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BIOLOGICAL CONTROL OF SUDDEN DECLINE DISEASE OF DATE PALM (PHOENIX DACTYLIFERA L.) IN THE UNITED ARAB EMIRATES

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Environmental Sciences

Under the Supervision of Professor Khaled A. El-Tarabily

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Declaration of Original Work

I, Khawla Juma Saif Zeail Alwahshi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Biological Control of Sudden Decline Disease of Date Palm (Phoenix Dactylifera L.) in the United Arab Emirates*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Prof. Khaled A. 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 Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions.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 orchards suffer from serious diseases, including sudden decline syndrome (SDS). The fungal pathogen *Fusarium solani* was associated with SDS on date palm in the United Arab Emirates (UAE). Application of Cidely® Top (difenoconazole and cyflufenamid) significantly inhibited the fungal mycelial growth *In vitro* and reduced SDS development on date palm seedlings pre-inoculated with *F. solani* under greenhouse conditions. This is the first report confirming that the chemical fungicide Cidely® Top is strongly effective against SDS on date palm. Fungal pathogens not only inhibit plant growth directly, but also induce the plant to synthesize stress ethylene. Much of the damage sustained by plants infected with fungal phytopathogens occurs as a result of the response of the plant to the increased levels of ethylene. The aim of the present study was to compare the effectiveness of antagonistic actinobacteria capable of producing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, with those incapables of producing ACC deaminase, on suppression and severity of SDS on date palm. To achieve this, actinobacteria-producing ACC deaminase isolated from the rhizosphere of date palm in the UAE were evaluated for their potential to produce antifungal metabolites and cell-wall degrading enzymes that can inhibit the pathogens growth *In vitro*. The most inhibitory isolates produced chitinase, diffusible antifungal metabolites, siderophores, and were able to lyse the hyphae of the three pathogens *In vitro*. Under greenhouse conditions, the ACC deaminase-producing isolates were significantly more effective in reducing the incidence and severity of SDS compared to ACC deaminase-non-producing isolate. This study demonstrated for the first time the superiority of antagonistic rhizosphere actinobacteria to enhance their effectiveness as biocontrol agents by their abilities to produce ACC deaminase in addition to their abilities to produce antifungal metabolites and cell-wall degrading enzymes. The results clearly showed the potential to enhance most if not all the biocontrol agents performance by including the ACC deaminase ability into the strains.

Keywords: Date Palm, Sudden Decline Syndrome, *Fusarium Solani*, Actinobacteria, ACC Deaminase, UAE.

Title and Abstract (in Arabic)

المكافحة البيولوجية لمرض الانخفاض المفاجئ لنخيل التمر في دولة الإمارات العربية المتحدة

الملخص

تعاني بساتين النخيل من أمراض خطيرة، بما في ذلك متلازمة الانخفاض المفاجئ (SDS). ارتبط الفطر *Fusarium solani* بمرض SDS في نخيل التمر في الإمارات العربية المتحدة. في الدراسة الحالية أدى استخدام Cidely® Top (difenoconazole و cyflufenamid) إلى تثبيط نمو الفطريات في المختبر بشكل كبير وخفض تطور SDS على شتلات نخيل التمر المحقونة مسبقًا بـ *F. solani* في ظروف البيوت البلاستيكية. تعتبر الدراسة الحالية هى الأولى من نوعها و التى تؤكد أن مبيد الفطريات الكيميائي Cidely® Top فعّال بشكل كبير ضد SDS على نخيل التمر. لا تمنع الفطريات الممرضة نمو النبات بشكل مباشر فحسب، بل تحفز النبات أيضًا على تخليق هرمون الاجهاد الفسيولوجى والمعروف باسم هرمون الإيثيلين. تحدث الكثير من الأضرار للنباتات المصابة بالفطريات نتيجة لاستجابة النبات لزيادة مستويات هرمون الإثيلين. كان الهدف من هذه الدراسة هو مقارنة فعالية الأكتينوبكتيريا القادرة على إنتاج إنزيم ديامينياز ACC، مع أولئك غير القادرين على إنتاج ديامينياز ACC، على تثبيط شدة SDS في نخيل التمر. لتحقيق ذلك، تم تقييم الأكتينوبكتيريا المُنتِجة لأنزيم ديايمينياز ACC والمعزولة من التربة حول جذور نخيل التمر في دولة الإمارات العربية المتحدة من حيث قدرتها على إنتاج مواد كيمائية قاتلة للفطر مسبب المرض وايضا على انتاج الإنزيمات المحللة لجدار الخلية والتي يمكن أن تمنع نمو الفطريات في المختبر عن طريق تحليل هذه الجدر الخلوية. أنتجت معظم العزلات المثبطة إنزيم الكيتينز بالإضافة إلى إنتاج مواد كيمائية قاتلة للفطريات، siderophores، كما كانت قادرة على تحليل خيوط الفطر الممرض في المختبر. تحت ظروف البيوت البلاستيكية، كانت العزلة المنتجة لـ إنزيم ديامينياز ACC أكثر فاعلية في تقليل حدوث وشدة SDS مقارنة بالعزلة غير المنتجة لـ إنزيم ديامينياز ACC. أثبتت هذه الدراسة لأول مرة تفوق الأكتينوبكتيريا المتنافسة في منطقة الجذور (rhizosphere) على تعزيز فعاليتها كعوامل تحكم بيولوجي من خلال قدرتها على إنتاج انزيم ديايمينياز ACC بالإضافة إلى قدرتها على إنتاج مواد كيمائية قاتلة للفطريات والإنزيمات المحللة للجدار الخلوي. أظهرت النتائج بوضوح القدرة على تعزيز معظم عوامل

المقاومة البيولوجية إن لم يكن كلها عن طريق تضمين قدرة إنتاج إنزيم دايامیناز ACC في السلالات المستخدمة فى المقاومة أو المكافحة البيولوجية أو الحيوية.

مفاهيم البحث الرئيسية: نخيل التمر، متلازمة الانخفاض المفاجئ، الفطر *Fusarium solani*، الأكتينوبكتيريا، إنزيم دايامیناز ACC، الإمارات العربية المتحدة.

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Dedication

To my beloved parents, family, and friends

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List of Abbreviations

ACC	1-Aminocyclopropane-1-Carboxylic Acid
AM	Arbuscular Mycorrhiza
ANOVA	Analysis of Variance
BCA	Biological (biocontrol) Control Agent
CAS	Modified Chrome Azurol Agar
CWDEs	Cell Wall Degrading Enzymes
DBF	Date Bunch Fading Disease
DF	Dworkin and Foster's Salts Minimal Agar Medium
DPI	Day Post Inoculation
DPT	Day Post Treatment
DSI	Disease Severity Index
FAO	Food and Agriculture Organization
FOA	*Fusarium oxysporum f. sp. Albedinis*
HDTMA	Hexadecyltrimethylammonium Bromide
HFMEA	Hussein's Fish-Meal Extract Agar
IDM	Integrated Disease Management
ISR	Induced Systemic Resistance
ISSA	Inorganic Salt Starch Agar
LY	Lethal Yellowing
OF	Organic Farming
OMYEA	Oat-Meal Yeast Extract Agar
PDA	Potato Dextrose Agar
PGPR	Plant Growth-Promoting Bacteria
RPW	Red Palm Weevil

SA	Salicylic Acid
SDS	Sudden Decline Syndrome
SEM	Scanning Electron Microscopy
SNA	Starch Nitrate Agar
UAE	United Arab Emirates
WPI	Week Post Inoculation

Chapter 1: Introduction

1.1 Overview

Throughout the history, date palm (*Phoenix dactylifera* L.) is considered as main cultivar that sustain human life in most of Arab countries. It has historical, traditional, nutritional as well as religious values. It possesses its value as it offers a wide range of products and services, which covers main necessities of life (El-Juhany, 2010).

Date palm withstand high temperatures, drought and relatively can tolerate salty and alkaline soil (Chao & Krueger, 2007). These characteristics allowed this cultivar to have wide geographical distribution. The majority are located in the Middle East and North Africa although the cultivar has been introduced in California, Arizona and Mexico in the Americas (El Hadrami & Al-Khayri, 2012). In the United Arab Emirates (UAE), date palm cover a huge agricultural areas, it retains the largest number of date palm in the world which reached up to 42 million trees (El-Juhany, 2010).

Unfortunately, date palm in UAE and many other countries are under threats as a result of pests and diseases which lead to huge economic loss. The nature and severity of the problems vary with cultivar, location, weather, and cultural practices (Carpenter & Elmer, 1978; Zaid, 2002). Pathogenic fungi are attributed to most reported date palm diseases (Chao *et al.*, 2007; Saleh *et al.*, 2017) and *Fusarium* spp. is the most important genus among them (Saleh *et al*., 2017). *Fusarium* spp. is a soil-borne fungus that cause sudden decline which is also known as *Fusarium* wilt. The

fungus attack date palm's root causing rot, then wilting and decline symptoms appear (Alwahshi *et al*., 2019).

A combination of cultural practices and fungicide applications are used to control this disease (Abul-soad *et al.*, 2011; Salim *et al.*, 2015). In the modern agricultural systems plant growth promoting bacteria and fungi are considered as a suitable alternative solution comparing with agrochemical products to control any diseases. Using diverse group of microorganisms which appear to have a potential for the biological control of *Fusarium* disease can provide a dual affect [(i.e. control plant disease and at the same time promote plant growth (Doumbou *et al.*, 2001; Whipps, 2001; El-Tarabily & Sivasithamparam, 2006).

1.2 Statement of the Problem

Date palm is one of the main agricultural crops that support different aspects of human life. It is known that maintaining healthy and productive date palm plantations encountered many obstacles, constraints and challenges. Controlling pests and diseases to be less than economic threshold considered as major issue (Chao *et al.*, 2007; El-Sabea *et al.*, 2009). One of which, *Fusarium* wilt or as known sudden decline syndrome (SDS) of date palm. This disease caused by *Fusarium spp.* which is the most causal fungal agent in date palm (El Hadrami I. & El Hadrami A., 2009). The disease is studied but need more investigations. Therefore, finding an alternative eco-friendly way to control the disease is in highly demand which support UAE future prospective. Thus, this study was conducted to determine the chemical control treatment using different available fungicides and determine the biological control treatment using antagonistic rhizosphere actinobacteria.

1.3 Origin and distribution of Date Palm

Date palm tree is one of the most important species in the palm family (Arecaceae), which include nearly 200 genera and more than 2,500 species. It is known that 14 species including *Phoenix dactylifera* belong to the genus *Phoenix*. It is considering to be one of the oldest planted and the oldest radiocarbon dated discovery of date seed was in 5110 B.C. on Dalma island, part of the Abu Dhabi Islands group species (El Hadrami I. & El Hadrami A., 2009; El Hadrami *et al.*, 2011).

Date palm is a crop capable of adapt in arid conditions where the annual precipitation rarely exceed 250 mm along with hot summers up to 50°C and cold winters down to -10°C (El Hadrami & Al-Khayri, 2012). Therefore, it is known as native tree in arid regions of the Arabian Peninsula, North Africa, and the Middle East. Additionally, new production areas such as Australia, India/Pakistan, Mexico, southern Africa, South America, and the United States were starting to introduce date palm during the past three centuries (Chao & Krueger, 2007).

1.4 Importance of Date Palm

Date palm has religious considerations and it is mentioned in several verses in Quran as tree with multiple uses and benefits. In many Arab and Muslim countries dates were consumed in daily basis. Nowadays, it has been used not only as fruits but also to produce and prepare other secondary food components such as baking products, confectionery and healthy food (Barreveld, 1993). In general, dates are rich in vitamins and other beneficial compounds that enhance human health.

It is known that date palm possesses many essential importance which support human since ages, it offered shade and protection for people, thus help them to settle

and establish an organized community. Moreover, it provided them with many necessities of life where its different parts like leaves, woody leaves midribs and trunk fibres have been used as a material component to made various objects including baskets, bags, mats and various building's material.

Environment protection is another important fact about date palm since it is controlling the desertification and create microclimate in the desert, enhancing the availability of vegetables and fruits which support people and their livestock (El Hadrami & Al-Khayri, 2012). In addition of that, date palm plays an important role in land reclamation generally in the Arabian Peninsula and precisely in UAE. In recent time, date palms used for ornamental and landscaping in southern Europe and some United states (Chao & Krueger, 2007).

