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BIOLOGICAL CONTROL OF FUSARIUM WILT IN DATE PALM (PHOENIX DACTYLIFERA L.) IN THE UNITED ARAB EMIRATES BY ENDOPHYTIC ACTINOBACTERIA

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Under the Supervision of Professor Khaled El-Tarabily

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Declaration of Original Work

I, Aisha Abdalla Darwish Shambeh Alblooshi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Biological Control of Fusarium Wilt in Date Palm (Phoenix dactylifera L.) in the United Arab Emirates by Endophytic Actinobacteria*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Khaled El-Tarabily, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Date palm trees suffers from many various serious diseases, including the sudden decline syndrome (SDS). External symptoms were characterized by whitening on one side of the rachis, progressing from the base to the apex of the leaf until the whole leaf dies; while the internal disease symptoms included reddish roots and highly coloured vascular bundles causing wilting and death of the tree. Although three *Fusarium* spp. (*F. oxysporum, F. proliferatum* and *F. solani*) were isolated from diseased root samples, the fungal pathogen *F. solani* was associated with SDS on date palm in the United Arab Emirates (UAE). *Fusarium* spp. were identified based on their cultural and morphological characteristics. The internal transcribed spacer regions and large subunit of the ribosomal RNA (ITS/LSU rRNA) gene complex of the pathogens was further sequenced. Pathogenicity assays and disease severity indices confirmed the main causal agent of SDS on date palm in the UAE as *F. solani*. In order to control SDS in the UAE using biological control methods, endophytic actinobacteria were isolated from within the roots of date palm in the UAE and were evaluated for their potential to secrete antifungal metabolites and cell-wall degrading enzymes that can inhibit the pathogen growth *in vitro*. The strongest two inhibitory endophytic biocontrol agents that produced cell wall degrading enzymes and lysed the hyphae of *F. solani* (BCA1) or secreted diffusible antifungal metabolites (BCA2) which completely inhibited *F. salani* growth were also tested under greenhouse conditions to study their effects on SDS reduction. The biocontrol agent BCA2 which produced only diffusible antifungal metabolites and without the production of cell wall degrading enzymes was significantly more effective in reducing the incidence and severity of SDS compared to biocontrol agent BCA1 which produced only cell wall degrading enzymes and without the production of diffusible antifungal metabolites. This study demonstrated the superiority of antagonistic endophytic actinobacteria (BCA2) capable of producing diffusible antifungal metabolites compared to antagonistic endophytic actinobacteria (BCA1) capable of producing cell wall degrading enzymes in reducing the severity of SDS on date palm under greenhouse conditions.

Keywords: Date palm, sudden decline syndrome, *Fusarium* wilt, biological control, endophytes, actinobacteria.

Title and Abstract (in Arabic)

المقاومة الحيوية لذبول النخيل في دولة الإمارات العربية المتحدة باستخدام البكتيريا التي تعيش داخل جذور النباتات

الملخص

تصاب أشجار النخيل بأمراض خطيرة للغاية من ضمنها متلازمة الذبول المفاجئ. تتميز الأعراض الخارجية بظهور اللون الابيض على جانب واحد من الورقة ، والذي يتقدم من القاعدة إلى قمة الورقة حتى تموت الورقة بأكملها، بينما تضمنت أعراض المرض الداخلية تلون الجذور بالأحمر وتصبغ الحزم الوعائية؛ مما يؤدي إلى ذبول وموت الشجرة. في هذه الدراسة تم عزل ثلاثة انواع من جنس فيوزاريوم (*F. oxysporum* و *F. proliferatum* و *F. solani*) من عينات جذور نخيل التمر المريضة، وكان الفطر الممرض *F. solani* مرتبطًا بذبول نخيل التمر في دولة الإمارات العربية المتحدة. تم تعريف الفطريات الثلاثة من خلال الخواص المورفولوجية عن طريق تنمية الفطريات على أطباق البيئات الغذائية المختلفة. تم ايضاً استخدام طرق تسلسل المناطق الفاصلة المنسوخة الداخلية والوحدة الفرعية الكبيرة لمركب الجين الريبوزومي RNA (*ITS*\LSU rRNA) لمعرفة مسبب المرض. تؤكد التجارب داخل البيوت البلاستيكية ومؤشرات شدة المرض أن المسبب الرئيسي لمتلازمة ذبول نخيل التمر المفاجئ في دولة الإمارات العربية المتحدة هو *F. solani*.

لدراسة تأثير المقاومة البيولوجية على هذا المرض؛ تم عزل مجموعة من الأكتينوبكتيريا من داخل جذور نخيل التمر في الإمارات العربية المتحدة وتم تقييم قدرتها على إنتاج و إفراز مواد مثبطة لفطر *F. solani* بالإضافة إلى قدرة عزلات اخرى من مجموعة الأكتينوبكتيريا على إفراز إنزيمات محللة للجدران الخلوية و التي بإمكانها أن تحد من نمو مسبب المرض في المختبر و داخل البيوت البلاستيكية. تم اختبار أقوى عزلتين من مجموعة الأكتينوبكتيريا أنتجتا مواد مثبطة لنمو الفطر أو إنزيمات قادرة على تحليل الجدار الخلوي للفطر الممرض في المختبر وفي داخل البيوت البلاستيكية. كانت الأكتينوبكتريا BCA2 و التي أنتجت مواد مثبطة مضادة للفطر الممرض فقط بدون إنتاج إنزيمات تحلل جدار الخلية أكثر فاعلية في الحد من انتشار وشدة ذبول النخيل مقارنة بالاكتينوبكتريا BCA1 والتي قامت بافراز إنزيمات تحلل جدار الخلية فقط بدون إنتاج مواد مثبطة لنمو الفطر. أوضحت هذه الدراسة تفوق الأكتينوبكتريا القادرة على إنتاج

مواد مثبطة لنمو الفطر على الاكتينوبكتريا القادرة على إنتاج إنزيمات تحطم جدران الخلايا في تقليل شدة متلازمة ذبول النخيل المفاجئ في نخيل التمر داخل البيوت البلاستيكية.

مفاهيم البحث الرئيسية: نخيل التمر، متلازمة الذبول المفاجئ، ذبول النخيل، المقاومة الحيوية، الإندوفايت، الأكتينوبكتيريا.

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Finally, I would like to express my appreciation to my friends. Thank you for sharing this beautiful journey with me, the days would not have passed easily without your presence. Thank you for your motivation and your beautiful spirit, I wish you all the happiness in your life.

Dedication

To my beloved parents and family

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List of Abbreviations

ACC	1-Aminocyclopropane-1-Carboxylate
BCA	Biological Control Agent
CFU	Colony Forming Unit
DPI	Days Post Inoculation
FAO	UN Food and Agriculture Organization
Foa	*Fusarium oxysporum* f. sp. *albedinis*
ITS	*Internal transcribed spacer*
LSU	Large Subunit
MAMPs	Microbe Associated Molecular Patterns
PB	Phosphate Buffer
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PGPS	Plant Growth Promoting Streptomyces
SDS	Sudden Decline Syndrome
TEF1-α	*Translational Elongation Factor 1-α*

Chapter 1: Introduction

1.1 Date Palms and Its Distribution

Date palm (*Phoenix dactylifera* L.) is one of the most ancient plants that occupies the world's semi-arid and arid regions (Tang et al., 2013). The oasis region provides a perfect environment for the date palms to grow with sun radiation and enough water (El-Juhany, 2010). It grows in dry and hot climate; tolerating temperature that reaches 50^{o}C and withstanding cold temperature of at least -5^{o}C (Chao & Krueger, 2007), beside its resistance to saline soil and water (Ashraf & Hamidi-Esfahani, 2011).

Date palm cultivation extends between latitudes 15^{o}N and 35^{o}N covering Morocco in the west and India in the east. The records showed that it was cultivated in North Africa and Middle East in more than 5000 years ago. Other records from Mesopotamia in Iraq revealed that the date culture introduced there at the beginning of the 3000 BCE, but the exact place of the origin was not identified as a result of its long history of cultivation and its widespread. Date palm cultivation spread from its original place all over the Arabian Peninsula, North Africa and the Middle East. The spread of Islam made date palms extends to Pakistan and Spain, then the Spaniards introduced it to America (Chao & Krueger, 2007). Recently, dates are cultivated in the Middle East, North Africa, areas of Central and South America, Southern Europe, India and Pakistan with more than 2000 varieties (Tang et al., 2013). The tree can grow, in addition to the previous places, in Australia, Mexico and North America particularly in California, Arizona and Texas (Al-Alawi et al., 2017).

1.2 The Date Palm's Reproductive Biology

Date palm is perennial, diploid (Chao & Krueger, 2007), Angiosperms and monocotyledonous plant that is classified among *Arecaceae* family. The *Arecaceae* family consists of approximately 200 genera and its species exceeds 2500 species. One of those genera are *Phoenix* that have nearby 14 species (including *Phoenix dactylifera* L.) occupying tropical and subtropical areas of south Asia and Africa (Al-Alawi et al., 2017). The name of the date palm (*Phoenix dactylifera* L.) species came from its fruit colour and appearance. *Phoenix* in Greek means purple or red and *dactylifera* was named because the fruit bunch look like fingers (Chao & Krueger, 2007).

Date palm is known as a dioecious tree with male's flowers bearing stamens and female's flowers bearing pistils. The pollination is needed for fruit development, and without it, fruits become parthenocarpic, which mean seedless, does not mature completely and are small in size. Naturally occurring pollination could be derived by winds which needs a huge number of male palms and it could be done manually where one male tree only can pollinate around 25 female date trees. The pollen source has an impact on date fruit quality and the amount of yields (Omar et al., 2014). Moreover, the shape and size of the fruit is determined by the cultivar, culture and environmental factors. Date palm begin fruit production at the age of 5 on average and it reach its maximum productivity in a period of 15 to 20 years. Forty kg of dates is the average fruit harvested per tree per year, but this number can increase to 100 kg with proper management or decrease to 20 kg or less in the case of bad or no management (Chao & Krueger, 2007). Others reported that date palm tree can produce roughly 400 to 600 kg of fruit per annum in different areas, this discrepancy is due to the fact that the

geographic distribution affects physical and chemical characteristics, weight, shape and size of the date fruit (Al-Alawi et al., 2017).

Palm trees can be propagated in three ways: the first way is the vegetative propagation of offshoot, which guarantee the delivery of the heritable traits of the native cultivar. It is considered the most widely used method; where the offshoot grows down on the trunk from the axillary buds and it can be detached after three to five years when it develops roots (Figure 1). The second way is to cultivate seedlings. The seedlings are the result of sexual crosses, so their genetic traits are different from their parents and the fruit amount, quality and the gender of the trees are not guaranteed. The third proliferation way is using Tissue Culture, which gives plants like the ones produced by offshoot methods. Nevertheless, somaclonal variation is a problem that arise from this method where different morphological variations could occur, for example: change in plant development or producing fruits without seeds (Chao & Krueger, 2007).

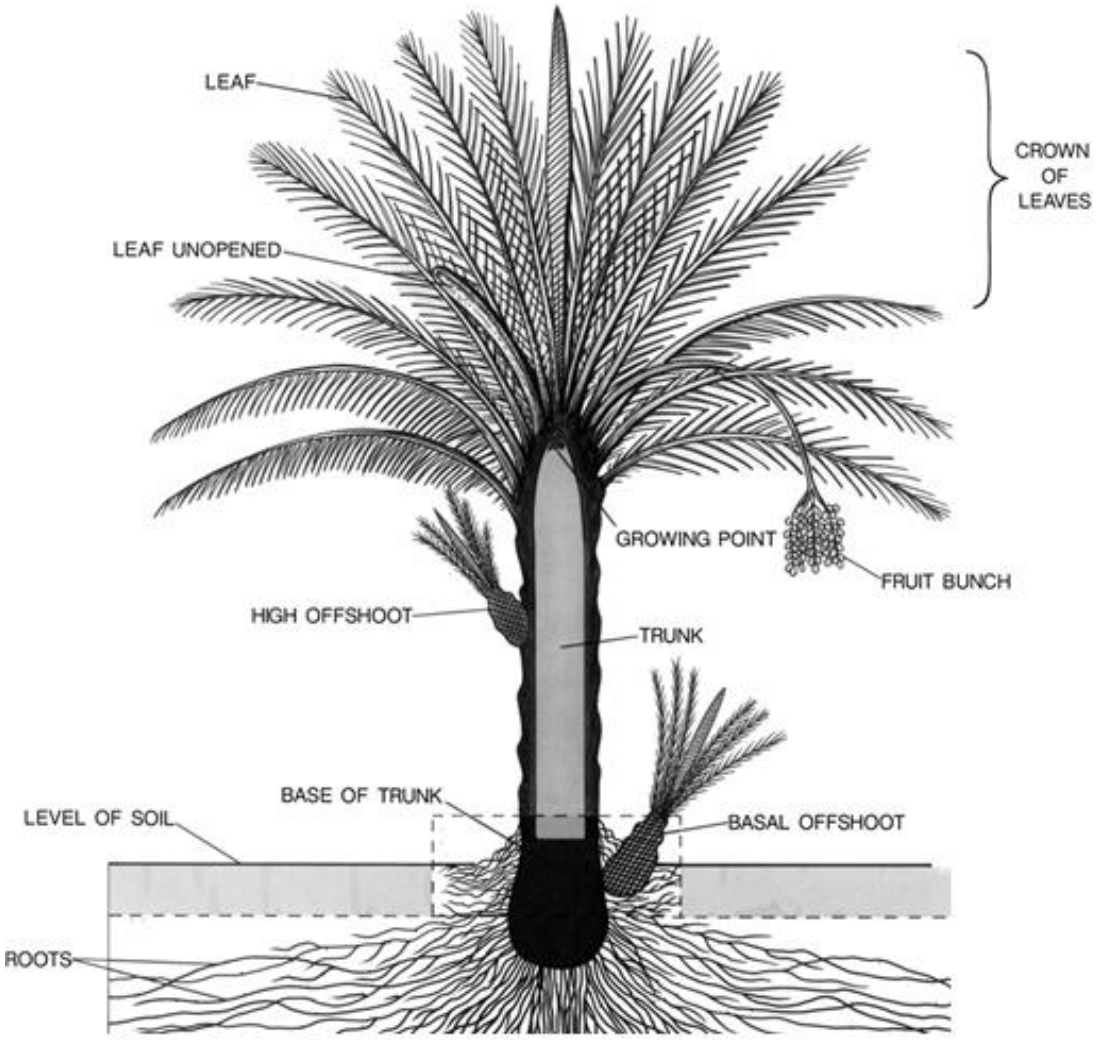


Figure 1: Date palm structure showing the offshoot attached to the parent tree (Chao & Krueger, 2007)

1.3 The Production of Date Palm Fruit

Date palm's productions in countries with oases and others with extreme climate conditions in arid and semiarid environments consider economically important. The worldwide production of date fruit reached 7.62 million tons in 2010; and the production increased since then as the demand increased (El-kinany et al., 2017). According to FAO (UN Food and Agriculture Organization), countries that lead the production of dates are Egypt, Saudi Arabia, Iran, United Arab Emirates, Algeria

(Tang et al., 2013) and Pakistan. Among the countries that produce this fruit crop, Arab countries are leading the world in the production of dates with about 75% of the production according to FAO statistics in 2009 (El-Juhany, 2010).

In the term of date consumption, the Asian countries mainly Saudi Arabia and United Arab Emirates are progressing the world. Africa ranked second in the consumption leaded by Libyan Arab Jamahiriya, Egypt and Algeria (Ashraf & Hamidi-Esfahani, 2011). There are approximately one hundred million palm trees spread around the world on circa 1.3 million hectares, with Asia having the biggest share of the world with 833,351 hectares. Africa comes next having 416,695 hectare with 392, 200 hectares in North Africa (Al-Alawi et al., 2017).

1.4 The Importance of Date Palms and Its Fruit

The date palm (*Phoenix dactylifera* L.) is a valuable tree in the desert and it supports the survival of the desert community. For its short replacement duration, it is recognized as a renewable resource and it can bear harsh environmental conditions like elevated temperatures, drought and salinity. It is considered a habitat for animals and people (El-Juhany, 2010); and it is used in controlling the desertification in countries like United Arab Emirates and other countries. Palm trees are not just a source of food, approximately all the parts of the tree are consumed by people for many purposes, for example, the trunk is used as a wood or as fuel. The trunk's fibres can also be considered a raw material for making ropes, mates and camel saddles. Besides, the palm oil can be used in making soaps and other products. Also, the terminal buds is eatable as the fruit (Chao & Krueger, 2007).

The date fruit provided a food security for people living in the desert, which supported their settling and inhabitation (El-Juhany, 2010). It is a highly energetic food

source for people and livestock as well. It is considered a valuable source of sugar with sugar content equivalent to 88% in some varieties (Hamad et al., 2015); particularly glucose sucrose and fructose. It also contains fibres like cellulose and lignin beside variety of minerals such as calcium, copper, magnesium, manganese, potassium, phosphorus, sodium, zinc, iron and fluorine (Tang et al., 2013). The date palm fruit is rich in vitamin B complex like thiamine (B1), riboflavin (B2), niacin (B3), Pantothenic (B5), Pyridoxine (B6) and folate (B9) (Al-Alawi et al., 2017).

In addition to its nourishing values, date fruits have a medical and pharmaceutical importance in the past and at the present days. Due to its richness in polyphenolic compounds and vitamins, it works as antioxidant and antimutagenic in the bodies of its consumers. Several studies reported that its extract can help in defeating bacteria and fungi (Hamad et al., 2015). In addition, extracts from different parts of the palm tree are used as a mouth wash, cure for kidney problems, astringent in intestinal problems, diarrhea and many other medical uses (El-Juhany, 2010).

1.5 Threats to Date Palms

There are many factors that threat the date palm and influence its production, some of them are biotics while others are abiotic factors. Among the abiotic factors, soil salinity, drought, changes in water quality and soil fertility, climate change and its consequences like desertification and pollution are the main problems that faces the tree. There are other circumstances associated with human activities such as immigration of the people from the rural areas to the cites seeking for jobs (El-Juhany, 2010). This affected the date production in some oasis areas, for instance in Kateef in Saudi Arabia. Additionally, the production reduced in countries like Iraq because of the war, which made the world lose one of its major producers. Moreover, drying and

contamination of wells, mainly in Arabian Peninsula, where the dates are socially and economically important is also an important issue (El-Juhany, 2010).

Moving to the biological factors, the date palm tree can be infected by insects, pests, bacteria or fungi; with the main diseases associated with fungi, each with different organisms and species based on their different geographical areas and climate. Among the pests, red palm weevils (*Rhynchophorus ferrugineus (Olivier)*) is one of the main pest that invade the date palms; and it spreads in the Middle East, South of Europe and North Africa in countries like UAE, Saudi Arabia, Kuwait, Oman, Egypt, Jordon, Iraq, Spain, Greece, Turkey, Italy, France, Qatar, Bahrain and Palestine (El-Juhany, 2010). It attacks 40 palm species around the world; and it is classified as category 1 of the pest that infect the date palm in Middle East by the FAO. This insect feeds on the palm tree causing dryness of the offshoots, tunnelling the palm tissues, drying the leaves and fruits and falling the trunk in severe tissues damage. Red palm weevil incurs date producers a lot of financial losses; in addition to the money paid to eradicate it (Al-Shawaf et al., 2013).

