepacia	*Penicillium spp*	ND	Wilson and Chalutz , 1989
	P. digitatum	Antibiosis, Competition for nutrient and space	Smilanick and Denis-Arrue, 1992
	Penicillium spp	Antibiosis	Wilson and Chalutz, 1989
Pseudomonas glathei	*P. digitatum*	Competition for nutrient and space, Induction of resistance	Huang *et al.,* 1995
Pseudomonas corrugata	*P. digitatum*	Antibiosis and competition for nutrients and space	Smilanick and Denis-Arrue, 1992
Pseudomonas fluorescens	*P. digitatum*	Antibiosis and competition for nutrients and space	Smilanick and Denis-Arrue, 1992

Paenibacillus polymyxa	*P. digitatum*	Antibiosis and competition for nutrients and space	Lai *et al.*, 2012
Entrobacter cloacae	*P. digitatum*	Competition for nutrient and space	Wilson and Chalutz, 1989
Bacillus subtilis	*P. digitatum*	Antibiosis	Singh and Deverall, 1984
	P. digitatum	Antibiosis	Leelasuphakul *et al.*, 2008
	P. digitatum *G. candidum*	Antibiosis	Yanez-Mendizabal *et al.*, 2011
Bacillus amyloliquefaciens	*P. crustosum*	Production of volatile compounds	Arrebola *et al.*, 2010
Bacillus thuringiensis	*Guignardia citricarpa*	Antibiosis, Induction of resistance	Lucon *et al.*, 2010
Bacillus pumilus	*P. digitatum*	ND	Huang *et al.*, 1992
Serratia plymuthica	*Penicillium spp*	Antibiosis and competition for nutrients	Meziane *et al.*, 2006
Pantoea agglomerans	*Penicillium spp*	ND	Canamas *et al.*, 2008
	P. digitatum	Triggers H2O2 production Triggers enzymatic activities	Torres *et al.*, 2011

Fungi :

Galactomyces citri-aurantii (avirulent)	*P. digitatum*	Antibiosis	Eayre *et al.*, 2003
Muscodor albus	*G. candidum* *P. digitatum*	Production of volatile compounds	Mercier and Smilanick, 2005
Aureobasidium pullulans	*P. digitatum*	Antibiosis	Liu, *et al.*, 2007
Verticillium lecanii	*P. digitatum*	Induction of resistance	Benhamou, 2004

Yeasts :

Pichia guilliermondii	*P. italicum*	Competition for nutrient and space, Directly parasitizing the pathogen	Arras *et al.*, 1998
	P. digitatum	Induction of resistance	Rodov *et al.*, 1994
	P. italicum	ND	Lahlali *et al.*, 2010
Pichia anomala	*Penicillium spp*	ND	Lahlali *et al.*, 2004
	P. digitatum	Competition for nutrient and space	Taqarort *et al.*, 2008
Pichia pastoris	*G. candidum*	Antibiosis	Ren *et al.*, 2011
P. membranefaciens	*Penicillium spp*	induction of resistance	Luo *et al.*, 2012
Candida saitoana	*P. italicum*	Competition for nutrient and space, direct parasitism	El-Ghaouth *et al.*, 2000

Candida famata	*P. digitatum*	Induction of resistance	Arras 1996
Candida guilliermondii	*P. digitatum*	Induction of resistance	Arras 1996
Candida oleophila	*P. digitatum*	Induction of resistance	Droby *et al.,* 2002
	Penicillium spp	ND	Lahlali *et al.,* 2004
Candida sake	*P. digitatum*	ND	Droby *et al.,* 1999a
Rhodotorula glutinis	*P. digitatum*	Competition for nutrient and space	Zheng *et al.,* 2005
Rhodosporidium paludigenum	*G. candidum*	Competition for nutrient and space	Liu *et al.,* 2010
Saccharomycopsis schoenii	*Penicillium spp*	ND	Pimenta *et al.,* 2008
Debaryomyces hansenii	*P. italicum*	Competition for nutrient and space	Droby *et al.,* 1989
	P. italicum		Hernández-Montiel *et al.,* 2010
	Penicillium spp	Competition for nutrient and space	Wilson and Chalutz ,1989
	Penicillium spp	ND	Chalutz and Wilson, 1990
	G. candidum	ND	
	P. digitatum	Competition for nutrient and space	Taqarort *et al.,* 2008
Cryptococcus laurentii	*P. italicum* *G. candidum*	Competition for nutrient and space	Liu *et al.,* 2010
Aureobasidium pullulans	*Penicillium spp*	Competition for nutrient and space	Wilson and Chalutz , 1989
Kloeckera apiculata	*P. italicum*	Competition for nutrient and space	Long *et al.,* 2006
Hanseniaspora guilliermondii	*P. digitatum*	Competition for nutrient and space	Taqarort *et al.,* 2008
Metschnikowia andauensis	*Penicillium spp*	Competition for nutrient and space	Manso and nunes , 2011
Wickerhamomyces anomalus	*P. digitatum*	Antibiosis	Platania *et al.,* 2012

ND : No determined

IV.1.4 Application methods of microbial antagonists

Infection of citrus fruit by pathogens can occur in the field prior to harvest, and it could be advantageous starting control at this point, as well as in the postharvest phase. So, microbial antagonists can be applied by two ways : preharvest and postharvest application. An important consideration for the application of antagonists at preharvest is their ability to

colonize the surface of fruit both in the field and during storage and to persist, for as long as possible, in sufficient numbers on the fruit surface to maintain an efficient decay control (Wisniewski and Wilson, 1992). Furthermore, to be successful in preharvest application, potential antagonists should be able to tolerate low-nutrient availability, UV rays, high temperature and dry conditions (Spadaro and Gullino, 2004). A large number of studies showed that several microbial antagonists are able to inhibit effectively postharvest diseases in citrus fruit. For example, preharvest application of the yeast *Pichia guilliermondii* was effective and controlled green mold (Droby *et al*., 1992). Similarly, preharvest application of *Pantoea agglomerans* provided an effective control for orange fruit against natural postharvest pathogen infections and artificial infections of *Penicillium digitatum* during storage (Canamas *et al.,* 2008). However, according to Sharma *et al.* (2009), this approach has still many limitations and did not provide commercially acceptable control of fruit diseases.

Unlike preharvest application, postharvest application of microbial antagonists is the most used and most practical method for controlling postharvest diseases of citrus fruit (Sharma *et al.,* 2009). In this case, microbial antagonists are applied either as postharvest spray, dip or drench applications (El-Ghaouth *et al.,* 2001; Mercier and Smilanick, 2005; Canamas *et al.,* 2008; Usall *et al.,* 2008; Lahlali *et al.,* 2010; Arrebola *et al.,* 2010). An unusual case is the control of citrus *Penicillium* decay by biofumigation with volatile compounds produced by grain cultures of the fungus *Muscodor albus* (Mercier and Smilanick, 2005).

Postharvest application of *Pseudomonas syringae, Pseudomonas cepacia, Bacillus subtilis, Trichoderma viride* and *Debaryomyces hansenii* resulted in control of *Penicillium digitatum* in citrus fruit (Singh and Deverall, 1984; Wilson and Chalutz, 1989; Borras and Aguilar, 1990; Smilanick and Denis-Arrue, 1992; Bull *et al.,* 1998). A significant reduction in storage decay of citrus, caused by *P. digitatum, P, italicum* and *Geotrichum candidum,* was achieved by bringing several yeast species in direct contact with wounds in the peel of harvested fruit (Chalutz and Wilson, 1990).

IV.1.5 Criteria for selecting a good antagonist

Few of the antagonists reported to control plant pathogens are successfully transferred from the laboratory into the commercial conditions. In the case of citrus fruits, among all tested antagonists on the control of postharvest decay, only three antagonists have been patented and evaluated for commercial use. Aspire (*Candida oleophila* strain 182; Ecogen

Inc., Langhorne, PA), Biosave (*Pseudomonas syringae* strain 10LP, 110, Eco Science Corporation, USA) and Yield Plus (*Cryptococcus albidus* : Anchor Yeast, Cape Town, South Africa) are available for the control of postharvest pathogens of citrus fruits (Droby *et al.*, 1998; Palou *et al.*, 2008). This shows the difficulty of marketing Biocontrol which must satisfy several conditions to be accepted for use in commercial conditions. For Biocontrol to be successful, it would be : (a) genetically stabile; (b) effective at low concentrations; (c) not fastidious in its nutrient requirements; (d) able to survive well under adverse environmental conditions (including storage environments); (e) efficacious against a wide range of pathogens on a variety of fruit and vegetables; (f) amenable to growth on an inexpensive medium in fermenters; (g) preparable in a form that can be effectively stored and dispensed; (r) non-productive of secondary metabolites that may be deleterious to humans : (i) resistant to pesticides; (j) compatible with other chemical and physical treatment of the commodity; and (k) non-pathogenic against the host (Wilson and Wisniewski, 1989). Additional criteria may include the ability to colonize wounded and sound fruit surfaces under various conditions, utilization of substrates occurring in fruit and resistance to synthetic fungicides applied postharvest to control decay. Also the conditions that favor a potential antagonist should be the same or similar to those that favors the pathogen (Janisiewicz and Korsten, 2002). Nevertheless, Even if an antagonist is found with these desirable characteristics, economic factors dictate whether it will be commercialized. If the potential market for the product is not large enough to generate a profit, then it cannot be commercialized which presents difficulty with antagonists for postharvest diseases of citrus fruits. However, the continuous withdrawal of fungicides used to control postharvest diseases and the growing public concern about chemical residues in fruit may encourage investing in the alternative of use of microbial antagonists.

IV.1.6 Use of antagonist mixtures

In the enhancement of a biocontrol system, work could focus on a promising approach, which is the development of antagonist mixtures. An effective biological control based upon a mixture of several complementary and non-competitive antagonists is more likely than a control based upon microorganism alone. Such mixtures have several advantages (Janisiewicz, 1988) : apart from a wider spectrum of activity (different fruits, cultivars and ripening stages), they can increase the efficacy (less biomass necessary), be more reliable and allow a reduction in application times and treatment costs. Moreover, they permit the combination of different genetic characteristics, minimising the need for genetic

engineering. Although the use of antagonistic mixtures offers more effective control, the economic viability of this approach appears to be a major obstacle for its adoption, as registration of two microbial antagonists will cause additional burden for the industry (Sharma *et al.*, 2009). Most of the work relates to grape and apples, with limited studies on other fruits, including citrus fruit. The enhancement of efficiency of microbial antagonists may be due to : better utilization of substrate, resulting in acceleration of the growth rate; removal of substances inhibitory to one organism by the other microbial antagonists; production by one microbe of nutrients that may be used by another; and formation of more stable microbial community that may exclude other microbes, including pathogens (Janisiewicz, 1998).

IV.2 Biological control of citrus diseases with natural plant products

With consumer trends for natural alternatives to chemical-based fungicides and changes in legislation, the use of natural products such as plant extracts may provide a solution for both industry and consumers. Recently, attention has been paid towards the exploitation of higher plant products as novel botanical fungicides in citrus diseases management. Indeed, higher plants contain a wide spectrum of secondary metabolites, such as phenolics, flavonoids, quinones, tannins, essential oils, alkaloids, saponins, sterols, with different biological properties (Tripathi and Dubey 2003; Tayel *et al.*, 2009). More than 1340 plant species are known to be potential sources of antimicrobial compounds and about 10,000 secondary plant metabolites have been chemically defined for their role as antimicrobials (Cowan, 1999; Tripathi and Dubey 2003). Plant extracts have the advantage of being biodegradable, not phytotoxic, are generally regarded as safe to mammals (GRAS) by the United States Food and Drug Administration (Ware and Whitacre, 2004), and are thought to have novel modes of action (Regnault-Roger *et al.*, 2005). Therefore, higher plants can be exploited for the discovery of new natural fungicides which can replace the synthetic ones. Some phytochemicals of plant origin have been formulated as botanical pesticides and are used successfully in integrated pest management programs (Tripathi *et al.*, 2004). In the available literature, research on biological control of postharvest diseases of citrus fruit by natural plant products focused mainly on the use of essential oils, volatile compounds, aqueous and organic solvent extracts and other products extracted from plants (Agnioni *et al.*, 1998; Droby *et al.*, 1999b; Arras and Usai, 2001; Du Plooy *et al.*, 2009; Liu *et al.*, 2009b; Gatto *et al.*, 2011; Talibi *et al.*, 2012a; Talibi *et al.*, 2012b).

IV.2.1 Use of essential oils

Essential oils are natural, volatile, complex compounds known for their antimicrobial, antioxidant and medicinal properties (Bakkali *et al.,* 2008). Essential oils are mostly a mixture of many different volatile compounds, their composition often varies between plant species (Mishra and Dubey, 1994) and exert differential effects depending on both the mode of action and the target organism (Liu *et al.,* 2006). The development of resistant strains of fungi against essential oils may not become a real issue as it is the case for many synthetic fungicides because several active components are often present in the final product and synergistic interactions may exist between the different components of the oils (Tripathi and Dubey 2003; Tripathi *et al.,* 2004; Dubey *et al.,* 2006;). Furthermore, most of the components of essential oils seem to have no specific cellular targets (Carson *et al.,* 2002). The volatility, ephemeral nature and biodegradability of essential oils compounds may be especially advantageous for treatment of postharvest citrus diseases because only low residues can be expected. These characteristics make them particularly suitable for application in their vapor phase by a new process defined as 'biofumigation'. Recently, packing systems that deliver volatile antimicrobials to the packaged product, known as 'antimicrobial active packaging', have been evaluated (Ayala-Zavala *et al.*, 2008).

Plaza *et al.* (2004a) reported that Thyme and cinnamon essential oils significantly reduced the incidence of green and blue molds of citrus. Also, thyme oil was reported to control most postharvest citrus rots, such as green mold, bleu mold and sour rot (Arras and Usai, 2001; Liu *et al., 2009b*). Many studies **(Table 1.4)** have documented the antifungal effects of plant essential oils against citrus fruit pathogens (Chebli *et al.,* 2003; Tripathi *et al.,* 2004; Alilou *et al.,* 2008; du Plooy *et al.,* 2009; Solaimani, *et al.,* 2009; Badawy *et al.,* 2011). Treatment of oranges with the essential oils of *Mentha arvensis*, *Ocimum canum* and *Zingiber officinale* has been found to control blue mold, thereby enhancing shelf life of fruit (Tripathi and Dubey 2003). Dixit *et al.* (1995) demonstrated the efficacy of *Ageratum conyzoides* essential oils for the protection of mandarins against blue mold. Essential oils of thyme, oregano, cinnamon, clove, dictamus or mint were very effective *in vitro* against *P. digitatum* and *P. italicum*, but results from *in vivo* experiments were contradictory and applications to citrus fruit were often ineffective or phytotoxic (Arras and Usai, 2001; Tripathi *et al.*, 2004). Application of Mentha oil (*Mentha arvensis*) reduced blue mold caused by *P. italicum* in orange and lime and enhanced the shelf life by 6 to 8 days (Tripathi *et al.*, 2004). The oil from *Thymus capitatus* exhibited strong fungicidal activity against *Penicillium digitatum*, *P.*

italicum, and *Alternaria citri* in vitro at 250ppm. The fungitoxicity of *T. capitatus* essential oil sprayed on healthy orange inoculated with *P. digitatum* was weak at atmospheric pressure, but in vacuum conditions conidial mortality on fruit exocarp was high (90–97%). The efficacy of this essential oil was comparable to that of thiabendazole (TBZ) at 2000 ppm concentration. Carvacrol was found to be the predominant compound, accounting for 81 to 83% of essential oil vapors present in *T. capitatus* and exhibited fungitoxicity (Arras and Usai, 2001). The antifungal activity of the essential oils suggests that they may be considered as a potential alternative to the synthetic fungicides for the control of postharvest citrus pathogens. However, despite their potent antifungal activity, commercial implementation of treatments with essential oils is strongly restricted in citrus because of problems related to potential phytotoxicity, intense sensory attributes or technological application as fumigants or in aqueous solutions (Palou *et al.,* 2008).

IV.2.2 Use of crude plant extracts

The preservative nature of some plant extracts has been known for centuries and there has been renewed interest in the antimicrobial properties of extracts from aromatic plants. In recent years, several studies have been focused on screening of plant extracts to develop new antifungal compounds that can be used to control postharvest citrus diseases. Aqueous or organic solvent extracts of plants from different origins are sources of antifungal activity against citrus postharvest pathogens under different experimental conditions (Obagwu and Korsten, 2003; Abd-El-Khair and Hafez, 2006; Ameziane *et al.,* 2007; Mekbib *et al.,* 2007; Gatto *et al.,* 2011; Talibi *et al.,* 2011a; Talibi *et al.,* 2012a; Talibi *et al.,* 2012b) **(Table 1.4)**. Talibi *et al.* (2012a) reported that aqueous extracts of *Cistus villosus*, *Halimium antiatlanticum* and *Ceratonia siliqua* reduced significantly the incidence and severity of citrus sour rot. Moreover, the aqueous extract of *Halimium umbellatum* and *Inula viscosa* were found to be effective against citrus blue mold caused by *P. italicum* (Askarne *et al.*, 2012). The aqueous extract of *Acacia nilotica* has also shown pronounced antifungal activity against *P. italicum* and enhanced the storage life of oranges for 6 days (Tripathi and Dubey, 2004). Aqueous extracts from plants can both inhibit and stimulate fungal growth of postharvest citrus pathogens (Ameziane *et al.,* 2007; Talibi *et al.*, 2012a).

Besides the aqueous extracts, organic solvent extracts of several plants were tested against citrus pathogens. Treatment of mandarin fruit by methanol extracts of *Cistus villosus*, *Halimium umbellatum* and *Ceratonia siliqua* successfully controlled the citrus sour rot (Talibi

et al., 2012b). Also of interest, methanol extracts from *Sanguisorba minor* showed good control of green mold (Gatto *et al.*, 2011). Tayel *et al.* (2009) showed that methanol and ethanol extracts of *Punica granatum* controlled citrus green mold and the antifungal activity of these extracts were higher than that of standard Imazalil. Also Mekbib *et al.* (2007) reported that wound inoculated fruit with *P. digitatum* and methanolic extract of *Withania somnifera* and *Acacia seyal* did not develop decay symptoms for up to 21 days of storage at 25°C. Ameziane *et al.* (2007) showed that methanol extract of *Cistus villosus* is more active against *P. digitatum* and *G. candidum* than the chloroformic extracts. Thus, the nature of extraction influence on the antifungal activity of plants tested. This difference in biological activity is due to the polarity of each solvent, i.e. the nature of the molecules extracted with each solvent. Methanol is a polar solvent which can extract several compounds with antimicrobial activities like alkaloids (Satish *et al.*, 2008), triterpene glycoside (Ismail *et al.*, 2008), tannins (Askun *et al.*, 2009), sesquiterpene lactones (Choi *et al.*, 2004) and phenolic compounds (Nicholson and Hammerschmidt, 1992; Tripathi and Dubey, 2004; Sisti *et al.*, 2008; Martini *et al.* 2009). Likewise, Askun *et al.* (2009) reported that Methanol provided more consistent antimicrobial activity. The potential use of crude plant extracts to control postharvest citrus diseases requires a detailed examination of their biological activity and dispersion in fruit tissues and the development of a formula which inhibits growth of pathogens without producing phytotoxic effects on fruit.

Table 1.4 : Plant extracts used for the control of citrus postharvest diseases

Plant specie	**Plant extract**	**Pathogen**	**Reference**
Cymbopogon sp.	Aqueous extract	*P. digitatum*	Abd-El-Khair and Hafez 2006
Lantana sp.	Aqueous extract	*P. digitatum*	Abd-El-Khair and Hafez 2006
Eucalyptus sp.	Aqueous extract	*P. digitatum*	Abd-El-Khair and Hafez 2006
Sanguisorba minor	Methanol extract	*P. digitatum* *P. italicum*	Gatto *et al.*, 2011
Borago officinalis	Methanol extract	*P.digitatum* *P.italicum*	Gatto *et al.*, 2011
Sonchus oleraceus	Methanol extract	*P.digitatum* *P.italicum*	Gatto *et al.*, 2011
Thymus sp	Essential oil	*G. candidum*	Liu *et al.*, 2009b
Thymus capitatus	Essential oil	*P.digitatum*,	Arras and Usai , 2001

Thymus vulgaris	Essential oil	*P.digitatum* *P.italicum* *A. citri*	Fatemi *et al.,* 2011
Zataria multiflora	Essential oil	*P.digitatum* *P.italicum*	Solaimani *et al.,* 2009
Chrysanthemum	Essential oil	*G. candidum* *P. digitatum,*	Chebli *et al.,* 2003
Cistus villosus	Aqueous extract	*G.candidum*	Talibi *et al.,* 2012a
	Methanol extract		Talibi *et al,* 2012b
Halimium antiatlanticum	Aqueous extract	*G.candidum*	Talibi *et al.,* 2012a
	Methanol extract		Talibi *et al,* 2012b
Halimium umbellatum	Aqueous extract	*G.candidum*	Talibi *et al.,* 2012a
		P.italicum	Askarne *et al.,* 2012
	Methanol extract	*G.candidum*	Talibi *et al.,* 2012b
Ceratonia siliqua	Aqueous extract	*G.candidum*	Talibi *et al.,* 2012a
	Methanol extract		Talibi *et al,* 2012b
Pistacia atlantica	Aqueous extract	*G.candidum*	Talibi *et al.,* 2012a
	Methanol extract		Talibi *et al,* 2012b
Inula viscosa	Aqueous extract	*G.candidum*	Talibi *et al.,* 2012a
		P. italicum	Askarne *et al.,* 2012
	Methanol extract	*G. candidum*	Talibi *et al.,* 2012b
Mentha spicata	Essential oil	*P.digitatum*	Du Plooy *et al.,* 2009
Lippia scaberrima	Essential oil	*P.digitatum*	Du Plooy *et al.,* 2009
Allium sativum	Aqueous and ethanol extracts	*P.digitatum*	Obagwu and Korsten, 2003
Mentha arvensis	Essential oil	*P. italicum*	Tripathi *et al.,* 2004
Zingiber officinale	Essential oil	*P. italicum*	Tripathi *et al.,* 2004
Ocimum canum	Essential oil	*P. italicum*	Tripathi *et al.,* 2004
Acacia nilotica	Aqueous extract	*P. italicum*	Tripathi *et al.,* 2002
Anvillea radiata	Aqueous extract	*P. italicum*	Askarne *et al.,* 2012
Punica granatum	Methanol extract	*P.digitatum*	Tayel *et al.,* 2009
Aloe barbadensi	Gel	*P.digitatum*	Saks and Barkai-Golan, 1995
Withania somnifera	Methanol extract	*P.digitatum*	Mekbib *et al.,* 2007
Acacia seyal	Methanol extract	*P.digitatum*	Mekbib *et al.,* 2007
Bubonium imbricatum	Essential oil	*P.digitatum*	Alilou *et al.,* 2008

Citrus sp.	Essential oil	*P.digitatum*	Badawy *et al.,* 2011
Ageratum conyzoides	Essential oil	*P.italicum*	Dixit *et al.,* 1995
Simmondsia chinensis	oil emulsion	*P.italicum*	Ahmed *et al.,* 2007
Foeniculum vulgare	Essential oil	*P.digitatum*	El-Tobgy *et al.,* 2010
Mentha piperita	Essential oil	*P.digitatum*	El-Tobgy, 2010
Carum carvum	Essential oil	*P.digitatum*	El-Tobgy, 2010

IV.2.3 Use of natural products extracted from plants

Higher plants contain a wide spectrum of secondary substances like as phenols, flavonoids, quinones, tannins, essential oils, alkaloids, saponins and sterols (Tripathi *et al.,* 2004). Among the numerous natural plant products with potential antimicrobial activity are : acetaldehyde, benzaldehyde, benzyl alcohol, ethanol, methyl salicylate, ethyl benzoate, ethyl formate, hexanal, (E)-2-hexenal, lipoxygenases, jasmonates, allicin, glucosinolates and isothiocyanates, etc (Utama *et al.,* 2002; Tripathi and Dubey, 2003; Palou *et al.,* 2008). Utama *et al.* (2002) demonstrated the efficacy of acetaldehyde, benzaldehyde, cinnamaldehyde, ethanol, benzyl alcohol, nerolidol and 2-nonanone as volatile fungitoxicants for the protection of citrus fruit against *P. digitatum.* Citral was reported to inhibit mycelial growth and spore germination of *P. digitatum* (Klieber *et al.,* 2002). Also Citral has been highlighted as an active compound in citrus fruit against decay caused by *Penicillium digitatum* (Fisher and Phillips, 2008). Production of citral in the flavedo of citrus fruit has been described as a preformed defense mechanism against infection by *P. digitatum* (Rodov *et al.,* 1995). Jasmonates (jasmonic acid and methyl jasmonate) has been found to be effective in postharvest control of *P. digitatum* either after natural or artificial inoculation of grapefruit (Droby *et al.,* 1999b). Exposure of fruit to jasmonates also effectively reduced chilling injury incidence after cold storage (Droby *et al.,* 1999b)**.** Since they are naturally occurring compounds and are given in low doses, jasmonates may provide a more environment-friendly means of reducing the current chemical usage. A naturally occurring compound isolated from the flavedo tissue of grapefruit (*Citrus paradisi*) identified as 7-geranoxy coumarin exhibited antifungal activity against *P. italicum* and *P. digitatum* during *in vitro* and *in vivo* tests (Agnioni *et al.,* 1998) **(Table 1.5)**. Also of interest, Smilanick *et al.* (1995) showed that a brief immersion of citrus fruit in a solution containing ethanol (10 % wt/vol) reduced green mold incidence without significant injury to the fruit.

Table 1.5 : Natural compounds tested against citrus postharvest pathogens

Compound	Causal agent	Reference
Benzaldehyde	*P. digitatum*	Wilson and Wisniewski, 1989
Cinnamaldehyde	*P. digitatum*	Wilson and Wisniewski, 1989
Acetaldehyde vapor	*P. digitatum*	Prasad and Stadelbacher, 1973
Heptanol	*G. candidum*	Suprapta *et al.*, 1997
Octanol	*G. candidum*	Suprapta *et al.*, 1997
Nonanol	*G. candidum*	Suprapta *et al.*, 1997
Decanol	*G. candidum*	Suprapta *et al.*, 1997
Geraniol	*G. candidum*	Suprapta *et al.*, 1997
Citronellol	*G. candidum*	Suprapta *et al.*, 1997
Citral	*G. candidum*	Suprapta *et al.*, 1997 Klieber *et al.*, 2002
	P. digitatum	Fisher and Phillips, 2008
Thymol	*P. digitatum*	Jafarpour and Fatemi, 2012
Menthol	*P. digitatum*	Jafarpour and Fatemi, 2012
7-geranoxy coumarin	*P. digitatum* *P. italicum*	Agnioni *et al.*, 1998
Jasmonic acid	*P. digitatum*	Droby *et al.*, 1999b
Methyl jasmonate	*P. digitatum*	Droby *et al.*, 1999b
Nerolidol	*P. digitatum*	Droby *et al.*, 1999b
2-nonanone	*P. digitatum*	Droby *et al.*, 1999b
Kaempferol	*P. italicum*	Tripathi *et al.*, 2002

IV.2.4 Mode of action of plant extracts

As the exploitation of natural plant products to protect the postharvest decay of citrus fruit is in its infancy, there is little information about their mechanism of action. Nevertheless, few attempts have been done to explain these modes of action. Considering the large number of bioactive chemical present in plant extracts, it is most likely that their antimicrobial activity is not attributable to one specific mechanism but to diverse modes of action (Skandamis *et al.*,

2001; Carson *et al.,* 2002). Droby *et al.* (1999b) reported that jasmonates suppress successively the development of *P. digitatum* in grapefruit. This control might be due to the induction of host resistance responses (Droby *et al.,* 1999b). Also, Tripathi and Dubey (2004) reported that jasmonates play an important role as signal molecules in plant defense responses against pathogen attack. The same authors showed that essential oils play a role in plant defense mechanisms against phytopathogenic micro-organisms and the synergism between their different components reduces the chance of development of resistant races of fungi. Also methanol extracts of *Withania somnifera* and *Acacia seyal* controlled green mold by stimulatory effect on the host defense mechanism (Mekbib *et al.,* 2007). These defense mechanisms resulted in : a) synthesis of cell wall that could serve as a physical and biological barrier to invading pathogens or b) the increase in the total soluble phenolics compounds concentration of orange peels (Mekbib *et al.,* 2007). Also of interest, phenolic compounds are known to alter membrane functionality of pathogens (Lanciotti *et al.,* 2004). However, more investigations on the mode of action of such plant products are required so as to recommend their formulation in control of citrus postharvest diseases.

IV.2.5 Application methods of plant extracts and criteria for selecting a good product

Keeping in view the merits of the botanicals as postharvest fungitoxicants, the products which are found efficacious during *in vitro* testings, should be properly tested for their practical potency based on *in vivo* trials, organoleptic tests and safety limit profile (Tripathi and Dubey, 2004). In *in vivo* trials, the efficacy of postharvest treatments of plant extracts on citrus fruits depended on their method of application. The incorporation of essential oils into fruit coatings primarily applied to retain moisture, is gaining popularity (Du Plooy *et al.,* 2009). Essential oil of *Simmondsia chinensis* (jojoba oil) was applied by Ahmed *et al.* (2007) as a coating for 'Valencia' oranges. They effectively maintained fruit quality for up to 60 days (Ahmed, 2007). Also Du Plooy *et al.* (2009) showed that the advantage of using coatings amended with essential oils, rather than vapour, is that there is closer contact between the essential oils and fruit surfaces, allowing exposure of each fruit to similar concentrations of inhibitor over a longer period. Another method of application of botanicals in controlling citrus postharvest diseases is the immersion of citrus fruits in plant solutions. Essential oil of Shiraz thyme showed antifungal activity against *P. digitutum* only in dipping application (Solaimani *et al.,* 2009)**.** The same authors reported that dipping method was significantly better than spray method on control of green mold. Also Tayel *et al.* (2009) demonstrated that

the *in vivo* prevention and control of *P. digitatum* invading harvested citrus fruit was successfully applied by immersing wounded fruit in pomegranate peel extract *(Punica granatum*) solutions. Keeping in view the merits of the botanicals as postharvest fungitoxicants and to ensure proper application of plant extracts, the products which are found efficacious during *in vivo* application must meet the following conditions : a) The product should be effective even for short duration treatment; b) The treatment should not have an effect on quality parameters such as acidity, flavour and aroma and c) The lowest suitable dose of the treatments for practical application should also be determined (Tripathi and Dubey, 2004).

IV.3 Control of citrus postharvest diseases by food additives and GRAS compounds

Antifungal compounds that leave low or non-detectable residues in the citrus fruit are actively sought in research programs. Organic and inorganic salts are widely used in the food industry; they are common food additives for leavening, pH control, taste and texture modifications (Smilanick *et al.,* 1999; Hervieux, *et al.,* 2002; Arslan *et al.,* 2009). These compounds have a broad spectrum of activity against bacteria and fungi, and are generally recognized as safe (GRAS) compounds for many applications, by European and North America regulations. In addition to their consistent antimicrobial activity, they are inexpensive, readily available, with favorable safety profile for humans and the environment and suitable for the postharvest handling practices (Foegeding and Busta, 1991; Olivier *et al.,* 1998; Hervieux, *et al.,* 2002; El-mougy *et al.,* 2008; Deliopoulos *et al.,* 2010). In recent years, interest in salts has increased and numerous studies on the antimicrobial activity of a wide range of organic and inorganic salts, against various pathogens of citrus fruit, have been reported (Kitagawa and Kawada, 1984; Sholberg, 1998; Biggs, 1999; Gabler *et al.,* 2001; Palou *et al.,* 2001; Hervieux, *et al.,* 2002; Plaza *et al.,* 2004b; Ilhan *et al.,* 2006; El-Mougy *et al.,* 2008; Smilanick *et al.,* 2008; Arslan *et al.,* 2009; Montesionos-Herrero *et al.,* 2010; Askarne *et al.,* 2011; Talibi *et al.,* 2011b**) (Table 1.6)**.

Table 1.6 : Salts and food additives used for the control of citrus postharvest diseases

Name of Salt	Causal agent	Remark with use	Reference
Acetic acid	*P. digitatum*	Fumigation at 99.99%	Sholberg, 1998
Formic acid	*P. digitatum*	Browning of the fruit peel	Sholberg, 1998
Propionic acid	*P. digitatum*	Fumigation at 99 to 100%	Sholberg, 1998

Sorbic acid	*G. candidum*	Good control at low pH	Kitagawa and Kawada, 1984
Benzoic acid	*G. candidum* *P.italicum* *P. digitatum*	Coating with 4% of salt	El-Mougy *et al.*, 2008
Boric acid	*G. candidum*	Effective at room temparature	Talibi *et al.*, 2011b
Potassium sorbate	*P. digitatum*	Effective when heated	Smilanick *et al.*, 2008 Montesinos-Herrero *et al.*, 2009
	G. candidum	Coating with 4% of salt	El-Mougy *et al.*, 2008
		Effective when heated at 50°C	Kitagawa and Kawada, 1984
		Partially effective when heated	Smilanick *et al.*, 2008
	P.italicum	Coating with 4% of salt	El-Mougy *et al.*, 2008
		Effective at 60°C	Montesinos-Herrero *et al.*, 2009
		Coating with 4% of salt	El-Mougy *et al.*, 2008
Sodium carbonate	*P.italicum*	Immersion at 45°C	Palou *et al.*, 2001
		Dip not accepted by industry	Plaza *et al.*, 2004b
	P.digitatum	Effective at 2% and 40°C	Smilanick *et al.*, 1999;
		Effective when heated	Smilanick *et al.*, 1997
		Dip not accepted by industry	Plaza *et al.*, 2004b
Sodium bicarbonate	*P.digitatum*	Effective at 2% and 40°C	Smilanick *et al.*, 1999
		Effective when heated	Smilanick *et al.*, 2008
	G. candidum	Partially effective when heated	
	P. italicum	Not effective at low concentrations	Palou *et al.*, 2001
Sodium benzoate	*G. candidum* *P.italicum* *P.digitatum*	Coating with 4% of salt	El-Mougy *et al.*, 2008
	P.digitatum	Effective at 2%	Hall, 1988
Sodium hypochlorite	*P.digitatum*	Combined with sodium bicarbonate	Cerioni *et al.*, 2012
Sodium salicylate	*G. candidum*	Effective at room temparature	Talibi *et al.*, 2011b
Sodium propionate	*P. digitatum*	Effective at 2%	Hall, 1988
Sodium tetraborate	*P.digitatum*	Effective when heated	Eckert and Sommer, 1967
Calcium Chloride	*P. digitatum*	Effective when combined with *P. Guilliermondii*	Droby *et al.*, 1997
calcium polysulfide	*P. digitatum*	Effective on lemon than orange	Smilanick and Sorenson, 2001
	G. candidum	Slightly effective	
Hydrogen peroxide	*P. digitatum*	Combined with sodium bicarbonate	Cerioni *et al.*, 2012

IV.3.1 Use of GRAS compounds and food additives

Among food preservatives, potassium sorbate has been evaluated for the control of citrus green and blue molds and sour rot (Kitagawa and Kawada, 1984; Hall, 1988; Palou *et al.,* 2002; El-Mougy *et al.,* 2008; Smilanick *et al.,* 2008). This compound, classified as a minimal risk active ingredient and exempt from residue tolerances, is more appropriate for application as aqueous solutions (Montesinos-Herrero *et al.,* 2010). However, its use did not become popular because its efficacy was sometimes low, and it was reported to delay, rather than stop, green mould infections (Smilanick *et al.,* 2008). Sodium benzoate and benzoic acid are known for their combination of bactericidal and bacteriostatic properties and their properties of being nontoxic and tasteless (El-Mougy *et al.,* 2008). Their effect on postharvest citrus diseases was reported against sour rot, green and blue molds incidence of stored citrus fruit (El-Mougy *et al.,* 2008; Hall, 1988). A comparison among the inhibitory effects of various food additives and low-toxicity chemicals against *P. digitatum* and *P. italicum* showed that potassium sorbate and sodium benzoate were the most effective on oranges and lemons (Palou *et al.,* 2002). In order to extend the shelf life of fresh fruit, many other salt compounds are actually used in many countries, aiming at the destruction of the pathogens or inhibition of their growth. Certain compounds, such as sodium bicarbonate or sodium carbonate, have been highly successful in controlling green mold (Smilanick *et al.,* 1999). Also, Smilanick *et al.* (2008) reported that sodium bicarbonate reduced the incidence of citrus sour rot. Moreover, these treatments poses a minimal risk of phytotoxicity to the fruit, and can be a useful tool in the management of fungicide resistant isolates, which have become particularly problematic (Smilanick *et al.,* 1999). A significant reduction in incidence of *P. digitatum* and *P. italicum* was noted in the case of oranges treated with ammonium molybdate and sodium molybdate (Palou *et al.,* 2002). Besides these salts, other compounds such as orthophosphoric acid, sodium propionate, calcium polysulfide, calcium chloride, EDTA, sodium salicylate and boric acid have been evaluated for the control of citrus green or blue molds or sour rot and show reduced incidence and severity of these diseases (Droby *et al.,* 1997; Smilanick and Sorenson, 2001; El-Mougy *et al.,* 2008; Hall, 1988; Askarne *et al.,* 2011; Talibi *et al.,* 2011b**) (Table 1.6).**

IV.3.2 Mode of action of salt compounds

Although many researchers have focused on the control of postharvest diseases of citrus fruit by the application of salt compounds, the mechanisms by which salts inhibit

microorganisms are not well understood. The various modes of action of salt compounds are through membrane disruption, inhibition of essential metabolic functions, stresses on pH homeostasis and through the accumulation of anions within the cell (Smilanick *et al.,* 2005). Bicarbonates are effective growth inhibitors of various phytopathogenic fungi *in vitro*. Most citrus fungal pathogens grow better in acidic to neutral conditions than in alkaline conditions. The principal mode of action of the bicarbonate ion is through its buffering capacity, whereby an alkaline environment is sustained. When this happens, pathogens, such as *P. digitatum* which require an acidic environment, expend more energy on fungal acid production than hyphal extension and therefore growth may be inhibited (Pelser *et al.,* 1977; Prusky and Yakoby, 2003). According to Corral *et al.* (1988), the anions $(HCO_3)^-$ or $(CO_3)^{2-}$ are primarily responsible for pathogen suppression by inorganic salts, and the cation plays only a minor role in the interaction. Inhibition of *G. candidum*, *P. digitatum* and *P. italicum* by sorbic acid and its salts may be caused by alteration of cell-membrane and cell transport functions, inhibition of enzymes and protein synthesis, and by uncoupling of the oxidative phosphorylation in mitochondria (Sofos 1986; El-Mougy *et al.*, 2008). The pH of the bicarbonate and carbonate solutions is important for the control of postharvest citrus diseases, because it directly affects the germination of conidia (Pelser *et al.,* 1977; Prusky *et al.,* 2004) and influences the virulence of pathogens through their colonization of host tissue (Prusky and Yakoby, 2003; Smilanick *et al.,* 2005). However, we and other workers showed that pH alone cannot explain the inhibitory effect of these compounds (Palmer *et al.,* 1997; Smilanick *et al.,* 1999; Talibi *et al.,* 2011b).

The effectiveness of calcium against *Penicillium digitatum* on grapefruit could be due to its direct effects on host tissue by making cell walls more resistant to pathogen penetration. Most of the calcium that penetrates into the host tissue seems to accumulate in the middle lamella region of the cell wall. The cations form bonds between adjacent pectic acids or between pectic acids and other polysaccharides, forming cross bridges which make the cell walls less accessible to the action of pectolytic enzymes of the pathogen (Droby *et al.,* 1997). Moreover some researchers reported that calcium ions enhance tolerance to abiotic diseases (Wisniewski *et al.,* 1995; Biggs *et al.,* 1997; campanella *et al.,* 2002). Ammonium molybdate affects metabolic processes in several organisms (Wang *et al.,* 1995; *Bodart et al.,* 1999). The basis of its biological activity was reported to be its ability to inhibit acid phosphatase which interferes with phosphorylation and dephosphorylation (Glew *et al.,* 1988), one of the most important processes of cell regulating (Remaley *et al.,* 1985; Hunter, 1995).