1.5 Major Pests and Diseases of Date Palm

Numerous pests threaten date palm cultivars, among of which diseases and arthropods pests which lead to 30% reduction in production (Gitau *et al*., 2009). Considerable date palm degradation related to mites and insects. Ten species are known as major date palm arthropods pests which are, the red palm weevil (RPW) (*Rhynchophorous ferrugineus*), Old world date mite (*Oligonychus afrasiaticus*), lesser date moth (*Batrachedra amydraula*), Dubas date bug (*Ommatissus binotatus*), green pit scale (*Palmapsis phoenicis*), carob moth (*Ectomyelois ceratoniae*), longhorn date palm stem borer (*Jebusaea hammerschmidti*), rhinoceros beetle (*Oryctes agamemnon*), fruit stalk borer (*Oryctes elegans*) and almond moth (*Cadra cautella*) (El-Shafie, 2012).

1.5.1 Red Palm Weevil as Key Arthropod Pest of Date Palm

According to the Food and Agriculture Organization (FAO), RPW was identified as a category-1 pest of date palm in the Middle East. In addition, Abraham *et al.* (1998) have pointed that RPW is a major pest of date palm. RPW was reported in the Northern Emirates of UAE in 1985, since then it has spread rapidly to all the countries of the Gulf region (El-Sabea *et al*., 2009). The adult exists in the buried part of the trunk, precisely in the soil where they aggregate, feed, mate and lay their eggs in the palm tissue. Then larvae from hatched eggs feed extensively on the surrounding soft tissue and weakening the structure of the trunk which might completely hollowed out and the trees dies (El-Sufty *et al*., 2011). Commonly the late detection of the infestation lead to the death of the tree. Abraham *et al.* (1998) mentioned that the infested date palm showed one of these symptoms according to the infestation stage: (1) existence of tunnels on the trunk and base of frond petiole, (2) chewing sound due to larval feeding, (3) presence of chewed plant tissues (frass) with fermentation smell, (4) presence of empty pupal cases and dead adults around a highly infested tree, (5) broken trunk or fallen crown in case of severe and (6) prolonged infestation and dryness of the infested offshoots.

In UAE, RPW severely damage date palm plantation causing adverse consequences. For instance, in Abu Dhabi Emirate, total number of RPW captured by 125,061 traps were reached approximately 3,982,847. While the number of date palm trees removed due to RPW infestation roughly reached 53.488 (ADAFSA, 2019). Figure 1 shows larval and adult stage of red palm weevil.

Figure 1: Larval and adult stage of red palm weevil (El-Sabea *et al*., 2009)

1.6 Diseases of Date Palm

Date palm afflicted with many diseases that affect its production. The well-known causes of these diseases are fungi and phytoplasma (Abdullah *et al*., 2010). On the other hand, nematodes have been reported in date palm however it is not been recognized as production limiting factor in regions with date as an ancient culture (Griffith *et al.,* 2005).

1.6.1 Diseases Caused by Phytoplasma

Only few studies have been revealed the occurrence of phytoplasma in date palm. Generally, there are four main diseases caused by phytoplasma which are Al Wijam, lethal yellowing, white tip die-back and slow decline (Abdullah *et al.,* 2010).

1- Al Wijam

Al Wijam disease along with RPW are mainly the most important pests and diseases that infest date palm in Saudi Arabia (El-Juhany, 2010). Both 16SrI, *Candidatus, Phytoplasma asteris* group and lethal yellowing like 16SrIV phytoplasma have been reported in Al-Wijam diseased palms in Al Hassa oasis eastern of Saudi Arabia. Another phylogenetic analysis in Al-Giza Governorate in Egypt showed that infested date palms have similar isolate clustered with the date palm phytoplasma

causing Al-Wijam disease in Saudi Arabia (Alkhazindar, 2014). The typical symptoms associated with the disease are leaf stunting, yellow streaking and a noticeable reduction in fruit and stalk size, which progresses to no fruit production in the final stages (Figure 2) (Alhudaib *et al*., 2008).

Figure 2: Typical symptoms of Al Wijam disease (Alhudaib *et al*., 2007)

2- Lethal Yellowing

Lethal yellowing (LY) is a phytoplasma disease which spread rapidly causing death of various palm species. The suggested name for the pathogen is *'Candidatus Phytoplasma palmae*' and planthopper *Myndus crudus* is the common cause of its dispersal (Harrison *et al*., 2008). As Abdullah *et al.* (2010) reported, both Al-Awadhi *et al.* (2002) in Kuwait and Ammar *et al.* (2005) detected phytoplasma associated with yellowing disease in date palm. Disease symptoms diverge according to the palm species and involve premature drop of the fruit, foliage discoloration, and death of the apical meristem. After the first recorded symptoms the diseased palms mainly die within 3 to 5 months (Harrison & Elliott, 2005).

3- White Tip Die-back

In this disease the phytoplasma attack young date palm about 5 to 8 years old causing severe chlorosis of the emerging leaf and at the tip of the pinnae of older fronds which change rapidly from green to dry white without showing yellowish symptoms in the crown (Cronjé *et al*., 2000a).

4- Slow Decline

The study showed similarity between the phytoplasma associated with slow decline and the phytoplasma associated with white tip-dieback however the phytoplasma in slow decline reported in mature date palms. Moreover, it survives 1 to 2 years until die after the first symptoms appear comparing with the phytoplasma associated with white tip-dieback which survives till 6-12 months since the symptoms appeared. Symptoms of the disease appear as yellowing in the outer fronds which progress to the central newly emerging fronds and leaves. Then all the infected fronds dry and appear white to pale brown till fall off the trunk. Generally, the disease considered with low economic importance (Cronjé *et al*., 2000b).

1.6.2 Diseases Caused by Fungal Pathogens

Fungal pathogens are the main cause of date palm diseases compared with phytoplasma. Fungal diseases vary in their economic importance according to the severity of the symptoms which consequently affect the production and the yield or cause death of the tree. For instance, there are many diseases caused by fungi with innocuous symptoms or consequences such as *Diplodia* and *Botryodiplodia* disease, brown leaf spot and balâat disease (Abdelmonem & Rasmy, 2007). While Bayoud, black scorch, khamedj and wilt or decline of date palm which caused by *Fusarium*

species are commonly known as diseases with major economic importance (Abdelmonem *et al.*, 2007; Abdullah *et al.*, 2010). Figure 3 shows symptoms of black scorch disease on Leaves, tilted apical top and the whole plant.

Figure 3: Symptoms of black scorch disease on (A) Leaves, (B) tilted apical top and (C) the whole plant (Saeed *et al.*, 2016)

1.7 *Fusarium* Species Associated with Wilt and Sudden Decline Diseases of Date Palm

As mentioned before date palm groves suffer from various fungal pathogen however, the danger precisely resides in the genus *Fusarium* which includes many economically destructive foot and root rot pathogenic species of diverse hosts. In addition, some *Fusarium* members produce mycotoxins that are highly toxic to plants and has the ability to disperse easily since it has been detected in soils, surface and drainage water (Desjardins *et al.*, 2006; Hartmann *et al.*, 2008). Moreover, numerous *Fusarium* species are soil-borne and, according to the ecological context, might be parasites, endophytes, or pathogens of healthy host plants (Aoki *et al.*, 2014).

1.8 Taxonomy of *Fusarium* Species

The first description of the genus *Fusarium* was in 1809 by Link based on production of its iconic fusiform multiseptate macroconidia and it was accepted later

in 1821 by Fries as follow: Kingdom: Fungi, Phylum: Ascomycota, Class: Sordariomycetes, Order: Hypocreales, Family: Nectriaceae, Genus: *Fusarium*.

Many species within the genus *Fusarium* represent notable differences in morphological, cultural and physiological characteristics. This capability could illustrate the pervasive existence of *Fusarium* in diverse ecological niches in most geographical areas around the world. Thus, developing firm and vastly accepted taxonomic system for this genus was difficult as a result of variation among the species (Toussoun & Nelson, 1975). However, general characteristics of *Fusarium* might aid in distinguishing species. To interpret, *Fusarium* species may produce three types of spores called macroconidia, microconidia, and chlamydospores (Nelson *et al*., 1983), hence morphology of the macroconidia, the presence or absence of microconidia, the morphology of the conidiophores bearing the microconidia, the presence or absence of chlamydospores can be take in consideration as a primary characteristic in *Fusarium* taxonomy. On the other hand, the morphology and pigmentation of the colony, including the presence or absence of sporodochia, sclerotia, or stroma are useful in describing species grown in culture under standard environmental conditions of light, temperature, and substrate as secondary characteristics in *Fusarium* taxonomy (Nelson *et al*., 1994). Lately, DNA analysis of species have been used for *Fusarium* identification, especially for species which cannot be featured based on morphological character (Rahjoo *et al*., 2008).

The recent estimation shows that *Fusarium* comprise at least 300 genealogically exclusive phylogenetic species in which most of them are soil-borne (Aoki *et al*., 2014). Many species among the genus *Fusarium* are pathogenic to many economically important crops including date palms, vegetables, and field crops. It can

persist in soil for decades and easily transferred by wind, water and equipment (Tuxbury *et al.*, 2014; Al-Sadi *et al.*, 2015). Generally, many *Fusarium* species have been recorded as date palm pathogens across the world, however there were three main species which include; *F. proliferatum*, *F. solani* and *F. oxysporum* (Saleh *et al*., 2017).

1. *Fusarium oxysporum*

Bayoud is the most devastating fungal disease of date palm. The causal agent *Fusarium oxysporum f.sp. albedinis* (FOA) is the most serious among *Fusarium* species (Zaid, 2002; Elkinany *et al*., 2017). The disease was first recorded in Morocco more than a century ago, and the name was derived from Arabic work "Abiadh" which means white because the symptoms appear as whitening in the infected palm fronds (Benzohra *et al*., 2015). Up to date the disease is restricted to eastern part of North Africa (Abdullah *et al.*, 2010) and lead to pernicious consequences. It was responsible for loss of more than 15,000,000 trees in Morocco and Algeria in less than century (Djerbi, 1982). In addition, Bayoud disease attack date palm with high quality dates with less resistance such as “Medjool and “Deglet Noor” which are no longer be grown commercially in Morocco and Algeria. For meantime, the disease is still spreading through North Africa countries (Djerbi, 1982; Mahmoudi *et al.*, 2008; El Hadrami I. & El Hadrami A., 2009). The disease evidenced by a progressive fading and wilting of the leaves in distinctive pattern where it turns to be grey then white from bottom to top. Additionally, the spines of the infected fronds whiten from base to top, then the whitening progress on other side of the fronds from base to apex, with a brown discoloration along the midrib of the leaves. Thereafter, the leaf appears arched, like a wet feather and hangs down along the trunk. Spreading of the disease symptoms

resulting in whitening and dying of the pinnae and leaves may take a few days to several weeks. Moreover, roots of the affected palm showed reddish colour, as well as the cutted out palm fronds which exhibits the external symptoms of the disease. Thus, a continuing of vascular symptoms is existing from roots of the palm to the tips of the fronds. The palm dies when the terminal bud is affected, and the death could be occurred between 6 weeks to 2 years after the appearance of the first symptoms according to the cultivar and the planting conditions. Finally, offshoots at the base of the palm tree are attacked and infected (Djerbi, 1982; Zaid, 2002; Abdullah *et al*., 2010; Benzohra *et al*., 2015).

Several studies showed that *F. oxysporum* can cause date palm root rot/necrosis symptoms (Mandeel, 2006; Al-Sadi *et al.*, 2012). Furthermore, in Iran (Ahwaz region), it has resulted in severe wilting disease which lead to reduction in dates production (Saremi & Saremi, 2013). Likewise, in Iraq it found that *F. oxysporum* was one of the prevalent fungus associated with date palm decline and death (Sarhan, 2001).

On the other hand, symptoms very similar to Bayoud disease appear on date palm of Al Qassim and Al Medina Al Monawara regions which located in Saudi Arabia. The investigation showed that the tested *F. oxysporum* strain that obtained from diseased date palm has limited virulence compared with other *Fusarium* strains. This may indicate the existence of saprophytic population in the soil (Abdullah *et al*., 2010).

2. Fusarium proliferatum

Many date palms growing countries such as Saudi Arabia, Spain, Canary Islands and USA have been reported *F. proliferatum* as date palm pathogen. The symptoms of the infected date palms including wilt and dieback (Abdalla *et al.*, 2000;

Armengol *et al.,* 2005; Hernández-Hernández *et al.*, 2010). A recent study in Saudi Arabia reveals that *F. proliferatum* is the most frequently isolated species from date palm tissues with fungal disease symptoms recovered from different areas around the country (Saleh *et al.*, 2017). The frequency of *F. proliferatum* isolation from diseased palms showed that it could be considered as a potentially dangerous pathogen of Saudi date palm (Abdullah *et al*., 2010).

In Iraq results of survey and investigations conducted in several date palm groves for almost six years, illustrated that *F. proliferatum* cause rapid death and complete dryness of the date palm. The fungus produces toxins and move through the xylem vessels of the trunk preventing the transmission of water and nutrients which leads to the quick death of the date palm (Alyasiri *et al*., 2016). Moreover, *F. proliferatum* was recognized in Iran as the causal agent of date bunch fading disease (DBF) (Mansoori, 2012). In addition, Hameed (2012) identified *F. proliferatum* as the causal of date palm inflorescence rot disease in southern part of Iraq.

