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CHEMICAL AND BIOLOGICAL CONTROL OF CANKER DISEASE OF *DELONIX REGIA* CAUSED BY THE FUNGUS *Neoscytalidium dimidiatum*

Seham Mohammed Suliman Mohammed Al Raish

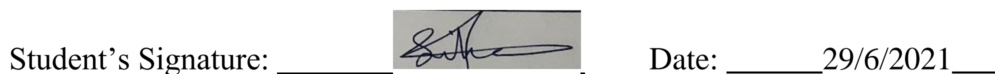
This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Professor Khaled El-Tarabily

June 2021

Declaration of Original Work

I, Seham Mohammed Suliman Mohammed Al Raish, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Chemical and Biological Control of Canker Disease of Delonix regia Caused by the Fungus Neoscytalidium dimidiatum*", hereby, solemnly declare that this dissertation is my own original research work that has been done and prepared by me under the supervision of Professor Khaled El-Tarabily, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions.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 dissertation.



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Abstract

In the United Arab Emirates (UAE), royal poinciana (*Delonix regia*) trees suffer from stem canker disease. Symptoms of stem canker can be characterized by branch and leaf dryness, bark lesions, discoloration of xylem tissues, longitudinal wood necrosis and extensive gumming. General dieback signs were also observed leading to complete defoliation of leaves and ultimately death of trees in advanced stages. The fungus, *Neoscytalidium dimidiatum* DSM 109897, was consistently recovered from diseased royal poinciana tissues; this was confirmed by the molecular, structural and morphological studies. Phylogenetic analyses of the translation elongation factor 1-a (TEF1-α) of *N. dimidiatum* from the UAE with reference specimens of Botryosphaeriaceae family validated the identity of the pathogen. To manage the disease, the chemical fungicides, Protifert®, Cidely® Top and Amistrar® Top, significantly inhibited mycelial growth and reduced conidial numbers of *N. dimidiatum* in laboratory and greenhouse experiments. The described “apple bioassay” is an innovative approach that can be useful when performing fungicide treatment studies. Under field conditions, Cidely® Top proved to be the most effective fungicide against *N. dimidiatum* among all tested treatments. The data suggest that the causal agent of stem canker disease on royal poinciana in the UAE is *N. dimidiatum*. In addition to the fungicides application, the current theory also studied the effect of biological control agents to control this disease. The current study also revealed that 47 actinobacterial isolates were obtained from rhizosphere soils of royal poinciana in the UAE, among which streptomycete actinobacteria were the predominant isolates. Three isolates, *Streptomyces rochei* UAE2, *S. coelicoflavus* UAE1 and *S. antibioticus* UAE1, exhibited powerful *in vitro* antifungal activity against *N. dimidiatum*. The antifungal action of *S. rochei* and *S. coelicoflavus* was mainly correlated with antibiosis and cell-wall-degrading enzymes production, respectively. *S. antibioticus* was, however, associated with both mode of actions. Using the novel apple fruit bioassay, these isolates suppressed lesion development on fruits inoculated with *N. dimidiatum*. Under greenhouse conditions, each of the potential Biological Control Agents (BCAs) showed greater efficacies against stem canker when applied before *N. dimidiatum* inoculation (preventive) than those when the same BCAs were applied after the pathogen (curative) or the pathogen alone (control). The curative effects of

Cidely® Top (a chemical fungicide) and *S. antibioticus* on disease symptoms were comparable on royal poinciana. Prior to pathogen inoculation, *S. antibioticus* was capable to suppress disease symptoms and prevent the pathogen spread, suggesting a relative superiority of *S. antibioticus* over all other chemical and biological treatments tested. In conclusion, the multiple mode of actions in *S. antibioticus* can be particularly effective to produce synergistic actions against the fungus. This study is the first to explore the potential to use both biocontrol and fungicides to further develop an integrated disease management strategy against stem canker disease in royal poinciana.

Keywords: Chemical fungicide, *Delonix regia,* Stem canker, UAE, Actinobacteria, *Neoscytalidium dimidiatum*.

Title and Abstract (in Arabic)

المقاومة الكيميائية و البيولوجية لمرض تسوس اشجار البونسيانا والذى يسببة فطر نيوسكايتليديوم دايميتديوم

الملخص

فى دولة الإمارات العربية المتحدة، تعاني أشجار البوينسيانا من مرض تسوس السيقان والاغصان. وتشمل اعراض المرض تقرح الساق مع ظهور جفاف فى الافرع والأوراق مع تغير لون أنسجة الخشب. ويصاحب المرض ايضا حدوث تسوس ونخر فى انسجة الخشب واللحاء ويظهر فى جميع الحالات افراز كميات من المواد الصمغية. وتؤدى الاصابة ايضا الى تساقط أوراق الاشجار بالكامل وفي النهاية موت الأشجار في مراحل متقدمة. تم عزل الفطر المسبب للمرض من انسجة الاشجار المصابة وتم تعريف الفطر باستخدام الطرق الميكروسكوبية وباستخدام التحليلات الوراثية وثبت انتمائه الى جنس نيوسكايتليديوم دايميتديوم. ومن اجل مقاومة الفطر وللقضاء على مرض تسوس اشجار البونسيانا، تم استخدام الطرق الكيمائية والطرق البيولوجية للقضاء على هذه الافة الخطيرة. فيما يخص الطرق الكيمائية باستخدام المبيدات القاتلة للفطريات، اثبتت الدراسة نجاح المبيدات سيدلى توب وايمستار توب وبروتيفرت على تثبيط نمو الفطر بشكل كبير ومعنوي مع الاقلال من اعداد جراثيم الفطر الممرض على اشجار البوينسيانا و ذلك فى التجارب والتي تم عملها داخل البيوت البلاستيكية. ولقد اثبتت الدراسة ايضا ان افضل هذه المبيدات الثلاثة هوالمبيد الفطرى سيدلى توب والذي نجح ايضا فى القضاء على المرض عند استخدامه على اشجار مصابة بالمرض فى الحدائق العامة فى الامارات العربية المتحدة. وفيما يخص بالمقاومة البيولوجية اثبتت الدراسة الحالية أيضًا أنه تم الحصول على 47 عزلة من الاكتينوبكتريا من التربة المحيطة بالجذور لاشجار البوينسيانا. أظهرت ثلاث عزلات تتبع جميعا جنس ستربتوميس وتم تعريفها كالتالى ستربتوميس رويشيى وستربتوميس كوليسوفلافس و ستربتوميس انتيبوتيكيس والتي اثبتت فعالية كبيرة فى قتل الفطر المسبب للمرض. كان السبب الرئيسى لتثبيط نمو الفطر هو افراز مضادات حيوية فقط فى جنس ستربتوميس رويشيى وافراز انزيمات محللة لجدران الفطر الممرض فقط فى جنس ستربتوميس كوليسوفلافس، ولكن لوحظ ان جنس ستربتوميس انتيبوتيكيس كان له القدرة على افراز مضادات حيوية وانزيمات محللة لجدران الفطر فى ان واحد. اثبتت الدراسة نجاح كبير فى الحد من المرض وذلك عند استخدام الثلاث عزلات داخل البيوت البلاستيكية واثبتت الدراسة ان العزلة الافضل وهي جنس ستربتوميس انتيبوتيكيس والذي قام بعمل اكثر من ميكانيكية لمقاومة الفطر الضار والتي اعطت

نتيجة فعالة وايجابية فى الحد من المرض، وكانت النتيجة مشابهة لحد كبير من استخدم المبيد الفطرى سيدلى توب. و بالتالي، توصي الدراسة بامكانية استخدام المقاومة البيولوجية كبديل قوي و فعال عن استخدام المبيدات الكيميائية. تعتبر هذه الدراسة هي الأولى من نوعها فى إمكانية استخدام كل من المكافحة الكيميائية والمقاومة البيولوجية لتطوير استراتيجية متكاملة لإدارة المرض ضد مرض تسوس اشجارالبوينسيانا مع الوضع فى الاعتبار افضلية استخدام المقاومة البيولوجية الصديقة للبيئة.

مفاهيم البحث الرئيسية: مبيدات الفطريات الكيميائية، البونسيانا، سرطان جذع النبات، دولة الإمارات العربية المتحدة، اكتينوبكتيريا.

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My special thanks are extended to my family, my friends and my country who supported me along this way.

Dedication

To my lovely family, myself and for my friends

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List of Abbreviations

°C	Degree Celsius
16 SrDNA	16S Ribosomal DNA
16 SrRNA	16S Ribosomal RNA
ACCD	1-Aminocyclopropane-1-Carboxylate Deaminase
Ag@Cs	Chitosan-Silver Nanoparticles
AL	Airone Liquido®
ANOVA	Analysis of Variance
AT	Amistar Top®
BCAs	Biological Control Agents
Ca	Calcium
CaCo3	Calcium Carbonate
Cd	Cadmium
Cm	Centimeters
CS	Chitosan
CT	Cidely® Top
Cu	Copper
d.	Day
D. regia	*Delonix Regia*
DFC	Dubai Festival City
DNA	Deoxyribonucleic Acid
dpi	Days Post Inoculation
dpi/wpi	Days/Weeks Post Inoculation
E.coli	*Escherichia Coli*
Fe	Iron

G+C	Guanine and Cytosine
h	Hours
H2So4	Sulfuric Acid
IAA	Indole-3-Acetic Acid
IDM	Implement integrated disease management
ISR	Induced Systemic Resistance
ITS	Internal Transcribed Spacer
JA	Jasmonic Acid
K	Potassium
Kg	Kilogram
M	Meter
MB	Methylene Blue
Mc	Colony Diameter (in mm) of Control Set
Mg	Magnesium
mg/L	Milligrams Per liter
MgCl2	Magnesium Chloride
Mi	Inhibition of the Mycelial Growth
Mi%	Growth Inhibition Rate
ML	Maximum Likelihood
Mm	Millimeters
Mn	Manganese
Mol%	Mole Percent
Mt	Colony Diameter (in mm) of the Target Fungus on the Medium with Fungicide
N. dimidiatum	*Neoscytalidium dimidiatum*
Na	Sodium
NaOCl	Sodium Hypochlorite

Ni (II)	Nickel(2+)
NSA	Non-*Streptomyces* Actinobacteria
NY	New York
ON	Ontario
P	Phosphorus
Pb	Lead
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PGPR	Plant Growth-Promoting Rhizobacteria
pH	Denoting Potential of Hydrogen
PPM	One Part Per Million
Pr	Protifert®
PR	Pathogenesis-Related Proteins
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SAS	Statistical Analysis System
SD	Standard Deviation
SDS	Sudden Decline Syndrome
TEF1-α	Translation Elongation Factor *1-a*
UAE	The United Arab Emirates
UK	United Kingdom
USA	United States of America
Vitamin E	RRR-alpha-tocopherol
VOCs	Volatile Organic Compounds
wpi	Weeks Post Inoculation

wpt	Weeks Post Treatment
Zi	Zineb
Zn	Zinc
μm	Micrometer

Chapter 1: Introduction

1.1 Overview

Chemically synthesized fungicide and fertilizers are usually composed of different chemicals compounds and minerals like potassium, phosphorus, and nitrogen were getting applied widely by many farmers for extend their harvest of their plants crop, but on the other hand their overabundant utilization has adversely affected the environment in direct and indirect way by inflicting the pollution to the soil, water and air, which can result in effect the agriculture sustainability (Wang *et al.,* 2020). One way for achieving the sustainable agriculture can be achieved by using a bio-based fungicide and fertilizers composed of living microorganisms and natural compounds rather than the agro-chemicals fungicide and fertilizers. Plants rhizosphere are hosting a wide diverse range of microorganism, and these plant-associated microorganisms could be classified into deleterious, beneficial and neutral microorganism according to their consequence on the plant health and growth. A useful bacterium that promotes the growth of the plant is called Plant Growth Promoting Rhizobacteria (PGPR) that affect the crop health as well as its growth in directly and indirectly way. The indirect promotion take place when PGPR decreases the harmful effects of phytopathogenic organisms or when Induced Systemic Resistance (ISR) occurred, while the direct promotion entails providing the plant with a compound that's synthesized by the PGPR, for example phytohormones like cytokinin, auxin, gibberellins and siderophore which facilitating the uptake of particular nutrients from the surrounding environment, like, phosphate solubilization, nitrogen fixation, and iron chelation (Wang *et al.,* 2020).

Streptomyces comprise a great clade of the phylum Actinobacteria, these filamentous prokaryotes are in the earth and are usually take place within the rhizosphere or plant roots. *Streptomyces* are famed to make secondary metabolites, which they take major place on the clinically antibiotics. The applications of *Streptomyces* in agriculture are principally considered as PGPR and as Biological Control Agent (BCA) or biocontrol agents, many BCA from *Streptomyces* are already being marketed. However, the promotion effects of *Streptomyces* on plant growth have not been adequately studied, some studies proved that *Streptomyces* promote plant growth by producing 1-AminoCyclopropane-1Carboxylate Deaminase (ACCD), siderophores and Indole-3-Acetic Acid (IAA) to scale back stress in plants. *Streptomyces* reside in the rhizosphere and react with different microorganisms community; however, very little information are understood especially on their interaction with the microbial community (Wu *et al.,* 2019; Wang *et al.,* 2020).

1.2 Botanic Description of *Delonix regia (D. regia)*

Delonix regia (*D. regia*) or as its generic name *'Delonix'* or (Royal Poinciana) had a several standard terms that derived from its enormous, flame-red flowers. In Arabic, it is called (Poinciana), while in English it is called (royal poinciana, flamboyant flame tree, peacock flower and flamboyant) and its trade name is (gold mohar) (Orwa *et al.,* 2009; Hosny, 2012).

D. regia is belong to the family Fabaceae (*Leguminosae*), and the subfamily Caesalpiniaceae, it is regarded as a fascinating decorative medium-sized tree with a broad slightly flat or bent crown and with fern-like leaves, its leaves are alternate, bipinnately combined, having ten to twenty pairs of significant leaflets and its fruits are 30 to 50 centimeters (cm) long, this pattern is found in three Malagasy genera

Delonix species (*D. regia, D. velutina and D. tomentosa*). In non-Malagasy species, the trees have umbrella-shaped trees reaching up to 12.20 meters high and with big red-orange flowers (Puy *et al.,* 1995; Jahan *et al.,* 2010; Suhane *et al.,* 2016).

This beautiful tree is currently widespread in both tropical and subtropical areas. It has been widely planted as avenue tree in almost every dampish and dried region, the genus *Delonix Raf.* consists of eleven species, nine of them are considered endemic to Madagascar. One of them which is (*D. elata (L.) Gamble*) is widespread in the geographical region of East Africa, Arabia and India and another one its (*D. baccal (Chiov.) Baker f.*) which is endemic to the northern Republic of Kenya and the African nation. The variation within the genus *Delonix* is found within the following characteristics: habit, trunk and bark characteristics; leaflet numbers and size; inflorescence position; petal size and colour; indumentum type and distribution and the degree of woodiness, size and form of the pods (Puy *et al.,* 1995).

1.2.1 Habit, Trunk and Bark

Generally, genus *Delonix* species are lithe trees with sort of umbrella-shaped crowns at maturity. It has slender trunks that often fork low down and have delicate grey bark (Rahman & Ahmad, 2010).

Some species, such as *D. regia*, have a slender trunk that often embranchment low down and has smooth grey bark. In contrast, others have swollen "cigar" formed trunks, constricted at the bottom, swollen higher than and tapering and branched solely at the top, with a 10-15 (max. 18) m high, massive trunk, soft bark with greyish-brown color and generally slenderly split and with many dots (lenticels); inner bark are brownness this characteristic is sometimes related to thin bark that flakes or peels off in papery fragments or strips, typically seasonally (Puy *et al.,* 1995).

1.2.2 Leaflet Number and Size

D. regia possess bipinnate leaves with abundant pinnae every bearing several tiny leaflets and with light green, feathery, 20 to 60 (cm) long; 10 to 25 leaflet pairs, 5-12 cm tall, each carrying 12-40 pairs of tiny oblong leaflets (Orwa *et al*., 2009).

1.2.3 Inflorescences

D. regia considers as a deciduous tree, the inflorescences of all species of genus *Delonix* are axillary racemes. These are commonly created at many consecutive nodes throughout the period, the lower racemes tending to be longer with the flowers ahead higher than the foliage, *D. regia* starts blossoms in its 4th or 5th years. In India, *D. regia* remain leafless between March and May and sometimes until June. In Egypt, the first leaves and flowers are mostly in April and last for several months. On the other hand, the flowering season of the United Arab Emirates is starting between May to July (Panga, 2014; Suhane *et al.,* 2016). In a humid area, before flowering season the tree starts to develop young foliage and doesn't prolifically bloom (Puy *et al.,* 1995). Planting the trees on dry land thereby promotes abundant flora. Every tree seems to follow its rhythm of leaves and flowering in regions with ample precipitation. Flowers are bisexual (Orwa *et al.*, 2009).

1.2.4 Calyx

All species of genus *D. regia* have subequal, valvate calyx, that is leathery and thickened, particularly towards the tips (Orwa *et al.*, 2009).

1.2.5 Petals

Most species of genus *Delonix* have flowers with five big, spreading petals, consisting of a somewhat rounded or ovate blade and a close claw, on thin stems 5-7.60 cm long. The Petals 2-3 cm wide orbicular and 5-6.50 cm long, rounded but wider than long, mostly spoon formed; petal margins are crisped and frilled when extended, sunbirds pollinate bright red-and-white flowers of *D. regia*. The white higher floral leaf also fades to pale yellow in this species. Corymbs 15-30 cm long, borne laterally close to the tip of the twig, with loose fit and little bit sweet-scented blossoms (Orwa *et al.*, 2009).

1.2.6 Sepals

Thick green outside, cherry-red with a yellow edge inside that reflects when the blossoms open, sharply, furry, about 2.50 cm long (Orwa *et al.*, 2009).

1.2.7 Stamens

All of the genera have spreading stamens, that are not clustered around the ovary, with slender custom almost 3 cm long (Orwa *et al.*, 2009).

1.2.8 Pods and Seeds

The pods of most Malagasy species of genus *Delonix* are big, linear rectangular, somewhat flattened, woody and pendulous, containing several rounded, pale brown, finely spotted seeds in individual chambers. *D. regia* has elongated, strap-shaped pods with additional varied, elongate-ellipsoidal seeds, pods of the previous year of *D. regia* tree are held till wind currents bring it wherever they rotate on the ground and release the seeds (Puy *et al.,* 1995; Rahman & Ahmad, 2010).

Seeds are the most commonly propagate way, seeds usually take longer time to start germinating, and it should be with specific characteristics such as it should be hard woody testa. Seeds can remain dormant in the soil without germination for up to 2-3 years, and it typically takes 12-349 days to start the germination process. To split this dormant seed, it can be cooked in hot water for at least one day and then grown in sunny pets, and germinates within a maximum of 10 days. Seedlings grow rapidly and later when it is reached 20-25 cm in height it can be replanted. Natural regeneration is very common. It can also be propagated by cutting process (Dauthal & Mukhopadhyay, 2016a; Wang *et al.,* 2016).

1.2.9 Roots

D. regia own a shallow-root superficial system that effectively competes with neighboring sapling and plants and makes the soil bare under its canopy. So that it must be planted far from other plants (Orwa *et al.*, 2009).

1.2.10 Fruits

When young, the fruits are flabby, turning into brown, tough and wooden pods, 30-75 cm long, 3.80 cm thick. This finished into a short beak when maturing, with several horizontally separated seed chambers within, rip finally into two elements (Puy *et al.*, 1995).

1.2.11 Distribution

D. regia is native to Madagascar and Zambia; however, currently it is considered a scarce tree in its native environment in Madagascar. Most of the Malagasy species were initially represented within the genera *Poinciana L.* or

Aprevalia Baill (Puy *et al.*, 1995). Exotic in many countries such as India, Brazil, Nigeria, Ethiopia, Burkina Faso, South Africa, United States of America, Egypt, Cyprus, Sudan and Tanzania. Although it was widely cultivated in the tropics since the 19th century, the scientist did not know the native habitat of the trees until the 1930s, thanks to the Botanist Wensel Bojer who discovers it, in its native Madagascar in the early nineteenth century (Puy *et al.,* 1995; Rahman & Ahmad, 2010; Orwa *et al.*, 2009; Hosny, 2012).

1.2.12 Suitable Soil Type for Cultivation

The *D. regia* species seems to adapt to a wide variety of clay and sandy soils; however, the *D. regia* prefers sandy soils most (Puy *et al.,* 1995; Rahman & Ahmad, 2010; Orwa *et al.*, 2009).

1.3 Products of *D. regia*

D. regia had a treasure trove of benefits, for example, there are many therapeutic uses that *D. regia* tree can be included on it. Concerning the chemical studies of this plant, several threptic, economic and environment important compounds could be isolated from different parts of this tree (Shabir *et al.,* 2011; Hosny, 2012; Salem, 2013; Suhane *et al.,* 2016; Pallavi, 2017).

In every blessed *D. regia* tree parts, there is a unique benefit and secretes for the environment, to write about every part its need separate section to give it its right.

1.3.1 Economical Uses of *D. regia*

D. regia could be contributed to the economic fields as it has many benefits end-products, for example., in a study where *D. regia* were compared with

Peltophorum pterocarpum (*Caesalpinioideae*) on the properties and also the compositions of the oils from *D. regia* and *Peltophorum pterocarpum*. The fatty acid distribution, proximate composition, chemical characteristics and levels (ppm) of selected trace metals (Mn, Cu, Pb, Fe and Zn) and macronutrients (K, Mg, Na and Ca) were determined within the seeds and seed oils. They found that *D. regia* includes a higher carbohydrate content (39.50%) than *Peltophorum pterocarpum* (38.30%). The crude protein is high, 45.20% for *D. regia* and 44.10% for *Peltophorum pterocarpum*. Neutral lipids were the predominant lipid part in oils with linoleic as the most ample fatty acid in both the oils. The physicochemical characterization, as well as the fatty acid profile of the oils, suggests an excellent industrial application of these oils (Adewuyi *et al.,* 2010).

On another study, they found that the seeds have many valuable nutrients such as minerals entails Na, Fe, Zn, Mn, Cu, K, P, Mg, and Ca, amino acids which are Glutamic acid and methionine, many vitamins like vitamin E, fatty acids like linoleic, palmitic, stearic oleic and ricinoleic acids and it was liquid at room temperature, and have sweet-smelling. Vitamin E and triacylglycerols accounted for the highest contributions to vitamins and acylglycerols, respectively, in the oil. These results showed that the seeds were nutritive and useful for both nutrition and industrial uses (Oyedeji *et al.,* 2017).

The fuel products that manufactured from the large pods and the tree bark as the wood is used for fuel in many places. On the other hand, the tree makes dense water-soluble mucilage of gum in a reddish brown or yellowish tear, the seeds produce gum that is ideal in the textile and food industries. The gum extracted from the dried seeds can be used in the production of the medical tablets as a binder. The heartwood is light brown, and the sapwood is pale yellow, it is thick, soft, weak, that if it has a

good finish, it will be durable and water resistant. The sapwood will also have an insect and moisture resistance, although it is highly suspected of being targeted by dry-wood termites, it is ideal for wood industry applications. Also, the long and hard seeds could be used as beads. The seeds contain many kinds of fatty oil that is used on industry (Orwa *et al.*, 2009; Panga, 2014; Suhane *et al.,* 2016).

It is planted as an ornamental tree in parks and streets in the subtropical and tropical regions. It is also an excellent tree for managing soil erosion in the semi-arid and arid regions. *D. regia* is helpful for the honey trade, as the flowers had a reputed produce bee forage (Orwa *et al.*, 2009; Panga, 2014; Suhane *et al.,* 2016).

Biogenic synthesis is a good, nontoxic and eco-friendly chemistry approach to synthesize metal nanoparticles which have many important properties and applications in numerous fields range between commercially and pharmacologically, important products are explored within the production of nanoparticles specifically silver nanoparticles for multiple uses in physics, medicine, and biological science. In research, they approve that with the help of *D. regia* leaf aqueous extract as bio-reducing and capping agent that could process Green synthesis of silver nanoparticles AgNPs which show anticancer activity and this protocol consider as environment-friendly protocol (Siddiquee *et al.,* 2020).

Antioxidants, carotenoids and their derivatives, as well as other phytochemicals including tannins and saponins, were also found in *D. regia* flowers (Pinakin *et al.,* 2020). In one study, they examine the opportunity to improve the oxidative stability of sunflower oil by enhancing it with carotenoids from the petals of *D. regia* flowers, and they found that with the enrichment of sunflower oil with carotenoid-rich extract the oil it is show improved in its oxidative stability by more than 50%. Therefore, the carotenoids extracted from the petals could be used as a

natural antioxidant in pharmaceuticals and in the food industry. These results propose that the investigated extract has the potential to be used as bio-preservative in food products as well (Table 1) (Golshany, 2019).

Table 1: Economical uses of *Delonix regia*

D. regia part	Application	References
Seed	In paracetamol tablets, the gum derived from the dried seeds can be applied as a binder.	(Adetogun & Alebiowu, 2009)
	Seeds had gum which is suitable for both food and textile industries used.	(Orwa *et al.*, 2009; Panga, 2014; Suhane *et al.,* 2016)
	Hard seeds are used as beads.	(Orwa *et al.*, 2009; Panga, 2014; Suhane *et al.,* 2016)
	Many types of fatty oil used in nutritional, industrial and pharmaceutical uses.	(Orwa *et al.*, 2009; Panga, 2014; Suhane *et al.,* 2016; Oyedeji *et al.,* 2017; Adejumo & Agboola, 2019)
Pods	Fuel products.	(Orwa *et al.*, 2009; Panga, 2014; Suhane *et al.,* 2016)
Bark	Fuel products.	(Orwa *et al.*, 2009; Panga, 2014; Suhane *et al.,* 2016)
	Wood industries.	(Orwa *et al.*, 2009; Panga, 2014; Suhane *et al.,* 2016)
Leaves	Leaf aqueous extract as bio-reducing and capping agent they could process Green synthesis of silver nanoparticles AgNPs.	(Siddiquee *et al.,* 2020)

Table 1: Economical uses of *Delonix regia* (Continued)

D. regia part	Application	References
Flowers	Produce bee forage.	(Orwa *et al.*, 2009; Panga, 2014; Suhane *et al.,* 2016)
	Antioxidants, carotenoids and other phytochemicals including tannins and saponins.	(Golshany, 2019; Pinakin *et al.,* 2020)
Tree	Plant as an ornamental tree in parks and streets in the subtropics and tropics area.	(Orwa *et al.*, 2009; Panga, 2014; Suhane *et al.,* 2016)
	Regulate the degradation of soil in semi-arid and arid regions.	(Orwa *et al.*, 2009; Panga, 2014; Suhane *et al.,* 2016)

1.3.2 Threptic Uses of *D. regia*

Mechanical properties and dissolution of paracetamol tablets prepared with *D. regia* seed gum as binder was tested, the use of the *D. regia* seed gum as a binder showed it is advance the balance between the binding and the disintegration properties of tablets if a quicker disintegration is required (Adetogun & Alebiowu, 2009).