IV.4 Use of combined strategy to control postharvest citrus diseases

Different disease management strategies that have to be applied, both at pre- and postharvest stages, have been integrated to provide more effective disease control than that possible with a single approach. As discussed in several works, the combination of microbial antagonists with other alternative control methods can be a promising approach to overcome some drawbacks in biocontrol activity, enhancing their efficacy (Huang *et al.*, 1995; Stevens *et al.*, 1997; Droby *et al.*, 1998; El-Ghaouth, *et al.*, 2000; Arras *et al.*, 2002; Janisiewicz and Korsten, 2002; Porat *et al.*, 2002; Plaza *et al.*, 2004c; Zhang *et al.*, 2004) **(Table 1.7)**. The combination of microbial antagonists with heat (Porat *et al.*, 2002), GRAS compounds (Usall *et al.*, 2001), and UV-C (Stevens *et al.*, 1997) produced a synergic effect and was superior to all the treatments alone in controlling green and blue molds. Such combined treatments can be easily implemented on a commercial scale in many citrus packinghouses because they are compatible with existing facilities and postharvest handling practices. In general, five objectives may be pursued by the integration of two or more treatments : additive or synergistic effects to increase the effectiveness or the persistence of individual treatments; complementary effects to combine preventive and curative activities; to delay the development of fungicide-resistant isolates, to control fungicide-resistant isolates already present within packinghouses and to facilitate a reduction in fungicide rates in order to minimize fruit residues and chemical costs (Palou *et al.*, 2008; Smilanick *et al.*, 2008). Several options of combination were mentioned such as combination of biocontrol agents with salts or food additives, low levels of conventional fungicides or physical control treatments.

IV.4.1 Combination of microbial antagonists with other control methods

As previously mentioned, the main shortcoming of the use of microbial antagonists has been inconsistency in their performance, especially when used as a stand-alone product to replace synthetic fungicides. Furthermore, as infection of citrus fruit occur either prior to harvest or during harvesting and processing, microbial antagonists are expected to display both a protective and curative activity comparable to that observed with synthetic fungicides. None of the proposed biological control agents, however, have been shown to control previously-established infections. The combination of biological control with other control methods is one of the most promising means of establishing effective integrated diseases management strategies (Schisler *et al.*, 2011) **(Table 1.7)**.

IV.4.1.1 Microbial antagonists combined with low risk substances

Various additives have been shown to increase the effectiveness of some antagonistic microorganisms in controlling postharvest decay. The addition of $CaCl_2$ to antagonistic yeast suspensions was found to enhance their biocontrol activity and reduce the populations of yeast cells required to give effective control (McLaughlin *et al.,* 1990; Droby *et al.,* 1997). A combination of the yeast antagonist, *Pichia guilliermondii* (10^7 cells ml^{-1}) with $CaCl_2$ in dip application, significantly decreased the incidence of green mold caused by *Penicillium digitatum* in grapefruit wounds (Droby *et al.,* 1997). Both spore germination and germ tube elongation of P. *digitatum* decreased with increasing $CaCl_2$ concentration. In addition, increased $CaCl_2$ concentration also resulted in the inhibition of pectolytic activity of a crude enzyme preparation of *P. digitatum.*

Enhancement of biocontrol of the green mold (*Penicillium digitatum)* on citrus has been achieved by adding 2-deoxy-D-glucose at 0.2% to suspensions of the yeast antagonist *Candida saitoana* applied to the fruits (El-Ghaouth *et al.,* 2001). This combination is more effective to control the disease than either 2-deoxy-D-glucose or *Candida saitoana* alone. Another combination aimed at improving the biocontrol of fruit decay by C. *saitoana* is that of the antagonistic yeast with glycolchitosan, a combination known as "a bioactive coating" (El Ghaouth *et al.,* 2000). When applied within 24 h after inoculation, the combination *Candida saitoana* with 0.2% glycolchitosan was more effective than the antagonistic yeast alone in controlling natural infection of oranges and lemons (mainly by P. *digitatum)* and the control level was equivalent to that achieved with 2000 ppm of imazalil (El Ghaouth *et al.,* 2000). The bioactive coating and Imazalil treatments offered consistent control of decay on Washington Navel oranges and Eureka lemons in early and late seasons.

The possibility of enhancing the antagonistic activity of the yeast *Rhodotorula glutinis* by addition of calcium chloride and lower cell concentration of biocontrol agent was explored. Calcium at 2% markedly enhanced the biocontrol potential of *Rhodotorula glutinis* inoculated at 10^7 and 10^6cells/ml, compared with *Rhodotorula glutinis* alone (Arras *et al.,* 1998). The enhancement in the effectiveness of biocontrol may be due to the toxicity of calcium to *P. italicum* by affecting the osmotic balance in the fungal cells and by inhibition of pectinolytic enzymes in the wound site (Arras *et al.,* 1998).

Others salt additives have also improved the bio-efficacy of some microbial antagonists in controlling postharvest decay of citrus. Among different salt additives, sodium bicarbonate, calcium propionate, sodium carbonate, potassium bicarbonate, potassium metabisulfite and ammonium molybdate etc., have been found very successful when used with microbial

antagonists for controlling postharvest diseases of fruits and vegetables more efficiently (Smilanick *et al.,* 1999; Teixidó *et al.,* 2001; Porat *et al.,* 2002; Obagwu and Korsten, 2003; Zhang *et al.,* 2004; Usall *et al.,* 2008) (**Table 1.7**). Smilanick *et al.* (1999) showed that control of green mold on oranges was maximized when dip treatments in sodium carbonate or bicarbonate were followed by the application of *P. syringae* strain ESC10, the active ingredient in the postharvest biological control BioSave™ products. Similar results were also observed with lemon fruits pretreated with sodium carbonate prior to treatment with *C. saitoana* (El Ghaouth *et al.,* 2000).

IV.4.1.2 Combination of microbial antagonists with low levels of conventional fungicides

Application of microbial antagonists by itself may not always provide commercially acceptable level of control of postharvest diseases of citrus. However, it is possible to increase the efficacy of microbial antagonists by combining with certain fungicides such as thiabendazole (TBZ) and imazalil (IMZ). Compatibility between a microbial antagonist and a synthetic fungicide offers the option of using the antagonists in combination with reduced level of the fungicide (Sharma *et al.,* 2009). For example, the combination of Aspire with thiabendazole (200 ppm) reduces the incidence of decay caused by *Penicillium digitatum* and *P. italicum* as effectively as a conventional fungicide treatment. Furthermore, Aspire is highly efficacious against sour rot caused by *Geotrichum candidum*, a decay not controlled by conventional treatment (Droby *et al.,* 1998). A combination of the yeast *Pichia guilliermondii* with reduced concentration of thiabendazole in dip application, significantly decreased the incidence of green mold to a level similar to that achieved by currently recommended concentration of TBZ application alone (Arras *et al.,* 2002). The combination of *Kloeckera apiculata* with Carbendazim at 40 ppm also resulted in improved control of citrus blue mold to a level equal to that of the commercial treatment at 200 ppm of Carbendazin (Long *et al.,* 2006**)**. Thus, such combination allows both maintaining very low level of chemical residue in the citrus fruit and improving protective properties of the treatment.

Table 1.7 : Combination of Biocontrol antagonists with other control methods

Antagonist	Combined with	Disease controlled	Reference
Combination with physical control methods :			
Candida oleophila	Hot-water	green mold	Porat *et al.*, 2002
	Ultraviolet light-C		D'hallewin *et al.*, 2004
Pseudomonas glathei	Heat treatment	green mold	Huang *et al.*, 1995
Bacillus subtilis	Hot-water	green mold	Obagwu and Korsten, 2003
Pantoea agglomerans	Heat treatment	green mold	Plaza *et al.*, 2004c
Debaryomyces hansenii	Ultraviolet light-C	green mold	Stevens *et al.*, 1997
Combination with low levels of conventional fungicides :			
Candida oleophila	Thiabendazole	Stem-end rot	Brown and Chambers, 1996
		Penicillium rots	Droby *et al.*, 1998
Pichia guilliermondii	Imazalil	Stem-end rot	Arras *et al.*, 2002
	Thiabendazole	Penicillium rots	
Kloeckera apiculata	Carbendazim	blue mold	Long *et al.*, 2006
Combination with food additives and other salts :			
Candida oleophila	Sodium bicarbonate	green mold	Porat *et al.*, 2002
Bacillus subtilis	Sodium bicarbonate	green mold	Obagwu and Korsten, 2003
Pseudomonas syringae	Sodium bicarbonate	green mold	Smilanick *et al.*, 1999
	Sodium carbonate		
Pantoea agglomerans	Sodium carbonate	green mold	Usall *et al.*, 2008
	Sodium bicarbonate		Teixidó *et al.*, 2001
Cryptococcus laurentii	Sodium bicarbonate	green mold	Zhang *et al.*, 2004
Pichia guilliermondii	Calcium chloride	green mold	Droby *et al.*, 1997
Candida saitoana	Glycolchitosan	green mold	El-Ghaouth *et al.*, 2000
	2-Deoxy-D-glucose		El-Ghaouth *et al.*, 2001
Kluyveromyces marxianus	Sodium bicarbonate	green mold	Geng *et al.*, 2011

IV.4.1.3 Combination of microbial antagonists with physical control treatments

Physical treatments could also be used in association with microbial antagonists, complementing their activity. Pathogens treated with such physical means might be weakened and become more vulnerable to the antagonist activity. Cold storage, curing, heat treatments and ultraviolet (UV) light have been the main physical methods used to improve the biocontrol efficacy of microbial antagonists (**Table 1.7**). For example, the bacterium *Pseudomonas glathei* was evaluated for its efficacy in reducing green mold decay of citrus caused by *P. digitatum*. The disease control was enhanced by treatment of citrus fruit with *P. glathei* cell suspension after heat treatment (Huang *et al.,* 1995). The antagonistic activity of *Pichia guilliermondii* against *P. digitatum* was significantly increased by combining controlled atmosphere with low storage temperature (Lurie *et al.,* 1995). Similarly, El Ghaouth *et al*. (2004) showed that the incidence of decay developed on grapefruits treated with hot water and *C. oleophila* 24 h after inoculation with *P. digitatum* was dramatically reduced in comparison to each treatment alone. Besides heat treatments, the ultraviolet light-C is also applied in association with the antagonistic yeasts *Candida oleophila* and *Debaryomyces hansenii* to control citrus green mold (Stevens *et al.,* 1997; D'hallewin *et al.,* 2004).

IV.4.2 Combination of salts and food additives with other control methods

Successful commercial control of postharvest diseases of fruits and vegetables must be extremely efficient, in the range of 95–98%, unlike the control of tree, field crop or soil borne diseases. Consistent performance to such levels of control cannot presently be achieved by alternatives to fungicides as stand-alone treatments, so strategies where they are combined are needed to attain commercially acceptable performance (Palou *et al.,* 2008). Among the strategies evaluated during the last few years, the combination of salts and food additives with low levels of conventional fungicides and physical control methods should be mentioned.

IV.4.2.1 Combination of salts and food additives with low levels of conventional fungicides

Due to the lack of preventive activity to control postharvest citrus diseases of some salt compounds, it was necessary to combine them with low levels of conventional fungicides. Several works have demonstrated the efficiency of this practice. The combination of sodium

bicarbonate with low levels of Imazalil (Smilanick *et al.*, 2005), Thiabendazole (Smilanick *et al.*, 2006b) or Pyrimethanil (Smilanick *et al.*, 2006a) improved their performance to control citrus green mold**.** Also Smilanick *et al.* (2008) reported that Green mold caused by an isolate of *P. digitatum* resistant to IMZ and TBZ was effectively controlled when potassium sorbate was added to a heated IMZ or TBZ solutions.

IV.4.2.2 Combination of salts and food additives with physical control treatments

Salts and food additives improve their efficacy when combined with curing and hot water treatments. Sorbate potassium was reported to control postharvest sour rot when it was applied as a hot water solution (Kitagawa and Kawada, 1984; Smilanick *et al.*, 2008). Palou *et al.* (2001) showed that temperature of sodium carbonate and sodium bicarbonate solutions influenced effectiveness of control of postharvest green and blue mold more than concentration or immersion period. Also of interest, Plaza *et al.*(2004b) reported that Dipping fruits in a sodium carbonate solution following a curing treatment satisfactorily reduced green and blue mold incidence during subsequent long-term storage. Besides heat treatments, salts are applied with wax coatings, a critical operation in citrus fruit packinghouses. (Youssef *et al.*, 2012) reported that wax mixed with sodium bicarbonate, potassium carbonate and potassium sorbate significantly reduced the incidence of postharvest green and blue molds.

V. Conclusion

From this review, it appears that some significant progress has been made toward biological and integrated control of postharvest diseases of citrus fruit. Some biofungicides are already on the market in a few countries, and will probably become more widely available as they are registered in more areas. Other microbial antagonists should reach the market soon. Postharvest conditions provide an ideal niche for microbial antagonists since they are less subject to sudden weather changes, and are often equipped with a sophisticated climate control system. However, so far, only a few products with high biocontrol potential have been made available on a commercial scale. With intensive research being carried out in various laboratories, the possibility of identifying potent microbes and developing suitable biocontrol products for commercial marketing appears to be bright. On the other hand, it's unrealistic to assume that microbial antagonists have the same fungicidal activity as fungicides. Optimized postharvest usage strategies of microbial antagonists include integration with other low risk treatments to optimize performance while allowing identification of methods that reduce the

use of conventional synthetic fungicides for the control of postharvest diseases of citrus fruit. In the development of these new strategies, emphasis was placed on minimizing human health risks and environmental toxicity. Research should provide appropriate tools (microbial antagonists, natural substances, GRAS compounds, etc.) to tailor a complete postharvest citrus diseases management strategy.

.

CHAPTER TWO

Screening of the antifungal activity of 43 plant extracts for the control of *Geotrichum candidum*

Publised in Crop Protection as :
Talibi, I., Askarne, L., Boubaker, H., Boudyach, E., Msanda, F., Saadi, B., Ait Ben Aoumar, A., 2012. **Antifungal activity of some Moroccan plants against *Geotrichum candidum*, the causal agent of postharvest citrus sour rot**. Crop Protection 35, 41-46.

Résumé

Les extraits aqueux de 43 échantillons de plantes récoltées dans différentes régions du sud du Maroc ont été testés pour évaluer leur pouvoir antifongique, *in vitro* et *in vivo*, contre *Geotrichum candidum*, agent causal de la pourriture amère des agrumes en post-récolte. Les extraits aqueux de ces plantes ont été évalués, d'abord, sur la croissance mycélienne de *G. candidum* en incorporant celles-ci dans le milieu de culture à base d'extrait de pomme de terre (PDA). Les résultats obtenus ont montré que la plupart des plantes testées ont réduit la croissance mycélienne de *G. candidum* avec des degrés d'inhibition qui différent selon l'espèce testée. Le meilleur degré d'inhibition de la croissance mycélienne a été obtenu avec les extraits aqueux de *Rubus ulmifolius*, *Ceratonia siliqua*, *Cistus monspeliensis* et *Halimium umbellatum* avec une inhibition totale de la croissance mycélienne de *G. candidum*. En outre, les extraits aqueux de *Cistus villosus*, *Pistacia atlantica*, *Halimium antiatlanticum*, *Inula viscosa*, *Ighermia pinifolia* et *Hammada scoparia* sont également efficaces contre *G. candidum* avec un pourcentage d'inhibition de la croissance mycélienne supérieure à 80%. Cependant, certains plantes testées dans cette étude ont stimulé la croissance de *G. candidum*, à savoir : *Mentha suaveolens*, *Psoralea bituminosa* et *Reseda alba*. L'effet des extraits aqueux des plantes les plus efficaces a été évalué aussi sur la germination des arthrospores de *G. candidum*. Les résultats obtenus ont révélé que parmi les extraits aqueux qui ont complètement inhibé la croissance mycélienne de *G. candidum*, seuls ceux de *C. villosus* et *H. antiatlaticum* ont également inhibé la germination des arthrospores à 5 et 2,5 mg/ml respectivement. Les concentrations minimales inhibitrices (CMI) et fongicides (CMF) relatives aux meilleures plantes ont été également déterminées. Les plus faibles CMI ont été enregistrées avec les extraits aqueux de *C. villosus* et *H. antiatlanticum* avec une CMI égale à 0,156 mg/ml, suivie de *C. siliqua*, *H. umbellatum* et *R. ulmifolius* avec une CMI de 0,312 mg/ml. Sur la base du screening *in vitro*, seuls les extraits qui ont réduit la croissance mycélienne de *G. candidum* de plus de 90% ont été retenus pour évaluer leur capacité à contrôler la pourriture amère. Les résultats obtenus ont montré que les extraits aqueux de sept espèces (*H. umbellatum*, *I. viscosa*, *R. ulmifolius*, *C. villosus*, *C.* siliqua, *H. antiatlanticum* et *P. atlantica*) ont significativement réduit l'incidence et la sévérité de la pourriture amère sans avoir des effets phytotoxique sur les fruits traités.

Mots clés : Activité antifongique, pourriture amère, Extraits de plantes, *Geotrichum candidu*, fruits d'agrumes.

Abstract

The powders and aqueous extracts of 43 plants species, harvested in different regions of southern Morocco, were screened for their *in-vitro* and *in-vivo* antifungal activity against *Geotrichum candidum*, the cause of citrus sour rot. Our results show that among the 43 plants tested, the powders of *Rubus ulmifolius*, *Ceratonia siliqua*, *Cistus monspeliensis* and *Halimium umbellatum* plants totally inhibited mycelial growth of *G. candidum.* Furthermore, the powders of *Cistus villosus, Pistacia atlantica, Halimium antiatlanticum, Inula viscosa, Ighermia pinifolia* and *Hammada scoparia* plants are also effective against *G. candidum* with a percent of inhibition of mycelial growth higher than 80%. The effect of plant aqueous extracts on arthrospores germination varied significantly ($P<0.05$) between tested plants. Aqueous extracts from *H. antiatlanticum* and *C. villosus* plants showed the strongest activity. The first plant has completely inhibited the arthrospores germination at 2.5 and 5 mg/ml, and the second plant at 5 mg/ml. The most active plants in *in vitro* studies were tested *in vivo* against sour rot on citrus fruit. Incidence of sour rot was lowered to 44.44 and 46.30 % when mandarin fruit where treated by *C. villosus* and *H. antiatlanticum* aqueous extracts, compared with 98.15% in the control. This study demonstrates that plants extracts have a high potential to control sour rot of citrus. Such natural products therefore represent a sustainable alternative to the use of chemical pesticides.

Keywords : Antifungal activity, sour rot, plant extracts, *Geotrichum candidum*, Citrus fruit.

I. Introduction

Citrus fruit cultivation is very important in Morocco, being the first exporting agricultural sector and playing a major role in the national economic development. The largest volume of citrus fruit for fresh consumption and export in the morocco is grown and shipped from packing houses in Souss-Massa-Draa (SMD) region (Boubaker *et al.,* 2009). The citrus fruits are exposed to many postharvest diseases during fruit processing, among which sour rot, caused by the yeast like fungus *Geotrichum candidum* Link ex Pers., is responsible for economically significant losses under favorable conditions for pathogen development, principally during fruit degreening and wet seasons (Eckert, 1978; Eckert and Brown, 1988; Cohen *et al.,* 1991). The decay is, also, more prevalent as fruit increases in maturity (Brown, 1979; Suprapta *et al.,* 1995). Fruits are infected by the fungus only through wounds. At favorable temperatures of 25-30°C, fruit will rot completely in 4 or 5 days, and the disease can spread by contact. In addition to citrus fruit, *G. candidum* is the causal agent of numerous decays of other fruits and vegetables like tomato, cucumber, carrot and stone fruit (Wells, 1997; Suprapta *et al.,* 1995; Palou *et al.,* 2009). The isolate which exhibits pathogenicity to citrus fruit was designated as *G. candidum* citrus race (Butler *et al.,* 1965) or *G. citri-aurantii* (Ferr.) Butler (Butler *et al.,* 1988). Sour rot is not controlled by postharvest chemical treatments, mainly imazalil and thiabendazole (Mercier and Smilanick, 2005), and only controlled by Guazatine (Brown, 1988), a chemical that is not authorized in several countries. The disease can be partially reduced by careful handling, harvesting of fruit before it becomes over-mature, and reducing fruit storage temperatures after harvest (Eckert and Brown, 1988).

The SMD region is one of the richest regions in Morocco in terms of plant diversity (Peltier, 1982; Msanda *et al.,* 2005). This diversity in flora provides a rich source of natural bioactive substances. Indeed, the scientific interest in these substances has increased today with the search of new antifungal compounds from plant source, due to increased resistance of postharvest fungal pathogens to fungicides, and growing concern for human safety and the protection of the environment (Suhr and Nielson, 2003; Smilanick *et al.,* 2008; Casals *et al.,* 2010). Plant extracts are very attractive as alternative or complementary control means because of their antifungal activity, non-phytotoxicity, systemicity, and biodegradability (Tripathi and Dubey, 2004; Kosalec *et al.,* 2005; Ameziane *et al.,* 2007; Gatto *et al.,* 2011). Limited information is available on the antifungal activity of plant extracts against *G. candidum.*

This study was aimed at the valorization of natural floral resources of SMD Valley, south of Morocco, by *in vitro* and *in vivo* screening of their antifungal activity against *Geotrichum candidum*, the causal agent of citrus sour rot.

II. Materials and methods

II.1 Collection of Plant Samples

Forty three fresh plant samples were collected from different locations in the Souss Massa valley (south of Morocco) between March and April 2008 and 2009. All samples were identified, and were deposited in the herbarium of the laboratory of Biotechnology and Valorization of the Natural Resources, Faculty of Sciences, Ibn Zohr University, Agadir, Morocco. Plant samples were cleaned, air dried in the shade, then grounde to a fine powder using a laboratory grinding mill (Polymix PX-MFC 90D, Switzerland) and stored in the dark at 4°C until use. The botanical name, family, and parts used of plant samples are summarized in **Table 2.1.** The 43 vascular plant species belong to 36 genera and 16 botanical families.

II.2 Preparation of plant extracts

Preparation of the plant samples for the *in vitro* antifungal screening against *Geotrichum candidum* was conducted as previously described by Ameziane *et al.* (2007). In brief, 10 grams of powders of each sample were added to 100 ml of molten Potato Dextrose Agar (PDA) medium. The resulting suspensions were stirred for 10 min, autoclaved for 15 min and subsequently filtered through four layers of sterile cheesecloth before being dispensed into 9-cm diameter Petri plates (Figure 2.1).

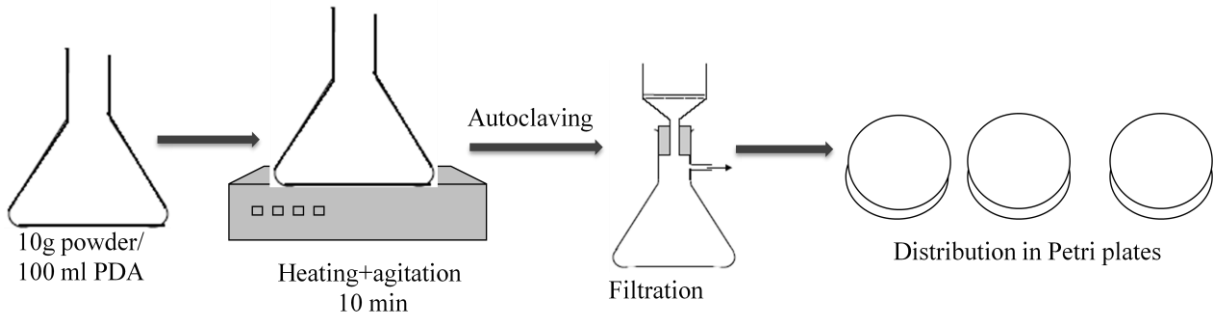


Figure 2.1 : Preparation of aqueous plant extracts for the *in vitro* antifungal screening

For the other tests (germination assay, Determination of MIC and MFC and the *in vivo* assay), aqueous plant extracts were used and prepared according to the method described by Abdel-Monaim *et al.* (2011). Ten grams of each plant powder were extracted by soaking in 100 ml

of boiling distilled water for 10 min in a magnetic hotplate stirrer. The aqueous extracts were then filtered through a Whatman no.1 filter paper, lyophilized, and stored at -20°C until use. Appropriate weights of each extracts were prepared in sterile distilled water to obtain the various concentration used for the experiments.

II.3 Pathogen

Geotrichum candidum was isolated from a decayed mandarin fruit and has been one of the most aggressive isolates in our collection. This isolate was deposited at the laboratory under the code SR10. The fungus was maintained on PDA plates at 5°C, with periodic transfers through citrus fruit to maintain its aggressiveness.

The pathogen inoculum consisted of aqueous arthrospores suspensions obtained from 7-days-old cultures plates incubated at 25°C. Arthrospores were harvested by flooding plates with 5 ml of sterile distilled water containing 0.05% (v/v) Tween 80, and passing the suspension through two layers of sterile cheesecloth to remove hyphal fragments. The arthrospore concentration was determined with the aid of a heamocytometer and adjusted to 10^6 arthrospores ml^{-1} with sterile distilled water.

II.4 Fruit

The fruit of mandarin (*Citrus reticulata* blanco) cv. "Clementine" was used. Fruits were harvested from orchards of the M'brouka cooperative, which used standard culture practices, in Souss-Massa-Draa region, south of Morocco. Only healthy and commercially mature fruits were used in the *in vivo* test. Freshly harvested or shortly stored (no longer than a week) fruits were used in the experiment.

II.5 Evaluation of antifungal activity of plant extracts

II.5.1 In vitro effect on mycelial growth of *G. candidum*

The incorporation method was used for determining the inhibition of mycelial growth of *G. candidum* by the plant powders. As described above, plant powders were tested at a concentration of 10% (w/v). Plates were inoculated with *G. candidum*, using a 5-mm diameter agar disk taken from one-week-old cultures, mycelium surface facing down. The agar plates were then incubated at 25°C for seven days. Control consisted of unamended PDA medium. Radial growth was determined by measuring colony size along two perpendicular axes. The antifungal activity was expressed in terms of percentage of mycelial growth inhibition and calculated according to the following formula :

% mycelial growth inhibition (MGI) = [(control diameter - plant extract diameter)/control diameter] ×100. Three replicate plates were used for each treatment.

To determine whether plant extracts have fungistatic or fungicidal effect on *G. candidum,* Plugs from treatment with no growth were transferred to unamended PDA medium, treatment in which mycelial growth did not occur after additional seven days of incubation were considered fungicidal (Alilou *et al.,* 2008). The experiments were performed twice.

II.5.2 Effect of aqueous extracts on arthrospores germination

The germination of arthrospores of *G. candidum* was determined in concentrations of 0.625, 1.25, 2.5, and 5 mg. ml^{-1} of aqueous extract for the plant that allowed a higher mycelial growth inhibition. Aliquots (40µl) of a arthrospores suspension (10^6 arthrospores. ml^{-1}) were aseptically transferred in triplicate to sterile depression slides containing 40 µl of 2 % sterile orange juice amended with different concentrations of aqueous extracts. Inoculated slides were placed on moist filter paper in Petri plates, sealed with Parafilm to avoid evaporation, and then incubated at 25°C for 24h. Each slide was then fixed with acid fuchsine solution to stop further germination (Smilanick *et al.,* 1999). Arthrospores germination was estimated under a microscopic equiped with a micrometer. At least 100 arthrospores within each replicate were observed. An arthrospore was scored as germinated if the germ tube length was equal or exceed to at least one time that of the spore body (Suprapta *et al.,* 1997). The results were expressed as percent spore germination inhibition and calculated by using the following formula : **GI (%) = [(Gc−Gt) /Gc)] ×100**, Gc and Gt represent the mean number of germinated spores in control and treated slides, respectively (Soylu *et al.,* 2010). Each treatment included three replicates and the experiment was conducted twice.

II.5.3 Determination of Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The minimum inhibitory concentrations (MICs) of the plant extracts were determined by the agar dilution method. Aqueous sterile plants extracts were first diluted to the highest concentration to be tested (5 mg/ml), and then serial two-fold dilution were made in a concentration range from 5 to 0.3125 mg/ml in 10 ml test tubes containing melted PDA medium. Culture plates with 24 microwells and a capacity of 4 ml per well were used. Aqueous plant extracts amended medium was dispended in the wells of culture plates (2 ml per well). Aliquots (10 µl) of an arthrospores suspension (10^6 spores/ ml) were then dropped onto the surface of the agar medium. Culture plates were incubated at 24°C (±1°C) for 48 h.

The MICs were recorded by reading the lowest aqueous extract concentration that allowed no visible growth of the pathogen (Phongpaichit *et al.*, 2005).

The minimum fungicidal concentrations (MFCs) were determined by taking agar plugs from well showing no visible mycelial growth and re-inoculating them on unamended PDA medium. MFC was regarded as the lowest concentration of the aqueous plant extract that prevented growth of the pathogen after the period of incubation, and indicating more than 99.5% killing of the original inoculums (Fabry *et al.*, 1996; Ali-Shtayeh and Abu Ghdeib, 1999). There were three replicates for each plant aqueous extract at each concentration and the experiment was conducted twice.

II.5.4 Effects of aqueous extracts on sour rot development in artificially inoculated and wounded fruit

Based on *in vitro* screening, only plant species that reduced the mycelial growth of *G. candidum* by more than 90% were retained. Mandarin fruits were washed, disinfected with 0.1% (v/v) sodium hypochlorite, rinsed three times in sterile distilled water and then air dried before wounding. One wound (2mm deep and 4mm wide) was made per fruit using a sterile needle at the equatorial side (Liu *et al.*, 2009b). The wounds were treated with 30 µl of aqueous plant extracts at a concentration of 50mg/ml. Controls were treated with the same volume of sterile distilled water under the same conditions. After two hours incubation at room temperature, each wound was inoculated with 20 µl of an aqueous suspension of arthrospores of *G. candidum* (10^6 arthrospores. $ml^{-1)}$. Treated fruits were placed on plastic tray in carton boxes and incubated at 26°C and 95% relative humidity (RH) (**Figure 2.2**). The number of the infected wounds and the lesion diameters of the overall treated fruit were determined daily. All treatments were arranged in a complete randomized block design. Eighteen fruits constituted a single replicate and each treatment was replicated three times. The experiment was conducted twice. The incidence and severity of disease were calculated as follows : **Disease incidence** (%) = [(number of rotten wounds / number of total wounds)] x 100.

Disease severity (%) = [(average lesion diameter of treatment / average lesion diameter of control)] x 100. In all experiments, the possible phytotoxic effect on mandarin fruit was examined.

II.6 Statistical analysis

All data were subjected to statistical analysis of variance (ANOVA) using STATISTICA software, version 6, Stat-Soft, 2001, France. Percentage values were subjected to arcsine-square root transformation before analysis of variance. Mean separation was performed following the Newman & Keuls test at $P < 0.05$.

Figure 2.2 : *In vivo* test of selected aqueous extracts : **a)** Injury of Mandarin fruits, **b)** Inoculation and treatment of fruits and **c)** incubation in growing room at 25°C and 95% HR

III. Results

III.1 *In vitro* effect on mycelial growth of *G. candidum*

Antifungal activity of 43 vascular plant species belonging to 16 botanical families (**Table 2.1**) has been evaluated *in vitro* against *G. candidum,* the causal agent of sour rot of citrus fruits. The powders of most tested plant species reduced colony growth of the fungus (**Table 2.1**). However, the inhibitory effect varied between plant species. The *in vitro* screening of plant powders has uncovered significant antifungal activity of *R. ulmifolius, C. siliqua, C. monspeliensis* and *H. umbellatum* plants. Indeed, these plants had completely (100 %) inhibited the mycelial growth of *G. candidum*. Some other plant powders from *C. villosus, P. atlantica, H. antiatlanticum, I. viscosa, I. pinifolia* and *H. scoparia* showed a higher degree of control (MGI> 80%; **Table 2.1** and **Figure 2.3**).

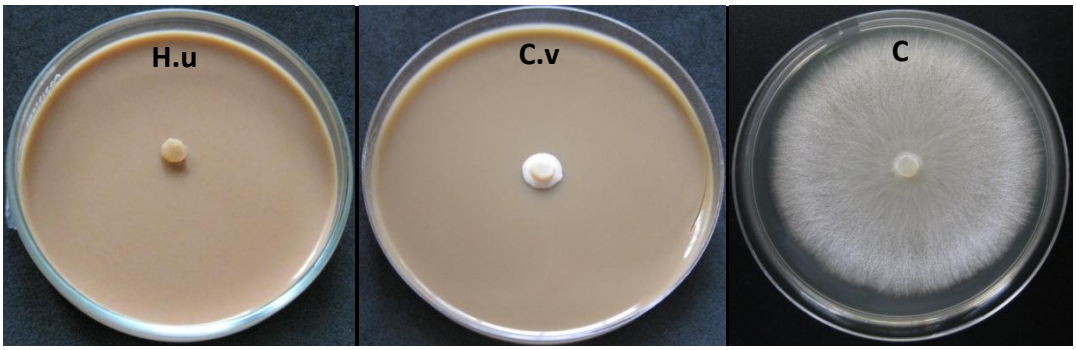


Figure 2.3 : Inhibition of mycelial growth of G. *candium* by aqueous extracts of *H. umbellatum* (H.u) and *C. villosus* (C.v) compared with the control (C).

According to their antifungal activity the tested plants were divided into three groups :

The first group combines the best plants (7 plants) for which the MGI percentage is greater than 95%. Among these plants, four (57%) belong to the family of Cistaceae (viz., *C. monspeliensis, C. villosus, H. umbellatum* and *H. antiatlanticum*). The second group includes plants for which the MGI percentage is between 87% and 52%. This group is represented by 13 plant species belonging to the families of Asteraceae (viz., *I. pinifolia, I. viscosa, A. radiata, A. graveolens*), Zygophyllaceae (viz., *F. harpago, Z. gaetulum, F. zilloides*), Chenopodiaceae (viz., *H. scoparia*), Lamiaceae (viz., *T. werneri, T. antiatlanticum*), Rosaceae (viz., *C. monogyna*), Boraginaceae (viz., *T. calcarata*) and Cistaceae (viz., *C. creticus*). The third group includes plants with a MGI percentage inferior to 45%, and some plants even enhanced the mycelial growth of *G. candidum* (e.g. *M. suaveolens, P. bituminosa, P.*

scoparius, *N. retusa*, and *R. alba*). These results indicate that the plant powders contain compounds with antifungal potential against *G. candidum*.

None of plants which had completely inhibited the mycelial growth of the fungus were found to be fungicidal at 10% (w/v); since plugs transferred from plant powder amended PDA to unamended PDA grow after additional 7 days incubation at 25°C (data not shown).

Table 2.1 : *In vitro* effects of plant powders on mycelial growth (MG) of *Geotrichum candidum,* agent of citrus sour rot

Family	**Scientific name**	**Part used**	**M.G. I %**
Rosaceae	*Rubus ulmifolius* Schott	Leaves+stem	100 ±0 **a**
Fabaceae	*Ceratonia siliqua* L.	Leaves	100 ±0 **a**
Cistaceae	*Cistus monspeliensis* L.	Leaves+stem	100 ±0 **a**
Cistaceae	*Halimium umbellatum* (L.) Spach	Leaves+stem	100 ±0 **a**
Cistaceae	*Cistus villosus* Auct.	Leaves+stem	98.26 ±0.07 **a**
Anacardiaceae	*Pistacia atlantica* Desf.	Leaves	97.68 ±0.04 **a**
Cistaceae	*Halimium antiatlanticum* Maire & Wilczek	Leaves+stem	95.94 ±0.04 **a**
Asteraceae	*Inula viscosa* (L.) Aiton	Leaves+stem	87.11 ±0.16 **b**
Asteraceae	*Ighermia pinifolia* (Maire & Wilczek) Wiklund	Leaves+stem	85.49 ±0,04 **b**
Chenopodiaceae	*Hammada scoparia* (Pomel) Iljin	Leaves+stem	80.39 ±0,09 **bc**
Asteraceae	*Anvillea radiata* Coss. Durieu	Leaves+stem	75.71±0 **cd**
Asteraceae	*Asteriscus graveolens* Forssk. Less.	Leaves+stem+Flowers	71.9 ±0.11 **cde**
Zygophyllaceae	*Fagonia harpago* Emb. Maire	Leaves+stem	70.98 ±0.09 **cde**
Zygophyllaceae	*Zygophyllum gaetulum* Emb. Maire	Leaves+stem	68.23 ±0.2 **def**
Lamiaceae	*Teucrium werneri* Emb.	Leaves+stem	67.55 ±0.11 **def**
Zygophyllaceae	*Fagonia zilloïdes* Humbert	Leaves+stem	65.78 ±0.04 **def**
Rosaceae	*Crataegus monogyna* Jacq.	Leaves+stem	61.96 ±0.04 **efg**
Lamiaceae	*Teucrium antiatlanticum* (Maire) Sauvage & Vindt	Leaves+stem	61.78 ±0.09 **ef**
Boraginaceae	*Trichodesma calcarata* Coss. Ex Batt.	Leaves+stem+Flowers	58.04 ±0.04 **fg**
Cistaceae	*Cistus creticus L.*	Leaves+stem	52.46 ±0.09 **g**
Asteraceae	*Artemisia inculta Delile*	Leaves+stem	44.44 ±0.09 **h**

Lamiaceae	*Lavandula coronopifolia* Poir.	Leaves+stem+Flowers	42.86 ±0.33 ^{h}
Lamiaceae	*Thymus satureioides* Coss.	Leaves+stem+Flowers	30 ±0.2 ^{ij}
Lamiaceae	*Lavandula stoechas L.*	Leaves+stem+Flowers	28.8 ±0.34 ^{ij}
Lamiaceae	*Thymus leptobotrys* Murb.	Leaves+stem	27.84 ±0.09 ^{ij}
Lamiaceae	*Marrubium deserti* (de Noé) Coss.	Leaves+stem	20.78 ±0.22 ^{jk}
Lamiaceae	*Ballota hirsuta* Benth.	Leaves+stem	20.44 ±0.98 ^{jk}
Plumbaginaceae	*Limoniastrum ifniense* (Caball.) Font Quer	Leaves + stem	15.71 ±0.07 ^{kl}
Rutaceae	*Ruta montana* L.	Leaves+stem	15.11 ±0.16 ^{kl}
Anacardiaceae	*Rhus pentaphylla* (Jacq.) Desf.	Leaves+seeds	14.76 ±1.09 ^{kl}
Rutaceae	*Ruta tuberculata* Forssk.	Leaves+stem	12.44 ±0.29^{klm}
Fabaceae	*Ononis natrix* L.	Leaves+stem	12 ±0.07 ^{klmn}
Asteraceae	*Cotula cinerea Delile*	Leaves+stem+Flowers	8.44 ±0.16 ^{lmno}
Thymelaeaceae	*Thymelaea antiatlantica* Maire	Leaves+stem	0 ±0 ^{no}
Fabaceae	*Lupinus angustifolius* L.	Leaves+stem	0 ±0 ^{no}
Asteraceae	*Pulicaria mauritanica Batt.*	Leaves+stem+Flowers	0 ±0 ^{o}
Capparaceae	*Maerua crassifolia* Forssk.	Leaves	0 ±0 ^{no}
Solanaceae	*Witania adpressa* Coss. Ex Batt.	Leaves	0±0 ^{no}
Lamiaceae	*Mentha suaveolens* Ehrh	Leaves+stem	-6.67±0 ^{o}
Fabaceae	*Psoralea bituminosa L.*	Leaves+stem	-6.67±0 ^{o}
Apiaceae	*Pituranthos scoparius* (Coss. Durieu) Benth. & Hook	Leaves+stem+Flowers	-6.67±0 ^{o}
Zygophyllaceae	*Nitraria retusa* Forssk. *Asch*	Leaves+stem	-19.42±0.18 ^{p}
Resedaceae	*Reseda alba* L.	Leaves+stem+Flowers	-21.74±0 ^{p}

[a]Values are means of three replicates ± standard deviation. Values followed by the same letters were not significantly different (P <0.05) according to Newman and Keels test. The negative values indicate a profungal activity against *G. candidum*.

III.2 Effect of aqueous extracts on arthrospores germination

The results shown in **Table 2.2** indicate that of the plant species tested; only the aqueous extracts of *H. antiatlanticum* and *C. villosus* at 5 mg/ml had the highest fungistatic effect (100% inhibition) on arthrospores germination of *G. candidum*. Indeed, tested at 2.5 mg/ml, the aqueous extracts of *H. antiatlanticum* and *C. villosus* have inhibited the

arthrospore germination by 100 and 75%, respectively. At a concentration of 1.25 mg/ml, the toxicity of aqueous extract of *C. villosus* to arthrospores was higher to that of the other tested plants. Since percent inhibition of germination in aqueous extract amended with 2 % sterile orange juice is higher than 70%. Tested at 5 mg/ml, the aqueous extract of *I. viscosa* reduced the percentage of arthrospores germination by more than 50%. The results obtained showed that the aqueous extracts of the others tested plants did not prevent or have slightly reduced arthrospore germination of *G. candidum,* even at the highest concentration tested (5mg/ml). Aqueous extracts from *C. siliqua* and *R. ulmifolius* reduced arthrospores germination of *G. candidum* by 32.67 and 15.0 %, respectively (**Table 2.2**).