3. Fusarium solani

F. solani also known as the third most important *Fusarium* species that is associated with date palm diseases worldwide. Although, it is not as dominant as *F. proliferatum*, it is identified as the main causal for several diseases in different countries (Saleh *et al.*, 2017). On the other hand, recent study found that *F. solani* was the most dominant pathogenic fungus associated with date palm root in UAE by (82%) (Al-Hammadi *et al*., 2019). In both Iraq and Iran, *F. solani* has been found to cause yellow death disease, a comparable disease symptom described as yellowing and death of fronds which lead to deterioration of date palm (Mansoori & Kord, 2006; Al-Yasiri *et al*., 2010). The disease called the yellow death (Mansoori & Kord, 2006).

Furthermore, *F. proliferatum* and *F. solani* were isolated from declined date palm in Iraq and Egypt (Rashed & Abdel Hafeez, 2001; Sarhan, 2001). Whereas in Saudi Arabia and Pakistan, *F. solani* was identified as the causal agent of sudden decline disease. The fungus is existing frequently in the rhizosphere causing yellowing of leaves. The most virulence strain may generate numb dates phenomenon due to the production of fungal toxins. The toxin weakening and ruining xylem vessels thus preventing the access of water and nutrients for the stalk (Maitlo *et al.*, 2013; Alyasiri *et al.*, 2016). According to Al-Sadi *et al.* (2015), *F. solani* cause root diseases of date palm in Oman. Figure 4 shows symptoms of sudden decline disease in Pakistan.

Figure 4: Symptoms of sudden decline disease in Pakistan (Maitlo *et al*., 2013)

1.9 Fusarium Diseases Control Measures

There are several pest management strategies which can be obtained to control *Fusarium* diseases. These strategies could be followed before, during or after the infection occurs (Mahmoudi *et al.*, 2008). Attain complete control of the disease is very difficult but minimizing the adverse consequences might be achieved through the following measures:

1. Prophylactic measures

Prevention is the first line of defence, thus creating an appropriate preventive measure may assist in avoiding fungal disease outbreaks (Mahmoudi *et al*., 2008). Saremi and Saremi (2013) pointed one of the evaluated approaches which is soil solarization which can help in developing management control strategies. Promising results have been demonstrated in *Fusarium* propagules reduction after the solarization process. In addition of that, strict hygienic precautions which include sterilization of trimming and pruning tools can mitigate disease outbreaks since *Fusarium* isolates can be transmitted during agricultural practices (Saremi & Saremi, 2013; Saleh *et al.*, 2017). Moreover, disposal of diseased palms, leaves and inflorescences considered as important implementation of prophylactic measures according to UN, 2003 in Mahmoudi *et al.* (2008). Adhere to strict quarantine which prohibit using infected seedlings and ensure planting the offshoots of healthy date palms as well as production of resistant cultivars known as essential practices can be used to prevent the occurrence and the spread of the disease (El Modafar, 2010; Ibrahim *et al.*, 2015). One of the most important prophylactic measure is controlling date palm parasitic insects especially those which could play role as disseminating factor of fungal spores such as *Oryctes elegans* (date palm borer) and *Rhynchophorus ferrugineus* (red palm weevil) (Khudhair *et al.*, 2015; Salgado-Neto *et al.*, 2016; Saleh *et al.*, 2017)

Finally, preventive methods are not favorable especially in areas where date palm groves were contaminated, yet the Tunisian palm groves which not infected by FOA are currently protected almost by prophylactic methods (Jaiti *et al*., 2007).

2. Cultural measures

Cultural measures are not recommended especially after the infection occurs. Nevertheless, the non-infected date palm can be isolated by digging a trench of 2 m deep around them. Moreover, providing the irrigation water through trough bridging which assure the total isolation of this non-infected trees. These cultural measures may secure healthy date palms for more than 10 years (Benzohra *et al.*, 2015).

3. Chemical measures

Control of soil-borne disease pathogens is uneconomic except in limited areas which is free of the disease (Abdullah *et al.*, 2010). However, chemical measures using fungicides are commonly used practice to control plant diseases. In the eighties, soil fumigation by methyl bromide was common practice as control measure in Algeria which is banned nowadays (Benzohra *et al.*, 2015). On the other hand, several conducted studies evaluated the sensitivity of *Fusarium* species to various fungicides. To illustrate, Rashed and Abdel Hafeez (2001) were pointed that Tposin M70 and corpus were the best fungicides to control date palm decline in Egypt. While Bavistin DF which belong to carbendazim group were found to be helpful in controlling sudden decline disease of date palm in parallel to proper sanitation irrigation and fertilization according to Maitlo *et al.* (2013). Additionally, it was mentioned that salicylic Acid (SA) protects date palm from *Fusarium* wilt and it was confirmed that SA stimulate the resistance of date palm by promoting induced chemical defenses (Dihazi *et al.*, 2003; Dihazi *et al.*, 2011). Lastly, it is important to shed a light on one of the side effects of using excess amount of fungicides which is the enhancement of *Fusarium* species resistance (Al-Sadi *et al.*, 2015). They also pointed out that some of *F. solani*

species obtained from date palm showed a resistance to hymexazol which is one of the most used fungicides to control *Fusarium* diseases.

4. Biological measures

Despite the fact of the effectiveness of chemical measures, implementation of chemical products is not recommended because of its negative impacts on environment and human health so finding an alternative measure is demanding (Elkinany *et al.*, 2017). Breeding resistant varieties to *Fusarium* is a necessity and can be consider as an effective and economic alternative way to control date palm *Fusarium* diseases (Saaidi, 1992; Alyasiri *et al.*, 2016). A scientific Moroccan and French collaboration achieved a rapid and efficient selection of Bayoud resistant individuals from the large number of date palm trees obtained by natural crosses which display good date quality. According to Quenzar *et al.* (2001) the diagnostic tool based on the presence or absence of two plasmids-like DNA (the S and R plasmid) in mitochondria as a credable molecular marker of resistance or susceptibility to Bayoud disease. Additionally, the pretreated seedlings of date palm with an hypoaggressive isolate of *Fusarium* species might be protected partially from further infection with the pathogen. The protection comprised biochemical interaction between the host plant and the disease pathogen (El Hassni *et al.*, 2004a).

Secondary metabolites are organic compounds which do not directly involved in primary metabolic processes of growth and development but are important components of resistance/tolerance to stress. To illustrate, the secondary metabolites play role in strengthen mechanically the cell walls and serve as defenses against herbivores, pathogens, ultraviolet radiation, and other abiotic stress. In addition, some of them have allelopathic activity and may adversely influence the growth of

neighboring plants (El Hadrami *et al.,* 2011). Moreover, secondary metabolites of date palm have been assured to be harmful to plant pathogens thus it can be considered as a biological solution. For instance, some of heaped secondary metabolites encompass the ability to cause significant alterations to the growth and development of the pathogens. Moreover, multiple studies have demonstrated that constitutive an induced hydroxycinnanates was involved in date palm resistance against Bayoud disease and other abiotic stresses such as salinity and drought (El Hassni *et al.,* 2004b; El Hadrami *et al.,* 2011).

Application of compost and/or its extract in the soil increased in the last years due to its ability to control several soilborne diseases pathogens including some *Fusarium* species (El-Masry *et al.,* 2002; Pane *et al.,* 2012). Microbiological properties are one of the main factors that affect the efficiency of the compost (Pane *et al.,* 2012). Microbes aggressivity demonstrated by microbiostasis, antibiosis and hyperparasitism and/or stimulate systemic resistance in host plants (Diánez *et al*., 2007). ElKinany *et al.* (2017) have pointed that *Fusarium* was suppressed efficiently *In vitro* by unsterilized compost extract. Furthermore, addition of arbuscular mycorrhiza (AM) fungi which colonize plant roots aid in the protection of date palm against Bayoud disease (Jaiti *et al.,* 2007).

Recent researches used microorganisms (bacteria including actinobacteria and fungi) which have antifungal activity to control diseases (Dihazi *et al.,* 2012; Saeed *et al.,* 2017a; Kamil *et al.,* 2018). This called biological control or biocontrol which means “The usage of living organism “biological control agent or BCA” to inhibit the impact or the density of other pest organism by reducing its population or the damage that may cause” (Eilenberg *et al.,* 2001).

1.10 Characteristics of Actinobacteria

Actinobacteria is a Gram-positive microorganism present abundantly in both terrestrial and aquatic ecosystems. It has an eminent morphological diversity features, since it varies from simple cocci to complex mycelial forms (Niu, 2018). In addityon, it has a distinct diversity in function. Some are pathogens of humans, animals, and plants; major agents of symbiotic nitrogen fixation (*Frankia* spp.); industrially important producers of amino acids, antibiotics, cell wall degrading enzymes. Genera such as *Streptomyces*, *Micromonospora*, *Saccharopolyspora* and *Actinoplanes* well identified as the richest natural source of antibiotics and other secondary metabolites; probiotic bifidobacteria; and agents of bioremediation, notably rhodococci (Ventura *et al.*, 2007; Chandra & Chater, 2014).

The rhizosphere actinobacteria called rhizobacteria which can be in general defined as those having mutual relationship with plants but not including the soil profile. These rhizobacteria can exist as free living with direct contact to plant surface or exist inside the root which called endophytic bacteria. Furthermore, these bacteria defined as plant growth-promoting bacteria (PGPR), as they have the ability to assist and stimulate plant growth (Kloepper *et al.*, 2004; Kang *et al.*, 2010; Parray *et al.*, 2016).

1.11 Mechanism of Action of Actinobacteria

Biological control agents including actinobacteria have various mechanisms to control pathogens, such as (i) competition with the pathogen over nutrients, (ii) physical displacement of the pathogen, (iii) secretion of siderophores or antibiotics (iv) inducing enzyme production to suppress pathogen growth (v) stimulate systemic resistance of the plant (vi) biosynthesis of stress related phytohormones like cytokinin

and enzymes such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Arora *et al.,* 2012; Parray *et al.,* 2016). Generally, mechanisms of action can be divided into direct and indirect as explained below:

1.11.1 Direct Mechanisms

Enhancement of plant growth in the absence of the pathogen, whereas the rhizobacteria facilitate water and nutrient uptake, affect root pattern and nurturing plant (Parray *et al.*, 2016). The rhizobacteria metabolized organic matter surrounding the root system producing carbon and nitrogen which can be utilized by plant for growth (Kang *et al.*, 2010). These microorganisms play an important role in nitrogen fixation and phosphate solubilization which make nitrogen and phosphate elements in a preferable form for plant uptake (Parray *et al.*, 2016).

Another indirect mechanism is siderophore secretion which act as solubilizing agents for iron from minerals and organic material, as well as, forming stable complexes with heavy metals and radionuclides which mitigate plant stresses and increase soluble metal concentration (Neubauer *et al.*, 2000; Indiragandhi *et al.*, 2008; Rajkumar *et al.*, 2010). Plants use iron from bacterial siderophores by chelation and release of iron or by ligand exchange reaction (Schmidt, 1999).

Additionally, most of microbial communities that lives in rhizosphere possess the ability of phytohormones production such as auxins, gibberellic acid, and cytokinins. Therefore, existence of these microorganisms increases root surface and number of root tips as well as induce shoot growth (Han *et al.*, 2005; Boiero *et al.*, 2007; Kloepper *et al.*, 2007).

The activity of ACC deaminase considered as indirect mechanism as well. Actinobacteria that have ACC deaminase enzyme support plant growth and development under abiotic stresses like drought and salinity. Likewise, ACC deaminase-producing bacteria alleviate biotic stresses such as effect of phytopathogens. Consequently, the noteworthy effects of ACC deaminase production in root system area are promotion of root and shoot growth, increases N, P and K uptake and mycorrhizal colonization in different cultivars (Nadeem *et al.*, 2007; Zahir *et al.*, 2008; Glick, 2012).

1.11.2 Indirect Mechanisms

Many Plant growth promoting bacteria (PGPB) have been commercialized as biocontrol strains since they can produce antibiotics that prevent the reproduction of certain fungal plant pathogens. Furthermore, some of these PGPB have the capability to lyse a portion of cell walls of various pathogenic fungi via production of lytic enzymes such as chitinase (Singh *et al.*, 1999; Mazurier *et al.*, 2009).

The colonization of non-pathogenic PGPB on plant root surface enhance their opportunity to use majority of available nutrients which complicate pathogen growth. This elucidation of indirect mechanism of competition (Glick, 2012).

PGPB can also triggered induced systemic resistance (ISR) phenomenon in plants. Substantially, plants with positive ISR have alerted defense mechanisms which enable the plant to react efficiently to suppress pathogenic attack (Glick, 2012).