The phytochemical investigation of the roots of *D. regia* yielded many compounds that showed great results for their antioxidant activities and cytotoxic activities against human promyelocytic leukaemia and human monocytic leukaemia (Hosny, 2012). Another study was carried out to assess its pharmacogenetic and antibacterial activity, using Gram-positive and gram-negative strains. The results showed significant antibacterial activity (Sama *et al.,* 2012). In a study where they test the effects of root extracted as a potential method against many types of harmful bacteria for human. The study was aimed at evaluating and comparing the antimicrobial spectrum of methanolic extract of leaves, roots, and a stem of *D. regia*.

Its antibacterial activity test was performed by using gram-negative bacteria *Escherichia coli (E.coli)* and Gram-positive bacteria *Staphylococcus* aureus by agar well diffusion method with Dimethyl sulfoxide as standard, it was evaluated by measuring the zone of inhibition in millimeters (mm), and they concluded that *D. regia* methanol extract had antibacterial activity, and it is effective against *E. coli* and *Staphylococcus*. It also indicates that the methanolic extract of the plant is potentially useful for the therapy of antibacterial-resistant bacteria (Pallavi, 2017). The root extract is not considered as new founding as the root of *D. regia* used in many traditional remedies against abdominal pain since a long time ago (Aye *et al.,* 2019).

Medicament and inhibitor activities of the methanolic extracted from *D. regia* barks were evaluated, and it showed it could be valuable for useful applications against harmful microorganism, the literature indicates that *D. regia* bark produces high concentrations through bark, flora and leaves from the ß-sitosterol, saponins, alkaloids, carotene, hydrocarbons phytotoxins and phenolic compounds. *D. regia* flowers have a wide variety of antibacterial and antifungal activities. Bark and flora have both been anti-inflammatory activities (Salem, 2013).

Besides its brilliant look, several ancient medications were made from *D. regia* tree. In many African countries it is utilized in the traditional medicine of anti-diarrheal, anti-constipation, inflammation, arthritis, hemiplagia, leucorrhoea, joint pains, flatulence, rheumatism also it is used as pill binder and anti-malarial (Sama *et al.,* 2012), *D. regia* were also used as traditional medication in many Asian nations as it is used as anthelmintic, diuretic, astringent, leucorrhoea and as antimicrobial and toxic activities of the various extractives (Jungalwala & Cama, 1962; Jahan *et al.,* 2010). Also, it has employed in many countries to arrange extracts with antimicrobial and antifungal properties (Hosny, 2012). *D. regia* leaf consumption is employed in

Bangladesh as they used it for the treatment of diabetes, and there is a study supported their traditional herbal medicine, this study was carried out to evaluate the possible glucose tolerance efficacy of methanolic extract of *D. regia* leaf using glucose-induced hyperglycemic mice. The statistical data indicated significant oral hypoglycemic activity on glucose-loaded mice at every dose. The methanolic extract of leaf of *D. regia* had substantial effects in reducing the elevated blood sugar level of hyperglycemic mice (Rahman *et al.,* 2010). Hepatocellular carcinoma is considered the third most common cause of cancer-related death worldwide. A study was designed to test the putative cytotoxic effect of *D. regia* besides its hepatoprotective activity, and they concluded that the extract of *D. regia* leaves possessed a significant anticancer impact, hepatoprotective and antioxidant activities due to the availability of flavonoids content (Azab *et al.,* 2013). In a study, where the research focus in the phytochemical, as the phytochemical analysis revealed the existence of flavonoids, phenols, alkaloids and carbohydrate. The research concluded that the crude extract of *D. regia* leaves and flowers extract is a rich source of secondary phytoconstituents which impart significant antioxidant potential. It is expected that the essential phytochemical properties recognized by this study in the indigenous medicinal plants will be beneficial in the curing of various diseases when taken along with the food (Aye *et al.,* 2019; Bhorga & Kamle, 2019). Leaves are used by traditional practitioners also, in cases of inflammatory joint disorders as a folklore remedy; also, leaves are used as anti-inflammation and antibacterial activity (Aye *et al.,* 2019).

Regarding the inflammatory activity for the *D. regia* leaves, in laboratory work done on Wistar albino rats (150–200 g) and mice (20–25 g), where the aim was to evaluate the anti-inflammatory activity of *D. regia* leaves, the ethanol that extracted from *D. regia* leaves was exhibited significant anti-inflammatory activity in their

research (Shewale *et al.,* 2012). From ethanolic flower extract of *D. regia* two separate amino acids, three sterols and four flavonoids were isolated. The ethanolic extract was tested for hepatoprotective activity against induced hepatic cell damage in rats. They suggest the use of the ethanolic extract of the flowers of *D. regia* as a chemo-preventive agent against the two leading causes of liver cell damage; liver toxicity by chlorinated agents and liver cancer (El-Sayed *et al.,* 2011). Many pharmaceutical companies now days depend on antioxidants substances as a main pharmaceutical compound, in scientific research, they show results suggest that the extract of ethyl acetate from the flowers of *D. regia* possesses significant antioxidant activity. The compounds are found to be ideal candidates for antioxidant therapy (Ramakrishnan, 2018). Also, many studies confirmed that *D. regia* flowers are packed with phytochemicals such as flavonoids, anthocyanins, phenolic acids, carotenoids, tannins and saponins and it is possessing various health benefits such as antioxidant, anti-inflammation, anticancer, antidiabetic, hepatoprotective and antimicrobial (Adejumo & Agboola, 2019; Aye *et al.,* 2019). Golshany (2019) extracted carotenoids from the petals of *D. regia* and assumed that it can be used as a natural inhibitor in pharmaceutical and in the food business as bio-preservative in the food product (Golshany, 2019). The using of *D. regia* flowers are not a new science, as there are many traditional herbal remedies for gynecological disorders that used *D. regia* flowers as treatment, and they are also used as a tablet binder; and for joint pains and in flatulence (Aye *et al.,* 2019).

Concerning the chemical studies of this plant, many compounds like flavonoid glycosides, anthocyanins, coumarins, fatty acids, sterols, triterpenes, condensed tannins, protein, amino acids as well as lectins are all isolated from entirely different parts of the *D. regia* (Table 2) (Hosny, 2012).

Table 2: Threptic uses of *Delonix regia*

D. regia part	Application	References
Seed	Antibacterial activity of ethanol extracts, antifungal potential and seeds which contain flavonoids are used as a wound-healing agent in households.	(Aye *et al.,* 2019)
Roots	Phytochemical isolated compounds showed great results of positive activity for their cytotoxic activities against human promyelocytic leukaemia and human monocytic leukaemia. The antioxidant activities were also found. The antimicrobial spectrum of methanolic extract against *E. coli* and *Staphylococcus*. Traditional remedies against abdominal pain.	(Hosny, 2012) (Bhokare *et al.*, 2018) (Aye *et al.*, 2019)
Bark	Methanolic extracted, saponins, alkaloids, carotene, ß-sitosterol ,hydrocarbons phytotoxins, flavonoids, antibacterial, antifungal activity and anti-inflammatory activity. The antimicrobial spectrum of methanolic extract from the stem against *E. coli* and *Staphylococcus*.	(Salem, 2013) (Bhokare *et al.*, 2018)
Leaves	Methanolic extracted, saponins, alkaloids, carotene, ß-sitosterol, hydrocarbons phytotoxins, flavonoids, antibacterial, antifungal activity and anti-inflammatory activity. Antibacterial activity of ethanol extracts and antifungal potential	(Salem, 2013) (Aye *et al.*, 2019)

Table 2: Threptic uses of *Delonix regia* (Continued)

D. regia part	Application	References
Leaves	The crude extract of flavonoids, phenols, alkaloids and carbohydrate is a rich source of secondary phytoconstituents which impart significant antioxidant potential.	(Bhorga & Kamle, 2019)
	In Bangladesh methanolic extract used for the treatment of diabetes glucose-induced hyperglycemic mice.	(Rahman *et al.,* 2010)
	Ethanol extract show anti-inflammatory activity on Wistar albino rats.	(Shewale *et al.*, 2012)
	In cases of inflammatory joint disorders as a folklore remedy; also, leaves are used as anti-inflammation and antibacterial activity.	(Aye *et al.*, 2019)
	The antimicrobial spectrum of methanolic extract against E. coli and Staphylococcus.	(Bhokare *et al.*, 2018)
Flowers	Carotenoids and in many Asian nations used as diuretic, anthelmintic, astringent, in leucorrhoea, antimicrobial and toxic activities of the various extractives.	(Jungalwala & Cama, 1962; Jahan *et al.,* 2010; Golshany, 2019)
	Phytochemicals such as flavonoids, anthocyanins, phenolic acids, carotenoids, tannins and saponins.	(Adejumo & Agboola, 2019)
	Antioxidant, anti-inflammation, anticancer, antidiabetic, hepatoprotective and antimicrobial.	(Adejumo & Agboola, 2019)

Table 2: Threptic uses of *Delonix regia* (Continued)

D. regia part	Application	References
Flowers	Ethanolic extract used as chemo-preventive agent against liver disease.	(El-Sayed *et al.*, 2011)
	Methanolic extracted, saponins, alkaloids, carotene, ß-sitosterol ,hydrocarbons phytotoxins, flavonoids, antibacterial, antifungal activity and anti-inflammatory activity.	(Salem, 2013)
	Isolation of three sterols and four flavonoids in addition to two different amino acid and ethanolic extract have positive against induced hepatic cell damage in rats.	(El-Sayed *et al.,* 2011; Azab *et al.,* 2013)
	Traditional herbal remedies for gynecological disorders treatment, and they are also used as a tablet binder; and for joint pains and in flatulence.	(Aye *et al.*, 2019)
	The extract of the ethyl acetate from the flowers of *D. regia* possesses significant antioxidant activity.	(Ramakrishnan, 2018)
	The crude extract of flavonoids, phenols, alkaloids and carbohydrate is a rich source of secondary phytoconstituents which impart significant antioxidant potential.	(Bhorga & Kamle, 2019)

Table 2: Threptic uses of *Delonix regia* (Continued)

D. regia part	Application	References
Tree	Antimicrobial agent for human.	(Hosny, 2012)
	Antifungal agent for human.	(Hosny, 2012)
	Flavonoid glycosides, anthocyanins, coumarins, fatty acids, sterols, triterpenes, condensed tannins, protein, amino acids as well as lectins are all isolated from entirely different parts of the *D. regia.*	(Hosny, 2012)
	In the traditional medicine of many African countries, as anti-diarrheal, anti-constipation, inflammation, arthritis, hemiplagia, leucorrhoea, joint pains, flatulence, rheumatism, also it is used as pill binder and anti-malarial.	(Sama *et al.*, 2012)
	Anticancer impact, hepatoprotective and antioxidant activities due to the presence of flavonoids content.	(Azab *et al.,* 2013)
	Antibacterial properties against a wide variety of both positive and negative gram strains.	(Sama *et al.,* 2012)

1.3.3 Environmental Uses of *D. regia*

Pyrolyzed *D. regia* pod carbon was used for adsorbent and removal of excess fluoride ions from contaminated water, the adsorbent was characterized both physically and chemically, the physicochemical properties and textural characters were analyzed, the results showed that pyrolyzed *D. regia* pod carbon is capable of maintaining high fluoride adsorption over an acidic pH range, so it will be useful in removing fluoride ion from polluted water or wastewater of lower pH. Hence, pyrolyzed *D. regia* pod carbon, a waste material from *D. regia* tree, will be a promising

adsorbent for fluoride removal (Patil & Shrivastava, 2010; Okoronkwo *et al.,* 2013; Angelina & Rajagopal, 2015). Also, it was used as a source of producing activated carbon to be used in waste-water treatment for removing organic and inorganic pollutants. In a research work, they addressed the development and utilization of sugarcane baggasse, waste paper and *D. regia* seed and pod derived adsorbents for the abatement of methylene blue from the aquatic environment (Dauthal & Mukhopadhyay, 2016a; Wang *et al.,* 2016). *D. regia* pods and leaves could be used as an environmentally friendly agent for many reasons, it is supports heavy metal elimination such as Pb, Ni, Cu and Cd from the polluted water and its play an excellent role in remediating the contaminated soils (Babalola, 2018).

D. regia can be considered as a great source of producing biodiesel, the researchers have made their best efforts to generate biodiesel from different edible and non-edible plant oil, oils residues and fats from animals' sources using a variety of tactics for the production of economically utilizable biodiesel, one of the sustainable, biodegradable alternatives energies for energy from fossil fuels. It had the benefit of a greater thermal stability and it is more safe for distribution, as well as being physically close to petro-diesel, and compatible with diesel engines (Callegari *et al.,* 2020; Karmakar, Ghosh *et al.,* 2020; Karmakar, Samanta *et al.,* 2020). In another research they conclude that low saponification value and higher molecular weight will make *D. regia* seed oil suitable for use as biodiesel feedstock (Adejumo & Agboola, 2019). Emissions of greenhouses gases are lower from *D. regia products* due to the low Sulphur content. There is a wide variety of feedstock and techniques for use in biodiesel processing. Karmakar, Ghosh *et al*. (2020) recorded research findings using carbonised and H2SO4 and *D. regia* pods were used for obtaining 97.04% return on

high-free Madhuca Indica fatty acids using methanol, in which raw *D. regia* pods were used as precursor material to produce carbon support (Karmakar, Ghosh *et al.,* 2020).

In another research, ß-sitosterol, epilupeol, lupeol, stigmasterol and P-methoxybenzaldehyde compounds were isolated from petroleum ether dichloromethane fractions of a methanolic extract of the stem bark of *D. regia* tree. The disc diffusion technique conducted antimicrobial screening of the various extracts and cytotoxicity (Jahan *et al.,* 2010).

Due to expanded risk connected with indiscriminate use of artificial herbicides, researchers are trying to find different weed management methods. In a study where it conducted to analyze *D. regia* aqueous, Ethyl acetate and lyophilized leaf extract against field bindweed (*Convolvulus arvensis L.)* and wheat (*Triticum aestivum L.).* It was concluded that lower to medium concentrations of *D. regia* leaf extract might be safely utilized for natural control of field bindweed in wheat, reducing reliance upon synthetic herbicides (Table 3) (Perveen *et al.,* 2019).

Table 3: Environmental uses of *Delonix regia*

D. regia part	Application	References
Seed	Seed pod derived adsorbents for the abatement of methylene blue from the aquatic environment. Biodiesel.	(Dauthal & Mukhopadhyay, 2016a; Wang *et al.,* 2016; Babalola, 2018). (Adejumo & Agboola, 2019)
Pods	Biodiesel. Pyrolyzed *D. regia* pod carbon was used for adsorbent and removal of excess fluoride ions from contaminated water. Removal of fluoride from aqueous solution.	(Karmakar, Ghosh *et al.,* 2020a; Karmakar, Samanta *et al.,* 2020) (Patil & Shrivastava, 2010; Okoronkwo *et al.,* 2013; Angelina & Rajagopal, 2015) (Angelina & Rajagopal, 2015)

Table 3: Environmental uses of *Delonix regia* (Continued)

D. regia part	Application	References
Bark	In another research, ß-sitosterol, epilupeol, lupeol, stigmasterol and P-methoxybenzaldehyde compounds were isolated from petroleum ether dichloromethane fractions of a methanolic extract of the stem bark of *D. regia* tree. The disc diffusion technique conducted antimicrobial screening of the various extracts and cytotoxicity.	(Patil & Shrivastava, 2010; Okoronkwo *et al.,* 2013; Angelina & Rajagopal, 2015)
Leaves	The silver nanoparticles were formed when the aqueous solution of leaf extract snatch the silver ion (Agþ) from the aqueous AgNO3 surroundings and reduce the ions into their corresponding pure elemental state and optimization and fabrication of gold nanoparticles (Au-NPs) with the help of various organic and phenolic compounds present in the extract.	(Dauthal & Mukhopadhyay, 2016a, 2016b; Wang *et al.,* 2016)
	Ethyl acetate and lyophilized used as weed management methods and leaf against field bindweed and wheat.	(Perveen *et al.*, 2019)
Tree	Heavy metal elimination such as Pb, Ni, Cu and Cd from the polluted water and contaminated soils.	(Babalola, 2018)
	Producing activated carbon to be used in waste-water treatment for removing organic and inorganic pollutants.	(Babalola, 2018)

1.4 Management of *D. regia* Tree

D. regia tree are planted in places where it has lower recommended amount of precipitation, as long as it is irrigated well. Pollarding is an acceptable method as *D. regia* is consider as a fast-growing tree. Good pruning is the key to a good design crown. The trees had a thin wood and shallow root systems, so they are likely to rip up and fall via heavy winds and severe storms. It is appear less appealing when the leaves are shed and their visible pods remain on the empty branches and nests of termites that

normally target this plant. The surface root system, often affecting sidewalks and walls, so some authorities identify *D. regia* as a tree that should not be planted (Orwa *et al.*, 2009; Panga, 2014).

1.4.1 Germplasm Management of *D. regia*

Seeds can be stored for a long time if an attack of insects is avoided, which can be achieved with the addition of ash to the seeds. Seed storage is recorded at room temperature for up to 9 years, and there are about 2000-3245 seeds/kg (Orwa *et al.*, 2009).

1.4.2 Pests and Diseases of *D. regia*

Poecilips sierralemensis larvaes and beetles made a bore in the pods to extract the seed. *D. regia* are vulnerable to shooting borers and are defoliated from time to time by caterpillars or insects (Orwa *et al.*, 2009).

The *Ganoderma lucidum* root rot attacks *D. regia*, especially in the rainforest regions of India, *Ganoderma tropicum* was increasingly isolated from a *D. regia* tree that not only parasite the tree but was relatively destructive and destroying the entire tree within1-5 years (Al-Bahry *et al*., 2005). An unknown *Ganoderma sp.* identified for attacking of *D. regia* seedlings in many countries (Al-Bahry *et al*., 2005; Fernando, 2008; Sujarit *et al.*, 2020). *Fusarium oxysporum* caused root rot in the northern Guinea region of Nigeria, with root and butt-rot disease marked by affected parts slowly enlarging and development of a thick, dark brown mycelial sheath all over the bases of infected trees. *Sphaerostilbe repens*, identified as root stinking, affects *D. regia*. Infection is caused by water based spore by root contact, reddish or dark brown rhizomorphs are formed under the root bark, the inner surface is bleached, and the

combined presence of the fungus and bacteria causes a strong odour (Orwa *et al.*, 2009; Witt & Luke, 2017).

Fungus, *Pleiochaeta setosa*, was recorded to cause wilted, discoloured of the leaves and growth brown mycelial mats on roots and basal stems, accompanied by death of *D. regia* in India. This attacks seedlings cotyledons and the leaves of young seedlings causing shriveling, death and shedding of the leave. The famous root red fungus *Armillaria mellea* is distributed worldwide and has a diverse variety of host systems, including *D. regia*. by making thick, white mycelia layer between the bark and the dead bark (Orwa *et al.*, 2009; Witt & Luke, 2017).

1.5 Stem Canker Disease of *D. regia*

D. regia species, especially under warm and moist conditions, are vulnerable to various diseases. Canker is infection caused by fungai with a non-specific symptom, caused by a number of plant pathogens and it cause most of mortality trees. It is considered as localized necrosis of the bark and cambium on stems, branches or twigs, often centered around a wound or branch stub (Elliott, 2015), canker disease could be linked to fungus *Neoscytalidium dimidiatum (N. dimidiatum)*. Stem canker was recorded in Oman on numerous trees, including *D. regia* (Elshafie & Ba-Omar, 2002). The signs of the branch wilt, dieback, canker, gummy, and deaths of infected trees can be immediately recognized as canker disease symptoms. In general stress factors such as heat and water stress may increase the intensity of the canker (Reckhaus, 1987; Elshafie & Ba-Omar, 2002). However, there have been no records of the causative agent of stem canker symptoms in *D. regia* or any other ornamental woody tree in the UAE until 2020 (Al Raish *et al.,* 2020).

1.6 *Neoscytalidium dimidiatum (N. dimidiatum)*

N. dimidiatum is a new genus in *Botryosphaeriaceae* and dematiaceous ascomycete, and has been documented in a wider range of woody plants and it is commonly consider as a plant disease in tropical countries. Symptoms of the disease include the branch wilt, decline and death of tree such as in citrus trees in Iran, while in India its caused leaf spot diseases and canker and dieback in Arizona of eucalyptus (Crous *et al*., 2006; Al-Bedak *et al.*, 2018).

1.6.1 Canker Disease and *N. dimidiatum*

Many research articles proved that there are many cases of canker disease in different plants linked with *N. dimidiatum*, many *D. regia* trees at Sultan Qaboos University campus get infected with *N. dimidiatum* and this was the first report of the fungus in Oman and in the world on *D. regia*. The strain of this fungus has not been found to cause human disease in Oman (Elshafie & Ba-Omar, 2002). In Malaysia during the year 2008 to 2009, stem canker has been found in all plantations of 40 dragon fruit (*Hylocereus polyrhizus*). On the other hand, A total of 40 *Scytalidium-like* fungus have been isolated and classified as *N. dimidiatum* based on morphology and Internal Transcribed Spacer (ITS) region sequences, which showed 99% similarity (Hawa *et al.,* 2009), *N. dimidiatum* is reported for the first time also in Australia associated with dieback of mango and common fig (Ray *et al.,* 2010). In July 2013, on pitahaya (*Hylocereus undatus*) in China the tree was attacked by *N. dimidiatum* according to morphological characteristics and phylogenetic analysis of ITS sequences (Cao *et al.,* 2018), in the same year *Ficus nitida* and *Ficus benjamina* were recorded in Assiut Governorate, Egypt for the first time in September 2013. Morphological analysis and the sequence of DNA have shown that the causative agent was *N.*

dimidiatum (Al-Bedak *et al.,* 2018), *Ficus benjamina L.* plants affected with branch dieback in 2014 with several strains of *N. dimidiatum* (Fernández-Herrera *et al.,* 2017). For the first time among 2014 and 2016, the fungus *N. dimidiatum* considered as an almond tree pathogen that causes canker, shoot blight and fruit rot in California (Nouri *et al.,* 2018). Fig (*Ficus carica L.*) were infected with canker in Iraq (Alwan, 2019), fruit rot caused by *N. hyalinum* on melon in Iran were also reported (Mirtalebi *et al.,* 2019), and in UAE in 2019 canker disease was linked to *N. dimidiatum* in *D. regia* tree (Al Raish *et al.,* 2020).

1.6.2 Treatment of *N. dimidiatum*

The conventional method used to fight fungus infection is the chemical control represented by various types of fungicides, and some researcher tries biological control as mentioned on the studies below.

1.6.2.1 Biological Control of *N. dimidiatum*

N. dimidiatum itself could be consider as a biological control agent against some pathogens as it showed in results of one study that it produced active antifungal compounds against the serious plant pathogenic fungi *T. cucumeris* and *G. zeae,* which are the causal agents of rice sheath blight and wheat head blight, respectively (Abdel-Motaal *et al.,* 2010).

A study where endophytic fungus from the stem of healthy dragon fruit (*Hylocereus spp.*) was successfully identified as *Trichoderma harzianum* T3.13. and *T. harzianum* T3.13 was shown to have the ability to produce antagonistic activity against *N. dimidiatum* U1, a pathogen fungus that attacked dragon fruit (Wan *et al.,* 2017). Another research proved the efficacy of eco-friendly biocontrols *Lactobacillus*

rhamnosus and *Azotobacter chroococcum* for boosting the plant growth and decrease the infection by *N. dimidiatum spp.* in fig (*Ficus carica L.*) saplings (Alwan *et al.,* 2019).

1.6.2.2 Chemical Control of *N. dimidiatum*

Chitosan-Silver Nanoparticles (Ag@CS) have been developed as a plant fungicide drug. In one study, the synergistic effect of Chitosan (CS), Silver Nanoparticles (AgNPs), and fungicide Zineb (Zi) were investigated as antifungal materials against *N. dimidiatum* in dragon fruit, the antifungal activity of Ag@CS has shown antifungal ability better than each component alone, analyzed by the zone of inhibition method against *N. dimidiatum* (Ngoc & Nguyen, 2018), on another study, they found the prepared Cu2 O-Cu NPs/alginate showed high antifungal efficiency (~100%) against *N. dimidiatum* with 31 Part Per Million (ppm) concentration copper (Du *et al.*, 2019).

In the United Arab Emirates (UAE), to manage the disease, the chemical fungicides, Protifert®, Cidely® Top and Amistrar® Top, significantly inhibited mycelial growth and reduced conidial numbers of *N. dimidiatum* in laboratory and greenhouse experiments on *D. regia* tree (Al Raish *et al.,* 2020).

In sooty canker disease of *Eucalyptus camaldulensis* trees in Kerbala Province, fungicide Beltanol-L (1 ml/ 1l) and Salicylic acid (1000 mg/ 1l) showed a significant role in full inhibition of the pathogen growth in PDA and green house (Al-Tememe *et al.*, 2019).

1.7 Actinobacteria

Actinobacteria are a group of organisms initially considered to be intermediates between fungi and bacteria. The application of the new taxonomic techniques has led to improvements in the classification and identification of actinobacteria genera and species. Actinobacteria are filamentous Gram-positive branch forming bacteria with high guanine and cytosine (G+C) content within their DNA (Barka *et al.,* 2016; Franco-Correa & Chavarro-Anzola, 2016), they are belonging to the phylum Actinobacteria and its order Actinomycetales. Most actinobacteria are commonly aerobic, chemoorganotrophic and mesophilic, actinobacteria isolates behave as neutrophils in culture, with a growth range, from pH 5.0 to 9.0 some acidophilic and alkalophilic actinobacteria have been detected and the optimum growth are near the pH around 7.0 and with optimal growth temperatures between 25 and 30°C although that some actinobacteria are thermophilic, the mycelia in some species may break to form rod- or coccoid-shaped forms reproduce by the production of spores in specialized areas of the mycelium or by fragmentation of the hyphae (Barka *et al.,* 2016; Franco-Correa & Chavarro-Anzola, 2016). Many genera also form spores; Among these groups of microorganisms, *Streptomyces* is dominant. The genus *Streptomyces*, appear to be an excellent candidate to manage various plant diseases (El-Tarabily *et al.,* 1996).

