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United Arab Emirates University

College of Science

Department of Biology

IDENTIFICATION OF PATHOGENS ASSOCIATED WITH MANGO DIEBACK DISEASE ON MANGO IN THE UNITED ARAB EMIRATES

Fatima Ali Hassan Kamil

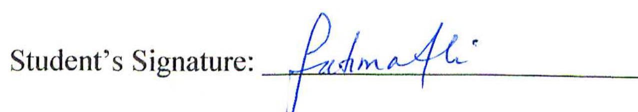
This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Dr. Khaled A. El-Tarabily

November 2018

Declaration of Original Work

I, Fatima Ali Hassan Kamil, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Identification of Pathogens Associated with Mango Dieback Disease on Mango 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 Dr. 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 Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions.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.

Student's Signature:  Date: 19/12/2018

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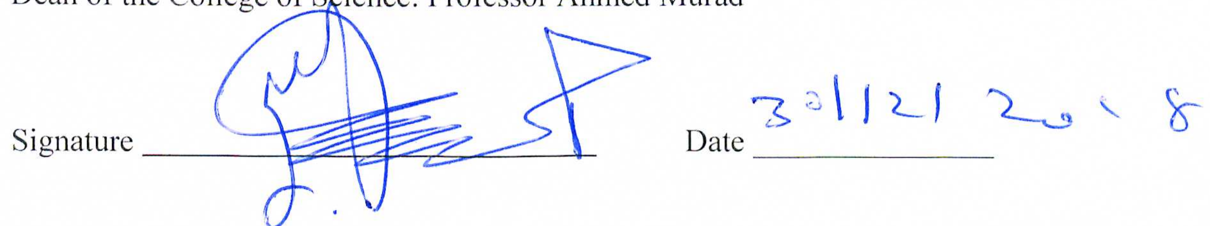

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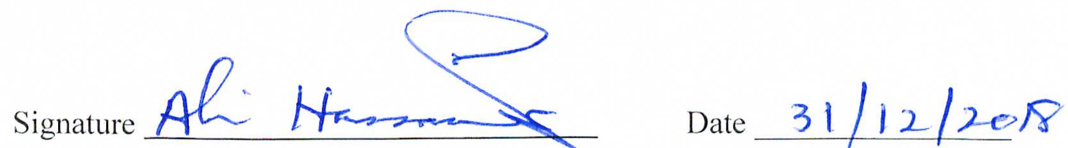

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Abstract

Dieback caused by the fungus *Lasiodiplodia theobromae* is an important disease on mango plantations in the United Arab Emirates (UAE). In this study, 53 actinobacterial isolates were obtained from mango rhizosphere soil in the UAE, of which 35 (66%) were classified as streptomycetes (SA) and 18 (34%) as non-streptomycetes (NSA). Among these isolates, 19 (12 SA and 7 NSA) showed antagonistic activities against *L. theobromae* associated with either the production of diffusible antifungal metabolites, extracellular cell-wall-degrading enzymes (CWDEs), or both. Using a "novel" mango fruit bioassay, all isolates were screened in vivo for their abilities to reduce lesion severity on fruits inoculated with *L. theobromae*. Three isolates, two belonging to *Streptomyces* and one to *Micromonospora* spp., showed the strongest inhibitory effect against this pathogen *in vitro* and were therefore selected for tests on mango seedlings. Our results revealed that the antifungal action of *S. samsunensis* UAE1 was related to antibiosis and the production of CWDEs (i.e., chitinase) and siderophores; whilst *S. cavourensis* UAE1 and *M. tulbaghiae* UAE1 were considered to be associated with antibiotic- and CWDE-production, respectively. Pre-inoculation in greenhouse experiments with the most promising actinobacterial isolates resulted in very high levels of disease protection in mango seedlings subsequently inoculated with the pathogen. This was evident by the dramatic reduction in the estimated disease severity indices of the mango dieback of individual biocontrol agent (BCA) applications compared with the pathogen alone, confirming their potential in the management of mango dieback disease. *L. theobromae*-infected mango seedlings treated with *S. samsunensis* showed significantly reduced number of defoliated

leaves and conidia counts of *L. theobromae* by 2- and 4-fold, respectively, in comparison to the other two BCA applications. This indicates that the synergistic antifungal effects of *S. samsunensis* using multiple modes of action retarded the *in planta* invasion of *L. theobromae*. This is the first report of BCA effects against *L. theobromae* on mango seedlings by microbial antagonists. It is also the first report of actinobacteria naturally existing in the soils of the UAE or elsewhere that show the ability to suppress the mango dieback disease.

Keywords: Actinobacteria, antibiosis, biocontrol, chitinase, dieback, mango, *Lasiodiplodia theobromae*, UAE.

Title and Abstract (in Arabic)

عزل وتعريف الفطريات المسببة لمرض الموت المتأخر لاشجار المانجو فى دولة الامارات العربية المتحدة

الملخص

يعد مرض الموت المتأخر للمانجوالذي يسببه الفطر *Lasiodiplodia theobromae* من الأمراض المهمة في مزارع المانجو في دولة الإمارات العربية المتحدة. في هذه الدراسة ، تم الحصول على 53 عزلة من البكتيريا الخيطية (الاكتينوبكتيريا) من تربة جذور المانجو في دولة الإمارات العربية المتحدة ، تم تصنيف 35 منها (66٪) على أنها ستاربتوميسيتيس (SA) و 18 (34٪) على أنها غير ستربتوميسيتات (NSA). بين هذه العزلات ، أظهرت 19 عزلة (12 SA و 7 NSA) الأنشطة العدائية ضد *L. theobromae* المرتبطة إما لإنتاج المضادات الحيوية المضادة للفطريات و القابلة للانتشار ،أو الأنزيمات المحللة للجدر الخلوية للفطر (CWDEs) ، أو كليهما. وباستخدام اختبار بيولوجي على فاكهة المانجو تم فحص جميع العزلات و قدراتهم على تقليل شدة الآفة على الفاكهة الملقحة ب *Lasiodiplodia theobromae* وأظهرت ثلاث عزلات ، اثنتان تنتميان إلى *Streptomyces* وواحدة إلى *Micromonospora* spp. ، أقوى تأثير مثبط ضد هذا الفطر في المختبر ، وبالتالي تم اختيارها للاختبارات على شتلات المانجو. أوضحت النتائج أن التأثير المضاد على *L. theobromae* باستخدام *S. samsunensis* UAE1 كان مرتبطاً بانتاج المضادات الحيوية وإنتاج CWDEs (chitinase) و siderophores. في حين اعتبرت *S. cavourensis* UAE1*M. tulbaghiae* UAE1 *M.* مرتبطتين بافراز المضادات الحيوية وإنتاج CWDE على التوالي. أدى التطعيم المسبق في تجارب البيوت البلاستيكية مع هذه الثلاث أنواع من الاكتينوبكتيريا إلى مستويات عالية جدًا من حماية المرض في شتلات المانجو التي تم تلقيحها

بعد ذلك مع الفطر الممرض. وكان هذا واضحا من خلال الانخفاض الكبير في مؤشرات شدة المرض التقديرية لمرض الموت المتأخرلاشجار المانجو في حالة استخدام المكافحة البيولوجية الفردية (BCA) مقارنة مع الفطر وحده. مما يؤكد إمكانية هذه الثلاث أنواع في مكافحة مرض موت المانجو. أظهرت شتلات المانجو المصابة و المعالجة بـ *S. samsunensis* انخفاضاً ملحوظاً في عدد الأوراق المتساقطة بمقدار 2 و 4 أضعاف ، على التوالي ، مقارنة بتطبيقات بقية BCA الأخرى. يشير هذا إلى أن التأثيرات المضادة للفطريات لـ *S. samsunensis* باستخدام أنماط متعددة من الإجراء قد أخرت غزو النبات ب *L. theobromae* هذه الدراسة هي أول دراسة عن استخدام كائنات حية نافعة في المقاومة البيولوجية ضد *L. theobromae* على شتلات المانجو. وهو أيضا أول دراسة عن البكتيرياالخيطية (الاكتينوبكتيريا) الموجودة طبيعياً في تربة الإمارات العربية المتحدة أو في أي مكان آخر والتي تظهر القدرة على قمع مرض الموت المتأخرلشتلات المانجو.

مفاهيم البحث الرئيسية: *actinobacteria* ، تضاد ، biocontrol، chitinase، الموت المتأخر، المانجو، *Lasiodiplodia theobromae* ، الإمارات العربية المتحدة.

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Finally, I would never forget all the chats and beautiful moments I shared with my friends. I greatly value their friendship. I have to give a special mention for the support given by Amna Hareb, my best friend, she was with me all the time and I would never forget all her support and kindness.

Dedication

To my beloved parents and family

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Chapter 1: Introduction

1.1 Origin and Distribution of Mango

Mango (*Mangifera indica* L.) fruit which belongs to the family of Anacardiaceae is cultivated in vast areas over the world, specifically in tropical regions. Mango fruit occupies the second position as a tropical crop after banana. (Jahurul *et al*., 2015). The genus Mangifera contains about 41 different valid species, but all mango cultivars fall under a single species of *Mangifera indica* L. The indigenous habitat of the genus is mainly Asia and particularly delimited to several regions between India and Malaysia (Figure 1). The economic importance of mango encouraged the other countries to introduce some species, especially *M. indica* L., into regions far distant from the original home, e.g. in America, Africa, Australia, China, Formosa, etc. (Mukerjee, 1953; Mukherjee, 1972).

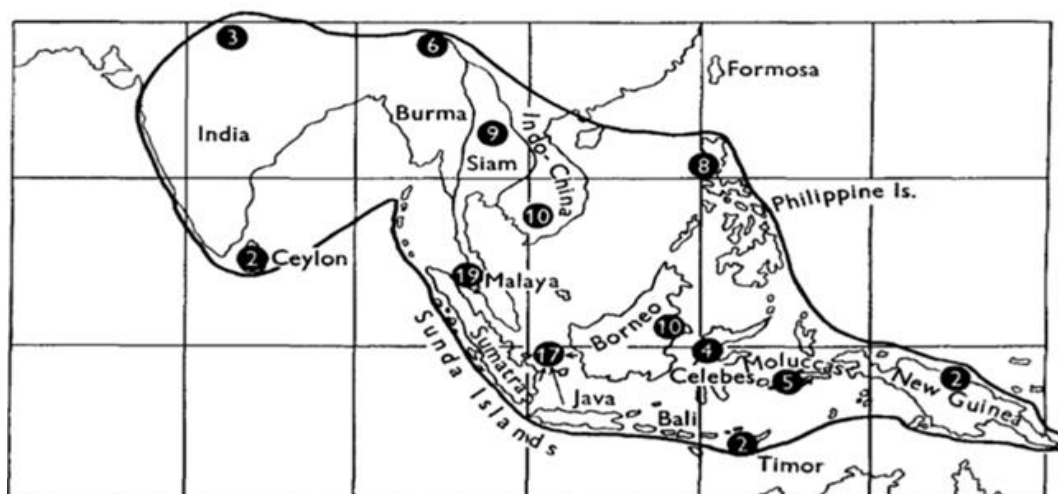


Figure 1: Distribution of mango. The thick line represents the limits of distribution and the numbers with in the circle indicate the number of species in different countries (Mukerjee, 1953).

1.2 Botanical Characteristics of Mango Tree

The most edible fruit belongs to the genus *Mangifera indica*, while other several species of *Mangifera* produce a lower quality fruit usually referred to as wild mangos. Although, mango tree has been cultivated widely, the optimum climate that promotes mango growth is warm climate with dry, frost-free winter. Humidity and rain during flowering season might affect the quality of crop yield. Mango trees are long-lived evergreen that can reach a height of 30 m (100 ft) or more in the indigenous habitats, but the average height is between 3 to 10 m (10-33 ft.) (Bally, 2006).

1.2.1 The Roots

The mango has a long taproot, forming between two to four main anchoring taproots that go deep to the water table and can reach up to 6 m (20 ft). The fibrous root which is known as feeder roots are detected form the surface down to 1 m (3.3 ft). The distribution of fibrous roots are subjected to seasonal changes depending on the moisture distribution in the soil (Bally, 2006).

1.2.2 The Flowers

The flowers of mango are of both polygamous and monoeicous. The flower has five sepals and petals and is highly pubescent (Singh & Sharma, 2008) Mango flowers panicles are borne at the end of the branchlets; each panicle is 4-24 in. long and can bear up to ten thousands individual flowers (Ramirez *et al*., 2010). The floral discs are 4-5 lobed, large with 5 stamens However, only one of the five stamens is fertile, the others are sterile staminodes that are capped by a small gland. The gynoecium is monocarpellous having a single ovule. Both monoeicous and

hermaphrodite flowers are coexisted within a single inflorescence. Often, the hermaphrodite flowers undergo proper pollination and fertilization and set fruit (Singh & Sharma, 2008).

Flowering is an important stage of serial events that set for a fruit production every season. The time and intensity of flowering has a great impact on the amount of fruit that produced at a certain season. The persistent efforts of scientists to investigate the mechanisms of mango flowering (flush) have revealed a better understanding of this sophisticated biological process in mango and other plant systems.

Flowering and pollinations play a vital role in mango fruit development. Mango pollination undergoes series of steps that contribute in development of healthy embryo. Both self and cross-pollination occur in mango. Although, scientists argue which of the two mechanisms is most successful, yet recent studies proofed that cross-pollination that mediated by insects, wind or vertebrate species is most reliable mechanism. On the other hand, supportive evidence by Gerke-Velez has proofed that self-pollination leads to the development of pollen tube and embryo sac, but in most cases, it produces atrophied embryos and aborted or malformed fruit (Ramirez *et al*., 2010; Gehrke-Vélez *et al*., 2012).

1.2.3 The Fruit

Mango fruit is known as "the king of fruit" for its delicious exotic taste. It is produced from panicles and belongs to the family Anacardiaceae and order spindles. The fruit classified as a drupe (fleshy with a single seed) differs in size, shape, color, aroma and flavor depending on the cultivars. The shape of the fruit may vary from

round, oval to oblong with a distinctive conical tip known as “beak” (Figure 2). Mango fruit has three main structures, the exocarp which is a waxy layer that covers and protects the flesh; initially it is green but changes to yellowish, orange or reddish while ripening depending on cultivars. The mesocarp represents the fleshy edible part and is always yellow due to the high content of carotenoids and the endocarp, which is a tough leathery coating of the seed (Bally, 2006; Sivakumar *et al*., 2011).

Mango fruit is climacteric and it matures at the week of eleventh to fourteenth after fruit sets. Therefore, it is extremely important to harvest the fruit during optimal maturity relatively defined as “mature green” stage for export markets. If the fruit are proharvested at immature stage will subsequently ripen with poor quality and might be exposed to disorders and malformation (Lebrun *et al*., 2008).

Figure 2: Popular varieties of mango: Haden (top left), Kensington (top right), Mapulehu (bottom left), Rapoza (bottom right) (Bally, 2006).

1.3 Importance of Mango

The mango is one of the most important fruit in tropical and subtropical regions of the world. This fruit occupies the second rank as the most traded tropical crops and the fifth in total production according to (FAOSTAD 2015). It is known as “king of fruit” because of its delicious exotic taste and pleasant aroma in addition to its high content of different valuable nutritional compounds (Torres-León *et al*., 2016). Due to the high nutritional value of mango it is regarded as an excellent supply of calories, fiber vitamins and minerals in human diet. The flesh and by products (peel and seed) of mangoes provide many bioactive compounds including both nutrient and non-nutrient substances that have great potential to improve human health and reduce the risk of chronic diseases (Ribeiro & Schieber, 2010; Keenan *et al*., 2015).

1.4 The Advantages of Mango’s Bioactive Compounds

Mango fruit is consumed in many food industries such as mango puree, slices in syrup, nectar, pickles and chutney. In addition, other parts of plant such as the leaves and tree bark together with the by-products of mango food industry (peel and seed) are considered an extremely important source of bioactive compounds. Bioactive compounds have aroused a great interest among scientific researches; these compounds are identified as rich source of antioxidants that provides health protection against many diseases. For example, mango seed kernel has total phenolic content of 21.9 to 447 mg g^{-1} dry weigh. This includes phenolic compounds such as (mangiferin, isomangiferin, homomangoferin, quercetin, kamepferol, anthocyanins) and phenolic acids (gallic, protocatechuic, ferulic, caffeic, cuomaric). Therefore, mango seed kernel extract has been used as a cancer alternative treatment. Abdullah *et al.* (2014) has reported that the extract of Water Lily of Malaysia and mango seed

kernel have high potential against breast cancer (Abdullah *et al*., 2014; Torres-León *et al*., 2016).

On the other hand, mango peels have attracted the attention in researchers community for their valuable content of phytochemicals, polyphenols, carotenoids, vitamin E and C. Mango peels are also rich in fiber, cellulose, lipids and proteins therefore they have been exploited in production of various food applications beside the pharmaceutical uses as anti-tumor, anti-oxidant and anti-cardiovascular diseases and hepatoprotective effects (Jahurul *et al*., 2015).

1.5 Diseases of Mango

Mango death or decay could be to two main factors, pathogenic or biotic disorders that mainly caused by different microbial agent or non-pathogenic or abiotic decay due to proharvest at immaturity stage or packaging processes.

1.5.1 Non-pathogenic Disorders of Mango

One of the serious problems that affect mango production is postharvest losses; especially most of the world production of mango is from developing countries. Initially mango fruit disorder is due to early proharvest at immature stage that makes the fruit more sensitive to the temperature variation that leads to inadequate ripening. On the other hand, harvesting at over mature stage makes the fruit sensitive to mechanical injury such as bruising, fruit softening, decay, and water loss. The method of harvesting fruit will affect the quality; using manual harvesting tools might cause physical damages such as scratches or bruising this in turn make the fruit susceptible to decay causing bacteria and fungi (Sivakumar *et al*., 2011).

1.5.2 Pathogenic Disease of Mango

Mango is one of the most important cultivars in tropic and subtropics regions. Recently, mango crops have been susceptible to a wide range of plant parasitic diseases that cause a drastic declining in mango tress worldwide. Below is listed some of the most significant diseases caused by different microorganisms.

1.5.2.1 Nematodes Associated with Mango Decline

Mango appears to be relatively free from severe nematodes damage, despite the long list of nematodes species associated with it probably the most widely distributed nematodes associated with mango is Hemicriconemoids mangifera which feed on mango roots together with the nematode *Xiphinema brevicolle* (Luc *et al*., 2005). Nematodes including *Criconemella sphaerocephala*, *Helicotylenchus dihystera*, *Hoplolaimus indicus*, *H. mangiferae, Meloidogyne spp., Pratylenchus brachyurus, R. reniformis, Trichodorus spp, Tylenchus filiformis* and *Tylenchorhynchus mashoodi* have also been found associated with mango decline trees (Anwar *et al*., 2012).

1.5.2.2 Bacteria Associated with Mango Diseases

Bacterial black spot or black canker is one of the significant problems that threaten mango orchards especially, if the trees are grown in poor soil and under unfavorable climates. Mainly *Xanthomonas campestris* pv. *Mangiferaeinicae* is the causal agent of the disease. It is a gram negative rods and motile by a single polar flagellum. These bacteria usually infect the young trees and all the parts are susceptible to infection. The major symptom observed on the fruit is a water-soaked halos around the lenticels or wounds and quickly raised and turn to black before a

cracking on the fruit surface (Dodd *et al*., 1997). The disease has been discovered in different regions over the world such as Australia, Japan, Kenya, Malaysia, Philippines, South Africa and United Arab Emirates (Ploetz, 2004).

Galal *et al*. (2006) has reported for the first time that *Bacillus pumilus* a rod-shaped, Gram positive, endo-spore formers and yellow pigment producers is a causal agent of leave blight and dieback of twigs in mango trees in El-Minia Governorate, Egypt (Galal *et al*., 2006).

1.5.2.3 Fungal Pathogens Associated with Mango Diseases

Fungal infection is one of the most devastating biotic diseases that affect the yield and vitality of many crops and in particular mango orchards. A major disease impacts mango tree in all mango-producing regions over the world is the mango dieback syndrome. The causal agent of decline syndrome was unknown until 1970s. In 1991 Ramos (Ramos *et al*., 1991) was the first one to report that *Botryosphaeria ribis* was the primary pathogen of this disease.

Other than *Botryosphaeria* spp. that cause mango decline, many fungi have associated with different types physiological disorders in mango with symptoms like bud necrosis, gummosis and vascular discoloration including *Alternaria alternata*, *Cladosporium sp*., *Colletotrichum gloeosporioides*, *Dothiorella dominicana*, *Fusarium spp*., *Lasiodiplodia theobromae*, *Penicillium sp*., *Pestalotiopsis sp*. and *Phomopsis spp (*Anwar *et al*., 2012).

1.6 Mango Dieback Disease

Lasiodiplodia theobromae belongs to the cosmopolitan Botryosphaeriaceae family. Members of Botryosphaeriaceae were discovered in 1820’s as a species of

Sphaeria. They form important pathogenic fungi of both woody trees and herbaceous plants; such as cassava, mango, apricot and peaches, cacao, coconut palm, citrus and many other economically important crops. They have been associated with gummosis, root necrosis, leaf spots and death of the host plant causing devastating losses all over the worlds (Slippers & Wingfield, 2007; Muniz *et al*., 2011; Netto *et al*., 2017).