1.12 Actinobacteria as Biological Control of Fungal Plant Pathogens

Actinobacteria can control different plant pathogens and act also as PGPB. These actinobacteria can be used as bioagent or its isolated metabolites and/or its derivatives (Chaurasia *et al.,* 2018).

The dispersal of pathogens with high resistance reveal the necessity of new control techniques instead of chemicals which induce this issue. Recently, several studies considered actinobacteria as eco-friendly alternative for chemicals in agriculture sector (Saeed *et al.,* 2017a; Kamil *et al.,* 2018).

Recent study showed the potential of actinobacteria as biocontrol agent against pathogenic fungi on date palm. To illustrate, black scorch disease caused by *Thielaviopsis punctulata*, is one of the most prevalent fungal disease that attack date palm plantation in UAE (Saeed *et al*., 2016). The first locally isolated actinobacterium *Streptomyces globosus* UAE1, repress symptoms of the disease as well as restrain the spread of the pathogen (Saeed *et al*., 2017a).

Several conducted studies demonstrate the ability of actinobacteria as biological control for various crops (El-Tarabily & Sivasithamparam, 2006; Chaurasia *et al.,* 2018). To illuminate some examples, endophytic actinobacteria isolated from either healthy or infected tomato's roots showed significant potential as biocontrol against tomato wilt diseases caused by fungal pathogen (Tan *et al.,* 2006; Cao *et al.,* 2016). Pathogenic *Botrytis cinerea* was minimized as causal of leaf infection when *Micromonospora spp.* strain inoculated in tomato roots, despite that it was isolated from alfalfa leguminous plant (Martínez-Hidalgo *et al.,* 2015). Therefore, potent actinobacteria isolated from certain plant can be used as biocontrol for a pathogen attacking another plant. Nevertheless, there are some actinobacteria that have broad

spectrum of activity enabled them to inhibit wide range of pathogen since they have almost same mode of action (Kunova *et al.*, 2016).

1.13 Aims of the Thesis

(1) To determine the chemical control treatment using different available fungicides.

(2) To determine the biological control treatment using antagonistic rhizosphere actinobacteria.

Chapter 2: Material and Methods

2.1 Methods

2.1.1 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 & Boyle, 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, 1978), 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.

2.2.2 *In Vitro* Evaluation of Fungicides Against *F. solani*

The fungicide experiment was carried out as previously described (Saeed *et al.*, 2016; Saeed *et al.*, 2017b). These fungicides selected were Baiclean® (Oligosaccharin; Baico, Beijing Multigrass Formulation Co., China), Uniform® (Azoxystrobin and Metalaxyl-M; Syngenta, USA) and Cidely® Top 125/15 DC (Difenoconazole and Cyflufenamid; Syngenta). Each of the obtained fungicide was dissolved in water to a final concentration of 0, 25, 75, 125, 250, 500, and 1000 ppm, and then introduced into sterilized PDA at 25°C. To inhibit the bacterial growth, penicillin-streptomycin antibiotics was also introduced. The solution was swirled to attain homogenization status. The mixtures were dispensed aseptically into sterile Petri dishes. A sterile cork-borer (8-mm in diameter) was used to introduce the tested pathogen onto the medium without fungicide (control) or with fungicide (treatment). Cultures were incubated at 25°C for 10 days and radial growth measurements were recorded daily. The percentage of the mycelial growth was measured and growth inhibition was calculated according to the equation: % Mi=(Mc−Mt)/Mc×100% where; Mi=Inhibition of the mycelial growth; Mc=colony diameter (in mm) of control set; and Mt=colony diameter (in mm) of the target fungus on the medium with fungicide.

2.2.3 *In vivo* Evaluation of Fungicides against *F. solani*

An *In vivo* evaluation of Cidely® Top, which was the most effective fungicide, was also carried out on six-month-old date palm seedlings (cv. Barhi) grown in 1.5-L plastic pots containing sterilized soil mixture under greenhouse conditions. Pots were kept in a greenhouse in continuous light at 700 µmol m-2 s-1, temperature of 25°C and

relative humidity of 60±5%. Seedlings were previously inoculated with 10 mL of inoculum on roots that were separately dipped for one hour in the inoculum suspension of *F. solani*, and were further kept in the greenhouse at 25°C for 10 days post inoculation (dpi) (until disease symptoms were evident). Similar to inoculated seedlings, control plants were dipped in 10 mL of sterile distilled water. *F. solani*-inoculated plants were then either sprayed with 250 ppm of Cidely® Top fungicide or with sterilized distilled water (*F. solani*). To estimate the number of conidia in the greenhouse experiment, conidia counts of known weight of affected tissues was homogenized in 5 mL of water and the suspended material was assessed using haemocytometer. For all inoculated seedlings, Disease severity index (DSI) was recorded for SDS symptoms at 7 and 25 days post treatment (dpt) (corresponding to 17 and 35 dpi with *F. solani*) 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%. All experiments were independently repeated three times with similar results.

2.2.4 Statistical Analysis for the Fungicide Experiment

For the *In vitro* evaluation of fungicides against *Fusarium* spp., eight plates for each treatment were used. For the DSI of the pathogenicity of *Fusarium* spp. and Cidely® Top treatment tests against *F. solani* in the greenhouse, three replicates for each treatment were examined and recorded. Data represent the mean ±SD from a minimum of 12 plants per replicate. Analysis of Variance (ANOVA) and Duncan's multiple range test were performed to determine the statistical significance at $p < 0.05$. All experiments were independently repeated three times with similar results. Similar

results were obtained in each replicate. SAS Software version 9 was used for all statistical analyses performed (SAS, 2011).

2.2.5 Isolation of Actinobacteria From Date Palm Rhizosphere

Four rhizosphere soil sample were indiscriminately collected from 30 cm-depth near healthy date palm trees roots. Rhizosphere soil samples were air-dried for 4 days at 28°C to decrease bacterial contaminant (Williams *et al.*, 1972), sieved with 5 mm mesh sieve and stored in sterile containers at 25°C in the dark, for a week before further analyses.

Soil samples were placed in dry oven and treated with heat under 100 ^{0}C for one hour. Soil dilution plate method was used for the isolation of actinobacteria (Johnson & Curl, 1972) on inorganic salt starch agar (ISSA), or as known also starch nitrate agar (SNA) (Küster, 1959) amended with cycloheximide and nystatin (each 50 µg ml^{-1}; Sigma–Aldrich) with specific soil pre-treatments (Hayakawa & Nonomura, 1987). Five replicate plates were used per dilution, which were incubated at 28°C in dark for 7 days. Later, actinobacterial colonies were purified and transferred onto oatmeal agar plates (ISP medium 3) supplemented with 0.1% yeast extract (OMYEA) (Küster, 1959).

2.2.6 Detection of the Antifungal and Cell wall Degrading Enzyme Activities

All actinobacterial isolates were categorized based on their ability to secrete diffusible antifungal metabolites active against *F. solani* using the cut-plug method (Pridham *et al.*, 1956). The actinobacterial isolates were inoculated on fish meal extract agar (El-Tarabily *et al.*, 1997) plates and incubated at 28°C in dark for 7 days. PDA-seeded plates were prepared by initially by cultivating *F. solani* on PDA slants at 28°C until sporulation, which were then flooded with 50 mM phosphate buffer (pH 6.8)

(Saeed *et al.*, 2017a). Spores and some mycelial fragments were homogenized at 4,000 rpm for 20 min; and the resulting supernatants were diluted in PDA plates. The inoculum consisted of approximately 10^8 cfu ml^{-1}. PDA-seeded plates with non-inoculated agar plugs served as control. Plugs were transferred from the actinomycetes cultures on fish meal extract agar with a sterilized 11 mm cork-borer onto PDA plates seeded with *F. solani* kept at 28°C in dark for 5 days. The diameters of zones of inhibition were determined. Five plates were used for each actinobacterial isolate. The most promising antifungal metabolite-producing isolates showing the largest zone of inhibition were selected for further experiments; and the rest of the isolates were not used in the following tests.

The most promising antagonistic actinobacterial isolates were also examined for their ability to secrete diffusible antifungal metabolites active against *F. solani* using the cup plate technique as previously described (Bacharach & Cuthbertson, 1948). Inocula for the preparation of the *F. solani*-seeded PDA plates were prepared, as described above, for the cut-plug method. Five plates were used for each actinobacterial isolate.

All actinobacterial isolates that showed the strongest diffusible antifungal metabolites were also tested for their potential to produce clearing zones on *F. solani* mycelial fragment agar as an indicator of preliminary production of cell wall degrading enzymes (CWDEs) according to Valois *et al.* (1996). Large (>30 mm) diameters exemplified high CWDE activities. Additionally, all isolates were evaluated for their ability to produce chitinase enzyme. Each isolate was inoculated onto colloidal chitin agar plates and incubated at 28°C in dark for 7 days (Gupta *et al.*, 1995). The clearing zones surrounding the colonies were measured and used to detect the chitinase activity.

Similarly, large (>30 mm) diameters represented high chitinase activities and five replicate plates were used for each actinobacterial isolate. The most promising, highly active CWDE-producing isolates showing the largest clearing zones on both mycelial fragment agar and colloidal chitin agar plates were picked for further experiments.

2.2.7 Production of Siderophores

Siderophore production was examined by the inoculation of chrome azurol S (CAS) agar plates developed by Schwyn & Neilands, (1987) with the BCAs and incubated at 28°C in dark for 7 days. Production of yellow-orange halo zone around the colony was considered positive for siderophore production.

2.2.8 Determination of CWDE Activities of the Actinobacterial Isolates

Minimal synthetic medium (Tweddell *et al.*, 1994) (50 ml) supplemented with 2 mg ml^{-1} of colloidal chitin, were placed in Erlenmeyer flasks. Flasks were inoculated with 2 ml of a 20% glycerol suspension of each BCA (10^8 cfu ml^{-1}), incubated on a rotary shaker (Model G76, New Brunswick Scientific, NJ, USA) at 250 rpm for 7 days, and further centrifuged at 12,000×g for 30 min. The supernatant was filtered using 0.22 µm Millipore membranes (Millipore Corporation, MA, USA) and used as a source of crude enzymes (El-Tarabily, 2003).

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

The effect of the crude culture filtrate of the selected actinobacterial isolates on the morphology of *F. solani* hyphae was evaluated (Sneh, 1981). At sampling, *F. solani* hyphae treated with the actinobacterial isolates was microscopically examined at 100X using a light microscope to examine hyphal lysis and any hyphal

abnormalities. *F. solani* mycelium incorporated with non-inoculated filter-sterilized fish meal extract broth served as control treatments. Three replicates were used at each sampling.

2.2.10 Qualitative Determination of ACC Deaminase Activity by Actinobacterial Isolates

The aim of this experiment was to screen the most promising actinobacterial isolates for their potential to produce ACC deaminase from ACC using the nitrogen-free Dworkin and Foster's salts minimal agar medium (DF) (Dworkin & Foster, 1958). The medium was supplemented with either 2 g $(NH_4)_2SO_4$ or 3 mM ACC (Sigma) l^{-1} as a sole nitrogen source. The heat-labile ACC was filter sterilized through sterile Millipore membranes (pore size 0.22 µm, Millipore Corporation, MA, USA) and the filtrate was added to the salt medium after autoclaving.

Five-day-old isolates grown on rich OMYEA were streaked in triplicate on DF agar medium plates amended with either $(NH_4)_2SO_4$ or ACC. The plates were incubated at 28±2°C in the dark for 7 days. Growth and sporulation of the isolates on DF agar medium amended with ACC (DF-ACC agar) was taken as an indicator of the efficiency of selected isolates to utilize ACC and to produce ACC deaminase.

2.2.11 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). 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.

2.2.12 *In vivo* Biological Control Experiment for the Evaluation of The Most Promising BCA

In vivo evaluations of the BCA were carried out on 6-month-old date palm plants (cv. Barhi). The set-up of the Experiment was as follows:

- Controls (C): Three seedlings without *F. solani* nor BCA.
- Infested controls (F): Three seedlings artificially infested with *F. solani.*
- BCA controls (BCA1) and (BCA2): six inoculated seedlings with BCA only, three for each BCA.
- Preventive treatment (BCA1+F) and (BCA2+F): six inoculated seedlings with BCA one week before *F. solani* inoculation, three for each BCA.