Table 2.2 : *In vitro* effect of some plants aqueous extracts on arthrospore germination of *Geotrichum candidum*

Plant species	**[a]Arthrospore germination inhibition (%)**			
	Concentration of aqueous extracts (mg/ml)			
	0.625	**1.25**	**2.5**	**5**
Halimium umbellatum	0.0± 0 ^{hi}	18.0 ± 2 ^{ef}	17.67 ± 2.33 ^{ef}	30.67 ± 1.67 ^{d}
Halimium antiatlanticum	4.67 ± 2.67 ^{fjh}	20.67 ± 1.67 ^{e}	**100.0 ± 0 ^{a}**	**100.0 ± 0 ^{a}**
Pistacia atlantica	0.0 ± 0 ^{hi}	0.0 ± 0 ^{hi}	0.67 ± 0.67 ^{hi}	1.33 ± 1.33 ^{hi}
Cistus villosus	20.33 ± 1.83 ^{e}	70.67 ± 1.83 ^{b}	75.67 ± 2.17 ^{b}	**100 ± 0 ^{a}**
Hammada scoparia	0.0 ±0 ^{hi}	0.0 ±0 ^{hi}	2.0 ± 0 ^{hi}	2.67 ± 0.67 ^{hi}
Rubus ulmifolius	0.0 ± 0 ^{hi}	6.67 ± 1.67^{fjh}	8.67 ± 0.83 ^{fjh}	15.0 ± 2.5 ^{fj}
Inula viscosa	1.33 ± 0.67 ^{hi}	2.67 ± 0.67 ^{hi}	20.67 ± 0.83 ^{e}	52.33 ± 0.83 ^{c}
Ceratonia siliqua	2.0 ± 1 ^{hi}	6.67 ± 1.67 ^{fjh}	9.33 ± 2.67 ^{jh}	32.67 ± 1.83 ^{d}
Control	0.0 ± 0 ^{hi}	0.0 ± 0 ^{hi}	0.0 ± 0 ^{hi}	0.0 ± 0 ^{hi}

[a]Each value represents the mean of three replicates ± standard deviation . Means followed by different letter (s) in each column are significantly different at $P < 0.05$.

III.3 . *MIC and MFC*

The MIC and MFC values of selected plant extracts are shown in **Table 2.3**. The MIC value for *C. villosus and H. antiatlanticum* aqueous extracts against *G. candidum*, was particularly low (0.156 mg/ml). On the other hand, the MIC value obtained by using aqueous extracts of *H. umbellatum, R. ulmifolius* and *C. siliqua* was 0.3125 mg/ml. *P. atlantica* and *H.*

scoparia aqueous extracts were the least effective for restricting *in vitro* mycelial growth (MIC>5). However, all tested plant aqueous extracts have a MFC value higher than 5mg/ml (**Table 2.3**).

Table 2.3 : Minimal inhibitory concentrations (MICs) and minimal fungicidal concentrations (MFCs) for the plant extracts investigated against *Geotrichum candidum*

Treatment	**MIC[a] (mg/ml)**	**MFC[b] (mg/ml)**
Halimium umbellatum	0.3125	>5
Rubus ulmifolius	0.3125	>5
Ceratonia siliqua	0.3125	>5
Cistus villosus	0.156	>5
Halimium antiatlanticum	0.156	>5
Inula viscosa	2.5	>5
Anvillea radiata	5	ND
Pistacia atlantica	>5	ND
Hammada scoparia	>5	ND

[a]Concentration that were fungistatic
[b]Concentration that were fungicidal
ND : not determined

III.4 . *In vivo* test

Data presented in the **figure 2.4** showed that all tested aqueous plant extracts significantly reduced the incidence of sour rot caused by *G. candidum* under the laboratory conditions. Percentages of rotted wounds were decreased by using all tested plant extracts compared with control. Mandarin fruit treated 2 hours before pathogen inoculation by *C. villosus* and *H. antiatlanticum* aqueous extracts resulted in the highest reduction in rot incidence compared with the control. *H. umbellatum*, *I. viscosa*, *C. siliqua*, and *P. atlantica* aqueous extracts had a moderate effect on sour rot, the percentage of rot incidence varying between 55.56 and 61.11 %. In contrast, the aqueous extract of *R. ulmifolius* showed the least effect on reduction of sour rot incidence (**Figure 2.4** and **Figure 2.6**). Also, data indicated that tested aqueous plant extracts exhibited significant reduction of disease severity compared with the control (**Figure 2.5** and **Figure 2.6**). They reduced disease severity from 98.5% in non-treated fruit to a minimum of 32% in *C. villosus* treated fruit (**Figure 2.5**). Furthermore, neither of the plants aqueous extracts showed any phytotoxic reaction on treated fruit, at the concentration tested (50 mg/ml).

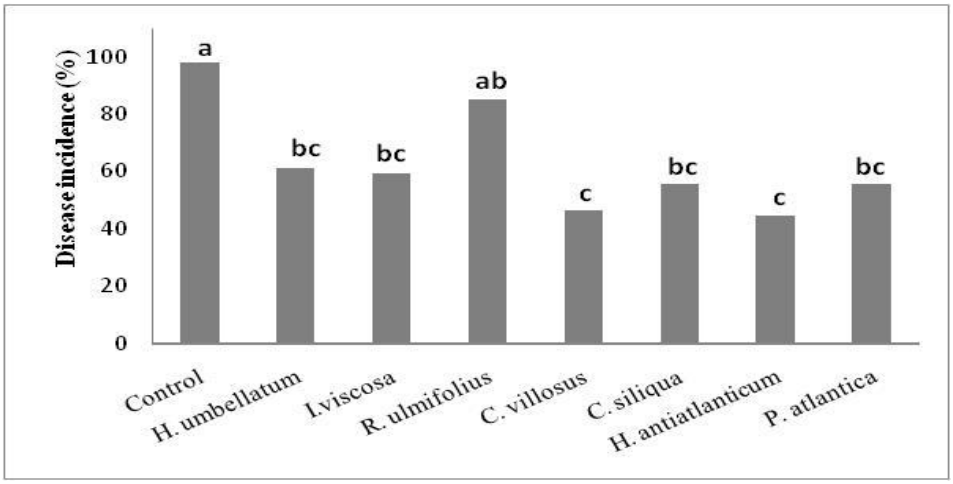


Figure 2.4 : Effect of aqueous plant extracts on sour rot incidence in mandarin fruit. Fruits were treated with aqueous extracts, inoculated with *G. candidum* and held for 7 days at 26°C. Significant differences (P < 0.05) between means were indicated by different letters above histogram bars.

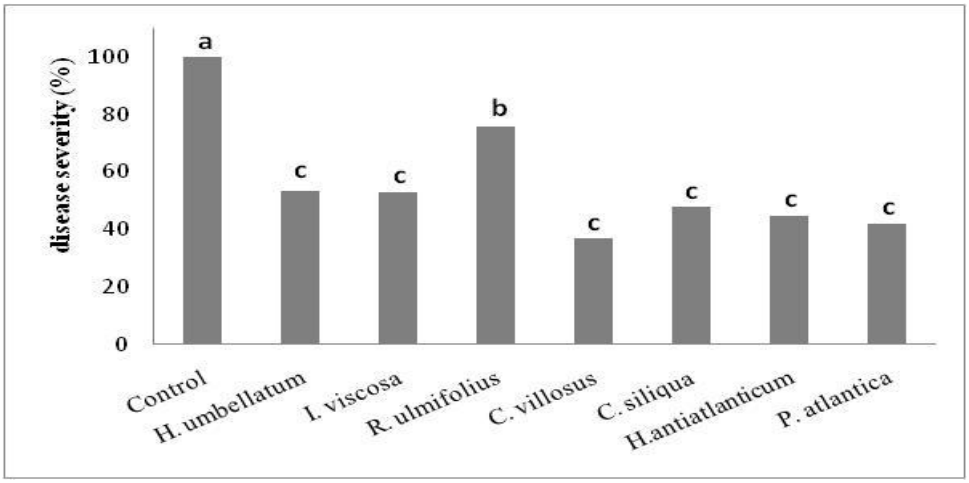


Figure 2.5 : Effect of aqueous plant extracts on sour rot severity in mandarin fruit. Fruits were treated with aqueous extracts, inoculated with *G. candidum* and held for 7 days at 26°C. Significant differences (P < 0.05) between means were indicated by different letters above histogram bars.

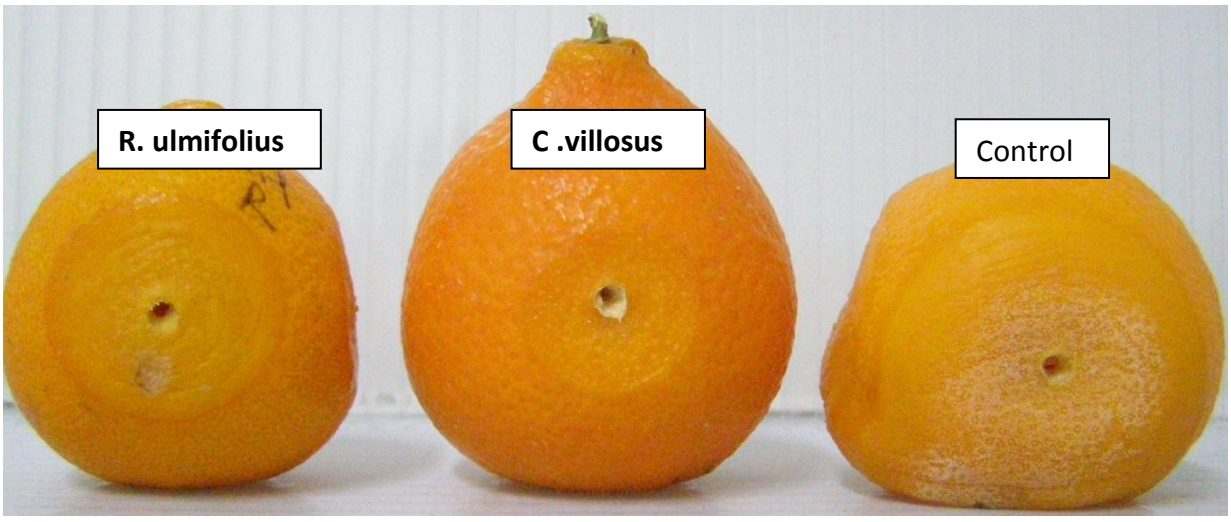


Figure 2.6 : Mandarin fruits treated with aqueous extracts of *R. ulmifolius* (R.u) and *C. villosus* (C.v) and inoculated with arthrospores suspension of *G. candidum*, after seven days of incubation.

IV. Discussion

The disease control strategies which have been studied as an alternative to currently used fungicides for the control of postharvest citrus fruit rots usually include the use of new fungicides (Smilanick *et al.,* 2006a; Kanetis *et al.,* 2007), antagonistic microorganisms (El-Ghaouth *et al.,* 2000; Taqarort *et al.,* 2008), and plant extracts (Ameziane *et al.,* 2007; Du Plooy *et al.,* 2009). In recent years, several studies have been focused on screening of plant extracts to develop new antifungal compounds that can be used to control postharvest citrus diseases (Arras and Usai, 2001; Soylu *et al.,* 2005). In this study the antifungal activity of crude and aqueous extracts of 43 plants species was evaluated against *G. candidum* under both *in vitro* and *in vivo* conditions.

The findings showed that most of tested plant powders reduced *in vitro* mycelial growth of *G. candidum* with different degrees depending on the type of plant species used. The response of pathogen to 10% (w/v) plant powders varied from a 21.74% promotion of mycelial growth to 100% inhibition. Several higher plants, belonging to different botanical families, have been found to possess excellent antifungal activity against mycelial growth of different fungal pathogens (Qasem and Abu-Blan, 1996; Arras and Usai, 2001; Wang *et al.,* 2004; Neri *et al.,* 2006). Previous reports mentioned that the effect of plant extracts on fungal pathogens may be attributed to their secondary metabolites (e.g., alkaloids, phenolic, flavonoids and terpenoids compounds) with known antifungal activity (Alilou *et al.,* 2008; Mohamed and El-Hadidy, 2008;).

The present study pointed out that plant powders may have potential antifungal activity and widened the list of allelopathic plants to *G. candidum* growth. Indeed, similar evaluation of 21 aromatic and medicinal plant species from Morocco showed that 14 of them reduced *in vitro* growth of *G. candidum* by more than 20%. Among them, powders from five plants (e.i, *Eucalyptus globulus*, *Juglans regia*, *Peganum harmala*, *Thymus leptobotrys* and *Cistus villosus*) completely inhibited mycelial growth of the pathogen (Ameziane *et al.,* 2007). The remainder of the plant powders tested exhibited much lower antifungal activities against *G. candidum*, and some even enhanced the mycelial growth of pathogen. A stimulatory effect on mycelial growth of *G. candidum* induced by some plant powders (e.g., *Lavandula multifida*, *Mentha pulegium*, and *M. rotundifolia*) was also reported by Ameziane *et al.* (2007).

Also of interest, we found that the powders of *R. ulmifolius* and *C. siliqua* completely inhibited the mycelial growth of *G. candidum.* In a previous study, Sisti *et al.* (2008) found

that the crude extract of *R. ulmifolius* possess potent antifungal activity against a wide range of human pathogens. According to these authors, the antifungal activity of the crude *R. ulmifolius* extract may be ascribed to the high content of tannins. Ben-Hsouna *et al.* (2011), have demonstrated the capacity of *C. siliqua* essential oil to control a panel of fungal strains, particularly, fungi responsible in biodeterioration of food during postharvest processing. Therefore, the antifungal activity of these plants extracts against postharvest decay microorganisms of citrus fruits needs to be investigated.

The *in vitro* trials in this study showed that *H. umbellatum, I. viscosa, R. ulmifolius, C. villosus, C. siliqua, H. antiatlanticum* and *P. atlantica* aqueous extracts gave consistent antifungal activity against *G. candidum*. In particular, *C. villosus* and *H. antiatlanticum* extracts proved to be the best mycelial growth and arthrospores germination inhibitors of the pathogen. Antifungal activity against *G. candidum* has previously been shown in trials *in vitro* or *in vivo* by some plant extracts and essential oils (Suprapta *et al.*, 1997; Liu *et al.*, 2009b), while as far as is known, *H. umbellatum, I. viscosa, R. ulmifolius, C. siliqua, H. antiatlanticum* and *P. atlantica* aqueous extracts have not been tested against this pathogen.

The effect of different plant powders and aqueous extracts was different according to the stage of development of *G. candidum.* Results revealed that among plants which completely inhibited mycelial growth of *G. candidum,* only *C. villosus* and *H. antiatlanticum* have inhibited strongly the arthrospores germination of the pathogen. This confirms that arthrospores germination is less sensitive to these plant extracts compared with mycelial growth. This finding is in agreement with that reported by Garduno-Pizana *et al.* (2010) for other plant extracts against *Fusarium oxysporum* f.sp. *gladioli*.

The aqueous extracts from *H. antiatlanticum* and *C. villosus* completely inhibited *G. candidum* arthrospores germination, whereas that of *I. viscosa, C. siliqua, H. umbellatum,* and *R. ulmifolius* reduced spores germination by 52.33, 32.67, 30.67 and 15.0%, respectively. Suprapta *et al.* (1997), reported that among three groups of volatile compounds tested, only alcohols and aldehydes compounds inhibited, by more than 50%, mycelial growth and arthrospores germination of *G. candidum*; while esters had no effect. These two crucial steps of the infection cycle of *G. candidum*, were inhibited by *C. villosus* and *H. antiatlanticum* to a high extent compared with others tested plants.

The MIC for *H. antiatlanticum* and *C. villosus* aqueous extracts against *G. candidum* was particularly low (0.156 mg/ml), followed by C. *siliqua, H. umbellatum,* and *R. ulmifolius*

aqueous extracts (0.3125 mg/ml). This antifungal effect was comparable to that reported by other findings. Bouamama *et al.* (2006) reported that leaf extracts of *C. villosus* and *C. monspeliensis* show antimicrobial properties with MIC values ranging from 0.78 to 50 mg/ml for bacteria (e.g., *Staphylococcus aureus* and *Pseudomonas aeruginosa)* and 0.19 to 200 mg/ml for yeasts and fungi (e.g., *Aspergillus fumigatus, Candida albicans, C. krusei* and *C. glabrata).* In the same study, the authors showed that *C. villosus* extracts exhibited more interesting antimicrobial activity than *C. monspeliensis (*Bouamama *et al.,* 2006). The genus *Halimium* is known to be rich in diterpene compounds (Urones *et al.,* 1995; Rodilla *et al.,* 1998), with antimicrobial activities (Kuzma *et al.,* 2007; Weckesser *et al.,* 2007). In previous studies, the antimicrobial active components from *P. lentiscus,* a species closely related to *P. atlantica,* were characterized as α-Pinene (Magiatis *et al.,* 1999), β-myrcene, carvacrol (Djenane *et al.,* 2011), verbenone, α-terpineol, linalool (Koutsoudaki *et al.,* 2005) and flavonic compounds (Benhammou *et al.,* 2008).

Although *in vitro* tests of plant extracts is an important first step in selecting plants with antifungal potential against postharvest citrus pathogens, *in vivo* tests are needed to check whether the positive results of the *in vitro* tests can be obtained, too (Gorris and Smid 1995; Tegegne *et al.,* 2008). Results obtained in this study indicated that aqueous extracts of seven plant species viz., *H. umbellatum*, *I. viscosa*, *R. ulmifolius*, *C. villosus*, *C. siliqua*, *H. antiatlanticum* and *P. atlantica* significantly decreased the incidence and severity of sour rot, without causing phytotoxic effects. In most commercial packing houses in SMD, pre-storage chemical control with guazatine is commonly applied, as postharvest drench, to reduce the incidence of sour rot in citrus fruit that are stored in cold before processing. However, this fungicide is not registered in several countries. According to Hao *et al.* (2010), the lack of registered fungicide in many countries for sour rot control is becoming a serious problem. In this study, mandarins treated with aqueous extracts of *C. villosus* and *H. antiatlanticum* plants were highly effective in reduction of sour rot incidence, compared with non-treated fruit. Several studies have tested different plants extracts in controlling the same pathogen or others and found similar effects (Neri *et al.,* 2006; Ribera *et al.,* 2008; Yahyazadeh *et al.,* 2008; Liu *et al.,* 2009b).

Treating mandarins with aqueous extracts of the seven plants significantly reduced the lesion diameters. However, none of these plant extracts have completely controlled the sour rot development. We found no publication on the antifungal activity of the selected plant species. Considering antifungal activity of *H. umbellatum*, *I. viscosa*, *R. ulmifolius*, *C.*

villosus, C. siliqua, H. antiatlanticum and *P. atlantica* against *G. candidum*, it is possible that extracts of these plants can be used as natural control agents. Most of these plants are widely distributed in SMD Valley and easy grown. Furthermore, the extraction method is simple using the most available solvent (water). Moreover, the use of a crude plant extract to protect citrus fruit against fungal attack may be acceptable in the organic production of citrus fruit.

V. Conclusion

This work is a part of an overall study that aims to determine the antifungal activity of natural floral resources of south of Morocco, against major postharvest citrus fungal pathogens. Among the 43 plants tested *C. villosus, H. antiatlanticum, H. umbellatum, P. lentiscus* and *I. viscosa* showed high antifungal activities against *Geotrichum candidum* both *in-vitro* and *in-vivo*. These plants possess potent antifungal activities with potential practical applications in the treatment of postharvest sour rot of citrus fruits and should be tested in future under degreening conditions. Moreover, new research should focus on the phytochemical analysis to identify the active principles responsible for the antifungal effect of each plant.

Chapter Three :

Evaluation of the effectiveness of organics fractions of the most active plants species, against *G. candidum*

Publised in Letters in applied microbiology as :
Talibi, I., Askarne, L., Boubaker, H., Boudyach, E.H., Msanda, F., Saadi, B., Ait Ben Aoumar, A., 2012. **Antifungal activity of Moroccan medicinal plants against citrus sour rot agent *Geotrichum candium***. Letters in applied microbiology 55, 155-161.

Résumé

Les plantes qui ont montré un fort pouvoir antifongique ont été retenues pour évaluer les effets de leurs fractions organiques contre *G. candidum.* Ceci est dans l'objectif d'identifier les fractions les plus actives et aussi pour améliorer leur activité antifongique contre l'gent pathogène. Les espèces suivantes : *C. villosus, H. antiatlanticum, H. umbellatum, P. atlantica, I. viscosa, A. radiata, R. ulmifolius et C. siliqua* ont été, successivement, extraites avec l'hexane, le chloroforme, l'acétate d'éthyle et le méthanol. Ceci nous a permis d'isoler quatre fractions organiques pour chaque plante. Ces fractions ont été évaluées pour leur activité antifongique, *in vitro* et *in vivo*, contre *G. candidum.*L'effet des fractions a été évalué sur la croissance mycélienne du pathogène par la méthode de diffusion au niveau des puits. Les résultats obtenus montrent que les espèces testées ont montré différents degrés d'inhibition de la croissance de *G. candidum*. En effet, le pouvoir inhibiteur varie avec l'espèce et le type de solvant d'extraction. . Les extraits méthanoliques de *C. villosus, H. umbellatum, C. siliqua, R. ulmifolius, H. antiatlanticum, P. atlantica* et les extraits d'acétate d'éthyle de *C. villosus*, *A. radiata* et *C. siliqua* ont fortement inhibé la croissance mycélienne du pathogène. En revanche, les extraits chloroformiques et hexaniques de toutes les plantes testées n'ont aucun effet sur la croissance mycélienne de *G. candidum*. De même, les fractions polaires des plantes testées (méthanolique et d'acétate d'éthyle) ont fortement inhibé la germination des arthrospores de *G. candidum* comparés aux fractions apolaires (hexanique et chloroformique). En effet, l'extrait méthanolique de *C. villosus, H. umbellatutm, H. antiatlanticum, C.siliqua, R. ulmifolius* et l'extrait d'acétate d'éthyle de *P. atlantica* et *A .radiata* ont inhibé la germination des arthrospores de *G. candidum* à plus de 92%. Les plus faibles concentrations inhibitrices (CMI) ont été obtenues par les extraits méthanoliques de *H. umbellatum* (0,156 mg/ml), *C. villosus* et *R. ulmifolius* (0,625 mg/ml). Les plus faibles concentrations minimales fongicides ont été enregistrées avec les extraits méthanoliques de *C. villosu, H. umbellatum* (2,5mg/ml) et *C. siliqua* (5mg/ml). Les autres extraits organiques (Hexanique, chloroformique et éthylique) n'ont montré aucun caractère fongicide aux concentrations testées. Sur la base de ces résultats, seuls les extraits méthanoliques et d'acétate d'éthyle (fractions polaires) des plantes testées ont été retenus pour évaluer leur capacité à réduire l'incidence et la sévérité de la pourriture amère. Les résultats obtenus ont montré que les extraits méthanoliques de *C. villosus, C. siliqua, H. umbellatum, H. antiatlanticum* et *R. ulmifolius* ont significativement réduit l'incidence et la sévérité de la pourriture amère, par rapport au témoin. En effet, le traitement en préventif des fruits de mandarine avec l'extrait méthanolique de *C. villosus* a totalement inhibé le développement de

l'agent pathogène. En plus, l'extrait méthanolique de *C. siliqua* et *H. umbellatum* ont respectivement réduit l'incidence de la pourriture amère à seulement 3,3 et 11,66%, par rapport au témoin (95%).

Mots clés : Activité antifongique, Extraits de plantes, pourriture amère, *Geotrichum candidum,* Fruits d'agrumes.

Abstract

The aim of this work was to find an alternative to the chemical fungicide currently used in the control of *Geotrichum candidum*, the cause of citrus sour rot. Here we evaluated methanol, ethyl acetate, chloroform and hexane extracts from eight plant species, harvested in different regions of southern Morocco, for their *in vitro* and *in vivo* antifungal activities against *G. candidum*. All plant species showed antifungal potential and the magnitude of their inhibitory activities was species and solvents dependent. Methanol and ethyl acetate extracts were found to inhibit the growth of *G. candidum* in a dose-dependent manner. *Cistus villosus*, *Ceratonia siliqua* and *Halimium umbellatum* methanol extracts proved to be the most effective inhibitors, totally inhibiting arthrospore germination of *G. candidum* at concentrations of 2.5 mg/mL and lower. The methanol extracts of *H. umbellatum*, *C. villosus* and *C. siliqua* exhibited strong antifungal activity with minimum inhibitory concentrations values ranged between 0.156 and 1.25 mg/mL, and minimum fungicidal concentrations values ranged between 2.5 and 5 mg/mL. The hexane and chloroform extracts of the studied species showed the lesser activity. The most active solvent extracts (methanol and ethyl acetate) in *in vitro* studies were tested *in vivo* against sour rot on citrus fruit. Incidence of sour rot was lowered to 0.00, 3.33 and 11.66% when mandarin fruit were treated with *C. villosus*, *C. siliqua* and *H. umbellatum* methanol extracts at 50 mg/mL, respectively, compared with 95% in the control. These plant species reduced disease severity from 100% in non-treated fruit to a 0% with *C. villosus*, 1.8% with *C. siliqua* and 8.23% with *H. umbellatum*. Poor control was recorded for ethyl acetate extracts. Phytochemical analysis of the most active fractions showed that they contain different classes of flavonoids and possess high levels of total phenols. These findings suggest that *C. villosus, H. umbellatum* and *C. siliqua* plants may be useful and effective agents for control of citrus sour rot. Such natural products therefore represent a sustainable alternative to the use of synthetic fungicides.

Keywords : Antifungal activity; Plant extracts; Sour rot; *Geotrichum candidum*; Citrus fruit

I. Introduction

Citrus is one of the most important fruit crop in Morocco, with an annual production of about 1.5 million tons from 76.500 ha under this crop. The largest volume of citrus fruit for fresh consumption and export in Morocco is grown and shipped from packing houses in the Souss-Massa-Draa (SMD) region (Boubaker *et al.,* 2009).

Fungal diseases are the major cause of postharvest rots of fresh citrus fruit during storage and transport (Eckert and Eaks, 1989). Sour rot, caused by *Geotrichum candidum* Link ex Pers. (*syn. G. citri-aurantii*), has been reported as an important postharvest disease of citrus fruit from most areas of the world (Eckert, 1978; Rippon and Morris, 1981; Brown, 1988; Hershenhorn *et al.,* 1992). In Morocco, production losses in commercial packing houses may be high under favorable conditions for pathogen development, especially during fruit degreening, wet and rainfall seasons. Fruit become more susceptible to sour rot as they increase in maturity (Brown, 1979; Baudoin and Eckert, 1985) and at favorable temperatures (25-30°C) for which fruit will rot completely in four to five days. The pathogen infects the rind through wounds made by insects or by mechanical means and causes internal disintegration and maceration of the whole fruit during storage (Brown, 1979). In addition to citrus fruit, *G. candidum* is the causal agent of numerous decays of other fruits and vegetables (Brown, 1979; Suprapta *et al.,* 1995; Wells, 1997; Palou *et al.,* 2009).

In humid conditions such as that during degreening, this fungal disease is difficult to control, and frequent applications of fungicides are used to prevent sour rot (Brown, 1979). The measures employed to manage postharvest citrus rot are not effective against *G. candidum*. This pathogen is not controlled by any of the fungicides (e.g., Imazalil and Thiabendazole) registered for use on citrus fruit (Brown and Miller, 1999; Liu *et al.,* 2009b). The disease can be partially reduced by sodium o-phenyl phenate (Rippon and Morris, 1981; Feng *et al.,* 2011), careful harvesting and handling of fruit, and by low-temperature storage (Eckert and Brown, 1988). In most commercial packing houses in the SMD Valley, pre-storage chemical control with guazatine is commonly applied as postharvest drench to reduce the incidence of sour rot in citrus fruit that are stored in cold before processing or before degreening operations. However, this fungicide is not registered in several countries. Currently, there is no potentially effective natural product to serve as an alternative in controlling sour rot. Therefore the challenge is to develop effective and safe strategies for the control of citrus sour rot. Over the last years, biological control using naturally-occurring

substances has emerged as an effective and safe strategy to control the citrus postharvest diseases. Natural products such as herbal extracts have gained popularity and scientific interest for their antibacterial and antifungal activity (Wilson *et al.,* 1991; Tripathi and Duby, 2004; Abd-El-Khair and Hafez, 2006; Abad *et al.,* 2007; Ameziane *et al.,* 2007; Liu *et al.,* 2009b; du Plooy *et al.,* 2009; Gatto *et al.,* 2011). Plant extracts are very attractive as alternative or complementary control means because of their antimicrobial activity, non-phytotoxicity, systemicity, and biodegradability (Fawcett and Spencer, 1970; Tripathi and Dubey, 2004; Kosalec *et al.,* 2005; Ameziane *et al.,* 2007; Gatto *et al.,* 2011; Talibi *et al.,* 2011a). In a recent screening study (Talibi *et al.,* 2012a), the powders and aqueous extracts of 43 plant species have been tested for their antifungal activity against *G. candidum*. Among these 43 plants, eight species showed the best results against *G. candidum* (Talibi *et al.,* 2012a). Very little work has been done to investigate the use of natural plant products to control citrus sour rot.

The aim of the present study is to determine the inhibitory effects of eight medicinal plants, extracted in different organic solvents (hexane, chloroform, ethyl acetate and methanol), on the growth of *G. candidum* under both *in vitro* and *in vivo* conditions, and to obtain preliminary information on the chemical composition of the plant extracts.

II. Materials and methods

II.1 Collection of Plant Samples

Eight fresh plant samples were collected from different locations in the Souss valley (Agadir, south of Morocco). The species of all samples were identified (**Table 3.1**), and were deposited in the herbarium of the laboratory of Biotechnology and Valorization of the Natural Resources, Faculty of Sciences, Ibn Zohr University, Agadir, Morocco. Plant samples were cleaned, air dried in the shade, then grounded to a fine powder using a laboratory grinding mill (Polymix PX-MFC 90D, Switzerland) and stored in the dark at 4°C until use. The botanical name, family, and parts used of plant samples are summarized in **Table 3.1**. The eight plant species belong to seven genera and five botanical families.

II.2 Preparation of plant extracts

Preparation of the plant samples for the *in vitro* antifungal screening against *G. candidum* was conducted as previously described by Duraipandiyan and Ignacimuthu (2007) and Bajpai *et al.* (2008). Briefly, powder (20g) of each plant species was extracted with 100 mL of hexane by maceration with stirring at room temperature for 48 h and then filtered through Whatman no. 1 filter paper to obtain particle free extract. The solvent of the filtrate was removed by evaporation to dryness under reduced pressure at 40°C. The remains of the plant material were extracted with chloroform, ethyl acetate and methanol sequentially in a similar manner (**Figure 3.1**) and yield was determined for each extraction (**Table 3.1**). Each dried plant extract was dissolved at a concentration of 200 mg/mL in the same solvent of extraction. The extracts were then sterilized by passing through 0.2µm pore diameter Millipore Swinex filters and kept at -20°C before assay. Solvents (analytical grade) for extraction were obtained from commercial sources.

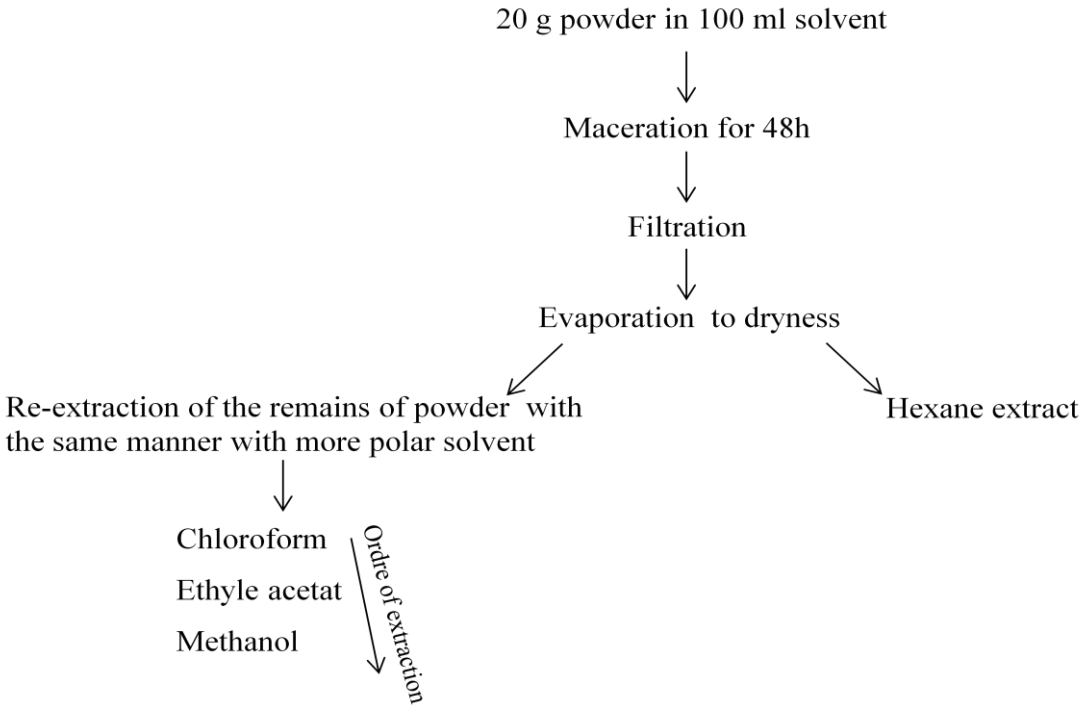


Figure 3.1 : Steps of preparation of organic fractions

II.3 Pathogen

The *G. candidum* isolate used in this study was obtained from a decayed mandarin fruit. It is one of the most aggressive isolates in our collection. This isolate was deposited at the laboratory of Biotechnology and Valorization of the Natural Resources under the code SR10.

The fungus was maintained on potato dextrose agar (PDA) plates at 5°C, with periodic transfers through citrus fruit to maintain its aggressiveness. The pathogen inoculum consisted of aqueous arthrospores suspensions obtained from seven-day old culture plates incubated at 25°C. Arthrospores were harvested by flooding plates with 5 mL of sterile distilled water containing 0.05% (v/v) Tween 80, and passing the suspension through two layers of sterile cheesecloth to remove hyphal fragments. The arthrospores concentration was determined with the aid of a heamacytometer and adjusted to 10^6 arthrospores/mL with sterile distilled water.

II.4 Fruit

Fruits of mandarin (*Citrus reticulata* Blanco cv. Clementine) were used. Fruits were harvested from orchards of the M'brouka cooperative, which used standard culture practices, in the Souss-Massa-Draa Valley, southern Morocco. Only healthy and commercially mature fruits were used in the *in vivo* test. Freshly harvested or briefly stored (no longer than a week) fruits were used in the experiment.

II.5 Evaluation of antifungal activity of plant extracts

II.5.1 *In vitro* effect of solvents extracts on mycelial growth of *G. candidum*

The solvent extracts were screened for their antifungal activity using the well-plate diffusion method. Aliquots (100 µL) of a spore suspension (10^6 arthrospores /mL) of *G. candidum* was overlaid on Petri plates (90 mm in diameters) containing 15 mL of PDA. Then five-mm diameter wells were made at three locations per dishes. The wells are then filled with 20 µL of solvent extract at concentration of 2.5, 5 and 10 mg/mL. Control plates consisted of wells filled with 20µL of the same solvent of extraction. The plates are stored at 4°C for 2 hours to allow the diffusion of extract, and then incubated at 26°C (**Figure 3.2**). After 48 hours incubation, the diameter (mm) of the inhibition zone was measured. By using eight plant species, four solvents of increasing polarities, and three concentrations, 96 treatments were generated. Three plates were used for each treatment as replications, and each experiment was repeated twice.

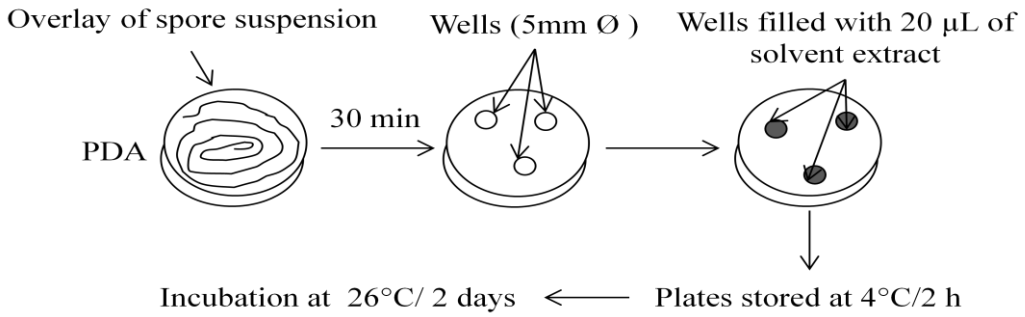


Figure 3.2 : *In vitro* effect of solvents extracts on mycelial growth of *G. candidum*

II.5.2 Effect of solvents extracts on arthrospores germination

The germination of arthrospores of *G. candidum* was determined in concentrations of 1.25, 2.5, 5, 10 and 20 mg/mL of each solvent extracts. Aliquots (40µL) of a spore suspension (10^6 arthrospores/mL) were aseptically transferred in triplicate to sterile depression slides containing 40µL of 2% sterile orange juice amended with different concentrations of solvent extracts. Inoculated slides were placed on moist filter paper in Petri plates, sealed with parafilm to avoid evaporation, and then incubated at 25°C for 24h. Each slide was then fixed with acid fuchsine solution to stop further germination (Smilanick *et al.,* 1999). Arthrospores germination was estimated under a microscope using a micrometer. At least, 100 arthrospores of each replicate were observed. An arthrospore was scored as germinated if the germ tube length was equal or exceed that of the spore (Suprapta *et al.,* 1997). The data were expressed as percent spore germination inhibition and calculated by using the following formula :

GI (%) = [(Gc−Gt) /Gc)] ×100, where Gc and Gt represent the mean number of germinated spores in control and treated slides, respectively (Soylu *et al.,* 2010). Each treatment included three replicates and the experiment was conducted twice.

II.5.3 Determination of Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The minimal inhibitory concentrations (MICs) of the plant extracts were determined by the agar dilution method. Organic plants extracts were first diluted to the highest concentration to be tested (5 mg/mL), and then serial two-fold dilution were made in a concentration ranging from 5 to 0.3125 mg/mL in 10mL test tubes containing melted PDA medium. Culture plates with 24 microwells and a capacity of 4 mL per well were used.

Solvent plant extracts amended medium was dispended in the wells of culture plates (2 mL per well). Aliquots (10µL) of an arthrospores suspension (10^6arthrospores/mL) were then

dropped onto the surface of the agar medium. Culture plates were incubated at 24°C (±1°C) for 48 h. The MICs were recorded by reading the lowest solvent extract concentration that allowed no visible growth of the pathogen (Phongpaichit *et al.,* 2005).

The MFCs were determined by taking agar plugs from well showing no visible mycelial growth and re-inoculating them on unamended PDA medium. MFC was regarded as the lowest concentration of the solvent plant extract that prevented growth of the pathogen after the period of incubation, and indicating more than 99.5% killing of the original inoculums (Fabry *et al.,* 1996; Ali-Shtayeh and Abu Ghdeib, 1999). There were three replicates for each plant solvent extract at each concentration and the experiment was conducted twice.

II.5.4 Effects of solvent extracts on sour rot development in artificially inoculated and wounded fruit

Based on *in vitro* screening results, only methanol and ethyl acetate extracts of the plant species were tested. Mandarin fruits were washed, disinfected with 0.1% (v/v) sodium hypochlorite, rinsed three times in sterile distilled water and then air dried before wounding. One wound (2mm deep and 4mm wide) was made per fruit using a sterile needle at the equatorial side (Liu *et al.,* 2009b). The wounds were treated with 30 µL of solvent extracts at a concentration of 50 mg/mL. Controls were treated with the same volume of sterile distilled water under the same conditions. After two hours incubation at room temperature, each wound was inoculated with 20 µL of an aqueous suspension of arthrospores of *G. candidum* (10^6 arthrospores/mL). Treated fruits were placed on plastic tray in carton boxes and incubated at 26°C and 95% relative humidity (RH). The number of the infected wounds and the lesion diameters of the overall treated fruit were determined daily. All treatments were arranged in a complete randomized block design. Eighteen fruits constituted a single replicate and each treatment was replicated three times. The experiment was conducted twice. The incidence and severity of disease were calculated as follows :

Disease incidence (%) = [(number of rotten wounds / number of total wounds)] x 100.

Disease severity (%) = [(average lesion diameter of treatment / average lesion diameter of control)] x 100.

In all experiments, the possible phytotoxic effect on mandarin fruit was examined.

II.6 Phytochemical analysis

The plant extracts who gave best control of sour rot were selected for their qualitative phytochemical analysis to identify the presence of alkaloids and flavonoids. The analysis was done using aluminium thin layer chromatography (TLC) plates (silica gel 60 F_{254} plates, 20 x 20 cm, Merck). The plates were developed with the eluent system ethyl acetate-methanol-water (40/5.4/5, v/v/v). Visualization of alkaloids and flavonoids compounds was achieved by spraying the plates with freshly prepared Dragendorff (alkaloids) and Neu (flavonoids) reagents, respectively. The principal phytochemical constituents were characterized with colorful reactions and by the establishment of their chromatographic profiles by TLC. Detection was carried out visually under UV light at 365 nm.