For the last decades, actinobacteria have been well-known as a key producers of numerous metabolites as they have broad-spectrum biological activities such as antibiotic especially from *Streptomyces* which consider as the primary producers of commercially antibiotics, antibacterial, antifungal, antiviral, antiparasitic, immunosuppressive, antitumor, insecticidal, anti-inflammatory, antioxidant, enzyme

inhibitory and diabetogenic. Those that produce antibacterial and antifungal metabolites are referred to as the antagonistic biological types. They are also essential for soil biodegradation and humus formation as nutrients are recycled. Furthermore, actinobacteria are well known as potent producers of extracellular hydrolytic enzymes, which is relevant for industrial applications (Doumbou *et al.,* 2001; Khasabuli & Kibera, 2014; Sharma & Vinayak, 2014; Mast & Stegmann, 2019).

In nature, it converts many compounds and mediates several biochemical reactions. It also participates in the composting process, their primary carbon sources in the soil are insoluble and polymeric. They can penetrate and solubilize these polymers, whether of the plant lignocellulose or animal chitin origin. In the aquatic environment, plant biomass and animal biomass is the carbon source for actinobacteria (Sharma & Vinayak, 2014; Barka *et al.,* 2016).

1.7.1 Nutrition of Actinobacteria

Actinobacteria are heterotrophic, often stringent and some form a parasite or mutual relationship with plants and animals. Actinobacteria are aerobic and neutrophilic. Some actinobacteria are assign to the non-actinobacteria and called the rare actinobacteria which defined as the strains less frequently isolated than other *Streptomyces* spp., even if they are not rare in the environment (Subramani & Sipkema, 2019). The actinobacteria occurring as an endosymbiont in various parts of the plant including stems, leaves and roots are called "endophytic actinobacteria ". Since the actinobacteria group is consider as diverse genera and physiologically and nutritionally distinctive, they have been observed in all forms of habitats. Actinobacteria can be found easily in both aquatic and terrestrial ecosystems but mostly found in soil

(Khasabuli & Kibera, 2014; Sharma & Vinayak, 2014; Barka *et al.,* 2016; Kuncharoen *et al.,* 2019).

1.7.2 Structure of Actinobacteria

The actinobacteria (singing actinobacteria) form branching hyphae and asexual spores and are a Gram-positive bacteria. In the *Streptomyces* species studies, the spores had a two-layered wall and the inner one stretched to form the germ-tube wall. The actinobacteria branch creates a network of hyphae developing on both the surface and under-surface of the agar when grown on an agar surface. Aerial hyphae are the on-the-surface hyphae and substrate hyphae are the under-surface hyphae. Typically, septa divide hyphae into long cells containing various bacterial chromosomes (nucleoids). This are the aerial hyphae that extend above and reproduce asexually over the substratum. Most actinobacteria are non-motile when motility is available, it is confined to flagellated spores (Sharma & Vinayak, 2014).

The cell wall of actinobacteria do not contain chitin or cellulose as fungi but it contains peptidoglycogens which is similar to Gram-positive bacteria cell wall, and about 10-20 nm thick and consist of peptidoglycan together with one or more associated polymers. Both the Peptidegycan and non-Peptidoglycan polymers are concentrated at the outer and inner surfaces of the cell walls, the structure of the cell wall differs considerably among the numerous classes in actinobacteria and its taxonomic significance. Four major cell wall types are distinguished in these based on the three features of peptidoglycan composition and structure (Sharma & Vinayak, 2014).

1.7.3 Occurrence of Actinobacteria in the Environment

The vast majority of cultures of aerobic actinobacteria have originated from the soil ecosystem; Actinobacteria represent a major contents of the soil microbial biomass. Environmental factors affect the aerobic actinobacteria type and population in the soil, such as the humidity, temperature, pH and plant diversity, for example, numbers of *streptomycetes* in grasslands was highest in summer and *Nocardiae* were most numerous in the pasture in winter (Doumbou *et al.,* 2001). In this context, several unusual or niche habitats have been explored for bioactive actinobacteria such as desert, cave, pristine forests, lakes, rivers, and other wetlands, high salt environments, marine ecosystems and endophytic niches (Fatahi-Bafghi *et al.,* 2019).

The aquatic actinobacteria are the wash-in forms from the soil actinobacteria. Predominant amongst them are the *Streptomyces*, *Nocardia, Actinoplanes, Micromonospora* and *Rhodococcus.* The aquatic environment will influence the nutrition, physiology and types of actinobacteria there. Since the aquatic environments differ significantly from the terrestrial habitats, the biological characteristics of aquatic actinobacteria and their distribution are expected to be different from those of soil actinobacteria as well (Sharma & Vinayak, 2014).

Marine actinobacteria were present in symbiosis with numerous marine invertebrates, in particular sponges, and their metabolites can degrade cellulose, lignin, alginate, laminarin and starch. Also, it can play a role as symbionts in the intestinal tract of animals that feed on the organic substances present in the soil. They have gained great attention as they have evolved unusual metabolic and physiological capacities that not only ensure longevity in intense environments, but also provide the prospect of developing antitumor compounds and other fascinating pharmacological

activities that would not be found in terrestrial microorganisms, possibly because of their near connections marine eukaryotic organisms including mammals (Sharma & Vinayak, 2014).

1.7.4 Novel Approaches in Isolation of Actinobacteria

For isolation of actinobacteria, sex basic stages for selective isolation of actinobacteria are recognized.

1.7.4.1 Selection of Material Containing Microbes

Choosing the location of actinobacteria , such as soils, could be built on the presumption that samples from widely varying locations are more likely to yield different novel isolates and, possibly, different novel metabolites. As the different location could be different in pH for example actinobacteria populations from acid soil could be different from those from neutral soil. Man's activities also can accidentally alter the community of natural habitats as well. The isolation of marine actinobacteria has proved to be a major source of new compounds and isolation from depths to deeper coastal sediments of the marine trench around the world shows the occurrence of actinomycetics in marine sediments, but at lower concentrations than in soil (Sharma & Vinayak, 2014).

1.7.4.2 Pre-treatment of Material

In order to improve the chances of isolation of the target microbes, the substance can be processed in different ways. For example, the number of bacteria on the actinobacteria plate is decreased by heat processing. Nutrient enrichment with chemicals, such as chitin provides more population of *Streptomyces*, the addition of

CaC03 increase the pH due to which alkalophilic *Streptomyces* could be isolated (Sharma & Vinayak, 2014).

1.7.4.3 Growth on Laboratory Media

The sensitivity of an isolation medium can be influenced by its nutrient composition, its pH and the addition of selective inhibitors. A wide range of media formulations has been recommended for the isolation of specific genera of actinobacteria (Sharma & Vinayak, 2014).

1.7.4.4 Incubation

Actinobacteria isolation plates are usually incubated at 25 to 30°C. Thermophiles are usually incubated between 40 to 45°C and psychrophiles between 4 to 10°C. The incubation period is variable, and commonly isolated genera grow between 7 to 14 days (Sharma & Vinayak, 2014).

1.7.4.5 Colony Selection

The colony selection relies on the screening programmers goals. The colonies may be multiple duplications. More appropriate ways must be used to separate micro-organisms. The site of sample collection, a knowledge of the secondary metabolite of an isolate, the isolation of new and potential isolates will result in objective enrichment techniques and objective culture formulations (Sharma & Vinayak, 2014).

1.7.4.6 Identification

There are various approaches for the identification of actinobacteria, the molecular approach are consider as the most influential taxonomy methods by study of the nucleic acids. Sequences of 16S rDNA have supplied the scientist with a full

detailed phyletic tree. Chemotaxonomy approach help a lot to identify the genera of actinobacteria. It is still widely used the classical approaches for classification of morphological, biochemical characters, and physiological which done by investigate the aerial mass color, melanoid pigments, reverse side pigments, soluble pigments, spore chain morphology and reproductive structure surface. The last approach is numerical taxonomic which includes reviewing numerous strains for a huge number of characters before assigning the test organism to a cluster based on linked characters, this approach gives us a valuable context for taxonomy and species identification for *Streptomyces* (Sharma & Vinayak, 2014).

1.7.5 Causal Agents of Disease

Actinobacteria have proved to be causal agents of many human and animal infections. In human, Hypersensitivity pneumonitis which is immune-mediated inflammatory lung disease and farmer's lung disease are both caused by repeated inhalation of antigenic organic particles in a variety of environmental such as thermophilic actinobacteria *Saccharopolyspora rectivirgula* (Cano-Jiménez *et al.,* 2016; Girard *et al.,* 2009; Kim *et al.,* 2010), while in the plant its mention that the cause of rootlets of apple trees was linked to actinobacteria which colonize and damage the rootlets of apple trees in many studies (Otto, 2017). In a review paper, they prove that chronic obstructive pulmonary disease of horses is a delayed hypersensitivity response to inhaled antigens, particularly the thermophilic moulds and actinobacteria that grow in damp hay (Robinson *et al.,* 1996).

1.7.6 Applications of Actinobacteria

Actinobacteria are responsible for the pleasant odour of the soil. In decomposition and bioremediation, it is also essential compounds. Actinobacteria, and especially streptomycetes, are also expected to have an important role to play in the antagonistic relationship with many earth microorganisms. This is why it is commonly used for biologic management of plant diseases and for the promotion of plant growth (Bizuye *et al.,* 2013; Sharma & Vinayak, 2014; Anwar *et al.,* 2016; Behie *et al.,* 2017; Chaurasia *et al.,* 2018).

1.8 Biological Control (Biocontrol)

Biological control (Biocontrol) is considered as a unique key for combat plant soil-borne pathogens that cause significant economic losses in crops; it can also be used to support plant growth, the beneficial plant microbes were seen as a better strategy to the use of chemical pesticides, fungicides and fertilizers because it's considered inoffensive for the environment by reducing the contaminations, cope the resistance to fungicides issues especially systemic fungicides and minimized the side effect on the beneficial microorganisms, on the other hand, many of these chemicals (fungicide and fertilizers) are known to induce tumors in experimental animals and also it could be considered as potential human carcinogens when present as residues in food and water (Lumsden & Papavizas, 1988; He *et al.,* 2016). Beneficial bacteria and fungi that are naturally associated with the plants and have a positive influence on Plant Growth-Promoting Agents (PGPA) by the alleviation of biotic and abiotic stresses were isolated and developed into Biological Control Agent (BCA) which is the organism that suppresses the pest or pathogen (Pal & Gardener, 2006), as PGPA (Puri *et al.,* 2020). Example of PGPA are the symbiotic nitrogen-fixing Rhizobium

species which influences the concentrations of Nitrogen-based (N-based) and Carbon-based (C-based) compounds that play a role in plant defense reactions, where induced resistance reactions inhibitive to aboveground antagonists and associated with increased activity of enzymes such as peroxidases and polyphenol oxidase, rhizobia are also known to trigger induced resistance reactions against other microbial pathogens and invertebrates that reside in plant roots (Pal & Gardener, 2006; Karoney *et al.*, 2020). Yeast, bacteria and fungi are considered as examples of biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters (Madbouly *et al.,* 2020).

From this bacteria, there are actinobacteria which have been studied for their biocontrol and plant growth promotion (Palaniyandi *et al.,* 2013). *Streptomyces* spp. in actinobacteria groups have been investigated mostly and less attention was giving for Non-*Streptomyces* Actinobacteria (NSA) categories (El-Tarabily & Sivasithamparam, 2006a, 2006b). More than half of biologically active compounds developed for agricultural use originated from *Streptomyces* species (Evangelista-Martínez, 2014).

A wide variety of biocontrols are available on the market, but further development and successful farmers adoption are required and more study of the complicated relationships between human, environment and biological interactions need to be investigated (Pal & Gardener, 2006).

1.8.1 The Forms of Interactivity Engaged in Biological Control

There are different interactions between the living organisms that happened under the soil; this could include:

1.8.1.1 Mutualistic

It is an association between two or more species, including the host plant where both are benefit in their way. It is sometimes seen as an obligatory, physical and biochemical interaction with direct contacts, and sometimes it is considered as an facultative and opportunistic relation.

1.8.1.2 Protocooperation

It is considered a type of mutualism, but all the organisms that engaged in the relations do not depend on each other exclusively for survival.

1.8.1.3 Commensalism

It is a long-term biological relationship as a symbiotic connection within living organisms, which benefits one organism and does not harm nor profit the other.

1.8.1.4 Neutralism

It is a biological connection when the organism had totally no effect on the other organism.

1.8.1.5 Antagonism

Hostile interrelationships between organisms and result in negative effects on one or both of them.

1.8.1.6 Competition

It could happens for many reasons, such as when they compete for space or nutrients, and this may end in decreased the fertility, activity and/or growth of the interacting organisms.

1.8.1.7 Parasitism

Parasitism is considered as a long time symbiotic relation between two phylogenetically unrelated microorganisms in which one benefits and the other is harmed, usually the physically big one is called the host which is injured and the other one called the parasite who is usually benefiting from this relation.

1.8.1.8 Predation

Refer to the murder of one microorganism by another microorganism for consumption and sustenance (Cook & Baker, 1983; Lumsden & Papavizas, 1988; Pal & Gardener, 2006).

1.8.2 Mechanisms of Biological Control

Plants and pathogens communicate with a wide range of species during their lifecycle. These interactions can have an enormous impact on plant health, biological control could occur from a wide variety organism reactions and there are three main antagonistic mechanisms, the first mechanism is the direct antagonism occurring from physical contact and/or a high degree of selectivity of the pathogen, while the second mechanisms are the indirect antagonisms resulting from activities that didn’t include targeting or sensing a pathogen by the BCA(s), and the last mechanism is the mixed-path antagonism (Pal & Gardener, 2006; Dasila *et al.*, 2020).

In this section, the three mechanisms: Indirect antagonism, Mixed-path antagonism and Direct antagonism will be discussed in full details.

1.8.2.1 Direct Antagonism

In the direct antagonism, the main type of mechanisms are Hyper-parasitism and Predation. In Hyper-parasitism, the pathogen is targeted directly by a particular BCA that destroys or propagates it (Pal & Gardener, 2006).

Hyper-parasitism including mycoparasitism, which is a fungal pathogen that categorized into biotrophs and necrotrophs. Biotrophic mycoparasites obtain their nutrients by directly invading a cell, forming haustoria and generally cause little harm to the host. In contrast, necrotrophic mycoparasites kill their hosts and then utilize the nutrients released from the dead hyphae. The mechanism of mycoparasitim in inhibition of fungal plant pathogen depends on the secretion of a broad range of fungal Cell-Wall Degrading Enzymes (CWDEs) and proteases that enable the parasite to invade the hyphae of the pathogen (Brimner & Boland, 2003). The degree of parasitism varies depending upon several factors including changes in the carbon: nitrogen ratio, light, pH, temperature and nutrients (Cook & Baker, 1983).

1.8.2.2 Mixed-Path Antagonism

The concept of the mechanisms Mixed-path antagonism are working around secretions from organism that could harm other organism in many ways; there are many mechanisms such as lytic enzymes, unregulated waste products, antibiotics, and chemical/physical interference.

1.8.2.2.1 Antibiotics

Antibiotics can be defined as the toxin’s excretion of one or more metabolites by an organism (by the BCAs), which at low concentrations could be toxins, inhibits the growth of particular pathogen and can have a deleterious effect on one or more

other organisms. In order for antibiotics to be able to succeed, enough quantity must be produced near the pathogen in order to create a biocontrol effect. However, due to the little amounts produced compared to the other least toxic organic compounds in the phytosphere, it is impossible to estimate the efficient adequate amounts (Pal & Gardener, 2006; Prajapati *et al.,* 2020; Sood *et al.*, 2020)

1.8.2.2.2 Lytic Enzymes and other Byproducts of Microorganism

Various microorganisms release and secrete another metabolites like lytic enzymes, which might lysis and/or stress the cell walls and decompose nonliving organisms, and generally cause plant residues and organic matter to decompose. Furthermore, certain products of lytic enzymes can help indirectly to eliminate many diseases. For instance, oligosaccharides which extracted from fungal cell walls are consider as strong powerful plants host defense activator, actinobacteria may also produce degrading enzymes for the cells walls that could hydrolyze a broad range of polymeric compounds, such as hemicellulose, proteins, chitin, cellulose, and other hydrolases which hydrolyze the glucan backbone of fungal cell walls (Kamil *et al*., 2018). Such enzymes may also interfere with the activities or growth of infectious agents (Pal & Gardener, 2006).

1.8.2.2.3 Unregulated Waste Products

There are many byproducts produced by organisms that may contribute to pathogen inhibition such as, large number of Volatile Organic Compounds (VOCs) produced by microorganisms including alkanes, alkenes, alcohols, esters, ketones, geosmics, sulfur compounds, ammonia, carbon dioxide, and isoprenoid compounds which can act as biofumigants to control various pathogens, nematodes, insects, and weeds (Sharma & Salwan, 2018). Hydrogen cyanide or as the other name Hydrocyanic

acid (HCN) considered as extremely lethal to all aerobic microorganisms at picomolar concentrations and it is produced by many rhizobacteria (Alström & Burns, 1989; Pal & Gardener, 2006).

1.8.2.2.4 Physical/Chemical Interference

The physical and chemical interference could also act as types of biological control of plant pathogens. This could include blockage of soil pores for example or the use of soil amendments which could be used to control pathogen in virtually way, for instance, the changes in the soil and rhizosphere environment chemically or physically by adding organic matter such as barley straw, sorghum stubble, fowl manure, lime, physical matter such as sawdust could affect the root environment and then the life cycle of pathogens (Chaube & Singh, 1991; Pal & Gardener, 2006).

1.8.2.3 Indirect Antagonism

Microbial biological control agents protect crops from damage by diseases via different Indirect way. They may induce or enhanced resistance in plant tissues without direct antagonistic interaction with the pathogen, another indirect interaction with pathogens is competition for nutrients, oxygen and space (Köhl *et al.,* 2019).

1.8.2.3.1 Competition

A living organism commonly competes for space and nutrient such as competition for nitrogen, carbon and other growth factors, aiming for utilization or removal of some resource of the environment, for the sake of nutrients, the organism can find it either on the host-supplied nutrients include plant surfaces, exudates, leachates or senesced tissue. Nutrients from other species like insects (example aphid on the surface of the leaf) and soil can also be obtained. The competition may also

include competition for other resources including siderophore (Cook & Baker, 1983; Pal & Gardener, 2006).

The degree of competition depends on the location. For example, the rhizosphere is a region of superabundant nutrients, more organisms, limited spaces and oxygen so the competition will be less for the nutrients, while in the upper part of the plant such as leaf surface, where the nutrients usually consider rare the competition will be rough (Cook & Baker, 1983; Pal & Gardener, 2006).

1.8.2.3.2 Induction of Host Resistance

Depending on the stimulating effect including light, physical stress, gravity, water, temperature and nutrient availability and environmental factors, plants as a living organism will response negatively or positively to the environment surrounding it. Plants often react to a wide varieties of chemical stimuli provided by microbes associated with soil and plants. Depending on the source, type, and number of stimulation, induction of host defenses may be systemic and/or local.

Inducing host defense mechanisms involves Systemic Acquired Resistance (SAR), a compound that is commonly generated after a pathogenic infection and usually contributes to the expression of Pathogenesis-Related (PR) proteins mediated by Salicylic Acid (SA). PR proteins contain many enzymes that can directly affect the lysis of invading cells, reinforce cell wall boundaries for resistance to the infection pathogen, and may also cause localized cell death. Another phenotype, known as Induced Systematic Resistance (ISR), which induced after infected by a certain of nonpathogenic rhizobacterias and mediate by Jasmonic Acid (JA), and/or ethylene. PGPR also encourages Induced Systemic Resistance (ISR) when the plant is properly induced by rhizobacteria; the roots of the plant will recognize these bacteria and

therefore increase resistance expressed during a pathogen infection. Interestingly, the defense pathways based on SA and JA can be mutually antagonistic and some bacterial pathogens use this to resolve the SAR.

Several strains of root-colonizing microbes were known as possible elicitors of plant host defenses. In some cases, Plant-Growth Promoters of Rhizobacterial (PGPR) inoculations have been successful in controlling multiple diseases induced by various pathogens, whereas PGPR strains, like salicylic acid, siderophore, and lipopolysaccharides, are likely to develop several chemical SAR or ISR emitters (Cook & Baker, 1983; Pal & Gardener, 2006).

1.9 Statement of the Problem

N. dimidiatum fungi is the leading cause of canker disease in many *D. regia* trees which lead to many economic and biological loss. Until now, there is no clear direction for treating this disease either by chemical or biological control.

1.9.1 Thesis Objectives

1. To isolate and identified the pathogen associated with infected *D. regia*.
2. To evaluate the efficacy of fungicides, and explore the most promising antagonistic actinobacteria isolate against the causal agent of stem canker *in vitro*.
3. To assess the potential fungicides and the most promising antagonistic actinobacteria isolates against the pathogen *in vivo* under greenhouse conditions.
4. To manage the disease of naturally infested plants in the field using the proper fungicide treatment.

5. To manage the disease of naturally infested plants in the field using the most promising antagonistic actinobacteria isolates treatment.
6. To develop and implement Integrated Disease Management (IDM) strategies using a combination of cultural, chemical and biological control with resistant to manage stem canker disease.

The overall objective of this project is to understand the suitable treatment for *N. dimidiatum* fungi by antagonistic actinobacteria and fungicide.

Chapter 2: Molecular Characterization and Disease Control of Stem Canker on Royal Poinciana (*Delonix regia*) Caused by *Neoscytalidium dimidiatum* in the United Arab Emirates

2.1 Introduction

Delonix regia ((Bojer ex Hook.) Raf.) is a beautiful flowering and shady branching tree. This member of the pea family (Fabaceae), which is also known as flamboyant, peacock or flame tree, can be recognized by the color of flowering cultivars, ranging from deep red to bright orange or yellow (Gledhill, 2008). It is a rapid growing tree that can reach to 6–12 m height, and bears compound leaves that reach 30–60 cm length and flat woody pod fruits of about 60 cm long (Kirtikar *et al.*, 1999). Despite it is native to Madagascar and tropical regions, this deciduous tree provides landscape with cooling shade during hot summers and warming-sunshine winters. In addition to the "umbrella" canopy it provides, *D. regia* can grow in a variety of soil conditions, and is highly tolerant to drought and salinity (Gilman & Watson, 2019).

For that reason, there is a growing interest in the plantations of *D. regia* in the United Arab Emirates (UAE), mainly in parks, sidewalks, streets, parking lots and open areas. Although, this tree does not often suffer from real problems, stem canker has currently become a serious disease affecting *D. regia*. Therefore, it is urgent to address this present threat to *D. regia* in the UAE and worldwide.

Like other ornamental and stone fruit trees, fungi can attack different parts or tissues of *D. regia* under certain favorable conditions to cause canker diseases (Cayley, 1923; Gilman & Watson, 2019). In general, cankers are destructive diseases which may cause damage to the whole or parts of trees such as branches, barks and woods. Fungi such as *Nectria galligena, Leptosphaeria maculans, Lasiodiplodia theobromae*

and *Teratospheria zuluensis* are among those associated with canker diseases on sweet birch tree (*Betula lenta*), oilseed rape (*Brassica napus*), eucalypt and pine trees (Anagnostakis & Ferrandino, 1998; Chungu *et al.*, 2010; Darge, 2017; Rouxel & Balesdent, 2005). *N. dimidiatum* is another fungal pathogen that causes cankers and has a wide geographical and host range, including plum, almond (*Prunus dulcis*), mango (*Mangifera indica*), pitahaya (*Hylocereus undatus*), Citrus, Musa, Populus, and Ficus spp. in Australia, China, Egypt, Niger, Tunisia and the USA (Al-Bedak *et al.*, 2018; Farr *et al.*, 2005; Hajlaoui *et al.*, 2018; Nouri *et al.*, 2018; Ray *et al.*, 2010; Reckhaus, 1987; Yi *et al.*, 2015).

In Oman, stem canker has been reported on different trees including *D. regia* (Elshafie & Ba-Omar, 2002). Symptoms can be recognized as branch wilt, dieback, canker, gummosis and death of infected trees. In general, severity of the disease caused by this fungus can be enhanced by stress factors such as water stress (Elshafie & Ba-Omar, 2002; Reckhaus, 1987). In the UAE, recent studies on tree diseases caused by fungi have reported black scorch disease and Sudden Decline Syndrome (SDS) on date palm, and dieback disease on mango caused by *Thielaviopsis punctulata, Fusarium solani* and *L. theobromae*, respectively (Alwahshi *et al.*, 2019; Saeed *et al.*, 2016, 2017a). So far, there are no reports about *D. regia-N. dimidiatum* interaction causing stem canker disease in the UAE.

Plant disease management mainly relies on the life cycle of the pathogen. *N. dimidiatum* produces two types of spores, pycniospores which are formed in pycnidia embedded in mature lesions and phragmospores which are formed by the breaking up of individual or groups of cells of mature hyphae in dead tissues of the lesion (Chuang *et al.*, 2012; Mohd *et al.*, 2013). In culture, only phragmospores are formed and produced. Typically, cultural and horticultural practices such as pruning and

fertilization may lower the risk of the pathogen, increase the vigor of the tree and extend its life (Gilman & Watson, 2019). On the other hand, such practices can be harmful due to the improper timing, unsterile tools, inexperienced persons or advanced stages of the pathogen's life cycle. Regardless of its ecological problems and human health concerns, the use of chemical fungicides is yet the main disease management tactic to attenuate the threat of crop diseases (Alwahshi *et al.*, 2019; Kamil *et al.*, 2018; Saeed *et al.*, 2016, 2017a). *In vitro* treatment with the chemical, Beltanol-L (8-hydroxyquinoline), effectively inhibited the growth of *N. dimidiatum in vitro* (Al-Tememe *et al.*, 2019). The same fungicide also reduced symptoms of canker lesions on the seedlings of *Eucalyptus camaldulensis* under greenhouse conditions. Application of any of the systemic fungicides, Elsa® (carbendizim), Mizab® (mancozeb) or Curzate® (cymoxamil),showed a significant inhibition to this fungus that causes wilt and canker diseases on cypress trees (Murad *et al.*, 2014). Hence, one should take into consideration the timing for minimum effective dose of the fungicide application to control the disease.

The long-term goal is to develop and implement Integrated Disease Management (IDM) strategies using a combination of cultural, chemical and biological control with resistant cultivars of *D. regia* to manage stem canker disease. In the present investigation, an attempt was made to explore the feasibility of using efficient chemical fungicide(s) for the management of stem canker of royal poinciana. Therefore, the objectives were to: (1) to isolate and identify the pathogen associated with infected plants; (2) to evaluate the efficacy of fungicides against the causal agent of stem canker *in vitro*;(3) to assess the potential fungicides against the pathogen *in vivo* under greenhouse conditions; and (4) to manage disease of naturally infested plants in the field using the proper fungicide treatment. Here, the assessment of

systemic chemical fungicide treatments against *N. dimidiatum in vitro*, in the greenhouse as well as in the field were reported. a short-term strategy to reduce the economic losses associated with stem canker disease were also developed. Future directions to employ research on Biological Control Agents (BCAs) to suppress the damaging activities of the pathogen and to lower the risk of the disease on *D. regia* will further cooperate in the development of effective IDM programs.