The preventive application a week before inoculation with BCA was chosen since it was the most effective in suppressing the pathogen invasion according to Saeed *et al.* (2017a). For the preparation of pathogen inoculum, millet (*Panicum miliaceum L.*) seed-based inoculum was prepared by adding 25 g of seeds to 40 mL of distilled water in 250 mL conical flasks. The flasks were autoclaved at 121°C for 30 min on three consecutive days (Saeed *et al.,* 2017a). Millet seeds were then aseptically inoculated with ten agar plugs (6 mm dia.) from the actively growing margins of *F. solani* colony and incubated at 25°C in the dark for 2 weeks. The flasks were periodically shaken to ensure uniformity of colonisation. Colonized and autoclaved millet seeds served as the control. Small amounts of the colonised and control millet seeds were plated onto PDA before use to confirm the presence or absence of *F. solani.*

For the preparation of BCA inoculum, the inoculum for each BCA was prepared by placing 50 g of moist oat bran into 500 mL conical flasks and autoclaved at 121°C for 20 min on three successive occasions as described (Saeed *et al.*, 2017a).

The substrate was aseptically inoculated with a 25 mL spore suspension (10% glycerol) of each BCA and incubated at 25°C in the dark for 3 weeks. The flasks were routinely shaken to ensure uniformity of colonisation. Colonised oat bran that had been similarly autoclaved served as the control. Prior to use, small amounts of both the colonised and control wheat bran were suspended in 50 mL of sterile distilled water. An aliquot (0.3 mL) of this suspension was spread onto oatmeal agar plates and incubated to confirm the presence or absence of the BCA, respectively.

For the soil infestation, soil was air dried, and passed through a 3 mm mesh sieve prior to use. The BCA colonised oat bran inoculum (1% weight of colonized wheat bran inoculum/weight of air-dried non-sterile soil) was added to each pot and dispersed through the soil. Pots were watered twice weekly. After one week, the pathogen was dispersed through the soil (1% weight of colonized millet seed inoculum/weight of air-dried non-sterile soil) by mixing in a plastic bag. The infested soil was put into 6 kg plastic free draining plant pots and date palm seedlings were transplanted into these pots. Pots were kept in a greenhouse in continuous light at 700 µmol $m^{-2}s^{-1}$, temperature of 25±2°C and relative humidity of 60±5%. Pots were watered twice weekly. Symptoms were observed and recorded in each week. Koch's postulates test was conducted to confirm that *F. solani* was the death causal of *F. solani* seedlings.

2.2.13 Spore Counts and Disease Severity Index in Inoculated Plants

The growth of *F. solani* in inoculated plants was determined based on the number of fungal spores (total spore counts) at eighth weeks post inoculation (wpi). Three roots from three inoculated seedlings per treatment were collected, cut into small pieces (2–5 mm in diameter), soaked in 10 ml distilled water and vigorously agitated

for 30 min. Harvested spores were counted using haemocytometer (Agar Scientific Limited, Essex, United Kingdom).

Disease severity index (DSI) was recorded at second and eighth wpi using a scale of 0–5: 0=no apparent symptoms, 1=1–10% necrotic or dark brown area around the point of infection, 2=11–25%, 3=26–50%, 4=51–75%, and 5=76–100% (Molan *et al.*, 2004).

2.2.14 Statistical Analyses for the Biological Control Experiments

For the *In vitro* evaluation of BCA against *F. solani*, data were analyzed using the analysis of variance (ANOVA) while means were separated using Duncan's multiple range test at 5% level of significance. These experiments were repeated in triplicates using five plates/treatment for each time with similar results.

For the fungal spore counts and DSI of the *In vivo* treatments against *F. solani*, three replicates for each group were examined. Data represent the mean ±SD from a minimum of six plants. ANOVA and Duncan's multiple range test were done to determine the statistical significance at $P<0.05$. Similar results were obtained in each replicate. SAS Software version 9 was used for all statistical analyses performed (SAS, 2011).

Chapter 3: Results

3.1 *In vitro* Evaluation of Selected Fungicides against *Fusarium* spp.

The effect of Baiclean® (Oligosaccharin), Uniform® (Azoxystrobin and Metalaxyl-M) and Cidely® Top (Difenoconazole and Cyflufenamid) fungicides on the mycelial growth of the three *Fusarium* spp. were evaluated *In vitro*. In PDA plates, a final concentration of 0, 25, 75, 125, 250, 500, and 1000 ppm of the selected fungicides were applied (Figure 5). Interestingly, mycelial growth increased in all isolates at higher concentrations of the fungicide Baiclean® (>250 ppm) compared with lower concentrations (<250 ppm). When different concentrations of Uniform® fungicide were applied, we noticed similar or minimal effect on the mycelial growth of the three fungal strains (Figure 5). On the other hand, Cidely® Top showed the highest mycelial growth inhibition on *Fusarium* spp. at all tested concentrations *In vitro*. Accordingly, the concentration of 250 ppm in the selected fungicides was further used and was considered as the most efficient concentration in the three fungicides (Figure 6).

At 250 ppm, three fungicides were statistically evaluated for their effectiveness to inhibit the growth of three potential isolates of *Fusarium* spp. of proven pathogenicity to date palm *In vitro*. After 10 days of inoculation, mycelial growth inhibition rate (%Mi) of *F. oxysporum*, *F. proliferatum*, and *F. solani* were significantly different among the three tested fungicides at 250 ppm (Figure 6). Thus, Cidely® Top fungicide increased fungal mycelial growth inhibition at 250 ppm and showed the highest zone of inhibition, ranging between 79.5–96.3% (Figure 6). This suggests that the systemic fungicide, Cidely® Top, inhibited the mycelial growth of *Fusarium* spp. and that 250 ppm is the recommended dosage of this fungicide to further applied in the greenhouse experiment.

Due to the pathogenicity of *F. solani* on date palm under greenhouse conditions and the effectiveness of 250 ppm of Cidely® Top *In vitro*, a microscopic examination was performed to find out the mode of action of this fungicide in inhibiting the growth of this pathogen. Our results revealed that Cidely® Top caused significant morphological alternations in *F. solani*. When Cidely® Top was applied, we observed unusual morphological abnormalities in cultures of *F. solani* in comparison to control (no fungicide) treatment. The fungicide not only caused septal malformations and cytoplasmic coagulation in the hyphal cells (Figure 6), but also conidial deformation in *F. solani* (Figure 6). Altogether, Cidely® Top inhibited the mycelial growth and induced morphological abnormalities of *F. solani* DSM 106836.

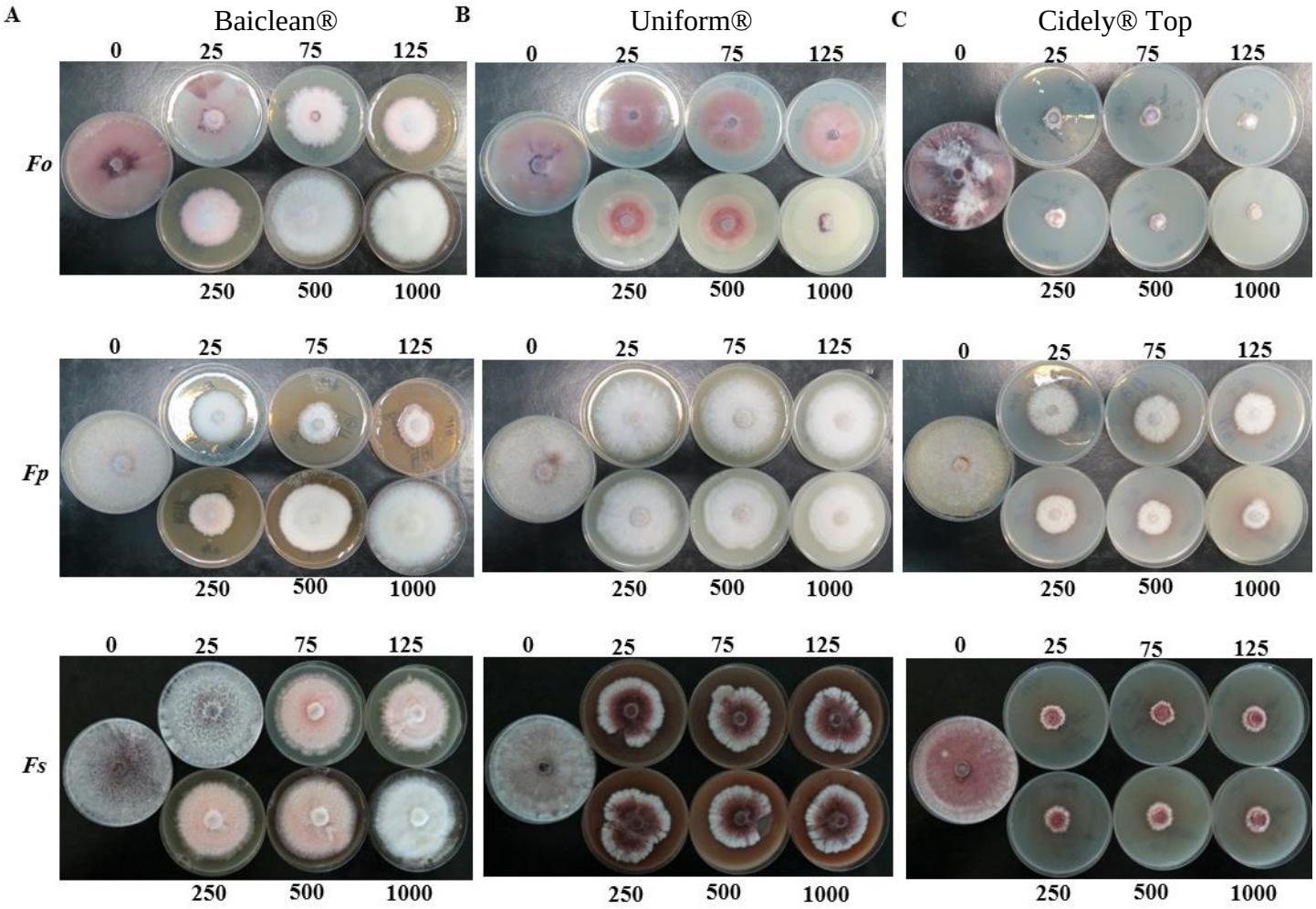

Figure 5: Growth inhibition effect of fungicides on *Fusarium* spp. Growth inhibitory effect on *Fusarium* spp. using different concentrations (in ppm) of (A) Baiclean®; (B) Uniform®; and (C) Cidely® Top, on PDA pales. Data were collected 10 days after inoculation. *Fo, F. oxysporum*; *Fp, F. proliferatum*; *Fs, F. solani.*

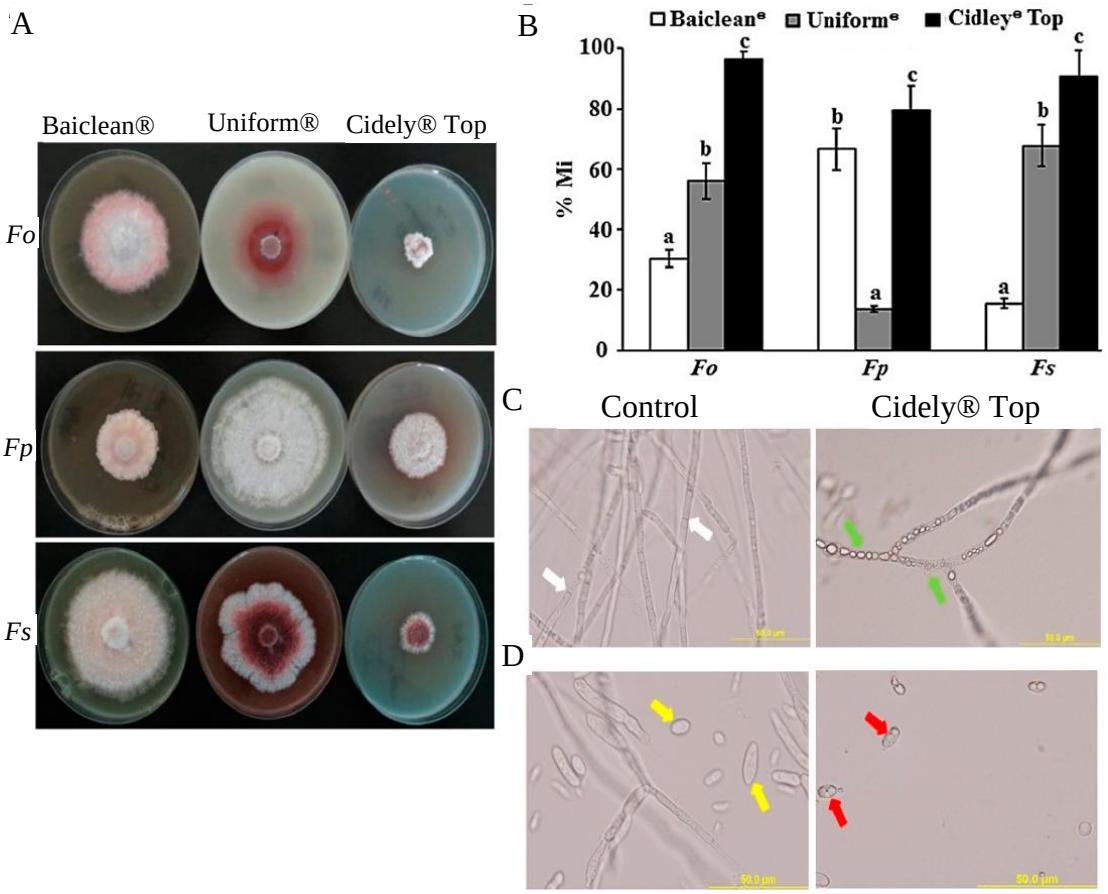

Figure 6: Efficacy of fungicides against *Fusarium* spp. *In vitro*. Effect of fungicides (250 ppm) on *In vitro* mycelial growth (A); growth inhibition rate (%Mi) of *Fusarium* spp. using 250 ppm of the fungicides after 10 days (B). Abnormalities in hyphal morphology, septum formation and cytoplasmic contents (C); and deformation of conidia (D) of *F. solani* following Cidely® Top treatment compared to control. In (B), values with different letters are significantly different from each other at p=0.05; In (C), white arrows indicate normal septate hyphal growth; and green arrows indicate formation of non-septate hyphal formation and cytoplasmic coagulation. In (D), yellow arrows indicate normal formation of conidia; and red arrows indicate deformation of conidia. *Fo*, *F. oxysporum*; *Fp*, *F. proliferatum*; *Fs*, *F. solani.*