Also, total phenolic content (TPC) of these extracts was measured by the Folin-Ciocalteu assay method **(**Slinkard and Singleton, 1977**)** with slight modifications. To 0.5 mL of a diluted methanolic extracts, 0.5 mL of Folin-Ciocalteau reagent was added and mixed vigorously. After five minutes, 0.5 mL of 20% sodium carbonate (Na_2CO_3) was added and mixed vigorously again. After 1min of shaken, the solution was brought up to 5 mL by adding distilled water. The control reaction contained all reagents except the plant extracts. The reaction mixture was then incubated in the dark at 25 °C for 90 min, and the absorbance of the resulting color was measured at 760 nm against a distilled water/sodium carbonate blank. The concentration of the TPC was estimated as mg of gallic acid equivalent (GAE) per gram of plant extract by using a standard gallic acid calibration curve. Values presented are the average of three measurements.

II.7 Statistical analysis

All data were subjected to statistical analysis of variance (ANOVA) using STATISTICA software, version 6 (Stat-Soft, 2001, France). Percentage values were subjected to arcsine square root transformation before analysis of variance. Mean separation was performed following the Newman & Keuls test at $P < 0.05$.

III. Results

III.1 *In vitro* effect of solvents extracts on mycelial growth of *G. candidum*

The eight plant species were extracted by four solvents of increasing polarities: hexane, chloroform, ethyl acetate and methanol. Methanol and ethyl acetate were the best solvents, extracting a larger quantity of material (**Table 3.1**). The highest yield was obtained for methanol extract of *Halimium antiatlanticum* (32.6%) and the lowest yield was recorded for hexane extract of *Rubus ulmifolius* (2.5%).

Table 3.1: Plants used in this study and yield extracted (%) with each solvent

Family	Plant species	Part used	Extracted yield (%)			
			Hex	**Chl**	**E.Ac**	**MeOH**
Cistaceae	*Halimium umbellatum*	Leaves+stem	10.9	7	12.3	27.5
Asteraceae	*Anvillea radiata*	Leaves+stem	3.4	27	13.4	16.3
Cistaceae	*Cistus villosus*	Leaves+stem	4.4	7	12.1	22.4
Asteraceae	*Inula viscosa*	Leaves+stem	4.4	10	13.9	19.6
Cistaceae	Halimium antiatlanticum	Leaves+stem	1.7	2.6	14	32.6
Rosaceae	*Rubus ulmifolius*	Leaves+stem	2.5	4.1	14.7	22.1
Fabaceae	*Ceratonia siliqua*	Leaves	6.2	5.5	18.5	29
Anacardiaceae	*Pistacia atlantica*	Leaves	6.4	8.9	12.4	32.4

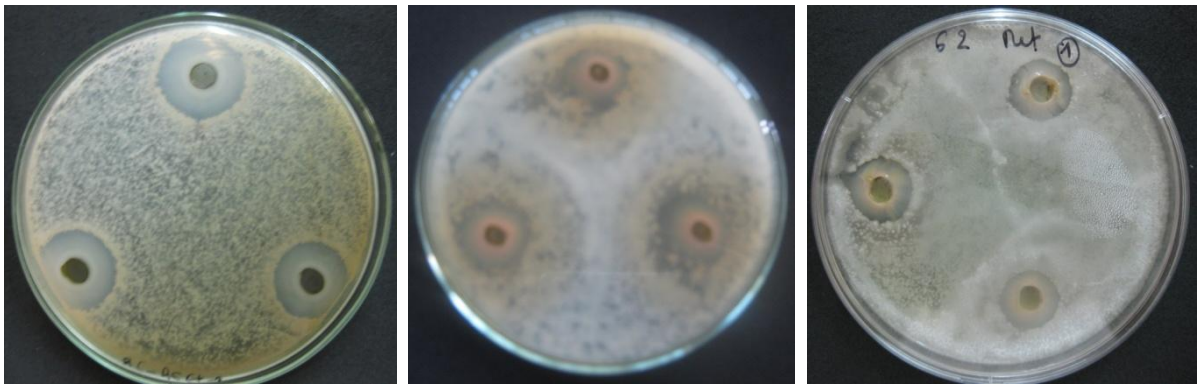
The results of the antifungal screening of methanol, ethyl acetate, chloroform and hexane extracts of eight plant species at different concentrations are given in **Table 3.2**. As shown, the methanol and ethyl acetate extracts were more active than the hexane and chloroform extracts. Both methanol and ethyl acetate plant extracts showed antifungal activity against *G. candidum* at concentration-related manner. At the concentration of 10 mg/mL, the diameter of the inhibition zones produced against *G. candidum* was ranged from 20.67 to 24 mm for the methanolic extracts from *Halimium umbellatum*, *Cistus villosus* and *Ceratonia siliqua,* and 20.7 to 22.7 mm for the ethyl acetate extracts from *Anvillea radiata* and *Pistacia atlantica* (**Figure 3.3**). It was observed that the antifungal activity resided mainly in the methanol and ethyl acetate extracts. Also, hexane and chloroform extracts from *P. atlantica* exhibited low antifungal effect with diameter zones of inhibition of 13.7 and 11.7 mm, respectively. However, no inhibitory effects of the hexane and chloroform extracts were observed against *G. candidum* for the others tested plant species (**Table 3.2**). In this screening, methanol and

ethyl acetate extracts were shown to exhibit, *in vitro*, a significant antifungal activity against *G. candidum.* This indicates that the two organic extracts contain active compounds which are responsible at least in part for the antifungal activity.

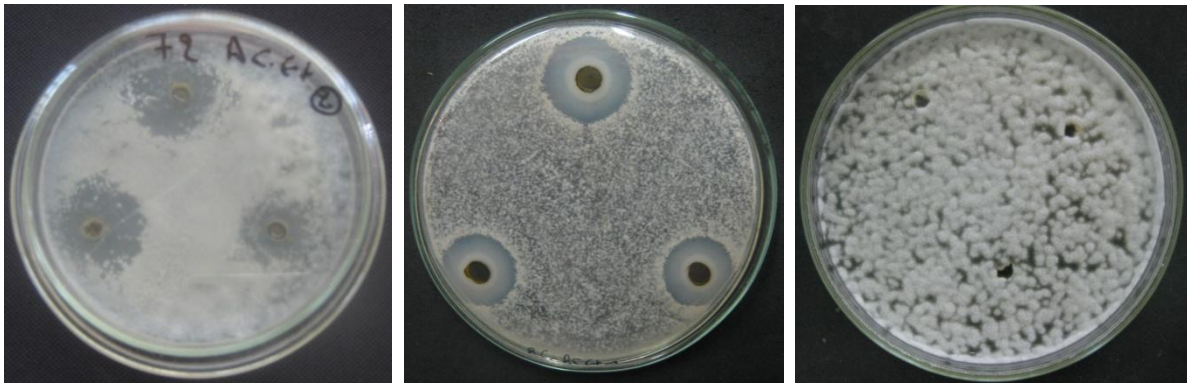
Table3.2. *In vitro* effects of solvents extracts on mycelial growth of *Geotrichum candidum*

Plant species	Extraction solvent	[a]Diameter of the inhibition zone (mm) Concentration of plant extracts (mg/mL)		
		2.5	**5**	**10**
Anvillea radiata	Hexane	0.00^{a}	0.00^{a}	0.00^{a}
	Chloroform	0.00^{a}	0.00^{a}	0.00^{a}
	Ethyl acetate	6.70^{bcde}	17.70^{qr}	22.70^{t}
	Methanol	0.00^{a}	6.00^{bcd}	7.30^{def}
Halimium umbellatum	Hexane	0.00^{a}	0.00^{a}	0.00^{a}
	Chloroform	0.00^{a}	0.00^{a}	0.00^{a}
	Ethyl acetate	11.70^{ij}	14.30^{lmn}	17.00^{pqr}
	Methanol	9.30^{gh}	18.00^{qr}	22.70^{t}
Ceratonia siliqua	Hexane	0.00^{a}	0.00^{a}	0.00^{a}
	Chloroform	0.00^{a}	0.00^{a}	0.00^{a}
	Ethyl acetate	0.00^{a}	11.30^{ij}	15.70^{nop}
	Methanol	8.70^{fg}	12.00^{ijk}	20.67^{s}
Cistus villosus	Hexane	0.00^{a}	0.00^{a}	0.00^{a}
	Chloroform	0.00^{a}	0.00^{a}	0.00^{a}
	Ethyl acetate	12.66^{jkl}	15.66^{nop}	21.00^{s}
	Methanol	16.33^{opq}	20.66^{s}	24.00^{t}
Pistacia atlantica	Hexane	4.30^{b}	7.70^{cdef}	13.70^{klm}
	Chloroform	0.00^{a}	8.70^{fg}	11.70^{ij}
	Ethyl acetate	14.30^{lmn}	15.70^{nop}	20.70^{s}
	Methanol	13.70^{klm}	17.70^{qr}	18.70^{r}
Rubus ulmifolius	Hexane	0.00^{a}	0.00^{a}	0.00^{a}
	Chloroform	0.00^{a}	0.00^{a}	0.00^{a}
	Ethyl acetate	0.00^{a}	6.86^{cde}	12.23^{ijk}
	Methanol	8.00^{efg}	12.86^{jkl}	17.56^{qr}
Halimium antiatlanticum	Hexane	0.00^{a}	0.00^{a}	0.00^{a}
	Chloroform	0.00^{a}	0.00^{a}	0.00^{a}
	Ethyl acetate	0.00^{a}	10.60^{hi}	15.00^{mno}
	Methanol	8.66^{fg}	14.00^{lmn}	16.66^{opq}
Inula viscosa	Hexane	0.00^{a}	0.00^{a}	0.00^{a}
	Chloroform	0.00^{a}	0.00^{a}	0.00^{a}
	Ethyl acetate	0.00^{a}	6.00^{bcd}	7.00^{cdef}
	Methanol	0.00^{a}	5.73^{bc}	7.16^{bcde}

[a] Values are means of three replicates. Values followed by the same letters were not significantly different (P <0.05) according to Newman and Keels test.



Methanol fraction of C. v | Methanol fraction of H.u | Methanol fraction of C.s



Ethyl acetat fraction of A.r | Ethyl acetat fraction of P.a | Control

Figure 3.3 : Inhibition zone of *G. candidum* around the wells filled with the organic fractions of *Cistus villosus (C.v), Hallimium umbellatum (H.u), Ceratonia siliqua (C.s), Anvillea radiata (A.r)* and *Pistacia atlantica (P.a)* compared with control

III.2 Effect of solvents extracts on arthrospores germination

The susceptibility of *G. candidum* arthrospore towards various concentrations of the organic extracts of eight plant species is summarized in **Table 3.3**. In general, methanol and ethyl acetate plant extracts presented higher inhibition of arthrospores germination than hexane and chloroform extracts. Indeed, methanol extracts from *C. villosus* and *C. siliqua* induced 100% inhibition of arthrospore germination at the lowest concentration tested (1.25 mg/mL) (**Figure 3.4**), while ethyl acetate extracts from *A. radiata* and *H. umbellatum*, and chloroform extract from *P. atlantica* required 10 mg/mL to bring the same effect. All applied concentrations of methanol extract from *H. umbellatum*, except 1.25 mg/mL, completely inhibited the arthrospores germination of *G. candidum.* Methanol extract from *H. antiatlanticum* exhibited less toxicity and only concentrations of 5 mg/mL and above reduced the percentage of arthrospores germination by more than 90%. For the hexane extracts, only extract of *P. atlantica* reduced the percentage of arthrospores germination by more than 50 % at 10 mg/mL (**Table 3.3**).

Table 3.3 : *In vitro* effect of solvents extracts on spore germination of *Geotrichum candidum*

Plant species	Extraction solvent	[a]Spore germination inhibition (%)				
		Concentration of plant extracts (mg/ mL)				
		1.25	2.5	5	10	20
Anvillea radiata	Hexane	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}
	Chloroform	0.00^{z}	1.00^{yz}	21.00^{op}	39.34^{kl}	59.34^{h}
	E. acetate	78.67^{f}	**82.67^{e}**	**93.34^{b}**	**100.00^{a}**	**100.00^{a}**
	Methanol	0.00^{z}	0.00^{z}	0.00^{z}	2.00^{yz}	3.00^{yz}
Halimium umbellatum	Hexane	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}
	Chloroform	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}
	E. acetate	0.00^{z}	0.00^{z}	16.67^{qr}	**100.00^{a}**	**100.00^{a}**
	Methanol	64.00^{h}	**100.00^{a}**	**100.00^{a}**	**100.00^{a}**	**100.00^{a}**
Ceratonia siliqua	Hexane	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}
	Chloroform	0.00^{z}	12.34^{stu}	17.34^{pq}	$18±0^{pq}$	18.67^{opq}
	E. acetate	0.00^{z}	0.00^{z}	2.00^{yz}	7.67^{vwx}	24.67^{n}
	Methanol	**100.00^{a}**	**100.00^{a}**	**100.00^{a}**	**100.00^{a}**	**100.00^{a}**
Cistus villosus	Hexane	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}
	Chloroform	2.34^{yz}	7.00^{wx}	9.00^{uvw}	10.67^{tuv}	12.34^{stu}
	E. acetate	0.00^{z}	0.00^{z}	0.00^{z}	10.00^{tuv}	19.00^{n}
	Methanol	**100.00^{a}**	**100.00^{a}**	**100.00^{a}**	**100.00^{a}**	**100.00^{a}**
Pistacia atlantica	Hexane	0.00^{z}	0.00^{z}	22.34^{n}	57.67^{h}	69.67^{g}
	Chloroform	**87.34^{d}**	**91.67^{b}**	**94.67^{b}**	**100.00^{a}**	**100.00^{a}**
	E. acetate	0.00^{z}	3.00^{yz}	31.67^{m}	$41.67±1.11^{k}$	**92.00^{b}**
	Methanol	0.00^{z}	0.00^{z}	0.00^{z}	$51.34±2.22^{j}$	69.34^{g}
Rubus ulmifolius	Hexane	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}
	Chloroform	0.00^{z}	0.00^{z}	5.00^{xy}	17.34^{pq}	36.34^{l}
	E. acetate	18.67^{pq}	36.00^{l}	61.34^{h}	69.34^{g}	**81.67^{e}**
	Methanol	8.67^{uvw}	38.67^{kl}	59.00^{h}	**90.40^{c}**	**97.00^{a}**
Halimium antiatlanticum	Hexane	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}
	Chloroform	0.00^{z}	0.00^{z}	8.00^{vwx}	13.34^{rst}	30.00^{m}
	E. acetate	0.00^{z}	19.67^{opq}	28.34^{m}	64.67^{h}	72.34^{g}
	Methanol	15.34^{qrs}	50.67^{j}	**90.67^{cd}**	**98.67^{a}**	**99.67^{a}**
Inula viscosa	Hexane	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}
	Chloroform	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}
	E. acetate	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}	0.00^{z}
	Methanol	0.00^{z}	0.00^{z}	1.34^{yz}	2.00^{yz}	4.00^{yz}

[a]Each value represents the mean of three replicates. Means followed by different letter (s) in each column are significantly different at $P < 0.05$. **Bold type** : % inhibition >80%.

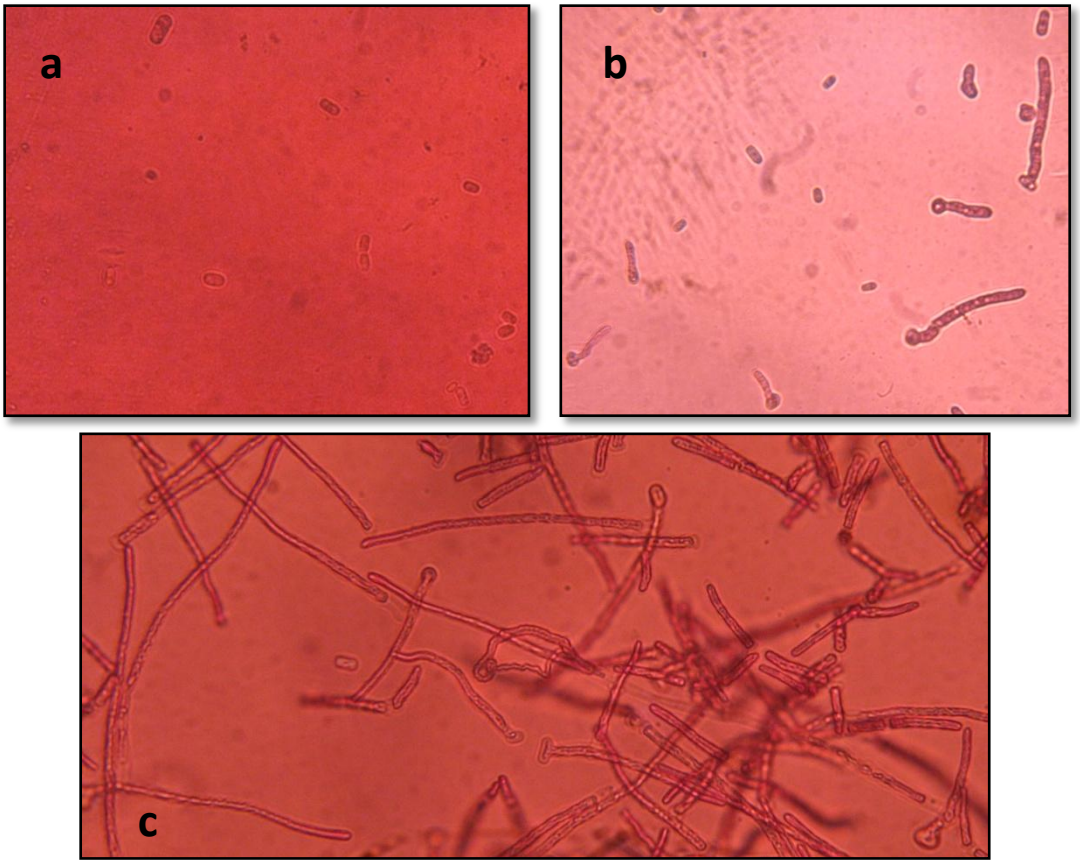


Figure 3.4 : Germination of arthrospores of *G. candidum* 24h after treatment with methanol extract of *C. villous* (a) and *H. umbellatum* (b) at 1.25 mg/ml compared with control (c)

III.3 MIC and MFC

The MIC values showed that the activity of the plant extracts varied depending on the solvent used for extract preparation (**Table 3.4**). Methanol extracts of *H. umbellatum*, *C. villosus*, *R. ulmifolius* and *C. siliqua* were the most active with MIC values of 0.156, 0.625, 0.625 and 1.25 mg/mL, respectively. For the others solvents, only chloroform and ethyl acetate extracts of *P. atlantica* have inhibited growth of *G. candidum* at 5 mg/mL (**Table 3.4).** On the other hand, the best MFC value was obtained by methanol extracts of *H. umbellatum* and *C. villosus* which are fungicidal at 2.5 mg/mL (**Table 3.4**).

III.4 *In vivo* test

The data presented in **Figure 3.5** showed that all tested methanol plant extracts significantly reduced the incidence of sour rot caused by *G. candidum* under the laboratory conditions. Percentages of rotted wounds were decreased by using all tested methanol extracts compared with control. Mandarin fruit treated 2 hours before pathogen inoculation by methanol extracts of *C. villosus*, *H. umbellatum* and *C. siliqua*, at 50 mg/mL, reduced the incidence of sour rot to 0.0, 3.33 and 11.66%, respectively (**Figure 3.5**). Methanol extracts of *R. ulmifolius* and *H. antiatlanticum* have also reduced the percentage of rot incidence to 30

and 30.66%, respectively, compared to the control (95%). *I. viscosa*, *P. atlantica* and *A. radiata* methanol extracts have a moderate effect on sour rot, the percentage of rot incidence varying between 50 and 65% (**Figure 3.5**).

Table 3.4 : Minimal inhibitory concentrations (MICs) and minimal fungicidal concentrations (MFCs) of selected plant species

Treatement	**[a]MICs and [b]MFCs (mg/** mL**)**							
	Hex		**Chl**		**Met**		**E. Ac**	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
H. umbellatum	> 5	> 5	> 5	> 5	< 0.156	2.5	> 5	> 5
A.radiata	> 5	> 5	> 5	> 5	5	> 5	> 5	> 5
C. villosus	> 5	> 5	> 5	> 5	0.625	2.5	> 5	> 5
I. viscosa	> 5	> 5	> 5	> 5	> 5	> 5	> 5	> 5
H. antiatlanticum	> 5	> 5	> 5	> 5	> 5	> 5	> 5	> 5
R. ulmifolius	> 5	> 5	> 5	> 5	0.625	> 5	> 5	> 5
C. siliqua	> 5	> 5	> 5	> 5	1.25	5	> 5	> 5
P. atlantica	> 5	> 5	5	> 5	> 5	> 5	5	> 5

[a] Concentrations that were fungistatic and [b] Concentrations that were fungicidal

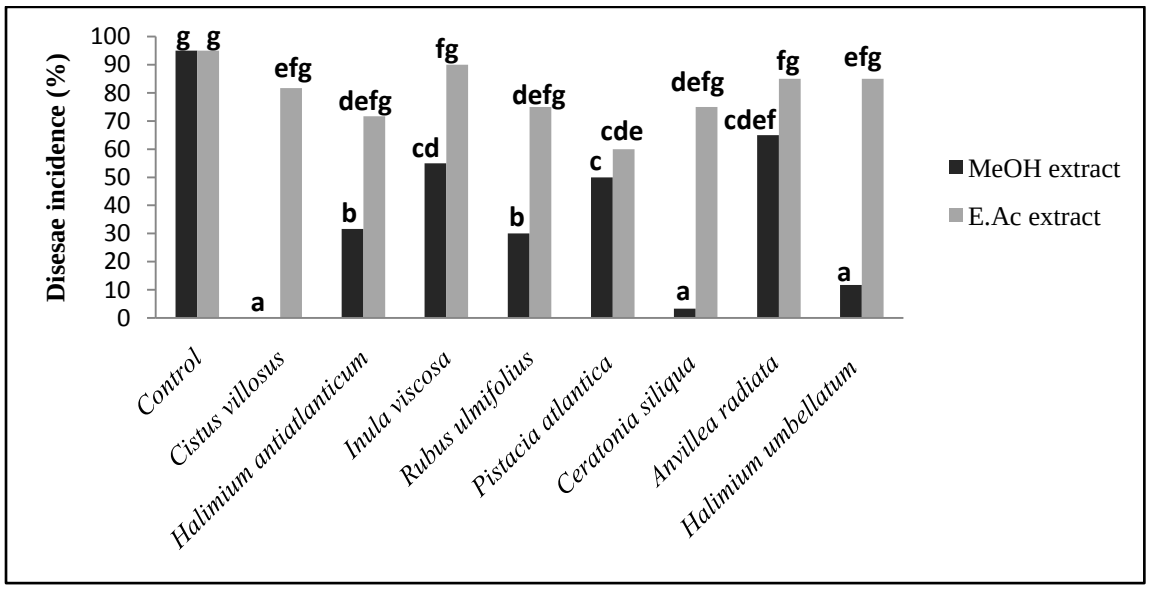


Figure 3.5 : Effect of solvents extracts on sour rot incidence in mandarin wounds. Fruit were treated with aqueous extracts, inoculated with *G. candidum* and held for 7 days at 26°C. Significant differences ($P < 0.05$) between means were indicated by different letters above histogram bars.

Also, the tested methanol extracts exhibited significant reduction of disease severity compared with the control (**Figure 3.6** and **figure 3.7**). They reduced disease severity from 100% in non-treated fruit to a 0% with *C. villosus*, 1.8% with *C. siliqua*, 8.23% with *H. umbellatum*, 22.27% with *R. ulmifolius*, 23% with *H. antiatlanticum* and 31.82% with *P. atlantica*. In contrast, ethyl acetate extracts of all tested plant species are less effective than methanol ones (**Figure 3.7**). The percent of infected fruits varied between 60 and 90% (**Figure 3.5**), and the disease severity between 38.63% (for *P. atlantica*) and 87.52% (for *I. viscosa*) (**Figure 3.6**). Furthermore, neither of the plant extracts showed any phytotoxic reaction on treated fruit, at the concentration tested (50 mg/mL).

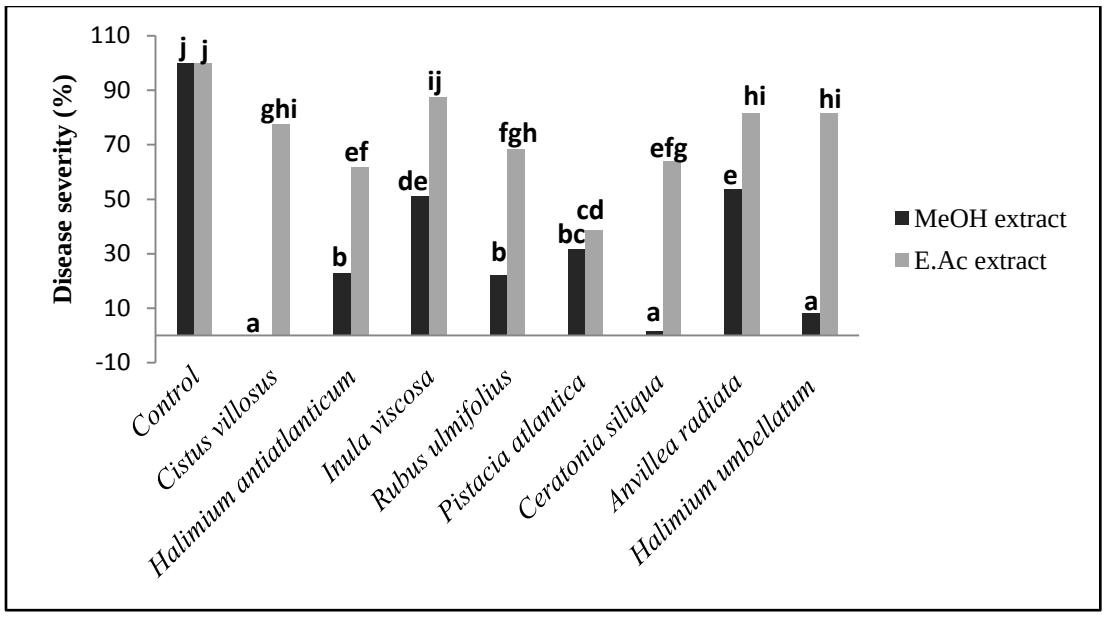


Figure 3.6 : Effect of solvents extracts on sour rot severity in mandarin wounds. Significant differences ($P < 0.05$) between means were indicated by different letters above histogram bars.

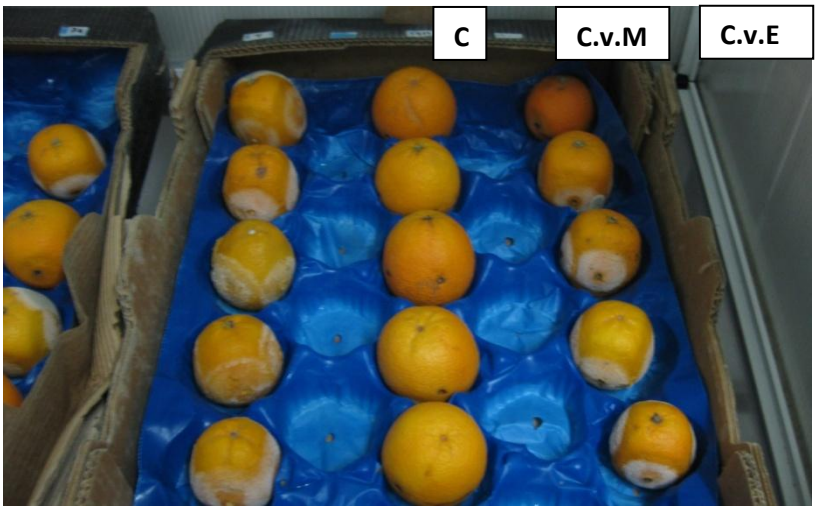


Figure 3.7: Control of sour rot by methanol extract of *Cistus villosus* (C.v.M) compared with Ethyl acetate extract of the same plant specie (C.v. E) and control (C).

III.5 Phytochemical analysis

The chemical screening of plant extracts, by using Neu reagent, permitted to detect flavonoids in *C. villosus*, *H. umbellatum*, *H. antiatlanticum*, *C. siliqua* and *R. ulmifolius*. However, for the alkaloids, Dragendorff reagent has revealed only minor bands with a light orange color in all extracts analyzed.

The total phenolic contents of methanol extracts of selected plant species as determined by Folin-Ciocalteu method are reported as gallic acid equivalent (GAE) (**Figure 3.8**). Among analyzed plant extracts, methanol extracts from *C. siliqua* showed the highest amount of phenolic compounds (165.2 mg GAE/g extract) followed by *H. umbellatum* and *C. villosus* (139.46 and 136.13 mg GAE/g extract, respectively).

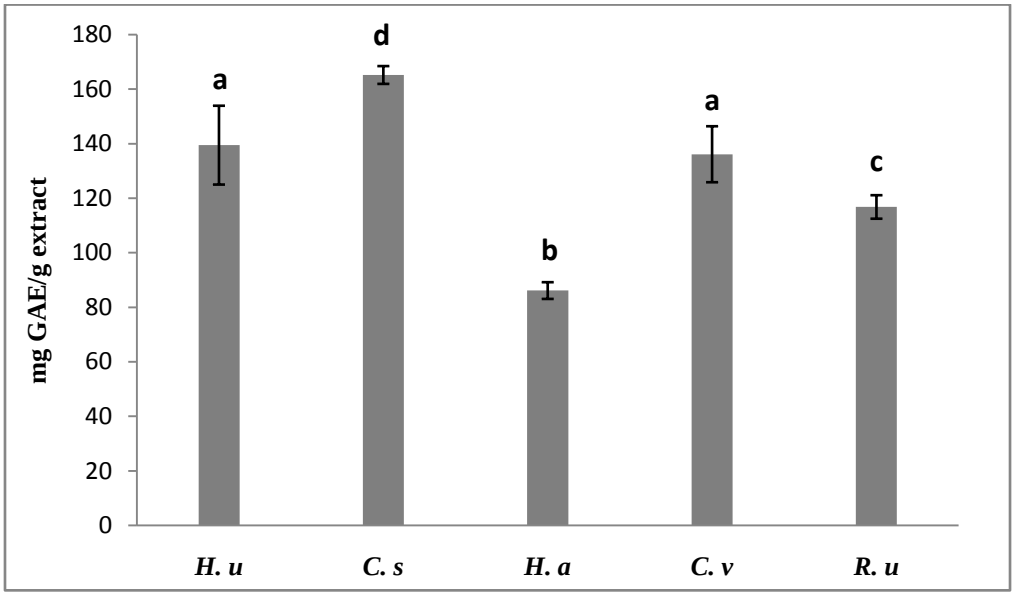


Figure 3.8 : Levels of total phenols present in selected plant species, H.u: *Hallimium umbellatum*, C.s : *Ceratonia siliqua*, H.a : *Hallimium antiatlanticum*, C.v : *Cistus villosus*, and R.u : *Rubus ulmifolius*. Results are expressed as mg of gallic acid per g of plant extract. The values represent the mean of triplicates ± standard deviation.

IV. Discussion

Management of fungal diseases of citrus fruit continues to be one of the greatest disease challenges in commercial packing houses. Currently novel tools, such as the use of new fungicides and salts (Smilanick *et al.*, 2006a; Kanetis *et al.*, 2007; Talibi *et al.*, 2011b), antagonistic microorganisms (Leelasuphakul *et al.*, 2008; Taqarort *et al.*, 2008; Lahlali *et al.*, 2010), and plant extracts (Ameziane *et al.*, 2007; Du Plooy *et al.*, 2009), are tested as an alternative to currently used fungicides for control of postharvest citrus fruit rots. In recent years, several studies have been focused on screening of plant extracts to develop new

antifungal compounds that can be used to control postharvest citrus diseases (Droby *et al.*, 1999b; Arras and Usai, 2001; Soylu *et al.*, 2005; Fisher and Phillips, 2008; Hao *et al.*, 2010; Talibi *et al.*, 2012a). At present, only a few measures for the control of sour rot, in particular in organic agriculture, are available. In this study, the antifungal activity of different organic extracts from eight plant species was evaluated against *G. candidum* under both *in vitro* and *in vivo* conditions.

The antifungal activity was confirmed in all the plant species tested, although the results showed that different plant extracts varied in their effectiveness in inhibiting *G. candidum* growth. Indeed, the inhibitory potential of the extracts was found to vary with the specific plant species as well as the solvent used for extract preparation. The *in vitro* trials showed that methanol extracts of *C. villosus, H. umbellatum, C. siliqua, R. ulmifolius, H. antiatlanticum, P. atlantica* and ethyl acetate extracts of *A. radiata, C. villosus* and *C. siliqua* proved to be the best mycelial growth inhibitors. These results concurred with those of Talibi *et al.* (2012a), who reported that aqueous extracts of *R. ulmifolius, C. siliqua,* and *H. umbellatum* had completely inhibited the mycelial growth of *G. candidum.* They also reported that aqueous extracts of *C. villosus* and *H. antiatlanticum* showed a higher degree of control (% inhibition > 92%). The hexane and chloroform extracts of the studied species showed the lesser antifungal activity. These results may not be entirely surprising considering that a greater variety of polar compounds could be included in the methanol and ethyl acetate fractions, while hexane and chloroform fractions are mainly composed of non-polar compounds. Thus, the antifungal bioactive components present in the polar solvents extracts need to be further characterized.

Among the extracts prepared with the different solvents having different polarities, methanol and ethyl acetate plant extracts exhibited higher inhibition of arthrospores germination than hexane and chloroform extracts. Indeed, methanol extracts from *C. villosus* and *C. siliqua* induced 100% inhibition of arthrospores germination at the lowest concentration tested (1.25 mg/mL). Talibi *et al.* (2012a), reported that the aqueous extracts of *C. villosus* and *H. antiatlanticum* had the highest fungistatic effect on arthrospores germination of *G. candidum*. This suggests that the active components of the extracts may be among the more polar compounds as they are found in both the aqueous and methanol extracts. Methanol is a general solvent and tends to provide a more complete extraction of polar and less polar compounds, while water may not extract some of the less polar compounds. Besides *G. candidum*, these plant extracts also showed an inhibitory effect on the

growth of *Clavibacter michiganensis* subsp. *michiganensis,* causal agent of the tomato bacterial canker (Talibi *et al.*, 2011a).

The MIC for *H. umbellatum* methanol extract against *G. candidum* was particularly low (0.156 mg/mL), followed by *C. villosus* and *R. ulmifolius* methanol extracts (0.625 mg/mL). This antifungal effect was comparable to that reported by other findings. Bouamama *et al.* (2006) reported that methanol leaf extract of *C. villosus* have MIC values ranging from 0.19 to 200 mg/mL for some yeasts and fungi. In the same study, the authors showed that *C. villosus* methanol extract exhibited more interesting antifungal activity than ethyl acetate extract (Bouamama *et al.,* 2006). In a recent work, Talibi *et al.* (2012a) reported that aqueous extracts of *C. villosus, H. antiatlanticum, H. umbellatum, R. ulmifolius* and *C. siliqua,* tested against *G. candidum,* had MIC values ranging between 0.156 and 0.3125 mg/mL. In the present investigation, the methanol extracts of *H. umbellatum* and *C. villosus* prove to have fungicidal action at 2.5 mg/mL; while, aqueous extracts of these two species exhibited only a fungistatic activity against *G. candidum*, even at 5 mg/mL (Talibi *et al.*, 2012a).

Although *in vitro* tests of plant extracts is an important first step in selecting plants with antifungal potential against postharvest citrus pathogens, *in vivo* tests are needed to check whether the positive results of the *in vitro* tests can be obtained too (Gorris and Smid, 1995; Tegegne *et al.*, 2008). Results obtained in this study indicated that methanol extracts of five plant species, viz. *C. villosus, C. siliqua, H. umbellatum, H. antiatlanticum* and *R. ulmifolius*, significantly decreased the incidence and severity of sour rot under *in vivo* conditions. In a previous study, Kivack *et al.* (2001) found that the methanol extract of *C. siliqua* possesses potent antifungal activity against a wide range of bacteria and yeast. Moreover, Sisti *et al.* (2008) found that the methanol extract of *R. ulmifolius* exhibited antimicrobial activity against a wide range of human pathogens. Also, Panizzi *et al.* (2002) demonstrate that methanolic extract of *R. ulmifolius* showed a wide range of activity against Gram-positive and Gram-negative bacteria and yeasts. According to these authors, the phenolic and tannins fractions of *R. ulmifolius* showed a high antimicrobial activity. Aqueous extracts of the same species tested in this study showed less control of citrus sour rot compared with the methanol extracts (Talibi *et al.,* 2012a). The findings of this study also corresponded with a previous report (Haouala *et al.,* 2008) on the antifungal activity of methanol and aqueous extracts of *Trigonella foenum-graecum* against *Rhizoctonia solani* and *Alternaria* sp.; methanol extract provided the best results. This could be due to difference in proportion of chemicals responsible for antifungal activity in the two extract types (solvent

and aqueous) of the plant species. *A. radiata* and *H. umbellatum* ethyl acetate extracts, which were active against *G. candidum* in the *in vitro* screening, did not significantly differ from the control in the *in vivo* experiment. Therefore, for these two plant species, the *in vitro* and *in vivo* tests presented opposing results. According to Gatto *et al.* (2011), numerous factors (e.g. degradation, hydrolysis, polymerization, etc.) can affect the biological activity of certain components of plant extract when in contact with fruit tissue.

In the present work, four organic solvents with increasing polarity were tested (Hexane, choloroform, ethyl acetate and methanol). Among them, methanol fraction possesses great control in both *in vitro* and in *vivo* tests. Askun *et al.* (2009) reported that methanol provided more consistent antimicrobial activity. Methanol extracts of several plant species have been found to possess excellent antimicrobial activity against different fungal pathogens and bacteria (Sato *et al.,* 2000; Choi *et al.,* 2004; Ameziane *et al.,* 2007; Bajpai *et al.,* 2008; Satish *et al.,* 2008; Mahlo *et al.,* 2010; Ahmadi *et al.,* 2010; Hajji *et al.,* 2010). These activities might depend on the compounds being extracted by the solvent (Askun *et al.,* 2009).The phytochemical analysis of methanol extract of plant species that reduced the incidence and severity of citrus sour rot showed that they contain different classes of flavonoids and possess high levels of total phenols. This result is backed by several studies who reported that methanol can extract several compounds with antimicrobial activities especially the phenolic compounds (Nicholson and Hammerschmidt, 1992; Cowan *et al.,* 1999; Tripathi and Dubey, 2004; Sisti *et al.,* 2008; Martini *et al.,* 2009). Also of interest, Cowan *et al.* (1999) reported that plants have an almost limitless ability to synthesize aromatic substances that most of which are phenols that play a role in plant defense mechanisms against pathogens. The relation between composition and antifungal activity of these plant extracts may be attributable both to their major bioactive compounds but also to that presents in small amount. It is possible that they may act synergistically to contribute to the activity of the whole plant extract.

The possibility of controlling sour rot with plant extracts appears of particular interest considering the lack of valid alternatives to guazatine. Plant species tested in our study proved to be useful for effective biocontrol of *G. candidum* on citrus fruit. *C. villosus*, *C. siliqua* and *H. umbellatum* methanol extracts successfully reduced the disease incidence and disease severity caused by *G. candidum*, and no phytotoxic effects were recorded on citrus fruit. The antifungal activity of these plant extracts gives new opportunities to improve control against postharvest citrus fruit pathogens, and, in particular, in organic agriculture.

If *in vivo* results can be confirmed under commercial conditions, *C. villosus*, *C. siliqua* and *H. umbellatum* methanol extracts would be a viable option for controlling sour rot. Plant products as antifungal agents present three main characters : the first is their natural origin which means more safety to the consumer and the environment, the second is that they have been considered at low risk for resistance development by pathogenic fungi and third, they represent a rich source of potential bioactive compounds (Tripathi *et al.,* 2008). It is believed that it is difficult for the pathogens to develop resistance to such a mixture of plant extracts components with, apparently, different modes of action (Daferera *et al.,* 2003). Crude plant extracts are generally a mixture of active and non-active compounds. The results obtained in this work are the first scientific *in vivo* evidence referred to antifungal activity against *G. candidum* of methanolic crude extracts of *C. villosus*, *C. siliqua* and *H. umbellatum.* However, the potential use of these plant extracts to control sour rot requires a detailed examination of their biological activity and the development of a formulation which inhibits the growth of *G. candidum* at non-phytotoxic concentrations.

V. Conclusions

Of the eight plant species screened, three- *C. villosus*, *C. siliqua* and *H. umbellatum*- demonstrated strong antifungal potential overall. Two others - *H. antiatlanticum* and *R. ulmifolius*- showed a significant degree of sour rot control compared to the control. Recently, there has been considerable interest in plant extracts with antifungal activity, which are more acceptable, ecological safe and less hazardous than synthetic fungicides. Most of these plants are widely distributed in SMD region and easy grown. Furthermore, the extraction method is simple. Moreover, the use of a plant extract to protect citrus fruit against fungal attack may be acceptable in the organic production of citrus fruit. Further research will explore commercial potential of these plant species under degreening conditions.