2.2 Materials and Methods

2.2.1 Fungal Culture and Isolation

Eight-year-old *D. regia* trees located in Dubai Festival City (DFC), Dubai, UAE (latitude/longitude: 25.22/55.36) were associated with longitudinal cankers on stems as shown in results. Cross-sections in trunks and branches were made, and drying leaves were gathered from diseased trees. All collected tissues were then transferred to the Plant Microbiology Laboratory, Department of Biology, United Arab Emirates University in Al Ain city, UAE, for isolation and identification purposes. To isolate the pathogen, affected tissues were cut into small pieces (3–5 mm long), washed and surface-sterilized with mercuric chloride 0.1% for 1 min, and 1.05% NaOCl for 5 min; followed by three consecutive washings in sterile distilled water. They were then transferred onto PDA (Lab M Limited, Lancashire, UK) plates, supplemented with 25 mg/L penicillin-streptomycin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) to inhibit bacterial contaminants. Petri dishes were incubated for five days at 25 ± 2 °C . Once grown out of the plated tissue, mycelia were aseptically sub-cultured on fresh PDA and purified using hyphal-tip isolation technique (Kirsop & Doyle, 1991). To characterize fungal structures, mycelia and conidia were observed using Nikon-Eclipse 50i light microscope (Nikon Instruments Inc., Melville, NY, USA). The

culture of the identified fungus, *N. dimidiatum* (Crous *et al.,* 2006), was deposited in Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany) under the accession number 109897.

2.2.2 Molecular Identification of the Pathogen

DNA of the pathogen isolated from diseased of stem, branch and leaf tissues was extracted from mycelia cultured for 10 d at 25°C on PDA plates, using the fungi DNA isolation kit (Norgen Biotek Corp., Thorold, ON, Canada). PCR was set up to amplify target regions of the ITS of the nuclear rDNA for *N. dimidiatum* using ITS1 and ITS4 primers (White *et al.,* 1990), partial 28S rDNA using LR0R and LR5 primers (Vilgalys & Hester, 1990), partial TEF1-α using EF1-728F and EF1-986R (Carbone & Kohn, 1999), and partial β-tubulin using Bt1a and Bt1b (Glass & Donaldson, 1995). PCR reactions (50 µL) contained 30-ng DNA template, 50 pmol of each primer, 200 µM of each dNTP, 2.5 unit of Taq DNA polymerase and 2.2 mM buffer (MgCl2). Each cycle of PCR was set as the following: 94°C for 1 min; 58°C for 1 min; and 72°C for 1 min (total of 32 cycles). All primer sequences can be found in Table 4. All protocols for amplification and sequencing were as described (White *et al.,* 1990).

Table 4: List of Polymerase chain reaction (PCR) primers (sequence 5' to 3') used in this study

Description	Left primer sequence	Right primer sequence
ITS	ITS1: TCCGTAGGTGAACCTGCGG	ITS4: TCCTCCGCTTATTGATATGC
28S DNA	LR0R: ACCCGCTGACTTAAGC	LR5: TCCTGAGGGAAACTTCG
B-tubulin	Bt1a: TTCCCCCGTCTCCACTTCTTCATG	Bt1b: GACGAGATCGTTCATGTTGAA CTC
TEF1-α	EF1F: TGCGGTGGTATCGACAAGCGT	EF2R: AGCATGTTGTCGCCGTTGAAG

The sequence of TEF1-α gene of the fungal isolate from the UAE was deposited in GenBank (accession number: MN447201). The phylogenetic tree using TEF1-α sequence, obtained from DSMZ, was constructed against other sequences of TEF1-α belonging to *N. dimidiatum spp.* retrieved from GenBank-NCBI (www.ncbi.nlm.nih.gov) (Crous *et al.,* 2006). ML analysis was performed for the estimation of the phylogenetic tree after all sequences were aligned. Phylogenetic trees were validated with a statistical support of the branches with 100 bootstrap resamples (Tamura *et al.,* 2013). The following isolates used in the analysis belong to *N. dimidiatum, N. novaehollandiae, N. hyalinum, Botryosphaeria dothidea* and *B. fusispora.*

2.2.3 *In Vivo* Pathogenicity Tests and Koch's Postulates

Pathogenicity tests were conducted on one-year-old healthy *D. regia* seedlings (n = 9), purchased from the local market. Using sterile scalpels, the bark of the main stem was wounded and inoculations under the wounded bark were performed at 30–50 cm above the soil surface (Al-Bedak *et al.,* 2018). An agar plug (8-mm-diameter) colonized by mycelium of 10-day-old culture of *N. dimidiatum* was placed into the wound, where the mycelium facing inner parts, and wrapped using parafilm. Control *D. regia* seedlings were inoculated with sterile agar plugs (no pathogen). Plants were maintained in the greenhouse (15 hours (h) day/9 h night at 25 ± 2°C) and were evaluated for symptoms and disease progression at 2 and 5 Weeks Post Inoculation (wpi). By the end of the experiment, the fungus was re-isolated from the point of infection on PDA and compared morphologically with the inoculated fungus.

Disease was assayed on disease-free apple fruits (*cv Granny Smith*), purchased from local fresh markets, to find out the effect of *N. dimidiatum*. Fruits (n = 8) were

washed with sterile distilled water, surface-sterilized with 70% ethanol and wounded with a sterilized scalpel (11 mm diameter) according to Hortova *et al.* (2014) with some modifications. On each fruit, one agar plug (11 mm in diameter) containing mycelium of *N. dimidiatum* (colonized mycelium facing down) and one agar control plug without pathogen was applied. Inoculated fruits were maintained in dark (at 25 ± 2°C and 80% relative humidity) and lesion size was rated for an interval of 5 d for 10 days (d). At 10 dpi, pieces from regions showing disease symptoms of inoculated fruit tissues were removed, surface sterilized, plated and incubated, as mentioned above. Structures of conidia and mycelium were morphologically compared with the inoculated fungus.

2.2.4 *In Vitro* Evaluation of Fungicides Against *N. dimidiatum*

The fungicide experiment was carried out according to the previously described procedures (Saeed *et al.,* 2016, 2017a; Alwahshi *et al.,* 2019).

The selected fungicides along with their active ingredients can be found in Table 5. Fungal growth was assessed on each fungicide with a final concentration of 0 (control), 250, 500, 750 and 1000 ppm aseptically introduced into sterilized PDA plates, supplied with penicillin-streptomycin antibiotics, at 25 ± 2°C. The tested fungal pathogen was introduced to PDA plates using a sterile cork-borer (8 mm diameter). Cultures were incubated at 25 ± 2°C for 10 days, and percentage of the mycelial growth inhibition was measured according to:

$$\% \text{ Mi} = (\text{Mc} - \text{Mt})/\text{Mc} \times 100\%$$ (Gledhill, 2008)

Where Mi, inhibition of the mycelial growth; Mc, colony diameter (in mm) of control set; and Mt; colony diameter (in mm) of the target fungus on the medium with fungicide.

Table 5: Labels, active ingredients, producing company and country of the fungicides used in this study

Fungicide	Active ingredients	Company	Country
Amistar Top®	Azoxystrobin 200 g/L + Difenoconazole 125 g/L	Syngenta	Switzerland
Uniform®	Azoxystrobin 322 g/L + Metalaxyl-M 124 g/L		
Cidely® Top	Difenoconazole 125 g/L + Ciflufenamide 15 g/L		
Protifert®	Copper sulfate pentahydrate 20% + Aminoacides 19% + Sulfur 8% + Total nitrogen 3.3%	Sicit 2000 S.P.A.	Italy
Airone Liquido®	Metal copper 272 g/L	Gowan Italia S.P.A.	
Proxanil®	Propamocarb hydrochloride 400 g/L + Cymoxanil 50 g/L	Arysta LifeScience	Belgium
Protoplant®	Propamocarb 722 g/L		
Penthiopyrad®	Penthiopyrad 40%	Mitsui Chemicals Agro Inc.	Japan
Previcur®	Propamocarb hydrochloride 530 g/L + Fosetyl-aluminium 310 g/L	Bayer	Germany

2.2.5 *In Vivo* Evaluation of Selected Fungicides

To determine the ability of fungicides to reduce lesion formation after *N. dimidiatum* inoculation under laboratory conditions, an apple fruit bioassay was developed. The apple fruit bioassay was modified according to previous bioassays on carrot and mango against *Pythium coloratum and L. theobromae*, respectively (El-Tarabily *et al.,* 1997; Saeed *et al.,* 2017a; Kamil *et al.,* 2018). Healthy apple fruits (*cv. Granny Smith)* were washed with sterile distilled water, surface-sterilized with 70% ethanol and placed in plastic trays on wet, sterile filter papers. Apple fruits were then inoculated using agar plugs (11 mm) colonized by the selected fungicide and/or *N. dimidiatum,* as described above, onto each apple fruit according to the following combinations: (i) two sterile non-inoculated PDA agar plug (control; C); (ii) *N. dimidiatum* alone with a sterile PDA agar plug below it; (iii) the fungicide alone with

a sterile PDA agar plug above it; and (iv) pairing *N. dimidiatum* and the fungicide together (the fungicide on the apple surface and *N. dimidiatum*-inoculated plug on top of the fungicide). All fungicides were introduced onto the apple surface 24 h before inoculation with the pathogen to have enough time for the active ingredients to disperse uniformly onto the apple surface. Each apple fruit was inoculated with the four combinations for each fungicide of five fruits/tray and was replicated three times. Trays were covered with aluminum foil and incubated in dark (at 25 ± 2°C and 80% relative humidity) for 10 d. Lesion diameters were measured (in mm) and averaged.

In a greenhouse experiment were assessed the impact of each fungicide on one-year-old *D. regia* seedlings were done. Seedlings were wounded and inoculated with agar plugs containing mycelium of *N. dimidiatum* in the stem of each plant as described above. Inoculated plants were maintained in the greenhouse at 25°C until symptoms were evident. At 2 wpi, seedlings were either sprayed with 250 ppm fungicide or water (control); and these treatments were designated as 0 Weeks Post Treatment (wpt). Symptoms on inoculated plants, conidia counts of the fungal pathogen and the number of falling leaves were recorded at 4 wpt (Saeed *et al.,* 2017a). The procedure of conidia counts involved homogenized weight of affected tissues placed in 5 mL of water, and the suspended material was assessed to estimate the number of conidia using haemocytometer (Agar Scientific Limited, Essex, UK).

Regarding the field experiments, trees were located in the same place described above. Cidely® Top (Syngenta International AG, Basel, Switzerland) was the only tested fungicide on six *D. regia* trees (8 years old). Each *N. dimidiatum* naturally infested tree was chosen to be surrounded by untreated corresponding trees to serve as a reservoir for recontamination. Trees were pruned and completely sprayed/treated

with the recommended dose of the fungicide (250 ppm). Experiments were repeated twice in February 2018 and February 2019 with similar results.

2.2.6 Statistical Analysis

For the pathogenicity assays, fruits (n = 5) and seedlings (n = 9) for each treatment were used. For the *in vitro* evaluation of fungicides against *N. dimidiatum*, 6 plates for each treatment were used. For the fungal conidia counts and the number of falling leaves in the *in vivo* evaluation of fungicides under greenhouse conditions, a minimum of 4 plants for each treatment was used. Data represent the mean ± SD. Analysis of Variance (ANOVA) and Duncan's multiple range test were performed to determine the statistical significance at $P<0.05$. All experiments were independently repeated three times with similar results. All statistical analyses were performed by using SAS Software version 9 (SAS Institute Inc., Cary, NC, USA).

2.3 Results

2.3.1 Symptoms of Stem Canker Disease on *D. regia*

Disease symptoms of stem/branch cankers associated with dieback were observed in the orchard of *D. regia* distributed in Dubai Festival City (DFC), UAE (Figure 1A). Apparently, the pathogen was able to attack different tissues of *D. regia*, and the trees were severely affected leading to progressive dieback. In general, cankers on branches were detected in young trees. Stem cankers were observed in old and mature trees, and were associated with pruning wounds and other wounds (Figure 1A).

Figure 1: Symptoms of stem canker on trees of *Delonix regia*.

(A) Severe symptoms of canker (left) and dieback (right); (B) typical longitudinal canker symptoms on stem; (C) gumming symptoms of the disease on the bark with fungal growth apparent beneath periderm; (D) main stem with the black stromata where the periderm has peeled away; (E) canker associated with internal symptoms in the trunk; and (F) affected vascular tissues. In (A–F), naturally infested royal poinciana trees with *N. dimidiatum* in Dubai Festival City (DFC), United Arab Emirates (UAE).

Cankers were developed longitudinally (Figure 1B), causing dark discoloration of xylem tissues and extensive gumming (Figure 1C). The main stem was often associated with black stromata, resulting the epidermis to peel away (Figure 1D). The discoloration continued outward, rotting symptoms led to spur and shoot blight was also observed. Sap was initially amber in color but later became dark. Internally, canker (Figure 1E) and affected vascular tissues (Figure 1F) were associated with this disease. Eventually, all *D. regia* trees were simultaneously found infected in the orchard (Figure 1A). These signs on *D. regia* are typical of stem canker that is known to be caused by a soil-borne wound pathogen. Therefore, attempts to isolate the

putative pathogen from diseased *D. regia* was the first step in identifying the causal agent of this disease.

2.3.2 Molecular Characterization and Identification of Pathogen

First, the fungus from different symptomatic tissues on Potato Dextrose Agar (PDA) were isolated. From the cultural characteristics, the fungus grew and colonized the plate rapidly. It produced cream to white effuse, hairy to woolly colonies after 2 days of incubation (Figure 2A). The colonies turned olive green, greyish to ochraceous yellow color after 4 days. The fungus showed dark grey to black pigmentation at 8 and 12 days (d.) of incubation, respectively (Figure 2A). Microscopically, mycelial growth were observed (Figure 2B) and production of *scytalidium*-like anamorph of different maturity stages of arthoconidia segmenting from the hyphae (Figure 2C). Various conidial shapes ranging from ellipsoid to ovoid, rod shaped or round shaped, to hyaline with an acutely rounded apex, truncate base were also noted. Conidia were initially aseptate and brownish; at maturity, 0- to 2-septate, central cells were darker than the end cells, measuring $11.02 \pm 0.33 \times 4.98 \pm 0.41$ µm (Figure 2C). Conidiogenous cells, or pycnidial anamorph, were described as hyaline and intermingled with paraphyses, forming pycnidiospores after 25 days of incubation (Figure 2D). Cultures also produced fusicoccum-like conidia in pycnidia (Figure 2E). Together, the cultural and morphological characteristics suggest that this fungal isolate may belong to *N. dimidiatum spp*. (Farr et al., 2005). Thus, molecular characteristics can identify the fungal specimen at the species level.

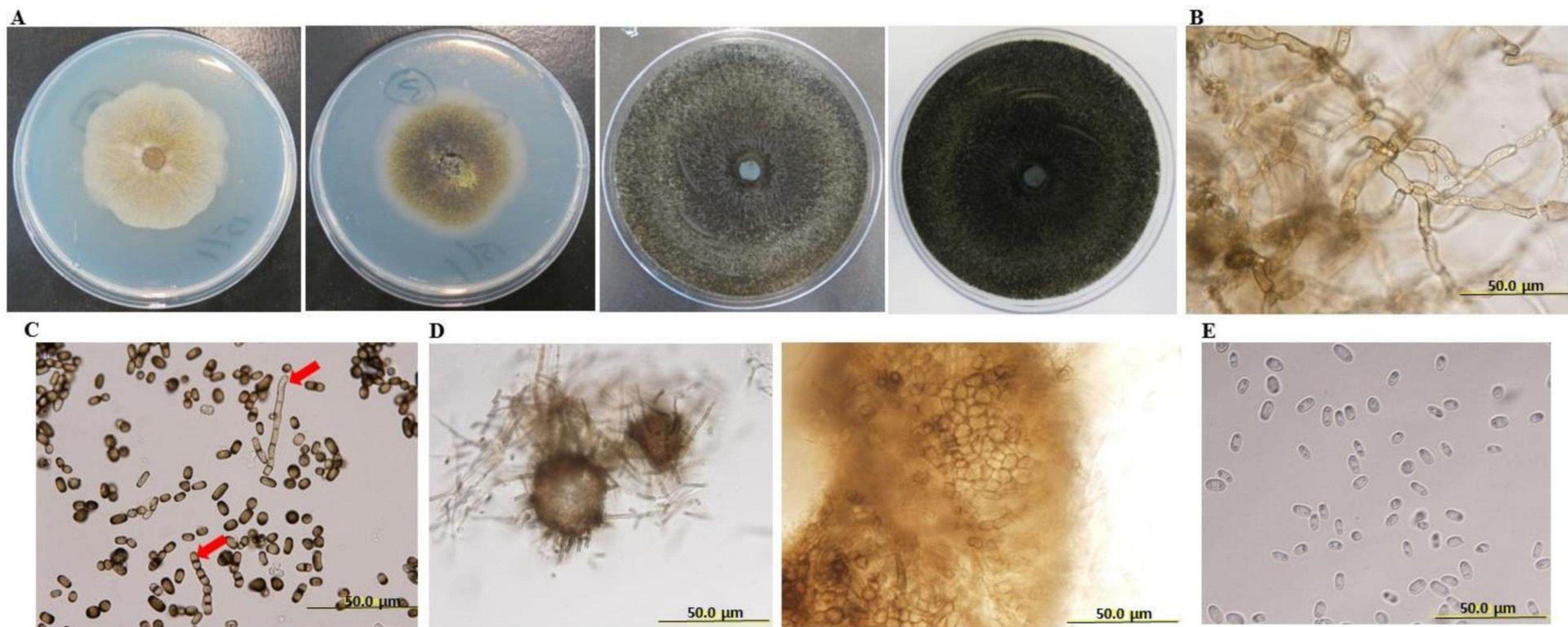


Figure 2: Cultural and morphological characteristics of *Neoscytalidium dimidiatum*

(A) Colonies on PDA (left to right: 2, 4, 8 and 12 days of incubation at 25 ± 2°C); (B) mycelia; (C) scytalidium-like anamorph showing various shapes and maturity stages of arthroconidia (red arrows) segmenting from hyphae; (D) pycnidia formed on a 25-day-old colony (left) and pycnidiospores (right) on PDA; and (E) fusicoccum-like pycnidial conidia (immature).

DNA-based methods are widely used to detect and identify plant pathogens. First, the fungal DNA were isolated from the PDA-grown mycelium from each tissue (stems, branches and leaves) sample. Polymerase Chain Reaction (PCR) amplification using primers targeting the genomic regions of Internal Transcribed Spacer (ITS), 28S rDNA region, Translational Elongation Factor 1-α (TEF1-α) and β-tubulin was performed. The amplification product of all tested genes was clearly generated in all tested specimens (Figure 3A). Because there were no available DNA sequences about the strain isolated from the UAE, the ITS and TEF1-α genes were further sequenced (White *et al.,* 1990). Sequences obtained from ITS/LSU rDNA and TEF1-α genes were also deposited in GenBank under the accession number, MN371844 and MN447201, respectively. The data suggest that *N. dimidiatum* spp. is probably the potential fungal pathogen commonly associated with stem canker disease symptoms on D. regia trees.

Second, a phylogenic tree using the obtained TEF1-α sequence was compared to other closely related sequences in order to determine the relationship with closely TEF1-α related sequences coming from other *N.dimidiatum spp.* the TEF1-α sequence of the strain isolated from the UAE grouped in a clade representing *N. dimidiatum* (Penz.) Crous & Slippers (Crous *et al.*, 2006) (Figure 3B). Results of the Maximum Likelihood (ML) tree indicated that the isolate, in the current study, showed >99% identity with the other isolates of *N. dimidiatum*. These isolates have been collected from different plant species such as *Juglan regia* (CBS 251.49), *Prunus sp.* (CBS 204.33), *pacific madrone* (Arbutus menziesii; CBS 204.33 and CBS 499.66), mango (*Megnifera indica*; CBS 499.66) and others (CBS 125616, CBS 125695 and DSM 104095). The identified fungal species and the other *N. dimidiatum* separately clustered from the two other species of *Botryosphaeriaceae*, *N. novaehollandiae* and *N. hyalinum*; thus, this isolate was identified as *N. dimidiatum*. Together, this suggests

that *N. dimidiatum* (DSM 109897) is most likely the causal species of stem canker disease on *D. regia* .

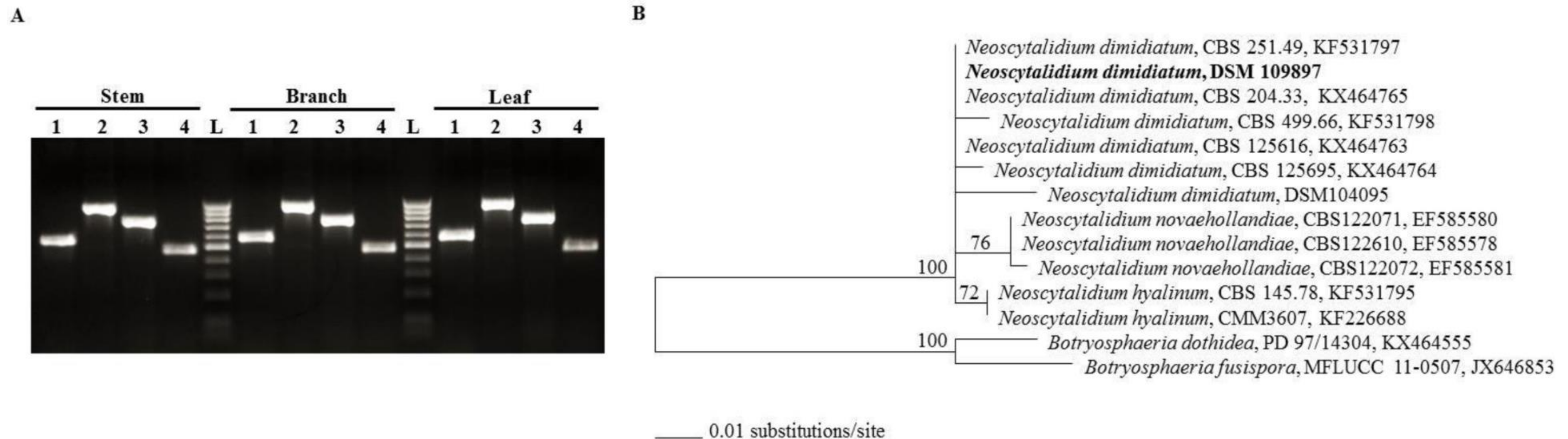


Figure 3: Molecular identification of *Neoscytalidium dimidiatum*

PCR amplification of specific genomic DNA regions of infected stem, branch and leaf tissues (A); and dendrogram showing phylogenetic relationships among *Neoscytalidium dimidiatum* (DSM 109897) identified in this study and other members of *Neoscytalidium dimidiatum* spp. prepared by the Maximum Likelihood (ML) method (B). In (A), lanes 1-4 correspond to amplifications of ITS, 28S rDNA region, TEF1-α and β-tubulin, respectively, in trunk (stem), branches and leaves. In (B), the ML tree was obtained from TEFα-1 sequence data. The specimens used in this study carry GenBank accession number, *Neoscytalidium dimidiatum* TEF1-α (MN447201). Numbers at the nodes are bootstrap values after 100 replicates are expressed as percentages (LnL = −603.684353). Only values above 70% are indicated. The scale bar on the rooted tree indicates a 0.01 substitution per nucleotide position. The strain of *Neoscytalidium dimidiatum* from this report is indicated in bold. *Botryosphaeria dothidea,* PD 97/14304 (KX464555) and *B. fusispora* MFLUCC 11-0507 (JX646853) were used as outgroups. ITS, internal transcribed spacer; 28S rDNA, large subunit (LSU) of rDNA; TEF1-α, translational elongation factor 1-α; L, DNA ladder.

2.3.3 Pathogenicity Tests of *N. dimidiatum* on *D. regia* Seedlings and Apple Fruits

Disease progress on one-year-old *D. regia* seedlings inoculated with 8-mm mycelial discs from 10-day-old pure culture of *N. dimidiatum* growing on PDA was regularly monitored in the greenhouse. Based on artificial inoculations, pathogenicity tests led to the development of disease symptoms on *D. regia* seedlings (Figure 4A–C). Typical symptoms of stem canker developed at the point of inoculation on the stem on plants following *N. dimidiatum* infection. At 2 Weeks Post Inoculation (wpi), dark brown lesions formed on the surface of the stem, leaves became pale, turned yellowish in color and dropped off (Figure 4A). The disease progressed upward along the stem with black, necrotic lesions appeared at the site of inoculation; subsequently the infected stem rotted at 5 wpi. In addition, a general dryness in the plant was recognized forcing the leaves to fall (Figure 4B). In contrast, no symptoms were noticed in control seedlings. The pathogen was consistently re-isolated from all inoculated tissues and identified by conidial morphology, fulfilling Koch′s postulates (Figure 4C).

Under laboratory conditions, apple fruits were also inoculated with the same pathogen. At 5 Days Post Inoculation (dpi), it is observed discoloration of apple tissues which expanded slowly underneath the PDA plugs containing the pathogen (Figure 4D). After 10 dpi, the fungus grew into apple tissues causing rapid spreading water-soaked lesions. By peeling away the skin from the discolored tissue and placing it on PDA Petri dishes, pure cultures recovered and conidia of *N. dimidiatum* were re-isolated (Figure 4E). No disease symptoms were evident on the same apple fruit under the control plug without the pathogen at 5 and 10 dpi (Figure 4D). Altogether, disease symptoms associated with the inoculated *D. regia* seedlings and apple fruits suggest

that the Koch's postulates are fulfilled and that *N. dimidiatum* is most likely the causal agent of the stem canker disease on *D. regia*.