3.2 Effect of Cidely® Top on Date Palm Seedlings Infected with *F. solani*

In the greenhouse experiments, we measured the efficacy of the most promising fungicide, Cidely® Top, at 7 and 25 days post treatment (dpt) on diseased date palm plants. We first inoculated seedlings with the fungal pathogen, *F. solani*, for 10 days until plants showed visible symptoms of SDS; followed by a treatment with Cidely® Top on diseased seedlings (designated as 0 dpt). At 7 dpt with the fungicide,

plants started to recover and prevented further disease progression at the end of the assessment period of 25 dpt (Figure 7). This contrasted with diseased plants that were sprayed with water (*F. solani*). Affected plants that were treated with Cidely® Top clearly showed vegetative growth recovery (Figure 7) and developed healthy root system (Figure 7) at 25 dpt comparable to control plants with no prior artificial infection.

We also determined the effects of the chemical fungicide, Cidely® Top, on the number of conidia progressing on diseased seedlings. As expected, a steep drop in the number of conidia was remarked in Cidely® Top-treated seedlings (Figure 7). In general, the number of conidia of *F. solani* recovered from the roots of treated-date palm plants was at least 20-fold reduced when compared with that in untreated ones. Based on the level of severity of necrosis in leaves or rotting in roots, DSI was also assessed on diseased- and recovered-seedlings (Figure 7). It is evident from our results that seedlings treated with Cidely® Top fungicide had significantly lower DSI than inoculated seedlings but without fungicide treatment at the same period of assessment (7 and 25 dpt). Together, this suggests that *F. solani* appeared to lose some of its aggressiveness as a pathogen and the severity of SDS was gradually suppressed as a disease when Cidely® Top fungicide was applied on date palm plants.

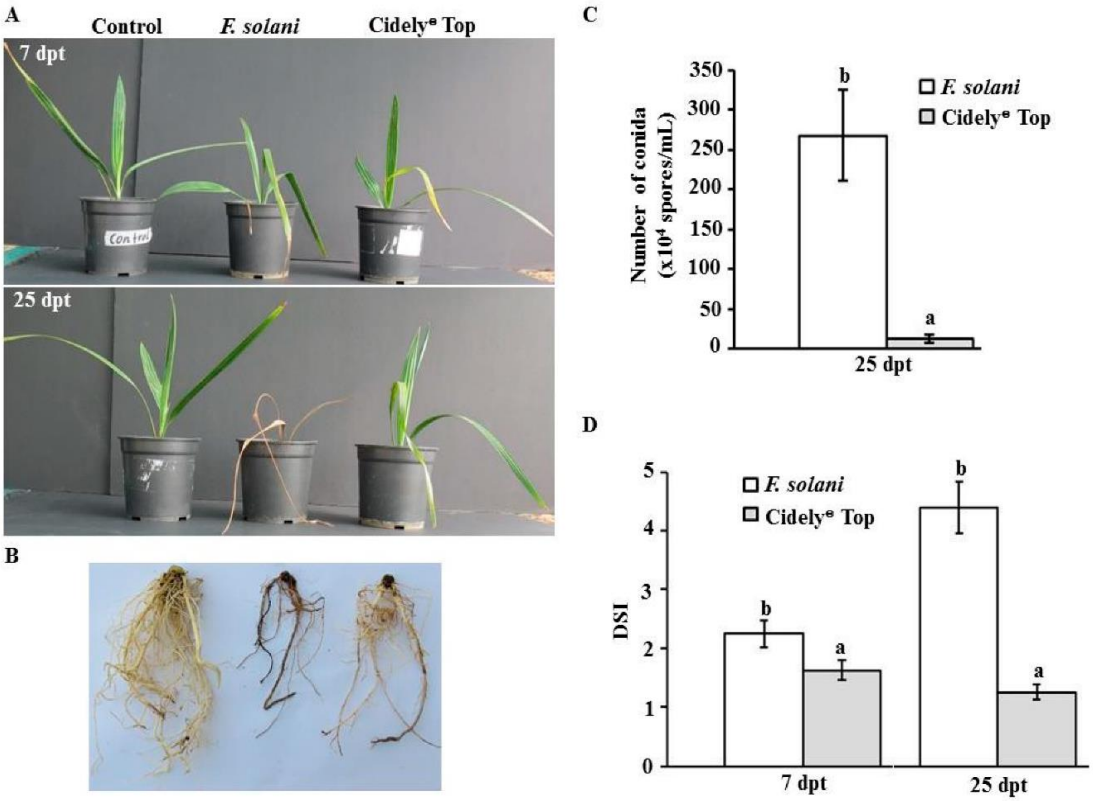

Figure 7: Effect of fungicide treatments on artificially inoculated date palm seedlings with *F. solani* in the greenhouse. Fungicidal suppression of *Fusarium* wilt disease on date palm seedlings cv. Barhi using Cidely® Top fungicide at 7 (top panel) and 25 (bottom panel) dpt (A); recovery of root tissues previously infected with *F. solani* using 250 ppm of Cidely® Top fungicide after 25 dpt (B). Number of conidia ($\times 10^4$ spores/mL) (C) and disease severity index (DSI) (D) after the application of Cidely® Top on date palm seedlings infected with *F. solani* (n=12). 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. In (C–D) Values with different letters are significantly different from each other at p=0.05. In (A–D), seedlings inoculated with *F. solani* at 10 days before the fungicide treatment. Control, non-inoculated seedling; *F. solani*, infected seedlings with *F. solani* only; Cidely® Top, infected seedlings with *F. solani* and sprayed with Cidely® Top; dpt, days post treatment.

3.3 Isolation, Identification and Screening for Actinobacterial Isolates

Thirty-three actinobacterial strains were isolated from date palm rhizosphere. All the isolates were screened through multiple *In vitro* tests in order to choose promising biocontrol agent (BCA) candidates (Figure 8). Isolates were identified according to the genus description of Shirling and Gottlieb (Shirling & Gottlieb, 1966)

indicated by chalky, discrete, powdery and velvety aerial mycelium which is a unique feature of the genus (Figure 8). The colony sizes ranged between small to medium with white, yellow, brown, and grey in colour. Each isolate was streak purified on starch nitrate agar (SNA) and on oatmeal agar medium for further morphological characterization and other metabolite activity.

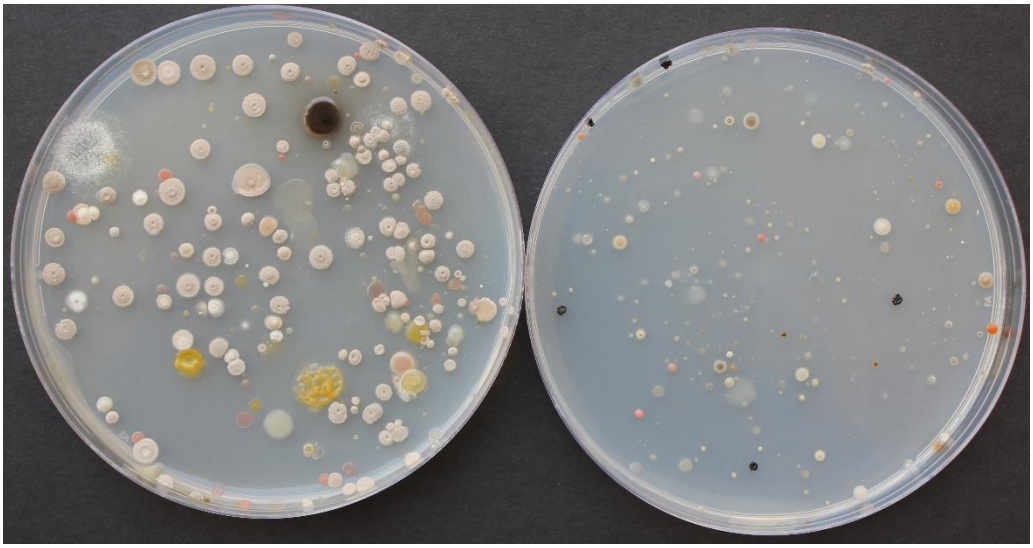

Figure 8: Colonies of actinobacteria isolated from date palm rhizosphere grown on inorganic salt starch agar plates

3.4 Production of Antifungal Metabolites, and CWDEs

Using Cut-plate method revealed that out of 33 isolates 17 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).

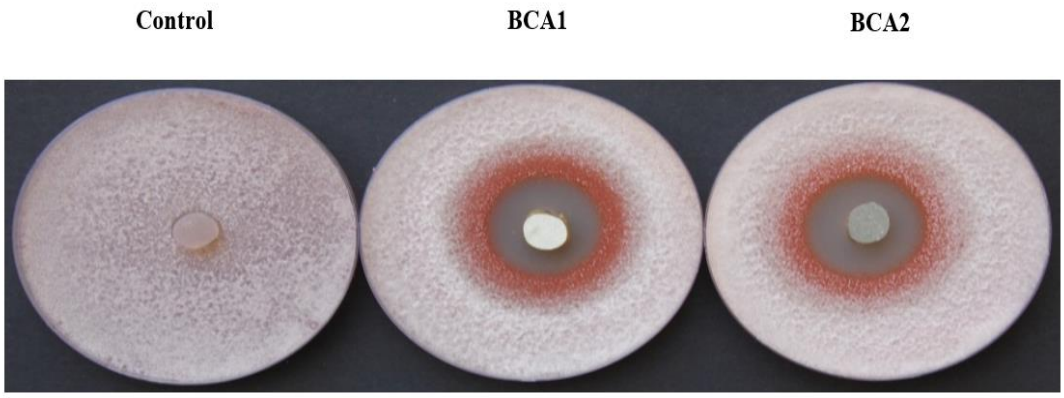

Figure 9: Inhibition of *F. solani* mycelial growth by the BCA1 and BCA2 using cut-plug method. Inhibition of *F. solani* mycelial growth was observed only by the diffusible antifungal metabolite-producing isolates BCA1 and BCA2.

Using Cup-plate method revealed that out of 17 isolates 10 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).

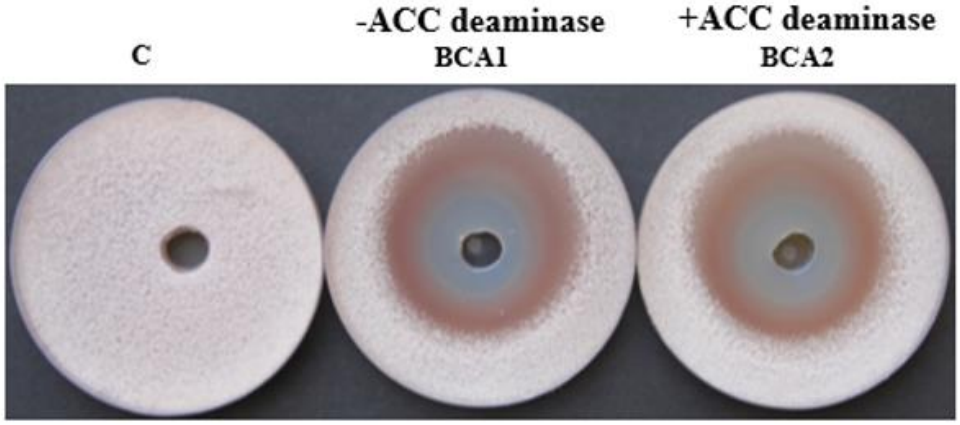

Figure 10: Effect of the BCA candidates on mycelial growth of *F. solani* using the cup plate method. Inhibition of *F. solani* mycelial growth was observed only by the diffusible antifungal metabolite-producing isolates BCA1 and BCA2. Wells were inoculated with either filter-sterilized fish meal extract broth (C), or filter-sterilized crude culture filtrates of isolates.