CHAPTER FOUR

Bioguided fractionation

Project of article :
Talibi, I., El Abed S., Boubaker, H., Boudyach, Ait Ben Aoumar.

Résumé

Dans l'objectif de purifier les meilleurs fractions organiques et de comprendre le mode avec lequel leurs composés agissent sur le pathogène, les extraits méthanoliques de *C. villosus* et *H. umbellatum* ont subi un fractionnement bio-guidé. Cette méthode consiste à fractionner l'extrait en question sur gel de silice (par chromatographie sur colonne) en utilisant des systèmes d'élution de polarité croissante. Les sous-fractions issues de chaque fraction ont été testées pour leur activité contre la croissance mycélienne de *G. candidum*. La sous-fraction F.7 issue de l'extrait méthanolique de *C. villosus* est la plus active contre *G. candidum* par inhibition de la croissance mycélienne du champignon avec un diamètre de la zone d'inhibition de 40,33 mm autour des puits inoculés avec la sous-fraction. Pour *H. umbellatum*, le meilleur degré d'inhibition de la croissance mycélienne est obtenu avec la sous-fraction F.6 avec un diamètre de la zone d'inhibition de 27 mm. Ces sous-fractions, les plus actives, ont subi un autre fractionnement et les sous-fractions obtenues ont été testées pour évaluer leur effet antifongique. Les résultats ont montré qu'il ya une diminution de l'activité antifongique des extraits au fur et à mesure du fractionnement.

Mots clés: Fractionnement bioguidé, Chromatographie sur colonne, Synergie.

Abstract

In the previous chapters we tested the antifungal activity, *in vitro* and *in vivo*, of four organic fractions (Hexane, Chloroform, Ethyl acetate and Methanol) of the eight best plants selected from the 43 plants tested initially. From these fractions we have selected the most active fractions (methanol extracts of *Cistus villosus* and *Halimium umbellatum*) for further purification by the bioguided fractionation method. Hence the objective of the present study was to investigate the most active subfraction from *C. villosus* and *H. umbellatum* against *G. candidum*. First of all, the methanolic extract of *C. villosus* and *H. umbellatum* were fractionated by open column chromatography and eight subfractions were obtained from each extract. The subfractions derived from each extract were tested for their antifungal activity against *G. candidum.* The subfraction F.7 from *C. villosus* is the most active against *G. candidum* by inhibiting the mycelial growth of the fungus, around wells inoculated with the subfraction, with 40.33 mm diameter. For *H. umbellatum*, the best level of inhibition is obtained with the subfraction F.6 with inhibition zone diameter of 27 mm. These subfractions underwent another fractionation and the resulting subfractions were tested for their antifungal effect. The results showed that there is loss of the biological activity with each fractionation.

Keywords : Bioguided fractionation, Columen chromatography, Synergism

I. Introduction

Fruit and vegetables have a number of constitutive and inducible compounds that are antimicrobial which can be used to produce safe marketable citrus fruits (Tripathi *et al.,* 2008; Tayel *et al.,* 2009). Although more than 1340 plants are known to be potential sources of antimicrobial compounds and about 10000 secondary plant metabolites have been chemically defined for their role as anti-pathogenic chemicals, many of these substances can play a fundamental role in biological control of postharvest disease (Cowan, 1999). Plant extracts have the potential to be natural fungicides and can replace the synthetic ones. Some phytochemicals of plant origin have been formulated as botanical pesticides and are used successfully in integrated pest management programs as botanical pesticides. (Tripathi *et al.,* 2004). Plant products as antifungal agents present three main characters : the first is their natural origin which means more safety to the consumer and the environment, the second is that they have been considered at low risk for resistance development by pathogenic fungi and third, they represent a rich source of potential bioactive compounds (Tripathi *et al.,* 2008). It is believed that it is difficult for the pathogens to develop resistance to such a mixture of plant extracts components with, apparently, different modes of action (Daferera *et al.,* 2003). In the available literature, researches on biological control of postharvest diseases of citrus fruits by plant extracts has explored various aspects such as essential oils, volatile compounds, aqueous and solvent extracts and some products extracted from plants (Droby *et al.,* 1999b; Arras and Usai, 2001; Du Plooy *et al.,* 2009; Liu *et al.,* 2009b; Gatto *et al.,* 2011; Talibi *et al.,* 2012a; Talibi *et al.,* 2012b). Among the plants tested, *Cistus villosus* and *Halimium umbellatum* are reported to control several plants pathogens (Bouamama *et al.,* 2006; Ameziane *et al.,* 2007; Askarne *et al.,* 2012; Talibi *et al.*, 2012a; Talibi *et al.,* 2012b).

This study is a continuity of works of Talibi *et al.* (2012b) who tested the effectiveness of four organic fractions (hexane, chloroform, ethyl acetate and methanol) of eight plants Against *Geotrichum candidum*, the causal agent of citrus sour rot. Among these fractions, the methanol extracts of *Cistus villosus* and *Halimium umbellatum* were the most active against the pathogen. Often, bioactive compounds constitute a very minor part of the crude extract; the bioassay-guided fractionation maximizes the possibility of discovering new compounds with biological activity. This methodology integrates the processes of compound isolation using various analytical methods. In the present work, this strategy has been used to obtaine compounds (s) from methanol fractions of *C. villosus* and *H. umbellatum* responsible (s) of the inhibition of *G. candidum*.

II. Material and Methods

II.1 Pathogen and arthrospores preparation

The *Geotrichum candidum* isolate used in this study was obtained from a decayed mandarin fruit. It is one of the most aggressive isolates in our collection. This isolate was deposited at the laboratory of Biotechnology and Valorization of the Natural Resources under the number SR10. The fungus was maintained on potato dextrose agar (PDA) plates at 5°C, with periodic transfers through citrus fruit to maintain its aggressiveness. The pathogen inoculum consisted of aqueous arthrospores suspensions obtained from seven-day old culture plates incubated at 25°C. Arthrospores were harvested by flooding plates with five mL of sterile distilled water containing 0.05% (v/v) Tween 80, and passing the suspension through two layers of sterile cheesecloth to remove hyphal fragments. The arthrospores concentration was determined with the aid of a heamacytometer and adjusted to 10^6 arthrospores mL^{-1} with sterile distilled water.

II.2 Preparation of plant extracts

Preparation of the extracts from *Cistus villosus* and *Halimium umbellatum* was conducted as previously described by Duraipandiyan and Ignacimuthu (2007) and Bajpai *et al.* (2008). Briefly, powder (20g) of each plant species was extracted with 100 mL of hexane by maceration with stirring at room temperature for 48 h and then filtered through Whatman no. 1 filter paper to obtain particle free extract. The solvent of the filtrate was removed by evaporation to dryness under reduced pressure at 40°C. The remains of the plant material were extracted with chloroform, ethyl acetate and methanol sequentially in a similar manner. The extracts were then sterilized by passing through 0.2µm pore diameter Millipore Swinex filters and kept at -20°C before assay. Solvents (analytical grade) for extraction were obtained from commercial sources. The bioguided chromatographic fractionation process began with the evaluation of the antifungal activity of these organic fractions. Methanol extracts are the most actives fractions (Data not shown). Further fractionation was carried out with the more active fractions and the process was repeated for subfractions until obtaining fraction with pure compounds exhibiting antifungal activity.

II.3 Open column chromatography

In order to localize the active fraction, methanol extract of *C. villosus* and *H. umbellatum* were purified using an open column chromatography. Silica gel 60 mixed with Dichloromethane was poured slowly into column (2.5 cm× 70 cm). Then, cotton-wool was

neatly placed on the top of gel to prevent disturbance at the surface during solvent introduction. The extract tested (Methanol extract of *C. villosus* or *H. umbellatum*) was then deposited neatly on top of the cotton-wool. Nine elution systems were added slowly in the order as in **table 4.1**. In order to select the best mobile phase for eluting the methanol extracts, 5 µl of each sample were developed in thin layer chromatography (TLC) (Merck, silica gel 60 F254) with various combination of solvents. The solvent that exhibited the most favorable separation of compounds was chosen. The vacuum was switched on and the solvent was allowed to run through the column, until the elution system had been collected in the tubes (**Figure 4.1**).

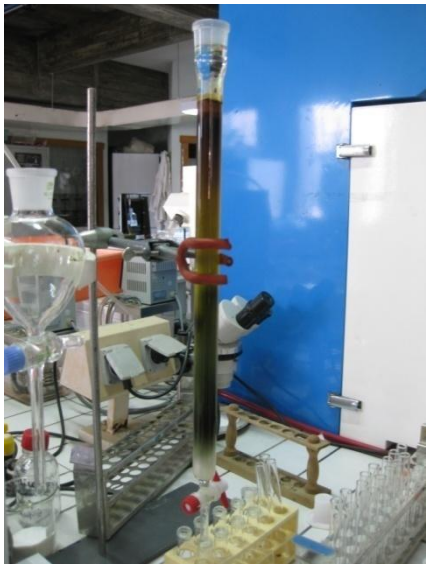


Figure 4.1 : Fractionation of methanol extract of *C. villosus* by the open columen chromatography

Table 4.1 : Solvents mixtures used in the first column chromatography (%)

System order	Hexan	Ethyl Acetate	Dichloromethane	Methanol
1	45	5	45	5
2	40	10	40	10
3	35	15	35	15
4	30	20	30	20
5	25	25	25	25
6	20	30	20	30
7	15	35	15	35
8	10	40	10	40
9	5	45	5	45

Fractions were analyzed by thin layer chromatography. From TLC results, fractions were combined, based on the similarity of their chemical profile **(Figure 4.2)**. Combined fractions were concentrated by removing the solvent by evaporation under reduced pressure at 40°C. Each fraction was tested for its antifungal activity and the active fractions were subjected to further column chromatography. The second and the third fractionations are carried out by using another elution system as indicated in **table 4.2**. The fractionation order of methanol extract of *C. villosus* and *H. umbellatum* is illustrated in **Figure 4.3** and **4.4** respectively.

Table 4.2 : Solvents mixtures used in the second and third column chromatography

	Portion of solvent (%)			
System order	**Chloroform**	**Ethyl Acetate**	**Methanol**	**Acitonitril**
1	30	40	20	10
2	10	40	30	20
3	0	40	40	20
4	0	20	50	30
5	0	10	60	30
6	0	0	70	30
7	0	0	80	20
8	0	0	90	10
9	0	0	100	0

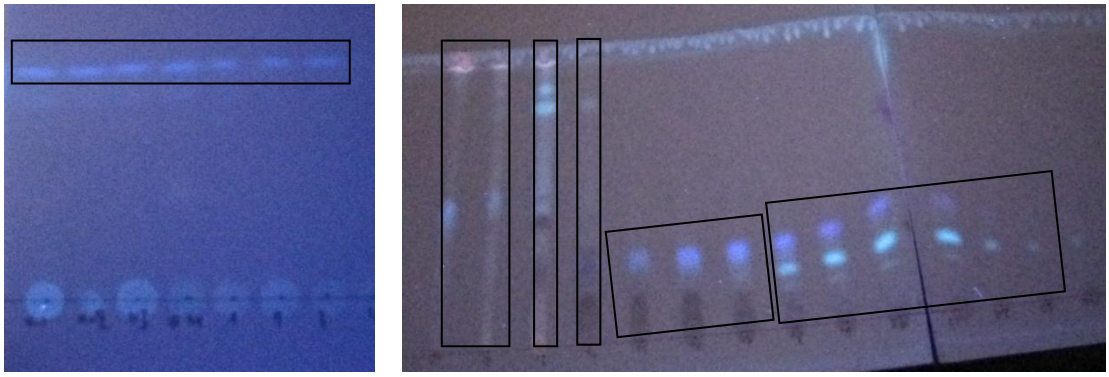


Figure 4.2 : Combination of fractions based on the similarity of their chemical profile (254 nm)

II.4 Antifungal assay

Each fraction was screened for their antifungal activity using the well-plate diffusion method. Aliquots (100 µL) of arthrospores suspension (10^6 arthrospores /mL) of *G. candidum* was overlaid on Petri plates (90 mm in diameters) containing 15 mL of PDA. The plates were

incubated at room temperature for 30 minutes to allow a good diffusion of the suspension in the culture medium. Then five-mm diameter wells were made at three locations per dishes. The wells are then filled with 20 µL of the fraction at concentration of 5 mg/mL. Control plates consisted of wells filled with 20µL of Methanol. The plates are stored at 4°C for 2 hours to allow the diffusion of extract, and then incubated at 26°C. After 48 hours incubation the diameter of the inhibition zone is measured. Three plates were used for each treatment as replications, and each experiment was repeated twice.

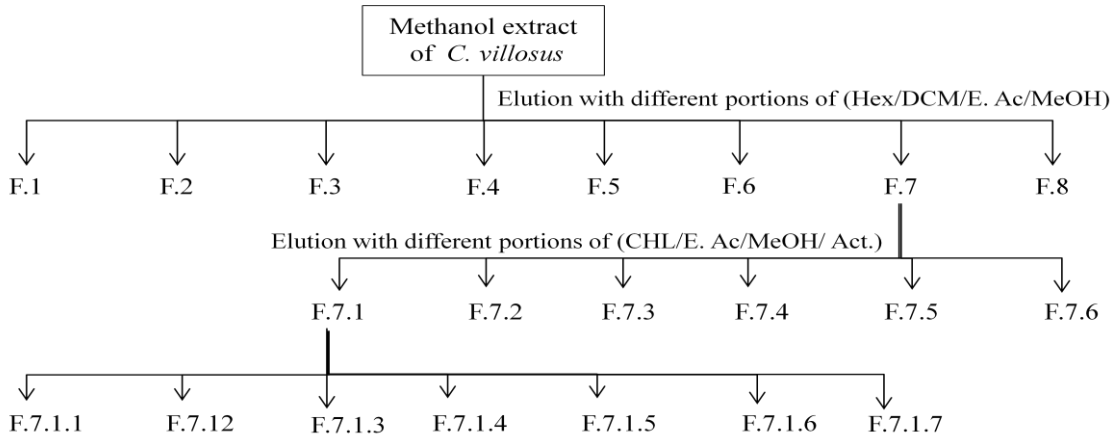


Figure 4.3 : Order of fractionation of the methanol extract of *C. villosus*. Hex : Hexan; DCM : Dichloromethan; CHL : Chloroform; E. Ac : Ethyl acetate; MeOH : Methanol; Act : Acitonitril

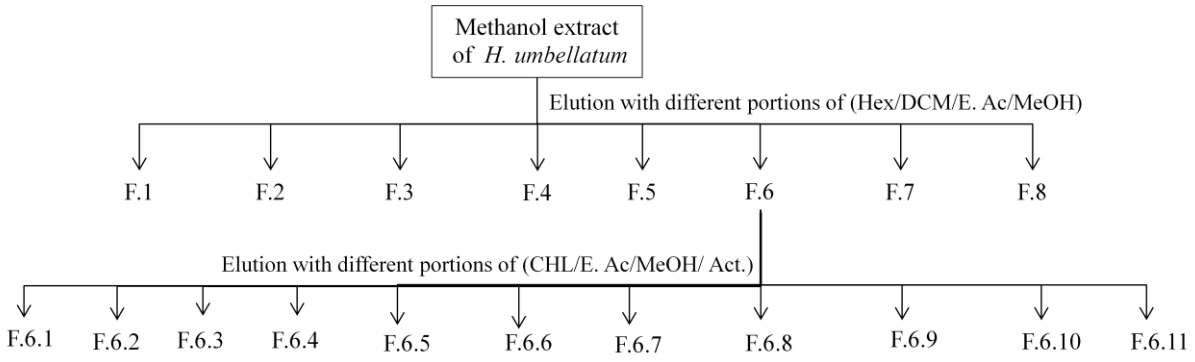


Figure 4.4 : Order of fractionation of the methanol extract of *H. umbellatum.* Hex : Hexan; DCM : Dichloromethan; CHL : Chloroform; E. Ac : Ethyl acetate; MeOH : Methanol; Act : Acitonitril

II.5 Statistical analysis

Results were subjected to statistical analysis of variance (ANOVA) using STATISTICA software, version 6, Stat-Soft, 2001, France. Mean separation was performed following the Newman & Keuls test at $P<0.05$.

III. Results

III.1 Bioguided fractionation of *Cistus Villosus*

The methanol extracts of *C. villosus* was fractioned by open column chromatography. These fractionations are guided by the antifungal activity assay of each fraction. Thus extract of *C. villosus* is fractioned three times (**Figure 4.3**).

The first fractionation was iluted with different portions of Hexan/Ethyl Acetate/ Dichloromethane/Methanol and yielding eight subfractions. Each subfraction was evaluated for their antifungal against *Geotrichum candidum* by the well-plate diffusion method and the diameter of inhibition zone was determined and data are showen in **figure 4.5**. Among the eight subfractions recovered, the fourth, sixth and seventh subfraction exhibited antifungal activity by 21.33; 4 and 40.33 mm of inhibition zone respectively (**Figure 4.5** and **4.6**).

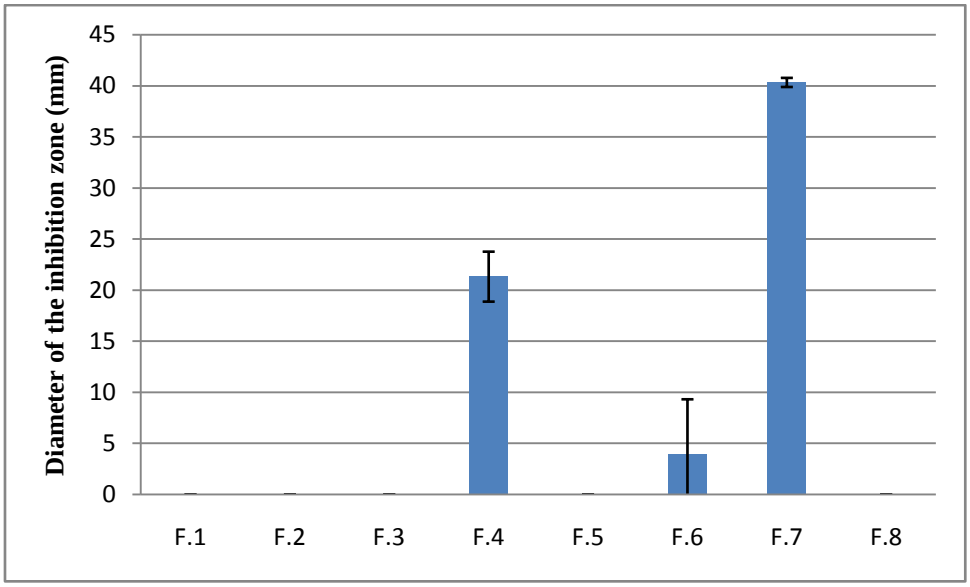


Figure 4.5 : *In vitro* effects of methanol oxtracts fractions of *C. villosus* on mycelial growth of *G. candidum*

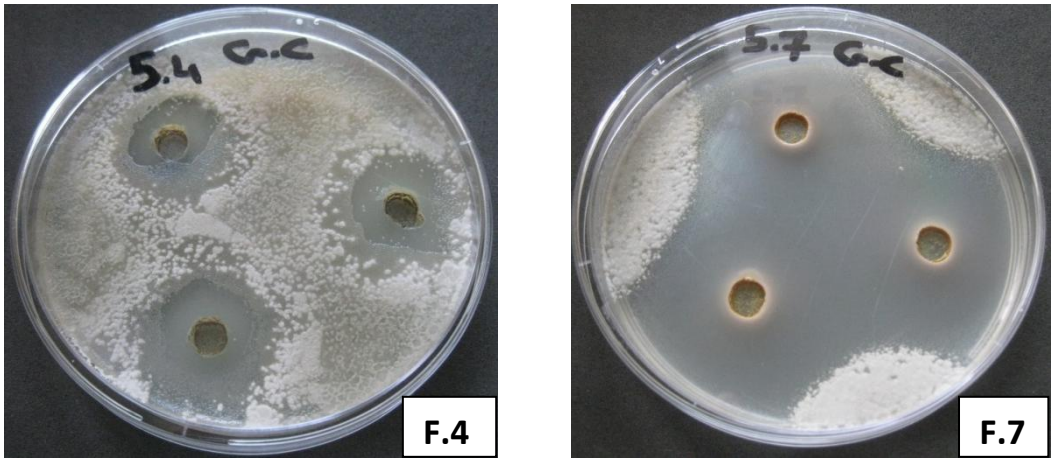


Figure 4.6 : Inhibition zone of *Geotrichum citri-aurantii* around wells amended with the subfractions of *Cistus villosus*

The TLC analysis of the subfraction N° 7 (F.7) eluted with the mixture DCM/CHL/ Et. Ac /MeOH (3/3/2/2) shows that it still contain many compounds (Data not shown). Therefore, this subfraction was subjected to further fractionation with different portions of Chloroform/Ethyl Acetate/ Methanol/Acitonitrile as elution system and yielding six subfractions (**Figure 4.3**). Then, each subfraction was tested on the mycelial growth of *G. candidum*. Results of the bioassay of thes subfractions showed that the antifungal activity was recorded in the subfractions F.7.1; F.7.2; F.7.3 and F.7.4 by 18; 8.66; 5.66 and 8.66 mm of inhibition zone respectively (**Figure 4.7** and **4.8**). The TLC analysis of the subfraction F.7.1 eluted with the mixture DCM/CHL/ Et. Ac /MeOH (3/3/2/2) shows six separated bands and thus, underwent another purification and yielding seven subfractions (**Figure 4.3).**

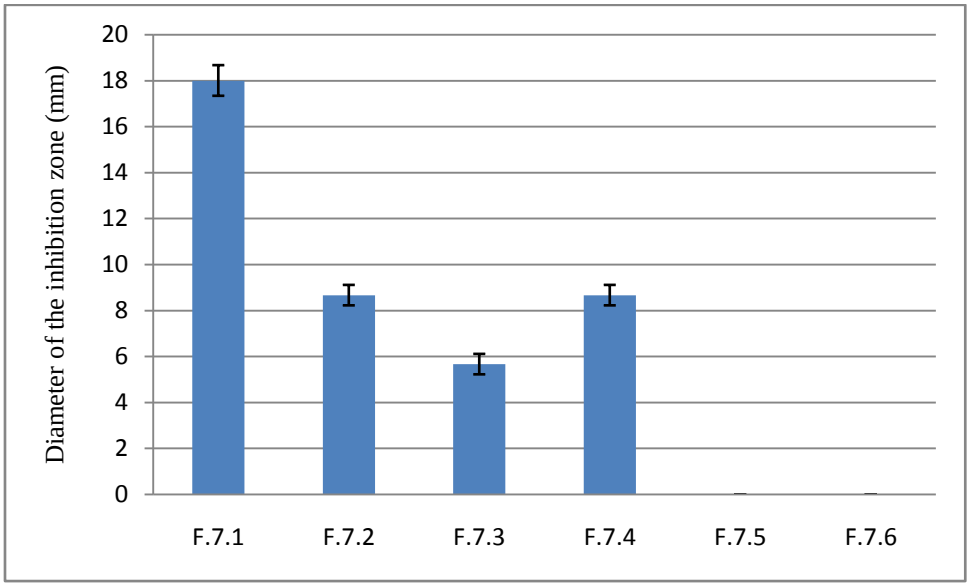


Figure 4.7 : *In vitro* effects of subfractions from the F.7 fraction of *C. villosus* on mycelial growth of *G. candidum*

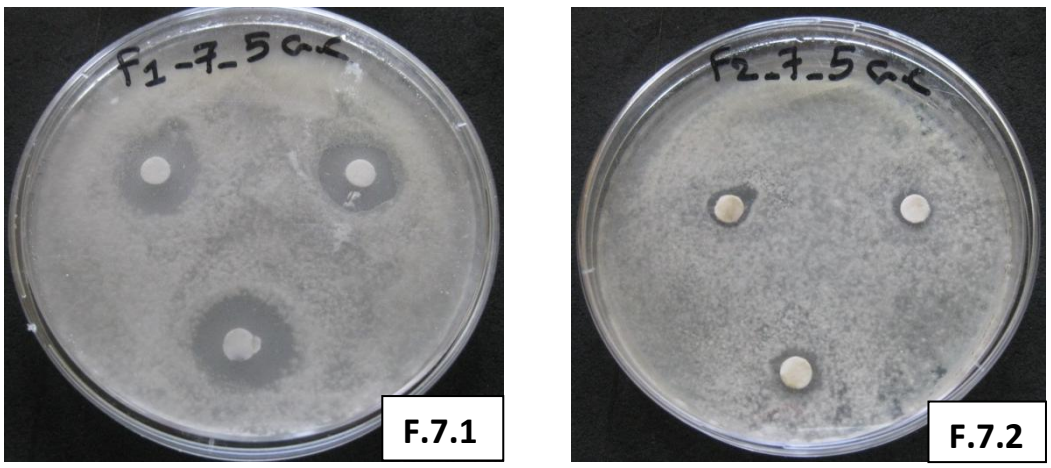


Figure 4.8 : Inhibition of zone around wells inoculated with the subfractions of *C. villosus*

Subfractions derived from this fractionation (F.7.1.1 to F.7.1.7) are tested against *G. candidum* and no subfraction has inhibited the mycelial growth of the fungus.

III.2 Bioguided fractionation of *Halimium umbellatum*

Like as *C. villosus*, the methanol extract of *H. umbellatum* was fractioned by open column chromatography and the results of its fractionation are detailed in **Figure 4.9**. The first subfractions derived from the methanol extracts of *H. umbellatum* have been tested on mycelial growth of *G. candidum*. The results indicate that of the subfractions tested, only fractions 6-7-8 have inhibited the mycelial growth of *G. candidum* by 27; 14.33 and 17 mm respectively **(Figure 4.9 and figure 4.10)**. The TLC analysis of the subfraction N°6 eluted with the mixture DCM/CHL/ Et. Ac/MeOH (3/3/2/2), revealed superimposed bands.

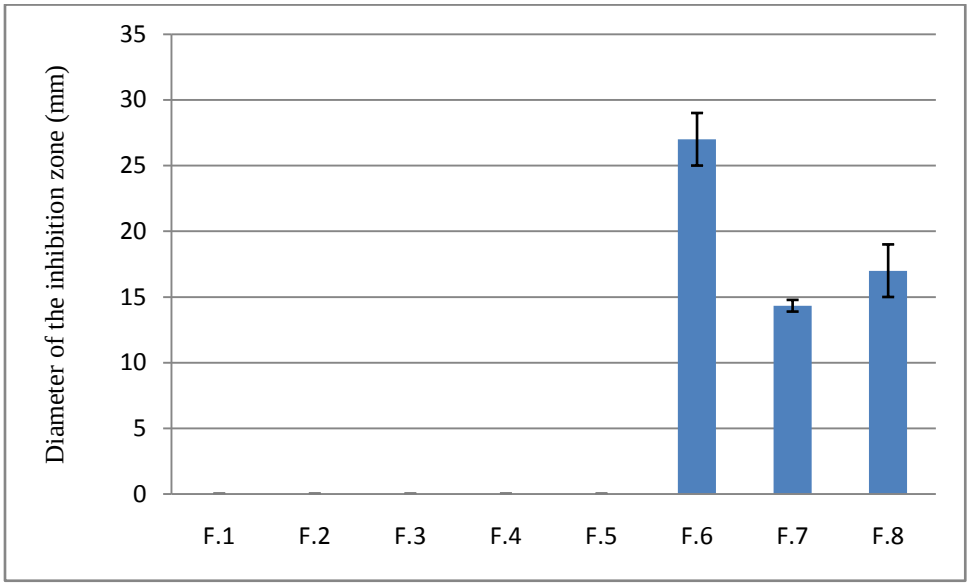


Figure 4.9 : *In vitro* effects of methanol extracts fractions of *H.umbellatum* on mycelial growth of *G. candidum*

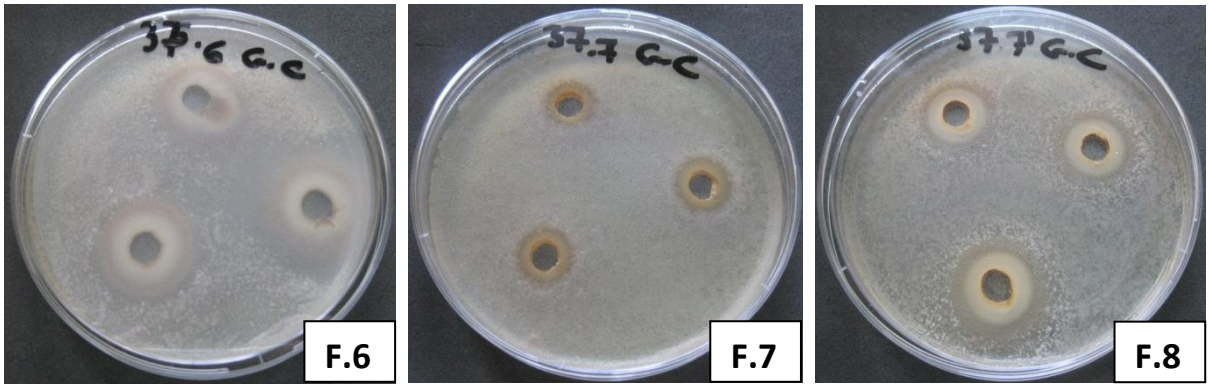


Figure 4.10 *:* Inhibition zone of *Geotrichum citri-aurantii* around wells amended with the subfractions of *Hallimium umbellatum*

The subfraction F.6 is allowed to further fractionation with the ilution system composed of different proportions of the mixture Chloroform/Ethyl Acetate/ Methanol/Acitonitrile (**Figure 4.4**). Eleven subfractions were obtained from this fractionation (F.6.1-11) and each subfraction was tested for its antifungal activity against *G. candidum.* Results presented in **figure 4.11** showed that subfractions F.6.1, F.6.2, F.6.5 and F.6.7 reduced the mycelial growth of *G. candidum* around inoculated wells by 8; 10; 6 and 6mm respectively.

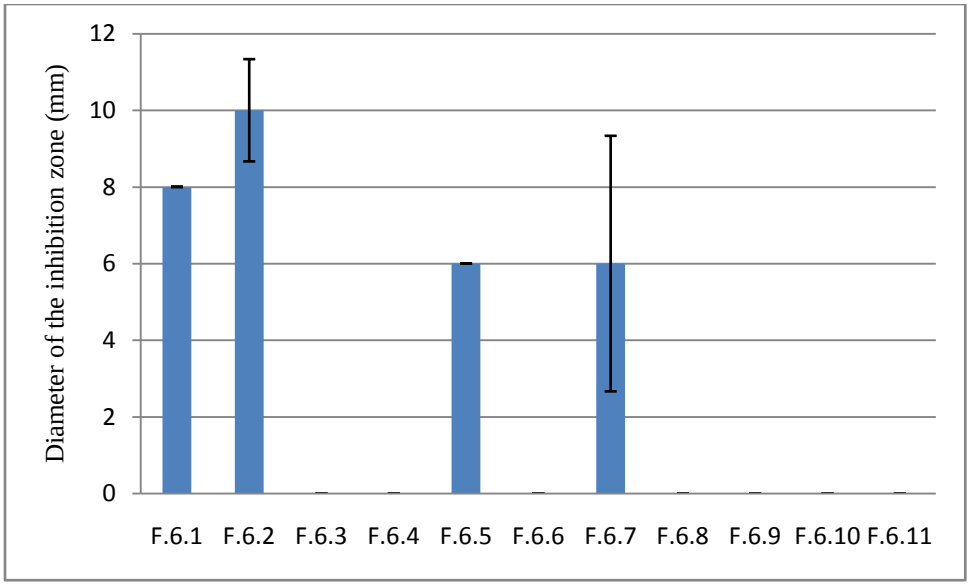


Figure 4.11 : *In vitro* effects of subfractions from the F.6 fraction of *H.umbellatum* on mycelial growth of *G. candidum*

IV. Discussion

From all of these results, we can deduce that more the extract is fractioned, the efficiency of the subfractions to inhibit the mycelial growth of the fungus decreases. So there is a loss of antifungal activity in the second and third fractionation compared to the first one. This can be explained by the decrease of the concentration of the active compound (s) and/or the loss of the synergistic action between any of the phytochemical constituents present in the original fraction. The synergistic effect of phytochemical compounds is also reported by several works (Ellof, 2004; Nenaah, 2010; Zakaria *et al.,* 2010; Suleiman *et al.,* 2012). Nenaah (2010) reported that when Harmala alkaloids are applied individually exhibited moderate antimicrobial effects. In contrast, the activity of the tested alkaloids was increased when tested as binary or total (crude) alkaloidal mixture suggesting a kind of synergism among these compounds (Nenaah, 2010). Likewise, Suleiman *et al.* (2012) reported the loss of more than 85% of the antimicrobial activity of fractions from the original crude extract of *Loxostylis alata.*

V. Conclusions

Therefore, we can conclude that compounds from *Cistus villosus* and *Halimium umbellatum* exhibit their antifungal activity by the synergism effect between their phytochemical compounds. So, the optimal effectiveness of plants is not, always, due to one main active constituent, but to the combined action of different compounds originally in the plant. Moreover, further research should focus on the phytochemical analysis to identify the chemical composition of the most actives fractions of each plant.

.

CHAPTER FIVE

Screening of organic and inorganic salts to control postharvest citrus sour rot

Published in Plant Pathology journal as :
Talibi, I., Askarne, L., Boubaker, H., Boudyach E.H., Ait Ben Oumar A., 2011. *In vitro* and *in vivo* Screening of organic and inorganic salts to control of postharvest citrus sour rot caused by *Geotrichum candidum* Plant Pathology journal 10, 138-145.

Résumé

Dans l'objectif de rechercher des alternatives saines et efficace aux fongicides chimique utilisés dans la lutte contre la pourriture amère des agrumes en post-récolte, nous avons testé l'efficacité de 34 sels organiques et inorganiques contre *G. candidum*. Les résultats du screening de l'activité antifongique de ces sels sur la croissance mycélienne ont montré que les concentrations minimales inhibitrices obtenues varient selon la nature du sel testé et de sa concentration. La CMI la plus faible (0,1%) est obtenue avec le carbonate d'ammonium et l'EDTA, suivie par le salicylate de sodium, le metabisulfite de sodium et l'acide borique avec une CMI de 0,25%. Les dix meilleurs sels, sélectionnés sur la base de leur CMI, ont été, ensuite, testés pour leurs capacité à réduire ou à inhiber la germination des arthrospores du pathogène. Les résultats obtenus ont montré que l'EDTA, l'acide borique, le metabisulfite de sodium, le carbonate de sodium, le sulfate de sodium et le thiosulfate de sodium ont totalement inhibé la germination des arthrospores à partir d'une concentration de 75 et 100 mM. En revanche, le salicylate de sodium, le carbonate d'ammonium et le carbonate de potassium se sont montrés peu efficaces sur la germination des arthrospores de *G. candidum*. Considérant que plusieurs sels peuvent influencer le pH du milieu, l'effet de ce dernier sur la croissance mycélienne de *G. candidum* a été déterminé. Les résultats obtenus ont montré que la croissance mycélienne est faiblement affectée par les modifications du pH dans la gamme de pH 4,0 à 12,0. Pour le test *in vivo*, des mandarines ont été traitées avec différentes concentrations de sels (1, 2 et 3% w/v), inoculés avec une suspension d'arthrospores de *G. candidum* puis incubées à 26°C et à une humidité relative élevée. Les résultats obtenus ont montré que seuls le salicylate de sodium, l'acide borique et l'EDTA ont réduit significativement l'incidence et la sévérité de la pourriture amère. En effet, testé à une concentration de 3%, l'incidence de la maladie a été réduite à 25,93% chez les fruits traités avec le salicylate de sodium ou l'acide borique et à 38,89% chez les fruits traités avec de l'EDTA. Le carbonate d'ammonium et le carbonate de sodium ont aussi significativement réduit l'incidence de la pourriture amère respectivement à 51,8 et 57,41%.

Mots clés : Agrumes, sels, *Geotrichum candidum*

Abstract

The aim of this study was to find an alternative to the chemical fungicide currently used in the control of postharvest citrus sour rot. Here, we screened thirty-four salt

compounds, considered as common food additives, for their activity against *Geotrichum candidum*, causal agent of citrus sour rot. The lowest Minimum Inhibitory Concentrations (MICs) values were obtained by ammonium carbonate and EDTA at a concentration of 0.1% (w/v) and boric acid, sodium carbonate and sodium metabisulfite at 0.25% (w/v). Over all, the medium-pH in the range of 4.0 to 12.0 did not influence the mycelial growth of the pathogen. The ten best salt compounds were tested for their ability to reduce the arthrospores germination of the fungi.The effect of salts varied significantly ($P<0.05$) between tested compounds and depended on their concentrations. The arthrospore germination was completely inhibited by EDTA, boric acid, sodium metabisulfite, sodium carbonate, sodium sulfate and sodium thiosulfate, both at 100 and 75 mM. The most active salts in *in vitro* studies were tested *in vivo* against sour rot on citrus fruit. Incidence of sour rot was lowered to 25.93% and 38.89%, when mandarin fruit where treated by sodium salicylate, boric acid and EDTA, compared with 100% in the control. However, only the application of boric acid at 3% (w/v) reduced disease severity by more than 70%. These results suggest that sodium salicylate, boric acid and EDTA may be useful and effective compounds for control of citrus sour rot. Such healthy products therefore represent a sustainable alternative to the use of guazatine mainly in organic production.

Key words : Citrus, salts, sour rot, *Geotrichum candidum*

I. Introduction

Sour rot caused by the fungus *Geotrichum candidum* is one of the major postharvest diseases of citrus fruits (Kitagawa and kawada, 1984). It is one of the most economically important postharvest diseases of citrus in arid growing regions of the world (Smilanick and Sorenson, 2001) and cause serious problems for harvested citrus fruits during handling, transportation, exportation and storage (El-mougy *et al.*, 2008). The organism is a wound pathogen, infecting fruit during harvest and subsequent handling, and requiring injury into the albedo for entry (Brown, 1979). Besides the injuries, the aggressiveness of the fungus increases especially during fruit degreening, wet and rainfall seasons (Eckert, 1978; Eckert and Brown, 1988; Cohen *et al.*, 1991, Liu *et al.*, 2009b).

The measures employed to manage postharvest citrus rot are not effective against *Geotrichum candidum.* This pathogen is not controlled by any of the fungicides (e.g., Imazalil and Thiabendazole) registered for use on citrus fruit (Eckert, 1978; Kitagawa and Kawada, 1984; Brown, 1988; Suprapta *et al.*, 1997; Mercier *et al.*, 2005; Smilanick *et al.*, 2008; Liu *et al.*, 2009b; Feng *et al.*, 2011). Guazatine is the only commercial fungicide that can control sour rot (Rippon and Morris, 1981; Brown, 1988). However, this fungicide is not authorized in several countries. The disease can be partially reduced by Sodium o-phenylphenate (SOPP) (Rippon and Morris, 1981; Feng *et al.*, 2011) which is found to be carcinogenic and has promoting activity towards the urinary bladder (Kitagawa and Kawada, 1984). Therefore, alternative treatments have become an essential requirement for the control of this disease. Furthermore, concerns about public health risks associated to fungicide residues and environmental issues have increased the need for these alternatives.

Use of organic and inorganic salts, generally recognized as safe (GRAS) compounds, is an interesting alternative to control postharvest disease of citrus fruits. Many of these salts have several advantages such as low mammalian toxicity, favorable safety profile for humans and environment, and a relatively low cost (Olivier *et al.*, 1998; Hervieux, *et al.*, 2002; Deliopoulos *et al.*, 2010). Moreover, these compounds have a broad-spectrum antimicrobial activity (Corral *et al.*, 1988; Olivier *et al.*, 1998; Deliopoulos *et al.*, 2010) and are usually used in the food industry for controlling pH, taste and texture (Smilanick *et al.*, 1999; Hervieux, *et al.*, 2002; Arslan *et al.*, 2009). Furthermore, several studies have reported the effectiveness of salts to control various pathogens of many crops. Potassium sorbate (KS) was

shown to reduce the incidence of sour rot under laboratory conditions (Kitagawa and Kawada, 1984; El-Mougy *et al.*, 2008; Smilancik *et al.*, 2008). Also, Sodium benzoate and KS were used to control postharvest decays caused by many fungi (Al-Zaemey *et al.*, 1993; Olivier *et al.*, 1998; El-Mougy *et al.*, 2008; Palou *et al.*, 2001). Palou *et al.* (2001) and smilanick *et al.* (1999) demonstrated that sodium carbonate reduced the incidence of citrus green and blue mold. Moreover, salt compounds also improve their performance when used in combination with other treatments like microbial antagonists (El-Ghaouth *et al.*, 2000; Nunes *et al.*, 2002; Zhang *et al.*, 2008; Sharm *et al.*, 2009), fungicides (Smilanick *et al.*, 2008) or hot water (Palou *et al.*, 2001; Porat *et al.*, 2002; Smilanick *et al.*, 2008).