1.6.1 Symptoms of Dieback Disease

Species of *Lasiodiplodia* is widely distributed in tropical and subtropical regions and could infect various plants. They can be saprobic, endophytic or pathogenic. In the past, it was believed that the infection by Botryosphaeriaceae occurred through wound or cracks on the plant. Later, a number of studies have proved that these fungi can also infect the host plant via lenticels, stomata or other natural opening on plants. However, endophytic infection could happen when the plant is under stress. Commonly all plant parts are exposed to infection by Botryosphaeriaceae including the bark, xylem of stems, branches, leaves, flowers fruits and seeds (Slippers & Wingfield, 2007).

In India an onset of spoilage symptoms due to *B. theobromae* infection on Alphonso mango, one of the most important cultivar, exhibiting a black discoloration of mango peel associated with swollen appearance due to softening of the pulp. When a section of the tissue is examined under microscope it was completely covered with the fungus mycelia (Mascarenhas *et al*., 1996).

The symptoms of dieback disease occurs in plants of all ages and are identified by shoot and branches necrosis, blight of the leaves, gummosis and stem

and branches canker by the time the disease will spread towards the root region causing the dieback of the plant (Figure 3) (Rodríguez-Gálvez *et al.*, 2017).

Figure 3: Dieback symptoms on *Mangifera indica* in Peru associated to *Lasiodiplodia* spp. (Rodríguez-Gálvez *et al*., 2017).

1.6.2 Causal Agent of Mango Dieback Disease

The mechanism of both dispersal and endophytic infection by Botryosphaeriaceae caused either by ascospores or conidia. Interestingly, some studies have shown that some species are probably obligately asexual such as *Diplodia pinea*. This fungus spread species exclusively via conidia while other species known to be sexual. The conidia or homothallic ascopores are an important, and often dominant, form of dispersal and infection in these fungi (Slippers & Wingfield, 2007).

The main morphological characteristics that identify the genus *Lasiodiplodia* from other closely related genera is the presence of pycnidia, paraphysis and longitudinal striations in mature conidia. More than 20 species have been recognized according to the morphology of conidia and paraphysis. However, the most accurate discriminatin of these species are based on the sequencing of the intergenic spacer regions of the rDNA (ITS) and elongation factor 1 alpha (EF-1) (Picos-Muñoz *et al*., 2015).

The basic features of ascocarp are dark brown to black with thick dark brown and hyaline wall in internal layers, 250-400 µm in diameter. The asca is bitunicated, stipulated, with 8 spores, 90-120 µm long. The ascospores are biseriadas, hyaline, aseptadas of 30-35 x 11-14 µm. The conidiomata is simple or aggregate, immersed in the host and once mature emerge from it, dark brown, unilocular, thick-walled or thin brown, often up to 5 mm wide. Hyaline paraphyses, cylindrical, septated, occasionally branched with the ends rounded to 55 µm long and 3-4 µm wide (Picos-Muñoz *et al*., 2015).

Conidiophores are hyaline, simple, sometimes septate, rarely branched and cylindrical. The conidia are subovoides to ellipsoidal, with widely rounded apices, which narrow to truncate the base, wider in the middle of the upper third, thick-walled, with granular content, initially hyaline and asepted, becoming dark brown once mature , with 1 septum, have melanin deposits on the inner surface of the wall arranged longitudinally giving a striated appearance with measurements of 21.5-31-5 x 13-17 µm and a ratio of 1.9 Length / Width (Figure 4) (Picos-Muñoz *et al*., 2015).

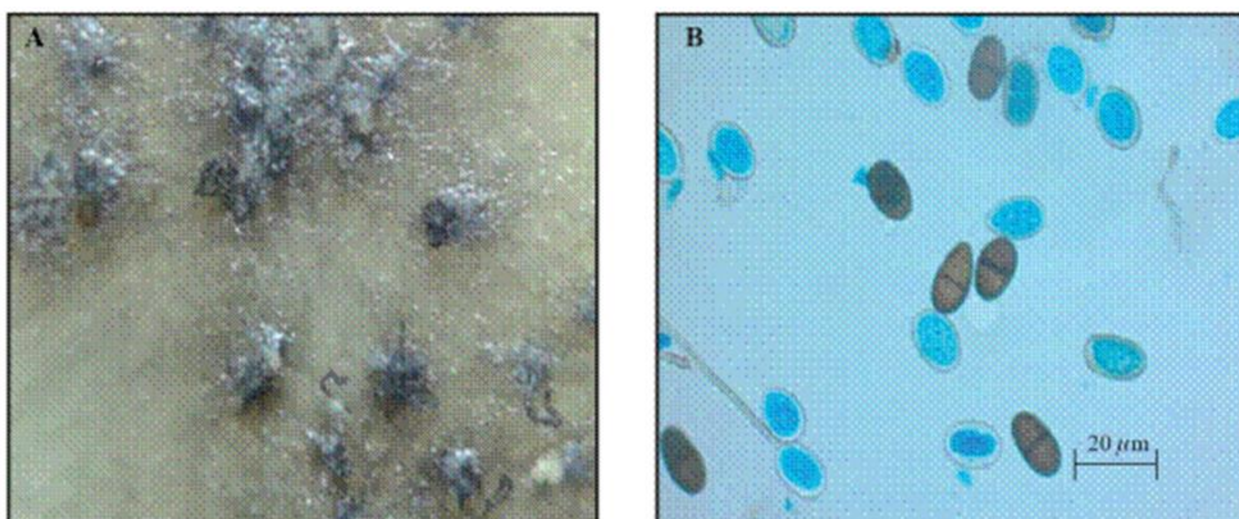


Figure 4: *Lasiodiplodia theobromae* in isolated from papaya. A) Mycelium and pycnidia in PDA at 14 days of growth. B) Mature and immature conidia at 14 days of growth (Picos-Muñoz *et al*., 2015).

1.6.3 Chemical Control of Mango Dieback Disease

Mango dieback disease is one of the threatening syndromes that affect negatively mango production and strike the economy of many countries that depends greatly on exporting of mango. Myriads of studies were conducted to fight the disease, which caused by a member of Botryosphaeriaceae particularly *L.theobormae.* Chemical fungicides up-to-date are one of the effective means in controlling pathogenic fungi in plan. In 1995 Ahmed *et al*. reported the effect of several fungicides (Topsin-M, Benlate, Daconil, Bordeaux mixture and Tilt) on mango nursery twigs. He concluded that Topsin-M and Benlate porved their efficiency in controlling the disease. In contrast, Tilt showed the minimum effect on the disease (Ahmed *et al*., 1995). Khanzada *et al*. reported that Carbendazim when applied in field experiment was found to be effective in ceasing the gum exudation dieback and wilting and showed great enhancement in vegetative growth of plant. (Khanzada, *et al*., 2005). Tovar Pedraza *et al*. (2013) has used physical and chemical treatments, washing and fungicides dip to control *Lasiodiplodia theobromae* in Mexican nursery during two grafting seasons. They concluded that washing reduced disease occurrence by 31%, while chemical treatment reduced up to 62 %. On the other hand, combinations of both treatments washing and thiabendazole dip resulted in an incidence decrease of 81% (Tovar Pedraza *et al*., 2013)

In UAE a research conducted by Saeed *et al*. (2017) has indicated that the application of chemical fungicides, Score®, Cidely® Top, and Penthiopyrad®, significantly inhibited the mycelial growth of *L. theobromae* both *in vitro* and in the

greenhouse. Among the used fungicides, Cidely® Top proved its efficincy against *L. theobromae* dieback disease also under field conditions (Saeed *et al*., 2017a).

1.7 Biological Classification of Actinobacteria

Actinobacteria are Gram-positive bacteria with distinctive morphological varieties, from cocci to filamentous mycelial forms. Among the different genera of Actinobacteria, Streptomyces are the most extensively studied mycelial actinobacteria.

Actinobacteria can usually reproduce by two means, the binary fission like most prokaryotes, or by formation of special spores or conidia and are distinguished by a non-segmented threads or mycelia. However, under certain condition septa or segments might be observed in some species. The mycelia could be branching or non-branching, straight or spiral shaped. The spores have different shapes spherical oval or cylindrical (Niu, 2018).

Actinobacteria are characterized by a rigid cell wall to maintain the shape of the cell and to stand the high osmotic pressure; the cell wall is composed of many different complex compounds such as peptidoglycan, teichoic and teichuronic acid and polysaccharides. The chemical structure of Actinobacteria cell wall resembles that of Gram positive bacteria but because of their well-developed hyphae, Actinobacteria have been considered as a separate group from other common bacteria (Niu, 2018).

According to Williams & Wellington (1982), those among Actinobacteria, recurrence of *Streptomyces* were 95.3%, *Actinoplanes* 0.2%, *Actinomadura* 0.1%, *Microbispora* 0.18%, *Micromonospora* 1.4%, *Nocardia* 1.98%, *Pseudonocardia*

0.06%, *Streptosporangium* 0.10%, *Thermoactinomyces* 0.14%, and *Thermomonospora* 0.22% (El-Tarabily & Sivasithamparam, 2006).

1.7.1 Distribution of Actinobacteria

The prevalence of saprophytic Actinobacteria is due to metabolic diversity and evolution of novel mechanisms for dispersal. Many Actinobacteria depend on air dispersal through their hydrophobic spores, other Actinobacteria seem to be adapted to water mediated dispersal including the range from *Actinoplanes* to *Nocardia*. *Micromonosporas* are similar to *Actinoplanete* but without aerial mycelium and produce single non-motile spores, these distinctive features of *Micromonosporas* contributes to their abundance in freshwater environments. Saprophytic Actinobacteria are mainly colonizers of soil, their typical abundance in soil is estimated between 10^6 to 10^9 cells per gram of soil; among the different species of Actinobacteria the genus *Strepromyces* is the most genus that dominating the soil, which accounts over 95% of the *Actinomycetales* strains. The growth of Actinobacteria is affected by several factors, such as temperature, pH, and soil moisture.

Like many other microbes, Actinobacteria are unequally distributed due to different environment parameters. The general preference of Actinobacteria for neutral to alkaline pH and absolute requirement for aerobic conditions are however established and clearly important in Actinobacteria ecology, the recovery of acidophilic Actinobacteria from soils of low pH and identification of a novel Actinobacteria population colonizing a salt marsh are good examples of the ability of Actinobacteria to adapt to a range of environmental factors (McCarthy & Williams, 1990).

1.7.2 Isolation and Cultivation of Actinobacteria

Actinobacteria are gaining special interest in the field of microbiology due to their unique niche in the environment. Their fungu-slike structure led to confusion between these ambiguous organisms and fungi. The progressive advances in microscopic and chemotaxonomic methods have greatly contributed in differentiation of the genera of Actinobacteria. For example, later studies on biochemical relationships of Actinobacteria have led to the differentiation of *Nocardia* and *Streptomyces* through chromatography of whole-cell hydrolysates. Therefore, an individual responsible for isolating and preserving Actinobacteria must have a basic knowledge of the properties of Actinomycete cultures (Dietz & Currie, 1996).

As mentioned earlier, the Actinobacteria are dominating bacteria over the mixed microflora in nature, but their isolation from other microflora is quite complicated; because of their characteristic slow growth relative to that of other soil bacteria. However, there are five basic procedures for the isolation of industrially important Actinobacteria. (i) Choice of substrate: whether the isolation of Actinobacteria from freshwater and marine environment or soil, as there are some differences between organisms existing in marine and terrestrial environments (Dilip *et al.*, 2013). (ii) Pre-heat treatment: allows the selective isolation of Actinobacteria found to be rare or absent in soils. Such pre-heat treatments significantly decrease the numbers of Gram-negative bacteria. Desiccation plus mild heat treatments associated with suitable selective media can yield well-separated bioactive Actinobacteria isolated from different environments. In addition to heat treatment, many antibiotics had been tested against a range of Actinobacteria, bacteria and fungi representing

types found in soil. “From these, nystatin (50 μg/ml), acetidione (50 μg/ml), polymyxin B sulphate (5 μg/ml) and sodium penicillin (1 μg/ml)” are generally selected for incorporation into a starch casein medium to achieve selective growth of Actinobacteria on soil dilution plates (Dilip *et al.*, 2013). (iii) Selective media: in order to suppress the growth of other bacteria and molds, both bacteriostatic and fungistatic additives such as phenol and sodium propionate would be integrated with the isolation media and thus favor the growth of Actinobacteria. Selective media such as chitin agar has shown superior selectivity upon other media for isolating Actinobacteria from water and soil. (iv) Incubation: normally antibiotic producing Actinobacteria grow between 25 to 30°C, while thermopiles are incubated at 40 to 45°C. The best incubation period for isolation plates are usually from 7 to 14 days. However, for isolation of new Actinobacteria incubation period may be extended for one month (Dilip *et al.*, 2013).

(v) Colony Selection: Selection of a colony is critical and time consuming step. It is related to the target of the screening project. Several intellectual ways must be utilized during isolation of Actinobacteria as there might be more duplication of the colonies. The majority of researchers now select candidate colonies by using a stereomicroscope and transferring growth with the aid of a pointed wooden cocktail stick. Many factors like, the site of sample collection, knowledge of the secondary metabolite of an isolate, objective enrichment techniques and objective culture media formulations contribute in a successful isolation of a novel and potential isolates (Dilip *et al.*, 2013).

There are many methods for handling Actinobacteria such as “lyophilization”, “soil” and “liquid nitrogen”. Thus restoration conditions (medium, temperature, air,

tube, plate, or shake flask cultivation) dramatically affect the viability and quality of the preserved culture. The importance of the restoration conditions is often overlooked; therefore the conditions used to restore the culture may vary from those used for the cultivation of the culture (Dietz & Currie, 1996).

1.8 Biological Control of Fungal Plant Pathogens

The general definition of the term "biological control" is the use of microbial antagonists to repress diseases, the organism that destroys the pest or pathogen is commonly referred to as biological control agent (BCA). The term biological control is also applied to secondary products extracted from the beneficial organism. The aim of conducting researches on biocontrol is to minimize the over consuming of agrochemicals and consequently the risks on human and animal health and the environment (Pal & Gardener, 2006; Ab Rahman *et al*., 2017).

1.8.1 Mechanisms of Biological Control

There are several modes of biocontol actions. Whether a single biocontrol mechanism, or in combination, directly or indirectly is applied; the beneficial microbe could suppresses plant diseases through one of the following mechanisms (i) competition with pathogen for space and nutrients, (ii) mycoparasitism,(iii) antibiosis, (iv) production of siderophores and (v) induced systemic resistance (Ghorbanpour *et al*., 2018).

An example of control measures taken to eliminate mango fruit diseases is the excessive use of copper oxychlroide, this lead to accumulation of copper in soils. The strict maximum residue level set by the European Union (EU) and the limitation of registered fungicides affect negatively the availability of chemical pesticides.

Therefore, a move towards another alternative to control diseases is persistence. One of the promising alternatives is biological control, which has shown a success on many crops including mango. Spraying the field with the antagonist *Bacillus licheniformis* have proved effective control on both Pre-harvest and post-harvest diseases in mango fields (Silimela & Korsten, 2007).

1.8.1.1 Competition for Nutrient and Space

When two or more organisms share the same resources for growth they start to compete for these resources to survive. The degree of competition is varied through different parts of plant. For example, the rhizosphere is a region of extensive microbial activity where the competition will be on the space and oxygen in a soil-borne pathogen that infects certain part of the roots. In upper part of the plant such as leaf surface, where the nutrients are in scarce, the competition will be tough. The competition for the same space is usually occurs between organisms that taxonomically related "(e.g. the fire blight bacterium *Ertutnia amglouora* and the saprophytic species *Erwinia Herbicola*)". The same thing happens in competition for nutrients, the antagonist with same nutrients requirements will dominate the pathogen. For example, *Pythium ultimum* the causal agent of seedling damping-off competes with rhizosphere bacteria for the same source of carbon, therefore the rhizosphere bacteria is an efficient biocontrol of *P. uttimum* (Stirling & Stirling, 1997).

1.8.1.2 Antibiosis

The term antibiosis includes the production of different antimicrobial elements by the BCA s that inhibits the growth of certain pathogen. This mechanism

has been observed in wide range of biocontrol agents such as *Trichoderma*, that produce multiple compounds with antagonistic properties like cell wall degrading enzymes (cellulase, xylanase, pectinase, glucanase, lipase, amylase, arabinase, and protease), volatile metabolites such as 6-n-pentyl-2H-pyran-2-one (6-PAP), and a number of antibiotics and formic aldehyde (Ghorbanpour, 2018).

1.8.1.3 Production of Siderophores

One of the most important elements for all organisms is iron. Although, iron is abundant in earth curst, its availability in insoluble form of ferric hydroxide makes it difficult for bacteria to take advantage of it, which require iron at micromolar concentration for their growth. To overcome this challenge, many microorganisms produce extracellular siderophores. Siderophores are low molecular weight high affinity Fe^{3+} chelators that transport iron into bacterial cells, most of aerobic and facultative anaerobic bacteria are found to produce siderophors that make them prominent in disease suppression and plant growth promotion. The plant growth promoting rhiczobacteria (PGPR) produce extracellular siderophors that chelate environmental iron making it unavailable to pathogenic microorganisms and suppress their growth (Sahu & Sindhu, 2011).

1.8.1.4 Induced Systemic Resistance

Induced systemic resistance (ISR) is referred to systemic resistance promoted by PGPR when the plant induced appropriately by rhizobacteria, the plant roots will recognize these bacteria and consequently elevate the level of resistance that expressed during subsequent infection by a pathogen. For example, *Arabidopsis thaliana* ecotype columbia plants (Col-0) treated with *Serratia marcescens* 90-66 and *Bacilus pumilis* strain SE-34 significantly reduced the symptom severity caused

by cucumber mosaic virus. The defensive mechanism results when the plant roots recognize the rhizobacteria this will enhance the capacity of plant to overcome the diseases upon subsequent infection by a pathogen (Ryu *et al*., 2004; Harish *et al*., 2008)

1.8.1.5 Mycoparasitism

Now a days, certain species or strains of fungi have been exploited to control plant diseases in agricultural, horticultural, and forestry systems. Many fungi have specific strategies in curing foliar and root diseases caused by other pathogenic fungi efficiently. One of these strategies is, mycoparasitism, whereby a certain species of fungus could destroy and feed on other fungi (Brimner & Boland, 2003). The exploration of mycoparasitism was dating back to 1930s. One of the most extensively studied fungi in this regards were several species of *Trichoderma* spp. late in 1980s, yeasts were discovered in minimizing the growth of pathogenic fungi, but most of the experiments required a close physical contact between the plant pathogen and mycoparasite. Most observations were depicted for petri dishes or detached plant tissue under controlled conditions. These findings have recently extended beyond *the in vitro* standardization to involve vegetable crops grown under glasshouse and field conditions; thereby these microbial agents have been registered as effective biocontrol agents (Punja & Utkhede, 2003). The mechanism of mycoparasitim in inhibition of fungal plant pathogen depends on secretion of a wide range of fungal cell-wall degrading enzymes (CWDEs) and porteases that enable the parasite to invade the hyphae of the pathogen. For example, chitinases and glucanases are the most effective CWDEs in *Trichoderma* spp. As chitin and glucan are the main fungal cell wall polysaccharides (Daguerre *et al*., 2014).

1.9 Aim of the Thesis

Dieback caused by the fungus *Lasiodiplodia theobromae* is one of the most threating and devastating disease on *mango* plantations in the United Arab Emirates (UAE). In this study, a number of actinobacterial isolates were obtained from mango rhizosphere soil in the UAE to test their antagonistic activities against *L. theobromae* associated with either the production of diffusible antifungal metabolites, extracellular cell-wall-degrading enzymes (CWDEs), or both.

1.10 Objective of the Thesis

The main objectives of this study are to:

(1) Develop a molecular identification of the pathogen(s) attacking mango.

(2) Test the actioncaterial isolates for their production of antifungal metabolites.

(3) Test the effective actinobacterial isolated on the identified pathogen *in vitro*.

(4) Apply the biocontorl agent on infected mango seedling, *in vivo*

Chapter 2: Material and Methods

2.1 Fungal Isolation

Diseased trees in Kuwaitat area in Al Ain city (Eastern region of Abu Dhabi Emirate; Latitude/Longitude: 24.21/55.74) with drying leaves on branches and twigs (Figure 5A-C) were studied in this investigation. A symptomatic tree (approximately 5 years old) was lifted and transferred to the Plant Microbiology Laboratory, Department of Biology, United Arab Emirates University in Al Ain city, for investigation. Longitudinal cross-sections were made of the diseased tree twigs and the pathogen was isolated from affected tissues (Figure 6). Tissues were cut into small pieces (2-5 mm long), washed and surface-sterilized with mercuric chloride 0.1% for 1 min, followed by three consecutive washings in sterile distilled water. They were then transferred onto PDA (Lab M Limited, Lancashire, UK) plates, pH 6.0; supplemented with penicillin-streptomycin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) used at a rate of 25 mg/L of the growth medium in order to inhibit the bacterial contaminants. Petri dishes were incubated in an incubator at 28°C ± 2°C for 5 days. After this period of incubation, the mycelia growing out of the plated tissue was aseptically sub-cultured on fresh PDA and lastly purified by using hyphal-tip isolation technique (Krisop & Doyle, 1991). The mycelium and conidia were observed using Nikon-Eclipse 50i light microscope (Nikon Instruments Inc., NY, USA) to characterize different fungal structures. A culture of the identified fungus, *L. theobromae* (Pat.) Griffon & Maubl. (Zamnettakis, 1954; Sutton, 1980) has been deposited in Leibniz-Institute DSMZ- Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) under the collection number DSM 105134.