Furthermore, date palms are susceptible to varied fungal diseases such as fruit rot induced by *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*, bending head triggered by *Ceratocystis paradoxa*, Black leaf spot arouses from *Chaetosphaeropsis* sp. and other diseases like Terminal bud rot, Inflorescence rot and Omphalia root rot (Bokhary, 2010). Amongst other pathogenic fungi, *Fusarium* is a significant threat to the date palm, causing several wilt diseases and root rot in different palm species. A disease known as bayoud disease, induced by *Fusarium oxysporum* f. sp. *albedinis* (Foa), has killed millions of palm trees in north Africa, mostly in Algeria and Morocco, putting the date fruit production at risk (Al-Sadi et al., 2012).

1.6 *Fusarium*

Fusarium spp. are widely distributed filamentous fungi that can be found in the soil, air, water and in the organic substrates as it can tolerate different environmental conditions. It can be found in human and animal bodies as an opportunistic pathogen resulting in sickness and even death; although it is greatly identified as soil borne fungi as it exist abundantly in the soil leading to agricultural and economic losses (Tupaki-Sreepurna & Kindo, 2018).

It is correlated to the root of the plant either as saprophyte or parasite. Some *Fusarium* produces several mycotoxins that settle in plants and their different parts like seeds; which allows it to enter the food chain, toxifying humans, animals and crops as well (Okungbowa & Shittu, 2012). Example of such poisonous secondary metabolites that was determined in the soil and water are Zearalenone, T-2 toxin, nivalenol, fusarins and fumonisins, with fumonisins recognized as strong carcinogen affecting humans and animals (Saleh et al., 2017).

1.6.1 *Fusarium* Taxonomy

Because most Fusaria's telomorphic stages are unknown, *Fusarium* taxonomy was based on anamorphic morphological features, including macroconidia size and shape, presence or absence of microconidia and chlamydospores, colony colour and conidiophore structure. The different strains of *Fusarium*, primarily *F. oxysporum* and others, either the pathogenic, saprophytic or the ones that works as a biocontrol agent are indistinguishable based on morphology. However, the pathogenic ones of them are very specific to certain host species or certain cultivars of the same plant species that they invade. This specificity is assigned as *formae speciales* and race, but it does not work for non-pathogenic species (Fravel et al., 2003).

Molecular tools are used for the phylogenetic analysis and identifying pathogenic strains and races in some situations, but the identification of pathogenicity remains largely dependent on bioassays. Fungus *forma specialis* can be determined by pathogenicity testing of the fungus on different species of plants, while race is determined by pathogenicity testing of single species of plants on different cultivars. While bioassays are very useful in pathogenicity testing, it cannot tell that a strain is non-pathogenic. The reason is that there are more than 120 *formae speciales* and races of *Fusarium* have been described, it is unattainable to examine them all against all cultivars. Since it is not possible to use the molecular and the biological method to classify the non-pathogenic strains, so researchers consider that the non-pathogenic strains are the ones that failed to induce disease on a limited number of plant species to which they were inoculated (Fravel et al., 2003).

1.6.2 *Fusarium* Species

Fusarium is a filamentous fungus that belongs to Ascomycetes class and Hypocreaceae family. The *Fusarium* genus includes more than 20 species, with teleomorphic state in some of its species. Its species can be distinguished by its different features that can be seen by eyes or by the microscope like the colony colour, existence or lack of chlamydospores, the macroconidia length and shape and the appearance of microconidia, its number and arrangement (Okungbowa & Shittu, 2012). Several molecular analyses can be used to classify *Fusarium* into species and subspecies such as 28S rRNA gene sequencing for instance. Other identification methods are detecting the presence of protein banding by SDS-PAGE, esterase isozyme electrophoresis used for determining protein banding arrangements and the use of PCR (Polymerase chain reaction) based on rDNA (Okungbowa & Shittu, 2012).

Fusarium is cultured on Sabouraud dextrose agar media forming flat or spreading colonies that has either woolly or cottony texture. The upper side of the colony is white, red, pink, cream, tan, pinkish to orange, cinnamon, yellow, violet, or purple. The underneath side could be colourless, red, purple tan or brown (Okungbowa & Shittu, 2012). Bokhary (2010) used Czapecdox agar (CZA), Potato dextrose agar (PDA) with two percent of yeast extract and Malt extract agar for culturing fungi causing date palm wilting including *Fusarium oxysporum* and *Fusarium solani* (Bokhary, 2010). The reason behind the high diversity distribution of this fungi refers to its ability to grows on numerous surfaces and environments. Also because of its formation of sclerotia under harsh environmental circumstances. The sclerotia is an asexual reproductive structure that arise from mass of hyphae that can stay dormant for a long time and germinate when conditions become favourable. Another mean of the *Fusarium* dispersal is the formation of spores. Three types of spores are formed that are macroconidia, microconidia and chlamydospores (Okungbowa & Shittu, 2012).

1.7 *Fusarium* Wilts

Fusarium wilt is a soil borne fugal disease induced by diverse strains of *Fusarium* that infect valuable crops around the world. *Fusarium* leads to plant wilts by producing mycelia and spores in the host vessels and also as a result of gels, gums and tyloses excreted by the plant defence as a result of its infection; this blocks the xylem, the water conducting vessel, making the plants wilts and eventually dies. Several species of *Fusarium* initiate this disease like *F. eumartii*, *F. oxysporum*, *F. avenaceum*, *F. solani*, *F. sulphureum* and *F. tabacinum*; However, the *F. oxysorum* is the commonest causal of the vascular wilting disease (Okungbowa & Shittu, 2012).

The pathogenic *Fusarium* that is responsible for wilting disease is highly specific organism and it is classified into 120 *formae specials* and races based on their host plant cultivars and the species that it attack (Fravel et al., 2003).

Fusarium species are considerable soil borne pathogen that can lead to multiple plant diseases like vascular wilts, root rots, crown rot, head blight, cankers, pokkan-boeng on sugarcane and bakanae disease of rice. However, not every *Fusarium* strain is pathogenic, many strains have been isolated from the roots of healthy plants and from soil that are suppressive to *Fusarium* wilting disease. In fact, those non-pathogenic *Fusarium* were used as controlling agents against the pathogenic strains by several researchers (Okungbowa & Shittu, 2012).

1.8 Host Plants

Fusarium causes wilting diseases for many economically valuable crops like banana; which is known as panama disease (Tupaki-Sreepurna & Kindo, 2018). It attacks also pea and chickpea, ginseng, mulberry tree (Šišić et al., 2018), maize, rice, asparagus (Abdalla et al., 2000), date palms (Abdalla et al., 2000; Al-Sadi et al., 2012; Maitlo et al., 2014; Saleh et al., 2017), tomato, sweet potato, legumes, cucurbits, cotton, heba (Okungbowa & Shittu, 2012), strawberry (Cha et al., 2016), wheat (Siegel-Hertz et al., 2018), flax and lentil (Merrouche et al., 2017).

1.9 *Fusarium* in Palm Trees

Fusarium wilts is widely dispersed disease that can infect many species of the date palm. The infected tree shows unusual frond death from single side of the tree or from the tree base or the centre of the tree. Also, the infected fronds show discolouration in the vascular bundles. One side of the pinnae and the spines of the

lower fronds dies first, leaving the top of the tree intact till the whole tree dies. If the tree became infected, it can take from two months to couple years until it dies. Using unsterilized tools and transplantation of infected tree are the main ways of transmitting the disease (Okungbowa & Shittu, 2012). The more frequent species that found to cause similar symptoms of wilting diseases are *Fusarium oxysporum, Fusarium monliforme*, *Fusarium proliferatum* and *Fusarium solani* (Maitlo et al., 2014).

Different *formae specials* of *Fusarium* are associated with different species of palm trees. *F. oxysprum* f. sp. *palmarum* invades the queen palm (Syagrus romanzoffiana) and the Mexican fan palm (Washingtonia robusta); while *F. oxysporum* f. sp. *canariasis* is the invader of the canary island date palm (*P.canariensis*). In addition, *F. oxysporum* f. sp. *elaeidis* is the creator of *Fusarium* wilting of (Elaeis guineensis and Elaeis oleifera), an oil palm trees (Saleh et al., 2017).

1.10 *Fusarium* Wilts in Date Palms (*Phoenix dactylifera* L.)

There are several *Fusarium* species that causes different forms of date palm's wilting symptoms. *Fusarium oxysporum* f. sp. *albedinis* is the most serious threat to the date palm in North Africa. In the last 100 years, it destroyed more than 10 million palm trees there. In Morocco, it damages about 75% of the date fruit produced (Dihazi et al., 2012).

In Saudi Arabia, the date palms displayed wilting symptoms similar to bayoud disease symptoms; specifically, in AlQassim and AlMedina AlMonawara. After isolating the fungi from the infected root and leaves, they found that the *F. proliferatum* is the main causal agent, besides *Fusarium oxysporum* and *Fusarium solani;* which were present to a lesser extent than *F. proliferatum* (Abdalla et al., 2000). Beside presence in Saudi Arabia, many surveys showed that *F. proliferatum* is

responsible for wilting diseases in Spain, Canary Islands, Iraq, Saudi Arabia, Iran, USA and Palestine, While *F. solani* was isolated from date trees in Iran, Pakistan, Egypt, Iraq and Oman (Saleh et al., 2017). Another study conducted by Saleh et al. (2017) in order to isolate and identify *Fusarium* pathogens that invade date palms in Saudi Arabia morphologically and molecularly, showed that the main pathogenic *Fusarium* there are *F. proliferatum*, *F. solani*, *F. brachygibbosum*, *F. oxysporum* and *F. verticillioides*. *F. proliferatum* was found in 6 out of 7 studied cites followed by *Fusarium solani* that was found in 5 regions; while other species was represented by one strain (Saleh et al., 2017). Also in Saudi Arabia, Bokhary (2010), isolated 11 distinct fungi causing date palm wilts from seeds of 6 date palm trees varieties. *Fusarium oxysporum* and *Fusarium solani* were the prevailing fungi acquired from all palm types (Bokhary, 2010).

In Pakistan, *Fusarium* wilts (also recognized as the sudden decline disease) was identified as major threat of the date palm orchards, with trees suffering from wilting symptoms similar to bayoud disease. The symptoms are: yellowing of the lowest outer leaves from the centre of the crown, whitening of the pinnae and spines (it take from days to weeks), discolouration of the rachis, yellowing and drying of the leaves, After the leaves dies the whole fronds die until it reaches to the terminal bud which makes the tree dies and also the root and the vascular bundles shows reddish to brownish colour (Maitlo et al., 2014). The dominant fungal agent isolated from different tree parts was *F. solani* according to bioassays (Abul-Soad et al., 2011).

In Oman, people were suffering from likeness wilting symptoms in the date palms. The infected trees displayed weakness in the growth, older leaves turned to yellow then the trees that have high infection dies. A research conducted by (Al-Sadi et al., 2012) covered 111 infected date tree in Oman belonging to 29 cultivars of the

date palm; showed that the *Fusarium solani* is the most frequent agent between the fungi and oomycetes with 27% in 4 regions and 14 cultivars. While *Fusarium brachygibbosum* padwick was recovered only in sharqiya area in only 6 date cultivars (Al-Sadi et al., 2012).

1.11 *Fusarium* Infection Process and Plant Defence Mechanism

The hyphae of the *Fusarium* have a tendency to grow towards plant roots because it is rich in exudates; and they proliferate on its surface before getting into the cortical tissue. The cuts and injuries of the root facilitate the entrance of the *Fusarium* to the cortex; afterward it moves to the vessels and then diffuse in the tree. In the cortex of the root, *Fusarium* can grow in the intercellular space, distinguishing the pits of the cell wall and extending towards it. It produces enzymes like cellulases to destroy the cell walls and to get into the cells. Then it adheres to the vessels. Plant react toward that by producing callose on sides of vascular parenchyma pits and vessel walls and excreting secondary metabolites that are toxic and destructive to the pathogen beside increasing the lignification and phytoalexins production. The two most important factors determine the *Fusarium* success in in entering the vessels and inhabiting the vascular system are the speed at which it can progress in the vascular tissue, avoiding plant defences and the characteristics of both the plant and the fungi. In the vessels, *Fusarium* creates microconidia and hyphae and continue to move in the transpiration stream (Smith, 2007).

Date palm defence system performs several actions when attacked by invading pathogens. One action is the induction of tyloses in vessel cells of date tree infested by a vascular disease. Tyloses are extension outgrow from parenchyma cells that contain phenolic compounds with antifungal activities. When the date palm gets attacked by

fungi like *Fusarium oxysporum*, the tyloses will fall and will diffuse its phenolic compound in the infected vessels. Another action is the induction of pathogenesis-related proteins; proteins that resist the proteolysis and withstand very low pH. In the date palm, chitinases and polygalacturonases were reported as pathogenesis-related proteins. The other action is the accumulation of caffeoylshikimic acid, a major phenolic compound that works as fungal toxin in the palm root (El Modafar, 2010). Two of the suppressive date palms cultivars to Foa was reported to produce caffeoylshikimic acid that is able to supress conidial germination and prohibit the fungal growth. One more action is cell-wall reinforcement by cell wall-bound phenols and lignification. *Fusarium oxysporum* produces cell wall degrading enzymes against palm roots involving polygalacturonases, pectinemethylesterases, and cellulases. The *Fusarium* suppressive cultivars interfere with the fungal hydrolytic enzymes, pectinolytic and cellulolytic, by producing lignin and cell wall-bound phenolic acid (El Modafar, 2010). This also can participate in inhibiting the mycelium growth and the production of hydrolytic enzymes by the Foa. Those compounds are sinapic acids, p-hydroxybenzoic, p-coumaric and ferulic. Both the strength and the rate of the cell wall-bound phenol accumulation and the lignification are the determinants of the date palm resistance to Foa (El Modafar, 2010).

1.12 *Fusarium* Wilts Management and Control

Different methods were applied in order to limit the spread of *Fusarium* wilts in date palms. Some of which are biotic, and others are not; each with different effect and limitations.

1.12.1 Planting Resistant Cultivars

Fusarium is among pathogens that are hard to defeat because it produces conidia and chlamydospores that can survive in the soil for up to 15 years (Merrouche et al., 2017). To limit *Fusarium* wilts dispersion in date palm groves, planting resistance cultivars toward the fungus is something can be done. In north Africa, resistance palm trees to bayoud disease were known for produces poor quality dates. Although Najda cultivar was recently known for resisting Foa and producing high fruit quality, cultivating large oasis areas with one palm genotype will tremendously threat the biodiversity (Dihazi et al., 2012). Also, the susceptible palm trees to Foa in Morocco are economically valuable. Therefore, the resistant genotypes are not a real solution. Likewise, Prophylactic methods are not of interest due to the contamination of several date palm groves and to their non-durable impact (El-kinany et al., 2017). Hence, to tackle *Fusarium* wilt integrated management plans are needed. These approaches must incorporate various substitutions to meet the high quality and ecological conditions (Dihazi et al., 2012). This may include cultural practices such as soil solarization, crop rotation, biological control and others (Merrouche et al., 2017).

1.12.2 Chemical Control

Of the chemical fungicides used to reduce the intensity of various crop wilting, Benzimidazole class was applied covering benomyl, fuberidazole, carbendazim, thiophanate-methyl thiabendazole and thiophanate. Its general mode of action includes cell division and hyphal growth interference, and it were effective against ascomycetes and basidiomycetes (Belgrove, 2008).

In Pakistan, 6 different fungicides were tested against sudden decline disease (*Fusarium* wilt) caused by *Fusarium solani in vivo* and *in vitro*; which are Bavistin

D.F, Topsin-M, Aliette, Ridomil gold, Mancozeb and Copper oxychloride. In *in vitro* experiment, Bavistin D.F was the best fungicide that defeated the *F .solani* at concertation of 150 ppm. Topsin-M and Aliette were the second and third at concentrations 100 and 50 ppm respectively. The same three fungicides were the most effective ones in the *in vivo* experiment; as a result, the infected plant recovered and produced date in its fruiting season (Maitlo et al., 2013).

In Algeria, a contaminated area with Foa was incinerated then the soil was sterilized using chloropicrin fungicide with the addition of methyl bromide. Recently, chloropicrin was prohibited for its hazardous effects and the limitation of its application (Benzohra et al., 2017).

The disadvantages of chemical monitoring that it has negative impact on human's health and the environment (Merrouche et al., 2017). They are also ineffective (Dihazi et al., 2012) and problematic in some cases as it can generate undefeatable fungi that are resistance to the majority of chemicals (Belgrove, 2008).

1.12.3 *Fusarium* Wilts Suppressive Soil

Disease suppressive soils are soils that establish low or no disease; or decrease the established infections resulted from soil-borne pathogen (Cha et al., 2016), in the presence of the climate conditions that support the pathogen progress. Examples of such plant pathogens are bacteria, fungi, oomycetes, nematodes and subterranean insects (Siegel-Hertz et al., 2018). This disease resistance is relied on the existence of microbial communities in plant roots and rhizosphere (Cha et al., 2016). Soil microorganisms are indicators of the soil health because of their function in biogeochemical cycles (nutrients cycles) and in the degradation and mineralization of organic matters (Siegel-Hertz et al., 2018). They interfere with disease causing agent

either by the competition for the nutrients or by direct antagonism (Cha et al., 2016). This interference can be general or specific to certain plant pathogen and crop (Cha et al., 2016; Siegel-Hertz et al., 2018).

Several experiments have been carried out in France where the *Fusarium wilt* caused by *F. oxysporum* is uncommon; to understand the reasons that made the soil there suppressiveness to *Fusarium wilt*. They found that this soil nature referred to the competition between all soil microbes for the nutrients and the competition among the pathogenic *Fusarium* with other harmless strains.

Various factors can aid in increasing the useful microbial communities in the soil; including good soil management which leads to healthy yields (Siegel-Hertz et al., 2018). The suppressive soil studies increased with increasing attention to the need of healthy organic agriculture that control diseases without chemicals and exotic controlling methods (Cha et al., 2016). To remove the supressiveness from the soil, different methods were used like sterilizing the soil, gamma radiation and steam pasteurization. Then, small quantity of natural suppressive soil is added to both earlier disinfected suppressive soil and natural conductive one (Siegel-Hertz et al., 2018).

1.12.4 Applications of Compost

Compost extracts are applied increasingly to overcome crops phytopathogens in recent years. Different reports demonstrated that animal compost have an ability to defeat pathogenic fungi such as *Alternaria alternata*, *Pythium debaryanum*, *Fusarium oxysporum* f. sp. *lycopersici* and *Botrytis cinerea*. This controlling ability is due to the presence of microorganisms in it and chemical compounds like salicylic acid, volatile fatty acids and phenolic compounds. Furthermore, compost can supress the pathogens by antibiosis, parasitism and inducing the host resistance. Aeriation, organic content,

temperature and microbial characteristics are important factors that stimuli the controlling efficiency (El-kinany et al., 2017).