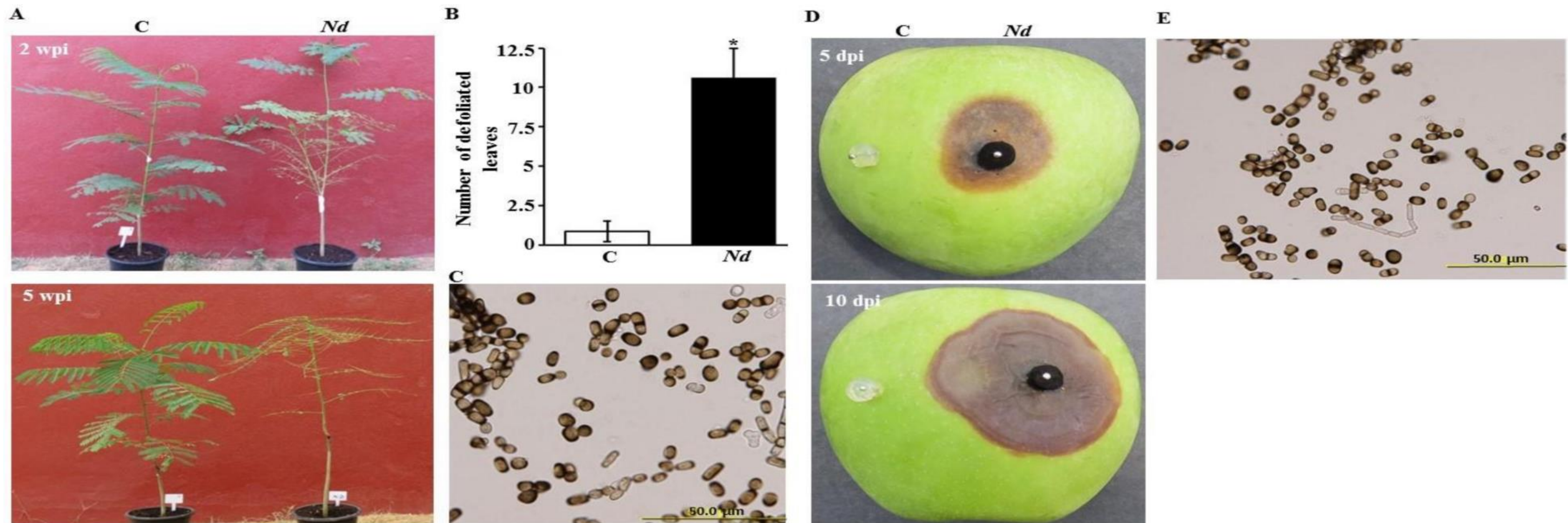


Figure 4: Development of canker on *Delonix regia* seedlings and apple fruits following artificial inoculation with *Neoscytalidium dimidiatum*

Pathogenicity test on *Delonix regia* seedlings inoculated (Nd; right) and non-inoculated (C; left) with *Neoscytalidium dimidiatum* at (A) 2 and 5 wpi; (B) number of defoliated leaves of inoculated and control seedlings; and (C) conidia after re-isolation of the pathogen from colonized stem tissues, at 5 wpi. Pathogenicity tests on (D) inoculated (right) and non-inoculated (left) apple fruits at 5 and 10 dpi; and (E) conidia of the pathogen from the inoculated apple fruits at 10 dpi. In (B), mean values followed by an asterisk are significantly different from control treatment at the tested time ($P<0.05$). Experiments were repeated at least three times with similar results. C, control (no *N. dimidiatum*); Nd, *N. dimidiatum*.; dpi/wpi, days/weeks post inoculation.

2.3.4 *In Vitro* Evaluation of Chemical Fungicides to *N. dimidiatum*

To determine their effects on the mycelial growth of *N. dimidiatum*, PDA plates containing a final concentration of 0, 250, 500 and 1000 ppm of the chemical fungicides -available in the market- were evaluated *in vitro* (Figure 5). In general, varied response of *N. dimidiatum* to the selected fungicides were noticed. For example, application of the fungicides, Penthiopyrad®, Proxanil®, Protoplant® and Previcur® at 250 ppm (the lowest tested concentration) showed minimal or no effect on the mycelial growth of the fungus (Figure 6A). When the chemical fungicides Amistar Top®, Uniform®, Cidely® Top, Protifert® and Airone Liquido® were, however, supplied in PDA medium, there was greater inhibition in the mycelial growth of *N. dimidiatum* at all the concentrations examined *in vitro* (Figure 5) including the concentration of 250 ppm (Figure 6A). These promising fungicides were also statistically ($P<0.05$) assessed at the concentration of 250 ppm for their efficacy to inhibit the growth of *N. dimidiatum in vitro*. Among the five fungicides, medium containing a final concentration of 250 ppm of either Cidely® Top or Protifert® demonstrated more than 85% inhibition in growth of *N. dimidiatum*, indicating that both fungicides were considered the most efficient fungicides (Figure 6B). Although the growth inhibition rate (Mi%) of *N. dimidiatum* at 5 dpi reached to 77–79% after the application of Amistar Top® and Airone Liquido® fungicides, Uniform® showed the lowest zone of inhibition (22%). This suggests that the latter fungicide is the least efficient; and therefore it is eliminated from further experiments. The fungal pathogen were also examined microscopically in order to figure out the mode of action of the effective fungicides against *N. dimidiatum*. Results revealed that three fungicides caused significant alternations in the fungal morphology. In comparison to control

treatment without any fungicide, application of either Amistar Top® or Cidely® Top at 250 ppm concentration to cultures led to lysis in hyphal wall and leakage in cytoplasm of *N. dimidiatum* (Figure 6C). It is also noticed that Airone Liquido® caused not only unusual morphological abnormalities in cultures, but also septal defects and cytoplasmic deformations in hyphal cells. Surprisingly, it was observed that normal, septate hyphal morphology in cultures containing Protifert® similar to those in control treatment.

N. dimidiatum produced not only reduced numbers of deformed conidia, but also absences of arthroconidia in Amistar Top®- or Cidely® Top-treated cultures (Figure 5D). Similar to control, cultures of Airone Liquido and Protifert® showed normal conidial formation and well-formed arthroconidial segmentation produced by hyphae of *N. dimidiatum*. Altogether, the chemicals, Amistar Top®, Cidely® Top and Airone Liquido, had a direct effects on *N. dimidiatum* DSM 109897 through the inhibition of mycelial growth and induction of morphological abnormalities; thus, the former two fungicides shared a common mechanism of action. The mode of action of Protifert® in competently inhibiting the mycelial growth of *N. dimidiatum* was not determined. Because there are many reports in which chemical control against plant pathogens has proven successful only under laboratory conditions, more reliable *in vivo* studies are needed for the reproducibility of the results obtained from those of *in vitro* testing.

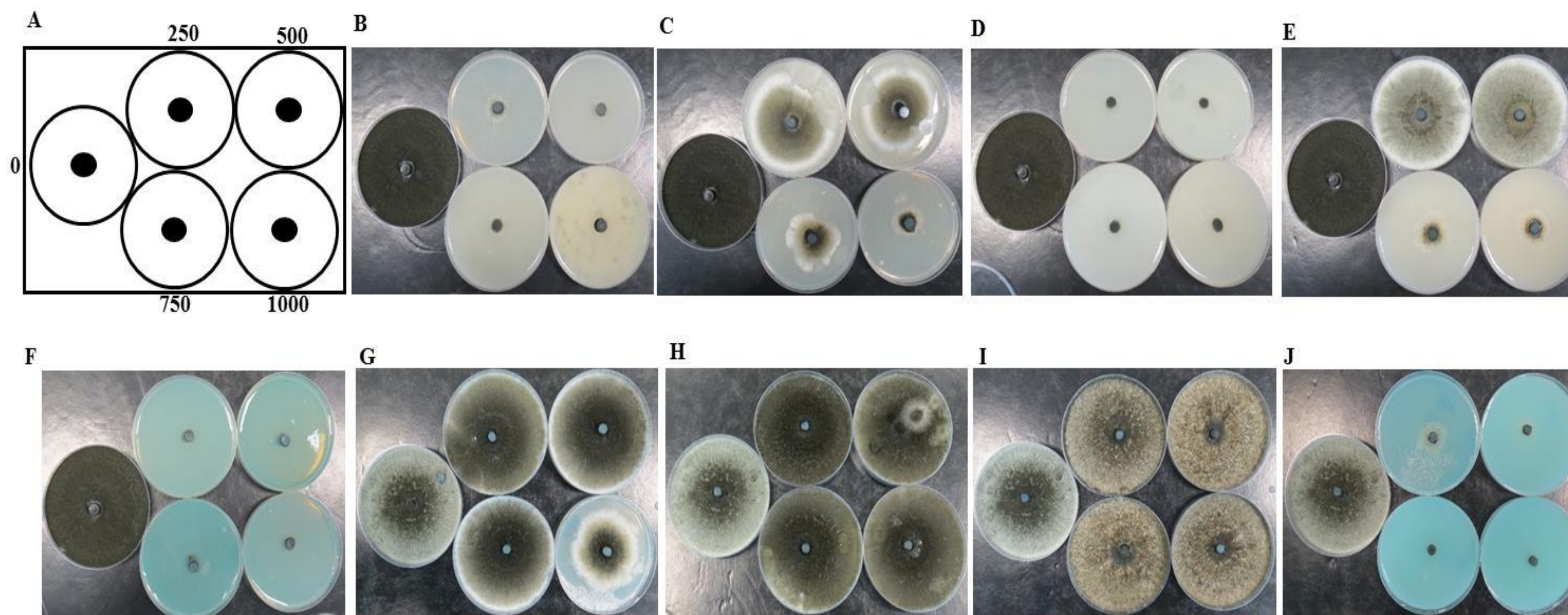


Figure 5: Growth inhibition effect of fungicides on *Neoscytalidium dimidiatum*

(A) An illustration showing inoculated-PDA plates containing different concentrations (in ppm) with the colonized *N. dimidiatum* agar plugs. Growth inhibitory effect on *Neoscytalidium dimidiatum* using the fungicides: (B) Amistar® Top; (C) Uniform®; (D) Cidely® Top; (E) Penthiopyrad®; (F) Protifert®; (G) Proxanil®; (H) Proplant®; (I) Previcur®; and (J) Airone Liquido® on PDA pales. In (A), black circles represent colonized *N. dimidiatum* agar plugs. In (B-J), photos were collected 5 days after inoculation.

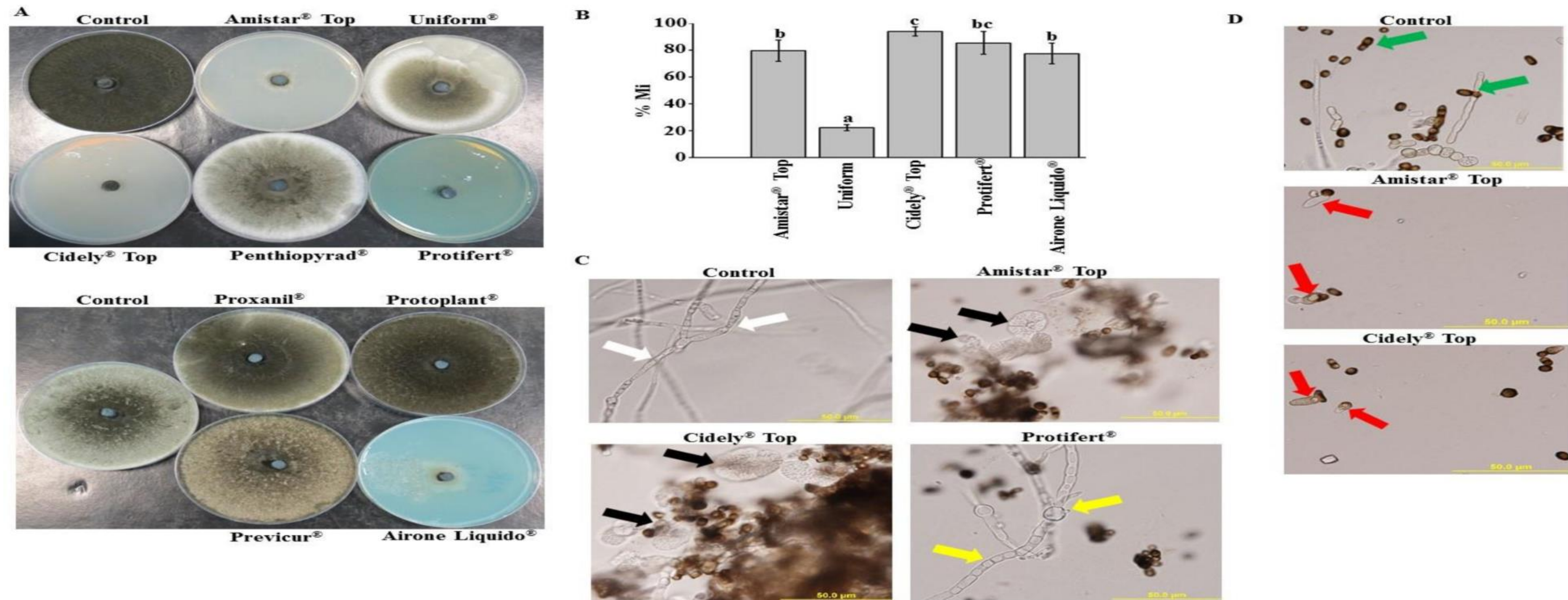


Figure 6: *In vitro* efficacy of fungicides against *Neoscytalidium dimidiatum*

(A) Effect of the fungicides Amistar Top®, Uniform®, Cidely® Top, Penthiopyrad®, Protifert® (top panel), Proxanil®, Proplant®, Previcur® and Airone Liquido® (bottom panel) at the c concentration of 250 ppm on *in vitro* mycelial growth; and (B) growth inhibition rate (% Mi) of *N. dimidiatum* using 250 ppm of the fungicides after 5 days. (C) Abnormalities in hyphal morphology, septum formation and cytoplasmic contents; and (D) deformation of conidia of *N. dimidiatum* following Amistar Top® and Cidely® Top treatments compared to control. In (B), values with different letters are significantly different from each other at $P<0.05$; In (C), white arrows indicate normal septate hyphal growth; black arrows indicate formation of non-septate hyphal formation and cytoplasmic coagulation; yellow arrows indicate lysis of hyphal wall and cytoplasm leakage. In (D), green arrows indicate normal formation of conidia and arthroconidia segmenting from hyphae; and red arrows indicate deformation of conidia and absence of arthroconidia.

2.3.5 Assessment of Chemical Fungicides on *N. dimidiatum* Using Apple Bioassay

To evaluate the most effective fungicides against *N. dimidiatum*, the apple fruit bioassay method were developed (Figure 7A). Placing the pathogen alone on apple fruits resulted in relatively large-sized, brown-colored lesions with distinct edges (Figure 7B). In contrast, none of the fungicides tested had negative effects against the pathogen. Excluding Airone Liquido®, when a plug containing any of the three fungicides paired with a plug of *N. dimidiatum* on the surface of the fruit, the particular fungicide completely suppressed the pathogen and no lesions were formed compared to the pathogen treatment alone (Figure 7B).

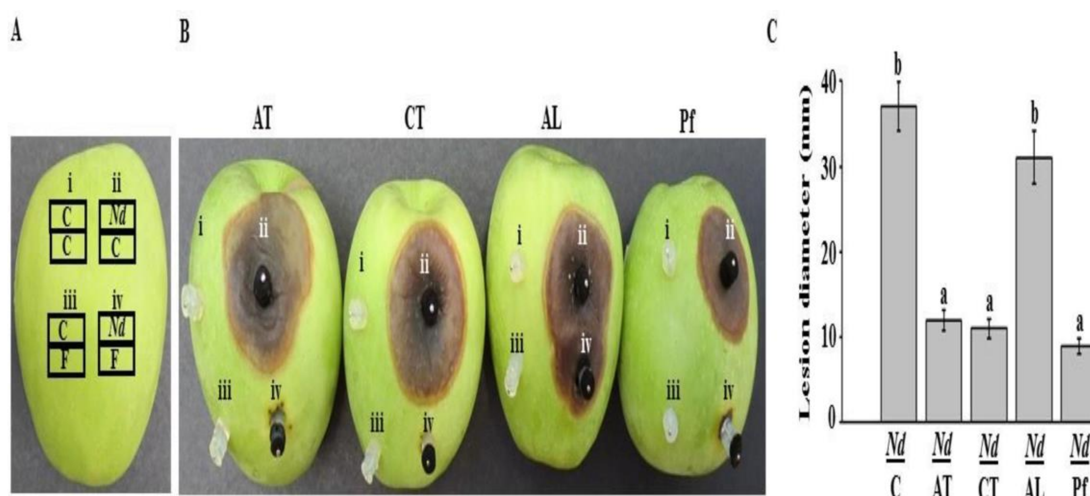


Figure 7: *In vivo* inhibitory effect of the chemical fungicides against *Neoscytalidium dimidiatum* using the “apple fruit bioassay”

An illustration showing (A) inoculated-apple fruit with the chemical fungicides and/or *N. dimidiatum* agar plugs in combinations; (B) apple fruit bioassays using chemical fungicides; and (C) lesion diameter of *N. dimidiatum* using 250 ppm of the fungicides after 10 dpi. In (A–B), (i) two sterile non-inoculated PDA agar plugs; (ii) *N. dimidiatum* inoculum alone with a sterile agar plug below it; (iii) the fungicide (F) alone with a sterile agar plug above it; and (iv) pairing *N. dimidiatum* and the fungicide together, with the fungicide on the apple surface and *N. dimidiatum*-inoculated plug on top of the fungicide. In (C), values with different letters are significantly different from each other at $P<0.05$. C, control (no *N. dimidiatum*); Nd, *N. dimidiatum*; F, fungicide; AT, Amistar Top®, CT, Cidely® Top; AL, Airone Liquido®; Pf, Protifert®; dpi, days post inoculation.

The fungicides Amistar Top®, Cidely® Top and Protifert® caused significantly (P<0.05) smaller lesion sizes than the positive control (*N. dimidiatum*) treatment (Figure 7C). However, there was no any significant (P<0.05) difference between the treatments of Airone Liquido® and the pathogen alone. Therefore, Airone Liquido® was excluded from further experiments. To greater extent, three chemical fungicides completely prevented lesion development on apple fruits. Overall, the novel apple fruit bioassay led to the selection of three prominent fungicides, Amistar Top®, Cidely® Top and Protifert®, which could have the potential to manage stem canker disease on *D. regia* seedlings.

2.3.6 Fungicide Effects on *D. regia* Infected with *N. dimidiatum*

In the greenhouse experiment, the efficacy of the most promising fungicides at 4 Weeks Post Treatment (wpt) on *N. dimidiatum*-inoculated *D. regia* plants were tested. Seedlings were artificially inoculated with the fungal pathogen for 2 weeks when symptoms of stem canker disease were easily recognized (Figure 8). Diseased plants were treated with a particular fungicide and this treatment was considered as 0 wpt. Disease progress or plant recovery of fungicide-treated plants was monitored until the end of the evaluation period of 4 wpt. In general, *N. dimidiatum*-inoculated plants that were sprayed with water only showed stem canker disease symptoms such as drying branches, falling leaves and discoloring stems, resulting in almost completely bare seedlings (Figure 8A). This was also clear in the longitudinal wood necrosis in these diseased plants (Figure 8B). In contrast, inoculated plants that were treated with Amistar Top®, Cidely® Top or Protifert® fungicide clearly showed vegetative growth recovery (Figure 8A) and developed relatively healthy wood (Figure 8B) at 4 wpt comparable to the negative control plants (no prior artificial infection). Affected plants

treated with Airone Liquido® showed similar disease symptoms as diseased plants (Figure 8A). Peeling away the periderm of the inoculated plants that were treated with Airone Liquido® revealed the presence of a black layer of fungal growth from which *N. dimidiatum* was re-isolated (Figure 8B).

The effects of each of the chemical fungicides were also determined according to the number of conidia progressing on diseased- and treated-seedlings. In general, there was a significant ($P<0.05$) difference between all treatments (Figure 8C). This was accompanied with a dramatic decrease in the number of conidia in Cidely® Top-treated seedlings that nearly reached to 6-fold reduction compared to that of untreated plants. It is noticed that the number of conidia of *N. dimidiatum* recovered from the stems of *D. regia* treated with Protifert® and Amistar Top® fungicides was 3.3- and 2-fold less than in the control, respectively (Figure 8C). Airone Liquido® was marked the least spore counts; and thus, it was considered the least effective among all tested fungicides.

The number of defoliated leaves was also assessed on diseased- and recovered-seedlings as an indication on the severity of disease symptoms on seedlings at 4 wpt. Based on these results, Cidely® Top treatment was comparable to the treatment without inoculation (Figure 8D). This was evident by the similar number of defoliated leaves per plant. On the other hand, the same plants showed significantly ($P<0.05$) less falling leaves than inoculated-seedlings without fungicide treatment at the same period of evaluation. At 4 wpt, defoliated leaves demonstrated 31–42% reduction on seedlings sprayed with Amistar Top® and Protifert®, respectively, in comparison to *N. dimidiatum*-inoculated seedlings without any fungicide treatment (Figure 8D). It was also clear that Airone Liquido® was not efficient enough, confirming the previous results on the number of conidia recovered from inoculated seedlings using the same

fungicide. The data imply that Cidely® Top seems to be the most effective fungicide because the severity of stem canker disease is gradually suppressed and the pathogen is more or less restrained.

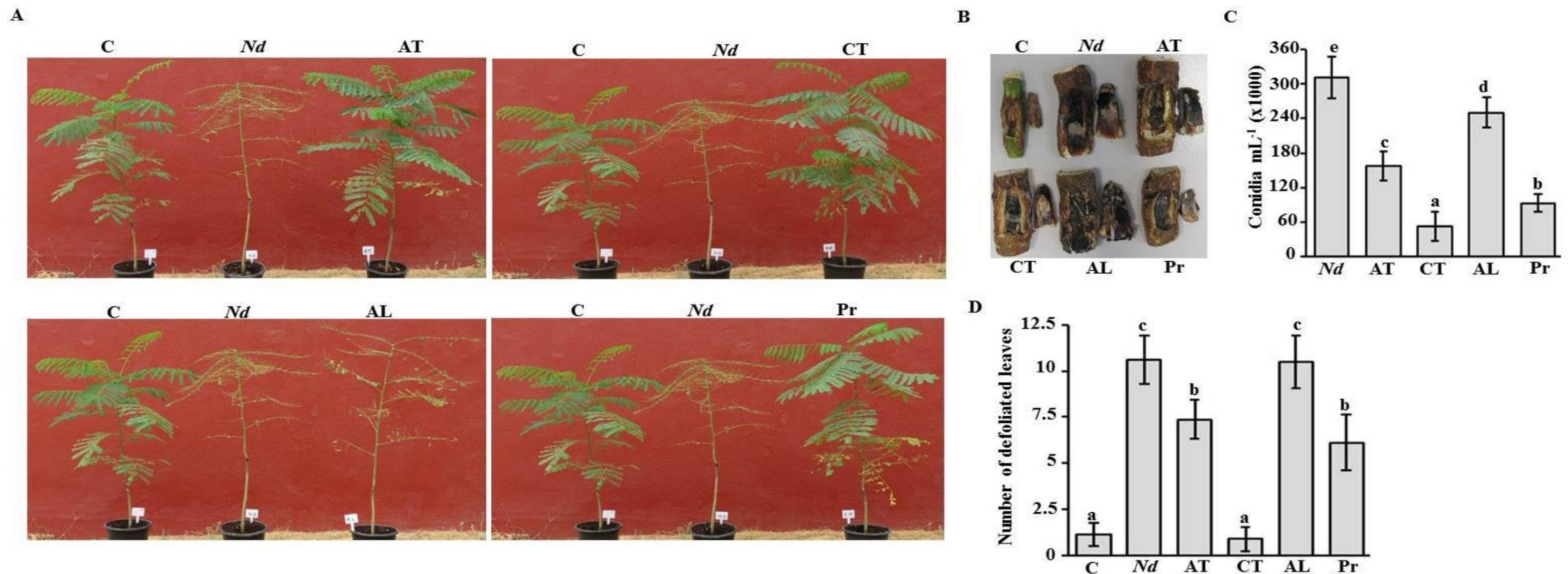


Figure 8: Effect of fungicide treatments on artificially inoculated *Delonix regia* seedlings with *Neoscytalidium dimidiatum* in the greenhouse

Fungicidal suppression of stem canker disease on *Delonix regia* seedlings using (A) potential chemical fungicides; (B) symptoms of inoculated regions; (C) number of conidia after recovery of the pathogen from stem tissues; and (D) number of defoliated leaves in inoculated seedlings sprayed with chemical fungicides at 4 wpt. In (A–D), seedlings were inoculated for 2 weeks with *N. dimidiatum* before the fungicide treatment. In (C & D), mean values with different letters are significantly different from each other at P<0.05. Experiments were repeated at least three times with similar results. C, control (no *N. dimidiatum*); Nd, *N. dim*idiatum; AT, Amistar Top®: CT, Cidely® Top; AL, Airone Liquido®, Pr, Protifert®; wpt, weeks post treatment.

2.3.7 Effect of Cidely® Top on *D. regia* Trees Naturally Infected with *N. dimidiatum*

The results obtained from the *in vitro* and *in vivo* experiments were confirmed by applying the promising fungicide Cidely® Top on *D. regia* trees naturally affected by stem canker under field conditions. *D. regia* trees were sprayed with 250 ppm of Cidely® Top, and severity of symptoms or recovery of the trees was monitored for 32 weeks. Typical disease symptoms were observed on the day of fungicidal application (0 wpt; Figure 9A). After 16 weeks of spraying with Cidely® Top, disease severity was remarkably decreased in the treated trees (Figure 9B). This was evident by diminishing trunk damage and developing new fresh shoots. It was also noted that trees treated with Cidely® Top fungicide increased their vegetative growth and were completely recovered at 32 wpt (Figure 9C). This suggests that the application with Cidely® Top results in disappearance of disease symptoms, ultimately leading to nice looking, healthy trees.