2.2 DNA Isolation, PCR and Sequencing

DNA of the pathogen from infected tissues of leaf, twig and apical shoot tips was extracted from mycelium cultured for 10-14 d at 28°C on PDA plates. DNA extractions were performed using the protocol of using the plant/fungi DNA isolation kit (Norgen Biotek Corp., Canada) with some modifications. PCR was set-up to amplify target regions of internal transcribed spacer (ITS) of the nuclear rDNA for *L. theobromae* using ITS1 and ITS4 primers (Alves, *et al*., 2008), and partial TEF1-α using EF1-728F and EF1-986R (Carbone & Kohn, 1999; Alves *et al*., 2008). All primer sequence sets can be found in Table 8. All protocols for amplification and sequencing were as described (Alves *et al*., 2008).

2.3 Phylogenetic Analysis

For the analysis of the phylogenetic placement of the fungal isolate the sequences of ITS rDNA and TEF1-α genes were used as single gene set and concatenated two-gene set, ITS/ TEF1-α. The obtained ITS and TEF1- α sequences were deposited in GenBank (accession number: MF114110 and MF097964, respectively) and were further combined for constructing the phylogenetic tree against Lasiodiplodia species database managed by the National Center for Biotechnology information (NCBI; www.ncbi.nlm.nih.gov). The ITS/TEF1-a sequence of the isolate from the UAE was aligned with sequences retrieved from GenBank, representing isolates that belong to about 18 species of the genus Lasiodiplodia (Alves *et al*., 2008; Ismail *et al*., 2012). All sequences were compared and aligned and Maximum Likelihood analyses were performed for estimation of the phylogenetic tree (Tamura *et al*., 2013). Phylogenetic trees were constructed and validated with a statistical support of the branches with 100 bootstrap resamples. These belong to isolates are: *L. plurivora, L. gilanensis, L. iraniensis, L.*

mahajangana, L. theobromae, L. hormozganensis, L. citricola, L. parva, L. egyptiacae, L. pseudotheobromae, L. crassispora, L. rubropurpurea, L. venezuelensis, L. gonubiensis, L. margaritaceae, Diplodia mutila, D. corticola and Phyllosticta capitalensis.

2.4 Isolation of Steptomycetes Actinobacteria (SA) and Non-Streptomycetes Actinobacteria (NSA) from Mango Rhizosphere

Five rhizosphere soil samples were collected from 30 cm depth under healthy mango trees in sealable plastic bags. The rhizosphere soil samples were air-dried for 4 days at 25°C (Williams *et al.*, 1972), passed through a 5 mm mesh sieve, and stored prior further analyses.

The soil dilution plate method was used to isolate SA from each rhizosphere sample using inorganic salt starch agar (Küster, 1959) supplemented with the antifungal antibiotics nystatin and cycloheximide (50 µg ml^{-1} each; Sigma-Aldrich). In order to increase and decrease the populations of filamentous actinobacteria and other bacteria, respectively, the soil pre-treatments described by Hayakawa and Nonomura (1987) was used. For each dilution, seven plates were used and incubated at 28°C in dark for 7 days.

For the NSA recovery, four polyvalent *Streptomyces* phages (El-Tarabily, 2006) were used to reduce the dominance of SA on inorganic salt starch agar plates (Kurtböke *et al.*, 1992). The stock phage suspension (1012 plaque forming units ml^{-1}) was prepared by mixing high-titer phage suspensions of each polyvalent *Streptomyces* phage. Seven plates were inoculated with 0.2 ml aliquots of the phage-treated soil suspension, dried and incubated in dark at 28°C for 14 days. Control treatments were considered as plates without phages.

Colonies of SA and NSA, expressed as $\log_{10}$ colony forming units (cfu) g^{-1} dry soil, were purified on oatmeal agar plates (ISP medium 3) amended with 0.1% yeast extract (Küster, 1959). Colonies were identified based on morphological features, distribution of aerial/substrate mycelia, presence/absence of aerial mycelia, and the stability/fragmentation of substrate mycelia (Cross, 1989).

2.5 Detection of The Antifungal and CWDE Activities

All actinobacterial isolates were characterized based on their ability to secrete diffusible antifungal metabolites active against *L. theobromae* 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 cultivating *L. theobromae* on PDA slants at 28°C until sporulation, which were then flooded with 50 mM phosphate buffer (pH 6.8) (Saeed *et al*., 2017b). 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 actinobacterial cultures on fish meal extract agar with a sterilized 11 mm cork-borer onto PDA plates seeded with *L. theobromae* 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 picked for further experiments; and the remaining of the isolates were not used in the subsequent tests.

All isolates were also tested for their abilities to produce clearing zones on *L. theobromae* mycelial fragment agar as an indicator of preliminary production of CWDEs according to Valois *et al*. (1996). Large (>30 mm) and small (<30 mm) diameters represented high and low CWDE activities, respectively. In addition, all obtained isolates were evaluated for their potential 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. Large (>30 mm) and small (<30 mm) diameters represented high and low chitinase activities, respectively. 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 chosen for further experiments.

2.6 Mango Fruit Bioassay

A novel mango fruit bioassay was developed in our laboratory to determine the ability of the most promising candidates to suppress or reduce disease development (lesion formation) following inoculation with *L. theobromae in vivo*. The mango fruit bioassay was modified according to previous tests of carrot bioassay against *Pythium coloratum* (El-Tarabily *et al*., 1997) and mango fruit pathogenicity against L. theobromae (Saeed *et al*., 2017a).

Mature mango fruits (cv. Badami) were placed in plastic trays on sterile paper towels moistened with sterile distilled water, and were inoculated by placing the agar plugs (11 mm) colonized by the actinobacterial isolates and/or L. theobromae, described above, onto each mango fruit according to the following combinations: (i)

a sterile non-inoculated PDA agar plug (control; C); (ii) the antagonist alone (BCA) with a sterile PDA agar plug above it; (iii) *L. theobromae* (Lt) alone with a sterile PDA agar plug below it; and (iv) pairing *L. theobromae* and the antagonists together (BCA+Lt), with the BCA on the mango surface and *L. theobromae*-inoculated plug on top of the BCA. The antagonists were inoculated onto the mango surface 24 h prior the pathogen in order to allow time for the secretion of antifungal metabolites and/or chitinase onto the mango surface. Each mango fruit was inoculated with the four treatment combinations for each BCA of four fruits/tray in triplicates. Trays were covered with aluminum foil and incubated under humid conditions in dark at 28°C for 4 days. The lesion diameters were measured in order to determine disease indices. To fulfill Koch's postulates, all diseased fruit tissues were incubated on PDA plates at 28°C in dark for 5 days.

2.7 Assay of Producing Diffusible Antifungal Metabolites or Chitinase

I assessed the three most promising antagonistic BCAs for their ability to secrete diffusible antifungal metabolites active against *L. theobromae* using the cup plate technique as previously described (Bacharach and Cuthbertson, 1948). Inocula for the preparation of the *L. theobromae*-seeded PDA plates were prepared, as described above, for the cut-plug method. In order to assess the inhibition of *L. theobromae* by the diffusible antifungal metabolites on fish meal extract agar (El-Tarabily *et al*., 1997) or by the chitinase on colloidal chitin agar (El-Tarabily *et al*., 2000), a dialysis membrane overlay technique (Gibbs, 1967) was used.

Briefly, dialysis membrane (Type 45311; Union Carbide Corporation, IL, USA) with adhering colonies were removed from the agar plates and the center of each plate was inoculated with a disc (5 mm diameter) of *L. theobromae* culture. At

the end of the incubation period, the colony diameter of *L. theobromae* was measured. The agar plugs were further transferred to a fresh PDA plate and incubated at 28°C for 5 days to determine whether the diffused metabolites/chitinase were fungistatic (pathogen growth from the plug) or fungicidal (no pathogen growth from the plug).

2.8 Volatile Antifungal Compounds, Hydrocyanic Acid and Siderophore Production

Production of volatile antifungal compounds (Payne *et al*., 2000) by the BCAs was examined using fish meal extract agar. For the production of hydrogen cyanide (hydrocyanic acid), the BCAs were inoculated on tryptic soy agar medium (Lab M Limited) supplemented with 4.4 g glycine l^{-1}. The plates were inverted and a piece of filter paper (soaked in 0.5% picric acid in 2% sodium carbonate) was placed in the lid of each Petri dish, and incubated at 28°C for 5 days (Bakker & Schippers, 1987). Discoloration of the filter paper to orange brown after incubation indicates production of hydrogen cyanide (Castric, 1975).

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

2.9 Determination of CWDE Activities of the BCA Candidates

Erlenmeyer flasks containing 50 ml of minimal synthetic medium (Tweddell *et al*., 1994) supplemented with 2 mg ml^{-1} of either *L. theobromae* cell wall fragments, colloidal chitin, or laminarin (Sigma-Aldrich) were prepared. Flasks containing each substrate 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).

Chitinase and ß-1,3-glucanase activities were determined by measuring the release of N-acetyl-D-glucosamine and the amount of reducing sugars liberated using dinitrosalicylic acid solution (Miller, 1959), respectively. The protein content of the enzyme solution was determined as described by Lowry *et al*. (1951) using Folin phenol reagent.

2.10 Effect of BCA Crude Culture Filtrates on Mycelia and Conidia of *L. theobromae*

The filter-sterilized crude culture filtrate for each BCA (section Determination of CWDE Activities of the BCA Candidates) using fish meal extract broth or colloidal chitin broth (Gupta *et al*., 1995) was proportionally poured in PDA plates. The medium was inoculated with a 5 mm diameter agar plug colonized with **L.** theobromae mycelium (placed upside down). The colony diameter (mm) of **L.** *theobromae* was measured after 5 days at 28°C.

The crude culture filtrate prepared from fish meal extract broth or colloidal chitin broth was also proportionally mixed with potato dextrose broth (PDB; Lab M) (Lorito *et al*., 1993). The PDB was inoculated with a 5 mm diameter agar plug colonized with *L. theobromae*. The dry weight of *L. theobromae* was measured after 10 days of incubation in dark at 28°C.

The effect of the crude culture filtrate of each BCA on mature conidia germination and germ tube elongation of *L. theobromae* was carried out in PDB

according to Lorito *et al*. (1993). The percentage spore germination and average germ tubes lengths were microscopically determined after 24 h at 40X using Nikon-Eclipse 50i light microscope (Nikon Instrument Inc., NY, USA) and compared with the control (non-inoculated filter-sterilized fish meal extract broth or colloidal chitin broth).

The effect of the crude culture filtrate of the three BCAs on the morphology of *L.* *theobromae* hyphae was assessed (Sneh, 1981). At sampling, *L. theobromae* hyphae treated with the BCA was microscopically examined at 100X using a light microscope. *L. theobromae* mycelium incorporated with non-inoculated filter-sterilized fish meal extract broth or colloidal chitin broth served as control treatments. Three replicates were used at each sampling.

2.11 Identification and Phylogenetic Analysis of the BCA Candidates

The identification of the three promising BCAs, BCA1 (isolate #12), BCA2 (isolate #29) and BCA3 (isolate #44), was carried out using 16*S* rRNA gene sequence analysis done by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, (DSMZ), Braunschweig, Germany, using the primers 900R (5′-CCGTCAATTCATTTGAGTTT-3′); 357F (5′-TACGGGAGGCAGCAG-3′) and 800F (5′-ATTAGATACCCTGGTAG-3′) (Rainey *et al*., 1996). Sequences for BCA1, BCA2 and BCA3 were deposited in Genbank with accession numbers MG548382, MG461691, and MF872601; respectively. Phylogenetic tree was constructed to predict the species level characterization of the studied isolates using the maximum likelihood method implemented in Molecular Evolutionary Genetics Analysis 7.0 (MEGA7) software (Felsenstein, 1981; Kumar *et al*., 2016)

after multiple alignments of the data by CLUSTAL_X (Thompson *et al.,* 1997). In each case, bootstrap values were calculated based on 1,000 resamplings.

Identification of BCA1 and BCA2 isolates was further confirmed based on cultural, morphological, and physiological characteristics as described by Locci (1989). Scanning electron microscopy (SEM) was carried out for the three BCA isolates (BCA1, BCA2, and BCA3) using Philips XL-30 SEM (FEI Co., Eindhoven, The Netherlands) to determine the morphology of the spore chains and surface.

2.12 Disease Assays and Greenhouse Trials

For disease assays on mango seedlings under greenhouse conditions, the growing tip region of the stem of 12-month-old mango (cv. Badami) seedlings were surface-sterilized with 70% ethanol, mechanically wounded, and inoculated with 5 mm PDA plugs colonized with *L. theobromae* culture or non-colonized plugs (controls) (Saeed *et al*., 2017a). The area of inoculation was covered with Parafilm. Inoculated seedlings were kept at 28°C under greenhouse conditions, and monitored for disease development.

In vivo evaluations of the BCAs were also carried out on mango seedlings. We aimed to investigate the efficacy of the three BCA treatments to manage dieback disease. Similar to the *L. theobromae* treatment described above, methods of inoculation with the pathogen and BCA application were used. All BCA treatments were preventive (seedlings treated with each BCA 1 week before *L. theobromae* inoculation). The treatments/groups used for this experiment were as follows:

Healthy controls (C): Non inoculated control seedlings;

Diseased controls (*Lt*): Seedlings inoculated with *L. theobromae* only;

Biocontrol treatment without pathogen (*Ss, Sc*, or *Mt*): Seedlings inoculated with either *S. samsunensis, S. cavourensis* or *M. tulbaghiae*, respectively;

Preventive biocontrol treatment (*Ss*+*Lt*; *Sc*+*Lt*, or *Mt*+*Lt*): Seedlings inoculated with either *S. samsunensis, S. cavourensis* or *M. tulbaghiae*, respectively, 1 week before *L. theobromae* inoculation.

For each treatment/group, six plants in separate pots, arranged in a completely randomized design, were used. Control (healthy and diseased) and inoculated seedlings were maintained under controlled greenhouse conditions of 15 h light/9 h dark under fluorescent lights (160 W mol^{-1} m^{-2} s^{-1}) at 28°C. Corresponding with the disease symptoms/recovery, disease severity index (DSI) was recorded at 3 and 9 weeks post inoculation (wpi) using the following scale: 0 = no apparent symptoms, 1 = 1–10%, 2 = 11–25%, 3 = 26–50%, 4 = 51–75%, and 5 = 76–100% necrotic or dark brown area around the point of inoculation (Saeed *et al*., 2017a). The number of falling leaves and the total number of fungal conidia in inoculated plants were determined at 6 and 9 wpi, respectively. Harvested conidia from three leaf bases of 6 inoculated seedlings per treatment were counted using a haemocytometer (Agar Scientific Limited, Essex, UK) (Saeed *et al*., 2017a).

2.13 Statistical Analysis

For mango fruit bioassay, the effect of actinobacteria on lesion formation was evaluated and analyzed using Analysis of Variance (ANOVA). Each mango fruit was inoculated with the four treatments and each tray contained four fruits with three replicate trays for each isolate. Significant differences between means at *P*= 0.05 were determined by Duncan's multiple range test.

ANOVA and Duncan's multiple range test at 5% level of significance were used to analyze the *in vitro* evaluation of BCA against *L. theobromae*. Experiments were repeated in triplicates using five plates per treatment for each time with similar results.

For the falling leaves and fungal conidia counts of the *in vivo* treatments against *L. theobromae* in the greenhouse trial, six plants were used for each treatment. ANOVA and Duncan's multiple range test were used to determine the statistical significance ($P < 0.05$). All experiments were repeated independently three times with similar results.

Three replicates for each group (6 plants each) were tested for the DSI of the *in vivo* treatments. ANOVA and Duncan's multiple range test were conducted to determine the statistical significance at $P < 0.05$. Similar results were obtained in each replicate. For all statistical analyses, SAS Software version 9 was used (SAS Institute, 2002).

Chapter 3: Results

3.1 Symptoms of Dieback Disease on Mango

Trees manifested with disease symptoms from Kuwaitat, Al Ain -Eastern region of Abu Dhabi Emirate, UAE- were reported. The pathogen was observed to attack different parts of the mango trees. First, I noticed the disease symptoms in all plant tissues, including leaves, twigs and apical tips. When the fungus attacks the leaves, their margins roll upwards (Figure 6) turning them to brownish color (Figure 5A). Later, a scorch-like appearance developed, followed by the dropping of the infected leaves. Moreover, twigs died from the tips back inwards (toward the vascular tissues) (Figure 5A), giving a scorched appearance to the branches (Figure 6). I observed browning in the vascular tissues when longitudinal cross-sections were made in diseased mango twigs (Figure 5B). We also determined the disease symptoms associated with dieback on whole trees in the field.

3.2 Morphological and Phylogenetic Identification of *L. theobromae* Associated with Dieback Disease

The isolate obtained on potato dextrose agar (PDA) and sporulation from affected tissues were microscopically examined. On PDA, colonies of *L. theobromae* (Pat.) Griffon & Maubl. (Zambettakis 1954; Sutton, 1980) had initial white aerial mycelia that turned greenish-gray mycelium with age (Figure 6). The mycelium produced dark brown to black conidia. We also observed mycelial growth and production of immature and mature conidia (Figure 5D). Immature conidia were subovoid or ellipsoid, thick-walled, hyaline and one-celled, turning dark brown, two-celled and with irregular longitudinal striations when at maturity. The size of mature

conidia averaged 26.6 ± 0.51 µm long and 12.9 ± 0.28 µm wide. This suggests that *L. theobromae* is most likely the causal organism of dieback on mango.

To establish a phylogenic analysis of the isolate obtained in this study, PCR amplification of internal transcribed spacers (ITS) of the rDNA gene was carried out from mycelium of infected tissues (Figure 5), plated and subcultured on PDA. Results from the PCR detected the gene ITS in all infected tissues (Figure 7A). This confirms that the pathogen *L. theobromae* is frequently associated with all dieback disease symptoms on mango trees. Because no DNA sequences of this species collected in the UAE were available in GenBank to check whether this fungus belongs to any isolated Lasiodiplodia isolate, I tried to identify this strain based on a phylogeny tree. For that purpose, the ITS rDNA and translational elongation factor 1-α (TEF1-α) gene (Alves *et al*., 2008) were used as a single gene set. The concatenated two-gene set (ITS and TEF1-α) were sequenced and deposited in GenBank (accession number: MF114110 and MF097964, respectively).

I also determined the relationship among those obtained and other closely related ITS/TEF1-α sequences (Zambettakis 1954; Ismail *et al*., 2012). All sequences were aligned and Maximum Likelihood analyses were performed for estimation of the phylogenetic tree. The adaptation to different plant hosts has led to the evolution of at least 13 cryptic species within the *L. theobromae* species complex (Ismail *et al.*, 2012). The generated ITS/TEF1-α sequence belonging to our strain clustered in one clade corresponding to *L. theobromae* from different sources, confirming its identity with this species (Figure 7B). Among the studied Lasiodiplodia species, our analysis revealed that this pathogen is placed adjacent to *L. theobromae* CBS130989, distinguishing the obtained isolate *from* those belonging to other species of

Lasiodiplodia, Diplodia or *Phyllosticta.* Our phylogenetic analysis supports that the species *L. theobromae* DSM 105134 dominates in the UAE causing dieback disease on mango trees.

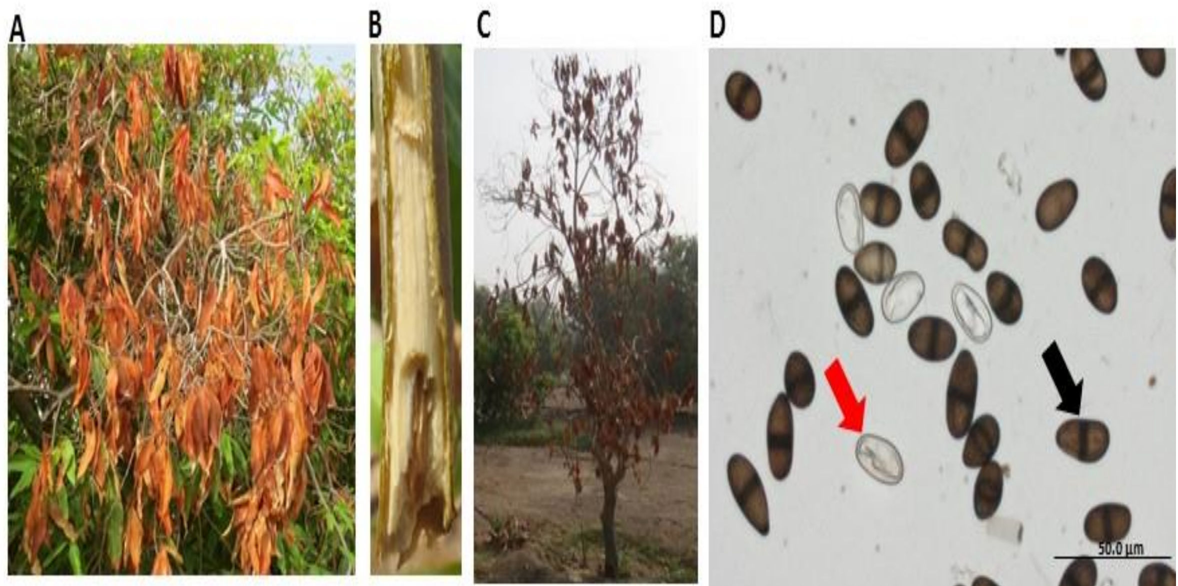


Figure 5: Mango trees showing symptoms of dieback disease and morphological phenotypes of *Lasiodiplodia theobromae* conidia. Symptoms on (A) leaves; (B) twigs; (C) whole tree; and (D) *L. theobromae* hyaline, aseptate immature (red arrow) and brown, 1-septate, thick-walled mature conidia (black arrow). In (A-C), naturally infested mango trees with *L. theobromae*. In (D), conidia from a 10-day old PDA.