Although most researchers have focused on controlling green and blue mold, little has been published about the control of sour rot. The present work was performed to evaluate the efficacy of a wide range of organic acids and salts, for *in vitro* and *in vivo* control of *G. candidum*, the causal agent of citrus sour rot.

II. Materials and Methods

II.1 Pathogen culture and chemicals

Geotrichum candidum was isolated from a decayed Clementine fruit and was one of the most aggressive isolates in our collection. The fungus was maintained on PDA plates at 5°C, with periodic transfers through citrus fruit to maintain its aggressiveness. The pathogen inoculum that consisted of aqueous arthrospores suspensions obtained from 7-day-old culture plates incubated at 25°C. Arthrospores were harvested by flooding plates with 5 ml of sterile distilled water containing 0.05% (v/v) Tween 80, and passing the suspension through two layers of sterile cheesecloth to remove hyphal fragments. The arthrospores concentration was determined with the aid of a heamacytometer and adjusted to 10^6 arthrospores ml^{-1} with sterile distilled water. A total of 34 salt compounds (**Table 5.1**) considered as common food additives were used in this study to evaluate their effectiveness against *G. candidum*.

II.2 Fruit

The fruit of mandarin (*Citrus reticulata* blanco) cv. "Clementine" was used. Fruits were harvested from orchards of the M'brouka cooperative, which used standard culture practices, in Souss-Massa-Draa region, Morocco. Only healthy and commercially mature fruits were used in the *in vivo* test. Freshly harvested or briefly stored (no longer than a week) fruits were used in the experiment.

Table 5.1 : The tested compounds, their chemical formula and molecular weight

Compounds	**Chemical Formula**	**Molecular weight**
Ammonium acetate	$(NH_4)C_2H_3O_2$	77.08
Ammonium carbonate	$(NH_4)_2CO_3$	96.09
Ammonium dihydrogen phosphate	$(NH_4)H_2PO_4$	115.03
Ammonium molybdate	$(NH_4)6Mo_7O_{24}$, $4H_2O$	1235.86
Ammonium sulfate	$(NH_4)_2SO_4$	132.14
Ascorbic acid	$C_6H_8O_6$	176.13
Aspartic acid	C_4H_7O4N	133.11
Boric acide	H_3BO_3	61.83
Calcium carbonate	$CaCO_3$	100.09
Calcium chloride	$CaCl_2$	110.98
Calcium Chloride	CaCl2, $2H_2O$	147.02
Calcium nitrate	$Ca(NO_3)_2 \cdot 4H_2O$	236.14
Citric acid	$C_2H_8O_7$, H_2O	210.14
Dipotassium hydrogen phosphate	K_2HPO_4	174.18
EDTA	$C_{10}H_{14}N_2Na_2O_8$, $2H_2O$	372.24
Glutaric acid	$C_5H_8O_4$	132.12
Magnesium chloride	$MgCl_2$	203.31
Magnesium sulfate	$MgSO_4$	246.48
Potassium acetate	$C_2H_3KO_2$	98.14
Potassium carbonate	K_2CO_3	138.21
Potassium chloride	KCl	74.55
Potassium phosphate dibasic	K_2HPO_4	174.18
Potassium sodium tartrate	$C_4H_4KNaO_6$, $4H_2O$	282.23
Sodium acetate	$C_2H_3O_2Na$	82.03
Sodium bicarbonate	$NaHCO_3$	84.01
Sodium carbonate	Na_2CO_3	105.99
Sodium chloride	NaCl	58.44
Sodium metabisulfite	$Na_2S_2O_5$	190.1
Sodium molybdate	Na_2MoO_4	241.95
Sodium salicylate	$C_7H_5NaO_3$	160.11
Sodium sulfate	Na_2SO_4	142.04
Sodium thiosulfate	$Na_2S_2O_3$, $5H_2O$	248.18
Sodium salicylate	$CuSO_4$, $5H_2O$	249.68
Sodium thiosulfate	$Na_2S_2O_3$ - $5H_2O$	248.2

II.3 Determination of Minimum Inhibitory Concentration (MIC)

The minimal inhibitory concentrations (MICs) of the 34 salts were determined by the broth dilution method using the Nutrient Yeast Dextrose Broth (NYDB : nutrient broth 8 g l^{-1}; yeast extract 5 g l^{-1}; dextrose 10 g l^{-1}) as culture medium. Each salt was tested at seven concentrations : 0.1, 0.25, 0.5, 0.75, 1, 1.75 and 2% (w/v). One millilitre of each aqueous

solution at desired concentration was transferred to test tubes containing 9 ml of NYDB. The medium without salt compounds served as control. Each tube was inoculated with 100 µl of a suspension of 10^6 arthrospores/ml of *G. candidum* and incubated at 25°C for 72 hours with shaking. The MICs were recorded by reading the lowest salt concentration that allowed no visible growth of the pathogen. There were three replicates for each salt at each concentration and the experiment was conducted twice.

II.4 Effect of pH on mycelial growth of *G. candidum*

Since some of the salt compounds could affect the pH of NYDB medium, we tested the effect of pH alone on mycelial radial growth of *G. candidum*. The pH tested varied from 2 to 12 and were adjusted with 1N HCl or NaOH. Hyphal plugs (5 mm diameter) were cut from the periphery of actively growing colonies (7 to 10 day-old) and transferred aseptically, mycelium down, to three replicate Petri plates containing NYDA at different pH. Radial growth was determined daily, by measuring colony size along two perpendicular axes. The experiment was performed with three replicate plates per treatment.

II.5 Effect of salts on arthrospores germination

To evaluate the impact of salt compounds on arthrospores germination, only salts with MIC value equal or lower than 0.5 % were tested. Aqueous solution of salt compounds was prepared in orange juice (2%) as nutrient medium. The germination of arthrospores of *G. candidum* was determined in concentrations of 25 mM, 50 mM, 75 mM and 100 mM of each salt. Aliquots (40µl) of an arthrospores suspension (10^6 arthrospores/mL) were aseptically transferred in triplicate to sterile depression slides containing 40 µl of 2 % sterile orange juice amended with different concentrations of salt (Droby *et al.,* 2003). The pH of solution was not modified; it was determined by the salt and its concentration. Inoculated slides were placed on moist filter paper in Petri plates, sealed with Parafilm to avoid evaporation, and then incubated at 25°C for 24h. Each depression slide was then fixed with acid fuchsine solution to stop further germination (Smilanick *et al.,* 1999). Arthrospores germination was estimated under a microscope equipped with a micrometer. At least 100 arthrospores within each replicate were observed. An arthrospore was scored as germinated if the germ tube length was at least equal or superior to the length of the spore body (Suprapta *et al.,* 1997). The results were expressed as percent spore germination inhibition and calculated by using the following formula : $\mathbf{GI\ (\%) = [(Gc - Gt)\ /Gc)]\ \times 100}$, Gc and Gt represent the mean number of germinated spores in control and treated slides, respectively (Soylu *et al.,* 2010). Each treatment included three replicates and the experiment was conducted twice.

II.6 Effects of salts on sour rot development in artificially inoculated and wounded fruit

Based on the *in vitro* antifungal activity, only salt compounds with a MIC equal or inferior to 0.5 % were retained. Mandarin fruits were washed, disinfected with 0.1% (v/v) sodium hypochlorite, rinsed three times in sterile distilled water and then air-dried before wounding. One wound (2mm deep and 4mm wide) was made per fruit using a sterile needle at the equatorial side (Liu *et al.,* 2009b). The wounds were treated with 30 µl of salt solution at concentrations of 1, 2 and 3% (w/v). Controls were treated with the same volume of sterile distilled water under the same conditions. After two hours incubation at room temperature, each wound was inoculated with 20 µl of an aqueous suspension of arthrospores of *G. candidum* (10^6 arthrospores. ml^{-1}). Treated fruits were placed on a plastic tray in cardboard boxes and incubated at 26°C and 95% relative humidity (RH). The number of the infected wounds and the lesion diameters of the overall treated fruit were determined daily. All treatments were arranged in a complete randomized block design. Eighteen fruits constituted a single replicate and each treatment was replicated three times. The experiment was conducted twice. The incidence and severity of disease were calculated as follows :

Disease incidence (%) = [(number of rotten wounds / number of total wounds)] x 100.

Disease severity (%) = [(average lesion diameter of treatment / average lesion diameter of control)] x 100. In all experiments, the possible phytotoxic effect on mandarin fruit was examined.

II.7 Statistical analysis

All data were subjected to statistical analysis of variance (ANOVA) using STATISTICA software, version 6, Stat-Soft, 2001, France. Percentage values were subjected to arcsine- square root transformation before analysis of variance. Mean separation was performed following the Newman & Keuls test at $P<0.05$.

III. Results

III.1 Preliminary screening of salts (MICs)

The mycelial growth of *Geotrichum candidum* is visualized in test tubes by the presence of a cloudy in the solution, which indicated the growth of the fungus (**Figure 5.1**). Two control tubes were made; postivie control corresponding to fungal culture alone and negative control corresponding to tubes with salts alone.

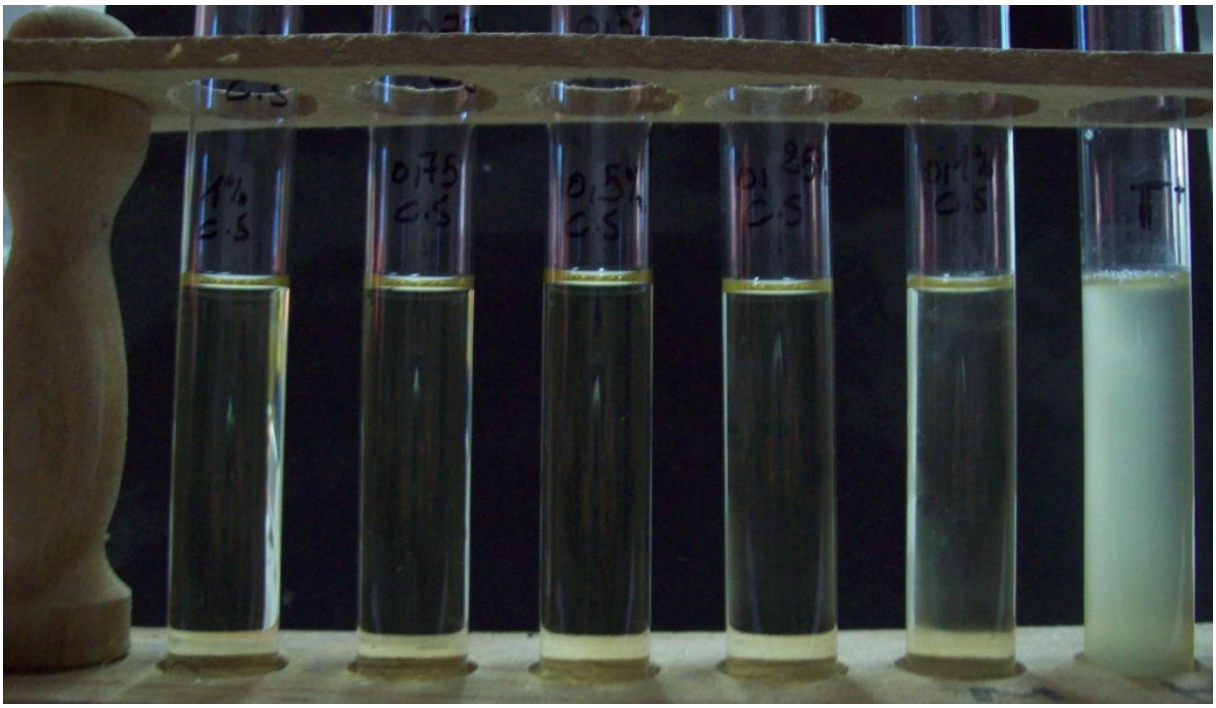


Figure 5.1 : Mycelial growth of *G. candidum* in test tubes containing NYDB medium and diffrents concentrations of sodium carbonate

The *in vitro* antifungal activity of 34 organic acids and salts was first examined at different concentrations, varying from 0.1 to 2 % (w/v), and showed a variable effects of tests compounds on *G. candidum* growth (**Table 5.2**). It was noticed that the reduction in growth was correlated to the increase in compounds concentration. The lowest MIC values were recorded for ammonium carbonate and EDTA tested at 0.1%. Tested at 0.25% boric acid, sodium carbonate and sodium metabisulfite inhibited completely the mycelial growth of *G. candidum*.

Table 5.2 : Minimum inhibitory concentrations of tested chemical compounds against *Geotrichum candidum*

Compounds	**MIC (w/v)**
Ammonium acetate	>2
Ammonium carbonate	0.1
Ammonium dihydrogen phosphate	>2
Ammonium molybdate	0.5
Ammonium sulfate	>2
Ascorbic acid	>2
Aspartic acid	>2
Boric acide	0.25
Calcium carbonate	>2
Calcium chloride	>2
Calcium Chloride	>2
Calcium nitrate	>2
Citric acid	2
Dipotassium hydrogen phosphate	>2
EDTA	0.1
Glutaric acid	>2
Magnesium chloride	>2
Magnesium sulfate	>2
Potassium acetate	>2
Potassium carbonate	0.5
Potassium chloride	>2
Potassium phosphate dibasic	>2
Potassium sodium tartrate	>2
Sodium acetate	>2
Sodium bicarbonate	0.75
Sodium carbonate	0.25
Sodium chloride	>2
Sodium metabisulfite	0.25
Sodium molybdate	2
Sodium salicylate	>2
Sodium sulfate	0.75
Sodium thiosulfate	0.5
Sodium salicylate	0.5
Sodium thiosulfate	>2

The third lowest MIC value was recorded for potassium carbonate, ammonium molybdate and sodium thiosulfate at 0.5%. At 0.75 % (w/v), only sodium sulfate and sodium bicarbonate completely inhibited the mycelial growth of *G. candidum*. The highest MIC value (2%) was obtained for citric acid and sodium molybdate. The others tested salt compounds are not effective against *G. candidum* even at 2% (**Table 5.2**). The data show that *G. candidum* has differential sensitivity to salts, as demonstrated by its varying rates for complete inhibition of growth.

III.2 Effect of pH on mycelial growth of *G. candidum*

To determine the effect of pH on the growth of *G. candidum*, NYDA medium at different pH values was used. The obtained results demonstrate that the fungus grew both on acidic and basic pH (**Figure 5.2**). The data indicate that pH from 4 to 12 does not significantly affect the growth of the fungus after 10 days of incubation at 25°C. Furthermore, only pH 2 reduced significantly the growth of the fungus in comparison with the control (**Figure 5.3**).

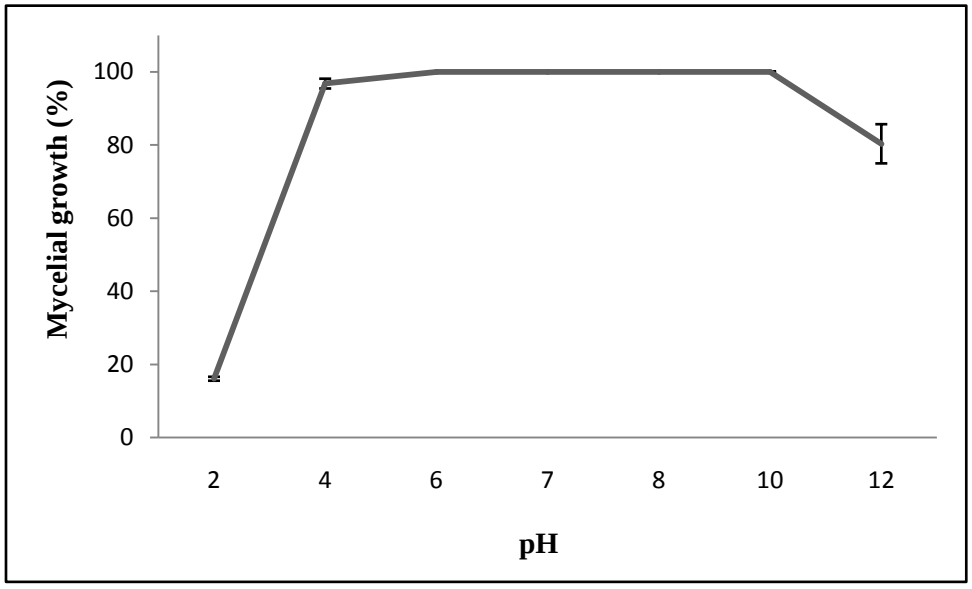


Figure 5.2 : Effect of pH on *in vitro* mycelial growth of *G. candidum*. Medium pH was adjusted with Hcl or NaOH. Bars represent standard deviations of the means.

III.3 Effect of salt compounds on arthrospores germination

Based on the previous results, only salt compounds that inhibited the growth of *G. candidum* at concentrations lower than 0.5% (w/v) were selected and evaluated for their potential to inhibit the arthrospores germination of the fungus. It is evident from the **table 5.3**, that the salt compounds tested against *G. candidum* showed a reduction or complete inhibition of arthrospores germination in a dose-dependent manner. The arthrospores germination was completely inhibited by EDTA, Boric acid, sodium metabisulfite, sodium carbonate, sodium sulfate and sodium thiosulfate both at 100 and 75 mM. Tested at 50 mM, sodium carbonate, EDTA, sodium sulfate, sodium metabisulfite and boric acid, strongly inhibited the arthrospore germination. At this concentration, the percentage of germination of arthrospores of *G. candidum* oscillates between 1%, for sodium carbonate, and 8.33%, for boric acid. At 25 mM, only sodium metabisulfite, boric acid and EDTA strongly inhibited arthrospores germination of *G. candidum*, since percent germination ranged between 11.66 and 33.33%.

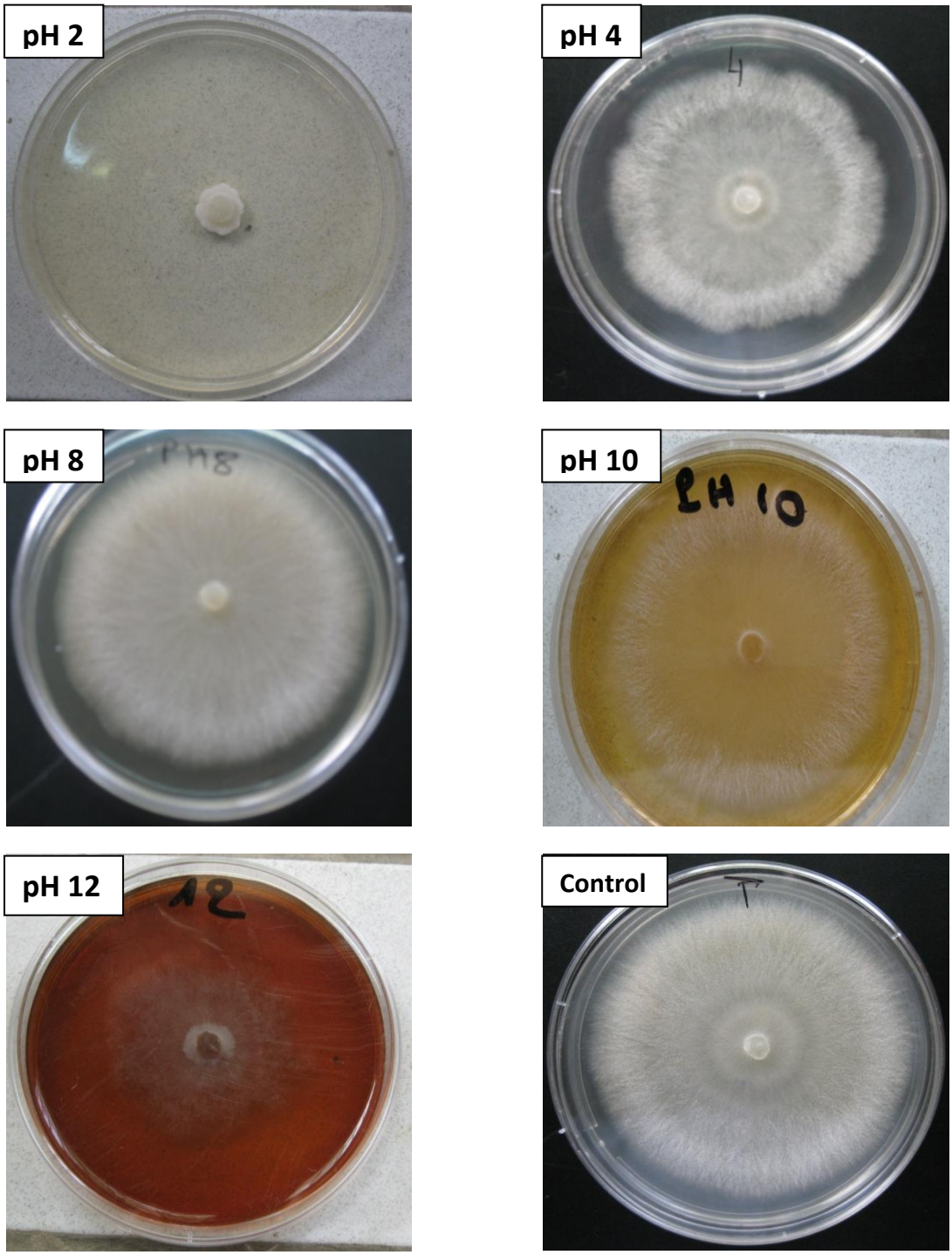


Figure 5.3 : Effect of pH on *in vitro* mycelial growth of *G. candidum*

In contrast, Sodium salicylate, ammonium carbonate and potassium carbonate, affects strongly the mycelial growth of the fungus, are less effective on arthrospores germination. The percentage of germination in salts amended medium varied between 50% for potassium carbonate at 75 and 100mM, and 100% for sodium salicylate at 25mM (**Table 5.3**).

Table 5.3 *: In vitro* effect of different chemical compounds on arthrospore germination of *Geotrichum candidum.*

Chemical tested	Arthrospore germination (%)			
	Concentration (mM)			
	25	**50**	**75**	**100**
EDTA	33.33±2.22^{g}	2±1.33^{ab}	0±0^{a}	0±0^{a}
Sodium salicylate	100±0^{p}	87.33±3.56^{o}	73±2 ^{m}	61.33±2.44^{k}
Sodium metabisulfite	11.66±2.22^{d}	6±1.33b^{c}	0±0^{a}	0±0^{a}
Boric acid	22.66±2.44^{e}	8.33±2.22^{cd}	0±0^{a}	0±0^{a}
Sodium carbonate	54.64±0.61^{ij}	1±0^{ab}	0±0^{a}	0±0^{a}
sodium sulfate	79.67±0.44^{n}	4.83±0.78^{abc}	0±0^{a}	0±0^{a}
Ammonium carbonate	99.23±0.51^{p}	95.37±1.71^{p}	86.58±2.88^{o}	56.63±5.79^{j}
Potassium carbonate	68.06±1.29^{l}	54.01±2.45^{ij}	50±1^{i}	50.63±1.04^{i}
Ammonium molybdate	99.33±0.44^{p}	80±1.33^{n}	42±1.33^{h}	12.66±1.55^{d}
Sodium thiosulfate	69.33±1.55l^{m}	28.33±3.55^{f}	0±0^{a}	0±0^{a}
Control	100±0^{p}	100±0^{p}	100±0^{p}	100±0^{p}

Means followed by different letter (s) in each column are significantly different at $P < 0.05$

III.4 Effect of salt compounds on disease development

Data presented in **Table 5.4** showed that all tested salt compounds significantly reduced the incidence of sour rot caused by *G. candidum* under the laboratory conditions. Percentages of rotted wounds were decreased by using all tested salts compared with control.

Table 5.4 : Effect of different chemical ccompounds on sour rot incidence on infected fruits

Chemical tested	Disease incidence (%)		
	Concentration (% w/v)		
	1%	**2%**	**3%**
EDTA	83.33f^{jhi}	50^{c}	38.89^{b}
Sodium salicylate	72.22^{ef}	46.3^{c}	25.93^{a}
Sodium metabisulfite	74.07^{ef}	66.67d^{ef}	68.52d^{e}
Boric acid	66.67^{de}	29.63^{a}	25.93^{a}
Sodium carbonate	72.22^{efg}	68.52^{def}	57.41^{cd}
sodium sulfate	92.59^{ijk}	77.78^{efg}	66.67^{de}
Ammonium carbonate	83.33^{hij}	64.81^{de}	51.85^{c}
Potassium carbonate	90.74^{hijk}	83.33^{hij}	64.81^{de}
Ammonium molybdate	88.89^{hijk}	79.63^{ghi}	72.22^{efg}
Sodium thiosulfate	94.44^{jk}	92.59^{ijk}	77.78^{fgh}
Control	100^{k}	100^{k}	100^{k}

Mandarin fruit treated 2 hours before pathogen inoculation by sodium salicylate and boric acid at 3% resulted in the highest reduction in rot incidence compared with the control. EDTA and ammonium carbonate had a moderate effect on sour rot, the percentage of rot incidence varying between 38.89 and 51.85. In contrast, sodium thiosulfate and ammonium molybdate showed the least effect on reduction of sour rot incidence (**Table 5.4**). Also, all the salts tested did not reduce effectively the disease incidence at concentration of 1%. On the other hand, data indicated that Boric acid, sodium salicylate and EDTA exhibited significant reduction of disease severity at 2 and 3% compared with the control.

The disease severity for these salts ranged between 30.97% (sodium salicylate at 3%) and 50% (EDTA at 2%) (**Table 5.5**). Also, there was a significant reduction of the rot severity from 100% in non-treated fruit to 51.77 and 53.1% respectively, in citrus treated fruits, by sodium carbonate and ammonium carbonate at 3%.

Table 5.5 : Effect of different chemical ccompounds on sour rot severity on infected fruits.

	Disease severity (%)		
	Concentration (% w/v)		
Salt	**1%**	**2%**	**3%**
EDTA	80.97^{ijk}	50^{de}	38.5^{bc}
Sodium salicylate	81.86^{ijk}	45.13^{cd}	30.97^{ab}
Sodium metabisulfite	65.93^{gh}	56.64^{efg}	54.42^{def}
Boric acid	63.72^{fg}	37.61^{bc}	22.574^{a}
Sodium carbonate	59.29^{efg}	55.75^{efg}	51.77^{de}
sodium sulfate	96.9^{lm}	73.45^{hi}	59.73^{efg}
Ammonium carbonate	78.76^{ijk}	59.73^{efg}	53.1^{de}
Potassium carbonate	88.94^{kl}	83.19^{ijk}	60.62^{efg}
Ammonium molybdate	87.17^{k}	84.07^{hij}	74.34^{jk}
Sodium thiosulfate	99.12^{m}	98.67^{m}	87.61^{kl}
Control	100^{m}	100^{m}	100^{m}

Fruit were treated with different concentrations of salt (1, 2 and 3%) , inoculated with *G. candidum* and held for 10 days at 26°C. Means followed by different letter (s) in each column are significantly different at $P < 0.05$

Although sodium salicylate was effective against citrus sour rot, it was phytotoxic to fruit rind at the three tested concentrations. A drying of the rind around the salt treated site was observed. The other salt compounds didn't lead any phytotoxic action on treated fruit at all tested concentrations.

IV. Discussion

The results showed that among the 34 salts tested for their minimum inhibitory concentrations, ten were most active against *Geotrichum candidum*. EDTA, sodium salicylate, sodium metabisulfite, boric acid, sodium carbonate, sodium sulfate, ammonium carbonate, potassium carbonate, ammonium molybdate and sodium thiosulfate have MIC values ranging between 0.1% and 0.5%. The present results are, therefore, consistent with those of Askarne *et al.* (2011) who showed that from 28 studied compounds tested against *Penicillium italicum*, causal agent of citrus blue mold, sodium metabisulfite, EDTA, ammonium carbonate, sodium carbonate and boric acid were the most active against the fungi, with MICs values ranged between 5 and 50 mM. Moreover, Palmer *et al.* (1997) showed that among 26 tested salts, ammonium carbonate, potassium carbonate, sodium carbonate, and sodium metabisulfite were effective against the mycelial growth of *Botrytis cinerea.* However, the same authors reported that sodium sulfate and sodium thiosulfate did not affect the mycelial growth of the fungi. Olivier *et al.* (1998) and Hervieux *et al.* (2002) showed that sodium carbonate, potassium carbonate and sodium metabisulfite have completely inhibited the mycelial growth of *Helminthosporium solani,* causal agent of silver scurf on potato tubers. Also, Droby *et al.* (2003) demonstrates that EDTA has a distinct inhibitory effect on the radial growth of *Botrytis cinerea* and *Penicillium expansum in vitro.*

The MIC test has allowed us to select the best salts that are effective against *G. candidum*. The ten best salts were further tested for their ability to reduce or inhibit the arthrospores germination of the pathogen. The results indicated that the arthrospores germination was completely inhibited by EDTA, boric acid, sodium metabisulfite, sodium carbonate, sodium sulfate and sodium thiosulfate both at 100 and 75mM. This finding corroborates with those of Askarne *et al.* (2011) showing that EDTA, boric acid, sodium metabisulfite and sodium carbonate completely inhibited spore germination of *Penicillium italicum.* Furthermore, sodium carbonate and sodium metabisulfite were demonstrated to inhibit completely the spore germination of *Helminthosporium solani* (Hervieux *et al.*, 2002). Also Smilanick *et al.* (1999) reported that sodium carbonate inhibit the spore germination of *P. digitatum*, causal agent of citrus green mold. Mills *et al.* (2004) reported that Sodium metabisulfite reduced significantly the spore germination of several phytopathogenic fungi. However, among the tested salts, sodium salicylate, ammonium carbonate and potassium carbonate are not effective on the arthrospores germination of the pathogen.

Considering that several salt compounds influenced medium pH, the effect of pH on G. *candium* growth was determined. The results showed that mycelial growth was not strongly affected by pH modification in the range of 4.0 and 12.0. This result agrees with those of Hervieux *et al.* (2002), and indicates that the inhibition obtained in the salt-amended medium cannot be due only to a direct effect of pH on pathogen growth.

For the *in vivo* test, the citrus fruits were treated with different concentrations of salt compounds (1, 2 and 3% w/v), inoculated with *G. candidum* and held for 10 days at 26°C. The results showed variable effects of tested compounds on disease incidence and severity. Sodium salicylate, boric acid and EDTA were the most effective against *G. candidum* in *in-vivo* conditions. At concentration of 3%, the disease incidence was reduced to 25.93% in fruits treated with sodium salicylate or boric acid and to 38.89% in fruits treated with EDTA. The present results are consistent with previous studies which demonstrated that EDTA was effective to control *P. digitatum* on oranges (Valencia-Chamorro *et al.,* 2008) and *B. cinerea* on apple fruit (Droby *et al.,* 2003). Moreover, Smilanick and Sorenson (2001) reported that immersion of citrus fruits in boric acid solution reduced significantly the incidence of citrus green mold. Also of interest, we found that ammonium carbonate and sodium carbonate have significantly reduced the incidence of sour rot to 51.8 and 57.41%, respectively. This result is in agreement with the finding of Palou *et al.* (2009) who reported that the same salts reduced the incidence of sour rot of stone fruits.

To understand the mechanisms by which fungi are tolerant or sensitive to salt compounds, several studies were carried out. It was found that inhibition of microorganisms by salts might be caused by reducing of the cell turgor pressure with collapse and shrinkage of hyphae and spore (Fallik *et al.,* 1997) or by alteration of cell-transport function, and inhibition of enzymes involved in the glycolytic pathway (Sofos *et al.,* 1986). However, the mechanisms by which salts inhibit *G. candidum* are not well understood.

V. Conclusion

Among the 34 compounds tested sodium salicylate, boric acid and EDTA showed high antifungal activities against *Geotrichum candidum* both *in-vitro* and *in-vivo*. These compounds possess potent antifungal activities with potential practical applications in the treatment of postharvest sour rot of citrus fruits and should be tested in future under degreening conditions.

DISCUSSION GÉNÉRALE
&
CONCLUSION

Discussion générale

Les stratégies de lutte contre les maladies des agrumes en post-récolte proposées comme alternative aux fongicides de synthèse comprennent généralement l'utilisation de nouveaux fongicides à faible risque (Smilanick *et al.,* 2006a; Kanetis *et al.,* 2007), des microorganismes antagonistes (El-Ghaouth *et al.,* 2000; Droby *et al.,* 2002; Taqarort *et al.,* 2008; Ren *et al.,* 2011), des extraits de plantes (Ameziane *et al.,* 2007; Mekbib *et al.,* 2007; Du Plooy *et al.,* 2009; Liu *et al.,* 2009b; Gatto *et al.,* 2011; Askarne *et al*., 2012), des sels organiques et inorganiques (Hall, 1988; Palou *et al.,* 2001; El-Mougy *et al.,* 2008; Smilanick *et al.,* 2008; Askarne *et al.*, 2011) ainsi que l'induction des mécanismes de résistance. Dans cette étude, l'activité antifongique des extraits aqueux et organiques de certaines plantes médicinales et aromatiques ainsi que celle d'une gamme de sels organiques et inorganiques a été évaluée *in vitro* et *in vivo* contre *Geotrichum candidum*, l'agent de la pourriture amère des agrumes en post-récolte.

Quarante trois échantillons de plantes fraîches ont été récoltés dans différentes régions du sud marocain durant la période 2008-2009. Les 43 plantes appartiennent à 16 familles botaniques et 36 genres. Les extraits aqueux de ces plantes ont été évalués, d'abord, sur la croissance mycélienne de *G. candidum* en incorporant celles-ci dans le milieu de culture à base d'extrait de pomme de terre (PDA). Les résultats obtenus ont montré que la plupart des plantes testées ont réduit la croissance mycélienne de *G. candidum* avec des degrés d'inhibition qui différent selon l'espèce testée. Le meilleur pourcentage d'inhibition de la croissance mycélienne a été obtenu avec les extraits aqueux de *Ceratonia siliqua, Halimium umbellatum, Pistacia atlantica, Halimium antiatlanticum, Cistus monspeliensis, Rubus ulmifolius* et *Cistus villosus.* En effet, les extraits de ces plantes ont montré une forte inhibition de la croissance mycélienne avec un pourcentage supérieur à 95%. Des résultats similaires ont été obtenus par Ameziane *et al.* (2007). D'après ces auteurs, l'extrait aqueux de *C. villosus* a complètement inhibé la croissance mycélienne de *G. candidum.* De même, l'évaluation de l'activité antibactérienne de quarante plantes du sud du Maroc a montré que les extraits aqueux de *R. ulmifolius*, *C. monspeliensis* et *P. atlantica* figurent parmi les meilleurs espèces qui ont inhibé la croissance de *Clavibacter michiganensis* subsp. *michiganensis*, agent du chancre bactérien de la tomate (Talibi *et al.,* 2011a). Cependant, certains plantes testées dans cette étude ont stimulé la croissance de *G. candidum*, à savoir :

Mentha suaveolens, Psoralea bituminosa et *Reseda alba.* Cet effet stimulant sur la croissance du pathogène a été également rapporté par Ameziane *et al.* (2007).

En plus de la croissance mycélienne, la germination des arthrospores est considérée comme étape cruciale du cycle d'infection de *G. candidum*. À cet égard, l'effet des extraits aqueux a été également évalué sur la germination des arthrospores de *G. candidum*. Les résultats obtenus ont révélé que parmi les extraits aqueux qui ont complètement inhibé la croissance mycélienne de *G. candidum*, seuls ceux de *C. villosus* et *H. antiatlaticum* ont également inhibé la germination des arthrospores. Ceci montre que l'activité des plantes testées différe selon le stade de développement du champignon. En effet, il ressort des résultats obtenus que la germination des arthrospores s'avère moins sensible aux plantes testées que la croissance mycélienne. Le même constat a été obtenu par Garduno-Pizana *et al.* (2010), qui ont testé l'effet de quinze plantes Mexicaines sur *Fusarium oxysporum*.

Nous avons également déterminé les concentrations minimales inhibitrices (CMI) et fongicides (CMF) relatives aux meilleures plantes. Les plus faibles CMI ont été enregistrées avec les extraits aqueux de *C. villosus* et *H. antiatlanticum* avec une CMI égale à 0,156 mg/ml, suivie de *C. siliqua*, *H. umbellatum* et *R. ulmifolius* avec une CMI de 0,312 mg/ml. Cet effet antifongique est comparable à celui rapporté dans d'autres travaux. Ainsi, Bouamama *et al.* (2006) ont rapporté que les extraits de *C. villosus* et *C. monspeliensis* possèdent des propriétés antifongiques avec des CMI qui varient entre 0,19 et 200 mg/ml. Les mêmes auteurs ont rapporté que l'extrait de *C. villosus* présente une activité antimicrobienne plus intense que celui de *C. monspeliensis* (Bouamama *et al.*, 2006).

Bien que la réalisation des tests *in vitro* est une étape importante dans le criblage des plantes médicinales et aromatiques sur la base de leur potentiel antifongique, les tests *in vivo* sont également essentiels pour confirmer, d'abord, les résultats obtenus *in vitro* et ensuite mettre au point des préparations dérivées des plantes qui sont susceptibles d'être appliquées à l'échelle semi-commerciale (Gorris and Smid, 1995; Tegegne *et al.*, 2008). Sur la base du screening *in vitro*, seuls les extraits qui ont réduit la croissance mycélienne de *G. candidum* de plus de 90% ont été retenus pour évaluer leur capacité à contrôler la pourriture amère. Les résultats obtenus ont montré que les extraits aqueux de sept espèces (*H. umbellatum*, *I. viscosa*, *R. ulmifolius*, *C. villosus*, *C.* siliqua, *H. antiatlanticum* et *P. atlantica*) ont significativement réduit l'incidence et la sévérité de la pourriture amère sans avoir des effets phytotoxique sur les fruits traités. A notre connaissance, il s'agit du premier rapport qui

montre l'efficacité de ces plantes à contrôler la pourriture amère des agrumes en post-récolte. Le meilleur degré de contrôle de la pourriture amère est obtenu avec l'extrait aqueux de *C. villosus* et *H. antiatlanticum,* qui ont réduit l'incidence de la maladie à respectivement 46 et 44 % par rapport aux fruits non traités (100%). Cependant, aucun des extraits aqueux des plantes testées n'a totalement contrôlé le développement de la pourriture amère.

Dans l'objectif d'identifier la fraction active des meilleures plantes et aussi pour améliorer leur activité antifongique contre *G. candidum,* nous avons procédé à un fractionnement de chaque plante en utilisant des solvants de polarité croissante. Les espèces suivantes : *C. villosus, H. antiatlanticum, H. umbellatum, P. atlantica, I. viscosa, A. radiata, R. ulmifolius et C. siliqua* ont été, successivement, extraites avec l'hexane, le chloroforme, l'acétate d'éthyle et le méthanol. Ceci nous a permis d'isoler quatre fractions organiques pour chaque plante. Ces fractions ont été évaluées pour leur activité antifongique, *in vitro* et *in vivo,* contre *G. candidum.*

L'effet des fractions a été évalué sur la croissance mycélienne du pathogène. Les résultats obtenus montrent que les espèces testées ont montré différent degré d'inhibition de la croissance de *G. candidum*. En effet, le pouvoir inhibiteur varie avec l'espèce et le type de solvant d'extraction. Les extraits méthanoliques de *C. villosus, H. umbellatum, C. siliqua, R. ulmifolius, H. antiatlanticum, P. atlantica* et les extraits d'acétate d'éthyle de *C. villosus, A. radiata* et *C. siliqua* ont fortement inhibé la croissance mycélienne du pathogène. Ces résultats sont similaires à ceux obtenus en utilisant les extraits aqueux des mêmes espèces (Talibi *et al.,* 2012a). Par contre, les extraits hexaniques et chloroformiques des plantes testées se sont révélés être moins efficaces. De même, les fractions polaires des plantes testées (méthanolique et d'acétate d'éthyle) ont fortement inhibé la germination des arthrospores de *G. candidum* comparés aux fractions apolaires (hexanique et chloroformique). En effet, l'extrait méthanolique de *C. villosus, H. umbellatutm, H. antiatlanticum, C.siliqua, R. ulmifolius* et l'extrait d'acétate d'éthyle de *P. atlantica* et *A .radiata* ont inhibé la germination des arthrospores de *G. candidum* à plus de 92%. Nous pouvons donc conclure que, contrairement aux extraits aqueux, l'extraction des plantes avec des solvants organiques a amélioré leurs propriétés antifongiques. En effet, les extraits méthanoliques des plantes se sont révélés être plus efficaces sur la croissance mycélienne et sur la germination des arthrospores de l'agent pathogène.

Les plus faibles concentrations inhibitrices (CMI) ont été obtenues par les extraits méthanoliques de *H. umbellatum* (0,156 mg/ml), *C. villosus* et *R. ulmifolius* (0,625 mg/ml). Des résultats comparables ont été obtenus par Bouamama *et al.* (2006) en testant les fractions organiques de *C. villosus* et *C. monspeliensis* contre une gamme de pathogènes des plantes. Dans cette même étude, l'extrait méthanolique de *C. villosus* s'est révélé plus efficace que l'extrait à l'acétate d'éthyle (Bouamama *et al.*, 2006). Par ailleurs, l'extrait méthanolique de *C. villosus* et *H. umbellatum* s'est révélé fongicide à partir d'une concentration de 2,5mg/ml ; alors que l'extrait aqueux de ces deux plantes n'a montré qu'une activité fongistatique vis-à-vis de *G. candidum* même à 5mg/ml.