1.12.5 Biological Control Agents of *Fusarium* Wilts

Biological control using antagonistic microorganisms that are able to supress the growth of the plant disease-causing agent is a promising controlling strategy. It depends on finding bacteria and\or fungi with antagonist behaviour against phytopathogens (Dihazi et al., 2012), and also have the ability to improve plant defence mechanisms. Several bacteria were reported as bio-controller of plant pathogens, involving *Fusarium*, such as *Gliocladium*, *Bacillus* ssp., *Pseudomonas* spp., *Trichoderma* spp. and others. Two mechanisms employed by the controlling agents toward the pathogens; which are indirect mechanism via enhancing plant defence and the direct interfering mechanism by the antagonism which include antimicrobial metabolites production, competition or parasitism (El Hassni et al., 2007).

Various studies indicated that actinobacteria are promising monitoring agents against agricultural pathogens, including *Fusarium* species. They are capable of excreting antifungal compounds and large scale of bioactive secondary metabolites. *Streptomyces* spp. is of the actinobacteria that produces high quantity of the antibiotics. Therefore, two bio-fungicides were manufactured from *Streptomyces* species and they are available commercially in order to treat seeds with them against *Fusarium* species; namely Mycostop® and Actinovate® (Merrouche et al., 2017). Actinobacteria, particularly *Frankia* and several *Streptomyces* spp., can colonize the internal plant tissue without causing disease symptoms. Those organisms are identified as endophytic actinobacteria. They have attracted researches attention for their

production of bioactive metabolites that promote plant growth and enhance plant resistance against invading pathogens (Qin et al., 2009).

1.13 Endophytes

Plants and microorganisms interaction has been studied broadly as this interaction affects the food production and plant development (Andreote et al., 2014). The presence of microorganism inside the plant tissues was first labelled as entophytae by the German botanist Heinrich Friedrich Link in 1809 were the term was referring to pathogenic fungi. In the 19th century, it was believed that healthy plants were free of microorganisms. However later, bacteria and fungi were isolated from vegetables were thought to be beneficial for their host, although this idea was not prevalent. Then, the isolation of nitrogen fixing bacteria from nodules of leguminous plants confirmed the existence of the beneficial microbes in plants (Hardoim et al., 2015). Recently, surveys have revealed that most of the microorganisms associated with the plants are nonpathogenic; and those microorganisms plays a major role in improving plant growth and supporting their survival (Andreote et al., 2014).

At present, endophytes are defined as a group of microorganisms (bacteria or fungi) that inhabit healthy plant tissues intracellularly and\or intercellularly without triggering any disease symptoms for their host (Nair & Padmavathy, 2014). They occupy approximately all plant tissues (Passari et al., 2015) and they were isolated from various plant parts like stems, roots, leaf, fruit, buds, seeds, hyaline cells (Gouda et al., 2016), bark, scale primordia, petiole, pine needles and meristem and resin ducts (Nair & Padmavathy, 2014). Its relationship with plants is either obligate or facultative. This relationship is considered as mutualistic in which this association

contributes in the survival and health of the host plant and the microbe (Khare et al., 2018). It also can exhibit antagonism interaction (Nair & Padmavathy, 2014).

Although it was hypothesized that those endosymbionts ascends from rhizosphere or seed microbes, studying their genomes showed that some of them have unique genes that set them apart from rhizosphere microflora and gives them the ability to produce novel compounds beneficial for plants (Khare et al., 2018).

1.13.1 Diversity of Endophytes

Frequent endophytic bacteria and fungi were isolated from about 300000 species of plants (Mefteh et al., 2017).

Endophytic bacteria including actinobacteria and mycoplasma, (Gouda et al., 2016) were isolated from monocots and dicots; extending from woody plants to herbaceous plants (Ryan et al., 2008). Proteobacteria is one of the predominant phyla of the endophytic bacteria including the classes α, β and γ- Proteobacteria. In addition, Firmicutes and Actinobacteria are among the common classes while Bacteroidetes, Planctomycetes, Verrucomicrobia and Acidobacteria classes are less common as endophytes. Moving to genera, *Pseudomonas*, *Bacillus*, *Burkholderia*, *Stenotrophomonas*, *Micrococcus, Pantoea* and *Microbacterium* are widespread among endophytic bacteria (Santoyo et al., 2016). Bacterial endophytes are in charge of producing valuable pharmaceuticals compounds and plant growth promoters (Nair & Padmavathy, 2014).

On the other hand, endophytic fungi are grouped into clavicipitaceous and the non-clavicipitaceous based on the phylogeny (Gouda et al., 2016), host plants and their functions in the environment. Clavicipitaceous endophytes usually colonize grasses; specifically, in the shoot and rhizome tissues and it can decrease the herbivory by

producing toxins for animals. Whereas the non-clavicipitaceous is a highly diverse group that can colonize symptomatic tissues of nonvascular plants, ferns and allies, conifers, and angiosperms; specifically, it can inhibit shoot, root and rhizome. It plays an important role in supporting the plant growth and development by helping the plant to adapt to biotic and abiotic stresses and by the improvement of the nutrient uptake (Rodriguez et al., 2009).

There are several circumstances that impact the endophytic communities in the plants such as plant health, plant growth stage, plant nutritional state and the nature of soil and its circumstances like its temperature, PH and moisture (Santoyo et al., 2016). Commonly, bacterial endophytes improve the plant growth by secreting growth hormones while endophytic fungi participate in phytoremediation, biodegradation and nutrient cycling (Nair & Padmavathy, 2014).

1.13.2 Colonization of the Endosphere

The rhizosphere zone is considered the main entrance for endophytes to get into plants; in spite of the stiff competition from phytopathogens and other microorganisms for nutrients (Santoyo et al., 2016). Root exudates are rich in bioactive molecules which selectively attracts endophytes and mutualistic microorganisms. Research regarding this showed that plants are able to know if the invading microbe is symbionts or not via nutrient monitoring. Exudates produced by plants includes amino acids, organic acids, sugar, phenolic compounds and other bioactive secondary metabolites (Khare et al., 2018). Some of those exudates are effective antimicrobial; which give a priority for the organisms that can produce specific detoxifying enzymes to inhabit the internal plant tissues (Compant et al., 2005).

Various techniques utilize by endophytes to enter the roots from cracks, wounds, lenticels or root hair (Santoyo et al., 2016). One strategy in order to pass plants defense, is to avoid the cognizance by the plant immune system. Microbe structures and molecules that are produced by the pathogenic and nonpathogenic microorganisms are recognized by the plant's pattern recognition receptors (PRRs) in the cell surface, which is the frontline of defense in the plant. This recognition is known as microbe associated molecular patterns (MAMPs). Among the identified MAMPs, are chitin, Beta-Glycan (GE), xylanase (EIX), peptidoglycan (PGN) and flagellin (Newman et al., 2013). Example of escaping mechanisms is the production of chitin deacetylases by endophytic fungi which deacetylate chitosan oligomers which is not recognized by plant receptors. Also, endophytes can protect themselves from reactive oxygen species by producing enzymes like catalases (CatA), peroxidases (POD), superoxide dismutases (SOD) and glutathione-S-transferases (GSTs). In addition to that, microbes can undergoes reduction in their genome, like the case of *Verrucomicrobia;* an endophytic bacteria showed a reduction in its genome size to the half compared to soil bacteria as a way of adaptation (Khare et al., 2018).

1.13.3 The Roles of the Endophytes

The symbiotic relationship between the endophytes and their host plant is crucial for the survival of both parties. While the plants afford the endophytes with nutrients, endophytes provides the plant with metabolites with antimicrobials, anticarcinogens, immunosuppressants, antioxidant (Martinez-Klimova et al., 2017) and insecticidal effectiveness. It is believed that the habitation of the plants by endophytes can minimize the infection by bacteria, viruses, fungi and the damage resulted from insects and nematodes (Ryan et al., 2008). There are two mechanisms in

which the endophytes can defend the plant against phytopathogens; the direct mechanism by releasing metabolites with antagonisms or lysing effects and indirectly by inducing host defense or promoting plant growth. Endophytes are producing antibiotics or lytic enzymes in order to avoid colonization of plant by insect, nematodes and phytopathogens (Alvin et al., 2014). Example of this is the inhibition of spore germination and germ tube elongation in Botrytis cinerea by the chitinase produced by *S. plymuthica* C48. Also, it has been proved that chitinase and laminarinase produced by *Pseudomonas stutzeri* can lyse mycelia of *F. solani*. In addition, endophytes are able to detoxify and degrade the pathogens virulence factors. For instance, strains of *B.cepacia* and *Ralstonia solanacearum* can hydrolyze the fusaric acid which is a phytotoxin excreted by *Fusarium* species (Compant et al., 2005). Likewise, endophytes can boost the plant growth by producing phytohormones, improving iron accessibility by the production of siderophores, fixing nitrogen, phosphate solubilization, producing volatile organic compounds (VOCs) and by manufacturing secondary metabolites that boost the plant growth (Liu et al., 2016).

1.14 Structures and Ecology of Actinobacteria

Actinobacteria are filamentous gram positive bacteria with high Guanine - Cytosine content in its DNA (Chaudhary et al., 2013). Its cell wall made of peptidoglycan and it produces mycelium; besides, many of them reproduce asexually via spores. Great majority of them are aerobic and they are widely distributed were they can inhibit aquatic environment, soil, plants and gastrointestinal tract. Also, they can exist as animal or plant pathogens.

Actinobacteria are found abundantly in the soil with preference for neutral to alkaline soil; and the one that is rich in organic matters and have moderate

temperatures where they live as a chemoautotroph and mesophiles. Beside the pH, temperature and nutrients (Barka et al., 2016), the density of actinobacteria can also influenced by the soil moisture, salinity, metal amount and biocides (Sharma & Vinayak, 2014).

Actinobacteria are among the most prevalent endophytes isolated from healthy surface sterilized tissues. Endophytic actinobacteria isolated from medicinal plants has the potential to produce several secondary metabolites and novel chemical compounds that are used in agricultural and pharmaceutical field because of its antitumor and antibiotics activities; besides its ability to produce plant growth hormones (Passari et al., 2015). It is known as the source of the earthy odor after rain because of its secretion of geosmin, a volatile organic compound (Sharma & Vinayak, 2014).

1.15 Isolation and Cultivation of Endophytic Actinobacteria

Endophytic actinobacteria were isolated from various plants including tomato, neem, banana, wheat and snakevine (Gangwar et al., 2014). According to Bergey's Manual of Systematic Bacteriology, actinobacteria is one of the 18 main lineages of the bacterial domain of the largest taxonomic unit, which involves 5 subclasses, 6 orders and 14 suborders. One of the widely studied genera of actinomycetes is Streptomyces (Chaurasia et al., 2018) of the family Streptomycetaceae (Sharma & Vinayak, 2014), since it is easily isolated, whereas the non-streptomyces was difficult to isolate and thought to be rare. Later with the development of specialized media, it appeared that the non-streptomyces are widespread as *Streptomyces*. Actinobacteria are known as bio-degrader of variety of recalcitrant compounds like herbicides and pesticides; it works also as biological pesticide and herbicide (Chaurasia et al., 2018).

Endophytic actinobacteria are isolated using surface disinfesting of plant organs by exposing them to ethyl alcohol and sodium hypochlorite. Then, the sterile organs are rinsed and triturated in a buffer with disinfected mortar and pestle under sterile conditions. After that, the slurry is filtered through a sterile cotton cloth, and serially diluted in buffer and cultivated on a selective agar medium (El-Tarabily & Sivasithamparam, 2006).

There are many factors that influence the isolation of endophytic actinobacteria, like the host and plant species, type and age of the plant tissue, sampling season, geographical distribution, tissue sterility and the culture media. Different culture media have been intended for culturing endophytic actinobacteria such as soybean, starch casein, starch casein nitrate (SCNA), chitin-vitamin B, humic acid vitamin B (HV), yeast extract casamino acid (YECA), modified Gausse and glycine–glycerol. The approaches that are currently used in classifying actinobacteria are morphology, physiology and molecular techniques, particularly 16S rRNA sequencing (Golinska et al., 2015).

1.16 Role of Actinobacteria in Controlling Phytopathogens

The emergence of new diseases and the expansion of pathogens resistance to drugs have led to the search for novel bioactive compounds and secondary metabolites. Endophytic actinobacteria have attracted people’s attention in their search for bioactive compounds against plant pathogens since they can produce antimicrobials and extracellular enzymes that protects the plants from environmental stresses; beside their production of plant growth promoters like siderophores and phytohormones (Gangwar et al., 2014).

It has been proved that endophytic actinomycetes can protect the plants from different soil borne phytopathogens such as *Rhizoctonia solani*, *Verticillium dahlia*, *Plectosporium tabacinum* and *Fusarium oxysporum* (El-Tarabily et al., 2009). Endophytic actinobacteria have different characters that enable them to protect the plants from the pathogenic agents and to promote plant growth, including those listed below:

1.16.1 Production of Antimicrobial Metabolites

Antimicrobial metabolites secreted by actinobacteria, mostly from Streptomyces genus, can limit the growth of phytopathogens and change microbial distribution in the rhizosphere (Olanrewaju & Babalola, 2019). Plants in turn are a niche for Streptomyces and it promote their growth via exudates production which support their mutualistic association. Various studies confirmed the effectiveness of those agents in controlling fugus plant pathogens like *Rhizoctonia solani*, *Pythium* spp., *Fusarium* spp., and *Phytophthora* spp.(Vurukonda et al., 2018), besides other pathogenic agents like bacteria and viruses.

6-prenylindole is an antifungal compound isolated from *Streptomyces* sp. TP-A0595; that is active against *Fusarium oxysporum*. 6-Prenylindole is firstly isolated from liverwort (*Hepaticae*) plants. Nonetheless, novel antimicrobial metabolites have been isolated from endophytic actinobacteria such as munumbicins A-D, celastramycins A-B, kakadumycins and demethylnovobiocins (Qin et al., 2011).

The problem that may arise is that those pathogens can develop resistance to selected antimicrobial compounds after exposure to high concentrations for a long time. Although there is no definite proof for resistance toward those compounds released by plant growth promoting *streptomyces* spp. (PGPS), antimicrobial

resistance gene can transfer horizontally and laterally among the rhizosphere microbes. Despite that fact, PGPS has other controlling modes of action like production of volatile compounds, enzymes and the production of different antimicrobial compounds by different PGPS. Researches are suggesting the use of biocontrol microorganisms instead of using their products, since the direct implication of antibiotics increases the pathogens resistance toward them; and also to gain advantages from other compounds released by PGPS such plant growth regulators like auxins, cytokinins and gibberellins (Olanrewaju & Babalola, 2019).

1.16.2 Hyperparasitism

Actinobacteria have the ability to eliminate and destroy soil-borne fungal microbes by hyperparasitism. Indeed, it has been reported that hyperparasitism and antimicrobial metabolites secretion can work together to destroy phytopathogens. Sabaou et al. (1983) described hyphal lysis of fungal pathogen *Fusarium oxysporum* f. sp. *albedinis* by a strain of *Nocardiopsis dassonvillei* by antibiotic and mycolytic secretion. Another research conducted by Upadhyay and Rai (1987), reported that a strain of *Micromonospora globosa* can break through the hyphae of *Fusarium udum in vitro* causing hyphal lysis, granulation and cytoplasm coagulation (Upadhyay & Rai, 1987). Actinobacteria can also parasitize the mycelium and the dormant oospores of oomycetes. Example of *in vitro* oospores parasitism is the parasitize of *P. megasperma* f. sp. *glycinea* and *Pythium* spp. By *Actinoplanes* spp., while the oospores of *Phytophthora megasperma* var. *sojae*, *Phytophthora cactorum*, *Pythium* sp., and *Aphanomyces euteiches* have been parasitized in the soil by *A. missouriensis* (El-Tarabily & Sivasithamparam, 2006).

1.16.3 Production of Lytic Enzymes

Actinobacteria are able to produce lytic enzymes with capability to breakdown complex nutrients in the soil, which will result in increasing soil fertility, enhancing nutrient cycles and improving the plant growth. Those enzymes include amylase, chitinase, cellulase, invertase, lipase, keratinase, peroxidase, pectinase, protease, phytase, and xylanase. Of those enzymes, chitinase secreted by endophytic actinobacteria, mostly from streptomycetes, recommend its agents as vital biological controlling organism against fungi. Since the cell wall of fungi is made of chitin (Vurukonda et al., 2018), which is a polysaccharide compound, endophytic actinobacteria can antagonize the fungi via producing chitinase that can degrade its cell wall. It has been reported that an endophytic actinobacteria called *Actinoplanes missouriensis* reduces conidial germination and cause hyphal lysis for fungal pathogens via chitinase production (Golinska et al., 2015).

1.16.4 Siderophores Production

Iron is an important mineral for living creatures including microorganisms and plants. Its concentration in the soil is low due to its low dissolvability, which increase the competition for it. Siderophores are iron-binding proteins produced by microorganisms to capture iron from the soil. This feature is possessed by Streptomyces and it uses it to inhibit the phytopathogens by competing with them for iron (Amaresan et al., 2018). Actinobacteria isolated from *Achillea fragrantissima* reveled notable repression to plant pathogens by producing siderophores (Golinska et al., 2015). Also, bacteria from pseudomonas group with the help of non-pathogenic *F. oxysporum* produces iron-chelating siderophores and antifungal phenazines to over compete with the pathogenic *Fusarium* for the iron (Siegel-Hertz et al., 2018).

1.16.5 ACC Deaminase Production

Ethylene is a hormone that regulates plant growth and development processes at low concentration and responses to biotic and abiotic stresses. Elevated levels of ethylene can suppress the plant growth processes such as root elongation. Bacteria that promote plant growth produces ACC (1-aminocyclopropane-1-carboxylate) deaminase, an enzyme that break the ethylene precursor ACC (Glick, 2005) into ammonia and 2-oxobutanoate which alleviate the stress level (Hardoim et al., 2015).

1.16.6 Induced Systemic Resistance (ISR)

Endophytes was reported to induce its host defense when attacked by pathogens. The plant reacts by rising the lignin of the cell wall, production of defense enzymes and by induction of pathogenesis-related (PR) protein (Dihazi et al., 2012). *Streptomyces* spp. were reported as inducer of the plant defense by colonizing the root and stimulating the defense against *F. oxysporum*, *E. carotovora*, *Botrytis cinerea* and *Heterobasidion abietinum* (Amaresan et al., 2018).