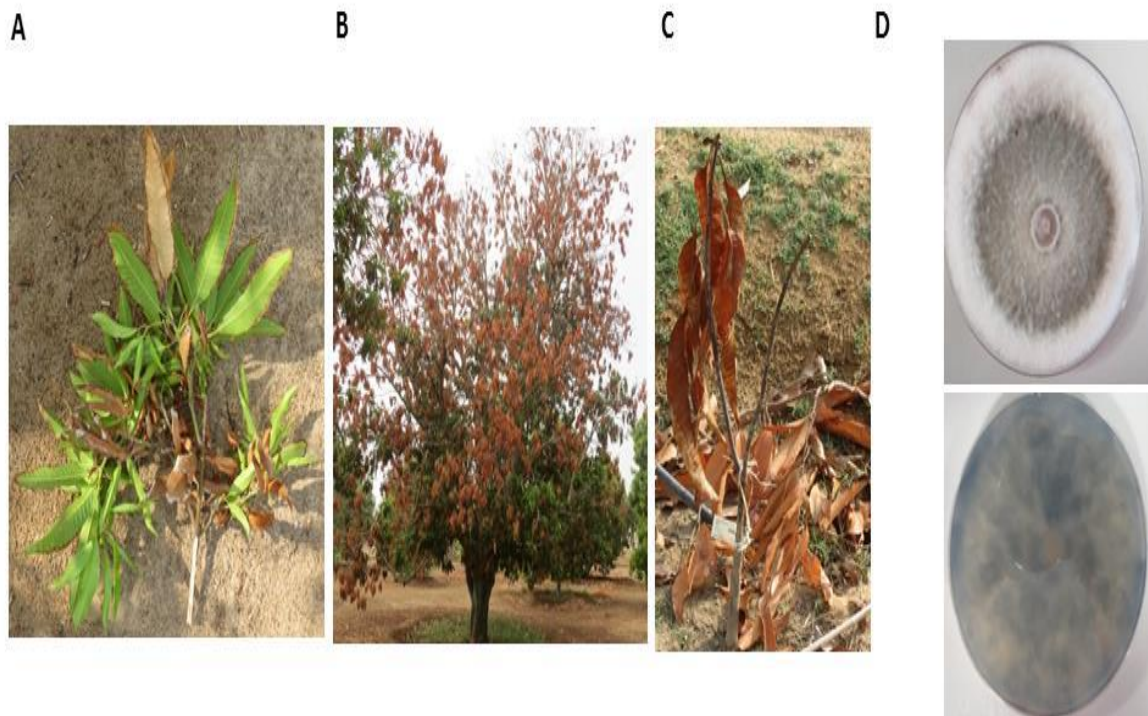


Figure 6: Additional symptoms of dieback disease on mango. Symptoms on (A) leaves; (B) whole tree; (C) disease affected small mango plant and (D) *L. theobromae* mycelia (top) with sporulation (bottom) on PDA plate. (A-C) naturally infested mango plants with *L. theobromae*; while (D) a 10-day old PDA culture.

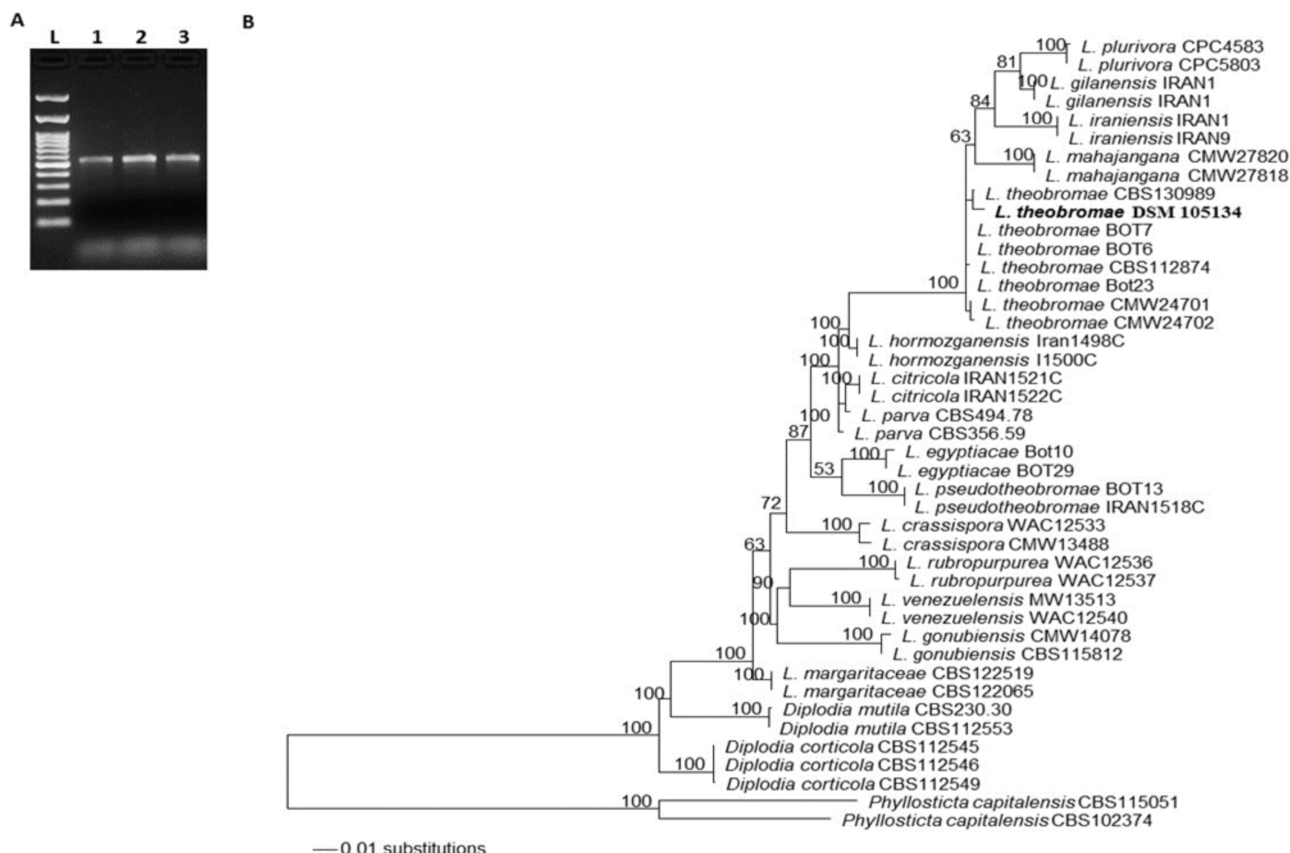


Figure 7: Molecular identification of *L. theobromae.* (A) PCR amplification of specific genomic DNA regions of infected tissues; and (B) Dendrogram showing phylogenetic relationships of the fungal sequence of the specimen used in this study (DSM 105134) with the most related *ITS* and *TEF1- α* sequences in GenBank, prepared by the neighbor-joining method. In (A), lanes 1-3 correspond to amplifications of *ITS* rDNA region in leaves, twigs and apical tips. In (B), the specimen used in this study carries GenBank accession number, MF114110. Maximum Likelihood tree obtained from combined *ITS*/*TEF1-α* sequence data. Numbers at the nodes are ML bootstrap values after 100 replicates are expressed as percentages (LnL = -3497.793130). The scale bar on the rooted tree indicates a 0.01 substitution per nucleotide position. The strain from this report is indicated in bold. *ITS*, *internal transcribed spacer*; *TEF1-α*, *translational elongation factor 1-α*; L, DNA ladder.

3.3 Production of Diffusible Antifungal Metabolites and CWDEs by BCAs

Fifty-three SA and NSA strains were isolated from mango rhizosphere, of which 35 SA (66.1%) and 18 NSA (33.9%) were obtained from inorganic salt starch agar plates. The Streptomyces phages with high polyvalency were used to facilitate the isolation of NSA from rhizosphere samples on inorganic salt starch agar plates (Figure 8). Consequently, the numbers of SA were significantly ($P < 0.05$) reduced, but the numbers of NSA increased on the plates treated with the four phages (Table 1). Therefore, SA and NSA (*Actinoplanes, Actinomadura, Microbispora, Micromonospora, Nocardia, Rhodococcus,* and *Streptosporangium* spp.) were readily isolated and identified to the genus level based on morphological features, distribution of aerial and/or substrate mycelia, presence/absence of aerial mycelia, and the stability or fragmentation of substrate mycelia.

It is found that 11 out of 53 of the rhizosphere actinobacterial (7 SA and 4 NSA) isolates were capable of producing strong antifungal metabolites active against *L. theobromae* using the cut-plug method (Table 2). Eleven isolates (#3, 7, 9, 12, 16, 21, 25, 29, 42, 49, and 50) produced large zones of pathogen inhibition (>30 mm), and were considered as the most promising BCA candidates (Table 2; Figure 9A); thus were selected for further analyses. The rest of the isolates that caused very low levels of inhibition (< 30 mm) were not included in the subsequent studies.

Of the 53 isolates, 8 SA and 4 NSA (isolates #3, 10, 12, 18, 31, 33, 41, 44, 45, 49, 50, and 51) were ranked as highly active chitinase-producing isolates. These 12 isolates produced large clearing zones (>30 mm) around the colony on colloidal chitin agar plates and on *L. theobromae* mycelial fragment agar (Table 2; Figure 9B).

The remainder of the isolates produced small clearing zones (< 30 mm) and were not further assessed. It is noteworthy to mention that four BCA candidates (isolates #3, 12, 49, and 50) produced both the diffusible antifungal metabolites and CWDEs (Table 2). This suggests that the isolated SA and NSA from the local mango rhizosphere soil samples may have antifungal activities of single or multiple modes of action against plant pathogens, including *L. theobromae*.

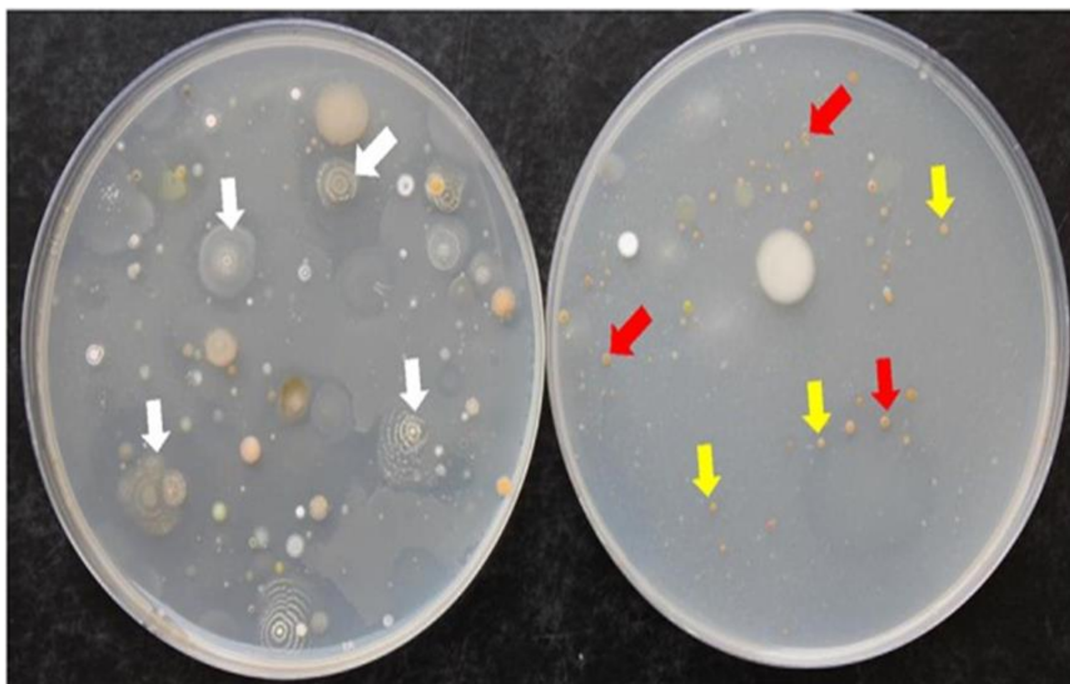


Figure 8: Colonies of actinobacteria isolated from mango rhizosphere grown on inorganic salt starch agar plates. Soil dilutions tubes were treated without (Left) and with (Right) four polyvalent *Streptomyces* phages. White arrows represent the dominance of streptomycete actinobacterial colonies (Left); whereas yellow and red arrows represent the dominance of non-streptomycete actinobacterial colonies i.e., *Actinoplanes* and *Micromonospora* spp., respectively (Right).

Table 1: The effect of introducing four polyvalent Streptomyces phages on the colony-forming units of steptomycete and non-streptomycete actinobacteria from mango rhizophere soil.

Actinobacteria	**Without phage**	**With phage**
	$\log_{10}$ cfu g soil^{-1}	
SA	5.96±0.45 *a*	2.04±0.31 *b*
NSA	1.86±0.18 *a*	3.70±0.26 *b*

Values are means of seven replicates ± SE of the mean. Within rows, values followed by the same letter are not significantly (P>0.05) different according to Duncan’s multiple range test.

SA, streptomycete actinobacteria; NSA, non-streptomycete actinobacteria.

Table 2: *In vitro* and *in vivo* antagonism shown by 19 isolates of streptomycete and non-streptomycete actinobacteria against *Lasiodiplodia theobromae*.

Treatment	**Isolate**	***In vitro***			***In vivo***
		Diameter of inhibition zone (mm) [a]	**Diameter of clearing zone (mm)** [b]	**Diameter of clearing zone (mm)** [c]	**Lesion diameter (mm)** [d]
Streptomyces sp.	3	46.41±0.49 *b*	46.10±0.98 *d*	48.79±0.35 *d*	15.88±1.03 *b*
	7	40.40±0.58 *e*	0.00±0.00 *g*	0.00±0.00 *g*	32.21±0.65 *a*
	10	0.00±0.00 *i*	54.50±2.16 *cb*	54.96±1.70 *c*	33.13±0.31 *a*
	12 (BCA1)	49.16±0.88 *a*	56.04±0.87 *b*	57.97±0.98 *b*	0.00±0.00 *e*
	18	0.00±0.00 *i*	52.59±0.45 *c*	54.80±0.65 *c*	33.32±0.49 *a*
	21	44.40±0.55 *c*	0.00±0.00 *g*	0.00±0.00 *g*	32.31±0.65 *a*
	29 (BCA2)	42.40±0.49 *d*	0.00±0.00 *g*	0.00±0.00 *g*	0.00±0.00 *e*
	31	0.00±0.00 *i*	35.98±0.72 *f*	37.52±0.55 *f*	0.00±0.00 *e*
	41	0.00±0.00 *i*	40.81±0.77 *e*	42.06±1.35 *e*	33.03±0.69 *a*
	42	37.10±0.86 *f*	0.00±0.00 *g*	0.00±0.00 *g*	12.73±1.74 *c*
	49	31.73±0.95 *h*	41.72±0.95 *e*	43.98±0.79 *e*	0.00±0.00 *e*
	51	0.00±0.00 *i*	56.22±1.05 *b*	57.29±0.53 *b*	32.38±0.42 *a*
Actinoplanes sp.	9	31.23±0.76 *h*	0.00±0.00 *g*	0.00±0.00 *g*	0.00±0.00 *e*
Microbispora sp.	16	49.60±0.60 *a*	0.00±0.00 *g*	0.00±0.00 *g*	32.81±0.73 *a*
Micromonospora sp.	25	47.13±0.84 *b*	0.00±0.00 *g*	0.00±0.00 *g*	7.45±0.57 *d*
	44 (BCA3)	0.00±0.00 *i*	63.90±1.43 *a*	65.62±1.30 *a*	0.00±0.00 *e*
Nocardia sp.	33	0.00±0.00 *i*	38.18±0.40 *f*	39.66±0.37 *f*	31.90±0.62 *a*
Rhodococcus sp.	45	0.00±0.00 *i*	52.18±1.04 *c*	54.37±1.18 *c*	32.22±0.98 *a*
Streptosporangium sp.	50	34.53±0.60 *g*	54.10±1.36 *cb*	56.15±1.29 *bc*	12.01±1.20 *c*
Diseased control (*L. theobromae*)		ND	ND	ND	32.51±0.57 *a*
Healthy control (no *L. theobromae*)		ND	ND	ND	0.00±0.00 *e*

[a] Production of diffusible antifungal metabolites active against *L. theobromae* using the cut-plug method.
[b] Production of chitinase on colloidal chitin agar.
[c] Production of cell-wall-degrading enzymes on mycelial fragment agar.
[d] Effect of the antagonistic BCA on *L. theobromae* using the *in vivo* mango fruit bioassay.
Values are means of three replicates ± SE for the *in vitro* experiments and *in vivo* experiments. Values within each column, followed by the same letter are not significantly (P>0.05) different according to Duncan's multiple range test.
Isolates #12, #29 and #44 represent *S. samsunensis* UAE1 (BCA1), *S. cavourensis* UAE1 (BCA2) and *M. tulbaghiae* UAE1 (BCA3); respectively.
ND, not determined.

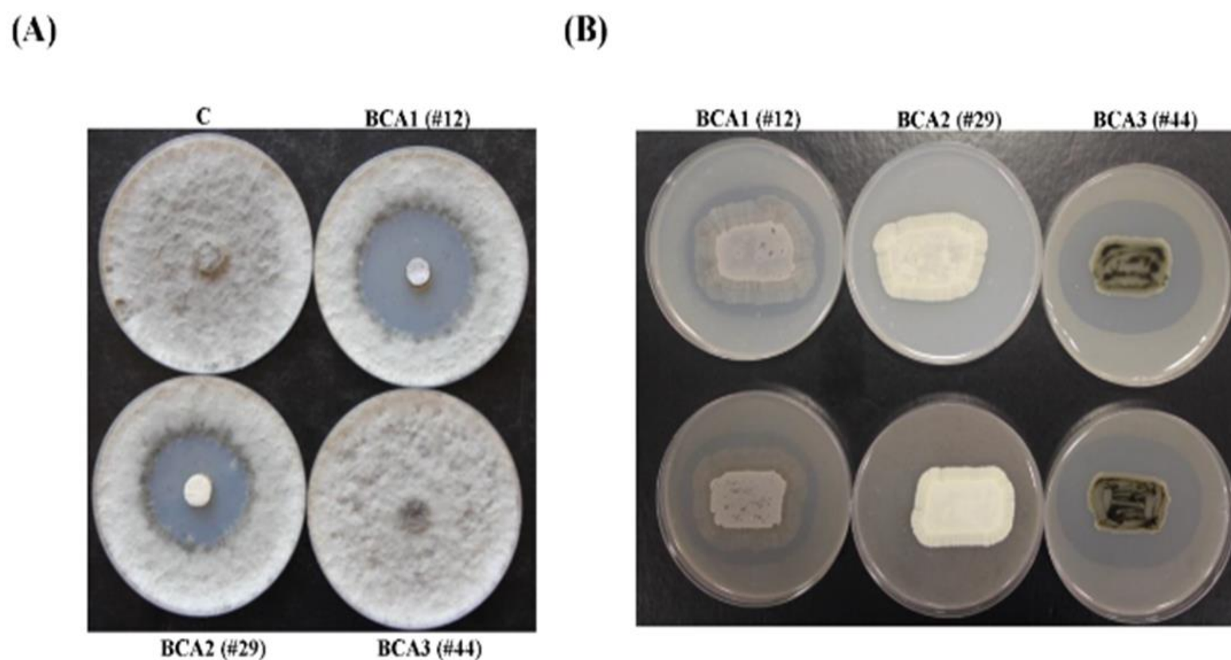


Figure 9: Production of diffusible antifungal metabolites and cell-wall-degrading enzymes by BCA candidates active against *Lasiodiplodia theobromae*. Inhibition of *L. theobromae* mycelial growth by the BCA1 and BCA2 using cut-plug method (A); and production of chitinase enzymes by BCA1 and BCA3 grown on colloidal chitin agar (upper panel) and production of CWDEs on mycelial fragment agar (lower panel) (B). In (A), the diffusible antifungal metabolite-producing isolate BCA1 (isolate #12; *Streptomyces samsunensis* UAE1) and BCA2 (isolate #29; *S. cavourensis* UAE1) compared to the non-diffusible antifungal metabolite-producing isolate BCA3 (isolate #44; *Micromonospora tulbaghiae* UAE1). In (B), production of CWDEs by BCA1 and BCA3 isolates compared to the non- CWDE producing BCA2 isolate. C, a sterile non-inoculated PDA agar plug (control).