À partir des résultats des tests *in vitro*, ce sont les fractions polaires (extraits méthanoliques, d'acétate d'éthyle) qui ont montré des propriétés antifongiques intéressantes comparés aux fractions apolaires (extraits héxaniques et chloroformiques). Ces résultats ne sont pas tout à fait surprenants du fait qu'une grande gamme de composés dotés d'activités antimicrobiennes (alcaloïdes, tannins, composés phénoliques, terpénoïdes…) ont une affinité pour les solvants polaires (Choi *et al.*, 2004; Tripathi and Dubey, 2004; Ismail *et al.*, 2008; Satish *et al.*, 2008; Askun *et al.*, 2009; Martini *et al.* 2009). Sur la base de ces résultats, seuls les extraits méthanoliques et d'acétate d'éthyle (fractions polaires) des huit plantes ont été retenus pour évaluer leur capacité à réduire l'incidence et la sévérité de la pourriture amère. Les résultats obtenus ont montré que les extraits méthanoliques de *C. villosus, C. siliqua, H. umbellatum, H. antiatlanticum* et *R. ulmifolius* ont significativement réduit l'incidence et la sévérité de la pourriture amère, par rapport au témoin. En effet, le traitement en préventif des fruits de mandarine avec l'extrait méthanolique de *C. villosus* a totalement inhibé le développement de l'agent pathogène. En plus, l'extrait méthanolique de *C. siliqua* et *H. umbellatum* ont respectivement réduit l'incidence de la pourriture amère à seulement 3,3 et 11,66%, par rapport au témoin (95%). Kivack *et al.* (2001) ont montré que l'extrait méthanolique de *C. siliqua* possède une puissante activité antibactérienne contre un large éventail de bactéries et de levures. En outre, Sisti *et al.* (2008) et Panizzi *et al.* (2002) ont montré que l'extrait méthanolique de *R. ulmifolius* est doté de propriétés antimicrobiennes. Comme dans le cas des tests *in vitro*, les extraits méthanoliques ont montré plus d'efficacité à contrôler la pourriture amère que les extraits aqueux des mêmes plantes. Ces résultats sont en concordance avec les travaux de Haouala *et al.* (2008), sur l'activité antifongique des extraits méthanoliques et aqueux de *Trigonella foenum-graecum* contre *Rhizoctonia solani* et *Alternaria sp.* Ceci pourrait être expliqué par la nature et la proportion des composés

responsables de l'activité antifongique dans chaque type d'extrait (aqueux et méthanolique). Les extraits à l'acétate d'éthyle de *H. umbellatum* et *A. radiata* bien qu'efficace *in vitro* contre *G. candidum* n'ont, cependant, aucun effet sur le développement de la pourriture amère (*in vivo*). Par conséquent, les tests réalisés *in vitro* et *in vivo* pour ces deux espèces ont montré des résultats opposés. D'après Gatto *et al.* (2011), de nombreux facteurs tel que la dégradation, l'hydrolyse ou la polymérisation, peuvent affecter l'activité biologique de certains composants de l'extrait de plante lorsqu'ils entrent en contact avec les tissus des fruits.

Dans la présente étude, quatre fractions organiques de polarité croissante ont été testées (hexane, chloroforme, acétate d'éthyle et le méthanol). Parmi elles, la fraction méthanolique possède le meilleur contrôle du pathogène à la fois *in vitro* et *in vivo.* D'après Askun *et al.* (2009), les extraits méthanoliques des plantes possèdent des propriétés antimicrobiennes plus consistantes. En effet, les extraits méthanoliques de plusieurs plantes ont été rapportés avoir une excellente activité antimicrobienne contre différents agents pathogènes des plantes (Sato *et al.,* 2000; Choi *et al.,* 2004; Ameziane *et al.,* 2007; Bajpai *et al.,* 2008; Satish *et al.,* 2008; Mahlo *et al.,* 2010; Ahmadi *et al.,* 2010; Hajji *et al.,* 2010). L'analyse phytochimique des extraits méthanoliques des plantes qui ont réduit l'incidence et la sévérité de la pourriture amère a montré qu'ils contiennent différents types de flavonoïdes et possèdent des niveaux élevés des phénols totaux. Ce résultat est appuyé par plusieurs études qui ont confirmé que le méthanol peut extraire plusieurs composés ayant des activités antimicrobiennes en particulier les composés phénoliques (Nicholson and Hammerschmidt, 1992; Cowan *et al.,* 1999; Tripathi and Dubey, 2004; Sisti *et al.,* 2008; Martini *et al.,* 2009). En outre, Cowan et *al.* (1999) ont signalé que les plantes ont une capacité presque illimitée à synthétiser des substances aromatiques, dont la plupart sont des phénols, qui jouent un rôle dans les mécanismes de défense des plantes contre les agents pathogènes.

Dans l'objectif de purifier les meilleurs fractions organiques et de comprendre le mode avec lequel leurs composés agissent sur le pathogène, les extraits méthanoliques de *C. villosus* et *H. umbellatum* ont subi un fractionnement bio-guidé. Cette méthode consiste à fractionner l'extrait en question sur gel de silice (par chromatographie sur colonne) en utilisant des systèmes d'élution de polarité croissante. Les sous-fractions issues de chaque fraction ont été testées pour leur activité contre la croissance mycélienne de *G. candidum*. La sous-fraction la plus active a subi à son tour un autre fractionnement réalisé de la même manière. Les résultats obtenus montrent que plus l'extrait est fractionné, plus l'activité antifongique des sous

fractions diminue. Ceci pourrait être expliqué par la perte de synergie ou l'effet additif entre les principes actifs présents dans la fraction initiale ou par leur redistribution dans les différentes sous fractions en fonction de leurs affinités pour les solvants utilisés. L'effet synergique entre les composés actifs des plantes a été également signalé dans plusieurs travaux (Ellof, 2004; Nenaah, 2010; Suleiman *et al.,* 2012; Zakaria *et al.,* 2010). Nenaah (2010) a rapporté que les alcaloïdes de *Peganum harmala* lorsqu'ils sont appliquées individuellement, montrent des effets antimicrobiens modérés. En revanche, l'activité antimicrobienne augmente lorsqu'ils sont combinés, suggérant donc une sorte de synergie entre ces composés (Nenaah, 2010). De même, Soliman *et al.* (2012) ont montré que le fractionnement de l'extrait brut de la plante *Loxostylis alata* entraine une diminution de l'activité antimicrobienne de plus de 85%.

Face à l'absence de moyens efficaces pour lutter contre cette maladie, et aux inquiétudes grandissantes à propos des impacts sur l'environnement et la santé humaine reliés à l'utilisation des fongicides synthétiques, il devient important de développer des composés antifongiques, qui soient efficaces et sains. De nombreux travaux menés au cours des deux dernières décennies ont démontré à cet effet que des sels organiques et inorganiques, utilisés en industrie alimentaire et/ou pharmaceutique, ont un potentiel intéressant pour le contrôle des maladies des plantes. L'utilisation de sels organiques et inorganiques apparaît donc comme une approche intéressante pour lutter contre la pourriture amère des agrumes. Dans ce contexte, nous avons opté pour l'évaluation, *in vitro* et *in vivo,* de l'efficacité de 34 sels contre *G. candidum.* Les résultats du screening de l'activité antifongique de ces sels ont montré que les CMIs obtenues varient selon la nature du sel testé. La CMI la plus faible (0,1%) est obtenue avec le carbonate d'ammonium et l'EDTA, suivie par le salicylate de sodium, le metabisulfite de sodium et l'acide borique avec une CMI de 0,25%. Ces résultats concordent avec ceux d'Askarne *et al.* (2011), qui ont montré que parmi les 28 sels testés contre *Penicillium italicum*, agent causal de la pourriture bleue des agrumes, le metabisulfite de sodium, l'EDTA, le carbonate d'ammonium et l'acide borique ont été les plus actifs contre ce champignon, avec des CMIs qui varient entre 5 et 50 mM. Des résultats similaires, rapportés par Olivier *et al.* (1998) et Hervieux *et al.* (2002), ont montré que le carbonate de sodium, le carbonate de potassium et le metabisulfite de sodium ont complètement inhibé la croissance mycélienne de *Helminthosporium solani.* En outre, Droby *et al.* (2003) ont démontré que l'EDTA a un effet inhibiteur sur la croissance mycélienne de *Botrytis cinerea* et *Penicillium expansum*.

Les dix meilleurs sels, sélectionnés sur la base de leur CMI, ont été, ensuite, testés pour leurs capacité à réduire ou à inhiber la germination des arthrospores du pathogène. Les résultats obtenus ont montré que l'EDTA, l'acide borique, le metabisulfite de sodium, le carbonate de sodium, le sulfate de sodium et le thiosulfate de sodium ont totalement inhibé la germination des arthrospores à partir d'une concentration de 75 et 100 mM. Ce résultat concorde avec ceux d'Askarne *et al.* (2011) qui ont indiqué que l'EDTA, l'acide borique, le metabisulfite de sodium et le carbonate de sodium ont complètement inhibé la germination des spores de *P. italicum* à des concentrations qui oscillent entre 20 et 200 mM. Également, Smilanick *et al.* (1999) ont rapporté que le carbonate de sodium a inhibé la germination des spores de *P. digitatum*, agent causal de la pourriture verte des agrumes. En revanche, le salicylate de sodium, le carbonate d'ammonium et le carbonate de potassium se sont montrés peu efficaces sur la germination des arthrospores de *G. candidum*. Considérant que plusieurs sels peuvent influencer le pH du milieu, l'effet de ce dernier sur la croissance mycélienne de *G. candidum* a été déterminé. Les résultats obtenus ont montré que la croissance mycélienne est faiblement affectée par les modifications du pH dans la gamme de pH 4,0 à 12,0. Ce résultat est similaire à celui de Nigro *et al.* (2006), qui ont démontré que le pH n'a qu'un rôle mineur dans le mode d'action des sels. En outre, Hervieux *et al.* (2002), ont indiqué que l'inhibition de la croissance des agents pathogènes dans des milieux additionnés de sels ne peut être due seulement à l'effet direct du pH. Par conséquent, c'est la nature même du sel qui affecte le développement de *G. candidum*.

Pour le test *in vivo*, des mandarines ont été traitées avec différentes concentrations de sels (1, 2 et 3% w/v), inoculés avec une suspension d'arthrospores de *G. candidum* puis incubées à 26°C et à une humidité relative élevée. Les résultats obtenus ont montré que seuls le salicylate de sodium, l'acide borique et l'EDTA ont réduit significativement l'incidence et la sévérité de la pourriture amère. En effet, testé à une concentration de 3%, l'incidence de la maladie a été réduite à 25,93% chez les fruits traités avec le salicylate de sodium ou l'acide borique et à 38,89% chez les fruits traités avec de l'EDTA. Ces résultats concordent avec des études antérieures qui ont montré que l'EDTA est efficace contre la pourriture verte des agrumes, due à *Penicilium digitatum* (Valencia-Chamorro *et al.,* 2008), et contre la pourriture grise des pommes, due à *Botrytis cinerea* (Droby *et al.,* 2003). Dans la présente étude, nous avons trouvé que le carbonate d'ammonium et le carbonate de sodium ont significativement réduit l'incidence de la pourriture amère respectivement à 51,8 et 57,41%. Ces résultats sont

en accord avec ceux de Palou *et al.* (2009) qui ont rapporté que les mêmes sels ont réduit l'incidence de la pourriture amère des fruits à noyaux.

En conclusion, nous avons démontré que les extraits aqueux et méthanoliques de *Cistus villosus, Ceratonia siliqua, Halimium umbellatum* et *H. antiatlanticum* sont efficaces contre la pourriture amère des agrumes, et sans causer aucune réaction phytotoxique sur les fruits traités. Compte tenu de l'activité antifongique de ces plantes, Il est donc possible de les utiliser, après de plus amples investigations, comme moyen de lutte alternatif à la guazatine. Ces plantes sont vivaces et largement répandues dans la région SMD. En outre, la méthode d'extraction est assez simple et l'utilisation des extraits des plantes pour protéger les agrumes contre les maladies fongiques est hautement souhaitable dans la production biologique des agrumes. Nous avons également démontré que parmi les sels testés, l'EDTA, l'acide borique et le salicylate de sodium sont dotés de propriétés antifongiques intéressantes pouvant avoir des applications pratiques dans le traitement de la pourriture amère. Ces produits sains représentent donc une alternative prometteuse et durable à l'utilisation de la guazatine principalement dans la production biologique.

Cette étude nécessite d'être complétée par un certain nombre de travaux. Ainsi, on se propose de :

- ✓ Effectuer des essais dans des conditions semi-commerciales en utilisant aussi bien les extraits de plantes, les sels ainsi que leurs combinaisons ;
- ✓ Identifier la composition chimique des fractions actives des plantes ;
- ✓ Explorer, d'une manière plus approfondie, les modes d'action des sels et des extraits de plantes sélectionnés ;
- ✓ Tester le pouvoir antifongique des huiles essentielles des plantes testées dans cette étude contre *G. candidum.*

REFERENCES

References

Abd-El-Khair, H., Hafez, O., 2006. Effect of aqueous extracts of some medicinal plants in controlling the green mould disease and improvement of stored" Washington" navel orange quality. J. Applied Sciences Research 2, 664-674.

Abdel-Monaim, M.F., Abo-Elyousr, K.A.M., Morsy, K.M., 2011. Effectiveness of plant extracts on suppression of damping-off and wilt diseases of lupine (*Lupinus termis* Forsik). Crop Protection 30, 185-191.

Abraham, A.O., Laing, M.D., Bower, J.P., 2010. Isolation and *in vivo* screening of yeast and *Bacillus antagonists* for the control of *Penicillium digitatum* of citrus fruit. Biological control 53, 32-38.

Agnioni, A., Cabras, P., Dhallewin, G., Pirisi, F., Reniero, F., Schirra, M., 1998. Synthesis and inhibitory activity of 7-geranoxy coumarin against *Penicillium* species in citrus fruits. Phytochemistry 47, 1521-1525.

Ahmadi, F., Sadeghi, S., Modarresi, M., Abiri, R., Mikaeli, A., 2010. Chemical composition, in vitro anti-microbial, antifungal and antioxidant activities of the essential oil and methanolic extract of Hymenocrater longiflorus Benth., of Iran. Food and Chemical Toxicology 48, 1137-1144.

Ahmed, D.M., El Shami, S., El Mallah, M.H., 2007. Jojoba oil as a novel coating for exported Valencia orange fruit part 1 : The use of trans (isomerized) jojoba oil. Amer. Eur. J. Agr. Environ 2, 173-181.

Ali Shtayeh, M., Abu Ghdeib, S.I., 1999. Antifungal activity of plant extracts against dermatophytes. Mycoses 42, 665-672.

Alilou, H., Akssira, M., Hassani, L., Chebli, B., EL Hakmoui, A., Mellouki, F., Rouhi, R., Boira, H., Blazquez, M.A., 2008. Chemical composition and antifungal activity of *Bubonium imbricatum* volatile oil. Phytopathologia Mediterranea 47, 3-10.

Al-Zaemey, A., Magan, N., Thompson, A., 1993. Studies on the effect of fruit-coating polymers and organic acids on growth of Colletotrichum musae in vitro and on post-harvest control of anthracnose of bananas. Mycological Research 97, 1463-1468.

Ameziane, N., Boubaker, H., Boudyach, H., Msanda, F., Jilal, A., Ait ben oumar, A., 2007. Antifungal activity of Moroccan plants against citrus fruit pathogens. Agronomy for sustainable development 27, 273-277.

Anonymous, 2011. Bilan Campagne agrumicole 2010/2011. Office Régional de Mise en Valeur Agricole du Souss-Massa, Service de production agricole.

Arras, G., 1996. Mode of action of an isolate of *Candida famata* in biological control of orange fruits. Postharvest Biology and Technology 8, 191-198.

Arras, G., Piga, A., Agabbio, M.C.S., 1996. Effect of TBZ, acetaldehyde, citral and *Thymus capitatus* essential oil on'Minneola'tangelo fruit decay. International Society of Citriculture.

Arras, G., De Cicco, V., Arru, S., Lima, G., 1998. Biocontrol by yeasts of blue mould of citrus fruits and the mode of action of an isolate of *Pichia guilliermondii*. J. Horticultural Science and Biotechnology 73, 413-418.

Arras, G., Usai, M., 2001. Fungitoxic activity of 12 essential oils against four postharvest citrus pathogens : chemical analysis of *Thymus capitatus* oil and its effect in subatmospheric pressure conditions. Journal of Food and Protection 174; 64, 1025-1029.

Arras, G., Scherm, B., Migheli, Q., 2002. Improving biocontrol activity of *Pichia guillermondii* against post-harvest decay of oranges in commercial packing-houses by reduced concentrations of fungicides. Biocontrol Science and Technology 12, 547-553.

Arrebola, E., Sivakumar, D., Korsten, L., 2010. Effect of volatile compounds produced by *Bacillus strains* on postharvest decay in citrus. Biological control 53, 122-128.

Arslan, U., Ilhan, K., Vardar, C., Karabulut, O.A., 2009. Evaluation of antifungal activity of food additives against soilborne phytopathogenic fungi. World Journal of Microbiology and Biotechnology 25, 537-543.

Askarne, L., Talibi, I., Boubaker, H., Serghini, M., A., Boudyach, E., H., Ait Ben Aoumar, A., 2011. Effects of Organic Acids and Salts on the Development of *Penicillium italicum*, the Causal Agent of Citrus Blue Mold. Plant Pathology Journal.

Askarne, L., Talibi, I., Boubaker, H., Boudyach, E., Msanda, F., Saadi, B., Serghini, M., Ait Ben Aoumar, A., 2012. *In vitro* and *in vivo* antifungal activity of several Moroccan plants against *Penicillium italicum*, the causal agent of citrus blue mold. Crop Protection 40, 53-58.

Askun, T., Tumen, G., Satil, F., Ates, M., 2009. *In vitro* activity of methanol extracts of plants used as spices against *Mycobacterium tuberculosis* and other bacteria. Food chemistry 116, 289-294.

Ayala-Zavala, J. F., del Toro-Sánchez, L., Alvarez-Parrilla, E., Soto-Valdez, H., Martín-Belloso, O., Ruis-Cruz, S., 2008. Natural antimicrobial agents incorporated in active

packaging to preserve the quality of fresh fruits and vegetables. Steward Postharvest Review, 3(9), 1-9.

Badawy, F.M.I., Sallam, M.A.N., Ibrahim, A., Asran, M., 2011. Efficacy of Some Essential Oils on Controlling Green Mold of Orange and their Effects on Postharvest Quality Parameters. Plant Pathology Journal 10, 168-174.

Bajpai, V.K., Shukla, S., Kang, S.C., 2008. Chemical composition and antifungal activity of essential oil and various extract of Silene armeria L. Bioresource technology 99, 8903-8908.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils-a review. Food and Chemical Toxicology 46, 446-475.

Batta, Y., 2004. Effect of treatment with *Trichoderma harzianum* Rifai formulated in invert emulsion on postharvest decay of apple blue mold. International Journal of Food Microbiology 96, 281-288.

Baudoin, A., Eckert, J., 1985. Influence of preformed characteristics of lemon peel on susceptibility to *Geotrichum candidum*. Physiological plant pathology 26, 151-163.

Ben-Hsouna, A., Trigui, M., Mansour, R.B., Jarraya, R.M., Damak, M., Jaoua, S., 2011. Chemical composition, cytotoxicity effect and antimicrobial activity of *Ceratonia siliqua* essential oil with preservative effects against Listeria inoculated in minced beef meat. International Journal of Food Microbiology 148, 66-72.

Benhamou, N., 2004. Potential of the mycoparasite, *Verticillium lecanii*, to protect citrus fruit against *Penicillium digitatum*, the causal agent of green mold : A comparison with the effect of chitosan. Phytopathology 94, 693-705.

Benhammou, N., Bekkara, F.A., Panovska, T.K., 2008. Antioxidant and antimicrobial activities of the *Pistacia lentiscus* and *Pistacia atlantica* extracts. African Journal of Pharmacy and Pharmacology 2, 022-028.

Biggs, A., El-Kholi, M., El-Neshawy, S., Nickerson, R., 1997. Effects of calcium salts on growth, polygalacturonase activity, and infection of peach fruit by *Monilinia fructicola*. Plant disease 81, 399-403.

Biggs, A.R., 1999. Effects of calcium salts on apple bitter rot caused by two *Colletotrichum* spp. Plant disease 83, 1001-1005.

Borras, A.D., Aguilar, R.V., 1990. Biological control of *Penicillium digitatum* by *Trichoderma viride* on postharvest citrus fruits. International Journal of Food Microbiology 11, 179-183.

Bouamama, H., Noel, T., Villard, J., Benharref, A., Jana, M., 2006. Antimicrobial activities of the leaf extracts of two Moroccan *Cistus* L. species. Journal of ethnopharmacology 104, 104-107.

Boubaker, H., 1993. Etude des problèmes phytosanitaires des fruits d'agrumes en post-récolte, Phytopathologie. Unv. Cadi Ayyad, Marrakech, p. 117.

Boubaker, H., Saadi, B., Boudyach, E., H, , Ait Ben Oumar, A., 2009. Sensitivity of *Penicillium digitatum* and *P. italicum* to Imazalil and Thiabendazole in Morocco. Plant Pathology Journal 8, 152-158.

Brown, G.E., 1979. Biology and control of *Geotrichum candidum*, the cause of citrus sour rot. Proc Fla State Hort Soc 92, 186-189.

Brown, G., E., 1988. Efficacy of guazatine and iminoctadine for control of postharvest decays of oranges. Plant disease 72, 906-908.

Brown, G., Eckert, J., 1988. Sour rot. Compendium of citrus diseases. American Phytopathological Society, St. Paul, MN, 37–38.

Brown, G.E., Chambers, M., 1996. Evaluation of biological products for the control of postharvest diseases of Florida citrus, pp. 278-282.

Brown, G., Miller, W., 1999. Maintaining fruit health after harvest. Citrus Health Management. LW Timmer and LW Duncan, eds. The American Phytopathological Society Press, St. Paul, MN, 175-188.

Bull, C., Wadsworth, M., Sorensen, K., Takemoto, J., Austin, R., Smilanick, J., 1998. Syringomycin E produced by biological control agents controls green mold on lemons. Biological control 12, 89-95.

Butler, E., Webster, R., Eckert, J., 1965. pathogenecity and physiological properties of the fungus causing sour rot of citrus. Phytopathology 55, 1262–1268.

Butler, E.E., Fogile, D., Miranda, M., 1988. *Galactomyces citri-aurantii* a newly found teleomorph of *Geotrichum citri-aurantii* the cause of sour rot of citrus fruit. Mycotaxon 33, 197-212.

Caccioni, D.R.L., Guizzardi, M., Biondi, D.M., Renda, A., Ruberto, G., 1998. Relationship between volatile components of citrus fruit essential oils and antimicrobial action on *Penicillium digitatum* and *Penicillium italicum*. International Journal of Food Microbiology 43, 73-79.

Campanella, V., Ippolito, A., Nigro, F., 2002. Activity of calcium salts in controlling Phytophthora root rot of citrus. Crop Protection 21, 751-756.

Cañamás, T.P., Viñas, I., Usall, J., Torres, R., Anguera, M., Teixidó, N., 2008. Control of postharvest diseases on citrus fruit by preharvest applications of biocontrol agent Pantoea agglomerans CPA-2 : Part II. Effectiveness of different cell formulations. Postharvest Biology and Technology 49, 96-106.

Carson, C.F., Mee, B.J., Riley, T.V., 2002. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. Antimicrobial Agents and Chemotherapy 46, 1914-1920.

Casals, C., Teixido, N., Vinas, I., Silvera, E., Lamarca., Usall, J., 2010. Combination of hot water, *Bacillus subtilis* CPA-8 and sodium bicarbonate treatments to control postharvest brown rot on peaches and nectarines. Eur J Plant Pathol 128, 51-63.

Cerioni, L., Rodríguez-Montelongo, L., Ramallo, J., Prado, F.E., Rapisarda, V.A., Volentini, S.I., 2012. Control of lemon green mold by a sequential oxidative treatment and sodium bicarbonate. Postharvest Biology and Technology 63, 33-39.

Chalutz, E., Wilson, C., 1990. Postharvest biocontrol of green and blue mold and sour rot of citrus fruit by *Debaryomyces hansenii*. Plant disease 74, 134-137.

Chebli, B., Achouri, M., Idrissi Hassani, L., Hmamouchi, M., 2003. Chemical composition and antifungal activity of essential oils of seven Moroccan Labiatae against *Botrytis cinerea* Pers : Fr. Journal of ethnopharmacology 89, 165-169.

Choi, G.J., Jang, K.S., Kim, J.S., Lee, S.W., Cho, J.Y., Cho, K.Y., Kim, J.C., 2004. *In vivo* antifungal activities of 57 plant extracts against six plant pathogenic fungi. Plant Pathology Journal 20, 184-191.

Cohen, E., Coggins, C., W., Eckert, J., W., 1991. Predisposition of citrus fruits to sour rot when submerged in water. Plant Dis 75, 166-168.

Corral, L.G., Post, L.S., Montville, T.J., 1988. Antimicrobial activity of sodium bicarbonate. Journal of food science 53, 981-982.

Cowan, M.M., 1999. Plant products as antimicrobial agents. Clinical microbiology reviews 12, 564-582.

Daferera, D.J., Ziogas, B.N., Polissiou, M.G., 2003. The effectiveness of plant essential oils on the growth of Botrytis cinerea, Fusarium sp. and Clavibacter michiganensis subsp. michiganensis. Crop Protection 22, 39-44.

D'hallewin, G., Arras, G., Venditti, T., Rodov, V., Ben-Yehoshua, S., 2004. Combination of ultraviolet-c irradiation and biocontrol treatments to control decay caused by

Penicillium digitatum in washington navel orange fruit. Acta Horticultura 682, 2007-2012, pp. 2007-2012.

Deliopoulos, T., Kettlewell, P.S., Hare, M.C., 2010. Fungal disease suppression by inorganic salts : A review. Crop Protection 29, 1059-1075.

Dixit, S., Chandra, H., Tiwari, R., Dixit, V., 1995. Development of a botanical fungicide against blue mould of mandarins. Journal of stored products research 31, 165-172.

Droby, S., Chalutz, E., Wilson, C., Wisniewski, M., 1989. Characterization of the biocontrol activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on grapefruit. Canadian Journal of Microbiology 35, 794-800.

Droby, S., Wisniewski, M., Cohen, L., Weiss, B., Touitou, D., Eilam, Y., Chalutz, E., 1997. Influence of CaCl2 on *Penicillium digitatum*, grapefruit peel tissue, and biocontrol activity of *Pichia guilliermondii*. Phytopathology 87, 310-315.

Droby, S., Cohen, L., Daus, A., Weiss, B., Horev, B., Chalutz, E., Katz, H., Keren-Tzur, M., Shachnai, A., 1998. Commercial testing of Aspire : a yeast preparation for the biological control of postharvest decay of citrus. Biological control 12, 97-101.

Droby, S., Lischinski, S., Cohen, L., Weiss, B., Daus, A., Chand-Goyal, T., Eckert, J.W., Manulis, S., 1999a. Characterization of an Epiphytic Yeast Population of Grapefruit Capable of Suppression of Green Mold Decay Caused by *Penicillium digitatum*. Biological control 16, 27-34.

Droby, S., Porat, R., Cohen, L., Weiss, B., Shapiro, B., Philosoph-Hadas, S., Meir, S., 1999b. Suppressing green mold decay in grapefruit with postharvest jasmonate application. Journal of the American Society for Horticultural Science 124, 184-188.

Droby, S., Vinokur, V., Weiss, B., Cohen, L., Daus, A., Goldschmidt, E., Porat, R., 2002. Induction of resistance to *Penicillium digitatum* in grapefruit by the yeast biocontrol agent Candida oleophila. Phytopathology 92, 393-399.

Droby, S., Wisniewski, M., El Ghaouth, A., Wilson, C., 2003. Influence of food additives on the control of postharvest rots of apple and peach and efficacy of the yeast-based biocontrol product Aspire. Postharvest Biology and Technology 27, 127-135.

Du Plooy, W., Regnier, T., Combrinck, S., 2009. Essential oil amended coatings as alternatives to synthetic fungicides in citrus postharvest management. Postharvest Biology and Technology 53, 117-122.

Duraipandiyan, V., Ignacimuthu, S., 2007. Antibacterial and antifungal activity of Cassia fistula L. : An ethnomedicinal plant. Journal of ethnopharmacology 112, 590-594.

Eayre, C., Skaria, M., Bull, C., Mackey, B., 2003. An avirulent Galactomyces species that controls green mold of citrus caused by *Penicillium digitatum*. trial 1, 98.

Eckert, J., Sommer, N., 1967. Control of diseases of fruits and vegetables by postharvest treatment. Annual Review of Phytopathology 5, 391-428.

Eckert, J.W., 1978. Pathological diseases of fresh fruits and vegetables. Journal of Food Biochemistry 2, 243-250.

Eckert, J.W., Ogawa, J.M., 1985. The chemical control of postharvest diseases : subtropical and tropical fruits. Annual Review of Phytopathology 23, 421-454.

Eckert, J.W., Brown, G.E., 1986. Postharvest citrus diseases and their control. Fresh citrus fruits, 315-360.

Eckert, J.W., Brown, G.E., 1988. Sour rot, In : Whiteside, J.O., Garnsey, S.M., Timmer, L.W. (Eds.), Compendium of Citrus Diseases. APS Press St Paul MN pp. 37-38.

Eckert, J.W., Ogawa, J.M., 1988. The chemical control of postharvest diseases : deciduous fruits, berries, vegetables and root/tuber crops. Annual Review of Phytopathology 26, 433-469.

Eckert, J.W., Eaks, I.L., 1989. Postharvest Disorders and Diseases of Citrus Fruits. The Citrus Industry : Crop protection, postharvest technology, and early history of citrus research in California 5, 179.

Eckert, J.W., 1990. Impact of fungicide resistance on citrus fruit decay control. ACS Publications, pp. 286-302.

El-Ghaouth, A., Smilanick, J., L., Wilson, C., L., 2000. Enhancement of the performance of *Candida saitoana* by the addition of glycochitosan for control of postharvest decay of apple and citrus fruit. Postharvest Biol Technol 19, 249-253.

El-Ghaouth, A., Smilanick, J.L., Brown, G.E., Ippolito, A., Wilson, C.L., 2001. Control of Decay of Apple and Citrus Fruits in Semicommercial Tests with *Candida saitoana* and 2-Deoxy-d-glucose. Biological control 20, 96-101.

El-Ghaouth, A., Wilson, C., Wisniewski, M., Droby, S., Smilanick, J.L., Korsten, L., 2002. Biological control of postharvest diseases of fruits and vegetables. Applied mycology and biotechnology 2, 219-238.

El-Ghaouth, A., Wilson, C., Wisniewski, M., 2004. Biologically-Based Alternatives to Synthetic Fungicides for the Control of Postharvest diseases of Fruit and Vegetables Diseases of Fruits and Vegetables : Volume II, In : Naqvi, S.A.M.H. (Ed.). Springer Netherlands, pp. 511-535.

El-Goorani, M., El-Kasheir, H., Kabeel, M., Shoeib, A., 1984. Resistance to benzimidazole fungicides of *Penicillium italicum* and *P. digitatum* isolated from packinghouses and orchards in Egypt. Plant disease 68, 100-102.

El-Mougy, N., El-Gamal, N., Abd-El-Kareem F., 2008. Use of organic acids and salts to control postharvest diseases of lemon fruits in Egypt. Archives of Phytopathology and Plant Protection 41, 467-476.

El-Tobgy, K., Mahmoud, G., Abo-El-Seoud, M., 2010. Biocides application as a natural and safe alternative for treating and protecting citrus fruits. Archives of Phytopathology and Plant Protection 43, 430-437.

Eloff, J.N., 2004. Quantification the bioactivity of plant extracts during screening and bioassay guided fractionation. Phytomedicine 11, 370-371.

Ezra, D., Hess, W., Strobel, G.A., 2004. New endophytic isolates of Muscodor albus, a volatile-antibiotic-producing fungus. Microbiology 150, 4023-4031.

Fabry, W., Okemo, P., Ansorg, R., 1996. Fungistatic and fungicidal activity of East African medicinal plants. Mycoses 39, 67-70.

Fallik, E., Ziv, O., Grinberg, S., Alkalai, S., Klein, J.D., 1997. Bicarbonate solutions control powdery mildew (*Leveillula taurica*) on sweet red pepper and reduce the development of postharvest fruit rotting. Phytoparasitica 25, 41-43.

FAOSTAT, 2011. Food And Agricultural Organization of United Nations : Economic And Social Department : The Statistical Division. http ://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor.

Fatemi, S., Jafarpour, M., Eghbalsaied, S., 2011. Study of the effect of Thymus vulgaris and hot water treatment on storage life of orange (*Citrus sinensis* CV. Valencia). Journal of Medicinal Plants Research 6, 968-971.

Fawcett, C., Spencer, D., 1970. Plant chemotherapy with natural products. Annual Review of Phytopathology 8, 403-418.

Feng, L., Wu, F., Li, J., Jiang, Y., Duan, X., 2011. Antifungal activities of polyhexamethylene biguanide and polyhexamethylene guanide against the citrus sour rot pathogen *Geotrichum* citri-aurantii in vitro and in vivo. Postharvest Biology and Technology 61, 160-164.

Fisher, K., Phillips, C., 2008. Potential antimicrobial uses of essential oils in food : is citrus the answer? Trends in Food Science & Technology 19, 156-164.

Foegeding, P., Busta, F., 1991. Chemical food preservatives. Disinfection, Sterilization and Preservation. Lea & Fehinger, New York, 802-832.

Gabler, F.M., Smilanick, J.L., 2001. Postharvest control of table grape gray mold on detached berries with carbonate and bicarbonate salts and disinfectants. American journal of enology and viticulture 52, 12-20.

Garduno-Pizana, C., Barrera-Necha, L., Gomez, Y., 2010. Evaluation of the fungicidal activity of leaves powders and extracts of fifteen Mexican plants against Fusarium oxysporum f.sp. gladioli. Plant Pathol J 9, 103-111.

Gatto, M.A., Ippolito, A., Linsalata, V., Cascarano, N.A., Nigro, F., Vanadia, S., Di Venere, D., 2011. Activity of extracts from wild edible herbs against postharvest fungal diseases of fruit and vegetables. Postharvest Biology and Technology 61, 72-82.

Geng, P., Chen, S., Hu, M., Rizwan-ul-Haq, M., Lai, K., Qu, F., Zhang, Y., 2011. Combination of Kluyveromyces marxianus and sodium bicarbonate for controlling green mold of citrus fruit. International Journal of Food Microbiology 151, 190-194.

Glew, R.H., Saha, A.K., Das, S. and Remaley, A.T.,1988. Biochemistry of the *Leishmania* species. Microbiological Reviews ,52, 412-432.

Gorinstein, S., Martin-Belloso, O., Park, Y.S., Haruenkit, R., Lojek, A., Cíz, M., Caspi, A., Libman, I., Trakhtenberg, S., 2001. Comparison of some biochemical characteristics of different citrus fruits. Food chemistry 74, 309-315.

Gorris, L., Smid, E., 1995. Crop protection using natural antifungal compounds. Pestic Outlook 6, 20-24.

Hajji, M., Jarraya, R., Lassoued, I., Masmoudi, O., Damak, M., Nasri, M., 2010. GC/MS and LC/MS analysis, and antioxidant and antimicrobial activities of various solvent extracts from Mirabilis jalapa tubers. Process Biochemistry 45, 1486-1493.

Hall, D.J., 1988. Comparative activity of selected food preservatives as citrus postharvest fungicides, pp. 184-187.

Hao, W., Zhong, G., Hu, M., Luo, J., Weng, Q., Rizwan-ul-Haq, M., 2010. Control of citrus postharvest green and blue mold and sour rot by tea saponin combined with imazalil and prochloraz. Postharvest Biology and Technology 56, 39-43.

Haouala, R., Hawala, S., El-Ayeb, A., Khanfir, R., Boughanmi, N., 2008. Aqueous and organic extracts of Trigonella foenum-graecum L. inhibit the mycelia growth of fungi. Journal of Environmental Sciences 20, 1453-1457.

Hernández-Montiel, L.G., Ochoa, J.L., Troyo-Diéguez, E., Larralde-Corona, C.P., 2010. Biocontrol of postharvest blue mold (*Penicillium italicum* Wehmer) on Mexican lime by marine and citrus Debaryomyces hansenii isolates. Postharvest Biology and Technology 56, 181-187.

Hershenhorn, J., Park, S.H., Stierle, A., Strobel, G.A., 1992. *Fusarium avenaceum* as a novel pathogen of spotted knapweed and its phytotoxins, acetamido-butenolide and enniatin B. Plant Science 86, 155-160.

Hervieux, V., Yaganza, E., Arul, J., Tweddell, R., 2002. Effect of organic and inorganic salts on the development of *Helminthosporium solani*, the causal agent of potato silver scurf. Plant disease 86, 1014-1018.

Holmes, G., Eckert, J., Pitt, J., 1994. Revised description of *Penicillium ulaiense* and its role as a pathogen of citrus fruits. Phytopathology 84, 719-727.

Holmes, G.J., Eckert, J.W., 1999. Sensitivity of *Penicillium digitatum* and *P. italicum* to postharvest citrus fungicides in California. Phytopathology 89, 716-721.

Huang, Y., Wild, B., Morris, S., 1992. Postharvest biological control of *Penicillium digitatum* decay on citrus fruit by *Bacillus pumilus*. Annals of applied biology 120, 367-372.

Huang, Y., Deverall, B.J., Morris, S.C., 1995. Postharvest control of green mould on oranges by a strain of *Pseudomonas glathei* and enhancement of its biocontrol by heat treatment. Postharvest Biology and Technology 5, 129-137.

Hunter, T., 1995. Protein kinases and phosphatases : the yin and yang of protein phosphorylation and signaling. Cell, 80, 225-236.

Ilhan, K., Arslan, U., Karabulut, O.A., 2006. The effect of sodium bicarbonate alone or in combination with a reduced dose of tebuconazole on the control of apple scab. Crop Protection 25, 963-967.

Ippolito, A., Nigro, F., 2000. Impact of preharvest application of biological control agents on postharvest diseases of fresh fruits and vegetables. Crop Protection 19, 715-723.

Ismail, M., Zhang, J., 2004. Post-harvest citrus diseases and their control. Outlooks on Pest Management 15, 29-35.

Ismail, H., Lemriss, S., Ben Aoun, Z., Mhadhebi, L., Dellai, A., Kacem, Y., Boiron, P., Bouraoui, A., 2008. Antifungal activity of aqueous and methanolic extracts from the Mediterranean sea cucumber, *Holothuria polii*. Journal de Mycologie Médicale/Journal of Medical Mycology 18, 23-26.

Jafarpour, M., Fatemi, S., 2012. Post harvest treatments on shelf life of sweet orange" Valencia. Journal of Medicinal Plants Research 6, 2117-2124.

Janisiewicz, W., 1987. Postharvest biological control of blue mold on apples. Phytopathology 77, 481-485.

Janisiewicz, W.J., 1988. Biocontrol of postharvest disease of apples with antagonist mixtures. Phytopathology 78, 194–198.

Janisiewicz, W.J., Korsten, L., 2002. Biological control of postharvest diseases of fruits. Annual Review of Phytopathology 40, 411-441.

Kanetis, L., Förster, H., Adaskaveg, J.E., 2007. Comparative efficacy of the new postharvest fungicides azoxystrobin, fludioxonil, and pyrimethanil for managing citrus green mold. Plant disease 91, 1502-1511.

Kinay, P., Mansour, M.F., Mlikota Gabler, F., Margosan, D.A., Smilanick, J.L., 2007. Characterization of fungicide-resistant isolates of *Penicillium digitatum* collected in California. Crop Protection 26, 647-656.

Kiss, L., 2003. A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. Pest management science 59, 475-483.

Kitagawa, H., Kawada, K., 1984. Effect of sorbic acid and potassium sorbate on the control of sour rot of citrus fruits, pp. 133-135.

Kivack, B., Mert, T., Tansel, H., 2001. Antimicrobial and cytotoxic activities of *Ceratonia siliqua* L. extracts. Turk. J. Biol 26, 197-200.

Klieber, A., Scott, E., Wuryatmo, E., 2002. Effect of method of application on antifungal efficacy of citral against postharvest spoilage fungi of citrus in culture. Australasian Plant Pathology 31, 329-332.

Kosalec, I., Pepeljnjak, S., Kustrak, D., 2005. Antifungal activity of fluid extract and essential oil from anise fruits (*Pimpinella anisum* L., Apiaceae). Acta Pharm 55, 377-385.

Koutsoudaki, C., Krsek, M., Rodger, A., 2005. Chemical composition and antibacterial activity of the essential oil and the gum of *Pistacia lentiscus* Var. chia. Journal of agricultural and food chemistry 53, 7681-7685.