1.16.7 Production of Phytohormones

Since actinobacteria are active root inhibitors, they are considered as biofertilizers for their nitrogen fixation, siderophores production and reducing plant tension by ACC deaminase secretion (Olanrewaju & Babalola, 2019). In the same way, actinobacteria are identified as plant growth promoters as they can excrete plants hormones such as auxins, gibberellins, abscisic acid and cytokinins. Those hormones play an effective role in regulating the cell development and physiological operations. Indole-3-acetic acid (IAA), of the auxin class, manufactured by actinobacteria was proved to enhance plant growth through improving root elongation, seed germination

and root dry weight on various crops like rice, wheat, tomato, beans and chili by different species like *S. kunmingenesis, S. nobilis, S. rimosus* and *S. globisporus* (Amaresan et al., 2018).

1.16.8 Mitigation of Plant Abiotic Stress

In addition to their contribution as nutrient suppliers and growth promoters, endophytes can also alleviate diverse plant stresses such as herbicides, heavy metals, salinity, dryness and reactive oxygen species. They are able to implement such tolerance in their host by expressing anti-stress genes, giving instructions for synthesising scavenger, antioxidants and secondary metabolite that sequestrate, immobilize and reduce stresses. Endophytic *Pseudomonas punonensis* of strain D1-6 was reported to prevent the accumulation of the herbicide 2, 4-dichlorophenoxyacetic acid in the plant tissue; as it contains metabolizing and resistance genes (Khare et al., 2018). Likewise, siderophores forming *Streptomyces* spp. were reported to reduce metal toxicity by accumulating metal on its cell wall. Other *Streptomyces* spp. were reported to adapt to water shortage and salinity; like *Streptomyces olivaceus, Streptomyces pada* and *Streptomyces niveus* (Amaresan et al., 2018).

1.17 The Aims of the Thesis

The main aim of the current thesis was to control sudden decline disease of the date palms in the UAE. This was achieved by:

1. Identifying the main causal agent of the sudden decline of the date palm in the UAE using cultural, morphological characteristics and molecular identification tools.

2. Assessing the pathogenicity of *Fusarium* spp., extracted from date palm trees in UAE on palm grasses.
3. Testing the efficiency of antagonistic actinobacteria isolated from healthy date palm roots in inhibiting the development of *Fusarium* wilt fungi *in vitro* and *in vivo.*

Chapter 2: Methods

2.1 Isolation, Purification, and Culture Maintenance

Fusarium spp. isolated from the roots of surface sterilized symptomatic date palm trees obtained from Al Wagan area in Al Ain city (Abu Dhabi Emirate; Latitude/Longitude: 24.19/55.76) were studied in this research. No specific permissions were required for the location. Sections were made of the diseased tree roots and the pathogens were isolated. The isolated fungi were maintained on PDA (Lab M Limited, Lancashire, UK) plates supplemented with 25 mg/L penicillin-streptomycin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and under growth conditions at pH 6.0 and temperature 25°C. After seven days of incubation, the mycelia growing from the plated tissues were sub-cultured on fresh PDA and lastly purified by using hyphal-tip isolation technique (Kirsop & Doyle, 1991). Later, sub-culturing on fresh slants was done at biweekly intervals to maintain the stock cultures and preserved at 25°C. Mycelia and conidia were observed using Nikon-Eclipse 50i light microscope (Nikon Instruments Inc., NY, USA) to characterize different fungal structures. The three *Fusarium* cultures obtained from the disease roots were sent to DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) for the full identification. The cultures of the identified *Fusarium* spp.: namely, *F. oxysporum* f. sp. *cumini* (Prasad & Patel), *F. proliferatum* (Matsushima) Nirenberg and *F. solani* (Martius) Saccardo (Patel et al., 1957; Nirenberg, 1976; Saccardo, 1881), has been deposited in Leibniz-Institute DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) under the collection numbers DSM 106834, DSM 106835, and DSM 106836, respectively. The strongest and virulent pathogenic isolate *F. solani* was used

in the present thesis as indicated in (Alwahshi et al., 2019). The pathogenicity test carried out by AlWahshi et al. (2019) indicated that the most severe pathogenic isolate was *F. solani.*

2.2 DNA Isolation, PCR, and Sequencing

DNA of the pathogen isolated from infected root tissues was extracted from mycelium cultured for 14 days on PDA plates at 25°C. DNA extractions were performed using the plant/fungi DNA isolation kit (Norgen Biotek Corp., Thorold, Canada). PCR reactions contained reaction buffer, 2.2 mM $MgCl_2$, 200 µM of each dNTP, 2.5 unit of Taq DNA polymerase, a 30-ng DNA template and 50 pmol of each primer, making to a final volume of 50 µL. PCR was set for 32 cycles; each cycle consisted of: 94°C for 1 min; 58°C for 1 min; 72°C for 1 min. For the three *Fusarium* spp., PCR amplified target regions of *internal transcribed spacer* (*ITS*) using ITS1 and ITS4 primers (White et al., 1990), *28S rDNA* using LRoR and LR5 (Schoch et al., 2012), *translational elongation factor* 1-α (*TEF1-α*) using EF1-728F and EF1-986R (Carbone & Kohn, 1999), and *β-tubulin* using Bt1a and Bt1b (Orbach et al., 1986). In addition, *pFalb11* and *pFalb28* belonging to *F. oxysporum* f. sp. *albedinis* were also amplified. All primer sequence sets can be found in Table1.

Table 1: List of PCR primers (sequence 5’ to 3’) used in this study

Description	Left primer sequence	Right primer sequence
ITS	ITS1: TCCGTAGGTGAACCTGCGG	ITS4: TCCTCCGCTTATTGATATGC
28S rDNA	LRoR: ACCCGCTGACTTAAGC	LR5: TCCTGAGGGAAACTTCG
TEF1-α	EF1-728F: TCATCGCAAGTCGAGAAGGT	EF1-986R: ACTTGAAGGAACCCTTACCG
β-tubulin	Bt1a: TTCCCCCGTCTCCACTTCTTCATG	Bt1b: GACGAGATCGTTCATGTTGAACTC
ITS/LSU	*ITS/LSU*F: TACGCCGCATCCTTGCCGAG	*ITS/LSU*R: TTCCGTAGGTGAACCTGCGG
pFalb11	FOA1: CAGTTTATTAGAAATGCCGCC	BIO3: GGCGATCTTGATTGTATTGTGGTG
pFalb28	FOA28: ATCCCCGTAAAGCCCTGAAGC	TL3: GGTCGTCCGCAGAGTATACCGGC

2.3 Phylogenetic Analysis

For the analysis of the phylogenetic placement of the fungal isolate the sequences of *ITS*/*LSU* rDNA and *TEF1-α* genes were used as single gene set and concatenated two-gene set, *ITS*/*TEF1-α*. The obtained *ITS/LSU* (accession numbers MH055398, MH055399, and MH055400) *TEF1-α* (accession numbers MH087478, MH087479, and MH087480) sequences were deposited in GenBank for *F. oxysporum*, *F. proliferatum*, and *F. solani*, respectively, and were further combined for constructing the phylogenetic tree against other *Fusarium* spp. database managed by NCBI.

The *ITS*/*TEF1-a* sequences were aligned with sequences retrieved from GenBank, representing isolates that belong to about 30 species of the genus *Fusarium* (El Hassni et al., 2007; Saeed et al., 2017). All sequences were compared and aligned and ML analyses were performed for estimation of the phylogenetic tree (Tamura et al., 2013). Phylogenetic trees were constructed and validated with a statistical support of the branches with 100 bootstrap resamples. The isolates used in the dendrogram

were: *F. proliferatum, F. conentricum, F. verticillioides, F. pseudocircinatum, F. nygamai, F. acutatum, F. udum, F. sacchari, F. oxysporum, F. foetens, F. commune, F. redolens, F. lacertarum, F. meridionale, F. graminearum, F. austroamericanum, F. culmorum, F. sporotrichioides, F. brachygibbosum, F. concolor, F. lateritium, F. solani,* other *Fusarium* spp. and Nectriaceae spp.

2.4 Disease Assays and Pathogenicity Tests

Fungal isolates grown in PDA plates as described above were prepared for conidial suspension for inoculation purposes. The inoculums were prepared by adding 10 mL of sterile distilled water to the 14-day-old fungal culture and scrapped by sterilized scraper. The harvested conidial solution was poured into a 50 mL Falcon tube after filtering the solution twice through sterile Miracloth. The conidial concentration was determined using hemocytometer (Agar Scientific Limited, UK) and adjusted to 1×10^6 conidia/mL according to (Elhassan, 2016).

Pathogenicity tests were conducted using Koch's postulates to confirm the *Fusarium* spp. as the causal agent of SDS of date palm orchard. Six-month-old date palm seedlings (cv. Barhi), obtained from the Date Palm Development Research Unit/UAEU (DPDRU) and showing no disease symptoms, were used. The *Fusarium* spp. used were the representative of isolates of *F. oxysporum* DSM 106834, *F. proliferatum* DSM 106835, and *F. solani* DSM 106836, which were successfully isolated and identified from SDS of date palm orchard. Seedlings (n = 12) were inoculated with 10 mL of inoculum on roots that were separately dipped for one hour in the inoculum suspension of each *Fusarium* spp. or altogether. The control plants were dipped in 10 mL of sterile distilled water. The inoculated seedlings were covered with transparent polyethylene sheet for 72 h. Seedlings in 1.5-L plastic pots containing

sterilized soil mixture (3:2:1 *v*/*v* ratio of top soil–peatmoss–sand) were grown randomly on the bench in a greenhouse with temperature of 25 °C and 60% RH, and watered two times weekly. Disease severity index on inoculated seedlings was recorded for SDS symptoms at 10 and 35 dpi (days post inoculation) using a scale of 0–5: 0 = no apparent symptoms, 1 = 1–10% necrotic or white area in leaves or rotting in roots, 2 = 11–25%, 3 = 26–50%, 4 = 51–75%, and 5 = 76–100% (Molan et al., 2004). All experiments were independently repeated three times with similar results.

To satisfy Koch's postulates, pieces of inoculated root tissues were removed from seedlings showing diseases symptoms at 35 dpi, surface sterilized with 70% ethanol and plated on PDA. Plates were incubated at 25 ± 2 °C and the subsequent growth was recorded.

2.5 Isolation of Endophytic Actinobacteria from Surface-Disinfested Date Palm Roots

Endophytic actinobacteria were isolated from the roots of one years old date palms. The roots were cut off from the stems and then were washed in the tap water for one hour to avoid surface contamination. The weight of the root samples were recorded then the roots were submerged for 10 minutes in sterile phosphate-buffered saline solution (PBS), adjusted to pH 7.0, to equilibrate osmotic pressure and to prevent passive diffusion of the chemical used below to go inside the roots (Rennie et al., 1982).

Sterilization process started by subjecting the roots to propylene oxide vapour for 25 minutes (Sardi et al., 1992) followed by submerging them in 70% of ethyl alcohol for 4 minutes and then they were soaked in 1.05% solution of commercial

bleach with hand mixing for 5 minutes. After that, the roots were rinsed in sterile phosphate buffer (PB) ten times with 5 minutes for each (Hallmann et al., 1997).

To assess the sterilisation process and to make sure that there are no microorganisms entered to the root from the surface during the previous step, roots impressions were taken for these tests (Sturz et al., 1998) and 0.2 ml from the final rinse was plated out on petri dishes of tryptic soy agar (TSA) (Lab M Limited, Lancashire, UK), potato dextrose agar (Lab M Limited, Lancashire, UK) and yeast-malt-peptone-dextrose agar (YMPDA) (Wickerham, 1951) adjusted with 250 µg ml^{-1} chloramphenicol (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). After 6 days of incubation, the absence of the microbial growth was an indication of sterility and the isolated yeasts had been considered endophytic.

Under aseptic conditions, roots were macerated in 100 ml of PB using a sterilized blender and then shaken for 1 hour using a wrist-action shaker. The macerate solution was filtered by using sterile filter papers and it was then serially diluted in PB to 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} (Hallmann et al., 1997). To count the numbers of endophytic actinobacteria, aliquots (0.2 ml) were spread on starch nitrate agar using a sterile plastic spreaders and the plates were dried in a laminar flow-cabinet for 10 minutes. Plates were incubated in the dark at 25°C for 7 days. Three plate per dilution were prepared for each root sample and the total numbers of endophytic bacteria was expressed as $\log_{10}$ colony forming units (CFU) g^{-1} fresh root weight (Hallmann et al., 1997). All endophytic actinobacterial isolates were cultured on oatmeal agar plates (ISP medium 3) supplemented with 0.1% yeast extract (Küster, 1959). Actinobacterial colonies were distinguished based on morphological characteristics and the formation of aerial and substrate mycelia as recommended by Cross (1989). The cells of all

isolates from four plates were stored at - 80°C in 20% glycerol as a cryo-protecting solution as suggested by Wellington and Williams (1978).

2.6 Screening Actinobacterial Isolates for the Production of Diffusible Antifungal Metabolites using the Cut Plate and the Cup Plate Methods

All endophytic actinobacterial isolates were screened based on their ability to produce diffusible antifungal metabolites active against *F. solani* using the cut-plug method as described by Pridham et al. (1957). The actinobacterial isolates were inoculated onto fish meal extract agar plates (El-Tarabily et al., 1997) and incubated for 7 days at 28°C in dark. *F. solani* was cultivated in PDA slants plates at 28°C until sporulation. After full sporulation, the slant was flooded with 50 mM phosphate buffer (pH 6.8) (Saeed et al., 2017b). Spores and fragments of the mycelium were blended at 4,000 rpm for 20 minutes and the supernatants resulted were diluted in PDA plates. The inoculum contained approximately 10^8 CFU ml^{-1} of PDA-seeded plates and the controlled one contained agar plugs without inoculation. The actinobacterial plugs were detached using 11 mm cork-borer and transferred from the fish meal extract agar onto PDA plates seeded with *F. solani* kept in dark at 28°C for 5 days. Five actinobacterial replicates were made and the diameters of the inhibition zones were recorded. The most effective antifungal metabolite-producing isolate exhibiting the largest inhibition zones were selected for further experiments; while isolates with low diameters were excluded.

The selected strongest antagonistic actinobacterial isolates were additionally examined for their ability to secrete diffusible antifungal metabolites active against *F. solani* using the cup plate technique (Bacharach & Cuthbertson, 1948). Inocula for the

preparation of the *F. solani*-seeded PDA plates were prepared, as explained above, for the cut-plug method. Five plates were used for each actinobacterial isolate.

2.7 Screening Actinobacterial Isolates for the Production of Cell Wall Degrading Enzymes

All endophytic isolates were additionally examined for their tendency to produce clearing zones on *F. solani* mycelial fragment agar as an indicator of preliminary production of cell wall degrading enzymes according to Valois et al. (1996). Likewise, they were examined for their ability to produce chitinase enzyme in colloidal chitin media. All isolates were cultivated on colloidal chitin agar plates and incubated for 7 days in dark at 28°C (Gupta et al., 1995). Five plates were used for each isolate. Isolates producing large clearing zone around the colony, with diameter greater than 30 mm, were correspond to high chitinase activities while the isolates with small diameter (< 30 mm) indicated low chitinase activity. The most effective isolates were further tested on the mycelial fragment agar similarly and the promising isolates have been selected.

2.8 Effect of Crude Enzyme Preparations on Living *F. solani* Hyphae

For crude enzyme preparation, 50 ml of minimal synthetic medium were poured in Erlenmeyer flasks (Tweddell et al., 1994) containing 2 mg ml^{-1} of colloidal chitin. Flasks containing the colloidal chitin substrate were inoculated with 2 ml of a 20% glycerol suspension of each biological control agent (BCA) (10^8 CFU ml^{-1}), incubated on a rotary shaker for 7 days at 250 rpm, and centrifuged at 12,000 × g for 30 minutes. The supernatant was filtered using 0.22 μm Millipore membranes

(Millipore Corporation, MA, USA) and used as crude enzymes source (El-Tarabily et al., 2003).

The effect of the crude culture of the two tested biological control agents on the morphology of *F. solani* hyphae was assessed (Sneh, 1981) by examining the impact at 100X with a light microscope (Olympus BH-2, Olympus Optical Co., Ltd, Tokyo, Japan). In the control experiment, *F. solani* mycelium incorporated with non-inoculated colloidal chitin broth was used. At each sampling, three replicates were used with eight independent replicates for each isolate.

2.9 Screening Actinobacterial Isolates for the Production of Siderophores

Plates of chrome azurol S (CAS) agar designed by Schwyn and Neilands (1987) were prepared for the screening of the BCA isolates for siderophore production. CAS agar plates were inoculated with the biocontrol agents and incubated in dark at 28°C for 7 days. The formation of yellow-orange halo zone around the colony was an indication for siderophore production.

2.10 Qualitative and Quantitative Production of Indole-3-Acetic Acid

The purpose of this experiment was to screen the obtained 2 endophytic BCA isolates, for their potential to produce indole-3-acetic acid (IAA) in glucose peptone broth (GPB) (Di Menna, 1957) supplemented with L-tryptophan (L-TRP). Erlenmeyer flasks (100 ml) each containing 20 ml of sterile GPB were amended with 5 ml of 5 % filter sterilized L-TRP as suggested by Khalid et al. (2004). The flasks were inoculated with 2 ml of each of the isolated BCA prepared from a 5-day-old shake GPB culture of around 1 x 10^8 CFU ml^{-1}, packed with aluminum foil and incubated on a shaker at 250 rpm in the dark for 7 days at 25°C. For the controlled experiment, non-inoculated

flasks were used. Following the incubation, the suspension from each flask was centrifuged at 12000 *g* for 30 minutes. The supernatant was filtered through sterile Millipore membranes and assembled in sterile tubes. The culture supernatants (3 ml) were pipetted into test tubes and 2 ml of Salkowski reagent (2 ml of 0.5 M $FeCl_3$ + 98 ml 35% $HClO_4$) were add up to it as suggested by Gordon & Weber (1951). The tubes were left for 30 minutes waiting for the red colour to reveal. The intensity of the colour was measured by optical density at 530 nm using a scanning spectrophotometer. Colour has also been utilized in IAA standard solutions and a standard curve has been produced and auxin compounds were expressed as IAA-equivalents (Gordon & Weber, 1951). Four replicates of each actinobacterial isolate were examined.

2.11 Identification of Actinobacterial Genera to Species Level

Identification of actinobacterial isolates **(**BCA1 and BCA2) isolates was confirmed based on cultural and morphological characteristics as described by Locci (1989). Scanning electron microscopy (SEM) was carried out for the two BCA isolates (BCA1 and BCA2) using Philips XL-30 SEM (FEI Co., Eindhoven, The Netherlands) to determine the morphology of the spore-chains and surface.

2.12 Disease Assays and Greenhouse Trials

2.12.1 Inoculum Production

Biological control agents inoculum were prepared for the greenhouse experiments by inoculating 4 ml of 20% glycerol suspension of each BCA into 250 ml of starch nitrate broth (SNB) in 500-mL Erlenmeyer flasks and the flasks were shaken for 5 days on a rotary shaker at 250 rpm.