3.4 Selection of the Most Promising Antagonistic BCA Candidates

I used the mango fruit bioassay method to evaluate the most effective 19 BCA candidates against *L. theobromae* (Table 2; Figure 10A). Lesions produced on the fruits by the pathogen (*Lt*) alone were relatively large, brownish, round to elliptical, water-soaked and depressed, with clear margins (Figures 10B–D). When certain isolates were paired with the pathogen (BCA+*Lt*) on the mango fruit surface, they completely suppressed the pathogen with no lesions formed compared with the treatment with the pathogen plug alone (*Lt*) (Table 2; Figure 10). Certain isolates significantly ($P < 0.05$) reduced lesion development in comparison to the treatment with the pathogen alone (Table 2).

In general, ten isolates (6 SA and 4 NSA) totally prevented or reduced lesion development to varying degrees, whilst the remaining 9 isolates failed to reduce lesion formation (Table 2). The isolates that showed production of antifungal metabolites and/or CWDEs, but failed to totally prevent lesion development on mango fruits were excluded. The mango fruit bioassay led to the selection of only three most promising antagonistic isolates: 2 SA (#12 and #29) and 1 NSA (#44) which completely prevented lesion formation on mango fruit.

My data suggest that the three BCA candidates selected are highly effective against *L. theobromae*, and that the preventive effect of isolates #12, #29 and #44 could have the potential to manage dieback disease on mango seedlings. The Actinobacteria tested alone (BCA treatment) did not cause any harmful effects on mango fruits (Figure 10).

This clearly showed that the antagonists and/or their metabolites(s) are able to inhibit the pathogen preventing it from producing lesions on the mango fruit surface.

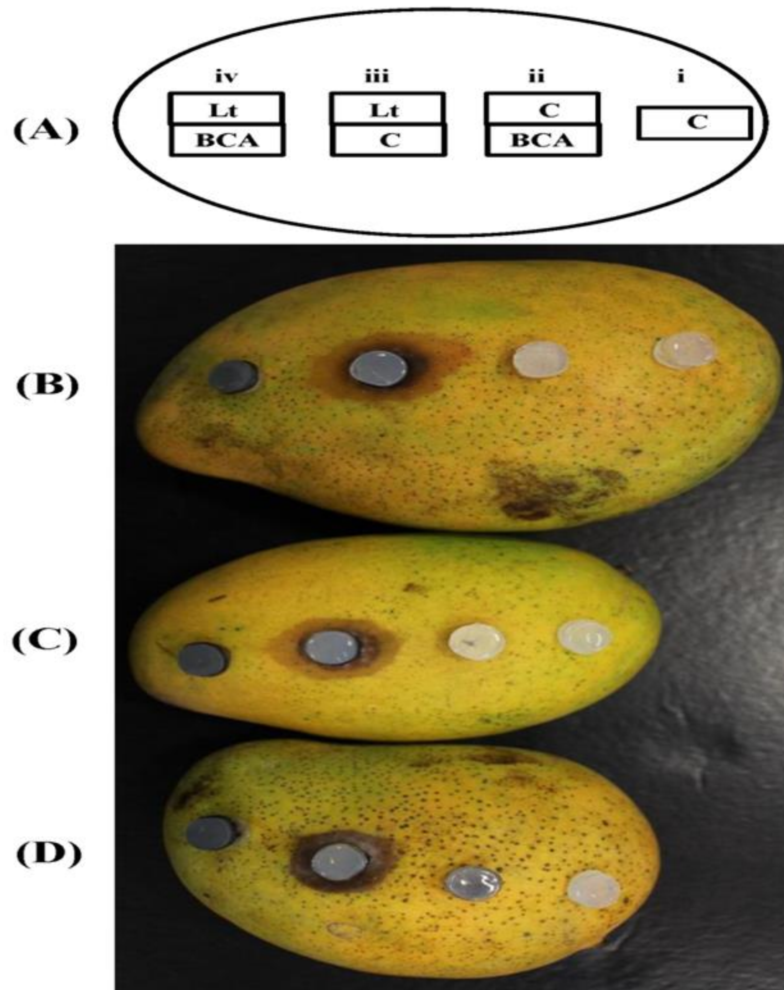


Figure 10: *In vivo* inhibitory effect of the BCA candidates against *Lasiodiplodia theobromae* using the *"*mango fruit bioassay". An illustration showing an inoculated-mango fruit with the colonized BCA and/or *L. theobromae* agar plugs in combinations onto each mango fruit (A). Mango fruit bioassays using BCA1 (isolate #12; *Streptomyces samsunensis* UAE1) (B); BCA2 (isolate #29; *S. cavourensis* UAE1) (C); or BCA3 (isolate #44; *Micromonospora tulbaghiae* UAE1) (D) as potential BCAs. (i) C, a sterile non-inoculated PDA agar plug (control); (ii) BCA, the antagonist alone with a sterile agar plug above it; (iii) *Lt*, *L. theobromae* inoculum alone with a sterile agar plug below it; and (iv) BCA+*Lt*, pairing *L. theobromae* and the antagonist together, with the BCA on the mango surface and *L. theobromae*-inoculated plug on top of the BCA. inoculum alone with a sterile agar plug below it; and (iv) BCA+*Lt*, pairing *L. theobromae* and the antagonist together.

3.5 *In vitro* Evaluation of Antagonistic Properties of the BCA Candidates

The filter-sterilized crude culture filtrate of either BCA1 or BCA2 introduced into the wells using the cup plate technique, caused significant ($P < 0.05$) retardation of the growth of *L. theobromae*, when compared to the antifungal metabolite non-producing BCA3 or control (Table 3; Figure 11). Notably, the effect of diffused antifungal metabolites by BCA1 was significantly ($P < 0.05$) higher than those produced by BCA2 (Table 3).

The growth of the pathogen was clearly inhibited by the diffused metabolites of BCA1 and BCA2 only after removing the dialysis membranes from the fish meal extract agar, compared to the control or BCA3 (Table 3; Figure 11). In addition, the pathogen failed to grow from the plugs transferred from the treatment plates to fresh PDA in the absence of diffused metabolites, confirming that the metabolites of BCA1 and BCA2 were clearly fungicidal to *L. theobromae*.

The diffused metabolites of BCA1 and BCA3 from the colloidal chitin agar plates inhibited the growth of *L. theobromae* inoculum; in contrast to isolate BCA2 or control, after removing the dialysis membranes (Table 3; Figure 11). The pathogen did not recover from the plugs when transferred from treated plates to fresh PDA. This indicated that BCA1 and BCA3 showed fungicidal activities to *L. theobromae*.

To determine whether the BCA produced volatile antifungal compounds, the three BCA candidates were grown on fish meal extract agar. BCA2 and BCA3 failed to produce any volatile antifungal compounds, capable to inhibit the growth of the pathogen (Table 3; Figure 12). However, BCA1 produced volatile antifungal compounds and caused complete suppression of the pathogen growth. None of the

three BCA candidates produced hydrogen cyanide; while only BCA1 produced siderophores (Table 3; Figure 12).

Chitinase production by BCA1 and BCA3 was significantly ($P < 0.05$) higher in the media amended with colloidal chitin or on media amended with the pathogen cell walls (Table 3). Both BCA1 and BCA3 also produced ß-1,3-glucanase when grown on media amended with laminarin or *L. theobromae* cell walls. The production of ß-1,3-glucanase was found to be significantly ($P < 0.05$) higher on laminarin-amended medium. On the other hand, there were no detectable levels of chitinase or ß-1,3-glucanase by BCA2 when it was grown in media containing either colloidal chitin or *L. theobromae* cell walls, or in media containing laminarin or *L. theobromae* cell walls, respectively (Table 3). The production of chitinase and ß-1,3-glucanase by BCA3 were, however, significantly ($P < 0.05$) higher than those produced by BCA1.

3.6 Effect of Crude Culture Filtrates of the BCA Candidates on *L. theobromae*

I showed that the filter-sterilized crude culture filtrates of either BCA1 or BCA2 from fish meal extract broth were effective in inhibiting growth of *L. theobromae* (Table 4). On PDA plates, the increasing levels of the BCA1 and BCA2, but not BCA3, crude culture filtrates significantly ($P < 0.05$) inhibited the colony and mycelial growth of *L. theobromae* (Table 4; Figure 13). Mycelial growth was totally inhibited when crude culture filtrates were incorporated into PDA at 75% or above. In PDB, the assay of the mycelial growth inhibition of the pathogen by BCA1 and BCA2 was similar to that in PDA plates. Crude culture filtrates of BCA1 and BCA2 from fish meal extract broth significantly decreased the mycelial dry weight of the pathogen when proportionally added into PDB (Table 4). When

compared with the control, the crude culture filtrates of BCA1 and BCA3 from colloidal chitin broth increasingly inhibited colony growth on PDA plates and mycelial dry weight of *L. theobromae* on PDB, with the increasing levels of crude culture filtrates after 5 days of incubation at 28°C (Table 4).

Table 3: *In vitro* antagonistic activities of the three BCA candidates against *Lasiodiplodia theobromae*

Activities	**BCA1**	**BCA2**	**BCA3**
Production of diffusible metabolites using the cup plate technique (diameter of zone of inhibition measured in mm).	67.97±0.91 *a*	53.82±0.93 *b*	0.00±0.00 *c*
Production of diffusible metabolites using the dialysis membrane technique from the fish meal extract agar plates [a]	+	+	-
Production of chitinase using the dialysis membrane technique from the colloidal chitin agar plates [a]	+	-	+
Production of volatile compounds [a]	+	-	-
Production of hydrogen cyanide [b]	-	-	-
Production of siderophores [b]	+	-	-
Chitinase from colloidal chitin (U ml^{-1}) [c]	5.56±0.17 *a*	0.00±0.00 *b*	7.75±0.22 *c*
Chitinase from *L. theobromae* cell wall (U ml^{-1}) [c]	3.73±0.14 *a*	0.00±0.00 *b*	5.60±0.21 *c*
ß-1,3-glucanase from laminarin (U ml^{-1}) [d]	3.57±0.16 *a*	0.00±0.00 *b*	5.54±0.18 *c*
ß-1,3-glucanase from *L. theobromae* cell wall (U ml^{-1}) [d]	2.79±0.20 *a*	0.00±0.00 *b*	3.76±0.21 *c*

[a] + = fungicidal effect; - = no fungicidal effect.
[b] + = produced; - = not produced.
[c] A unit of chitinase was expressed as the amount of the enzyme that released 1 µmol of N-acetyl-D-glucosamine mg^{-1} protein h^{-1}.
[d] A unit of ß-1,3-glucanase was expressed as the amount of the enzyme that released 1 µmol of glucose mg^{-1} protein h^{-1}.
Values are means of three replicates ± SE. Values with the same letter within a row are not significantly (P>0.05) different according to Duncan's multiple range test.
BCA1, isolate #12; *Streptomyces samsunensis* UAE1; BCA2, isolate #29; *S. cavourensis* UAE1; BCA3, isolate #44; *Micromonospora tulbaghiae* UAE1.

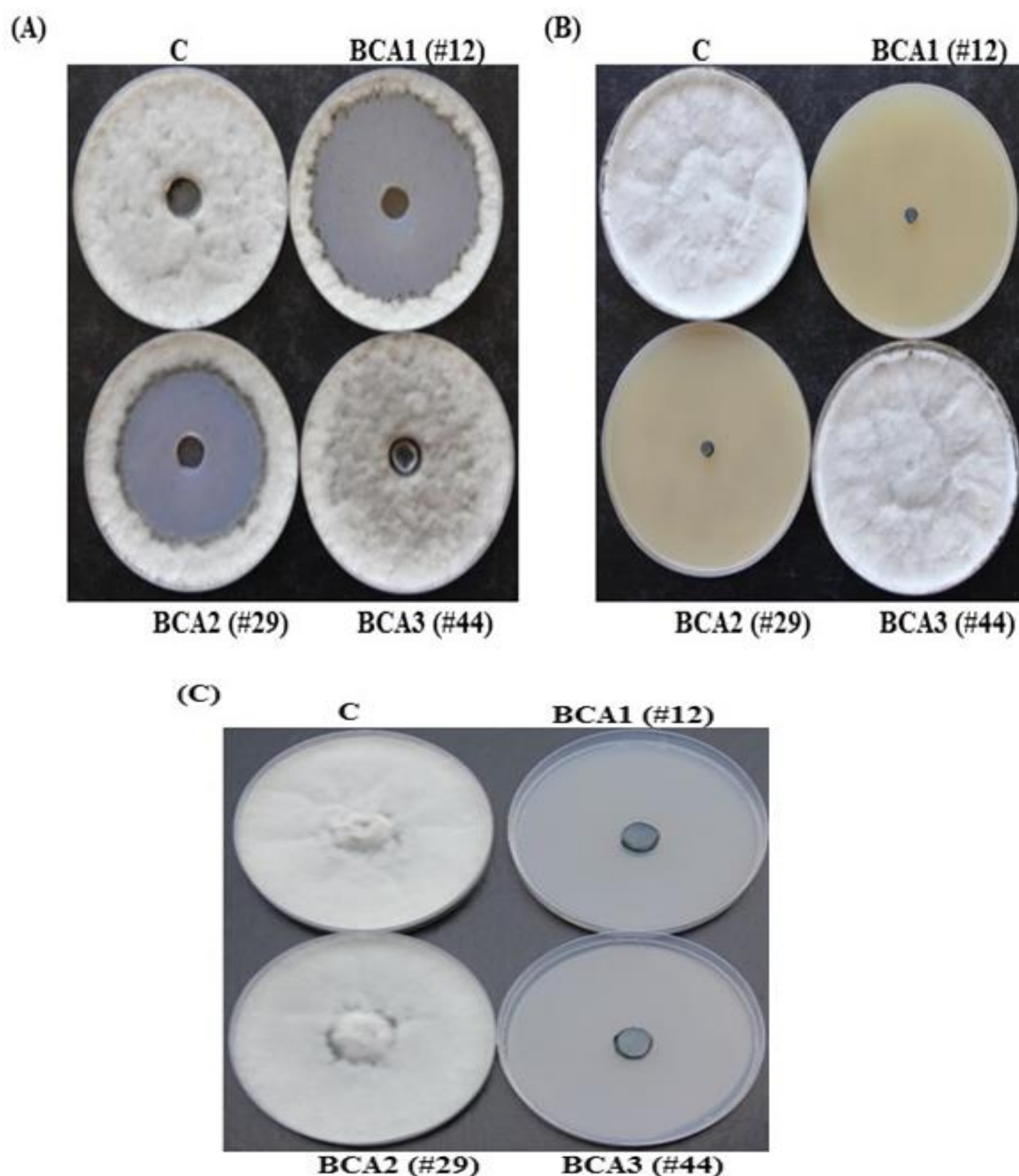


Figure 11: Effect of the BCA candidates on mycelial growth of *Lasiodiplodia theobromae*. Cup plate method (A); and dialysis membrane overlay technique using (B) fish meal extract agar or (C) colloidal chitin agar plates. In (A & B), inhibition of *L. theobromae* mycelial growth was observed only by the diffusible antifungal metabolite-producing isolate *Streptomyces samsunensis* UAE1 (BCA1; isolate #12) and *S. cavourensis* UAE1 (BCA2; isolate #29) compared to the non-diffusible antifungal metabolite-producing isolate *Micromonospora tulbaghiae* UAE1 (BCA3; isolate #44). In (C), inhibition of *L. theobromae* mycelial growth were only by the chitinase-producing isolates #12 and #44 compared to the chitinase non-producing isolate #29. In (A), wells were inoculated with either filter-sterilized fish meal extract broth (C), or filter-sterilized crude culture filtrates of isolates #12, #29 or #44; while in (B & C), fish meal extract agar or colloidal chitin agar plates are either colonized by no BCA (C), or isolates #12, #29 or #44.

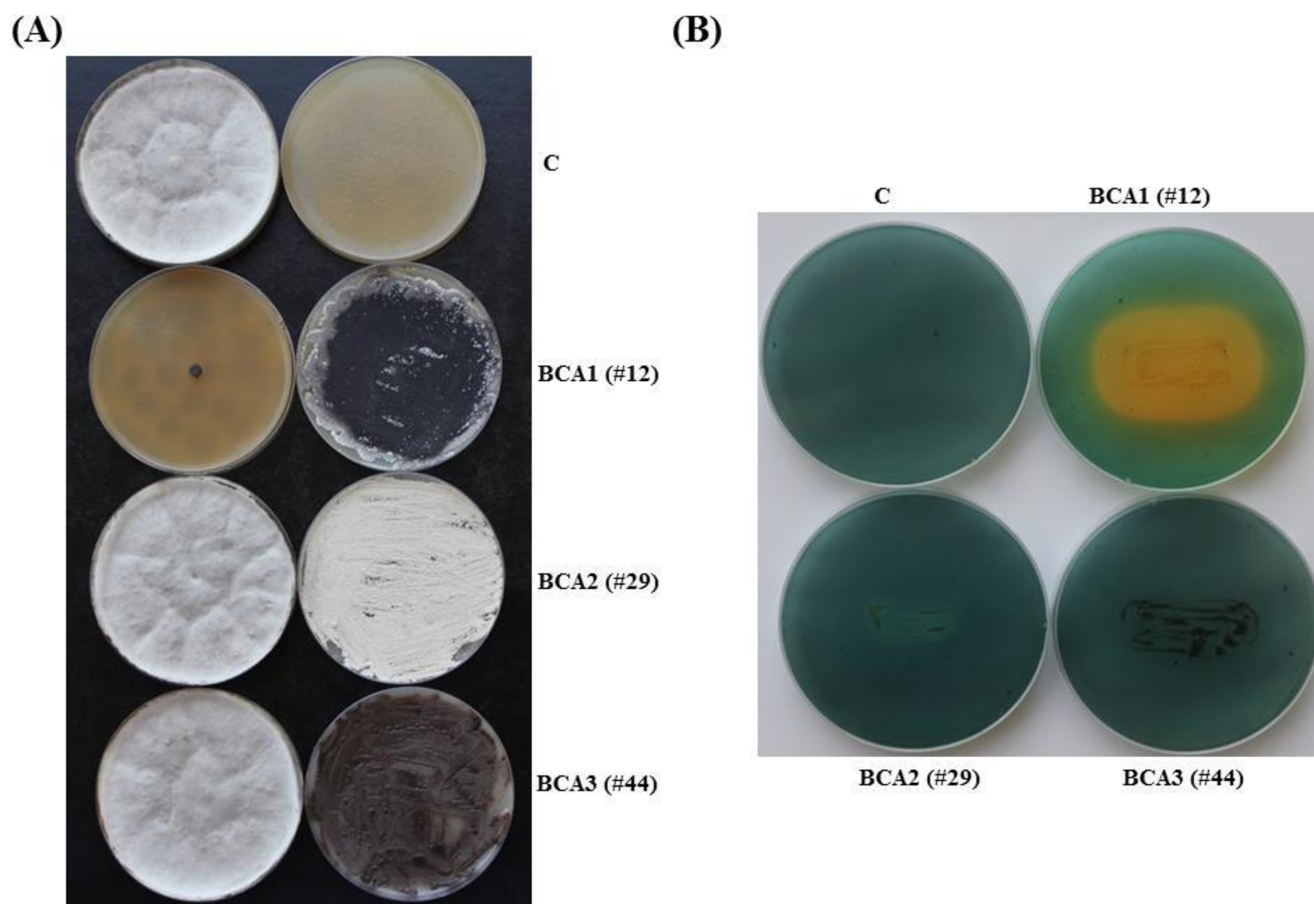


Figure 12: Effect of the BCA-producing volatile antifungal compounds on *Lasiodiplodia theobromae* and siderophore production. (A) Inhibition of *L. theobromae* mycelial growth by the volatile antifungal compound-producing isolate *Streptomyces samsunensis* UAE1 (BCA1; isolate #12) compared to the non-volatile antifungal compounds producing isolate *S. cavourensis* UAE1 (BCA2; isolate #29) and *Micromonospora tulbaghiae* UAE1 (BCA3; isolate #44). (B) Siderophore production by isolate #12 compared to the siderophore non-producing isolates #29 and #44 on chrome azurol S agar plates. In (A), from upper to lower panel, fish meal extract agar plates (right) are either colonized by no BCA (C), or isolates #12, #29 or #44. In (B), the yellow halo surrounding the colony indicates the excretion of siderophore.

Table 4: Inhibition of mycelial growth, spore germination and germ tube elongation of *Lasiodiplodia theobromae* by the crude culture filtrate of the three BCA candidates either obtained from fish meal extract broth or colloidal chitin broth.