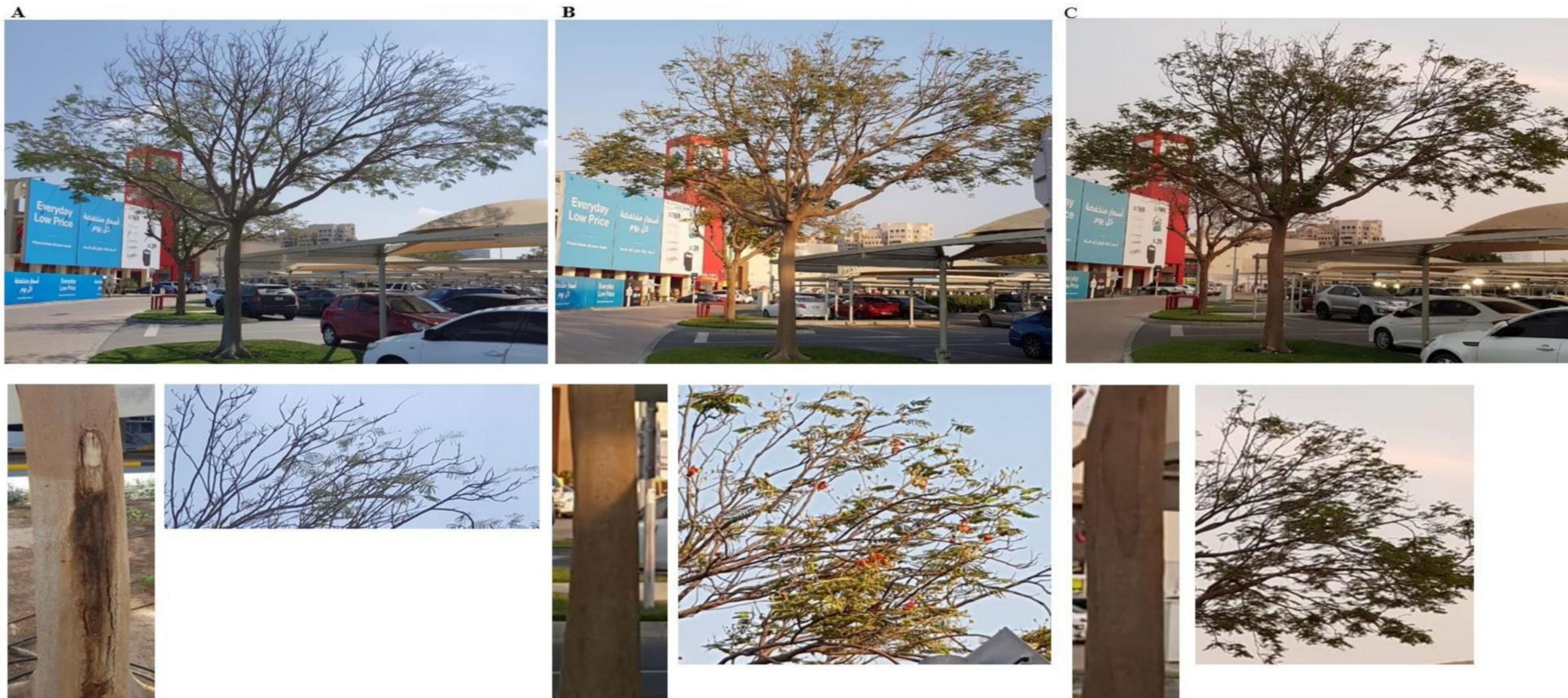


Figure 9: Effect of Cidely® Top on *Delonix regia* trees naturally infected with *Neoscytalidium dimidiatum* in the field in Dubai Festival City, United Arab Emirates

Fungicidal suppression of stem canker disease symptoms on (A) *Delonix regia* trees (n = 6); followed by treatment with the fungicide Cidely® Top at (A) 0 (B) 16 and (C) 32 weeks post treatment. In (A–C), photos showed the severe disease symptoms and the recovery of the same whole tree (upper panel), trunk (left, bottom panel) and branches (right, bottom panel).

2.4 Discussion

D. regia is a large deciduous tree species prevalent in subtropical and tropical areas of the world. It is valued as a local street tree and is widely planted in open areas (Gledhill, 2008). In the last decade, this beautiful flowering plant has become widespread in urban and agricultural areas of the UAE. Although it is known for its ability to withstand severe conditions, diseases are major factors that affect the health of *D. regia* (Cayley, 1923; Gilman & Watson, 2019). Many of the phytopathogens can cause diseases on host plants, including *D. regia* (Anagnostakis & Ferrandino, 1998; Chungu *et al.*, 2010; Darge, 2017; Rouxel & Balesdent, 2005). Therefore, careful attention should be attained to the causal agent of stem canker disease on *D. regia*, taking into account the frequency of disease incidence, the geographical distribution and the environmental conditions favorable to the disease occurrence.

In efforts to identify the pathogen linked with the diseased trees, first detected the symptoms of stem canker on *D. regia* were done. In general, dieback, canker and gummosis, which ultimately led to complete dryness and death of *D. regia* trees were noticed (Figure 1). Although some studies have reported several fungi to cause cankers on plant species (Anagnostakis & Ferrandino, 1998; Chungu *et al.*, 2010; Darge, 2017; Rouxel & Balesdent, 2005), others have recorded *N. dimidiatum* on almond, dragon fruit, eucalyptus, fig and plum, displaying disease symptoms of canker and dieback in different places of the world (Al-Bedak *et al.*, 2018; Du *et al.*, 2019; Hajlaoui *et al.*, 2018; Nouri *et al.*, 2018). In general, environmental stress has negative impact on the severity of disease, depending on the level and duration of the stress, and the sensitivity and developmental stage of the plant species. In hot summers, sooty canker invades trees and ornamentals of mulberry, ash, walnut, fig, sycamore, apple, apricot, poplar,

eucalyptus and olive in Iraq (Hassan *et al.*, 2009, 2011). In Oman, significant damage due to dieback, witling and death of *D. regia* has been reported to be caused by *N. dimidiatum* and symptoms are even worsened when trees are exposed to heat (up to 45 °C) and shortage of water (Elshafie & Ba-Omar, 2002). All previously mentioned reports are in agreement with the findings of the current study. Yet, there are no reports about the causal agent of the disease symptoms of stem canker on *D. regia* or any other ornamental woody tree in the UAE. Previously, the fungal pathogens *T. punctulata* and *F. solani* have been shown to cause black scorch disease and SDS on date palm, respectively (Al-Hammadi *et al.*, 2019; Alwahshi *et al.*, 2019; Saeed *et al.*, 2016), and *L. theobromae* to cause dieback disease on mango (Saeed *et al.*, 2017b). Therefore, accurate fungal identification was carried out, along with proper chemical fungicide treatment to manage the devastating damage of this disease on *D. regia.*

The fungal pathogen was constantly isolated from all symptomatic tissues examined from trees of *D. regia*, and it was characterized based on its morphology, phylogeny and pathogenicity assays. On PDA, a rapid growth of mycelia filling the entire plate was observed within 8 days. The culture was effuse, hairy to wooly, started as white with creamy, ochraceous-yellowish color that turned to dark greyish or blackish color by day 12. Similar observations have been previously reported on *N. dimidiatum* isolated from diseased trees of eucalyptus (Al-Tememe *et al.*, 2019). Microscopic examination of the pathogen demonstrated branched and septate hyphae with no conidiophores. Consistent with Mohd *et al.* (2013), arthroconidia were thick-walled and barrel-shaped that could be found individually or in chains, ranging 5–15 x 3–6 µm in size (Figure 2). Old cultures, of 25 days, developed hyaline pycnidial conidia when young, and dark brown central regions when aged. Cultures also produced *fusicoccum*-like conidia in pycnidia (Figure 2) (Pavlic *et al.*, 2008). Because

N. dimidiatum spp. are very close and difficult to discriminate, molecular characterization was followed to avoid misleading conclusions about the pathogen. For that reason, phylogenetic analysis using TEF1-α sequence (MN447201) was generated and proved the identity of the fungus as *N. dimidiatum. N. dimidiatum* was closely related to both *N. novaehollandiae* (Polizzi *et al.*, 2009), and *N. hyalinum* (Madrid *et al.*, 2009), confirming previous findings (Al-Bedak *et al.*, 2018; Alwan & Hussein, 2019 The data indicated that the isolate of *N. dimidiatum* in the current study was morphologically and genetically similar to other isolates of *N. dimidiatum* from Juglan regia, Prunus sp., mango and others. Therefore, isolate DSM 109897 in the present study belonged to *N. dimidiatum* and was the main causal agent of stem canker on *D. regia* in the UAE. Observations were showed on the symptoms and the pathogen associated with stem canker disease on *D. regia* are similar to a previous report on the same tree in Oman (Elshafie & Ba-Omar, 2002). This suggests that *N. dimidiatum* may possibly have been introduced from this neighboring country to the UAE.

The existence of the pathogen and the progression of the disease in tissues of the whole *D. regia* seedlings and apple fruits were further verified via pathogenicity tests. The results obtained from the greenhouse experiment on young healthy plants after inoculation were similar to the disease symptoms on trees of *D. regia* located in the field, and that was confirmed by Koch's postulates when *N. dimidiatum* was frequently recovered from the inoculated seedlings. The data match those in other trials using artificial inoculation of the same pathogen on *D. regia* (Elshafie & Ba-Omar, 2002), or other plant species (Al-Bedak *et al.*, 2018; Chuang *et al.*, 2012; Du *et al.*, 2019; Hajlaoui *et al.*, 2018; Nouri *et al.*, 2018). Pathogenicity assays on seedlings of *D. regia* (Figure 4), *F. benjamina* and *F. nitida* (Al-Bedak *et al.*, 2018), and eucalyptus, poplar and olive (Hassan *et al.*, 2011), clearly described that discoloration of vascular

tissues, and drying and defoliation of leaves, were symptoms associated with stem canker caused by *N. dimidiatum*. There has been a rise in reports about *N. dimidiatum* causing diseases on fruits of pitahaya, plum and almond (Hajlaoui *et al.*, 2018; Nouri *et al.*, 2018; Yi *et al.*, 2015). Apple fruit bioassays have been conducted to determine the effects of the fungal pathogen associated with canker diseases (Alwan & Hussein, 2019; Anagnostakis & Ferrandino, 1998). Therefore, pathogenicity tests were performed on healthy apple fruits and monitored the disease progress.

There are some examples of using BCAs effective against *N. dimidiatum* or other pathogens (AbuQamar *et al.*, 2017; Mengiste *et al.*, 2010; Wan *et al.*, 2017); yet these studies have not been assessed *in vivo*. For example, *Trichoderma harzianum* T3.13 revealed *in vitro* antagonistic activities to *N. dimidiatum* (Wan *et al.*, 2017). Although chemical fungicides have adverse effect on human health, food and environment (Al-Tememe *et al.*, 2019; Budzinski & Couderchet, 2018), these agents are commonly used due to their relatively low cost, rapid acting, long lasting, high stability and ease of application (Kuai *et al.*, 2017). Under laboratory conditions, four of the tested chemicals, Amistar Top®, Cidely® Top, Protifert® and Airone Liquido®, showed suppression in the growth of *N. dimidiatum.* This was evidenced by the abnormalities seen in hyphal morphology, septal formation, cytoplasmic contents and the deformation of conidia following fungicide treatments (Figure 6). Previously, Cidely® Top exhibited the strongest inhibition of mycelial growth of *T. punctulata* and *L. theobromae* in petri dish experiments (Saeed *et al.*, 2016, 2017b). The same fungicides were further evaluated *in vivo* using apple fruit bioassay (Figure 7). In general, Amistar Top®, Cidely® Top and Protifert®, significantly reduced the lesion size on apple fruits when 250 ppm of the fungicide was applied concurrently with the pathogen. On contrast, Airone Liquido® was not effective against this

pathogen on apple and was carried out in further experiments as a negative control. Claimed were done that the novel apple bioassay is a small-scale reference of what may occur in the greenhouse/field. *In vivo* experiments using carrot roots and mango fruits have previously been implemented to assess growth retardation of *Pythium coloratum* and *L. theobromae* by BCAs, respectively (El-Tarabily *et al.*, 1997; Kamil *et al.*, 2018).

Recent reports have shown that *in vitro* tests along with greenhouse experiments are essential to determine the sensitivity of plant pathogens to chemical and/or biological treatments (Alwahshi *et al.*, 2019; Kamil *et al.*, 2018; Saeed *et al.*, 2016, 2017a, 2017b). According to the greenhouse experiments, Cidely® Top, followed by Protifert® and then Airone Liquido® were effective on diseased seedlings of *D. regia*. It is known that the organic foliar fertilizer, Protifert®, is a good source of minerals, essential traces, amino acids and peptides necessary for plant growth and development. In this study, it is also showed that Protifert® not only provided vigorous and healthy seedlings, but also it served as a protection to trees from fungal infections i.e., *N. dimidiatum*. Under greenhouse conditions, the most significant reduction in disease symptoms of stem canker was found in Cidely® Top-treated seedlings of *D. regia* at 4 wpt. This was clear in seedlings sprayed with Cidely® Top possessing the lowest conidial counts and the least number of defoliated leaves, indicating that this fungicide could be a potent fungicide for the management of *N. dimidiatum* affecting *D. regia* trees. The result of Cidely® Top is in agreement with previous studies indicating high effectiveness of this fungicide against a number of fungal pathogens attacking trees such as *T. punctulata, L. theobromae* and *F. solani* that were almost completely inhibited (Alwahshi *et al.*, 2019; Kamil *et al.*, 2018; Saeed *et al.*, 2017b). To a lesser extent, Amistar Top® was not as effective as Protifert® or Cidely® Top in

reducing the pathogenic activities of *N. dimidiatum* in greenhouse trials. Even though Amistar Top® and Cidely® Top were difenoconazole-based fungicides sharing the same concentration of the active ingredient; the superior efficiency of Cidely® Top over Amistar Top® could be attributed to the presence of cyflufenamid as an additional active ingredient leading to increased inhibition levels of *N. dimidiatum*. Difenoconazole was ineffective against *Fusarium magniferae* (Iqbal *et al.*, 2010), but it was significantly capable for managing other diseases (Khan *et al.*, 2004; Saeed *et al.*, 2017a, 2017b; Yanase *et al.*, 2013), including stem canker on *D. regia* in the current study (Figure 8). This can be disputed to the fungicide application methods, active ingredient concentrations, plant growth conditions or pathogen responses. Airone Liquido® (metal copper), on the other hand, is not recommended to manage the disease.

So far, there are no reports to evaluate Cidely® Top or any systemic fungicide on *D. regia* trees infected with *N. dimidiatum* under field conditions. Thus, the same fungicide was found to be highly effective against plant pathogenic fungi on date palm and mango (Alwahshi *et al.*, 2019; Saeed *et al.*, 2017a, 2017b). Accordingly, a field experiment was carried out to assess the efficacy of Cidely® Top on naturally infested *D. regia* plants. Apparently, the entire trees showed “more or less” full recovery that was mainly observed in newly developed inflorescences (branches with flower clusters) and reduced disease symptoms on trunks of *D. regia* trees sprayed with Cidely® Top at 16 and 32 wpt (Figure 9). This suggests that Cidely® Top can possibly serve as a competent element of IDM of stem canker on *D. regia*. The symptoms here were reported, the pathogen as well as the proper chemical treatment to manage stem canker as the first step toward planning IDM programs against this devastating disease on *D. regia* in the UAE or elsewhere. In the current study, the phenotype i.e.,

symptoms associated with the disease can be considered as a starting point for future comparative ‘omic’ analyses including genomes and responses to environmental variation (AbuQamar *et al.*, 2016). A combination of different methods to achieve suitable IDM practices is on top of priorities. Investigations for cultural (pruning), chemical (Cidely® Top and Protifert®) and BCAs as IDM to manage stem canker on *D. regia* are in progress for environmental sustainability.

Chapter 3: Evaluation of Streptomycete Actinobacterial Isolates as Biocontrol Agents Against Royal Poinciana Stem Canker Disease Caused by *Neoscytalidium dimidiatum*

3.1 Introduction

Royal poinciana (*Delonix regia*), also known as flamboyant tree, is a beautiful flowering tree of the pea family (Fabaceae) (Gledhill, 2008). Due to its colorful flowers, rapid growing and cooling shade during hot summers, *D. regia* has increasingly been planted not only in its native regions, but also in frost-free and tropical countries such as the United Arab Emirates (UAE) (Kirtikar & Basu, 1999).

In general, fungi can attack many hardy and ornamental trees causing canker diseases (Gilman *et al.,* 2019; Ali *et al.,* 2020). Cankers associated with the fungal pathogen, *Neoscytalidium dimidiatum*, have been recognized in many countries affecting a wide range of hosts. This includes almond (*Prunus dulcis*) in the USA (Nouri *et al.,* 2018), fig (*Ficus carica*) in Australia (Ray *et al.,* 2010), mango (*Mangifera indica*) in Niger (Reckhaus, 1987), and plum (*Prunus domestica*) in Tunisia (Hajlaoui *et al.,* 2018). In Oman and the UAE, stem canker caused by *N. dimidiatum* is a serious disease affecting shade and ornamental trees such as lebbeck (*Albizia lebbeck*) and *D. regia* (Elshafie & Ba-Omar, 2002; Al Raish *et al.,* 2020).

Stem canker disease symptoms of *D. regia* is mainly associated with progressive dieback that has been detected on young and old trees (Elshafie & Ba-Omar, 2002). Dark discoloration of xylem, extensive gumming and epidermis peeling from the stem are among the advanced signs of stem canker; eventually leading to the death of the tree (Al Raish *et al.,* 2020). In general, unfavorable environmental conditions and poor field management can also increase the disease progression (Reckhaus, 1987). Stem canker disease of *D. regia* causes significant damages to trees

and devastating losses of orchards. Consequently, actions in research and innovation should be immediately taken to control this devastating disease and avoid the spread of the fungal pathogen.

Nowadays, fungicides and chemical approaches play a predominant role in plant protection (Saeed *et al.,* 2016; 2017b, Behlau *et al.,* 2021; Yeng & Pahirulzaman, 2021). Thus, the abusive application of fungicides in agriculture has shifted the public concerns because of their harmful impact on the environment and the food chain representing a risk to ecosystems and human health. Eco-friendly horticultural and natural biological control practices can be used alternatively to or alongside of chemical compounds in Integrated Disease Management (IDM) strategies (Lopez-Escudero & Mercado-Blanco, 2011; Saeed *et al.,* 2017a; Kamil *et al.,* 2018; Vinchira-Villarraga *et al.,* 2021). As such, native natural enemies of plant diseases, also known as Biological Control Agents (BCAs), can effectively kill or lower pathogen populations (AbuQamar *et al.,* 2017; Ab Rahman *et al.,* 2018). *In vitro* studies were previously applied against *N. dimidiatum* using the filamentous fungus *Trichoderma harzianum* (Rusmarini *et al.,* 2017), and *Streptomyces* is the largest genus of actinobacteria that are biologically active in the soil (Barka *et al.,* 2016). Although some *Streptomyces* spp. are considered as BCAs (Saeed *et al.,* 2017b; Kamil *et al.,* 2018; Torabi *et al.,* 2019); others are recognized as Plant Growth Promoters (PGPs) (Ayswaria *et al.,* 2020; El-Tarabily *et al.,* 2020). Several mechanisms have been linked with the biological control of plant fungal pathogens by actinobacteria. Inhibition of pathogen growth can be associated with the production of Cell-Wall-Degrading Enzymes (CWDEs), antifungal metabolites and/or siderophores (El-Tarabily *et al.,* 2000; El-Tarabily 2006; Palaniyandi *et al.,* 2013), hyperparasitism (El-Tarabily *et al.,* 1997), and competition for nutrients (Cook & Baker, 1983). As soil-borne beneficial

microorganisms, actinobacteria can also induce resistance wherein pre-infection of plants can result in resistance against subsequent challenge of phytopathogens; this is termed as Induced Systemic Resistance (ISR) (Martínez-Hidalgo *et al.,* 2015). By increasing the productivity of biocontrol-based means, this creates more stable populations of BCAs, which in turn, ensures plant protection for sustainable and green agriculture.

Actinobacteria were intentionally selected as potential BCAs in the present study due to their bioactivity in the soil microflora in arid and semi-arid environments (Goodfellow & Williams, 1983; Barka *et al.,* 2016). Hypothesize made that Streptomycete Actinobacteria (SA) isolates can potentially serve as BCAs to manage stem canker disease on royal poinciana. The objectives of this research were to: (i) screen all isolated actinobacteria for their capability to produce diffusible antifungal metabolite(s) and/or CWDEs *in vitro* and to successfully suppress *N. dimidiatum*, (ii) select the potent SA isolates using apple fruit bioassays to evaluate their potential to reduce disease progression on fruits, and (iii) test the promising SA isolates with the particular mode of action(s) for their efficacies in controlling stem canker disease on *D. regia* seedlings under greenhouse conditions. The results demonstrated the potential to use selected SA isolates with more than a single mode of action as antagonists to be incorporated into sustainable IDM strategies to manage stem canker disease in *D. regia* orchards in the UAE and elsewhere.

3.2 Materials and Methods

3.2.1 Fungal Culture

The fungal pathogen *N. dimidiatum* (DSM 109897) was previously identified as the causal agent of *D. regia* stem canker disease in the UAE (Al Raish *et al.,* 2020).

The pathogen was cultured on Potato Dextrose Agar (PDA; Lab M Limited, Lancashire, United Kingdom) plates, supplemented with 25 mg l^{-1} ampicillin (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) to suppress bacterial contaminants. The fungus was sub-cultured every 10 days on PDA plates at 28°C.

3.2.2 Isolation of Streptomycete (SA) and Non-Streptomycete (NSA) Actinobacteria

Six different rhizosphere soil samples were collected from 30 cm depth under healthy *D. regia* trees, and air-dried for 5 days at 25°C (Williams *et al.,* 1972). The SA from each rhizosphere soil sample was isolated using the standard dilution plate method procedure on inorganic salt starch agar (Küster, 1959) supplemented with nystatin and cycloheximide at 50 µg ml^{-1} (Sigma-Aldrich) to eliminate growth of fungal contaminants. In order to increase the populations of SA and decrease other non-actinobacterial isolates, pre-treatments of soils were followed (Hayakawa & Nonomura, 1987). Five plates per dilution from each rhizosphere soil sample were incubated at 28°C in dark for 7 days.

For the recovery of Non-Streptomycete Actinobacteria (NSA) on inorganic salt starch agar plates, four polyvalent *Streptomyces* phages were used (Kurtböke *et al.,* 1992; El-Tarabily, 2006). The stock phage suspension of about 10^{12} plaque forming units ml^{-1} was prepared by mixing each of the individual high-titer phage suspension of each polyvalent *Streptomyces* phage. Five plates per dilution from each rhizosphere soil sample were inoculated with 0.2 ml aliquots of the phage-treated soil suspension, dried and incubated in dark at 28°C for 14 days. Plates without phages were considered as control.

Colonies representing SA and NSA (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). These SA and NSA colonies were tentatively identified according to their morphological characteristics, distribution of aerial and substrate mycelia, presence of aerial mycelia and stability of substrate mycelia (Cross, 1989).

3.2.3 *In Vitro* Bioassays for the Diffusible Antifungal and Cell Wall Degrading Enzymes (CWDE) Activities

Antifungal activities of all isolates were characterized based on the secretion of diffusible antifungal metabolites against *N. dimidiatum* using the cut-plug method (Pridham *et al.,* 1956). Actinobacterial isolates were inoculated on fish meal extract agar plates and incubated at 28°C in dark for 7 days (El-Tarabily *et al.,* 1997; Saeed *et al.,* 2017b). Briefly, plugs from the actinobacterial cultures growing on fish meal extract agar were transferred on PDA plates seeded with *N. dimidiatum* using a sterilized 11-mm cork-borer and kept at 28°C in dark for 5 days. The diameters of the inhibition zones were determined. For each actinobacterial isolate, six plates were used. Isolates showing the largest inhibition zone on plates were considered the most promising diffusible antifungal metabolite-producers and selected for further experiments; whereas the rest of isolates were discarded.

For preliminary assessment of CWDEs production, all isolates were tested for their abilities to produce clearing zones on *N. dimidiatum* mycelial fragment agar (Valois *et al.,* 1996). High or low CWDE activities were associated with large (>30 mm) or small (<30 mm) diameters, respectively. All obtained isolates were also evaluated for the production of the 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, where diameters of >30 mm (large clearing zone) and <30 mm represented high and low chitinase activities, respectively. Six replicate plates were used for each isolate. Only the highly

active CWDE-producing isolates showing the largest clearing zones on both mycelial fragment agar and colloidal chitin agar plates were chosen for further analyses.

3.2.4 *In Vivo* Apple Fruit Bioassay

To determine the ability of the candidate isolates to reduce lesion formation as an indication of disease suppression after inoculation with *N. dimidiatum*, an *in vivo* apple fruit bioassay was performed according to Al Raish *et al.* (2020). Mature apple fruits (cv. Granny Smith), placed in plastic trays on sterile paper towels moistened with sterile distilled water, were inoculated with agar plugs (11 mm) colonized by the isolates and/or *N. dimidiatum* onto each apple fruit as the following: (i) two sterile, non-inoculated PDA agar plugs on each other (control; C); (ii) the antagonist alone (BCA) with a sterile PDA agar plug on top of it; (iii) *N. dimidiatum* (*Nd*) alone with a sterile PDA agar plug underneath it; and (iv) pairing *N. dimidiatum* and the antagonists together (BCA+*Nd*), with the BCA on the apple surface and *N. dimidiatum*-inoculated plug on top of the BCA. The antagonists were inoculated onto the apple surface 24 h prior the pathogen to give the actinobacterial isolate the time to secret antifungal metabolites and/or CWDEs onto the apple fruit. Trays were covered with aluminum foil to maintain humidity in dark at 28°C for 5 days and lesion diameters were measured. All diseased fruits were incubated on PDA plates at 28°C in dark for 5 days to fulfill Koch's postulates. The four combinations were applied on each fruit; where five fruits/tray were randomly selected and evaluated.

3.2.5 Assays for Antifungal Metabolites, Chitinolytic Activity and Production of Other Antifungal Compounds

The cup plate technique (Bacharach & Cuthbertson, 1948), was used to assess the three potential antagonistic BCAs for the ability to secrete diffusible antifungal

metabolites against *N. dimidiatum*. The diameter of the inhibition zone was determined for each BCA.

To evaluate the inhibition of *N. dimidiatum* by the diffusible antifungal metabolites or the CWDE on fish meal extract agar or colloidal chitin agar, respectively (El-Tarabily *et al.,* 1997; 2000), a dialysis membrane overlay technique (Gibbs, 1967), was used. The dialysis membrane (Type 45311; Union Carbide Corporation, IL, USA) with adhering BCA colonies were removed from the agar plates and the center of each plate was inoculated with a disc (5 mm diameter) of *N. dimidiatum* culture. At the end of the incubation period, the colony diameter of *N. dimidiatum* was measured. The agar plugs were further transferred to a fresh PDA plate and incubated at 28°C for 5 days to determine if the diffused metabolites/chitinase were fungistatic (pathogen growth from the plug) or fungicidal (no pathogen growth from the plug). The promising BCAs were also tested on fish meal extract agar for the production of volatile antifungal compounds (Payne *et al.,* 2000).

For the production of hydrogen cyanide, plates of tryptic soy agar medium (Lab M Limited) supplemented with 4.4 g glycine l^{-1} were inoculated with the BCAs. Plates were inverted and a piece of filter paper, soaked in 0.5% picric acid and 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 hydrogen cyanide production (Castric, 1975). For the production of siderophore, plates of Chrome Azurol S (CAS) agar developed by Schwyn & Neilands (1987), were inoculated with the concerned BCAs and incubated at 28°C in dark for 7 days. Development of yellow-orange halo zone around the colony represents a siderophore-producing isolate (Appendix).