Cells were obtained by centrifugation for 15 minutes at 20°C (12000 g) and the pellet suspended in 10 mL PB and centrifuged again. A dilution was prepared of each suspension in PB, and 0.1 ml each of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions was dispersed onto SNA. The plates were incubated for 5 days before determining the CFU mL^{-1}. The concentration that was used for each isolate inoculum was 10^{8} CFU mL^{-1}.

2.12.2 *In Vivo* Biological Control under Greenhouse Conditions

The effectiveness of each endophytic BCA for the biological control experiment against *F. solani* was further evaluated *in vivo*.

2.12.3 Preparation of the Pathogen Inoculum

For the preparation of the *F. solani* inoculum, a 100 g of millet seeds were added to 500-ml Erlenmeyer flasks containing 50 ml of distilled water. The flasks were then autoclaved for three days at 121°C for 30 minutes (El-Tarabily et al., 1997). The millet seed flasks were inoculated with 10 agar plugs (5 mm in diameter) from 14 days old culture of *F. solani* colony grown on PDA. For the control experiment, flasks containing 100 g of millet seeds autoclaved twice were used. The flasks were shaking at regular intervals in order to assure full colonization of the millet seeds by the hyphae. Small amounts of the inoculated and control millet seeds were suspended in 50 ml of distilled sterile water prior to use. Suspensions (0.2 ml) were spread onto PDA and incubated to confirm the colonization of the pathogen.

2.12.4 Soil Infestation

Soil was obtained from the same date palm field as described above. Soils were sieved using 1 cm mesh sieved then dried by air. The chemical characteristics of the

soil were assessed as illustrated previously by El-Tarabily et al. (1996). The characteristics of the soil were: pH of 7.4 (in 0.01 M $CaCl_2$); electrical conductivity 2.357 dS m^{-1}; organic carbon 1.7%; 520 mg kg^{-1} soil of bicarbonate extractable potassium and 35 mg kg^{-1} soil of phosphorus; 28 mg kg^{-1} soil of nitrate nitrogen, 75 mg kg^{-1} soil of ammonium nitrogen; 510 mg kg^{-1} soil of sulfate and 930 mg kg^{-1} iron.

The pathogen inoculated in the soil by mixing in a mixer (0.03 g colonized millet seeds inoculum g^{-1} soil). In the control experiment, soil supplemented with the same amount of autoclaved millet seeds were used. Thirty-cm diameter of plastic pots were filled with 8 kg of soil: either with or without the pathogen. The pots were moved to the greenhouse and were irrigated twice a week. The controlled date palm seedlings and the ones specified for inoculation with biocontrol agents had been planted one week later. Three-mm of the seedlings root out of 25 mm were trimmed using a sterilized scalpel (Pleban et al., 1995; Downing & Thomson, 2000) to facilitate the uptake of the endophytic actinobacterial inoculum. The seedlings were put in sterilized containers for 4 hours at 25°C, with their roots immersed in the actinobacterial inoculum at 10^8 CFU mL^{-1} (Pleban et al., 1995; Downing & Thomson, 2000). Seedlings with cut roots tips treated with autoclaved inoculum were used as control. After that, all seedlings were sown in the 30 cm in diameter pots filled with 8 kg of soil: including BCA and non-containing BCA seedlings.

Six treatments were conducted in total, as follows: (1) Control seedlings (2) non-inoculated seedlings in soils inoculated with *F. solani*, (3) seedlings inoculated with the endophytic cell wall degrading enzymes producing biocontrol agent (BCA1) in soils inoculated with *F. solani*, (4) seedlings inoculated with the endophytic antifungal metabolites producing biocontrol agent (BCA2) in in soils inoculated with

F. solani, (5) seedlings inoculated with the endophytic cell wall degrading enzyme producing biocontrol agent (BCA1) in soils not inoculated with *F. solani* and (6) seedlings inoculated with the endophytic antifungal metabolites producing biocontrol agent (BCA2) in soils not inoculated with *F. solani*. In all the treatments not including *F. solani*, the soil was mixed with colonized but autoclaved millet seeds. The pots were placed in an evaporative cooled greenhouse and kept for 8 weeks at 25°C ± 2°C. The pots were watered daily and fertilized weekly with inorganic fertilizer and the plants were daily monitored for symptom progression.

2.12.5 Disease Assessment

Disease severity index on inoculated seedlings was recorded for SDS symptoms at 10 and 35 dpi using a scale of 0–5: 0 = no apparent symptoms, 1 = 1–10% necrotic or white area in leaves or rotting in roots, 2 = 11–25%, 3 = 26–50%, 4 = 51–75%, and 5 = 76–100% (Molan et al., 2004). All experiments have been repeated three times independently with similar results.

2.12.6 Spore Counts and Disease Severity Index in Inoculated Plants

F. solani growth in inoculated seedlings was determined based on the total number of spores counts after 8 weeks of inoculation. The roots of the inoculated seedlings were cut into pieces from 2 to 5 mm in diameter and soaked in 10 ml distilled water and vigorously agitated for 30 minutes. Haemocytometer (Agar Scientific Limited, Essex, United Kingdom) was used in spores count.

2.13 Statistical Analysis

All treatments for all experiments were arranged in a randomized complete block design. *In vitro* experiments were replicated three times and each experiment was repeated four times independently. Population data were converted into fresh root weight of $\log_{10}$ CFU g^{-1}. Percentage data of spore germination were arcsine transformed before analysis of variance was completed. This transformation improved normality of the distribution of the data and made group variances homogenous. ANOVA and Duncan's multiple range test at 5% level of significance were used to evaluate the *in vitro* evaluation of BCA against *F. solani*. Experiments were repeated in triplicates using five plates per treatment for each time with similar results. SAS Software version 9 was used for all statistical analyses (The SAS System, 2002).

Chapter 3: Results

3.1 Symptoms of SDS on Date Palm

Symptoms of SDS were noticed on manifested date palm trees from Al Wagan, Al Ain, UAE (Figure 2A). Although the pathogen was observed to attack different parts of the date palm trees, plant tissues were severely affected. The symptoms started with orange yellowish colouring for the rachis (frond's midrib), followed by the leaflets (pinnae). This disease usually progresses from outer frond whorls toward central younger fronds, and from base to apex (Figure 2B). In general, drying begins from the terminal part in a single frond to cover the whole frond (Figure 2C). The affected leaves turn pale green and then to a yellowish colour. The discolouration continues outward and the colour becomes white on one side of the frond until the whole leaf is completely white (Figure 2C).

Under severe conditions, roots show reddish colour (Figure 2D, E), fronds start drying up and the terminal bud is affected. In most cases, external symptoms are usually associated with highly coloured vascular bundles (Figure 2F). Eventually, the entire tree dies within few months. Similar symptoms of decline then begin to appear on other leaves and pinnae. Thus, all palms are rarely found infected in an orchard at same time (Figure 2A). These symptoms on date palm are typical of the SDS that is known to be caused by a soil-borne fungus *Fusarium* spp.

Figure 2: Date palm trees showing symptoms of sudden decline syndrome. Symptoms on a group of trees (A); and whole tree (B) of date palm. Progress of symptoms on fronds (C) and roots (D) of diseased plants. Brown-coloured, dry roots (E); and redness of vascular tissues (F). In (A–F), naturally infested date trees with *Fusarium* spp.

3.2 Cultural and Morphological Identification of *Fusarium* spp. Associated with SDS

The sporulation of the isolated pathogens from affected tissues were microscopically examined on potato dextrose agar (PDA) plates. Based on the colour of colonies and conidial morphology identified on PDA, the culture of the identified isolates may be predicted to belong to *Fusarium* spp. Colonies of the first isolate were woolly to cottony with cream to white aerial mycelium and purple pigment (Figures 3, 4). Mycelial growth and production of microconidia and macroconidia was also observed in this study. Conidiophores had simple or branched monophialides (Figure

3B). Microconidia are generally small, 1–2 celled, hyaline with oval to oblong or reniform with slightly curved shape, measuring 9.2–11.8 × 3.3–4.8 µm (Figure 3C). On the other hand, macroconidia have 3−5 septa, and are boat-shaped to oblong and 28.0–30.5 × 3.5–5.3 µm in size. Together, this suggests that this fungal isolate could be *F. oxysporum* f. sp. *cumini* (Patel et al., 1957).

F. proliferatum (Matsushima) (Nirenberg, 1976) was also isolated in the current study. On PDA, colonies grew rapidly, aerial mycelia were white to dark vineaceous or purple (Figure 3A; 4). Conidiophores were densely branched arising laterally from aerial hyphae (Figure 3B). Although chlamydospores were absent, macroconidia were distinctly abundant (Figure 3C). The 3−5 septated, nearly straight macroconidia had a size of 25.0−56.6 × 2.6−4.6 µm. Microconidia were clavate measuring 2.2−3.0 × 6.8−8.6 µm.

Colonies on PDA of the third isolate were fast-growing with white and purple, fluffy aerial mycelia with undersurface showing a dark violet colour (Figure 3A; Figure 4). Hyphae were septate and hyaline; conidiophores were unbranched (Figure 3B). The produced microconidia were thin walled, hyaline, and oval to kidney-shaped, and were measured 10.8–15.4 × 2.0–4.0 µm (Figure 3C). Macroconidia had 3–5 septa with 24.6–44.2 × 3.2–4.2 µm, with stout and falcate (curved) shaping. *F. solani* (Martius) (Saccardo, 1881) was speculated to be one of the fungal pathogens isolated from root tissues of SDS symptomatic date palm trees. The cultural and morphological analyses suggest that *Fusarium* spp. caused SDS. Thus, molecular identification on an isolated specimen would remove any kind of controversial identification at the species level.

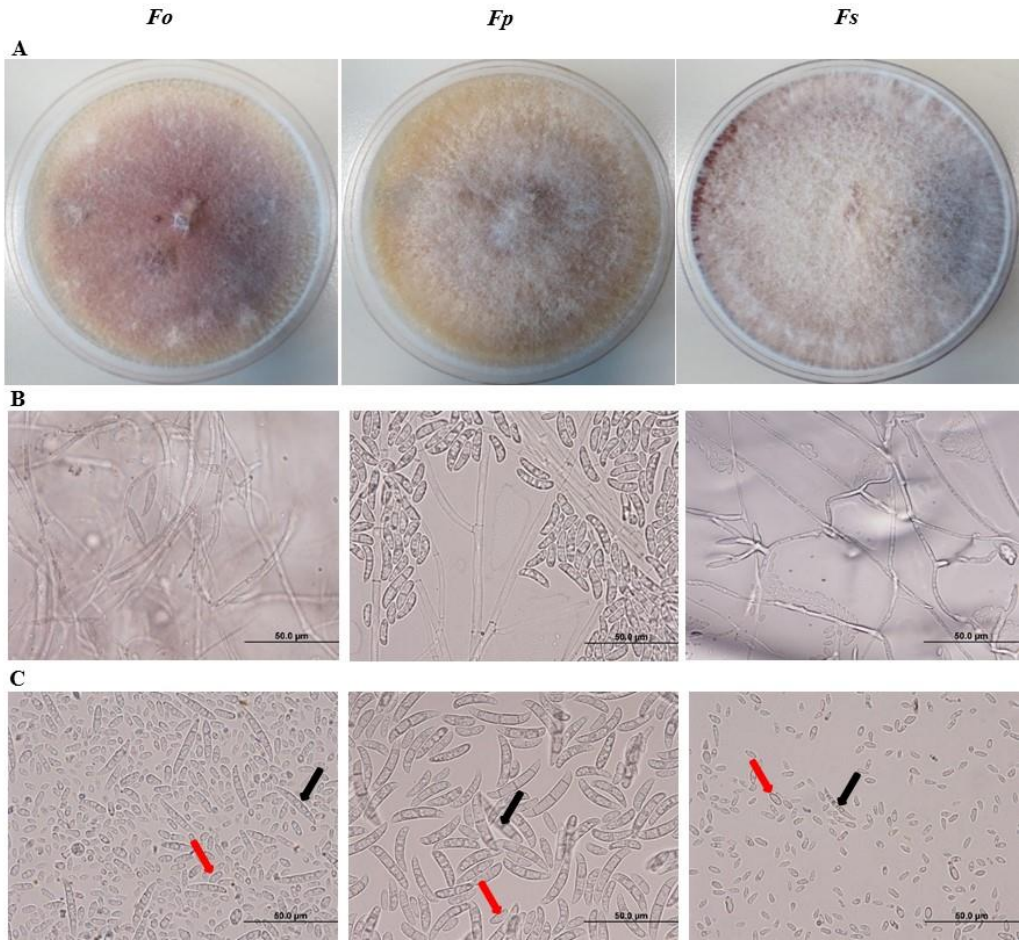


Figure 3: Morphological phenotypes mycelium and conidia of *Fusarium* spp. Mycelia of *Fusarium* spp. growing on a 10-day old PDA culture plate (A). Mycelium growth (B); and microconidia (red arrow) and macroconidia (black arrow) (C) of *Fusarium* spp. In (A–C), mycelia and conidia are from a 10-day old PDA culture. *Fo*, *F. oxysporum*; *Fp*, *F. proliferatum*; *Fs*, *F. solani*

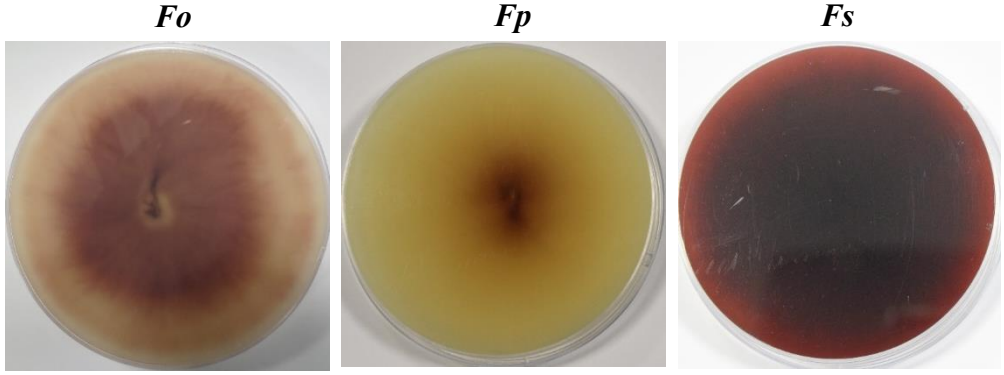


Figure 4: Sporulation of *Fusarium* spp. on a 10-day old PDA culture plate. *Fo*, *Fusarium oxysporum*; *Fp*, *F. proliferatum*; *Fs*, *F. solani*

3.3 Molecular and Phylogenetic Classification of *Fusarium* spp.

A phylogenic analysis on the isolates obtained in this study was established. To identify the fungi generated from the infected tissues (Figure 3), DNA isolated from the PDA-grown mycelium of each sample was PCR amplified. Successful amplification with primer targeted to the genomic regions of *internal transcribed spacer* (*ITS*), 28S rDNA region and *translational elongation factor 1-α* (*TEF1-α*) was obtained from all putative *Fusarium* spp., and also the amplification product of *β-tubulin* gene was visible in putative *F. solani* and *F. oxysporum* specimens (Figure 5A). This confirms that *Fusarium* spp. are the pathogens frequently associated with all SDS symptoms on date palm trees. Until this report, no DNA sequences of the species collected in the UAE were available in GenBank. Therefore, the *ITS* rDNA and *TEF1-α* genes (Alves et al., 2008) were further sequenced and deposited in GenBank: *F. oxysporum ITS/LSU* rDNA (MH055398) and *TEF1-α* (MH087478), *F. proliferatum ITS/LSU* rDNA (MH055399) *TEF1-α* (MH087479), and *F. solani ITS/LSU* rDNA (MH055400) and *TEF1-α* (MH087480). This research also has tried to amplify the flanking DNA regions of the *Fot1* insertion sites of the pathogen *F. oxysporum* f. sp. *albedinis* from these samples. By using *pFalb*11 and *pFalb*28 primer pairs (Fernandez et al., 1998), none of the samples used in the PCR assays thus provide evidence of any diagnosis for *F. oxysporum* f. sp. *albedinis* isolates in the UAE (Figure 6).

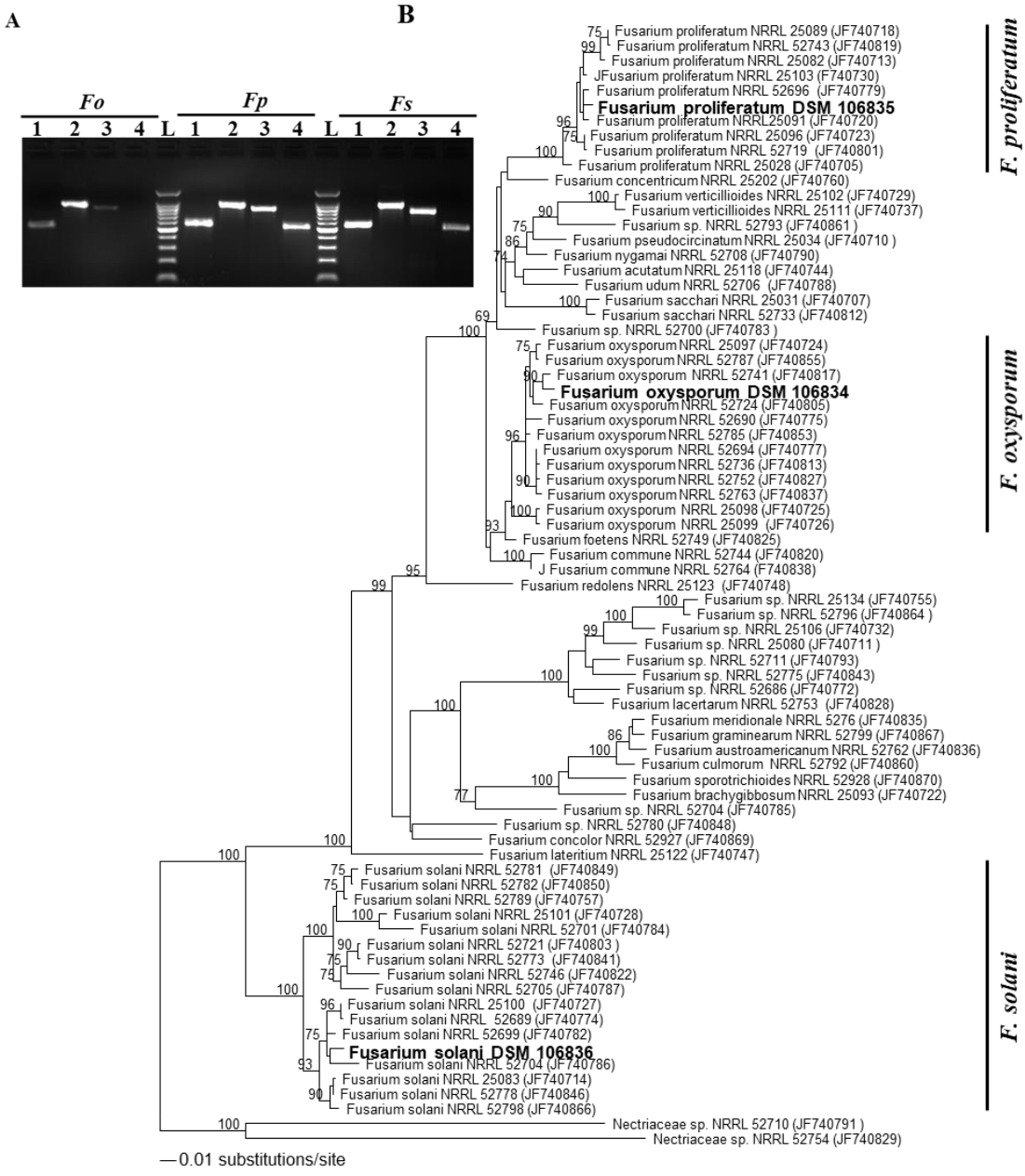


Figure 5: Identification of *Fusarium* spp. at the molecular level. PCR amplification of specific genomic DNA regions of infected root tissues (A); and dendrogram showing phylogenetic relationships among *F. oxysoprum* (*Fo*) species complex (DSM 106834), *F. proliferatum* (*Fp*) (DSM 106835), and *F. solani* (*Fs*) species complex (DSM 106836) identified in this study and other members of *Fusarium* spp. prepared by the maximum likelihood (ML) method (B). In (A), lanes 1–4 correspond to amplifications of *ITS*, 28S rDNA region, *TEF1-α* and *β-tubulin* in roots. In (B), the ML tree was obtained from combined *ITS/LSU* rDNA and *TEFα-1* sequence data. The specimens used in this study carry GenBank accession numbers, *Fo ITS/LSU* rDNA (MH055398), *Fo TEF1-α* (MH087478), *Fp ITS/LSU* rDNA (MH055399), *Fp TEF1-α* (MH087479), *Fs ITS/LSU* rDNA (MH055400), *Fs TEF1-α* (MH087480). Numbers at the nodes are ML bootstrap values after 100 replicates are expressed as percentages (LnL = −8908.252933). The scale bar on the rooted tree indicates a 0.01 substitution per nucleotide position. The strains from this report are indicated in bold.