BCA	**Culture filtrate (%)**	**Colony diameter (mm)**	**Mycelial dry weight (g)**	**Conidia germination (%)**	**Germ tube length (µm)**
(A) Fish meal extract broth					
BCA1	0	99.60±0.22 *a*	81.05±2.76 *a*	90.06±1.77 *a*	59.86±0.74 *a*
	10	37.76±1.39 *b*	28.40±2.86 *b*	34.25±1.74 *b*	47.24±0.92 *b*
	25	18.87±0.95 *c*	9.86±1.08 *c*	21.31±1.34 *c*	29.35±0.94 *c*
	50	9.72±0.84 *d*	3.88±0.76 *d*	8.96±0.36 *d*	18.59±1.58 *d*
	75	0.00±0.00 *e*	0.40±0.10 *d*	3.19±0.32 *d*	11.81±0.84 *e*
	100	0.00±0.00 *e*	0.00±0.00 *d*	0.54±0.09 *d*	3.50±0.61 *f*
BCA2	0	99.51±0.12 *a*	78.60±3.60 *a*	89.17±1.91 *a*	53.26±1. 53*a*
	10	39.72±1.83 *b*	35.87±2.08 *b*	40.36±3.03 *b*	36.09±1. 91*b*
	25	21.57±0.89 *c*	15.92±2.35 *c*	26.12±1.41 *c*	28.17±1. 79*c*
	50	11.33±1.21 *d*	7.82±1.15 *d*	16.19±0.91 *d*	16.36±0. 01*d*
	75	0.00±0.00 *e*	3.15±0.59 *de*	6.68±0.37 *e*	6.32±1. 27*e*
	100	0.00±0.00 *e*	0.15±0.08 *e*	0.97±0.02 *f*	0.42±0.14 *f*
(B) Colloidal chitin broth					
BCA1	0	98.70±0.68 *a*	80.39±2.97 *a*	93.10±2.13 *a*	60.69±1.77 *a*
	10	48.13±2.05 *b*	40.49±1.66 *b*	49.49±2.86 *b*	36.58±1.85 *b*
	25	26.94±1.14 *c*	22.80±1.82 *c*	28.55±0.90 *c*	25.11±1.28 *c*
	50	16.25±1.23 *d*	13.58±1.03 *d*	18.87±0.93 *d*	13.21±1.29 *d*
	75	0.00±0.00 *e*	4.24±0.92 *e*	8.48±0.75 *e*	3.41±0.51 *e*
	100	0.00±0.00 *e*	0.48±0.16 *e*	2.19±0.42 *f*	0.00±0.00 *e*
BCA3	0	99.84±0.12 *a*	83.27±2.64 *a*	93.32±1.47 *a*	66.34±1.81 *a*
	10	29.27±1.45 *b*	30.84±2.79 *b*	28.04±0.86 *b*	42.55±1.49 *b*
	25	16.79±1.05 *c*	13.12±1.34 *c*	18.42±1.03 *c*	17.44±1.30 *c*
	50	6.85±1.00 *d*	4.96±0.95 *d*	10.03±0.81 *d*	6.07±0.83 *d*
	75	0.00±0.00 *e*	0.00±0.00 *e*	2.49±0.45 *e*	0.00±0.00 *e*
	100	0.00±0.00 *e*	0.00±0.00 *e*	0.00±0.00 *e*	0.00±0.00 *e*

Values are means of three replicates ± SE. Values with the same letter within a column for each BCA are not significantly ($P>0.05$) different, according to Duncan's multiple range test.
BCA1, isolate #12; *Streptomyces samsunensis* UAE1; BCA2, isolate #29; *S. cavourensis* UAE1; BCA3, isolate #44; *Micromonospora tulbaghiae* UAE1.

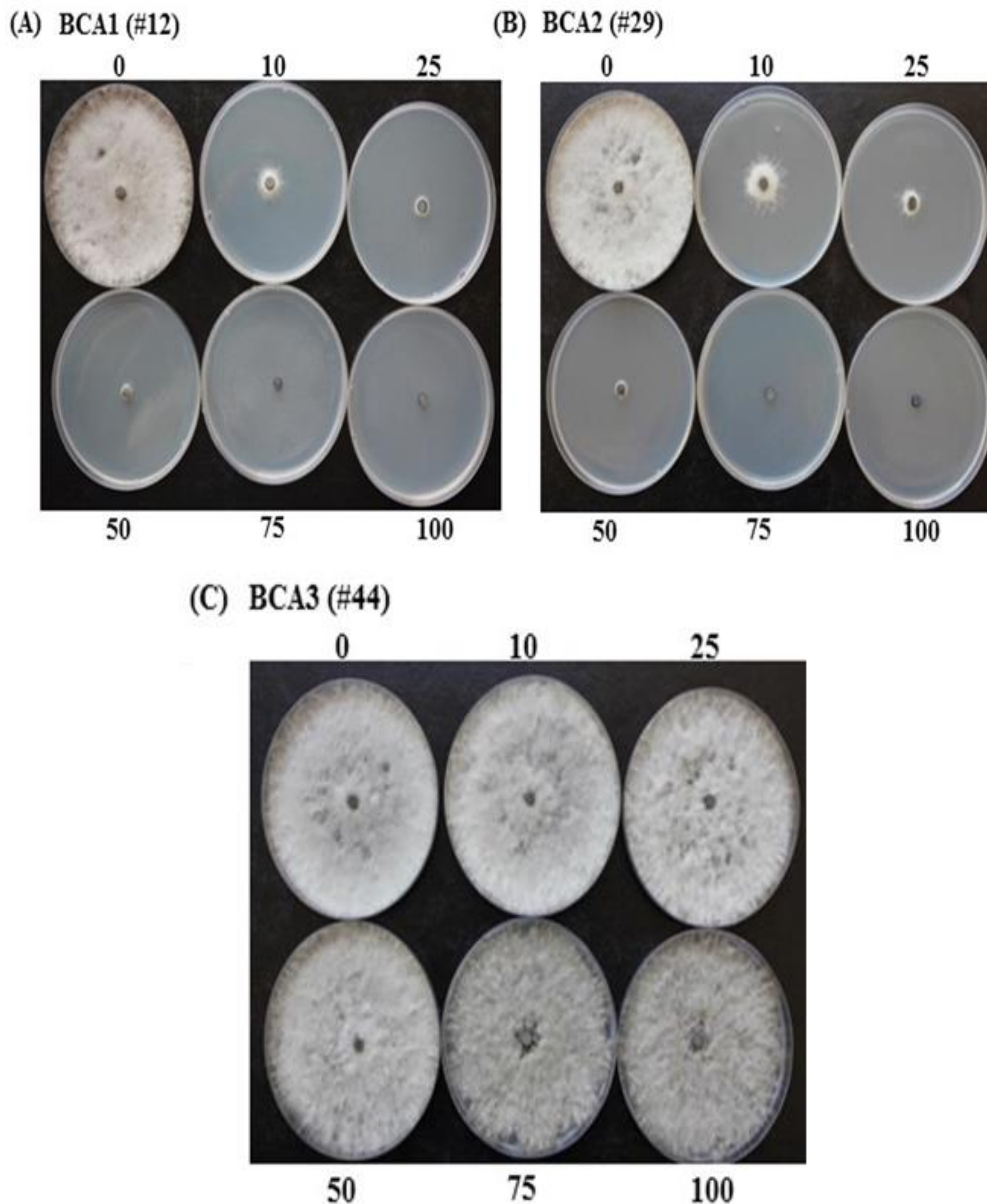


Figure 13: Effect of filter-sterilized crude culture filtrates of BCA candidates on *Lasiodiplodia theobromae*. Gradual inhibition of *L. theobromae* colony growth on PDA plates containing different proportions (%) of crude culture filtrate obtained from fish meal extract broth only with the antifungal metabolite-producing isolates (A) *Streptomyces samsunensis* UAE1 (BCA1; isolate #12) and (B) *S. cavourensis* UAE1 (BCA2; isolate #29) compared to (C) the non-diffusible antifungal metabolite-producing isolate *Micromonospora tulbaghiae* UAE1 (BCA3; isolate # 44).

Similarly, a significant ($P < 0.05$) reduction in the germination of the thick walled, mature conidia and the average length of germ tubes produced by *L. theobromae* was found when the pathogen was exposed to the crude culture filtrate of BCA1 and BCA2 (in fish meal extract broth), and BCA1 and BCA3 (in colloidal chitin broth) after 24 h of incubation (Table 4). This indicates that the crude culture filtrates of BCA1, BCA2, and BCA3 inhibited not only mycelial growth, but also spore germination and germ tube elongation of *L. theobromae*.

We also observed hyphal abnormalities such as hyphal swelling (ballooning), cytoplasmic coagulation and hyphal lysis in *L. theobromae* treated with the crude culture filtrate of BCA1 obtained from fish meal extract broth and colloidal chitin broth, respectively (Figure 14). There were hyphal abnormalities, including hyphal swelling and cytoplasmic coagulation without hyphal lysis in *L. theobromae* exposed to the crude culture filtrate of BCA2 from fish meal extract broth (Figure 11A). The pathogen treated with the crude culture filtrate of BCA3 obtained from colloidal chitin broth showed only hyphal lysis (Figure 14B). Mycelial mats in control flasks remained healthy and unaffected (Figure 14).

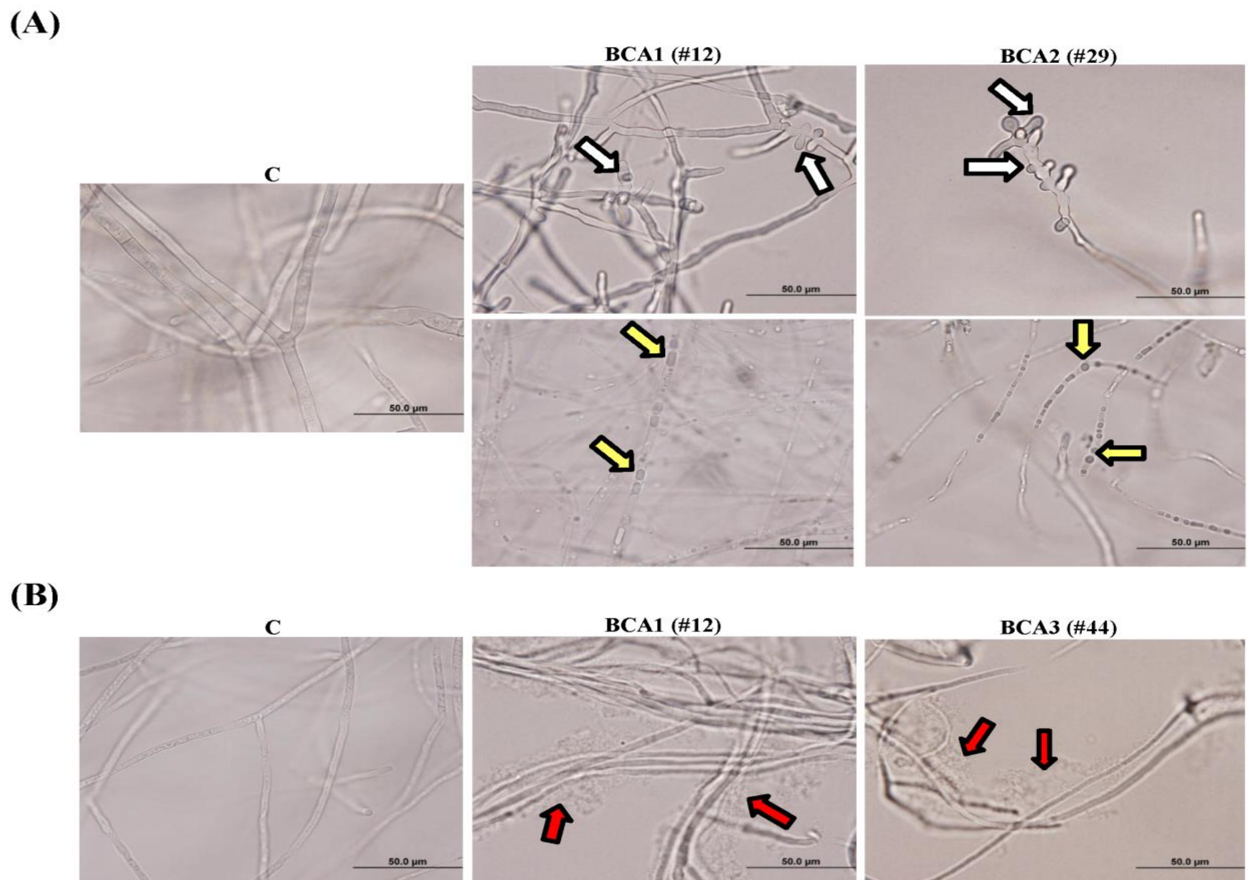
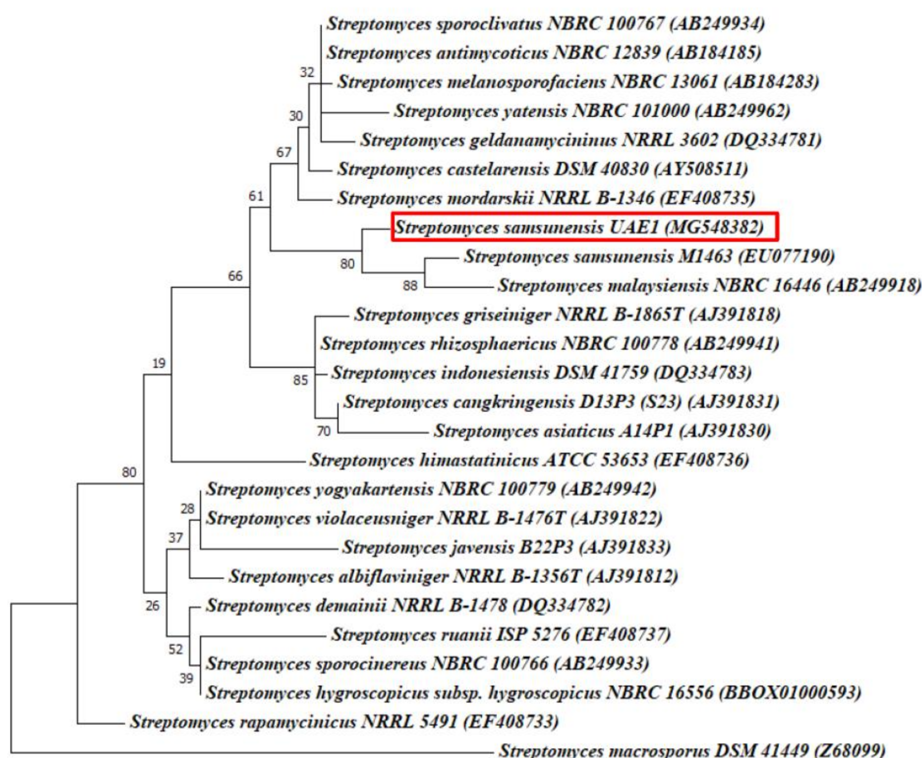


Figure 14: Effect of the BCA candidates on hyphae and cytoplasm of *Lasiodiplodia theobromae***.** Abnormalities observed in hyphal morphology and cytoplasmic contents of *L. theobromae*, following exposure to (A) filter-sterilized crude culture filtrate of BCA1 (isolate #12; *Streptomyces samsunensis* UAE1) and BCA2 (isolate #29; *S. cavourensis* UAE1) on fish meal extract broth, or (B) BCA1 and BCA3 (isolate #44; *Micromonospora tulbaghiae* UAE1) on colloidal chitin broth compared to control. White arrows point to hyphal septum malformation and branch deformation; while yellow and red arrows point to cytoplasmic coagulation and lysis of cytoplasm, respectively.

3.7 Identification of the Promising BCA Candidates to the Species Level

The promising antagonists BCA1, BCA2, and BCA3 were identified by determining the nucleotide sequence of their 16*S* rRNA gene. The 16*S* rRNA gene sequences of BCA1 (*Streptomyces samsunensis*; GenBank accession number MG548382), BCA2 (*S. cavourensis*; MG548383) and BCA3 (*Micromonospora tulbaghiae*; MG548384) were compared with that of other actinobacteria. Comparisons of the 16*S* rRNA gene of BCA1, BCA2 and BCA3 with sequences in the GenBank database showed that these BCA candidates were streptomycete spp. for isolates #12 (BCA1) and #29 (BCA2) and a non-streptomycete sp. for isolate #44 (BCA3). BCA1 showed above 99% similarity to *S. samsunensis* (EU077190) and *S. malaysiensis* (AB249918) (Figure 15A), although, the remaining isolates of *Streptomyces* spp. showed less than 98.8% similarities. The phylogenetic analysis of BCA2 showed 100% similarity to both *S. cavourensis* (AB184264) and *S. albolongus* (AB184425) (Figure 16A); while the rest showed < 99.6% similarity with the strain of interest. This may suggest that BCA1 may possibly be either *S. samsunensis* or *S. malaysiensis*; while BCA2 could be either *S. cavourensis* or *S. albolongus*; thus it was necessary to obtain a more reliable identification of these isolates. Based on the 16*S* rRNA gene comparisons, the third identified BCA3 was considered as *Micromonospora tulbaghiae* (Kirby & Meyers, 2010) Strain UAE1, due to the high similarity (100%) with *M. tulbaghiae* (DSM 45142). The rest of the *Micromonospora* spp. showed 99.4% or less similarity than that with this specific strain (Figure 17A).

(A)



0.0050

(B)

(C)

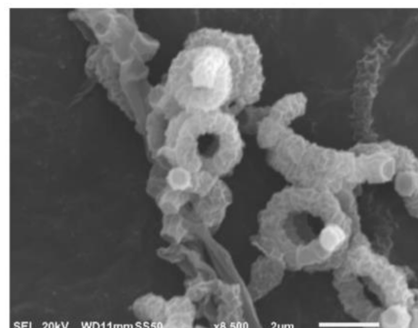


Figure 15: Taxonomic determination of *Streptomyces samsunensis* UAE1, based on phylogenetic, cultural and morphological characteristics. (A) The tree showing the phylogenetic relationships between *S. samsunensis* UAE1 (*MG548382*; 1475 bp) and other members of *Streptomyces* spp. on the basis of 16*S* rRNA sequences. (B) Greyish black aerial mycelia (left) and greyish yellow substrate mycelia (right) growing on ISP medium 3 supplemented with yeast extract, (C) Scanning electron micrograph (8,500X) showing the spiral chains of rugose ornamented spores of *S. samsunensis* UAE1 (BCA1; isolate #12). In (A), numbers at nodes indicate percentage levels of bootstrap support based on a maximum likelihood analysis of 1000 resampled datasets. Bar, 0.005 substitutions per site. *S. macrosporus DSM 41449* (Z69099) was used as an outgroup. GenBank accession numbers are given in parentheses.

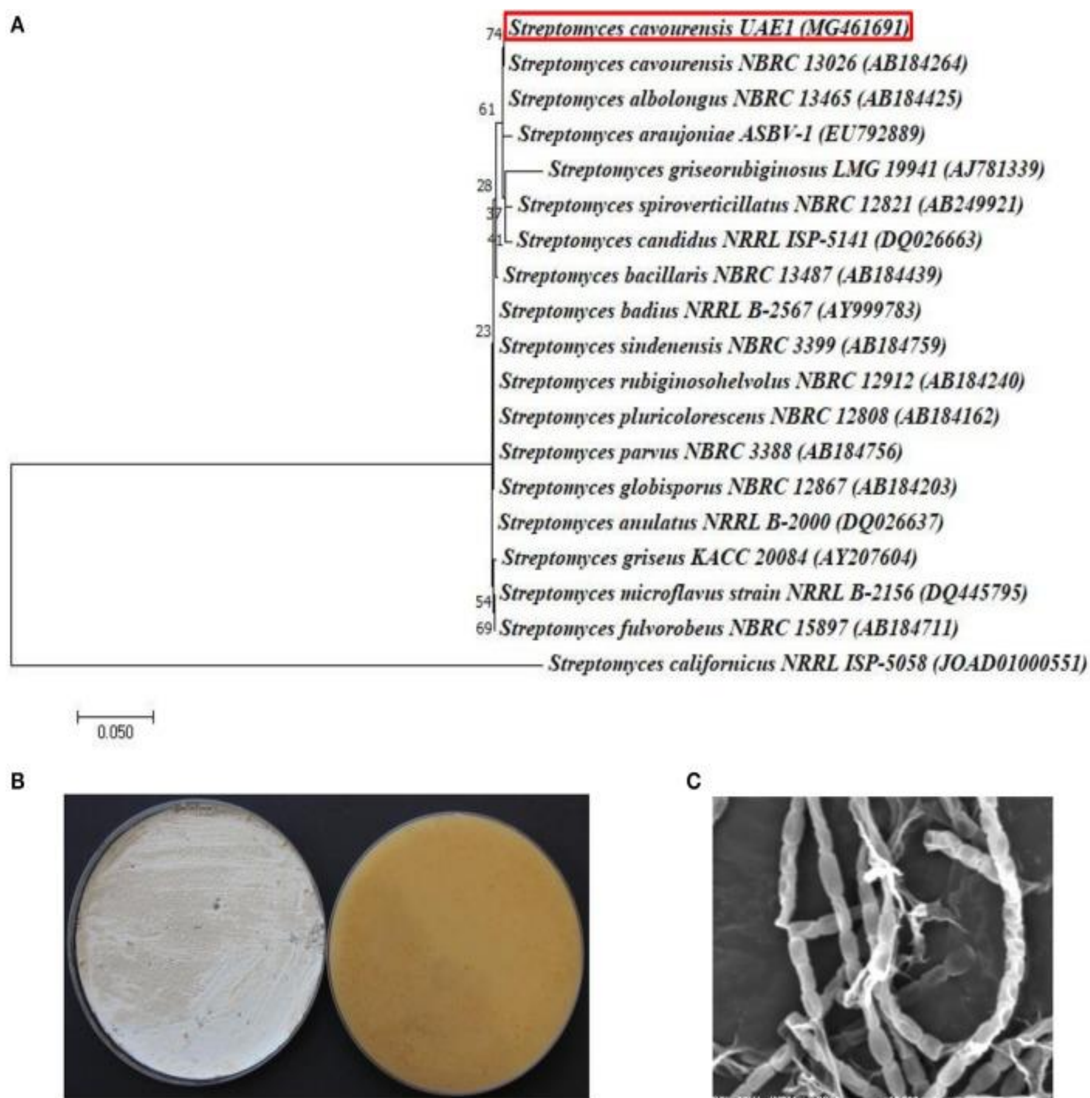


Figure 16: Identification of *Streptomyces cavourensis* UAE1, based on phylogenetic, cultural and morphological characteristics. (A) The tree displaying the phylogenetic relationships between *S. cavourensis* UAE1 (*MG461691*; 1484 bp) and other members of *Streptomyces* spp. on the basis of 16*S* rRNA sequences. (B) Yellow aerial mycelia (left) and yellowish-brown substrate mycelia (right) growing on ISP medium 3 supplemented with yeast extract, and (C) Scanning electron micrograph (10,000X) of the straight to flexuous (Rectiflexibiles) chains and smooth-surfaced spores of the strain of *S. cavourensis* UAE1 (BCA2; isolate #29). In (A), numbers at nodes indicate percentage levels of bootstrap support based on a maximum likelihood analysis of 1000 resampled datasets. Bar, 0.05 substitutions per site. *S. californicus* NRRL ISP-5058 (JOAD0100051) was used as an outgroup. GenBank accession numbers are given in parentheses.