The 10 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 8 of them produce large clearing zone around their colonies (more than 30 mm) (Figure 11). Overall, eight isolates were produced both antifungal metabolites and cell-wall degrading enzyme (i.e. chitinase).

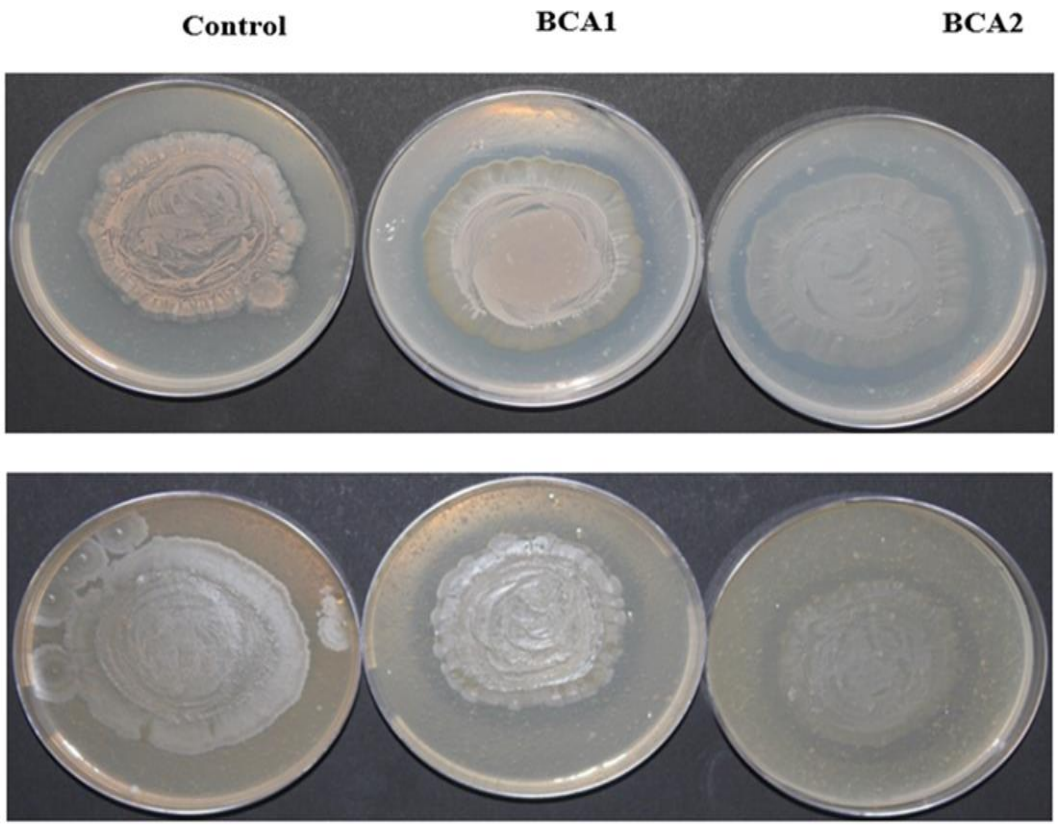

Figure 11: Production of cell-wall-degrading enzymes by BCA candidates active against *F. solani*. Production of chitinase enzymes by BCA1 and BCA2 grown on colloidal chitin agar (upper panel) and production of CWDEs on mycelial fragment agar (lower panel).

3.5 Production of ACC Deaminase

The eight promising isolated candidates subsequently tested for their ability to produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, the immediate precursor of the stress hormone ethylene.

In order to study the effectiveness of ACC deaminase against *F. solani*, DF agar plates were used and only two isolates were chosen between the eight candidates. One of the chosen isolates producing ACC deaminase and the other isolates non-producing ACC deaminase (Figure 12).

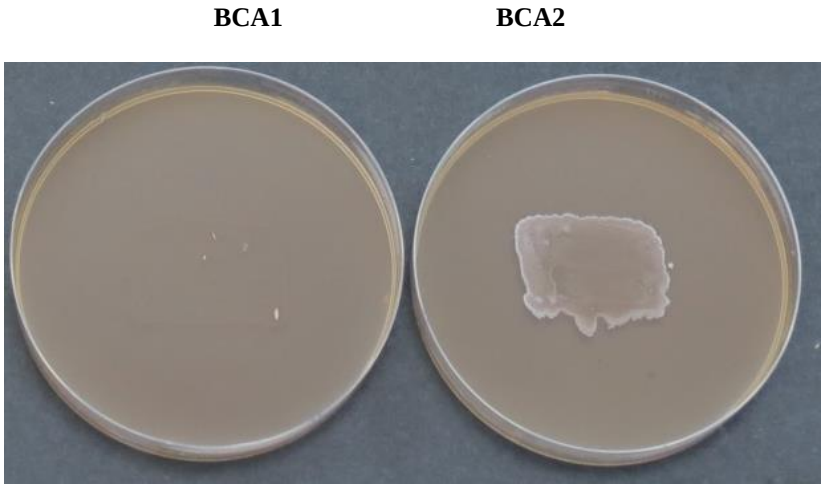

Figure 12: Production of ACC deaminase. Isolates were tested in N-free Dworkin and Foster's salts minimal agar medium amended with ACC; where growth and sporulation indicated the efficiency to utilize ACC and the production of ACCD.

3.6 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 13).

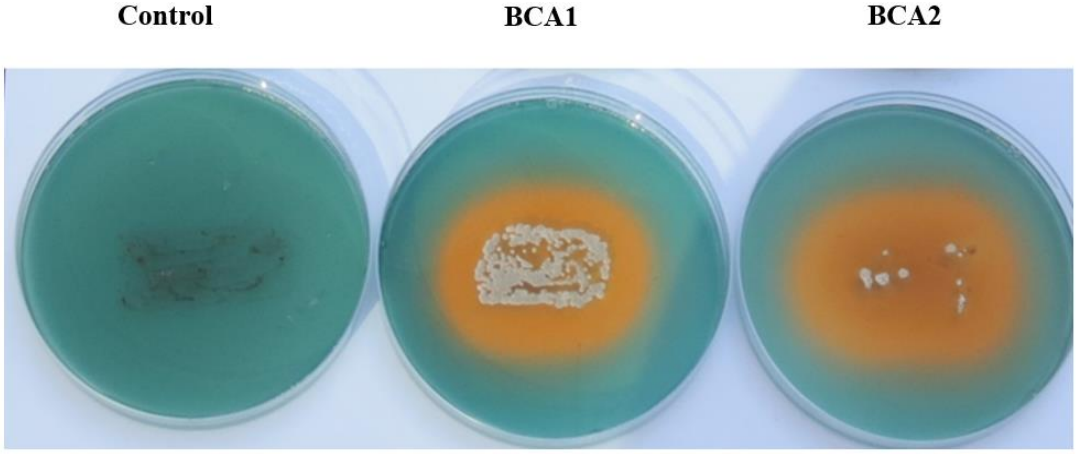

Figure 13: Yellow-orange halo zone indicate siderophore production

3.7 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 14). Mycelial mats in control flasks remained healthy and unaffected (Figure 14).

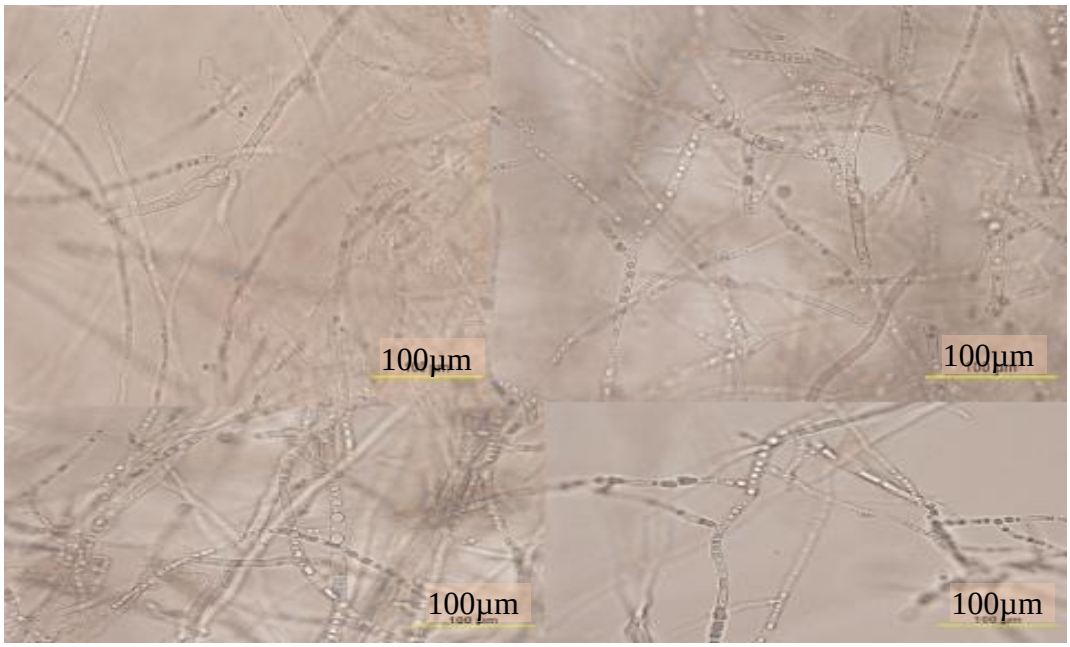

Figure 14: Effect of the BCA candidates on hyphae and cytoplasm of *F. solani*. Abnormalities observed in hyphal morphology and cytoplasmic contents of *F. solani* following exposure to filter-sterilized crude culture filtrate of BCA1 and BCA 2.

3.8 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*.

3.9 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 ($ACCD^+$) comparing with the treatments of ($ACCD^-$) which showed slight symptoms only (dryness of the leaves) after thirty days. Control seedlings did not develop any disease symptoms likewise the seedlings which inoculated separately with both BCA1 and BCA2 only. The ACC deaminase producing BCA2 ($ACCD^+$) performed superior to the ACC deaminase-non-producing biocontrol agents BCA1 ($ACCD^-$). This indicates that the ability to produce ACC deaminase clearly enhances the performance of the antagonist to effectively suppress the disease (Figure 15).

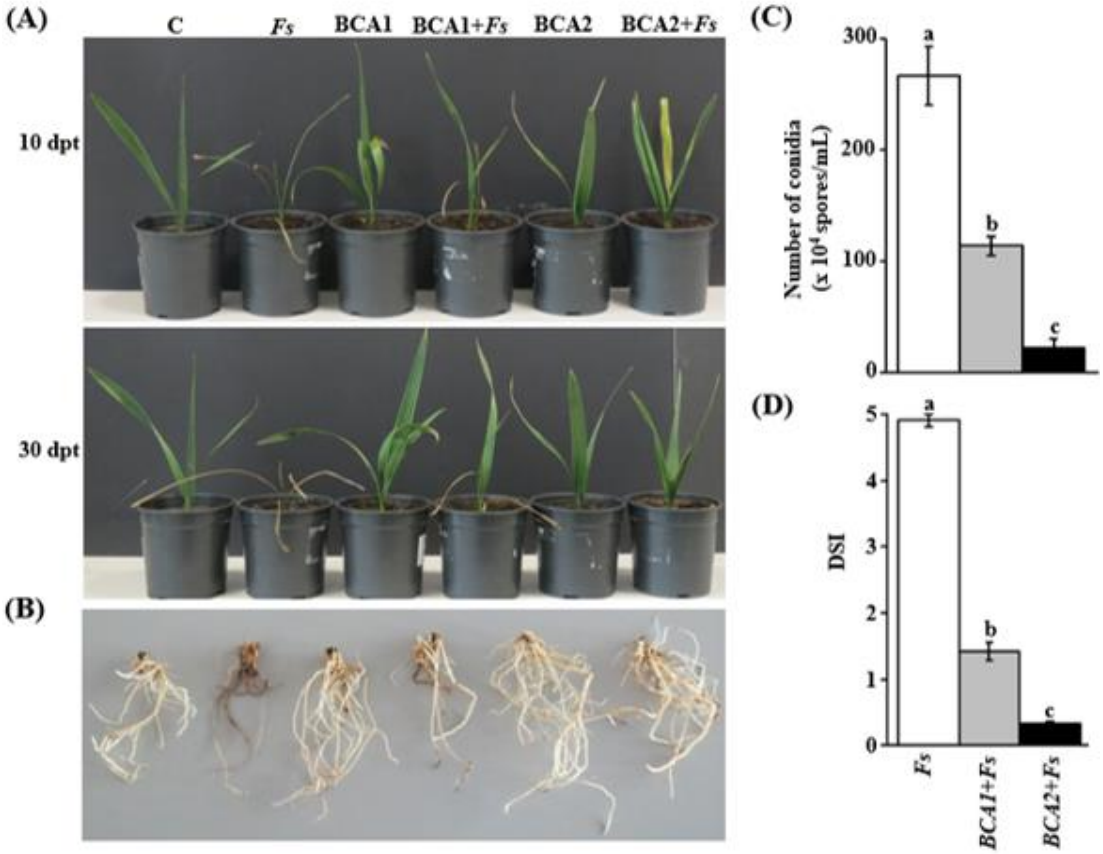

Figure 15: Preventive effect of BCA treatments on artificially inoculated date palm seedlings with F. solani in the greenhouse. Suppression of *Fusarium* wilt disease on date palm seedlings cv. Barhi using BCA1 and BCA2 at 10 (top panel) and 30 (bottom panel) dpt (A); and recovery of root tissues infected 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. Control, non-inoculated seedling; Fs, infected seedlings with *F. solani* only; BCA1+Fs and BCA2+Fs, seedlings inoculated with the individual BCA1 and BCA2, respectively, 5 days prior to *F. solani* inoculation; dpt, days post treatment. C=Control, *Fs*=Inoculation with *F. solani*, BCA1=Inoculation with the rhizosphere-competent non-producing ACC deaminase isolate, BCA1+*Fs*=Application of BCA1 5 days prior to inoculation with *F. solani*, BCA2=Inoculation with the rhizosphere-competent producing ACC deaminase isolate, BCA2+*Fs*=Application of BCA2 5 days prior to inoculation with *F. solani*.