Nectriaceae spp. NRRL 52710 (JF740791) and NRRL 52754 (JF740829) were used as outgroups. *ITS, internal transcribed spacer*; 28S rDNA, large subunit (LSU) of rDNA*; TEF1-α, translational elongation factor 1-α*; L, DNA ladder

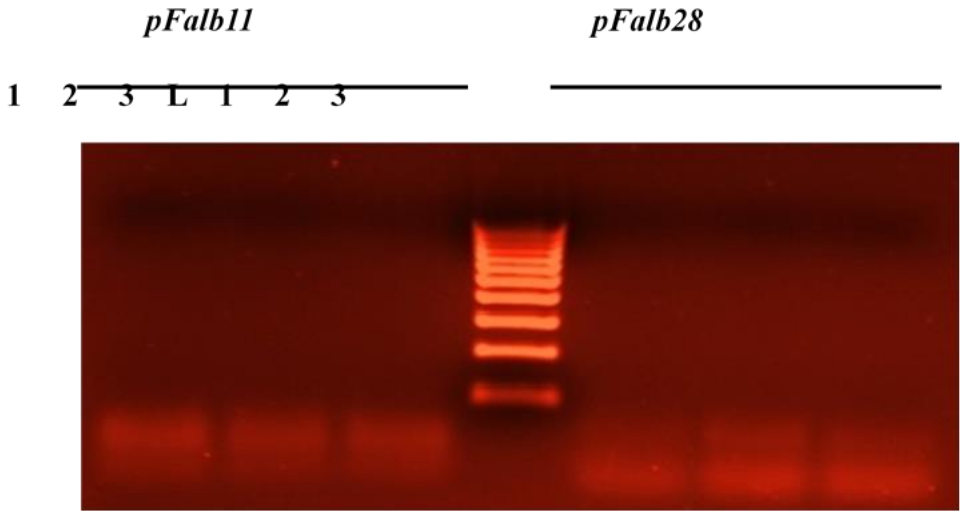


Figure 6: PCR amplification of specific genomic DNA regions of infected root tissues. Lanes 1-3 correspond to amplifications of specific primers of *F. oxysporum* f. sp. *albedenis* clone *pFalb11* and *pFalb28* in leaf tissues of seedling 1, 2 and 3. L, DNA ladder

In addition, the molecular analysis of combined sequence data of *ITS* regions and *TEF-1α* was used to determine the relationship among the obtained *Fusarium* spp. and other closely related *ITS*/*TEF1-α* sequences for the generation of the phylogenetic tree (Watanabe, 2013; Saleh et al., 2017). For the estimation of the phylogenetic tree, sequences were aligned and maximum likelihood (ML) analyses were performed. The adaptation to different plant hosts has led to the evolution of over 30 cryptic species within the *Fusarium* spp. complex (El Hassni et al., 2007). The generated *ITS*/*TEF1-α* sequence belonging to the isolated strains clustered separately representing three different clades corresponding to *F. oxysporum*, *F. proliferatum* and *F. solani* from different sources (Figure 5B). Phylogenetic tree analysis demonstrated that three strains isolated from the UAE were placed in *F. oxysporum*, *F. Proliferatum*, and *F.*

solani groups with a strong bootstrap support (100, 93, and 100%, respectively). This confirms the identity of these isolates with *Fusarium* spp. Among the studied species, the analysis done in this study revealed that the isolates *F. oxysporum* DSM 106834, *F. proliferatum* DSM 106835 and *F. solani* DSM 106836 had a sister relationship with *F. oxysporum* NRRL 52741, *F. proliferatum* NRRL 25091 and *F. solani* NRRL 52704, respectively, which distinguishes the obtained isolates from those belonging to other *Fusarium* or Nectriaceae spp. The phylogenetic analysis supports that *F. oxysporum* DSM 106834, *F. proliferatum* DSM 106835 and *F. solani* DSM 106836 dominate in the UAE. Altogether, this suggests that at least one of the *Fusarium* isolates is probably the causal species of SDS on date palm.

3.4 Pathogenicity Tests of *Fusarium* spp. on Date Palm Seedlings

To confirm the results, pathogenicity tests using individual and combined isolated pathogens on healthy date palm seedlings (cv. Barhi) were performed and monitored for disease progress. Plants were inoculated with the conidial suspension in the root system using a 10-day old pure culture of *F. oxysporum*, *F. proliferatum*, or *F. solani* grown in PDA, while control seedlings were inoculated with sterile distilled water. The roots were also treated with the three combined conidial suspensions to determine whether SDS symptoms on date palm seedlings are similar to or stronger than in inoculations of individual *Fusarium* spp. under greenhouse conditions. Following inoculation with *F. oxysporum* or *F. proliferatum*, seedlings did not develop SDS symptoms on stem and leaf tissues (Figure 7A). However, typical symptoms of SDS such as white colour and dryness of the stem and leaves appeared at 10 dpi with *F. solani*. Moreover, the disease progressed rapidly along the stem and leaves in the following days. At 35 dpi, symptoms often expressed as complete dryness of leaves

and characterized by tissue necrosis in *F. solani*-inoculated plants (Figure 7A, B). Minor effect on seedlings inoculated with any of the other two *Fusarium* spp. was observed after 35 days. Similar to *F. solani*-inoculation, symptoms developed quickly and seedlings showed complete discolouration and necrosis of tissues by the end of the experiment when the three species of *Fusarium* were combined (Figure 7A, B). Control leaf tissues remained symptomless. To fulfill Koch's postulates, the pathogens were consistently re-isolated from the disease affected tissues; thus, detected symptoms were associated with the inoculation with the pathogen *F. solani* (Figure 7C).

Based on visual observations (Figure 7A), it has been speculated that the three combined *Fusarium* spp. might have a synergetic effect on date palm seedlings. Disease severity index (DSI) on date palm seedlings inoculated with the three *Fusarium* spp. was not significantly different from those inoculated with *F. solani* throughout the greenhouse experiment (Figure 7D). However, pathogenic ability of *F. oxysporum* or *F. proliferatum* had less effect on the seedlings. This is evident from DSI which predicted reduced scores of affected seedlings of date palm with *F. oxysporum* or *F. proliferatum* when compared with that of *F. solani* or the combinations of *Fusarium* spp. at 10 and 35 dpi. The obtained data suggest that *F. solani* causes the disease on different tested tissues of date palm.

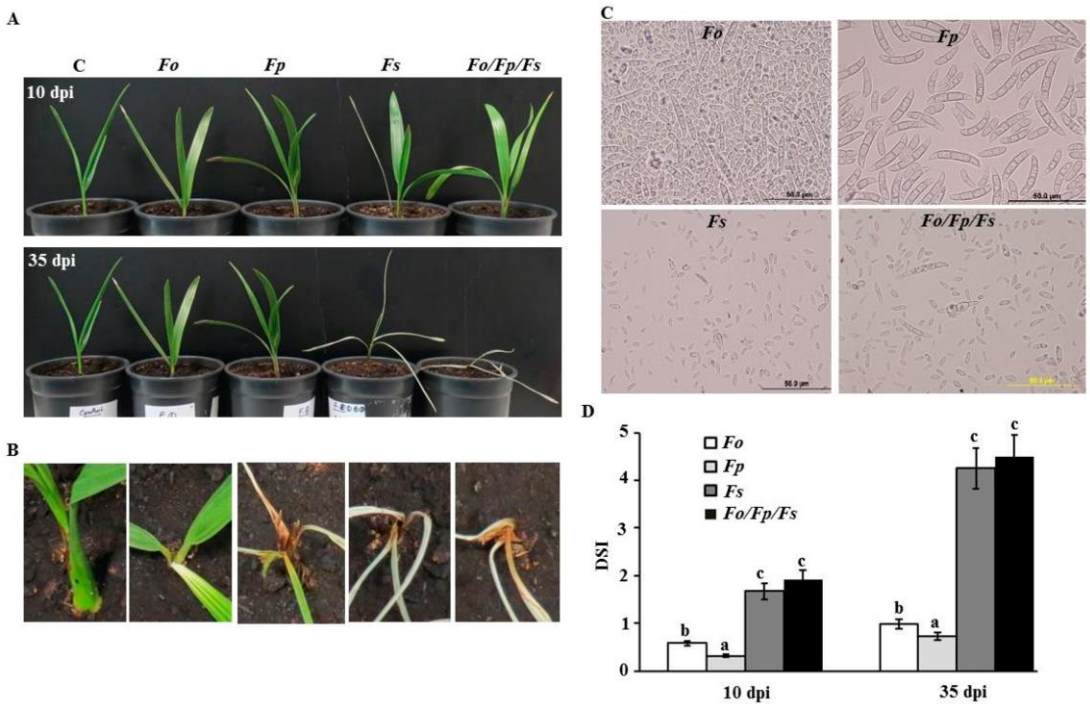


Figure 7: Pathogenicity and Koch's postulate testing with *Fusarium* spp. Pathogenicity test on inoculated and non-inoculated date palm seedlings cv. Barhi at 10 dpi (top) and 35 dpi (bottom) (A); symptomatic tissues of the inoculated area of the seedling at 35 dpi (B); conidia after re-isolation of the pathogen from colonized tissues at 35 dpi (C); and disease severity index (DSI) of affected seedlings (n = 12) at 10 and 35 dpi (D). In (B), close-up views of infected stem and frond at 35 dpi of *Fusarium*-inoculated and non-inoculated seedlings. Young seedlings showing general whitening and dryness of stem and leaf tissues in *F. solani* and the three *Fusarium* spp. In (D), DSI is on a scale of 5: 0 = no infection, 1 = 1–10%, 2 = 11–25%, 3 = 26–50%, 4 = 51–75%, and 5 = 76–100% damage including necrosis, white area in leaves or rotting in roots. Values with different letters are significantly different from each other at p = 0.05. C, control (no infection); *Fo*, *F. oxysporum*; *Fp*, *F. proliferatum*; *Fs*, *F. solani*; *Fo*/*Fp*/*Fs*, all three *Fusarium* spp.; dpi, days post inoculation

3.5 Isolation, Identification and Screening of Actinobacterial Isolates

Twenty-four actinobacterial strains were isolated from date palm roots. All the isolates were screened through multiple *in vitro* tests in order to choose promising biocontrol agent (BCA) candidates (Figure 8).

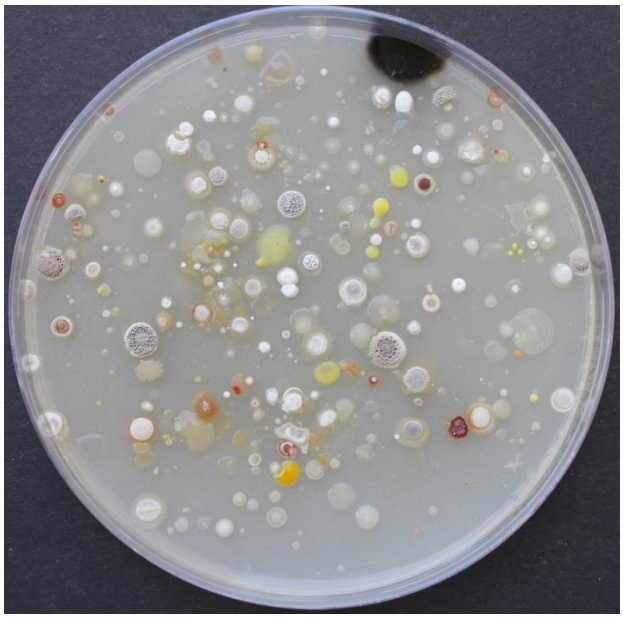


Figure 8: Colonies of endophytic actinobacteria isolated from date palm roots grown on inorganic salt starch agar plates

3.6 Production of Antifungal Metabolites and CWDEs

Using Cut-plate method revealed that out of 24 isolates 12 were able to produce active antifungal metabolites against *F. solani*. The colony and the mycelial growth of *F. solani* were inhibited significantly and the pathogen inhibition zone was more than 30 mm (Figure 9).

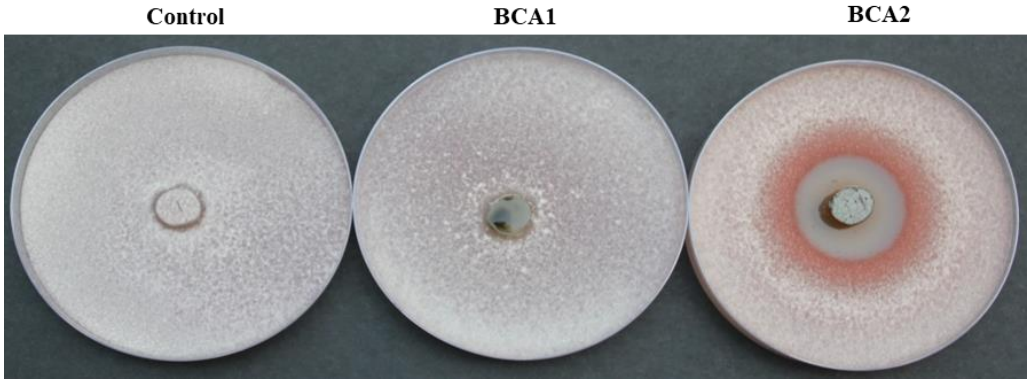


Figure 9: Inhibition of *F. solani* mycelial growth by the BCA1 and BCA2 using cut-plug method. Inhibition of *F. solani* mycelial growth was observed only by the diffusible antifungal metabolite-producing isolates BCA2 and not BCA1

Using Cup-plate method revealed that out of 12 isolates 4 were able to produce strong active antifungal metabolites against *F. solani*. The colony and the mycelial growth of *F. solani* were inhibited significantly and the pathogen inhibition zone was more than 30 mm (Figure 10).

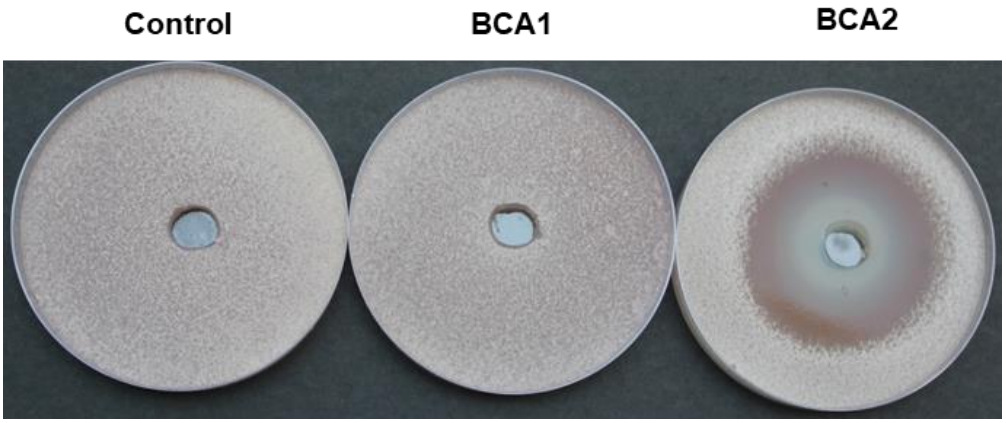


Figure 10: Effect of the BCA candidates on mycelial growth of *F. solani* using the cup plate method. Inhibition of *F. solani* mycelial growth was observed only by the diffusible antifungal metabolite-producing isolates BCA2 and not BCA1. Wells were inoculated with either filter-sterilized fish meal extract broth (C), or filter-sterilized crude culture filtrates of isolates

The 12 BCA isolates that produced the strongest diffusible antifungal metabolites active against *F. solani* were further tested on *F. solani* mycelial fragment agar and on colloidal chitin agar. Among the tested isolates only 4 of them produce large clearing zone around their colonies (more than 30 mm) (Figure 11).

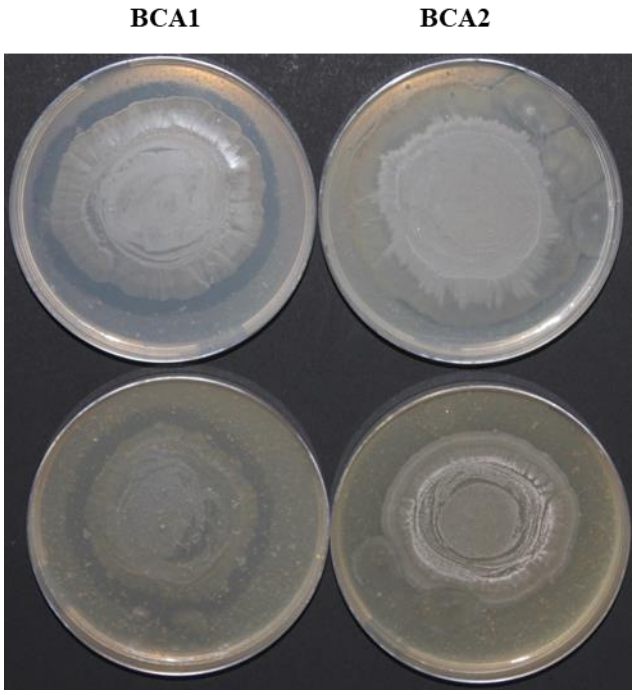


Figure 11: Production of cell-wall-degrading enzymes by BCA1 candidates active against *F. solani*. Production of chitinase enzymes by BCA1 only grown on colloidal chitin agar (upper panel) and production of CWDEs on mycelial fragment agar (lower panel)

3.7 Production of Siderophores

Both BCA1 and BCA2 develop yellow-orange halo zone around their colonies in CAS agar which indicate the production of siderophores (Figure 12).