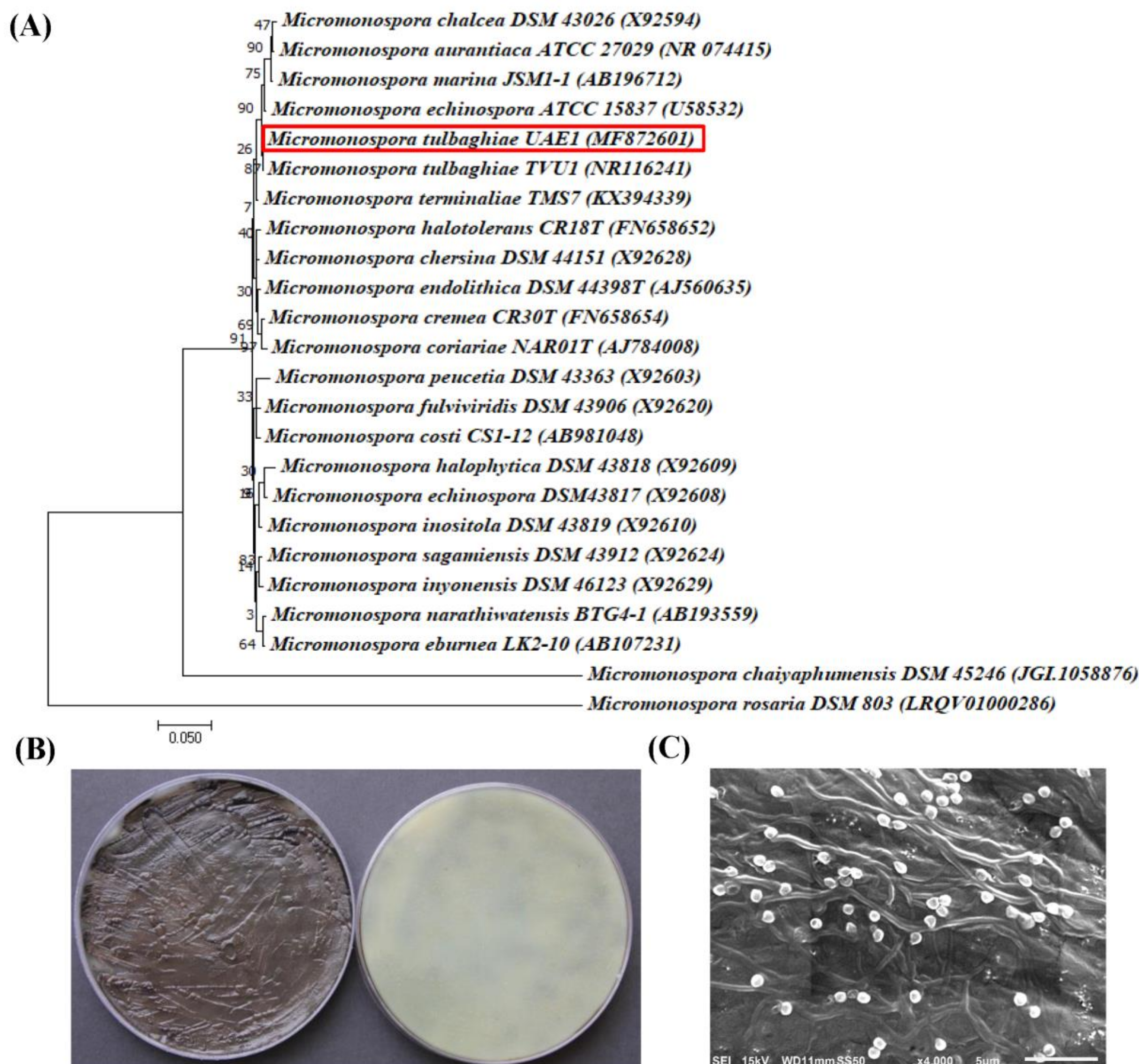


Figure 17: Identification of *Micromonospora tulbaghiae* UAE1 using phylogenetic, cultural and morphological characteristics**.** (A) The tree shows the phylogenetic relationships among *M. tulbaghiae* UAE1 (*MF872601*; 1486 bp) and other members of *Micromonospora* spp. on the basis of 16*S* rRNA sequences. (B) brownish-black charcoal-like substrate mycelia without the formation of aerial mycelium (left) and the brownish-black color of the substrate mycelia (right) growing on ISP medium 3 supplemented with yeast extract, and (C) Scanning electron micrograph (4,000X) of the single oval to spherical smooth-surfaced spores of the strain of *M. tulbaghiae* UAE1 (BCA3; isolate #44). In (A), numbers at nodes indicate percentage levels of bootstrap support based on a maximum likelihood analysis of 1000 resampled datasets. Bar, 0.05 substitutions per site. *M. rosaria* DSM 803 (LRQV01000286) was used as an outgroup. GenBank and Joint Genome Institute (JGI) accession numbers are given in parentheses

To confirm the identity of BCA1, the pure cultures produced grayish black aerial mycelia with grayish yellow substrate mycelial growth on ISP medium 3 for 14 days (Figure 15B). Using SEM, the configuration of the spore chains showed spiral chains of rugose ornamented spores (Figure 15C). Together, the genotypic, morphological, cultural and phenotypic data showed that BCA1 can be recognized as *Streptomyces samsunensis* (Sazak *et al*., 2011). Strain UAE1 (Table 5).

On the other hand, typical yellow aerial mycelia and yellowish-brown substrate mycelia were observed when BCA2 was cultivated (Figure 16B). BCA2 showed straight to flexuous (Rectiflexibiles) chains and smooth-surfaced spores (Figure 16C). Our data demonstrated that BCA2 (isolate #29) can be recognized as *Streptomyces cavourensis* (Giolitti, 1958) (in Waksman, 1961) Strain UAE1 (Table 6). Pure cultures of BCA3 (*M. tulbaghiae*) on ISP medium 3 produced typical brownish-black charcoal-like substrate mycelia without the formation of aerial mycelia (Figure 17B); with the formation of single oval to spherical smooth-surfaced spores (Figure 17C).

3.8 Effect of The Promising BCA Candidates on *L. theobromae* in the Greenhouse

The responses of the pathogen to these selected antagonists *in vitro* clearly indicated the three BCA candidates to be effective against mango dieback caused by *L. theobromae*. With an aim of evaluating and comparing the outcome of the application of the BCAs in suppressing *L. theobromae* using different antifungal activities, an *in vivo* experiment involving greenhouse grown plants was conducted. For this purpose, “preventive” treatments with the three BCA candidates 1 week before inoculation with *L. theobromae* were applied to determine their impact on mango dieback.

Initially, a pathogenicity test was carried out to determine the effect of inoculation with *L. theobromae* on mango seedlings. Symptoms typical of the mango disease after 3 wpi with *L. theobromae* (*Lt*) were observed (Figure 18A-C). The disease progressed with time, initially with leaves of infected seedlings showing distinct defoliation at 6 wpi. No disease symptoms were evident in any of the plants inoculated with BCA candidate alone (*Ss, Sc* or *Mt*) or on non-inoculated seedlings (C) (Figure 18A-C). Secondly, we individually applied the BCA candidates *S. samsunensis*, *S. cavourensis* or *M. tulbaghiae* on seedlings one week before inoculation with *L. theobromae*, designated as *Ss*+*Lt, Sc*+*Lt* or *Mt*+*Lt*, respectively. Plants inoculated with the BCA candidates prior to inoculation with *L. theobromae* (*Ss*+*Lt, Sc*+*Lt* or *Mt*+*Lt*) recovered when compared with seedlings inoculated with *L. theobromae* only (*Lt*) at all time points of assessments. These plants appeared to be healthy and were comparable to those that were inoculated with any of the corresponding BCA candidate alone (*Ss*, *Sc* or *Mt*) or without infection with *L. theobromae* (C) (Figure 18A-C; Figure 19). This suggests that these BCA candidates effectively inhibit *L. theobromae* growth *in vivo.*

Table 5: Comparison of morphological, cultural and phenotypic characteristics that distinguish BCA1 (isolate #12) from very closely related species *Streptomyces samsunensis* and *S. malaysiensis*

Characteristics	Isolate #12	*S. samsunensis*	*S. malaysiensis*
Morphological			
Morphology of the spore chains	Closed spiral	Closed spiral	Closed spiral
Spore surface	Wrinkled (Rugose)	Wrinkled (Rugose)	Wrinkled (Rugose)
Cultural			
Color of aerial mycelium on ISP3	Greyish black	Greyish black	Smoky black
Color of substrate mycelium on ISP3	Greyish yellow	Greyish yellow	Yellow brown
Color of diffusible pigment on (ISP3)	None	None	None
Production of melanin pigments on peptone-yeast extract-iron agar	-	-	+
Production of melanin pigments on tyrosine agar	-	-	+
Phenotypic			
Growth on sole carbon sources (%, w/v):			
Adonitol (1.0)	-	-	+
L-Arabinose (1.0)	+	+	+
Dextrin (1.0)	+	+	+
Lactose (1.0)	+	+	-
Salicin (1.0)	-	-	+
myo-inositol (1.0)	-	-	+
Raffinose (1.0)	-	-	+
Xylose (1.0)	-	-	+
Sodium propionate (0.1)	-	-	+
Growth on sole nitrogen sources (0.1 %, w/v):			
L- Methionine	+	+	-
L-Serine	+	+	+
Degradation tests (%, w/v):			
Aesculin hydrolysis (0.1)	-	-	+
Arbutin hydrolysis (0.1)	+	+	-
Gelatin hydrolysis (0.4)	-	-	+
Hypoxanthine hydrolysis (0.4)	-	-	+
L-Tyrosine (0.4)	-	-	+
Tween 80 (1.0)	+	+	+
Reference	This study	Sazak *et al.*, 2011	Al-Tai *et al.*, 1999

+, growth or positive reaction; -, no growth or negative reaction.

Table 6: Comparison of morphological, cultural and phenotypic characteristics that distinguish BCA2 (isolate #29) from very closely related species *Streptomyces cavourensis* and *S. albolongus.*

Characteristics	**Isolate #29**	***S. cavourensis***	***S. albolongus***
Morphological			
Morphology of the spore chains	Straight to flexuous (Rectus-flexibilis)	Straight to flexuous (Rectus-flexibilis)	Straight to flexuous (Rectus-flexibilis)
Spore surface	Smooth	Smooth	Smooth
Cultural			
Color of aerial mycelium on ISP3	Yellow	Yellow	White to pale beige
Color of substrate mycelium on ISP3	Yellow-brown	Yellow-brown	Yellow-brown
Color of diffusible pigment on (ISP3)	Yellow-brown	Yellow-brown	None
Production of melanin pigments on peptone-yeast extract-iron agar	+	+	+
Production of melanin pigments on tyrosine agar	+	+	+
Phenotypic			
Growth on sole carbon sources (%, w/v):			
Glucose (1.0)	+	+	+
L-Arabinose (1.0)	-	-	+
Fructose (1.0)	+	+	-
Rhamnose (1.0)	-	-	-
Mannitol (1.0)	+	+	-
myo-inositol (1.0)	-	-	-
Raffinose (1.0)	-	-	+
Xylose (1.0)	+	+	+
Reference	This study	Giolotti, 1958 (in Waksman, 1961)	Tsukiura *at al.,* 1964

+, growth or positive reaction; -, no growth or negative reaction.

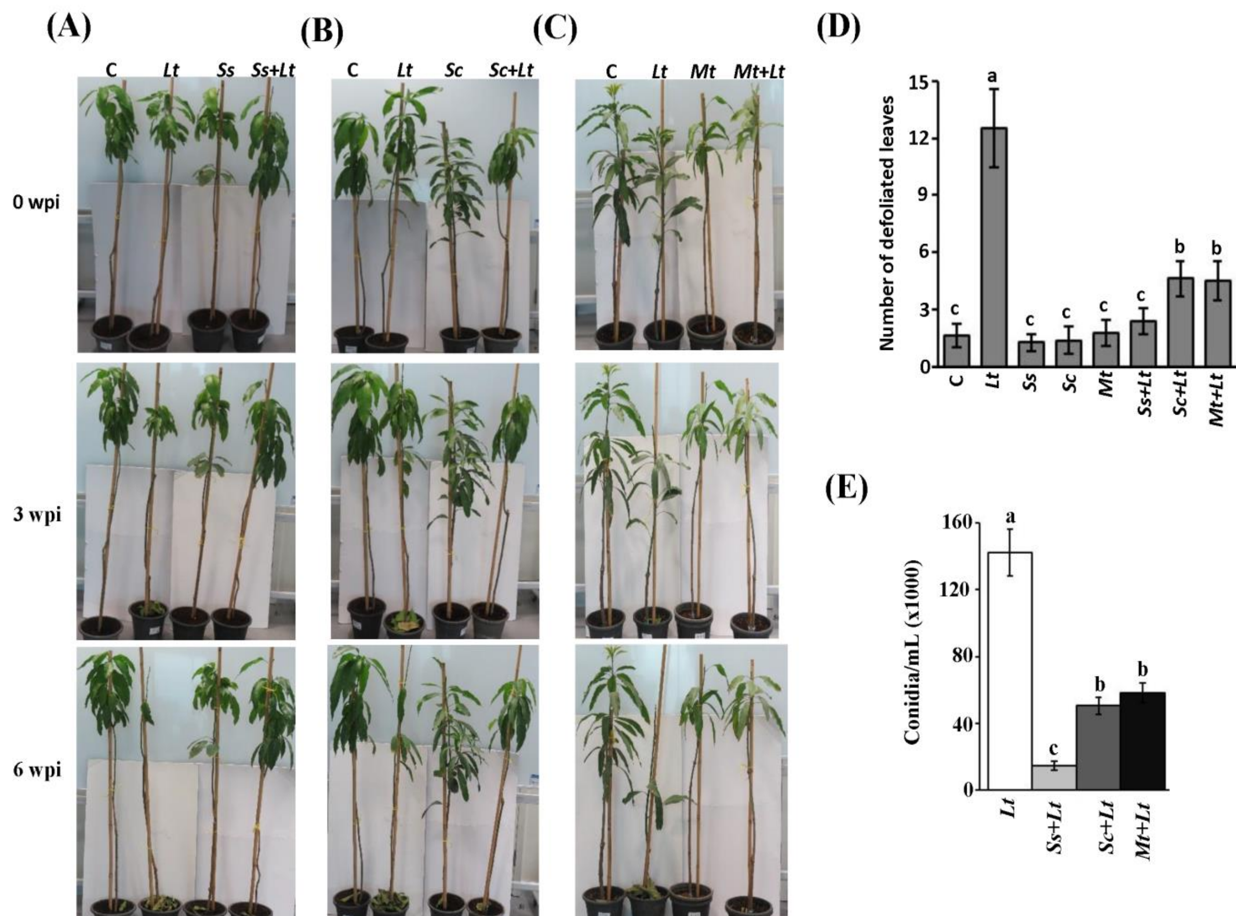


Figure 18: Antagonistic effect of BCA candidates against mango dieback disease under greenhouse conditions**.** Effect of preventive biocontrol treatment of (A) *Streptomyces samsunensis* UAE1 (BCA1; isolate #12); (B) *S. cavourensis* UAE1 (BCA2; isolate #29); and (C) *Micromonospora tulbaghiae* UAE1 (BCA3; isolate #44). The number of (D) defoliated leaves; and (E) conidia after recovery of the pathogen from affected mango stem tissues (cv. Badami) at 6 and 9 wpi with *L. theobromae*, respectively. In (D & E), values with different letters are significantly different from each other at $P<0.05$. C, non-inoculated control seedlings, *Lt*, seedlings inoculated with *L. theobromae* only; *Ss*, *Sc* or *Mt,* seedlings inoculated with only *S. samsunensis* UAE1, *S. cavourensis* UAE1 or *M. tulbaghiae* UAE1, respectively; *Ss*+*Lt*, *Sc*+*Lt* or *Mt*+*Lt*, seedlings inoculated with the individual BCA, *S. samsunensis* UAE1*, S. cavourensis* UAE1 or *M. tulbaghiae* UAE1, respectively, one week prior to *L. theobromae* inoculation; wpi, weeks post inoculation.

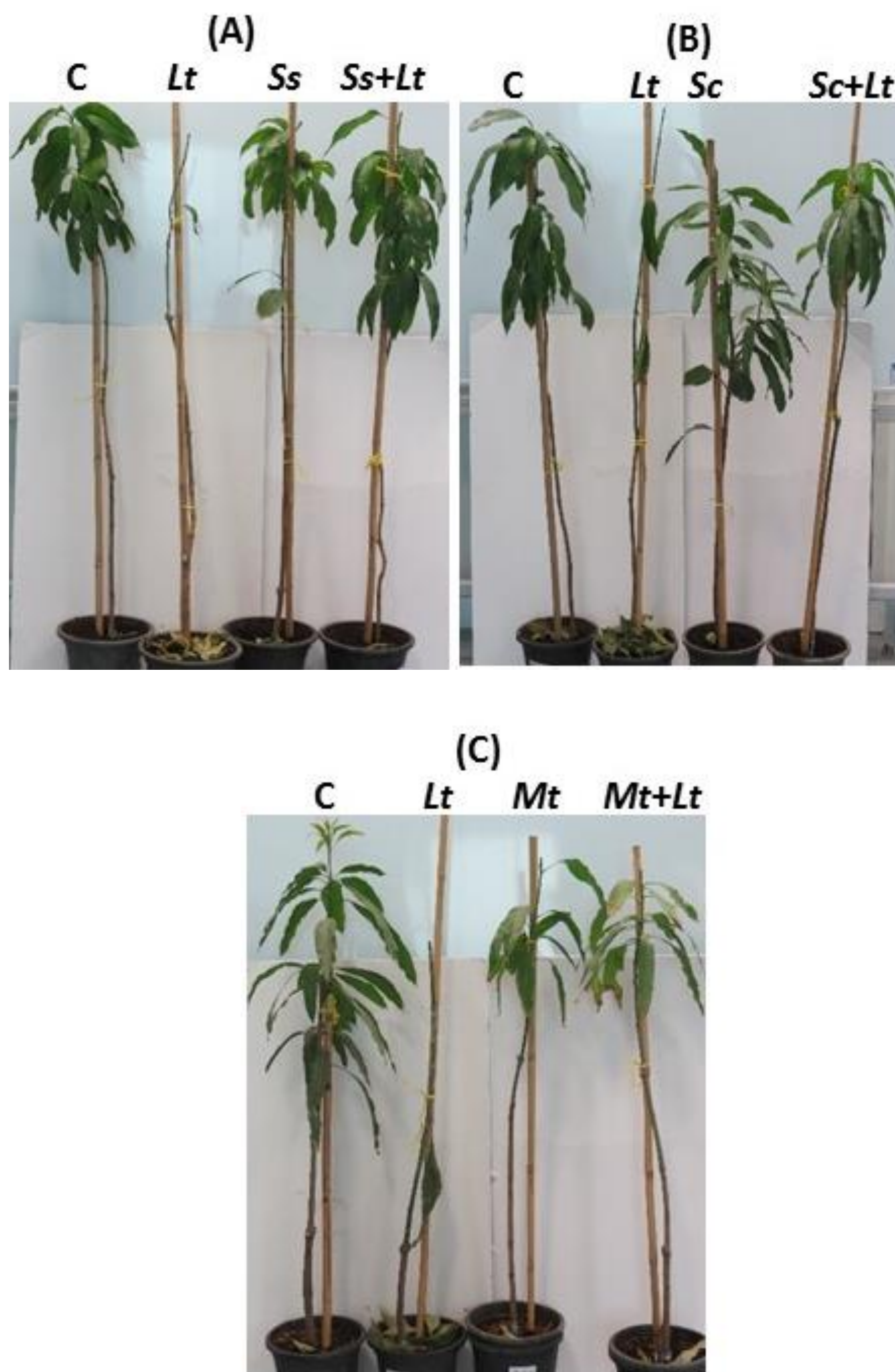


Figure 19: Antagonistic effect of BCA candidates against mango dieback disease caused by *Lasiodiplodia theobromae* in the greenhouse. Effect of preventive biocontrol treatment of (A) *Streptomyces samsunensis* UAE1 (BCA1; isolate #12); (B) *S. cavourensis* UAE1 (BCA2; isolate #29); (C) *Micromonospora tulbaghiae* UAE1 (BCA3; isolate #44) on mango seedlings at 9 wpi with *L. theobromae*. C, non-inoculated control seedlings, *Lt*, seedlings inoculated with *L. theobromae* only; *Ss*, *Sc* or *Mt,* seedlings inoculated with only *S. samsunensis*, *S. cavourensis* or *M. tulbaghiae*, respectively; *Ss*+*Lt*, *Sc*+*Lt* or *Mt*+*Lt*, seedlings inoculated with the individual BCA, *S. samsunensis, S. cavourensis* or *M. tulbaghiae*, respectively, one week prior to *L. theobromae* inoculation. wpi, weeks post inoculation.