3.2.6 *In Vitro* CWDE Activities of BCA Isolates on *N. dimidiatum*

Two mg ml^{-1} of either *N. dimidiatum* cell-wall fragments, colloidal chitin or laminarin (Sigma-Aldrich) were placed flasks containing 50 ml of minimal synthetic medium (Tweddell *et al.,* 1994). Each substrate in flasks was inoculated with 2 ml of a 20% glycerol suspension of each BCA (10^8 cfu ml^{-1}), placed on a rotary shaker (Model G76, New Brunswick Scientific, NJ, USA) for 7 days at 250 rpm speed, followed by 12000 x *g* centrifugation for 30 min. The supernatant which was used as a source of crude enzymes (El-Tarabily, 2003), was filtered using 0.22 µm Millipore membranes (Millipore Corporation, MA, USA).

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

3.2.7 Effect of BCA Crude Culture Filtrates on Mycelia and Conidia of *N. dimidiatum*

For each BCA, the filter-sterilized crude culture filtrate (described above) using fish meal extract broth or colloidal chitin broth (Gupta *et al.,* 1995), was proportionally poured in PDA plates. The medium was inoculated with an agar plug of 5 mm diameter, colonized with *N. dimidiatum* mycelium by placing it upside down. The colony diameter (mm) of the pathogen (*N. dimidiatum*) was measured after 5 days at 28°C. In addition, the prepared crude culture filtrate from fish meal extract broth or colloidal chitin broth was proportionally mixed with potato dextrose broth (PDB; Lab M) (Lorito *et al.,* 1993). Similarly, the PDB was inoculated with a 5-mm diameter agar plug of the pathogen; and the dry weight of *N. dimidiatum* was measured after 10 days of incubation in dark at 28°C.

The effect of the crude culture filtrate of the BCAs on mature conidia germination and germ tube elongation of *N. dimidiatum* on PDB were also tested (Lorito et al., 1993). By microscopically determined and compared the percentage of spore germination and the average of germ tubes length using 40X Nikon-Eclipse 50i light microscope at 40X after 24 h (Nikon Instrument Inc, NY, USA) with the non-inoculated fish meal extract broth or colloidal chitin broth (control).

The crude culture filtrate of BCAs was assessed on the hyphal morphology of *N. dimidiatum* (Sneh, 1981). Using a light microscope (100X), *N. dimidiatum* hyphae treated with the BCA was examined. Control treatments of *N. dimidiatum* mycelium incorporated with non-inoculated filter-sterilized fish meal extract broth/colloidal chitin broth were also investigated.

3.2.8 Species Identification and Phylogenetic Analysis of the Promising BCAs

The three promising BCAs were identified based on their 16S rRNA gene sequence analysis using the primers 900R (5’-CCGTCAATTCATTTGAGTTT-3’); 357F (5’-TACGGGAGGCAGCAG-3’) and 800F (5’-ATTAGATACCCTGGTAG-3’) (Rainey *et al.,* 1996), done by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, (DSMZ), Braunschweig, Germany. All ssequences were deposited in Genbank with accession numbers MW527431 for BCA1 (#25), MW527429 for BCA2 (#23) and MW527430 for BCA3 (#17). The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment. To construct species-level phylogenies, the tree was based on Maximum Likelihood method and Tamura-Nei model (Tamura & Nei, 1993) implemented in Molecular Evolutionary Genetics Analysis version X (MEGAX) software (Kumar *et*

al., 2018), and were applied after data alignments by CLUSTAL_X (Thompson *et al.,* 1997). In each case, bootstrap values were calculated based on 500 resamplings.

Cultural, morphological, physiological and biochemical characteristics of BCA isolates were also determined (Locci, 1989). The morphology of the spores for each of the three promising BCA isolates was observed using Scanning Electron Microscopy (SEM) of XL-30 from Phi`lips (FEI Co., Eindhoven, The Netherlands).

3.2.9 Biological and Chemical Control Experiments in the Greenhouse

The pathogenicity test was conducted on 1-year-old healthy *D. regia* seedlings-purchased from the local market. Under-bark inoculation was performed at 30-35 cm above the root collar using a sterile scalpel to wound the bark, and placing an agar plug (8 mm diameter) colonized by mycelium of a 10-day-old culture of *N. dimidiatum* into the wound with mycelium facing the stem (Al Raish *et al.,* 2020). Each wound was wrapped in parafilm to maintain moist conditions. Seedlings of *D. regia* were inoculated with sterile agar plugs as negative controls. Plants were monitored for symptoms at 5 Weeks Post Inoculation (wpi). The fungus was reisolated from the infected stems on PDA at the end of the experiment and morphologically compared with the inoculated fungus.

In the greenhouse, the evaluation of the BCAs was also carried out on *D. regia* seedlings. The aim was to test the efficacy of the three BCAs prior and post infection with *N. dimidiatum* on royal poinciana. Similar to the pathogenicity (described above), inoculation methods with the pathogen and BCA application were used. For BCA preventive treatments, seedlings were treated with each BCA 7 days before *N. dimidiatum* inoculation; whereas in curative treatments, seedlings were treated with each BCA one week after inoculation of *N. dimidiatum*.

In these experiments, the treatments used were as the following:

- Healthy controls (C): Non-inoculated control seedlings.
- Diseased controls (*Nd*): Seedlings inoculated with *N. dimidiatum* only.
- Biocontrol treatment without pathogen (*Sr*, *Sc* or *Sa*): Seedlings inoculated with either *S. rochei, S. coelicoflavus* or *S. antibioticus,* respectively.
- Chemical control treatment (*Nd*+CT): Seedlings inoculated with 250 part per million (ppm) of Cidely® Top 1 week after *N. dimidiatum* inoculation.
- Curative biocontrol treatment (*Nd*+*Sr*; *Nd*+*Sc* or *Nd*+*Sa*): Seedlings inoculated with either *S. rochei, S. coelicoflavus* or *S. antibioticus*, respectively, 1 week after *N. dimidiatum* inoculation.
- Preventive biocontrol treatment (*Sr*+*Nd*; *Sc*+*Nd* or *Sa*+*Nd*): Seedlings inoculated with either *S. rochei, S. coelicoflavus* or *S. antibioticus*, respectively, 1 week before *N. dimidiatum* inoculation.

For each treatment/group, six separate pots each containing one seedling were arranged in a completely randomized design. Greenhouse experiments were independently repeated twice. Control and inoculated seedlings were maintained in the greenhouse (15 h day/9 h night; 160 W mol^{-1} m^{-2} s^{-1} fluorescent lights; 25 ± 2°C). To determine disease symptoms/recovery of inoculated plants, conidia counts of the fungal pathogen and the number of falling leaves were recorded at 4 wpt (Al Raish *et al.,* 2020). Conidia were harvested from affected tissues (three leaf bases of 6 inoculated seedlings per treatment) in 5 ml of water, and counted using haemocytometer (Agar Scientific Limited, Essex, UK).

3.3 Statistical Analyses

All *in vitro* experiments were repeated independently six times. Similar results were obtained in each replicate. For *in vitro* evaluation of BCA against *N. dimidiatum*, ANOVA and Duncan's multiple range test at P = 0.05 were used using six plates/treatment. For apple fruit bioassays, each fruit was inoculated with the four treatments and each tray (5 trays were used) containing five fruits. The effect of BCAs on lesion formation was analyzed using Analysis of Variance (ANOVA), and significant differences between means at P = 0.05 were determined by Duncan's multiple range test.

All greenhouse experiments were independently repeated twice with similar results, and the obtained data were combined and analyzed. For the falling leaves and fungal conidia counts of the *in vivo* treatments against *N. dimidiatum* in the greenhouse trial, 12 plants (6 replicates from each experiment) were used for each treatment. ANOVA and Duncan's multiple range test were used to determine the statistical significance ($P<0.05$). For all statistical analyses, SAS Software version 9 was used (SAS Institute Inc., NC, USA).

3.4 Results

3.4.1 Identification of SA and NSA Isolates Producing Diffusible Antifungal Metabolites and CWDEs

In the efforts to isolate actinobacteria from *D. regia* rhizosphere, a total of 31 strains of which 26 SA (83.9%) and 5 NSA (16.1%) were obtained successfully on inorganic salt starch agar plates without the application of *Streptomyces* phages (Figure 10). When *Streptomyces* phages with high polyvalency were used, the numbers significantly ($P<0.05$) increased for NSA but decreased for SA (Table 6). The use of

the polyvalent phages allowed an additional 16 NSA isolates from *D. regia* rhizosphere for further studies. Consequently, 31 SA and 16 NSA isolates without and with the application of *Streptomyces* phages, respectively, were identified to the genus level according to their morphology, presence of aerial mycelia, and the stability of substrate mycelia and distribution of aerial and substrate mycelia.

Figure 10: Colonies of actinobacteria isolated from *Delonix regia* rhizosphere grown on inorganic salt starch agar plates without the application of the *Streptomyces* phages. Note the dominance of *streptomycete* actinobacterial colonies

Table 6: The effect of introducing four polyvalent Streptomyces phages on the colony-forming units of streptomycete and non-streptomycete actinobacteria from *Delonix regia* rhizosphere soil

Actinobacteria	Without phage	With phage
	$\log_{10}$ cfu g soil^{-1}	
SA	6.04±0.60 *a*	2.36±0.57 *b*
NSA	2.12±0.24 *a*	4.88±0.36 *b*

Values are means of six 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.

By using the cut-plug method, 6 SA and 4 NSA isolates displayed strong production of diffusible antifungal metabolites against *N. dimidiatum* (Table 7). Ten isolates produced large zones of pathogen inhibition (>30 mm) and were considered the promising BCA candidates. Isolates #2, #3, #7, #11, #17, #21, #24, #25, #28 and #31 (Table 7; Figure 11a) were selected for further analyses. The rest were not included in subsequent studies because they showed <30 mm zone of inhibition.

Table 7: *In vitro* and *in vivo* antagonism shown by 18 isolates of streptomycete and non-streptomycete actinobacteria against *Neoscytalidium dimidiatum*

		In vitro			*In vivo*
	Isolate	Diameter of inhibition zone (mm) [a]	Diameter of clearing zone (mm) [b]	Diameter of clearing zone (mm) [c]	Lesion diameter (mm) [d]
	#3	43.90±0.15 *c*	0.00±0.00f*g*	0.00±0.00 *g*	31.18±0.18 *a*
	#4	0.00±0.00 *i*	34.78±0.78 *e*	36.15±0.34 *f*	0.60±0.02 *e*
	#6	0.00±0.00 *i*	51.87±0.34 *c*	53.35±0.18 *c*	32.43±0.91 *a*
	#7	36.01±0.17 *f*	0.00±0.00 *f*	0.00±0.00 *g*	11.17±0.88 *c*
Streptomyces sp.	#11	45.33±0.65 *b*	45.00±1.01 *d*	47.56±0.78 *d*	14.77±0.97 *b*
	#17 (BCA3)	48.07±0.08 *a*	55.13±0.99 *b*	56.19±1.10 *b*	0.19±0.00 *e*
	#19	0.00±0.00 *i*	55.13±1.13 *b*	56.02±0.56 *b*	31.17±0.61 *a*
	#21	39.38±0.55 *e*	0.00±0.00 *g*	0.00±0.00 *g*	31.18±0.55 *a*

Table 7: *In vitro* and *in vivo* antagonism shown by 18 isolates of streptomycete and non-streptomycete actinobacteria against *Neoscytalidium dimidiatum* (Continued)

	Isolate	*In vitro*			Isolate
		Diameter of inhibition zone (mm) [a]	Diameter of clearing zone (mm) [b]	Diameter of clearing zone (mm) [c]	Lesion diameter (mm) [d]
Streptomyces sp.	#23 (BCA2)	0.00±0.00 *i*	62.85±1.54 *a*	64.76±1.43 *a*	0.53±0.01 *e*
	#25 (BCA1)	41.90±0.59 *d*	0.00±0.00 *f*	0.00±0.00 *g*	0.58±0.01 *e*
	#29	0.00±0.00 *i*	53.44±1.93 *cb*	54.38±1.56 *c*	32.86±0.18 *a*
Actinoplanes sp.	#2	30.32±0.67 *h*	0.00±0.00 *f*	0.00±0.00 *g*	0.55±0.02 *e*
Microbispora sp.	#24	46.31±2.04 *b*	0.00±0.00 *f*	0.00±0.00 *g*	6.54±0.75 *d*
Micromonospora sp.	#10	0.00±0.00 *i*	37.81±0.30 *e*	38.48±1.07 *f*	32.00±0.26 *a*
	#28	48.11±0.58 *a*	0.00±0.00 *g*	0.00±0.00 *g*	31.18±0.37 *a*
Nocardia sp.	#22	0.00±0.00 *i*	50.09±1.22 *c*	54.02±1.03 *c*	5.96±68 *d*
Rhodococcus sp.	#31	33.35±0.44 *g*	53.87±1.96 *cb*	55.56±2.29 *bc*	11.67±1.06 *c*
Streptosporangium sp.	#15	0.00±0.00 *i*	51.18±2.02 *c*	53.73±1.88 *c*	31.22±1.04 *a*
Diseased control (*N. dimidiatum*)		ND	ND	ND	31.12±0.36 *a*
Healthy control (no *N. dimidiatum*)		ND	ND	ND	0.00±0.00 *e*

[a] Production of diffusible antifungal metabolites active against *N. dimidiatum* 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 *N. dimidiatum* using the *in vivo* apple fruit bioassay.
Values are means of six replicates ± SE for all the *in vitro* experiments and five replicates ± SE for *in vivo* fruit bioassay. Values within each column, followed by the same letter are not significantly (P>0.05) different according to Duncan's multiple range test. Isolates #25, #23 and #17 represent *Streptomyces rochei* (BCA1), *S. coelicoflavus* (BCA2) and *S. antibioticus* (BCA3); respectively. ND, not determined.

In addition, eleven (7 SA and 4 NSA) isolates produced large clearing zones (>30 mm) around the colony on colloidal chitin agar plates and on *N. dimidiatum* mycelial fragment agar (Table 6; Figure 11b). These isolates were #4, #6, #10, #11,

#15, #17, #19, #22, #23, #29 and #31. The remainder were not assessed further due to the production of small clearing zones of <30 mm. it was noticed that isolates #11, #17 and #31 showed antifungal activities against *N. dimidiatum* by producing both diffusible antifungal metabolites and CWDEs (Table 7). This suggests that SA and NSA isolated from the *D. regia* rhizosphere soils found in the UAE possessed one or more mode of actions can have potential antifungal effects on plant pathogens (*example N. dimidiatum*).

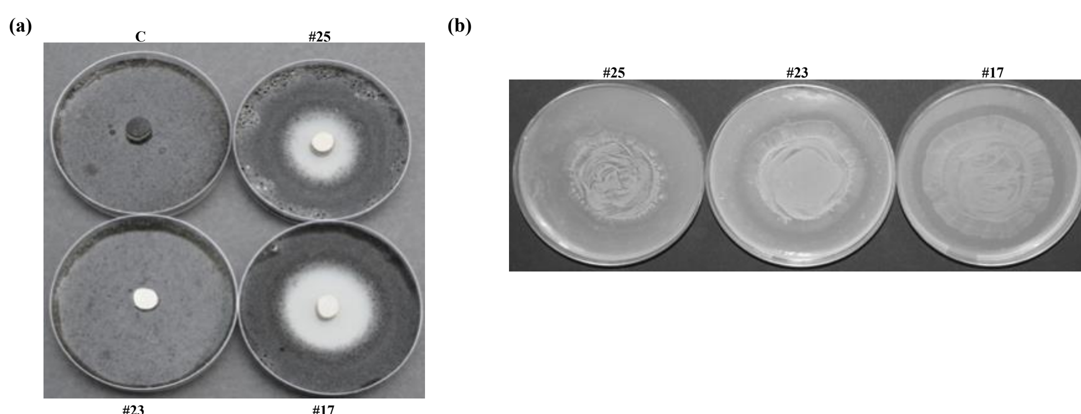


Figure 11: Production of diffusible antifungal metabolites and cell wall degrading enzymes by biological control agents candidates active against *Neoscytalidium dimidiatum*

(a) Inhibition of *Neoscytalidium dimidiatum* mycelial growth using cut-plug method; and (b) production of cell wall degrading enzymes on mycelial fragment agar by the three.

3.4.2 Selection of the Best BCA Candidates to *N. dimidiatum* Using Apple Fruit Bioassay

The 18 BCA candidates (11 SA and 7 NSA) (Table 7) were checked for their effect against *N. dimidiatum* using an apple fruit bioassay method (Table 7; Figure 12a). On apple fruits, it was observed relatively large, brownish, round and water-soaked lesions with clear margins that were produced by the pathogen (*Nd*) alone or with isolate #5 (non-antagonistic *Streptomyces* sp.) used as a positive control (Figure

12b). Ten actinobacterial (6 SA and 4 NSA) isolates (#2, #4, #7, #11, #17, #22, #23, #24, #25 and #31) paired with the pathogen (BCA+*Nd*) on the surface of the fruit significantly ($P<0.05$) reduced lesion formation compared with the pathogen treatment (Table 7); while the other 8 isolates (5 SA and 3 NSA) did not significantly ($P>0.05$) succeed in the reduction of lesion formation (Table 7) and were excluded. From the 10 identified isolates, 2 SA and 3 NSA (#7, 11, 22, 24 and 31) were excluded as they reduced lesion development on apple fruits to varying degrees; whereas 4 SA and 1 NSA isolates (#2, #4, #17, #23 and #25) almost completely prevented lesion formation (Table 7).

It is noteworthy to mention that isolate #2 produced less diffusible antifungal metabolites than isolates #17 and #25, and isolate #4 produced lower quantity of CWDEs than isolates #23 and #25. Thus, these two isolates were eliminated. Accordingly, only three outstanding antagonistic SA isolates (#17, #23 and #25) were further selected as BCA candidates according to the *in vivo* apple bioassay as well as the production of high levels of diffusible antifungal metabolites and/or CWDEs (Table 7; Figure 12b).

Clearly, the antifungal activities and the mode of actions/metabolites of these BCA isolates were able to inhibit the pathogen on the apple fruit surface. All individual BCA treatments did not cause any deleterious effects on apple fruits (Figure 12b). Overall, the three selected BCA candidates (#17, #23 and #25) were greatly effective against *N. dimidiatum*, and that their preventive effect could potentially manage stem canker disease on *D. regia* seedlings.

Figure 1: *In vivo* inhibitory effect of the biological control agents against *Neoscytalidium dimidiatum* using the "apple fruit bioassay"

An illustration showing a inoculated-apple fruit with the BCAs and/or *N. dimidiatum* agar plugs in combinations; and b apple fruit bioassays using BCAs after 5 days incubation at 28°C. (i) two sterile non-inoculated PDA agar plugs; (ii) *N. dimidiatum* inoculum alone with a sterile agar plug below it; (iii) BCA alone with a sterile agar plug above it; and (iv) pairing *N. dimidiatum* and BCA together, where BCA was placed on the apple surface and *N. dimidiatum*-inoculated plug on top of BCA. C, control (no *N. dimidiatum* or BCA); *Nd*, *N. dimidiatum*; #5, isolate #5 (non-antagonistic *Streptomyces* sp. used as a positive control), BCA1, *Streptomyces rochei* UAE1 (#25); BCA2, *S. coelicoflavus* UAE1 (#23); BCA3, *S. antibioticus* UAE1 (#17).

3.4.3 *In Vitro* Evaluation of Antagonistic Properties of the BCA Candidates

The cup plate method was also carried out to determine the effect of the culture filtrate of the three selected BCAs from fish meal extract broth on *N. dimidiatum* growth *in vitro*. When applied into the wells, the filter-sterilized crude culture filtrate of BCA1 or BCA3 caused significant ($P<0.05$) retardation of the pathogen growth compared to that in the control (no BCA) or BCA2 (Table 8; Figure 13). Thus, the effect of BCA3 was significantly ($P<0.05$) higher than BCA1, suggesting more effective diffused antifungal metabolites in BCA3. Similar results were observed when the dialysis membranes obtained from the fish meal extract agar of BCA1 and BCA3 clearly inhibited growth of *N. dimidiatum* compared to those of control or BCA2 (Table 8; Figure 13). The pathogen did not recover from the plugs when transferred

from treated plates to fresh PDA, confirming that the metabolites of BCA1 and BCA3 were clearly fungicidal to *N. dimidiatum*.

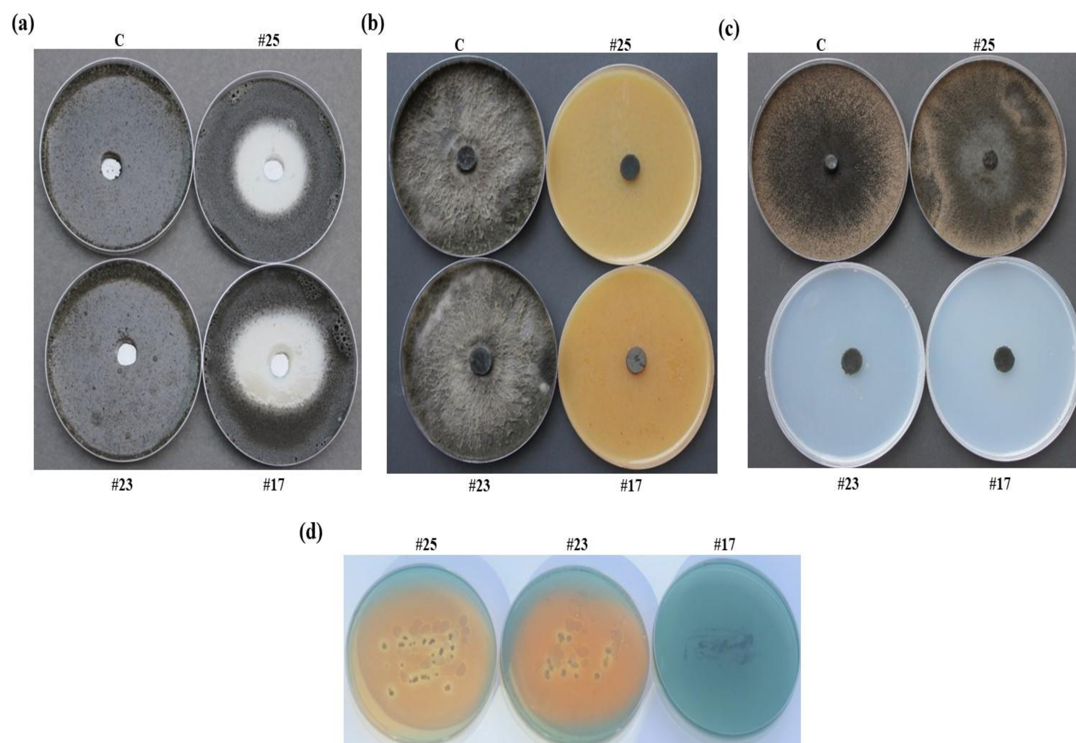


Figure 13: *In vitro* effect of biological control agent candidates on mycelial growth of *Neoscytalidium dimidiatum*

Inhibition of *N. dimidiatum* mycelial growth using a cup plate method; and dialysis membrane overlay technique using b fish meal extract agar; or c colloidal chitin agar plates; and (d) production of siderophores by BCAs. In (a) & (b), inhibition of *N. dimidiatum* mycelial growth was observed only by the diffusible antifungal metabolite-producing isolate #25 (BCA1) and #17 (BCA3) compared to the diffusible antifungal metabolite non-producing isolate #23 (BCA2) and control. In (c), inhibition of *N. dimidiatum* mycelial growth by the chitinase-producing isolates #17 and #23 compared to the chitinase non-producing isolate #25 and control. In (d), isolates #23 and #25 were considered as siderophore-producing isolates compared to the non-sideophore-producing isolate #17. In a, wells were inoculated with either filter-sterilized fish meal extract broth (C) or filter-sterilized crude culture filtrates of isolates #17, #23 or #25. In (d), isolates were tested on chrome azurol S agar plates; and yellow halo surrounding the colony indicates the excretion of siderophores. Isolates #25, #23 and #17 represent *Streptomyces rochei* (BCA1), *S. coelicoflavus* (BCA2) and *S. antibioticus* (BCA3); respectively

Table 8: *In vitro* antagonistic activities of the strongest Biological Control Agent (BCA) candidates showing antagonism to *Neoscytalidium dimidiatum*

Activities	BCA1	BCA2	BCA3
Production of diffusible metabolites using cup plate (diameter of inhibition zone in mm)	51.98±1.12 *a*	0.00±0.00 *c*	66.86±1.19 *b*
Production of diffusible metabolites using dialysis membrane from fish meal extract agar plates [a]	+	-	+
Production of chitinase using dialysis membrane from 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]	0.00±0.00 *c*	6.57±0.34 *b*	4.65±0.38 *a*
Chitinase from *N. dimidiatum* cell-wall (U ml^{-1}) [c]	0.00±0.00 *c*	4.06±0.42 *b*	2.87±0.44 *a*
ß-1,3-glucanase from laminarin (U ml^{-1}) [d]	0.00±0.00 *c*	4.68±0.48 *b*	2.98±0.32 *a*
ß-1,3-glucanase from *N. dimidiatum* cell-wall (U ml^{-1}) [d]	0.00±0.00 *c*	2.86±0.30 *b*	1.97±0.16 *a*

[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 six 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 (#25), BCA2 (#23) and BCA3 (#17) represent *Streptomyces rochei*, *S. coelicoflavus* and *S. antibioticus*; respectively.

On colloidal chitin agar plates, the diffused CWDEs of BCA2 and BCA3 inhibited *N. dimidiatum* growth; thus, otherwise was observed for the BCA1 isolate or control treatment after the removal of dialysis membranes (Table 8; Figure 12). The pathogen also failed to grow from plugs transferred from the treatment plates to fresh PDA in the absence of diffused CWDEs, indicating that BCA2 and BCA3 showed fungicidal activities to *N. dimidiatum*.

To determine the production of volatile antifungal compounds, the three BCA candidates were grown on fish meal extract agar. Except of BCA1, both BCA2 and

BCA3 failed to produce volatile antifungal compounds to suppress the pathogen growth (Table 8). Although BCA1 and BCA2 produced siderophores, none of them produced hydrogen cyanide (Table 8; Figure 12).

The production of chitinase on the amended media with colloidal chitin or *N. dimidiatum* cell-walls was significantly ($P<0.05$) higher in BCA2 and BCA3 than in BCA1 (Table 8). The same two BCA candidates produced significantly ($P<0.05$) higher ß-1,3-glucanase; whereas no ß-1,3-glucanase was detected by BCA1 when grown in media containing either laminarin or *N. dimidiatum* cell-walls (Table 8). The production of chitinase and ß-1,3-glucanase by BCA2 was significantly ($P<0.05$) higher than by BCA3.