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, 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.*, 2019). 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.*, 2019).

Because immediate and appropriate management for this destructive disease was highly required, it is aimed in this work to search for a cost-effective solution for the potential risk of SDS in the UAE. Since there are several cases of chemical control of plant pathogens successfully reported under laboratory conditions, but when repeated at a greenhouse-scale, they fail to have the predicted impact (O'Callaghan, 2016). This research was further extended to evaluate systemic and non-systemic fungicides to potentially control the pathogen *In vitro* as well as in the greenhouse. In order to find a successful fungicide to inhibit *F. solani*, the efficacy of three fungicides, Baiclean®, Uniform® and Cidely® Top were tested under *In vitro* conditions. Although all tested chemical treatments inhibited mycelial growth of *F. solani* in the controlled laboratory experiments, the systemic fungicide, Cidely® Top (difenoconazole and cyflufenamid), was the most effective chemical at a concentration of 250 ppm. Similarly, Cidely® Top has also proven to be effective against a number of pathogens

such *T. punctulata* and *L. theobromae* (Saeed, *et al.*, 2017a; Saeed *et al.*, 2017b). Other systemic fungicides, such as Bavistin D.F. (Carbendazim), have been reported to suppress *F. solani* and *F. mangiferae* (Bhanumathi & Rai, 2007; Iqbal *et al.*, 2010; Maitlo *et al.*, 2013). Despite there is a body of evidences that the non-selective use of chemicals poses a perceived risk to human health and adverse effects to the environment (Aktar *et al*., 2009), chemicals are still widespread to limit the damage on crops (Ganesan *et al*., 2010). This is because of the ease of use, effectiveness, fast activity, and relatively low cost. Searching for eco-friendly strategies is crucial to control diseases in crops and reduce growth of pathogens. For example, integrated disease management (IDM) combines two or more antagonistic strategies of horticultural practices, fungicides, BCA and natural compounds (AbuQamar *et al*., 2017; Saeed *et al.*, 2017b; Kamil *et al*., 2018). To date, research is limited on the effectiveness of fungicide treatment to manage SDS because of the unpredictable nature of the disease (Haudenshield *et al.*, 2015). Performances of the same treatments under similar laboratory–greenhouse–field studies are rare in plant science.

The obtained data demonstrated that Cidely® Top inhibited *F. solani* at 250 ppm by affecting hyphal development, septum formation and cytoplasmic integrity. In addition, conidial formation of the fungal pathogen was also altered. In general, Cidely® Top was highly effective in reducing the pathogenic activities of *F. solani*. Similar to the *In vitro* observations, Cidely® Top showed a significant reduction in disease symptoms in relation to the conidia counts in Cidely® Top-treated seedlings at 25 dpt in the greenhouse trials. This indicates that Cidely® Top can be considered as a candidate fungicide for the management of *F. solani*-affected date palm trees. The result obtained for Cidely® Top seems to be in agreement with previous findings that this fungicide inhibits the growth of plant pathogens (Saeed *et al.*, 2017b). Cidely®

Top, on the other hand, does not have a retarded effect on *F. magniferae* (Iqbal *et al*., 2010). This could be attributed to many factors such as fungicide application methods, growth conditions and the nature of fungal species. The fungicide Cidely® Top contains the active substance, cyflufenamid, which is known to act in the developmental stages of mycelia and spores, which may possibly contribute to the increased extent of inhibition of *F. solani*. To the best of our knowledge, this is the first study relating the assessment of Cidely® Top on date palm trees infected with *F. solani*. Farmers in the UAE, the region and other date production areas suffering from this devastating disease will directly benefit from this study. Thus, the same fungicide was fairly known to be successful on other pathogenic fungi including *T. punctulata* on date palm and *L. theobromae* on mango (Saeed *et al.*, 2017a; Saeed *et al.*, 2017b). Therefore, a future field experiment must be deployed to test the efficacy of Cidely® Top in 'real' infected date palm orchards.

In this regard the study concluded that Cidely® Top was a favorable chemical means to inhibit the growth of the pathogen on date palm. Future directions for seeking environmentally-friendly applications i.e., integrated disease management (IDM) to manage SDS in date palm is on my priorities. The long-term goal 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.

Plant diseases possess serious economic threats to the agricultural sector. Almost 10% of food production is lost worldwide by plant disease (Arora *et al*., 2012). Management of plant disease has been obtained by fungicide application since

decades. However, utilization and frequent application of such chemicals comprise adverse environmental and human health consequences (Saeed *et al.*, 2017a). Subsequently finding an alternative for chemicals to be introduced into an IDM programs is a necessity. The replacement of chemicals applications should be reliable environmentally and economically as well as enhancing soil fertility and plant production (Aghighi *et al*., 2004). Using microbial agents such as actinobacteria can significantly support the IDM programs and eco-friendly farming practices (Chaurasia *et al*., 2018), and it is aligned with all recent global environmental and sustainability approaches and suggestions.

Organic farming (OF), have increased importance globally and locally, and the application of biocontrol agent considered as appropriate solution for disease management in OF, since chemical cannot be used (Van Bruggen *et al*., 2016). Therefore, the aim in this study is to isolate actinobacterial strains from date palm rhizosphere soil and evaluate its efficacy against *F. solani* under *In vitro* and *In vivo* conditions to be used as sustainable and environmentally friendly alternative.

Many studies have been conducted to evaluate the competency and the efficacy of different biocontrol agents that can aid in promoting plant growth, production and enhancing its resistance against pathogens (Parray *et al*., 2016; Chaurasia *et al*., 2018; Ab Rahman *et al.*, 2018). The evaluation of isolated antagonistic actinobacterial strains in our study subjected to series screening steps to select proper candidates. *In vitro* assessment which include evaluation of antifungal activity, production of CWDEs, siderophore secretion and ACC deaminase availability revealed distinctive preliminary results. Several microorganisms have hyperparasitic activity, attacking pathogens by lytic enzyme production which lyses pathogen’s cell wall (Chernin &

Chet, 2002). According to Osherov and Yarden (2010), the cell walls of filamentous fungi consist mostly of chitin and ß-glucans. Hence, the large clearing zone around the selected isolates on both mycelial fragment agar and on colloidal chitin agar, indicated their ability to secrete ß-1,3-glucanases and chitinase which hydrolyzed glucans and chitin present in the pathogen cell wall and helped to lyse *F. solani* hyphae. This could explain the morphological abnormalities in the hyphae of *F. solani* under microscopic observation as mentioned in previous studies (Saeed *et al.*, 2017a; Kamil *et al.*, 2018).

Over the past two decades, the base of antibiosis as biocontrol mechanism of actinobacteria has become well investigated and increasingly better understood (Whipps, 2001; Parray *et al*., 2016). Glick (2012) have stated that actinobacteria are mostly capable to prevent the proliferation of plant pathogens specifically fungi by synthesis of a range of different antibiotics. Results agree with other studies that recognized some actinobacterial strains which can produce chitinase and other hydrolyses enzymes and identified as biocontrol agents against different plant pathogenic fungi such as *Fusarium oxysporum f. sp. cucumerinum*, *Phytophthora* spp., *Botrytis cinerea, Thielaviopsis punctulate* and *Lasiodiplodia theobromae* (Singh *et al.*, 1999; Frankowski *et al.*, 2001; Kim *et al.*, 2010; Saeed *et al.*, 2017a; Kamil *et al.*, 2018)

In this study, all isolations have been screened for siderophores production. The siderophores produced by actinomycetes have a much greater affinity for iron than fungal pathogens. Thereby presence of siderophores prevent some phytopathogens from acquiring a sufficient amount of iron which limit their ability to proliferate (O'sullivan & O'Gara, 1992; Glick, 2012). According to Kang *et al.* (2010), the gaseous hormone "ethylene" play an important role in controlling several

physiological processes in plants, on the other hand, its usually inhibits plant growth. Actinobacteria that possess the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase obverse ethylene effects and assist plant development by diminishing ethylene level (Nadeem *et al*., 2007; Zahir *et al*., 2008). The effects of phytopathogenic microorganisms (viruses, bacteria, and fungi), can be relieved by ACC deaminase producers as suggested by Lugtenberg and Kamilova (2009).

In prior study, El-Tarabily *et al*. (1997) indicated that the ability of actinomycetes to produce antifungal metabolites in agar did not necessarily confirmed the recurrence of the same performance on plant material. Therefore, *In vivo* assessment in greenhouse was carried out for only two isolates with and without ACC deaminase based on previous screening results.

As shown in the outcomes of this study both isolates suppress disease symptoms. However, there was a significant difference between $ACCD^+$ (isolate BCA2) and $ACCD^-$ (isolate BCA1) since the $ACCD^+$ clearly reduced SDS incidence in comparison with $ACCD^-$. Many studies support the finding of this study which mentioned that main obvious effects of seed/root inoculation with ACC deaminase-producing rhizobacteria are plant root elongation, promotion of shoot growth, and enhancement in rhizobial nodulation and N, P, and K uptake (Nadeem *et al*., 2007, 2009; Shaharoona *et al*., 2008; Glick, 2012).

Chapter 5: Conclusion

In conclusion, this study demonstrated for the first time that the chemical fungicide Cidely® Top was strongly effective against SDS on date palm as well as it was the first study to demonstrate the superiority of antagonistic actinobacteria to enhance their effectiveness as biocontrol agents by their ability to produce ACC deaminase, antifungal metabolites and cell-wall degrading enzymes.

Our long-term goal from the current thesis is to achieve the protection of date palm health and its productivity and improve environment sustainability. Plant biotechnology, modern molecular biology technique's and the conventional breeding, could also generate breakthroughs for the production of new date palm resistant types against SDS. Additionally, efforts will continue to find new solutions for applying ACC deaminase producing rhizospheric actinobacteria in the field as one of the most promising and eco-friendly agricultural practices.

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List of Publications

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Appendix

Used 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. Modified chrome azurol agar (CAS agar).
6. Potato Dextrorse Agar (PDA) (Lab M Limited, Lancashire, UK).
7. Dworkin and Foster’s salts minimal agar medium (DF) (Dworkin & Foster, 1958)

Composition of media

1- Inorganic salt-starch agar (starch nitrate agar) (SNA)

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

Twenty grams of oat-meal were steamed for 20 min 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

Preparation of colloidal chitin

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 min 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)

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- Modified chrome azurol agar (CAS agar)

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 FeCl3 (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.

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

Distilled water	1 L
PDA Powder	39 g

7- Dworkin and Foster's salts minimal agar medium (DF) (Dworkin & Foster, 1958)

Di- hydrogen potassium phosphate	4.0 g
Di-sodium hydrogen phosphate	6.0 g
Magnesium sulfate	0.2 g
Ferrous sulfate	1.0 g
Boric acid	10 µg
Manganese sulfate	10 µg
Zinc sulfate	70 µg
Copper sulfate	50 µg
Molybdenum oxide	10 µg

Glucose	2.0 g
Gluconic acid	2.0 g
Citric acid	2.0 g
Agar	20 g
Distilled water	1 L