Significant differences were found between treatments when the DSI was assessed. Plants inoculated with *L. theobromae* (*Lt*) showed disease progression until 9 wpi (Table 7).

Seedlings not inoculated with *L. theobromae* (control; C) showed no disease symptoms at any of the assessment time-points. There was a dramatic decrease in DSI in all *L. theobromae*-infected seedlings that were previously treated with any of the BCA candidates at 3 and 9 wpi, when compared with plants inoculated with the pathogen alone. In comparison with the *L. theobromae*-infected seedlings, the DSI of the preventive applications of individual BCA candidate (*Ss*+*Lt, Sc*+*Lt*, and *Mt*+*Lt*) dropped from 2.83 to 0.17 at 3 wpi and from 4.33 to 0.17 at 9 wpi; providing 94.0 and 96.1% reduction in disease development, respectively. The three preventive treatments with the BCA candidates at 3 and 9 wpi did not show significant ($P >$ 0.05) difference among the DSI measurements, and the BCA-treated plants without the pathogen. This suggests that the preventive treatment with BCA candidates a week before inoculation with *L. theobromae* effectively suppresses the pathogen invasion.

It is evident that the DSI values in the case of BCA applications were significantly lower than those with *L. theobromae*; however, the differences of the DSI were indistinguishable among these BCA treatments. For that reason, we compared the responses of the biological control treatments to determine their effects on leaf defoliation and the production of conidia on the host plant. The number of falling leaves in seedlings inoculated with *L. theobromae* dramatically increased compared to any BCA-inoculated or non-inoculated seedlings at 6 wpi (Figure 18D). Mango seedlings treated with BCA before *L. theobormae* infection significantly

reduced the number of defoliated leaves; thus seedlings of *Ss*+*Lt* showed the lowest number of falling leaves among the other two treatments and were comparable to its corresponding Ss treatment. In addition, conidia counts of *L. theobromae* at the leaf base of treated mango seedlings were made. The BCA treatment (*Ss*+*Lt*) caused a greater reduction in the number of conidia, followed by the other BCA-treated plants, *Sc*+*Lt* and *Mt*+*Lt* at the end of the greenhouse experiment (Figure 18E). At least 4-fold reduction in total conidia numbers of *L. theobromae* in *S. samsunensis*-treated plants was measured when compared with other BCA treatments (Figure 18E). In general, the pathogen appeared not to be sufficiently aggressive to support disease progression in the presence of *S. cavourensis* or *M. tulbaghiae*, while a strong inhibitory effect on the pathogen was observed in the case of the *S. samsunensis* treatment. Together, this suggests that *S. samsunensis*, which exhibited multiple modes of action, successfully controlled mango dieback disease.

Table 7: Disease severity index (DSI) of *Lasiodiplodia theobromae* inoculated-mango seedlings (cv. Badami) inoculated with each BCA candidate at 3 and 9 wpi (*n* = 6).

Treatment	**DSI** [a]	
	3 wpi	**9 wpi**
C	0.00 *a*	0.00 *a*
Lt	2.83 *b*	4.33 *b*
Ss	0.00 *a*	0.00 *a*
Ss+Lt	0.17 *a*	0.17 *a*
Sc	0.00 *a*	0.00 *a*
Sc+Lt	0.17 *a*	0.17 *a*
Mt	0.00 *a*	0.00 *a*
Mt+Lt	0.17 *a*	0.17 *a*

[a] 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 (necrotic or dark brown area around the point of inoculation). Values with similar letters are not significantly different at $P = 0.05$.
C, control (no inoculation or treatment); *Lt*, seedlings inoculated with *L. theobromae* only; *Ss, Sc* or *Mt*, seedlings inoculated with a particular BCA. *Ss+Lt*, *Sc+Lt* or *Mt+Lt*, seedlings inoculated with a particular BCA one week before *L. theobromae* inoculation; *Ss*, *Streptomyces samsunensis* UAE1; *Sc*, *S. cavourensis* UAE1; *Mt*, *M. tulbaghiae* UAE1. wpi, weeks post inoculation.

Chapter 4: Discussion

The pathogen was isolated and identified morphologically and phylogenetically. Microscopy demonstrated that the pathogen is a prolific producer of immature and mature conidia on PDA (Figure 5D). Consistent with Punithalingam (Punithalingam, 1980), immature conidia were initially hyaline, unicellular, ellipsoid to oblong, thick walled with granular contents. We also observed that with age, mature conidia became 2-celled, dark brown, with longitudinally striated appearance and an average size of 26.6 x 12.9 µm. On maturity, the size of conidia is about 20-30 X 10-15 µm (Punithalingam, 1980; Khanzada *et al.*, 2005) In addition, the morphological characteristics of conidia were similar to those previously described (Sutton, 1980). The assessments of spore biology are important to distinguish the fungal survival, dispersal and pathogenicity among closely related species within Botryosphaeriaceae spp. (Slippers *et al*., 2005; Alves *et al*., 2008) , though we argue about the difficulty in identifying the species of the pathogen based merely on its conidial characteristics.

Specific genomic regions of *L. theobromae* that correspond to the two widely used loci ITS and TEF1-α were amplified and sequenced. Phylogenetic analysis of DNA sequences combining, ITS and TEF1-α (Alves *et al*., 2008; Ismail *et al.,* 2012; Cruywagen *et al*., 2017), as also performed to discriminate between Lasiodiplodia species, and to identify the causal agent of the dieback disease on mango in the UAE. The adaptation to different plant hosts and environments has led to the evolution of at least 13 cryptic species within the *L. theobromae* species complex (Ismail *et al*., 2012). The identified *L. theobromae* DSM 105134 from the UAE fits into one clade with several *L. theobromae* strains from different sources. The most closely related ITS/TEF1-α was *L. theobromae* CBS 130989 (= BOT4), an isolate from mango in

Egypt (Ismail *et al*., 2012), which demonstrated an identity of 100%. Our data also showed that the ITS/TEF1-α identified in this study clustered together with *L. theobromae* isolates BOT 6, BOT 7 and BOT 23 from mango in Egypt (Ismail *et al*., 2012; Cruywagen *et al*., 2017) .The isolate CBS 112874 of *L. theobromae* was reported to infect grapes in South Africa (Pavlic, *et al*., 2004). Similarly, the ITS/TEF1-α which belongs to *L. theobromae* collected from the UAE showed 99% identity with that of both CMW 24701 and CMW 24702 strains isolated from Eucalyptus sp. in China (Chen *et al*., 2011). None of the ITS/TEF1-α sequences that belong to *L. theobromae* including the pathogen from this study, clustered with other Lasiodiplodia spp. reported worldwide. This provides strong evidence that the isolate DSM 105134 in the current study belongs to *L. theobromae* sp. complex and is the main causal agent of dieback on mango in the UAE. Yet, it is probable this destructive strain of the fungal pathogen may have been introduced from Egypt.

Nowadays, seeking an alternative strategy to the use of chemicals by native microorganisms as a source material for plant growth promotion and disease amelioration in agriculture has become a priority (Saeed *et al*., 2017b; Ab Rahman *et al*., 2018). Actinobacteria could be potential targets as BCA candidates since they have many properties that control diseases, increase nutrient supply and enhance growth of plants (Doumbou *et al*., 2001; Barka *et al*., 2016). Ultimately, this approach can be employed as a main component in IPM, and may be combined with others to prevent losses and damages caused by plant diseases (Saeed *et al*., 2017b; Ab Rahman *et al*., 2018). These outcomes led us to explore local UAE soils for the actinobacterial communities and screen their potential under *in vitro* and *in vivo* greenhouse conditions for their effectiveness in the control of the mango dieback disease caused by *L. theobromae*. We hypothesized that exposure to

microbial metabolites produced by the soil-inhabiting actinobacteria can be exploited for the management of this devastating mango disease.

In this study, I aimed at identifying SA and NSA isolates that are capable of restricting invasions of *L. theobromae* on mango plantations. A total of 53 actinobacterial strains were obtained from the rhizosphere of healthy mango plants. Due to their dominance as a biologically active component of the soil microflora, many researchers have successfully isolated SA from soil environments (Goodfellow & Williams, 1983; Palaniyandi *et al*., 2013). The NSA, however, are rarely isolated actinobacteria whose isolation frequency using commonly used techniques is usually lower than the numbers of SA (Jose & Jebakumar, 2013). For that reason, the isolation of the uncommon actinobacteria was achieved by the application of *Streptomyces*polyvalent phages that selectively permitted the appearance of the genera of NSA on isolation plates, a technique not commonly used in screening for BCA candidates. Some studies have recommended the use of *Streptomyces* phages to omit "weedy" *Streptomyces* colonies (Kurtböke *et al*., 1992; Kurtböke, 2012). In the current study, the isolated NSA group comprised of *Actinoplanes, Actinomadura, Microbispora, Micromonospora, Nocardia, Rhodococcus*, and *Streptosporangium* spp. (Table 1).

The development of new BCA and/or biocontrol products for use against plant diseases requires screening of large numbers of antagonistic candidates (Köhl *et al*., 2011). In our effort to conduct an appropriate performance evaluation of BCA candidate(s) against *L. theobromae*, a series of screening steps were performed. A first round of *in vitro* screening on agar plates allowed rapid and clear discriminating results. Secondly, a selection of antagonists to *L. theobromae*, using the novel *in*

vivo mango fruit bioassay was also evaluated. Thirdly, selected candidates were identified to the genus and species levels. Finally, an assessment of the feasibility of the selected BCA candidates in controlling dieback disease on mango seedlings under greenhouse conditions was carried out. Accordingly, 11 were regarded as highly diffusible antifungal metabolite-producing isolates on fish meal extract agar plates. In addition, an agar medium incorporating *L. theobromae* mycelial fragments helped to select 12 antagonists which had the required enzymes to destroy the components of *L. theobromae* cell wall. Mycelial fragment agar has been previously used to isolate glucanolytic BCA candidates against *Phytophthora fragariae* (Valois *et al*., 1996) and *Pythium aphanidermatum* (El-Tarabily, 2006). Many promising antagonistic isolates obtained in the current investigation that showed clearing zones on *L. theobromae* mycelial fragment agar and on colloidal chitin agar, may have secreted ß-1,3-glucanases and chitinase which hydrolyzed glucans and chitin present in the pathogen cell wall and helped to lyse *L. theobromae* hyphae. This study clearly indicates that SA and NSA can serve as potential BCAs against *L. theobromae*.

Because testing of isolates under greenhouse and field conditions for efficacy requires additional labor and time, the antagonistic potential of candidates were further assessed in a second screening round of laboratory bioassays. I argue that the mango fruit antagonism bioassay would provide a “clear-cut” prediction of what may occur in the field. Such laboratory *in vivo* assays using plant material are of value since they provide a rapid screening of large numbers of antagonistic candidates in the presence of the pathogen. Thus, the results must be confirmed by greenhouse/field trials. Previous studies have used similar “bioassay” approach as a practical step in the selection of BCAs for major pathogens and pests on crops. In assays carried out *in vitro* as well as on the carrot roots or mango fruits, the BCAs

used were found to be capable of almost complete inhibition of *P. coloratum* or *L. theobromae*, respectively (El-Tarabily *et al*., 1997; Seethapathy *et al*., 2016). Seethapathy *et al*. (2016) have demonstrated that dual culture technique of bacterial anatogonists using *Pseudomonas fluorescens* (Pf1) and *Bacillus subtilis* (EPCO16) reduced the pathogen population *in vitro*; and further strengthened the cell-wall structures of mango fruits against *L. theobromae* infection. This is consistent with the present study that many isolates killed the pathogen *in vitro*; whereas certain isolates completely arrested development of lesions, with others reducing only the lesion size or having no effect on the disease in the mango fruit bioassay (Table 2). According to El-Tarabily *et al*. (1997), the failure of isolates to reduce lesion diameter in the mango fruit bioassay indicated that their ability to produce antifungal metabolites in agar did not necessarily address that this performance would be reproducible on plant material. Our data indicate the importance of the *in vitro* as well as the mango fruit bioassay for the selection of potential antagonists prior to their screening on plants in the greenhouse.

The efficacious isolates from the *in vitro* and *in vivo* assays were further identified. The three candidates represent 5.7% of the total isolated actinobacteria from the soil of healthy mango rhizosphere. In this study, the two identified isolates (#12 and #29) belonging to *Streptomyces* spp. confirm the findings from previous reports that SA are predominant among actinobacteria appearing in isolation plates and more often produce useful antibiotics and active secondary metabolites (Thenmozhi and Krishnan, 2011; Barka *et al*., 2016). Isolates #12 (BCA1) and #29 (BCA2) were identified as *S. samsunensis* and *S. cavourensis*, respectively, while BCA3 (isolate #44) was considered as *M. tulbaghiae*. The mechanisms involved in disease reduction or prevention of lesion development appeared to be antibiosis

(diffusible antifungal metabolites and volatile compounds) for *S. samsunensis* and *S. cavourensis*, and the production of CWDEs such as chitinase and ß-1,3-glucanases for *S. samsunensis* and *M. tulbaghiae*. Several actinobacteria have been demonstrated to inhibit the growth of soil-borne plant pathogens such as *Pythium ultimum, Rhizoctonia solani* (Yuan and Crawford, 1995), *P. coloratum* (El-Tarabily *et al*., 1997) and *Thielaviopsis punctulata* (Saeed *et al*., 2017b) *in vitro* via the production of inhibitory diffusible antifungal metabolites.

The cell walls of filamentous fungi consist largely of chitin and ß-glucans (Osherov and Yarden, 2010), it is probable that ß-1,3-glucanase and chitinases produced by the antagonistic actinobacteria in this study may be involved in the pathogen suppression. The exposure of phytopathogenic fungi to CWDEs can result in the lysis and degradation of the fungal cell walls (Doumbou *et al*., 2001; Whipps, 2001; Berini *et al*., 2018). Therefore, the production of chitinase and ß-1,3-glucanase was set, in this study, as a criteria for selection of potential BCA against *L. theobromae*. Chitinase-producing actinobacteria tested previously under *in vitro* conditions have included *Streptomyces viridicans* (Gupta *et al*., 1995), *S. viridodiasticus* (El-Tarabily *et al*., 2000), and *Streptomyces* spp. (Singh and Gaur, 2016). In addition, several chitinase-producing SA such as *Streptomyces* spp. (Singh *et al*., 1999), and NSA such as *Micromonospora carbonacea* (El-Tarabily *et al*., 2000) and *Actinoplanes missouriensis* (El-Tarabily, 2003) were used for the management of cucumber wilt caused by *Fusarium oxysporum* f.sp. *cucumerinum*, lettuce basal drop caused by *Sclerotinia minor*, and lupin root rot caused by *Plectosporium tabacinum*, respectively. Valois *et al*. (1996) have reported some ß-glucanase-producing actinobacterial isolates that hydrolyzed cell wall glucans,

caused hyphal lysis and resulted in the suppression of root rot disease of raspberry caused by *Phytophthora fragariae*.

Beside its ability to produce diffusible and volatile inhibitory antifungal compounds and siderophores, *S. samsunensis* produced CWDEs. Better control of mango dieback disease by *S. samsunensis* in comparison to the other BCA candidates (*S. cavourensis* or *M. tulbaghiae*) may indicate that this response could be a result of synergetic effects of the multiple modes of action i.e., co-antagonism. Our results are in agreement with other reports that *Streptomyces spiralis* and *Actinoplanes campanulatus* which produced diffusible inhibitory antifungal metabolites and CWDEs were superior on *Micromonospora chalcea* which produced CWDEs only in controlling root rot and crown rot of cucumber caused by *P. aphanidermatum* (El-Tarabily *et al.*, 2009).

Bailey and Falk (2011) have stated that less than 1% of candidate microorganisms isolated by routine isolations make successful antiobiotic products and secondary metabolites. Among these, *Streptomyces griseoviridis* K61 (Mycostop®), *S. lydicus* strain WYEC108 (Actinovate®, Actino-iron®, or Micro108® and *S. saraceticus* KH400 (YAN TEN) are commercial BCA products that reduce spore germination and inhibit hyphal growth of plant fungal pathogens (Minuto *et al.*, 2006; Elliott *et al.*, 2009; Palaniyandi *et al.*, 2013). In the UAE, *Streptomyces globosus* UAE1 has recently been reported as the first effective BCA against black scorch disease in date palm plantations (Saeed *et al.*, 2017b). On the other hand, few NSA have been recognized as BCA and/or PGP (El-Tarabily *et al.*, 1997; El-Tarabily and Sivasithamparam, 2006). This suggests that the SA and NSA strains isolated in our study may also serve as producers of potentially useful

antifungal products active against *L. theobromae*. Therefore, efforts to be among the first to manage mango dieback by the three antagonists were aimed in greenhouse trials.

One should take cautions when assuming a correlation between *in vitro* inhibition and greenhouse or field performance (Fravel, 2005; Parnell *et al*., 2016). In the present study, the actinobacteria were inoculated at the apices of the mango stems 1 week before inoculation with the pathogen. The gap in the incubation periods may favor the establishment of the introduced actinobacteria prior to the exposure to the pathogen, and/or to enable them to propagate on the mango stem or to activate the mechanism(s) of antagonism (Rothrock and Gottlieb, 1984). Prevention of infection using actinobacteria is a highly recommended management strategy in plant-pathogen interaction systems (Saeed *et al*., 2017b). It was clear that the application of any of the BCAs prior to pathogen invasion helped to establish the required biomass of the BCA; and thus delay the systemic invasion by *L. theobromae* within the host plant (Figure 8). The results obtained from the DSI indicated that *S. samsunensis, S. cavourensis*, or *M. tulbaghiae* have good potential as BCAs of the mango dieback caused by *L. theobromae*; yet it was difficult to choose the most effective BCA. With all the successful BCA applications, a significant reduction in disease symptoms regarding the number of falling leaves at 6 wpi and the conidia counts in affected tissues at 9 wpi in BCA1-treated seedlings was found. This suggests that *S. samsunensis* is the most efficient BCA among the tested strains, and may serve as a candidate biofungicide for the control of *L. theobromae*-affected mango orchids. To a lesser degree, *S. cavourensis* and *M. tulbaghiae* were also notably effective in reducing the pathogenicity of *L. theobromae* in the greenhouse.

Biological control can be used as an alternative method to agrochemicals if the BCA or its product survives temporarily adverse conditions and preferably improves plant performance (El-Tarabily & Sivasithamparam, 2006). The antagonistic *Streptomyces* and *Micromonospora* spp. identified in this study are safe, inexpensive, long lasting and well-suited to extreme harsh conditions, meeting the expectations previously reported in literature (Goodfellow & Williams, 1983; Ningthoujam *et al*., 2009). These indigenous strains of BCA are well-adapted to the local conditions of the UAE of dry soils and arid environments. In addition, actinobacteria are capable of producing spores resisting heat and drought stresses (Goodfellow & Williams, 1983), making them adequately suitable for being implemented as a prime component in IPM leading to sustainable agriculture in the future. In general, it is well-known that the CFU of actinobacteria remains high as soils dry out; while the relative incidence of bacteria is adversely affected as they lack tolerance to arid conditions (Alexander, 1977).

Together, the diffusible compounds and/or CWDEs produced by the BCA candidates were closely associated with the inhibition, suppression and destruction of *L. theobromae* within the plant host. The mechanisms identified are most likely to have significantly contributed to the relative success of these selected strains as BCAs. Other factors, such as the production of compounds capable of inducing host resistance (e.g., ISR) to the pathogen by the BCA (Martínez-Hidalgo *et al*., 2015) could also be attributed to the reduction of the mango dieback incidence detected. This form of resistance by the BCA candidates was not investigated in this research, but will surely be looked at in future studies. The present study provided the first record of SA and NSA as microbial antagonists to control a *Lasiodiplodia* disease. To our knowledge, the results demonstrate, for the first time, the isolation,

identification and confirmation of the biocontrol potential of actinobacteria from soil native to the UAE, and likely to be naturally suited to be suppressive to *L. theobromae*. In order to make biocontrol more effective, future research focusing on development of novel formulations, broadening of host range targets, and increasing biomass production of BCAs in association with the use of biotechnology in improvement of BCA mechanisms will effectively develop schemes to widely manage the dieback disease using environmentally sustainable strategies.

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