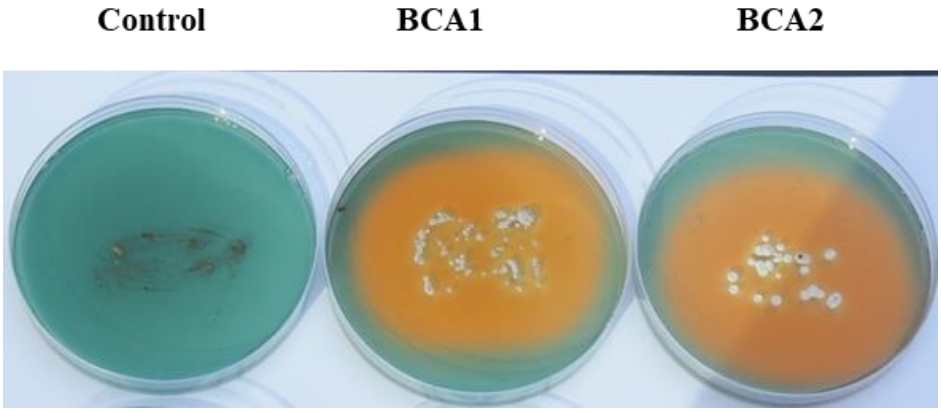
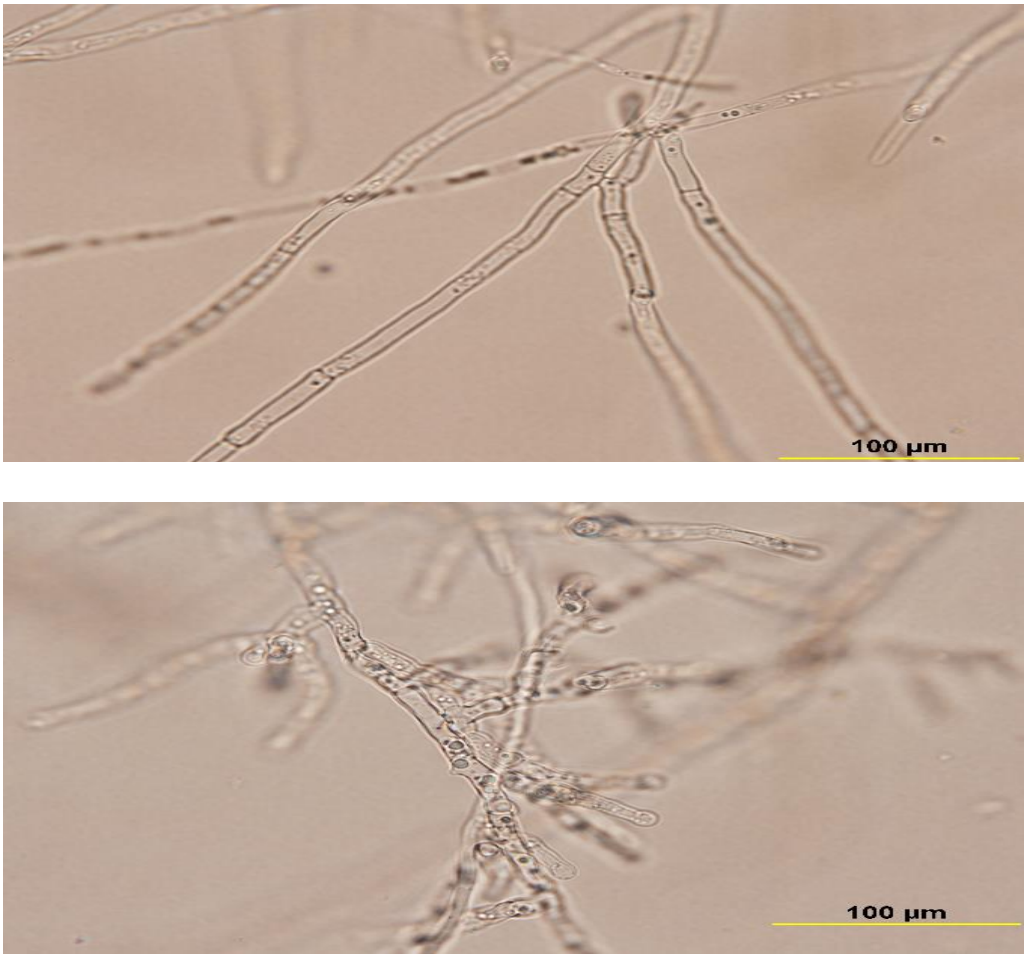


Figure 12: Yellow-orange halo zone indicates siderophore production

3.8 Effect of Actinobacterial Isolates Crude Culture Filtrates on Mycelia and Conidia of *F. solani*

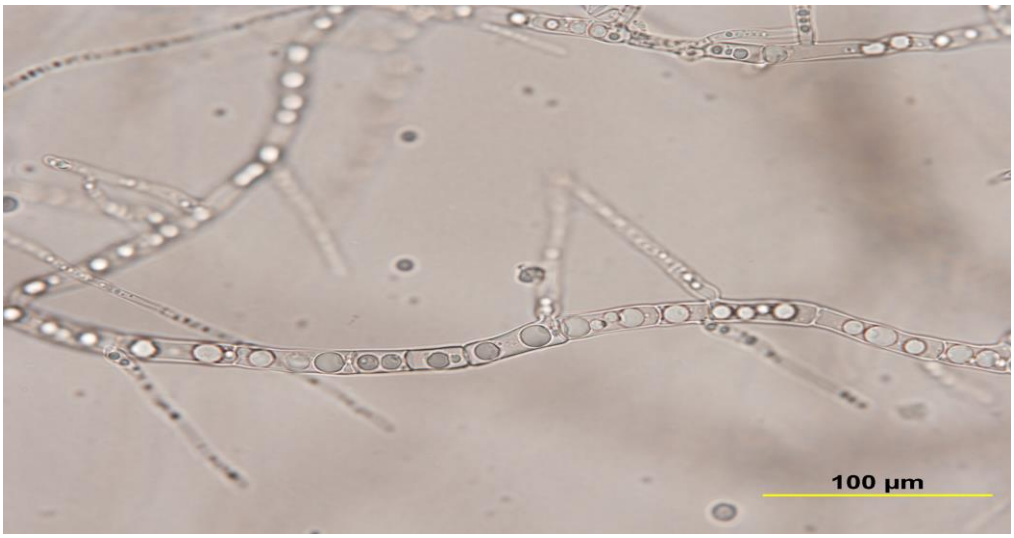
Hyphal abnormalities such as hyphal swelling (ballooning), cytoplasmic coagulation and hyphal lysis in *F. solani* treated with the crude culture filtrate of BCA1 and BCA2 obtained from fish meal extract broth and colloidal chitin broth, respectively (Figure 13). Mycelial mats in control flasks remained healthy and unaffected (Figure 13).



Fs

BCA1

Figure 13: Effect of the BCA candidates on hyphae and cytoplasm of *F. solani*. Abnormalities observed in hyphal morphology and cytoplasmic contents of *F. solani* following exposure to filter-sterilized crude culture filtrate of BCA1 and BCA2. *Fs* = *F. solani*, BCA1 = *F. solani* + endophytic isolate producing chitinase, BCA2 = *F. solani* + endophytic isolate



BCA2

Figure 14: Effect of the BCA candidates on hyphae and cytoplasm of *F. solani.* Abnormalities observed in hyphal morphology and cytoplasmic contents of *F. solani* following exposure to filter-sterilized crude culture filtrate of BCA1 and BCA2. *Fs* = *F. solani*, BCA1 = *F. solani* + endophytic isolate producing chitinase, BCA2 = *F. solani* + endophytic isolate (continued)

3.9 Identification of the Actinobacterial Isolates

Identification of actinobacterial isolates (BCA1 and BCA2) isolates was confirmed based on cultural, morphological, and physiological characteristics as described by (Locci, 1989). The two isolates BCA1 and BCA2 belonged to the genus *Streptomyces*.

3.10 Qualitative and Quantitative Production of Indole-3-Acetic Acid

The efficiency of the two BCA isolates in their capability for IAA production varied greatly. The isolates that formed dark red colour after the addition of Salkowski reagent were considered as IAA-producing isolates (BCA2), whilst failure to produce dark red colour after the addition of Salkowski reagent were considered as non- IAA-producing isolates (BCA1) (Figure 14). Auxins expressed as (IAA equivalents in µg ml^{-1}) using the colorimetric analysis were detected only in liquid cultures of BC2 and

not BCA1. The amount of auxins produced by BCA2 were found to be 26.58 IAA equivalents in µg ml^{-1} compared to 0.00 IAA equivalents in µg ml^{-1} for BCA1.

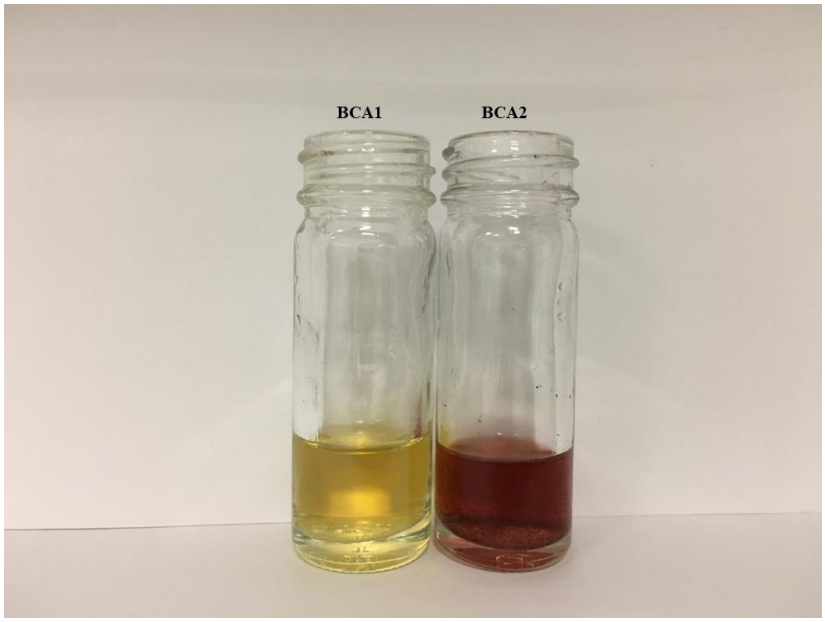


Figure 15: *In vitro* production of Indole-3-Acetic Acid by the two selected biocontrol agents (BCA1 and BCA2). The addition of Salkowski reagent to cultures grown in inorganic salt starch broth amended with L-tryptophan; and the formation of red colour indicated the production of the auxin, IAA

3.11 Effect of The Promising BCA Candidates Against *F. solani In Vivo*

All the artificially infected seedlings with *F. solani* showed disease progression from the tenth day till eventually died after thirty days. There was a noticeable significant suppression of the disease in the treatments of (BCA2) comparing with the treatments of (BCA1) which showed slight symptoms only (dryness of the leaves) after thirty days (Figure 15). Control seedlings did not develop any disease symptoms likewise the seedlings which inoculated separately with both BCA1 and BCA2 only. The BCA2 (Only diffusible antifungal metabolites), and no cell wall degrading enzymes production performed superior to the BCA1 which produced only cell wall degrading enzymes producing isolate (no diffusible antifungal metabolites) (Figure

15). This indicates that the ability to produce diffusible antifungal metabolites clearly enhances the performance of the antagonist to effectively suppress the disease. This demonstrated the superiority of antagonistic endophytic actinobacteria capable of producing diffusible antifungal metabolites compared to antagonistic endophytic actinobacteria capable of producing cell wall degrading enzymes in reducing the severity of SDS on date palm under greenhouse conditions.

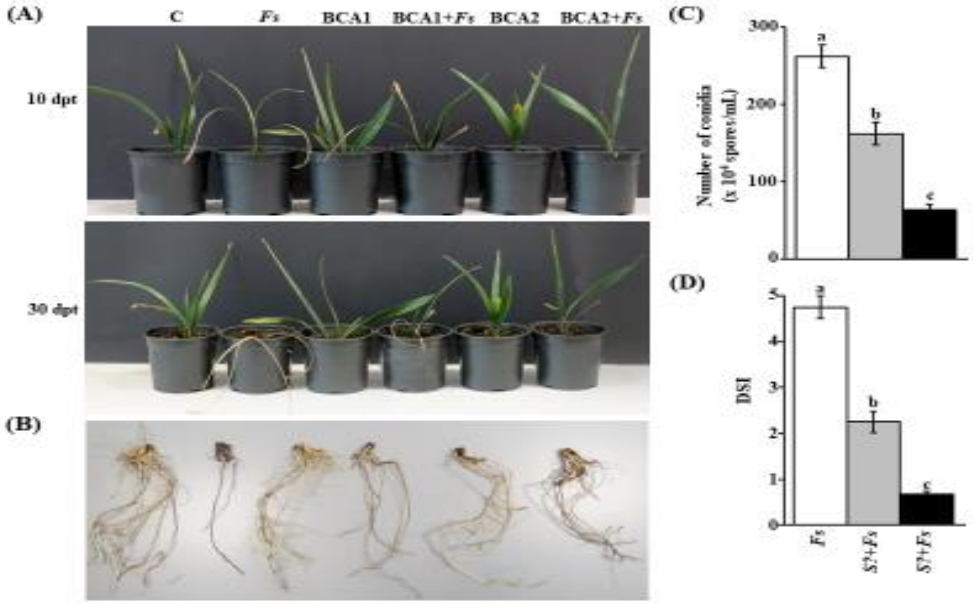


Figure 16: Preventive effect of BCA treatments on artificially inoculated date palm seedlings with *F. solani* in the greenhouse. Suppression of *Fusarium* wilt disease on date palm seedlings cv. Barhi using (BCA1 and BCA2) at 10 (top panel) and 30 (bottom panel) dpt (A); and recovery of root tissues infected with *F. solani* and previously inoculated with BCA at 30 dpt (B). Number of conidia (x10^4 spores/mL) (C) and disease severity index (DSI) (D) after recovery of the pathogen from affected date palm root tissues (n = 12) treated with BCAs at 30 dpt. In (A-D), seedlings inoculated with *F. solani* at 5 days after BCA treatment. In (C-D) values with different letters are significantly different from each other at P = 0.05. In (D), DSI is on a scale of 5: 0 = no infection, 1 = 1-10%, 2 = 11-25%, 3 = 26-50%, 4 = 51-75% and 5 = 76-100% damage including necrosis, white area in leaves or rotting in roots. C = Control, *Fs* = Inoculation with *F. solani*, BCA1 = Inoculation with the endophytic isolate producing chitinase, BCA1 + *Fs* = Application of BCA1 5 days prior to inoculation with *F. solani*, BCA2 = Inoculation with the endophytic isolate producing antibiotic + plant growth promoters, BCA2+*Fs* = Application of BCA2 5 days prior to inoculation with *F. solani*. BCA1 = Only cell wall degrading enzymes producing isolate (NO diffusible antifungal metabolites), whilst, BCA2 = Only diffusible antifungal metabolites), and No cell wall degrading enzymes production

Chapter 4: Discussion

Date palm (*Phoenix dactylifera* L.) is an important and economic fruit crop in the tropical and subtropical areas and is widely grown in the UAE. Diseases, including those caused by fungal pathogens, are among the major factors that affect marketing and hinder the yield of dates (Zaid et al., 2002). Under local conditions of the UAE, roots of date palm are liable to attack by several pathogenic soil-borne fungi that cause destructive diseases such decline, wilt, neck bending and root rot (Saeed et al., 2016; Al-Hammadi et al., 2018). Recent studies have linked these diseases with *F. solani*, *Lasiodiplodia theobromae*, *L. hormozganensis*, *Thielaviopsis punctulate*, and *T. paradoxa* being the most common in the UAE (Saeed et al., 2016; Saeed et al., 2017b; Al-Hammadi et al., 2018). In the UAE, farmers might be confused between disease symptoms of black scorch and SDS on date palm. Symptoms of black scorch disease are hard black lesions on leaves, inflorescence blight, and trunk rot (Zaid et al., 2002; Saeed et al., 2016). Death of trees occurs from tips or terminal buds back inwards. Typical disease symptoms of SDS in the UAE are the orange-yellowish fronds, and the drying of leaflets. In contrast to black scorch, drying usually starts from older, lower fronds toward younger, central fronds. Regrettably, the end result of untreated palm trees is death within few months in both cases.

Although several fungi have been recorded as causal pathogens of SDS on date palm worldwide (Mansoori & Kord, 2006; El-Deeb et al., 2007; Abdullah et al., 2009; Salim et al., 2015), many of these studies have reported the isolation of *Fusarium* spp. from roots, fronds, and trunks of date palm trees showing wilt and decline (Abul-Soad et al., 2011; Salim et al., 2015). For example, *F. oxysporum*, *F. proliferatum*, and *F. solani* have been most frequently found in roots displaying decline symptoms on date

palm in different regions of Iraq (Sarhan, 2001; Hameed, 2012). In Egypt, declined date palm trees have been associated with two *Fusarium* spp., *F. solani* and *F. monliforme* (Rashed & El-Hafeez, 2001). A serious disease of yellowing and death of the fronds in date palm groves in Iran has also been reported to be caused by *F. solani* (Mansoori & Kord, 2006). Three *Fusarium* spp. have been isolated from the infected fronds and roots of date palm trees, and have been identified as *F. oxysporum*, *F. proliferatum*, and *F. solani* in Saudi Arabia, particularly in Al Qassim and Al-Medina Al-Monawara regions (Abdalla et al., 2000). In the UAE, 82% of the isolated fungal pathogens from date palm roots is known to be *F. solani* (Al-Hammadi et al., 2018). All previously mentioned reports are in agreement with the findings of the current study; however, no reports have linked any *Fusarium* spp. as the causal agent of SDS on date palm in the UAE. Therefore, an investigation of the causal agents of SDS on date palm and the remedy of this disease has to be done.