Kuzma, L., Rozalski, M., Walencka, E., Rozalska, B., Wysokinska, H., 2007. Antimicrobial activity of diterpenoids from hairy roots of *Salvia sclarea* L. : Salvipisone as a potential anti-biofilm agent active against antibiotic resistant *Staphylococci*. Phytomedicine 14, 31-35.

Ladaniya, M.S., 2008. 16 - Postharvest diseases and their management, Citrus Fruit. Academic Press, San Diego, pp. 417-XIX.

Lahlali, R., Serrhini, M., Jijakli, M., 2004. Efficacy assessment of *Candida oleophila* (strain O) and *Pichia anomala* (strain K) against major postharvest diseases of citrus fruits in Morocco. Communications in agricultural and applied biological sciences 69, 601.

Lahlali, R., Hamadi, Y., Jijakli, M.H., 2010. Efficacy assessment of *Pichia guilliermondii* strain Z1, a new biocontrol agent, against citrus blue mould in Morocco under the influence of temperature and relative humidity. Biological control 56 (2011) 217–224

Lai, K., Chen, S., Hu, M., Hu, Q., Geng, P., Weng, Q., Jia, J., 2012. Control of postharvest green mold of citrus fruit by application of endophytic *Paenibacillus polymyxa* strain SG-6. Postharvest Biology and Technology 69, 40-48.

Lanciotti, R., Gianotti, A., Patrignani, F., Belletti, N., Guerzoni, M., Gardini, F., 2004. Use of natural aroma compounds to improve shelf-life and safety of minimally processed fruits. Trends in food science & technology 15, 201-208.

Leelasuphakul, W., Hemmanee, P., Chuenchitt, S., 2008. Growth inhibitory properties of *Bacillus subtilis* strains and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of citrus fruit. Postharvest Biology and Technology 48, 113-121.

Liu, X., Wang, J., Gou, P., Mao, C., Zhu, Z.R., Li, H., 2007. *In vitro* inhibition of postharvest pathogens of fruit and control of gray mold of strawberry and green mold of citrus by aureobasidin A. International Journal of Food Microbiology 119, 223-229.

Liu, B., Qiao, H., Huang, L., Buchenauer, H., Han, Q., Kang, Z., Gong, Y., 2009a. Biological control of take-all in wheat by endophytic *Bacillus subtilis* E1R-j and potential mode of action. Biological Control 49, 277-285.

Liu, X., Wang, L., Li, Y., Li, H., Yu, T., Zheng, X., 2009b. Antifungal activity of thyme oil against *Geotrichum citri aurantii in vitro* and *in vivo*. Journal of applied microbiology 107, 1450-1456.

Liu, X., Fang, W., Liu, L., Yu, T., Lou, B., Zheng, X., 2010. Biological control of postharvest sour rot of citrus by two antagonistic yeasts. Letters in applied microbiology 51, 30-35.

Long, C.A., Deng, B.X., Deng, X.X., 2006. Pilot testing ofKloeckera apiculata for the biological control of postharvest diseases of citrus. Annals of microbiology 56, 13-17.

Lucon, C., Guzzo, S., de Jesus, C., Pascholati, S., de Goes, A., 2010. Postharvest harpin or *Bacillus thuringiensis* treatments suppress citrus black spot in 'Valencia'oranges. Crop Protection 29, 766-772.

Luo, Y., Zeng, K., Ming, J., 2012. Control of blue and green mold decay of citrus fruit by *Pichia membranefaciens* and induction of defense responses. Scientia Horticulturae 135, 120-127.

Magiatis, P., Melliou, E., Skaltsounis, A.L., Chinou, I.B., Mitaku, S., 1999. Chemical composition and antimicrobial activity of the essential oils of *Pistacia lentiscus* var. chia. Planta medica 65, 749-750.

Mahlo, S., McGaw, L., Eloff, J., 2010. Antifungal activity of leaf extracts from South African trees against plant pathogens. Crop Protection 29, 1529-1533.

Manso, T., Nunes, C., 2011. *Metschnikowia andauensis* as a new biocontrol agent of fruit postharvest diseases. Postharvest Biology and Technology.

Martini, S., D'Addario, C., Colacevich, A., Focardi, S., Borghini, F., Santucci, A., Figura, N., Rossi, C., 2009. Antimicrobial activity against *Helicobacter pylori* strains and antioxidant properties of blackberry leaves (*Rubus ulmifolius*) and isolated compounds. International Journal of Antimicrobial Agents 34, 50-59.

Mekbib, S.B., Regnier, T.J.C., Korsten, L., 2007. Control of *Penicillium digitatum* on citrus fruit using two plant extracts and study of their mode of action. Phytoparasitica 35, 264-276.

Mercier, J., Wilson, C.L., 1994. Colonization of apple wounds by naturally occurring microflora and introduced *Candida oleophila* and their effect on infection by *Botrytis cinerea* during storage. Biol. Control 4, 138-144.

Mercier, J., Smilanick, J.L., 2005. Control of green mold and sour rot of stored lemon by biofumigation with *Muscodor albus*. Biological control 32, 401-407.

Meziane, H., Gavriel, S., Ismailov, Z., Chet, I., Chernin, L., Hofte, M., 2006. Control of green and blue mould on orange fruit by *Serratia plymuthica* strains IC14 and IC1270 and putative modes of action. Postharvest Biology and Technology 39, 125-133.

Mills, A., Platt, H., Hurta, R., 2004. Effect of salt compounds on mycelial growth, sporulation and spore germination of various potato pathogens. Postharvest Biology and Technology 34, 341-350.

Mishra A.K., Dubey N.K., 1990. Fungitoxicity of essential oil of *Amomum subulatum* against *Aspergillus flavus*, Econ. Bot 44, 530–533.

Mohamed, N., H., El-Hadidy, A., M., 2008. Studies of biologically active constituents of *Verbascum eremobium* Murb. and its inducing resistance against some diseases of cucumber. Egypt. J Phytopathol 36, 133-150.

Montesinos-Herrero, C., Smilanick, J.L., Tebbets, J.S., Walse, S., Palou, L., 2010. Control of citrus postharvest decay by ammonia gas fumigation and its influence on the efficacy of the fungicide imazalil. Postharvest Biology and Technology.

Morris, S., 1982. Synergism of *Geotrichum candidum* and *Penicillium digitatum* in Infected Citrus Fruit. Phytopathology 72, 1336-1339.

Msanda, F., El Aboudi, A., Peltier, J., 2005. Biodiversité et biogéographie de l'arganeraie marocaine. Cah Agric 14, 357-364.

Nenaah, G., 2010. Antibacterial and antifungal activities of (beta)-carboline alkaloids of *Peganum harmala* (L) seeds and their combination effects. Fitoterapia 81, 779-782.

Neri, F., Mari, M., Brigati, S., 2006. Control of *Penicillium expansum* by plant volatile compounds. Plant pathology 55, 100-105.

Nicholson, R.L., Hammerschmidt, R., 1992. Phenolic compounds and their role in disease resistance. Annual Review of Phytopathology 30, 369-389.

Norman, C., 1988. EPA sets new policy on pesticide cancer risks. Science 242, 366-367.

Nunes, C., Usall, J., Teixidó, N., Vinas, I., 2002. Improvement of Candida sake biocontrol activity against post harvest decay by the addition of ammonium molybdate. Journal of applied microbiology 92, 927-935.

Obagwu, J., Korsten, L., 2003. Control of citrus green and blue molds with garlic extracts. European journal of plant pathology 109, 221-225.

Okigbo, R., Ikediugwu, F., 2000. Studies on biological control of postharvest rot in yams (Dioscorea spp.) using *Trichoderma viride*. Journal of Phytopathology 148, 351-355.

Olivier, C., Halseth, D.E., Mizubuti, E.S.G., Loria, R., 1998. Postharvest application of organic and inorganic salts for suppression of silver scurf on potato tubers. Plant disease 82, 213-217.

Osman, M.S., Sivakumar, D., Korsten, L., 2011. Effect of biocontrol agent *Bacillus amyloliquefaciens* and 1-methyl cyclopropene on the control of postharvest diseases and maintenance of fruit quality. Crop Protection 30, 173-178.

Palmer, C.L., Horst, R.K., Langhans, R.W., 1997. Use of bicarbonates to inhibit *in vitro* colony growth of *Botrytis cinerea*. Plant disease 81, 1432-1438.

Palou, L., Smilanick, J.L., Usall, J., Viñas, I., 2001. Control of postharvest blue and green molds of oranges by hot water, sodium carbonate, and sodium bicarbonate. Plant disease 85, 371-376.

Palou, L., Usall, J., Smilanick, J.L., Aguilar, M.J., Vinas, I., 2002. Evaluation of food additives and low toxicity compounds as alternative chemicals for the control of *Penicillium digitatum* and *Penicillium italicum* on citrus fruit. Pest management science 58, 459-466.

Palou, L., Smilanick, J.L., Droby, S., 2008. Alternatives to conventional fungicides for the control of citrus postharvest green and blue moulds. Stewart Postharvest Review 4, 1-16.

Palou, L., Smilanick, J.L., Crisosto, C.H., 2009. Evaluation of food additives as alternative or complementary chemicals to conventional fungicides for the control of major postharvest diseases of stone fruit. Journal of Food Protection 174; 72, 1037-1046.

Panizzi, L., Caponi, C., Catalano, S., Cioni, P., Morelli, I., 2002. *In vitro* antimicrobial activity of extracts and isolated constituents of *Rubus ulmifolius*. Journal of ethnopharmacology 79, 165-168.

Pelser, P. du T., Eckert, J. W., 1977. Constituents of orange juice that stimulate the germination of conidia of *Penicillium digitatum*. Phytopathology, 67,747-754.

Phongpaichit, S., Subhadhirasakul, S., Wattanapiromsakul, C., 2005. Antifungal activities of extracts from Thai medicinal plants against opportunistic fungal pathogens associated with AIDS patients. Mycoses 48, 333-338.

Pimenta, R.S., Silva, F.L., Silva, J.F.M., Morais, P.B., Braga, D.T., Rosa, C.A., Corrêa Jr, A., 2008. Biological control of *Penicillium italicum*, *P. digitatum* and *P. expansum* by the predacious yeast *Saccharomycopsis schoenii* on oranges. Brazilian Journal of Microbiology 39, 85-90.

Platania, C., Restuccia, C., Muccilli, S., Cirvilleri, G., 2012. Efficacy of killer yeasts in the biological control of *Penicillium digitatum* on Tarocco orange fruits (*Citrus sinensis*). Food Microbiology 30, 219-225.

Plaza, P., Usall, J., Torres, R., Lamarca, N., Asensio, À., Viñas, I., 2003. Control of green and blue mould by curing on oranges during ambient and cold storage. Postharvest Biology and Technology 28, 195-198.

Plaza, P., Torres, P., Usall, J., Lamarca, N., Vinas, I., 2004a. Evaluation of the potential of commercial post-harvest application of essential oils to control citrus decay. Journal of horticultural science & biotechnology 79, 935-940.

Plaza, P., Usall, J., Torres, R., Abadias, M., Smilanick, J.L., Vinas, I., 2004b. The use of sodium carbonate to improve curing treatments against green and blue moulds on citrus fruits. Pest management science 60, 815-821.

Plaza, P., Usall, J., Smilanick, J.L., Lamarca, N., Vinas, I., 2004c. Combining *Pantoea agglomerans* (CPA-2) and curing treatments to control established infections of *Penicillium digitatum* on lemons. Journal of Food Protection 174; 67, 781-786.

Porat, R., Daus, A., Weiss, B., Cohen, L., Droby, S., 2002. Effects of combining hot water, sodium bicarbonate and biocontrol on postharvest decay of citrus fruit. Journal of horticultural science & biotechnology 77, 441-445.

Prasad, K., Stadelbacher, G.J., 1973. Control of post harvest decay of fresh raspberries by acetaldehyde vapor. Plant Dis 57, 795-797.

Prusky, D., Yakoby, A. N., 2003. Pathogenic fungi : leading or led by ambient pH? Mol. Plant Pathology, 4,509-516.

Prusky, D., McEvoy, J. L., Saftner, R., Conway, W. S., Jones, R., 2004. Relationship between host acidification and virulence of *Penicillium* spp. on apple and citrus fruit. Phytopathology, 94,44-51.

Qasem, J., Abu-Blan, H., 1996. Fungicidal activity of some common weed extracts against different plant pathogenic fungi. Journal of Phytopathology 144, 157-161.

Ren, X., Kong, Q., Wang, H., Yu, T., Zhou, W., Zheng, X., 2011. Biocontrol of fungal decay of citrus fruit by *Pichia pastoris* recombinant strains expressing cecropin A. Food chemistry.

Ribera, A., Cotoras, M., Zúñiga, G.E., 2008. Effect of extracts from in vitro-grown shoots of Quillaja saponaria Mol. on Botrytis cinerea Pers. World Journal of Microbiology and Biotechnology 24, 1803-1811.

Rippon, L.E., Morris, S.C., 1981. Guazatine control of sour rot in lemons, oranges and tangors under various storage conditions. Scientia Horticulturae 14, 245-251.

Rodilla, J.M.L., De Mendonça, D.I.M., Urones, J., Moro, R., 1998. Hydroxylated diterpenoids from Halimium viscosum. Phytochemistry 49, 817-822.

Rodov, V., Ben-Yehoshua, S., Fang, D., D'hallewin, G., Castia, T., 1994. Accumulation of phytoalexins scoparone and scopoletin in citrus fruits subjected to various postharvest treatments, pp. 517-525.

Saks, Y., Barkai-Golan, R., 1995. Aloe vera gel activity against plant pathogenic fungi. Postharvest Biology and Technology 6, 159-165.

Sánchez-Torres, P., Tuset, J.J., 2011. Molecular insights into fungicide resistance in sensitive and resistant *Penicillium digitatum* strains infecting citrus. Postharvest Biology and Technology 59, 159-165.

Satish, S., Raghavendra, M., Mohana, D., Raveesha, K., 2008. Antifungal activity of a known medicinal plant Mimusops elengi L. against grain moulds. Technology 4, 151-165.

Sato, J., Goto, K., Nanjo, F., Kawai, S., Murata, K., 2000. Antifungal activity of plant extracts against *Arthrinium sacchari* and *Chaetomium funicola*. Journal of Bioscience and Bioengineering 90, 442-446.

Schisler, D.A., Janisiewicz, W.J., Boekhout, T., Kurtzman, C.P., 2011. Chapter 4 - Agriculturally Important Yeasts : Biological Control of Field and Postharvest Diseases

Using Yeast Antagonists, and Yeasts as Pathogens of Plants, The Yeasts (Fifth Edition). Elsevier, London, pp. 45-52.

Sharma, R.R., Singh, D., Singh, R., 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists : A review. Biological control 50, 205-221.

Sholberg, P., 1998. Fumigation of fruit with short-chain organic acids to reduce the potential of postharvest decay. Plant disease 82, 689-693.

Singh, V., Deverall, B.J., 1984. *Bacillus subtilis* as a control agent against fungal pathogens of citrus fruit. Transactions of the British Mycological Society 83, 487-490.

Sissay, B., 2007. Identification of citrus (*Citrus sinensis*) Postharvest Pathogens from Ethiopia and their Control, Faculty of Natural and Agricultural Sciences. Univ. Pretoria-Mekbib, Pretoria, p. 220.

Sisti, M., De Santi, M., Fraternale, D., Ninfali, P., Scoccianti, V., Brandi, G., 2008. Antifungal activity of *Rubus ulmifolius* Schott standardized *in vitro* culture. LWT 41, 946-950.

Skandamis, P., Koutsoumanis, K., Fasseas, K., Nychas, G.J.E., 2001. Inhibition of oregano essential oil and EDTA on E. coli O157 :H7. Italian Journal of Food Science 13, 55-65.

Slinkard, K., Singleton, V.L., 1977. Total phenol analysis : automation and comparison with manual methods. American journal of enology and viticulture 28, 49-55.

Smilanick, J., Denis-Arrue, R., 1992. Control of green mold of lemons with *Pseudomonas* species. Plant disease 76, 481.

Smilanick, J., Mackey, B., Reese, R., Usall, J., Margosan, D., 1997. Influence of concentration of soda ash, temperature, and immersion period on the control of postharvest green mold of oranges. Plant disease 81, 379-382.

Smilanick, J.L., Margosan, D.A., Mlikota, F., Usall, J., Michael, I.F., 1999. Control of citrus green mold by carbonate and bicarbonate salts and the influence of commercial postharvest practices on their efficacy. Plant disease 83, 139-145.

Smilanick, J., Sorenson, D., 2001. Control of postharvest decay of citrus fruit with calcium polysulfide. Postharvest Biology and Technology 21, 157-168.

Smilanick, J., Mansour, M., Margosan, D., Gabler, F.M., Goodwine, W., 2005. Influence of pH and NaHCO3 on effectiveness of imazalil to inhibit germination of *Penicillium digitatum* and to control postharvest green mold on citrus fruit. Plant disease 89, 640-648.

Smilanick, J., Mansour, M., Gabler, F.M., Goodwine, W., 2006a. The effectiveness of pyrimethanil to inhibit germination of *Penicillium digitatum* and to control citrus green mold after harvest. Postharvest Biology and Technology 42, 75-85.

Smilanick, J., Mansour, M., Sorenson, D., 2006b. Pre-and postharvest treatments to control green mold of citrus fruit during ethylene degreening. Plant disease 90, 89-96.

Smilanick, J.L., Mansour, M.F., Gabler, F.M., Sorenson, D., 2008. Control of citrus postharvest green mold and sour rot by potassium sorbate combined with heat and fungicides. Postharvest Biology and Technology 47, 226-238.

Sofos, J., Pierson, M., Blocher, J., Busta, F., 1986. Mode of action of sorbic acid on bacterial cells and spores. International Journal of Food Microbiology 3, 1-17.

Solaimani, B., Ramezani, S., Rahemi, M., Saharkhiz, M.J., 2009. Biological control of postharvest disease caused by *Penicillium digitutum* and *P. italicum* on stored citrus fruits by shiraz thyme essential oil. Advances in Environmental Biology 3, 249-254.

Sommer, N.F., 1982. Postharvest handling practices and postharvest diseases of fruit. Plant disease 66, 357-364.

Soylu, E., Tok, F., Soylu, S., Kaya, A., Evrendilek, G., 2005. Antifungal activities of the essential oils on postharvest disease agent *Penicillium digitatum*. Pakistan J Biol Sci 8, 25-29.

Soylu, E., M., Kurt, S., Soylu, S., 2010. *In vitro* and *in vivo* antifungal activities of the essential oils of various plants against tomato grey mould disease agent Botrytis cinerea. International Journal of Food Microbiology 143, 183–189.

Spadaro, D., Gullino, M.L, 2004. State of the Art and Future Prospects of the Biological Control of Postharvest Fruit Diseases. International Journal of Food Microbiology 91, 185-194.

Spotts, R.A., Cervantes, L.A., Facteau, T.J., Chand-Goyal, T., 1998. Control of brown rot and blue mold of sweet cherry with preharvest Iprodione, postharvest Crptococcus infirmo-miniatus, and modified atmosphere packaging. Plant Dis., 1158–1160.

Stevens, C., Khan, V.A., Lu, J.Y., Wilson, C.L., Pusey, P.L., Igwegbe, E.C.K., Kabwe, K., Mafolo, Y., Liu, J., Chalutz, E., Droby, S., 1997. Integration of Ultraviolet (UV-C) Light with Yeast Treatment for Control of Postharvest Storage Rots of Fruits and Vegetables. Biological control 10, 98-103.

Strobel, G.A., Dirkse, E., Sears, J., Markworth, C., 2001. Volatile antimicrobials from Muscodor albus, a novel endophytic fungus. Microbiology 147, 2943.

Suhr, K., I., Nielson, P., V., 2003. Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. J Appl Microb 94, 1-11.

Suleiman, M., Naidoo, V., Eloff, J., 2012. Preliminary screening of some fractions of Loxostylis alata (Anacardiaceae) for antimicrobial and antioxidant activities. African Journal of Biotechnology 11, 2340-2348.

Suprapta, D., Arai, K., Iwai, H., 1995. Distribution of *Geotrichum candidum* citrus race in citrus groves and non-citrus fields in Japan. Mycoscience 36, 277-282.

Suprapta, D., N,, Arai K, Iwai H, Matsuo T, 1996. Change in susceptibility of satsuma mandarin fruit to sour rot pathogen (*Geotrichum candidum* citrus race) with relation to biochemical changes during maturation and storage. Mycoscience 37, 209-216.

Suprapta, D., N., Arai, K., Iwai, H., 1997. Effects of volatile compounds on arthrospore germination and mycelial growth of *Geotrichum candidum* citrus race. Mycoscience 38, 31-35.

Talibi, I., Amkraz, N., Askarne, L., Msanda, F., Saadi, B., Boudyach, E., H., Boubaker, H., Bouizgarne, B., Ait Ben Aoumar, A., 2011a. Antibacterial activity of moroccan plants extracts against *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of tomatoes' bacterial canker. Journal of Medicinal Plants Research 5, 4332-4338.

Talibi, I., Askarne, L., Boubaker, H., Boudyach E.H., Ait Ben Oumar A., 2011b. *In vitro* and *in vivo* Screening of organic and inorganic salts to control of postharvest citrus sour rot caused by *Geotrichum candidum* Plant Pathology journal 10, 138-145.

Talibi, I., Askarne, L., Boubaker, H., Boudyach, E., Msanda, F., Saadi, B., Ait Ben Aoumar, A., 2012a. Antifungal activity of some Moroccan plants against *Geotrichum candidum*, the causal agent of postharvest citrus sour rot. Crop Protection 35, 41-46.

Talibi, I., Askarne, L., Boubaker, H., Boudyach, E.H., Msanda, F., Saadi, B., Ait Ben Aoumar, A., 2012b. Antifungal activity of Moroccan medicinal plants against citrus sour rot agent *Geotrichum candidum*. Letters in applied microbiology 55, 155-161.

Taqarort, N., Echairi, A., Chaussod, R., Nouaim, R., Boubaker, H., Benaoumar, A.A., Boudyach, E., 2008. Screening and identification of epiphytic yeasts with potential for biological control of green mold of citrus fruits. World Journal of Microbiology and Biotechnology 24, 3031-3038.

Tayel, A., El-Baz, A., Salem, M., El-Hadary, M., 2009. Potential applications of pomegranate peel extract for the control of citrus green mould. Journal of Plant Diseases and Plant Protection (JPDP) 6, 252-256.

Tegegne, G., Pretorius, J., Swart, W., 2008. Antifungal properties of *Agapanthus africanus* L. extracts against plant pathogens. Crop Protection 27, 1052-1060.

Teixidó, N., Usall, J., Palou, L., Asensio, A., Nunes, C., Viñas, I., 2001. Improving control of green and blue molds of oranges by combining Pantoea agglomerans (CPA-2) and sodium bicarbonate. European journal of plant pathology 107, 685-694.

Torres, R., Teixidó, N., Usall, J., Abadias, M., Mir, N., Larrigaudiere, C., Viñas, I., 2011. Anti-oxidant activity of oranges after infection with the pathogen *Penicillium digitatum* or treatment with the biocontrol agent Pantoea agglomerans CPA-2. Biological control.

Tripathi, P., Dubey, N.K., Pandey, V.B., 2002. Kaempferol : the antifungal principle of *Acacia nilotica* Linn. Del. J. Indian Bot. Soc. 81, 51-54.

Tripathi, P., Dubey, N., 2004. Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. Postharvest Biology and Technology 32, 235-245.

Tripathi, P., Dubey, N., Banerji, R., Chansouria, J., 2004. Evaluation of some essential oils as botanical fungitoxicants in management of post-harvest rotting of citrus fruits. World Journal of Microbiology and Biotechnology 20, 317-321.

Tripathi, P., Dubey, N., Shukla, A., 2008. Use of some essential oils as post-harvest botanical fungicides in the management of grey mould of grapes caused by *Botrytis cinerea*. World Journal of Microbiology and Biotechnology 24, 39-46.

Urones, J.G., Marcos, I.S., Oliva, I.M., Garrido, N.M., Hagget, J., Humphreys, V.M., 1995. Labdane diterpenes from *Halimium viscosum*. Phytochemistry 38, 663-666.

Usall, J., Smilanick, J., Palou, L., Denis-Arrue, N., Teixidó, N., Torres, R., Viñas, I., 2008. Preventive and curative activity of combined treatments of sodium carbonates and *Pantoea agglomerans* CPA-2 to control postharvest green mold of citrus fruit. Postharvest Biology and Technology 50, 1-7.

Utama, I.M.S., Wills, R.B.H., Ben-yehoshua, S., Kuek, C., 2002. *In vitro* efficacy of plant volatiles for inhibiting the growth of fruit and vegetable decay microorganisms. Journal of agricultural and food chemistry 50, 6371-6377.

Valencia-Chamorro, S.A., Palou, L., del Ri o, M.A., Pe rez-Gago, M.B., 2008. Inhibition of *Penicillium digitatum* and *Penicillium italicum* by Hydroxypropyl Methylcellulose-Lipid Edible Composite Films Containing Food Additives with Antifungal Properties. Journal of agricultural and food chemistry 56, 11270-11278.

Van Loon, L., Bakker, P., Pieterse, C., 1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology 36, 453-483.

Verma, R., Naosekpam, A.S., Kumar, S., Prasad, R., Shanmugam, V., 2007. Influence of soil reaction on diversity and antifungal activity of fluorescent pseudomonads in crop rhizospheres. Bioresource technology 98, 1346-1352.

Wang, W., Ben-Daniel, B., Cohen, Y., 2004. Control of plant diseases by extracts of *Inula viscosa*. Phytopathology 94, 1042-1047.

Weckesser, S., Engel, K., Simon-Haarhaus, B., Wittmer, A., Pelz, K., Schempp, C., 2007. Screening of plant extracts for antimicrobial activity against bacteria and yeasts with dermatological relevance. Phytomedicine 14, 508-516.

Wells, J., 1997. Sour Rot of Peaches Caused by *Monilia implicata* and *Geotrichum candidum*. Phytopathology 67, 404-408.

Wild, B., McGlasson, W., Lee, T., 1976. Effect of reduced ethylene levels in storage atmospheres on lemon keeping quality. HortScience 11, 114-115.

Wilson, C.L., Chalutz, E., 1989. Postharvest biological control of *Penicillium* rots of citrus with antagonistic yeasts and bacteria. Scientia Horticulturae 40, 105-112.

Wilson, C.L., Wisniewski, M.E., 1989. Biological Control of Postharvest Diseases of Fruits and Vegetables : An Emerging Technology. Annual Review of Phytopathology 27, 425-441.

Wilson, C.L., Wisniewski, M.E., Biles, C.L., McLaughlin, R., Chalutz, E., Droby, S., 1991. Biological control of post-harvest diseases of fruits and vegetables : alternatives to synthetic fungicides. Crop Protection 10, 172-177.

Wisniewski, M.E., Wilson, C.L., 1992. Biological control of postharvest diseases of fruits and vegetables : recent advances. HortScience 27, 94-98.

Wisniewski, M., Droby, S., Chalutz, E., Eilam, Y., 1995. Effects of Ca2+ and Mg2+ on *Botrytis cinerea* and *Penicillium expansum in vitro* and on the biocontrol activity of Candida oleophila. Plant pathology 44, 1016-1024.

Yahyazadeh, M., Omidbaigi, R., Zare, R., Taheri, H., 2008. Effect of some essential oils on mycelial growth of *Penicillium digitatum* Sacc. World Journal of Microbiology and Biotechnology 24, 1445-1450.

Yánez-Mendizábal, V., Usall, J., Viñas, I., Casals, C., Marín, S., Solsona, C., Teixidó, N., 2011. Potential of a new strain of *Bacillus subtilis* CPA-8 to control the major postharvest diseases of fruit. Biocontrol Science and Technology 21, 409-426.

Youssef, K., Ligorio, A., Nigro, F., Ippolito, A., 2012. Activity of salts incorporated in wax in controlling postharvest diseases of citrus fruit. Postharvest Biology and Technology 65, 39-43.

Zakaria, Z., Desa, A.M., Ramasamy, K., Ahmat, N., Mohamad, A., Israf, D., Sulaiman, M., 2010. Lack of antimicrobial activities of Dicranopteris linearis extracts and fractions. African Journal of Microbiology Research 4, 071-075.

Zhang, J., Swingle, P., 2003. Control of green mold on Florida citrus fruit using bicarbonate salts, pp. 375-378.

Zhang, H.Y., Fu, C.X., Zheng, X.D., He, D., Shan, L.J., Zhan, X., 2004. Effects of Cryptococcus laurentii (Kufferath) Skinner in combination with sodium bicarbonate on biocontrol of postharvest green mold decay of citrus fruit. Botanical Bulletin of Academia Sinica 45.

Zhang, H., Ma, L., Wang, L., Jiang, S., Dong, Y., Zheng, X., 2008. Biocontrol of gray mold decay in peach fruit by integration of antagonistic yeast with salicylic acid and their effects on postharvest quality parameters. Biological control 47, 60-65.

Zheng, X.D., Zhang, H.Y., Sun, P., 2005. Biological control of postharvest green mold decay of oranges by Rhodotorula glutinis. European Food Research and Technology 220, 353-357.

APPENDICES

Appendix 1 : Cultur mediums

NYDA : Nutrient Yeast Dextrose Agar :

Nutrient browth	8g
Yeast extracts	5g
D-glucose	10g
Bacteriological agar	15g
Distilled water	1000ml

NYDB : Nutrient Yeast Dextrose Broth :

Nutrient browth	8g
Yeast extracts	5g
D-glucose	10g
Distilled water	1000ml

PDA : Potato Dextrose Agar :

Potato extract	4g
D-glucose	20g
Bacteriological agar	15g
Distilled water	1000ml

Fuchsine acide :

Fuchsine acide	0.2% w/v
Acetic acide	1 volume
Ethanol 95%	1 volume

Appendix 2 : Bilan agrumicole de la campagne 2010/2011

I- SUPERFICIE :

Le verger agrumicole du Souss occupe actuellement une superficie d'environ 36 285 ha. Les nouvelles plantations s'élèvent à 637 ha et concernent surtout la Nuless (169 Ha) et Afourar (152 Ha). La superficie réservée aux petits fruits a atteint 18 163 ha, contre 17 081 ha pour le groupe des oranges, soit 50% du verger agrumicole régional.

DESIGNATION	SUPERFICIE (Ha) 2009/10 (1)	ARRACHAGE (Ha) 2010/2011 (2)	NOUVELLES PLANTATIONS (Ha) 2010/2011 (3)	SUPERFICIE TOTALE ACTUELLE (Ha) (1) + (3) - (2)	SUPERFICIE PRODUCTIVE (Ha)
PETITS FRUITS	**17565**	**0**	**598**	**18163**	**16201**
Clémentine	8038	0	14	8052	7756
Nuless	2993	0	169	3161	2377
Nour	4440	0	64	4503	4224
Afourar	211	0	152	363	160
Nova	299	0	85	384	289
Fortune	29	0	0	29	29
Ortanique	303	0	0	303	303
Autres petits fruits	1253	0	115	1368	1063
ORANGES	**17059**	**16**	**39**	**17081**	**16508**
Maroc-Late	8763	8	23	8778	8499
Navel	3241	0	15	3257	3133
Navelina	15	0	0	15	15
Navel Late	162	0	0	162	161
Navel Lane Late	766	0	0	766	739
Salustiana	897	8	0	889	873
W. Sanguine	415	0	0	415	337
Autres Oranges	2800	0	0	2800	2751
AUTRES	**1048**	**8**	**0**	**1040**	**1023**
Citron	1033	8	0	1025	1008
Autres	15	0	0	15	15
TOTAL	**35672**	**24**	**637**	**36285**	**33733**

II- CLIMAT :

- Pluviométrie :

Le cumul des précipitations au cours de la campagne 2010/2011 a atteint 362 mm contre 543 mm pour la campagne 2009/2010, soit une diminution de 33%. Ces précipitations sont réparties selon les stations comme suit :

STATION/MOIS	SEP10	OCT	NOV	DÉC	JANV11	FEV	MARS	AVR	MAI	JUIN	JUIL	AOUT	CUMUL
AIT MELLOUL													
09/10	0	0	0	197	77	244	12	0	0	2	0	42	**572**
10/11	0	16	58	87	73	2	67	41	83	0	0	0	**426**
MASSA													
09/10	0	0	0	105	62	167	25	0	0	0	0	0	**358**
10/11	0	21	45	23	45	0	60	17	90	0	0	0	**300**
OD TEIMA													
09/10	0	0	0	249	37	236	0	0	0	0	0	22	**544**
10/11	0	5	93	96	57	0	74	42	75	0	0	0	**442**
TAROUDANT													
09/10	0	0	0	239	28	243	81	3	0	0	0	41	**633**
10/11	0	4	80	108	52	0	82	35	81	0	0	0	**442**
AOULOUZ													
09/10	0	0	0	201	26	274	30	13	0	0	0	33	**577**
10/11	10	6	149	42	59	0	94	19	67	0	0	0	**444**
MOYENNE													
09/10	**0**	**0**	**1**	**198**	**46**	**233**	**29**	**3**	**0**	**0**	**0**	**32**	**542**
10/11	**1**	**15**	**67**	**49**	**52**	**0**	**68**	**24**	**84**	**0**	**0**	**0**	**362**

- Température :

STATION/MOIS	SEP10	OCT	NOV	DÉC	JANV11	FEV	MARS	AVR	MAI	JUIN
AIT MELLOUL										
max absolue	38	30	28	28	24	23	29	35	37	41
min absolue	18	14	11	12	9	7	9	13	13	19
moy max	29.2	25.8	22.0	23.5	21.8	19.2	21.6	25.4	27.8	28.8
moy min	21.9	18.1	14.1	15.0	11.5	10.2	13.0	17.8	20.7	21.9
TAROUDANT										
max absolue	43	37	30	30	25	27	33	37	38	44
min absolue	13	12	8	8	6	3	3	10	10	3
moy max	33.4	27.9	23.4	23.2	21.4	21	21.9	26.8	28.9	35.5
moy min	18.8	15.6	11.4	12.4	8.3	7.2	9.2	13.5	17.3	19.3

III- DEROULEMENT DE LA CAMPAGNE 2010/2011:

1- Production :

La production brute a été estimée à 698 300 T, soit une augmentation de 8% par rapport à la campagne précédente (646 300 T).

VARIETES	PRODUCTION (T)		VARIATION %
	2010/2011	2009/2010	
- Clémentine	253 100	218 000	1,16
- Navel	93 100	85 000	1,10
- Maroc Late	161 300	170 000	0,95
- Autres	190 800	173 300	1,10
TOTAL	**698 300**	**646 300**	1,08

2- Exportation :

Les exportations totales ont atteint au 30 Juin 2011 365 629 T contre 345 924 T en 2009/2010, soit une augmentation de 6%. La répartition de ces exportations par variété se présente comme suit:

PRODUITS	(1) 30/06/2011 (T)	(2) 30/06/2010 (T)	(1)/(2) EVOLUTION	TOTAL NATIONAL	% PAR RAPPORT AU NATIONAL
PETITS FRUITS	**242 609**	**232 432**	**104**	**3491 39**	**69**
. Clémentines	139735	138847	101	215209	64,9
. Nour	67496	74626	90	79151	85,3
. Mandarine				84	0,0
. Marisol	0	0		916	0,0
. Nova	5500	4591	120	6514	84,4
. Afourer	21213	10793	197	37931	55,9
. Muska				57	0,0
. Ortanique	8665	3575	242	9277	93,4
. Wilking					
ORANGES	**118 584**	**110 399**	**107**	**173 258**	**68**
. Navel	15594	14029	111	23897	65,3
. Maroc Late	71398	71288	100	98599	72,4
. Salustiana	20465	17094	120	25172	81,3
. W. sanguine	11127	7988	139	25590	43,5
AUTRES	**4436**	**3093**	**143**	**5080**	**87**
. Pomelos	439	383	115	672	65,3
. Citron	3997	2710	147	4408	90,7
TOTAL	**365 629**	**345 924**	106	**527 477**	**69**

IV- PREVISION DE LA PRODUCTION DES AGRUMES 2011/2012:

Suite aux premières prospections effectuées dans les différentes zones de production , il ressort les principaux points suivants :

- Augmentation de la production de :
  - 10 à 20% pour le groupe clémentine ;
  - 10% pour la Navel et la Maroc Late;
  - 5% pour la salustiana ;
- Diminution de la production de :
  - 30% pour la Nour ;
  - 10% pour la Washington Sanguine et l'Ortanique;
- Pour les autres variétés, le rendement à l'hectare est supposé identique à celui de la campagne écoulée

Les prévisions de la production agrumicole seraient de l'ordre de 744 000T contre 698 300 T au cours de la campagne écoulée, soit une augmentation de 7%. Elles sont réparties comme suit :

VARIETES D'AGRUMES	2011/2012			2010/2011			% (1)/ (2)
	SUPERFICIE PRODUCTIVE Ha	RENDEMENT T/Ha	PRODUCTION T (1)	SUPERFICIE PRODUCTIVE Ha	RENDEMENT T/Ha	PRODUCTION T (2)	
Clémentine	10133	29,32	297100	9928	25,49	253100	1,17
Maroc-Late	8499	20,90	177600	8489	19,00	161300	1,10
Navel	4048	25,30	102400	4048	23,00	93100	1,10
Autres	11053	15,11	167000	10953	17,42	190800	0,88
TOTAL	**33733**	**22,06**	**744100**	**33418**	**20,90**	**698300**	**1,07**

La répartition par variété se présente comme suit :

VARIETES D'AGRUMES	2011/2012			2010/2011			% (1)/ (2)
	SUPERFICIE PRODUCTIVE Ha	RENDEMENT T/Ha (3)	PRODUCTION T (1)	SUPERFICIE PRODUCTIVE Ha	RENDEMENT T/Ha (4)	PRODUCTION T (2)	
PETITS FRUITS	**16201**	**24,2**	**392503**	**15848**	**23,6**	**373877**	**1,05**
Clémentine	7756	29,3	227448	7623	25,5	194375	1,17
Nuless	2377	29,3	69701	2305	25,5	58782	1,19
Nour	4224	16,1	68004	4058	23,0	93342	0,73
Afourar	160	14,0	2240	6	10,0	60	37,33
Nova	289	24,3	7031	289	27,0	7813	0,90
Fortune	29	19,0	551	29	19,0	551	1,00
Ortanique	303	27,0	8171	303	30,0	9079	0,90
Autres p. fruits	1063	8,8	9357	1235	8,0	9877	0,95
ORANGES	**16508**	**20,8**	**342657**	**16547**	**19,1**	**316289**	**1**
Maroc-Late	8499	20,9	177631	8489	19,0	161285	1,10
Navel	3133	25,3	79268	3133	23,0	72062	1,10
Navelina	15	25,3	387	15	23,0	352	1,10
Navel Late	161	25,3	4064	161	23,0	3694	1,10
Navel Lane Late	739	25,3	18700	739	23,0	17000	1,10
Salustiana	873	34,7	30248	873	33,0	28808	1,05
W. Sanguine	337	36,0	12139	337	40,0	13488	0,90
Autres Oranges	2751	7,4	20219	2800	7,0	19599	1,03
AUTRES	**1023**	**8,8**	**9006**	**1023**	**8,0**	**8184**	**1,10**
Citron	1008	8,8	8873	1008	8,0	8064	1,10
Autres	15	8,8	132	15	8,0	120	1,10
TOTAL	**33733**	**22,1**	**744165**	**33418**	**20,9**	**698351**	**1,07**

SUPERFICIE ACTUELLE PAR VARIETE

DESIGNATION	SUPERFICIE (Ha) 2009/10 (1)	ARRACHAGE (Ha) 2010/2011 (2)	NOUVELLES PLANTATIONS (Ha) 2010/2011 (3)	SUPERFICIE TOTALE ACTUELLE (Ha) (1) + (3) - (2)	SUPERFICIE PRODUCTIVE (Ha)
PETITS FRUITS	**17565**	**0**	**598**	**18163**	**16201**
Clémentine	8038	0	14	8052	7756
Nuless	2993	0	169	3161	2377
Nour	4440	0	64	4503	4224
Afourar	211	0	152	363	160
Nova	299	0	85	384	289
Fortune	29	0	0	29	29
Ortanique	303	0	0	303	303
Autres petits fruits	1253	0	115	1368	1063
ORANGES	**17059**	**16**	**39**	**17081**	**16508**
Maroc-Late	8763	8	23	8778	8499
Navel	3241	0	15	3257	3133
Navelina	15	0	0	15	15
Navel Late	162	0	0	162	161
Navel Lane Late	766	0	0	766	739
Salustiana	897	8	0	889	873
W. Sanguine	415	0	0	415	337
Autres Oranges	2800	0	0	2800	2751
AUTRES	**1048**	**8**	**0**	**1040**	**1023**
Citron	1033	8	0	1025	1008
Autres	15	0	0	15	15
TOTAL	**35672**	**24**	**637**	**36285**	**33733**