Due to the drying manner of fronds that displayed similar symptoms and disease severity of bayoud (*Fusarium* wilt) as in North Africa (Killian & Maire, 1930; Djerbi, 1982), molecular diagnostic techniques using PCR (Fernandez et al., 1998) can eliminate the occurrence of bayoud disease. Here, it was confirmed that there is no bayoud disease and the fungus, *F. oxysporum* f. sp. *albedinis*, does not exist in the UAE. In view of the fact that three *Fusarium* spp., *F. oxysporum* f. sp. *cumini* (Prasad & Patel), *F. proliferatum* (Matsushima) Nirenberg and *F. solani* (Martius) Saccardo (Patel et al., 1957; Nirenberg, 1976; Saccardo, 1881) were isolated from root tissues of infested date palm trees showing SDS symptoms. The cultural, morphological, and molecular phylogenetic analyses revealed that *F. oxysporum* f. sp. *cumini* DSM 106834, *F. proliferatum* DSM 106835, and *F. solani* DSM 106836 were the potential isolates associated with SDS on date palm in the UAE. Microscopic examinations of

the hyphal and conidial morphology of these isolates were consistent with those previously reported (Adame-García et al., 2015; Abd-Elsalam et al., 2016; Nawade et al., 2017). In this study, individual sequences generated from the three isolates were compared with those maintained in the National Center for Biotechnology information (NCBI; www.ncbi.nlm.nih.gov) and relevant sequences were included in the subsequent phylogenetic inference. Phylogenetic congruencies of the single gene set *ITS/TEF1-α*, clustered *Fusarium* isolates in three different clades corresponding to *F. oxysporum, F. proliferatum,* and *F. solani*. These isolates were further selected for pathogenicity assays.

Although there are few reports about the antagonistic effects of multiple *Fusarium* spp. on plants (Lemanceau et al., 1993); many others have shown synergistic effects (Lamichhane & Venturi, 2015; Kuzdraliński et al., 2017). The four *Fusarium* spp., *F. graminearum, F. culmorum, F. poae, and F. sporotrichioides*, causing foot and crown rot diseases on wheat (*Triticum aestivum* L.) have been reported to be more virulence when they work together (Kuzdraliński et al., 2017). Given the evidence for an arsenal of virulence factors of *Fusarium* spp., this study hypothesized that interactions of the three species would lead to additive effects in pathogenicity on date palm. It is likely that synergism among multiple pathogens leads to more severity in disease symptoms than an individual pathogen (Lamichhane & Venturi, 2015). Pathogenicity assays and DSI scores on healthy date palm seedlings inoculated with either *F. oxysporum* or *F. proliferatum* in the greenhouse did not provide evidence that these isolates cause SDS. This does not rule out the possibilities that these isolates may lead to minor disease symptoms, symptoms appearing at later stages of infection, or causing diseases under certain environmental conditions. Weather conditions are important parameters determining the production of mycotoxins such as

deoxynivalenol by *Fusarium* spp. to cause diseases in plants (Landschoot et al., 2012). After analysing the morphological phenotypes amongst the isolates, no significant difference was found in the DSI between seedlings inoculated with *F. solani* vs. the three combined *Fusarium* spp. This suggests that *F. solani* is most probably the *Fusarium* species that plays a major role in establishing the SDS in date palm plantation in the UAE. In this regard, *F. solani* was identified as the main causal pathogen of SDS on date palm in the UAE.

Root infectious diseases triggered by fungal pathogens such as wilt, root rot, collar rot, and foot rot are gaining significance from day to day globally and for which no direct control techniques have been developed until now (Whipps, 2001; Schumann & D'Arcy, 2006). *Fusarium* species are a widespread soil-borne phytopathogens and are the causal agents of wilt disease of many economically important crops (Jones et al., 1991). They are also considered a limiting factor for the plant yield production in most agricultural fields. Wilt disease of date palm is a common problem in almost all fields. At present, a combination of cultural practices and fungicide applications are used to control the diseases (Alwahshi et al., 2019).

In recent years, public and scientific concern regarding the application of chemical herbicides and pesticides in food industry has increased (Smith & Thomson, 2003; Vinale et al., 2008). Plant treatment with hazardous chemical fungicides is growing concern in developed and developing countries for its environmental effects (Smith & Thomson, 2003). Chemical pesticides and fungicides have been proven to contaminate the environmental and to result in health hazards to humans and other living organisms (Horrigan et al., 2002). The development of fungi resisting fungicides (Spotts & Cervantes, 1986) and the increasing public concern about human health and

environmental hazards associated with the fungicides have resulted in the development of environmental friendly alternative for controlling plant diseases (Vinale et al., 2008).

Biological control using beneficial organism, their genes, and/or their products, like the metabolites, that diminish the negative impacts of phytopathogens and promote positive responses by the plant is gaining more interests nowadays. Disease inhibition by biocontrol agents, is a result of the interactions between the plant, pathogens and other microbial community in the soil (Vinale et al., 2008). Plant researches went through screening for potential biocontrol agents and testing them *in vitro*, under greenhouse conditions and under the field conditions (Doumbou et al., 2002; El-Tarabily & Sivasithamparam, 2006; Vinale et al., 2008). Biological control using microbial antagonists has emerged as one of the most promising method, together with the integrated control strategy, to reduce usage of chemical pesticides (Whipps, 2001; Doumbou et al., 2002; Vinale et al., 2008).

The present thesis clearly shows the potential for the application of actinobacteria for maneging date palm wilt disease caused by *F. solani* in the UAE. The two isolates were antagonistic to *F. solani* when applied individually, and significantly reduced disease incidence under controlled greenhouse conditions. The disease reduction was most pronounced in the presence of the antifungal metabolites producing isolate compared to the chitinase-producing isolate.

The two-promising antagonistic actinobacterial isolates used in this study were identified on the basis of morphological, and cultural characteristics. The isolates were tentatively identified and grouped to the genus level on the basis of their standard morphological criteria and according to the absence or presence of aerial mycelium,

and form of any spores present and fragmentation of substrate mycelium (Cross, 1989). Identification to species levels was based on characteristics specific for each species as presented in Bergey's Manual of Systematic Bacteriology (Williams et al., 1989). Both isolates were identified as *Streptomyces* spp.

In the present study, only BCA1 produced large clear zones on colloidal chitin agar and were effective producers of chitinase and ß-1,3, glucanases which hydrolysed glucans from the pathogen cell wall and lysed living hyphae of *F. solani*. BCA1 failed to produce any diffusible inhibitory antifungal metabolites active against *F. solani*. Accordingly, the main mechanisms involved in disease reduction by BCA1 appears to be the production of chitinases and ß-1,3-glucanase. This conclusion was reached after extensive screening procedures to determine the production of diffusible antifungal metabolites, and a variety of enzymes for each antagonist. In addition to the ability of *Streptomyces* isolate (BCA1) to produce chitinase, ß-1,3-glucanase and to lyse the hyphae of *F. solani*, it also produced siderophores. The *in vitro* assays indicated that BCA1 in the final selection used siderophores and cell wall degrading enzymes as mechanisms of biocontrol.

Since the cell walls of *F. solani* consist largely of chitin and ß-glucans (Bartnicki-Garcia & Lippman, 1982), it was suggested that chitinase and ß-1,3-glucanase produced by the antagonist BCA 1 could be involved in disease control. The production of chitinases and ß-1,3- glucanase were therefore used as the criteria for selection of potential biocontrol agents against *F. solani*. Microbial chitinolytic enzymes have been considered effective in the biological control of several plant pathogens as they are able to degrade fungal cell walls (Nawani et al., 2002; El-Tarabily et al., 2003; Vinale et al., 2008). The exposure of phytopathogenic fungi to

lytic enzymes such as chitinases, proteases, or glucanases can lead to degradation of the structural matrix of fungal cell walls (Whipps, 2001; Doumbou et al., 2002). Valois et al. (1996) reported 13 actinomycete isolates with capability to produce ß-1,3, ß-1,4 and ß-1,6 glucanases hydrolyzed glucans from *Phytophthora fragariae* cell walls and caused hyphal lysis. They also confirmed that 11 strains also significantly reduced the root-rot disease of raspberry caused by *P. fragariae* (Valois et al., 1996).

Many bacterial genera such as *Serratia* (Sneh, 1981; El-Tarabily et al., 2000), *Aeromonas* (Inbar & Chet, 1991), *Chromobacterium* (Park et al., 1995), *Enterobacter* (Chernin et al., 1997), *Arthrobacter* (Sneh, 1981) and *Paenibacillus* (Singh et al., 1999) have been characterized to be successful biological control agents of several soil-borne phytopathogens. Chitinolytic actinomycetes previously used for *in vitro* experiments have included *Streptomyces* spp. (Gupta et al., 1995; El-Tarabily et al., 2000; Nawani et al., 2002), and non-streptomycete actinomycetes (*Actinoplanes* sp.) (El-Tarabily et al., 2003). Singh et al. (1999) used a chitinolytic *Streptomyces* sp. for the inhibition of cucumber wilt caused by *F. oxysporum*. Also, research carried out under greenhouse conditions, showed that a ß-1,3-glucanase-producing *Micromonospora carbonacea* decreased the incidence of *Sclerotinia minor*, the causal agent of basal drop disease of lettuce in the United Arab Emirates (El-Tarabily et al., 2000). This antagonist agent produced high levels of chitinase and ß-1,3-glucanase, and caused extensive hyphal plasmolysis, cell wall lysis (El-Tarabily et al., 2000).

In the present study BCA2 did not produce large clear zones on colloidal chitin agar and was not effective producers of chitinase and ß-1,3, glucanases and did not hydrolyse glucans from the pathogen cell wall also did not lyse living hyphae of *F. solani* when it was grown on colloidal chitin agar. However, BCA2 produced

diffusible inhibitory antifungal metabolites active against *F. solani*. Accordingly, the main mechanisms involved in disease reduction by BCA2 appears to be the production of diffusible inhibitory antifungal metabolites. It was also able to produce siderophore and auxin which participates in promoting plant growth. This conclusion was achieved after extensive screening procedures to determine the production of diffusible antifungal metabolites, and a variety of enzymes for each antagonist.

The cut plate method on fish meal extract agar used in this study gave a satisfactory result in terms of selecting potential inhibitory antagonists against *F. solani in vitro*. This technique is routinely used to detect *in vitro* antagonism between pathogens and antagonists (Crawford et al., 1993; Yuan & Crawford, 1995). The principle of this method is based on the production of active metabolites by the antagonist, which diffuses into the agar medium and in turn inhibits the growth of the pathogen. This method allows observations to be made on the effects of the antagonist on the growth and survival of a pathogen in agar culture.

Non-volatile antifungal metabolites produced by some bacteria and fungi are recognized to be inhibitory to particular soil-borne pathogens, have been extensively studied by means of the cut plate method (El-Tarabily et al., 1997). In the present study only BCA2 grown on fish meal extract agar using the cup plate method was effective in the deformation of the hyphae of *F. solani* and also lysed living hyphae of *F. solani*.

Although the two *Streptomyces* spp. (BCA1 and BCA2) were capable of suppressing the pathogen, they appear to do so employing different mechanisms. BCA1 was only capable of producing cell wall degrading enzymes and not producing antifungal metabolites, whilst BCA2 was only capable of producing antifungal

metabolites and not producing cell wall degrading enzymes. This may explain why BCA2 was superior in disease suppression, compared to BCA1.

The biocontrol activity of the antagonistic actinomycetes *in vivo* was positively correlated with *in vitro* inhibition trends. The two isolates were antagonistic to *F. solani* when applied individually, and significantly reduced wilt disease under controlled greenhouse conditions.

Chapter 5: Conclusion

Date palm tree is susceptible to serious soil-borne diseases including *Fusarium* wilts. This study identified *F. Solani* as the causal agent of *Fusarium* wilts of date palms in the UAE among two other *Fusarium* species isolated from infected tree. While concerns about the application of chemical fungicides increases, the use of biocontrol antagonistic agents to control phytopathogens is a promising alternative for disease control. In the current thesis, two antagonistic actinobacteria have proven their effectiveness in inhibiting *Fusarium* wilt causing agent *in vivo* and *in vitro* by producing antifungal metabolites and cell-wall degrading enzymes.

The long-term goal after this study is to achieve the protection of date palm health and its productivity and improve environment sustainability. Conventional breeding, plant biotechnology, and modern molecular tools (AbuQamar et al., 2017) could also generate breakthroughs for the production of new resistant varieties to SDS. There is also a need to test these treatments, on a field scale, to determine the feasibility of such a recommendation.

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List of Publications

Alwahshi, K. J., Saeed, E. E., Sham, A., Alblooshi, A. A., Alblooshi, M. M., El-Tarabily, K. A., & AbuQamar, S. F. (2019). Molecular identification and disease management of date palm sudden decline syndrome in the United Arab Emirates. *International Journal of Molecular Sciences*, *20*(4), 923. https://doi.org/10.3390/ijms20040923

Appendix

Media

The following media have been used in the present study. The media composition is listed below

1. Inorganic salt-starch agar (starch nitrate agar) (SNA) (Küster, 1959).
2. Oat-meal yeast extract agar (OMYEA) (Küster, 1959).
3. Colloidal chitin agar (Gupta et al., 1995).
4. Hussein's fish-meal extract agar (HFMEA) (El-Tarabily et al., 1997).
5. Water agar.
6. Modified chrome azurol agar (CAS agar) (Alexander & Zuberer, 1991).
7. Potato Dextrorse Agar (PDA) (Lab M Limited, Lancashire, UK).
8. Glucose peptone broth (GPB) (Di Menna, 1957).

Composition of Media

1. Inorganic salt-starch agar (starch nitrate agar) (SNA) (Küster, 1959)

Soluble starch	10 g
Di-potassium hydrogen phosphate	1 g
Magnesium sulfate	0.5 g
Sodium chloride	0.5 g
Potassium nitrate	2 g
Calcium carbonate	3 g

Ferrous sulfate	0.01 g
*Trace salt solution	1 mL
Cycloheximide (Sigma)	50 µg mL^{-1}
Nystatin (Sigma)	50 µg mL^{-1}
Distilled water	1 L
Agar	20 g

*Trace salt solution (Pridham et al., 1957) composed of: 0.1 mg per liter of each of the following salts: ferrous sulfate, magnesium chloride, copper sulfate and zinc sulfate.

2. Oat-meal yeast extract agar (OMYEA) (Küster, 1959)

Twenty grams of oat-meal were steamed for 20 minutes in 1 liter of distilled water and it was filtered through cheese cloth. Distilled water was added to proceed the filtrate to 1 liter. 1 gram of yeast extract (Sigma) and 20 grams of agar (Sigma) were added, and the final medium pH has been adjusted to 7.2.

3. Colloidal chitin agar (Gupta et al., 1995)

Preparation of colloidal chitin (Hsu & Lockwood, 1975)

Crude chitin (from crab shells, Sigma) was washed for 24 hours in 1 N NaOH and 1 N HCl (Sigma) alternately, for five times each. It was then washed with 95% (v/v) ethanol four times.

15 grams of the purified white chitin was then dissolved in 100 mL of concentrated HCl and stirred for 20 minutes in an ice bath. Then, the mixture was filtered through glass wool and poured into cold distilled water to precipitate the chitin.

The remaining insoluble chitin on the glass wool was treated with HCl again, and the process was repeated until no more precipitate was gained when the filtrate was added to the cold water. The colloidal chitin was allowed to settle overnight, and the supernatant was decanted. The remaining suspension was neutralized with NaOH to pH 7.0. The precipitated chitin had been centrifuged, washed with distilled sterile water, and stored at 4°C as a paste. The medium contained:

Colloidal chitin (Dry weight)	2 g
Calcium carbonate	0.02 g
Ferrous sulfate	0.01 g
Magnesium sulfate	0.05 g
Potassium chloride	1.71 g
Di-sodium hydrogen phosphate	1.63 g
Distilled water	1 L
Agar	20 g
pH	7.2

4. Hussein's fish-meal extract agar (HFMEA) (El-Tarabily et al., 1997)

Fish-meal extract	20 g
Glucose (Sigma)	20 g
Peptone (Sigma)	5 g
Sodium chloride	0.5 g

Calcium carbonate	3 g
Distilled water	1 L
Agar	20 g

5. Water agar (WA)

Distilled water	1 L
Agar	20 g

6. Modified chrome azurol agar (CAS agar) (Alexander & Zuberer, 1991)

CAS agar was prepared by mixing four separately sterilized solutions. Solution 1 which is the Fe-Chrome azurol S indicator solution was prepared by mixing 10 mL of 1 mM $FeCl_3$ (in 10 mM HCl) with 50 mL of an aqueous solution of Chrome azurol S (CAS) (1.21 mg mL^{-1}) (Sigma). The resulting dark purple mixture was slowly added to 40 mL of an aqueous solution of hexadecyltrimethylammonium bromide (HDTMA) (1.82 mg mL^{-1}) (Sigma) with continues with stirring. The resulting solution was then autoclaved separately and cooled down to 50°C.

Solution 2 which is the buffer solution was prepared by dissolving 30.24 g of PIPES buffer (Piperazine-N,N-bis[2-ethanesulonic acid) (Sigma) in 750 ml of a slat solution containing 0.3 g potassium di-hydrogen phosphate, 0.5 g sodium chloride, and 1 g ammonium chloride. The pH was set to 6.8 and water was added to raise the volume to 800 ml. 15 grams of agar was added and then the solution was autoclaved and then cooled to 50°C.

Solution 3 was made by adding 2 g glucose, 2 g mannitol, 493 mg magnesium sulfate, 11 mg calcium chloride, 1.17 mg manganese sulfate, 1.4 mg boric acid, 0.04 mg copper

sulfate, 1.2 mg zinc sulfate, and 1 mg sodium molybidate in 70 ml of water and then autoclaved and cooled to 50°C separately. Solution 4 is composed of 30 ml of Millipore membrane sterilized (pore size 0.22 µm, Millipore Corporation, MA, USA) 10% (w/v) casamino acids (Sigma). For getting the final solution, solution 3 was then added to solution 2 and the solution 4. Last, solution 1 was added with enough mixing to mix the ingredients of the four solutions without creating bubbles. The resulting medium was dark blue after adding the 4 solutions.

7. Potato Dextrorse Agar (PDA) (Lab M Limited, Lancashire, UK).

Distilled water	1 L
PDA Powder	39 g

8. Glucose peptone broth (GPB) (Di Menna, 1957)

Glucose (Sigma)	10 g
Peptone (Sigma)	5 g
L-Tryptophan (L-TRP) (Sigma)	5 mL of 5%
Distilled water	1 L

Root samples

A date palm roots were collected from a farm at Al-Wagan area in Al Ain city in United Arab Emirates (Abu Dhabi Emirate; Latitude/Longitude: 24.19/55.76), in January 2018.

Plant Material

Date palm seedlings were used in the present research.

Inorganic Liquid Fertilizer

(Thrive®) (Arthur Yates & Co Limited, Milperra, NSW, Australia). The chemical analysis (%) of the fertilizer was as follows: N as NO_3^{-3}, N as NH_4 2.6, N as NH_2CONH_2 21.4, P as water soluble 5.5, K as KNO_3 9, Mg as $MgSO_4$ 0.15, S as SO_4 0.22, Cu as $CuSO_4$ 0.005, Zn as $ZnSO_4$ 0.02, B as $Na_2B_4O_7$ 0.005, Mn as $MnSO_4$ 0.04, Fe as chelated Fe 0.18, and Mo as Na_2MoO_4 0.002.