3.4.4 Effect of Crude Culture Filtrates of the BCA Candidates on *N. dimidiatum*

The increasing levels of the crude culture filtrates of BCA1 and BCA3 collected from fish meal extract broth significantly ($P<0.05$) inhibited the colony and mycelial growth of *N. dimidiatum* on PDA plates, when compared with that of BCA2 (Table 9). This was confirmed when the crude culture filtrates of BCA1 and BCA3 incorporated into PDA at ≥75%, mycelial growth was completely inhibited. Similar to that in PDA plates, inhibition of the pathogen using the assay of the mycelial growth by BCA1 and BCA3 was noticed in PDB. In general, crude culture filtrates of BCA1 and BCA3 from fish meal extract broth experiment significantly decreased the mycelial dry weight of *N. dimidiatum* when proportionally added into PDB (Table 8). This suggests that the filter-sterilized crude culture filtrates of either BCA1 or BCA3 on fish meal extract broth were effective in inhibiting growth of *N. dimidiatum* (Table 9).

Table 9: Effects of the crude culture filtrate of the three Biological Control Agent (BCA) candidates obtained from fish meal extract broth and colloidal chitin broth on the mycelial growth, conidia germination and germ tube length of *Neoscytalidium dimidiatum*

BCA	Culture filtrate (%)	Colony diameter (mm)	Mycelial dry weight (g)	Conidia germination (%)	Germ tube length (µm)
(a) Fish meal extract broth					
BCA1	0	98.78±0.84 *a*	77.54±2.98 *a*	88.56±1.80 *a*	50.78±1.04 *a*
	10	39.72±1.83 *b*	35.87±2.08 *b*	40.36±3.03 *b*	47.24±0.92 *b*
	25	20.16±1.60 *c*	14.30±3.02 *c*	25.92±2.04 *c*	28.28±1.12 *c*
	50	10.14±0.96 *d*	6.28±1.56 *d*	15.78±1.12 *d*	16.60±1.34 *d*
	75	0.00±0.00 *e*	0.006±0.00*e*	5.86±0.44 *e*	10.18±0.72 *e*
	100	0.00±0.00 *e*	0.00±0.00 *e*	1.04±0.14 *f*	2.40±0.50 *f*
BCA3	0	98.88±0.64 *a*	80.95±2.07 *a*	89.28±2.08 *a*	52.45±2.34 *a*
	10	37.76±1.39 *b*	28.40±2.86 *b*	34.25±1.74 *b*	36.09±1.91 *b*

Values are means of six 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 (#25), BCA2 (#23) and BCA3 (#17) represent *Streptomyces rochei*, *S. coelicoflavus* and *S. antibioticus*; respectively.

Table 9: Effects of the crude culture filtrate of the three BCA candidates obtained from fish meal extract broth and colloidal chitin broth on the mycelial growth, conidia germination and germ tube length of *Neoscytalidium dimidiatum* (Continued)

BCA	Culture filtrate (%)	Colony diameter (mm)	Mycelial dry weight (g)	Conidia germination (%)	Germ tube length (µm)
(a) Fish meal extract broth					
	25	17.18±1.02 *c*	8.90±0.98 *c*	20.56±1.45 *c*	27.10±1.80 *c*
	50	8.92±0.95 *d*	2.96±0.65 *d*	7.96±1.34 *d*	15.42±0.16 *d*
	75	0.00±0.00 *e*	0.00±0.00 *d*	2.48±0.43 *d*	5.46±1.04 *e*
	100	0.00±0.00 *e*	0.00±0.00 *d*	0.42±0.12 *d*	0.34±0.20 *f*
(b) Colloidal chitin broth					
BCA2	0	97.78±0.86 *a*	79.92±3.10 *a*	92.44±1.28 *a*	59.70±1.80 *a*
	10	48.13±2.05 *b*	40.49±1.66 *b*	28.04±0.86 *b*	36.58±1.85 *b*
	25	25.88±1.44 *c*	21.78±2.08 *c*	17.58±1.12 *c*	24.22±1.32 *c*
	50	15.28±1.40 *d*	12.56±0.99 *d*	9.82±0.88 *d*	5.76±0.78 *d*
	75	0.00±0.00 *e*	0.00±0.00 *e*	2.49±0.45 *e*	0.06±0.00 *e*
	100	0.00±0.00 *e*	0.00±0.00 *e*	0.00±0.00 *e*	0.00±0.00 *e*
BCA3	0	98.78±2.22 *a*	82.22±2.46 *a*	94.90±1.98 *a*	65.14±2.90 *a*
	10	29.27±1.45 *b*	30.84±2.79 *b*	49.49±2.86 *b*	42.55±1.49 *b*
	25	15.88±2.02 *c*	12.46±1.78 *c*	27.46±1.02 *c*	16.56±1.66 *c*
	50	5.18±0.92 *d*	3.66±1.00 *d*	17.76±1.04 *d*	12.32±1.22 *d*

Values are means of six 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 (#25), BCA2 (#23) and BCA3 (#17) represent *Streptomyces rochei*, *S. coelicoflavus* and *S. antibioticus*; respectively.

Table 9: Effects of the crude culture filtrate of the three BCA candidates obtained from fish meal extract broth and colloidal chitin broth on the mycelial growth, conidia germination and germ tube length of *Neoscytalidium dimidiatum* (Continued)

BCA	Culture filtrate (%)	Colony diameter (mm)	Mycelial dry weight (g)	Conidia germination (%)	Germ tube length (µm)
(b) Colloidal chitin broth					
	75	0.00±0.00 *e*	0.00±0.00 *e*	7.36±0.56 *e*	2.42±0.38 *e*
	100	0.00±0.00 *e*	0.00±0.00 *e*	1.82±0.34 *f*	0.00±0.00 *e*

Values are means of six 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 (#25), BCA2 (#23) and BCA3 (#17) represent *Streptomyces rochei*, *S. coelicoflavus* and *S. antibioticus*; respectively.

In comparison to BCA1, the crude culture filtrates of BCA2 and BCA3 from colloidal chitin broth significantly (P<0.05) inhibited colony growth on PDA plates as well as mycelial dry weight of the pathogen on PDB, when the levels of crude culture filtrates were increased from 0 to 100% (Table 9).

In general, the reduction in the germination of the thick walled, mature conidia and the average length of germ tubes produced by the pathogen was significantly (P<0.05) observed when *N. dimidiatum* was exposed to the crude culture filtrate of BCA1 and BCA3 in fish meal extract broth, and BCA2 and BCA3 in colloidal chitin broth (Table 9). The results confirmed that the crude culture filtrates of the three BCA candidates not only inhibited mycelial growth, but also conidia germination and germ tube elongation of *N. dimidiatum* (Table 9).

When exposed to the crude culture filtrate of BCA1 from fish meal extract broth, *N. dimidiatum* showed hyphal abnormalities of hyphal swelling (ballooning) and cytoplasmic coagulation (Figure 14a). The same fungal pathogen treated with the crude culture filtrate of BCA2 obtained from colloidal chitin broth demonstrated hyphal lysis only (Figure 14b). The crude culture filtrate of BCA3 obtained from fish

meal extract broth and colloidal chitin broth caused hyphal swelling and cytoplasmic coagulation, and hyphal lysis in *N. dimidiatum*, respectively (Figure 14). Mycelial mats of all control treatment remained unaffected and intact (Figure 14).

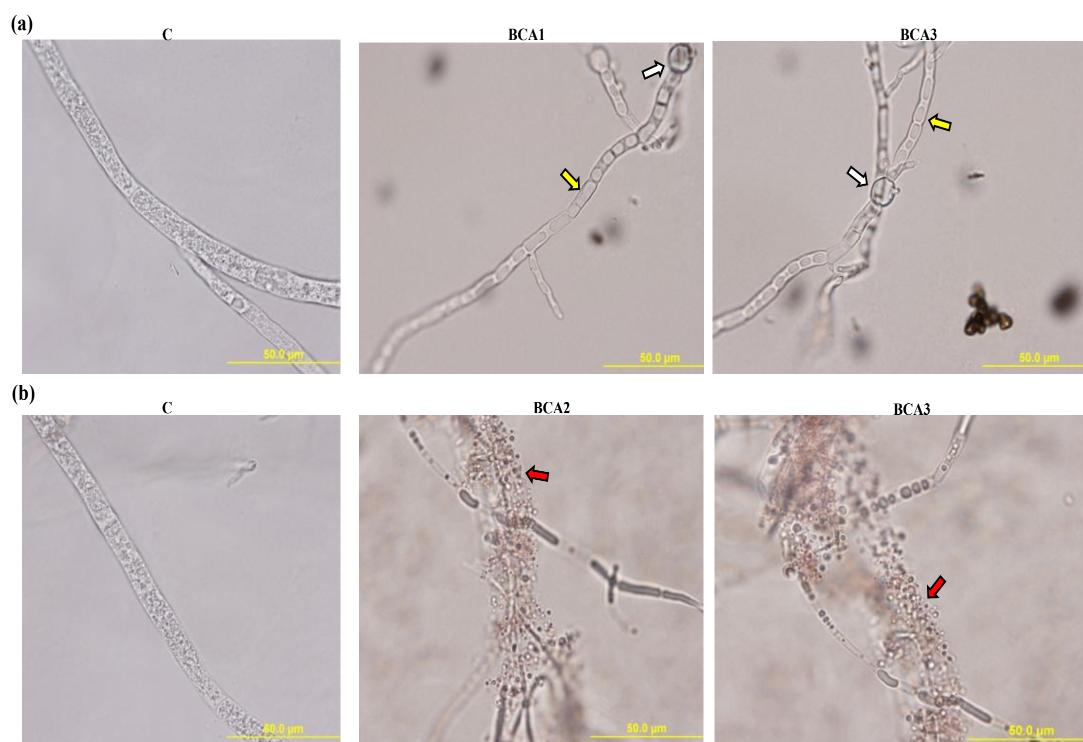


Figure 14: Effect of the biological control agent candidates on hyphae and cytoplasm of *Neoscytalidium dimidiatum*

Abnormalities observed in hyphal morphology and cytoplasmic contents of *N. dimidiatum,* following exposure to (a) filter-sterilized crude culture filtrate of BCA1 (#25) and BCA3 (#17) from fish meal extract broth, or (b) BCA2 (#23) and BCA3 (#17) from colloidal chitin broth compared to control. White and yellow arrows point to hyphal septum malformation and branch deformation, and cytoplasmic coagulation, respectively. Red arrows point to lysis of cytoplasm. Light microscopy images were taken at 1000x magnification. C, control (no BCA was applied). Isolates #25, #23 and #17 represent *Streptomyces rochei* (BCA1), *S. coelicoflavus* (BCA2) and *S. antibioticus* (BCA3); respectively

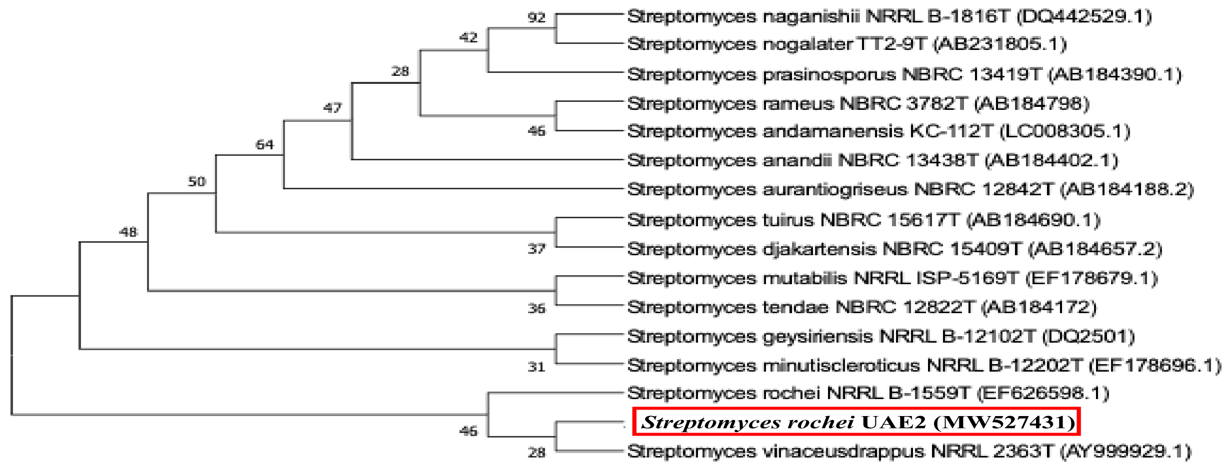
3.4.5 Phylogenetic Analysis and Characteristics of the Candidate BCA Species

The three promising antagonists were identified according to their molecular, cultural and morphological of spores. The 16S rRNA gene sequences of BCA1 (GenBank accession number MW527431), BCA2 (MW527429) and BCA3

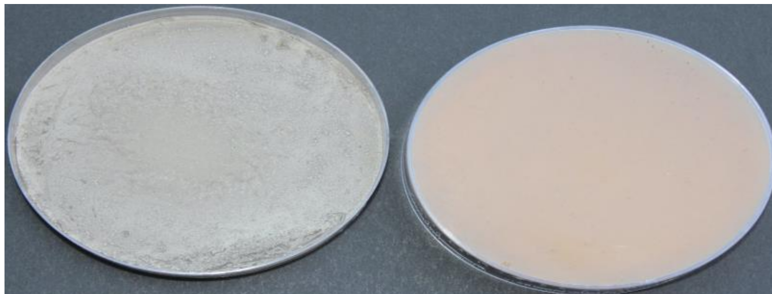
(MW527430) were compared with other streptomycete spp. sequences in the GenBank database.

The 1518-bp sequence of BCA1 (#25) showed 100% similarity with the 16S rRNA nucleotide sequences of *Streptomyces rochei* strain NRRL B-1559^T (accessin number EF626598) (Figure 15a). To confirm the phylogenetic identification, the pure cultures produced grayish aerial mycelia with grayish yellow substrate mycelial groth on ISP medium 3 (Figure 15b). No distinctive pigment was observed on the same medium. By using SEM, the strain displayed smooth, oval-rod shaped spores forming straiht to flexuous long chains (section: rectiflexibiles) of 20-35 spores per chain (Figure 15c). CA1 was designated as *Streptomyces rochei* Strain UAE2 (Berger *et al.,* 1953).

(a)



(b)



(c)

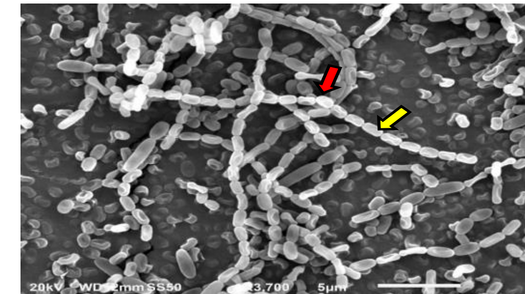


Figure 15: Taxonomic identification of *Streptomyces rochei* UAE2.

(a) The dendrogram showing the phylogenetic relationships between *S. rochei* UAE2 (isolate #25; MW527431) and other members of *Streptomyces* spp. on the basis of 16S rRNA sequences. (b) Gray aerial (left) and grayish yellow substrate (right) mycelia growing on ISP3 medium supplemented with yeast extract; and (c) scanning electron micrograph (3700X) of the rectiflexibiles spore chains (red arrow) and smooth-surfaced spores (yellow arrow) of *S. rochei* UAE2. In (a), numbers at nodes indicate percentage levels of bootstrap support based on a neighbor-joining analysis of 500 resampled datasets. GenBank accession numbers are given in parentheses

On the other hand, the phylogenetic analysis of the 1521-bp 16S rRNA of BCA2 showed 100% similarity to *S. coelicoflavus* NBRC 15399^{T} (AB184650) (Figure 16a); while the rest of other *Streptomyces* spp. showed <99.5% similarity with the isolate-of-interest. Typical grey aerial mycelia and grayish pink substrate mycelia were observed when BCA2 was cultivated (Figure 16b). BCA2 showed spiral (section: spirales) chains and smooth-surfaced spores (Figure 16c). The data demonstrated that BCA2 (#23) can be recognized as *Streptomyces coelicoflavus* (ex Ryabova and Preobrazhenskaya) (Terekhova, 1986; Gause *et al.,* 1983) Strain UAE1.

With 100% similarity to 16S rRNA gene sequence of *S. antibioticus* NRRL B-1701^{T} (accession #: AY999776) (Figure 17a), the other *Streptomyces* strains showed less than 98.9% similarity with BCA3 (1521 bp). On ISP medium 3, pure cultures of BCA3 produced dark grayish greenish substrate mycelia with grayish aerial mycelia (Figure 17b); with smooth-surfaced mature spores forming spore chains in rectiflexibiles section (straight to flexuous long chains of spores) (Figure 17c). The third BCA3 could be identified as *Streptomyces antibioticus* (Waksman and Woodruff 1941) Strain UAE1. Together, the results indicated that the molecular marker for identification of actinobacterial spp. should rely on a robust method (example 16S rRNA) in addition to the cultural and morphological characteristics.

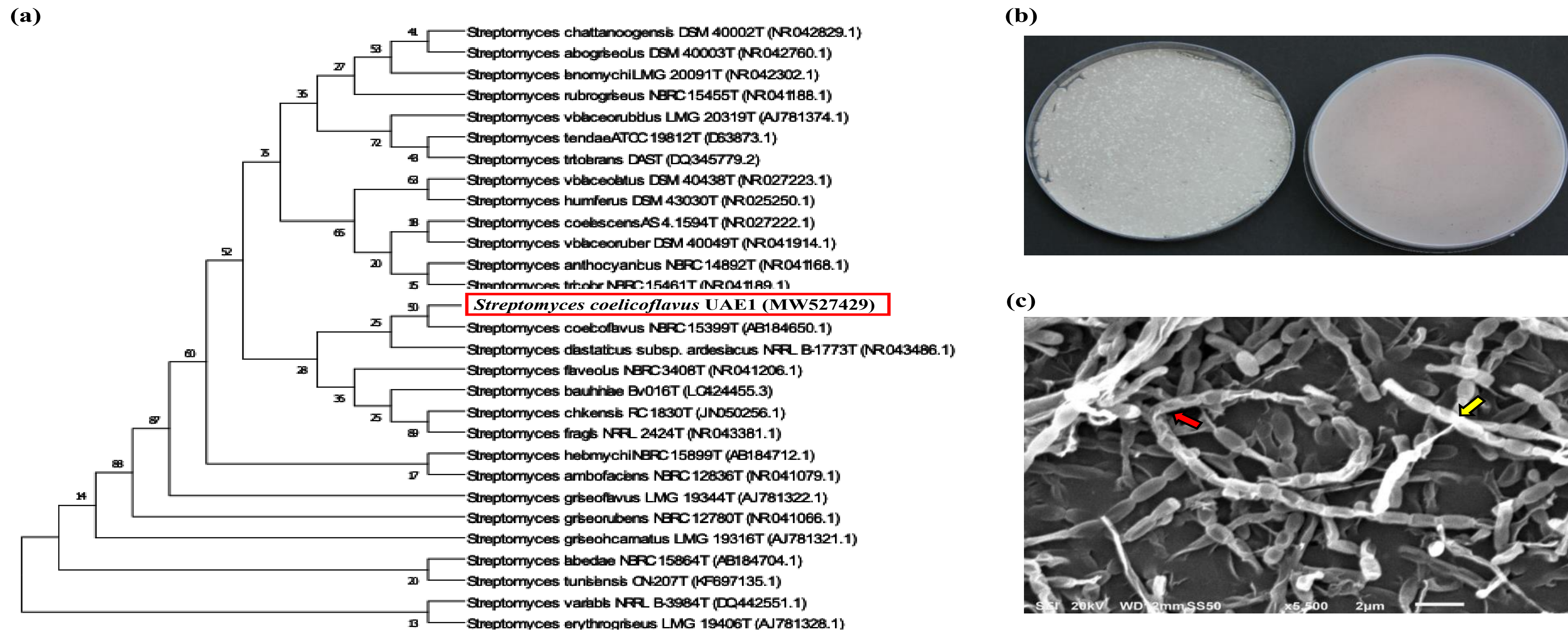


Figure 16: Taxonomic identification of *Streptomyces coelicoflavus* UAE1

(a) The dendrogram showing the phylogenetic relationships between *S. coelicoflavus* UAE1 (isolate #23; MW527429) and other members of *Streptomyces* spp. on the basis of 16*S* rRNA sequences. (b) Gray aerial (left) and grayish pink substrate (right) mycelia growing on ISP3 medium supplemented with yeast extract; and c scanning electron micrograph (5500X) of the spiral spore chains (red arrow) and smooth-surfaced spores (yellow arrow) of *S. coelicoflavus* UAE1. In (a), numbers at nodes indicate percentage levels of bootstrap support based on a neighbor-joining analysis of 500 resampled datasets. GenBank accession numbers are given in parentheses

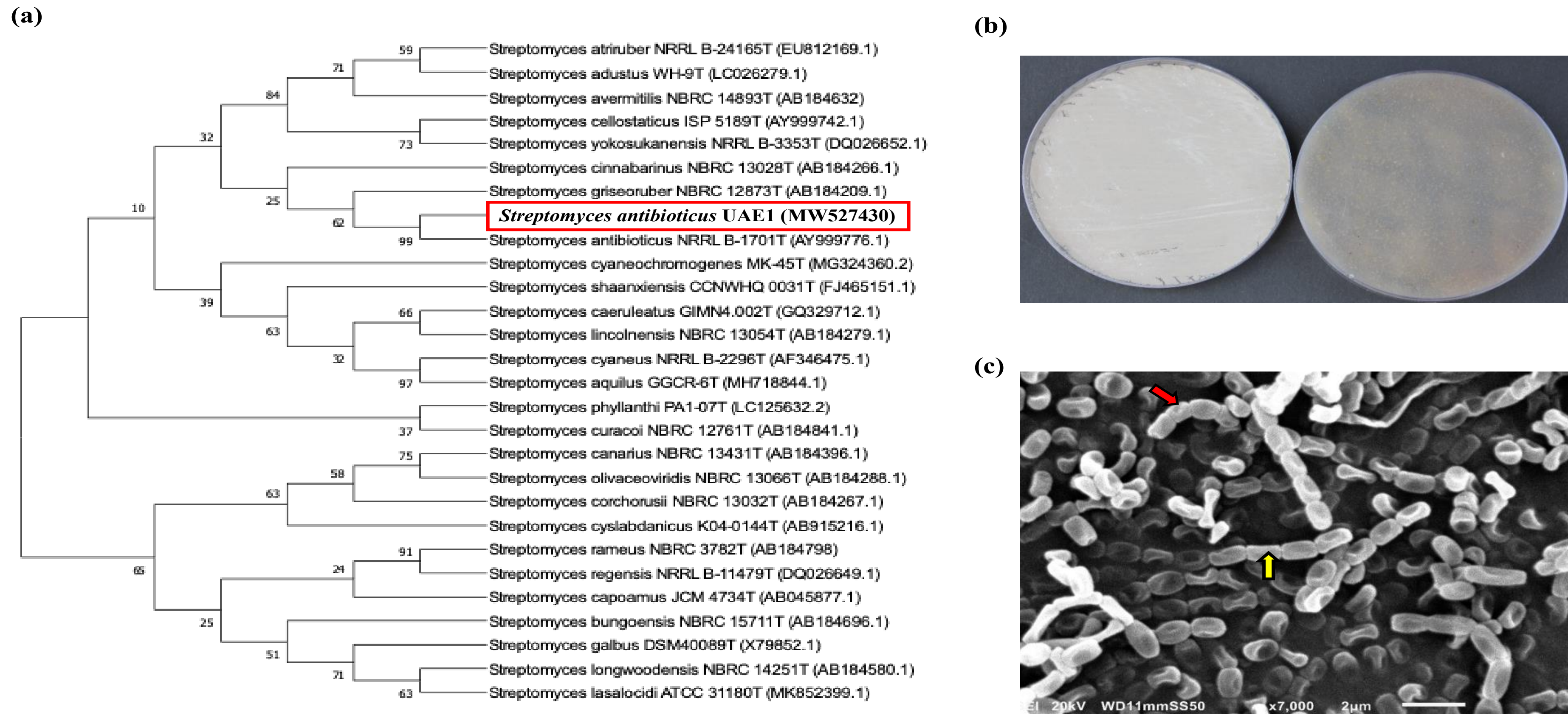


Figure 17: Taxonomic identification of *Streptomyces antibioticus* UAE1

(a) The dendrogram showing the phylogenetic relationships between *S. antibioticus* UAE1 (isolate #17; MW527430) and other members of *Streptomyces* spp. on the basis of 16*S* rRNA sequences. (b) Gray aerial (left) and dark grayish greenish substrate (right) mycelia growing on ISP3 medium supplemented with yeast extract; and c scanning electron micrograph (7000X) of the rectiflexibiles spore chains (red arrow) and smooth-surfaced spores (yellow arrow) of *S. antibioticus* UAE1. In (a), numbers at nodes indicate percentage levels of bootstrap support based on a neighbor-joining analysis of 500 resampled datasets. GenBank accession numbers are given in parenthese

3.4.6 *In Vivo* Evaluation of BCA Applications on *N. dimidiatum* in the Greenhouse

A previous study by Al Raish *et al.* (2020) has clearly indicated that the chemical fungicide, Cidely® Top, was found to be effective at the concentration of 250 ppm against stem canker *D. regia* caused by *N. dimidiatum*. In order to compare the efficacy of Cidely® Top with that of the three BCAs in disease suppression, an *in vivo* experiment was carried out on *D. regia* in the greenhouse. First, the pathogenicity of *N. dimidiatum* on *D. regia* seedlings (*Nd*) were determined. Classical symptoms of stem canker disease, such as falling leaves and discoloration of stems, were observed on seedlings until they became completely bare at the end of the experiment (Figure 18a). This was also confirmed as longitudinal necrosis on woods was clearly observed in diseased plants (Figure 18b). After spraying with Cidely® Top on diseased seedlings (*Nd*+CT) at 4 wpt (corresponding to 5 wpi with *N. dimidiatum*), there was not major differences in responses of plants compared with the negative control treatment where no pathogen infection occurred (Figure 18a). Thus, these plants started to recover, which was in contrast to *N. dimidiatum*-inoculated plants (Figure 18a, b). Similar to the curative Cidely® Top (*Nd*+CT) treatment, individual SA isolates representing the three BCA candidates on *D. regia* seedlings after one-week of inoculation with *N. dimidiatum* were applied. Following inoculation with the pathogen, plants inoculated with either BCA1 (*Nd*+*Sr*) or BCA2 (*Nd*+*Sc*) showed partial recovery (Figure 18a) and stems/branches seemed not completely healthy (Figure 18b) when compared with seedlings inoculated with *N. dimidiatum* only at the tested time point. On the other hand, diseased plants inoculated with BCA3 (*Nd*+*Sa*) looked healthy with minor effect of *N. dimidiatum* on seedlings (Figure 18a). This was also confirmed as the longitudinal wood necrosis in *Nd*+*Sa* treatment was comparable to that when seedlings

were sprayed with the chemical fungicide (Figure 18b). Even though the three BCA candidates had the potential to manage stem canker disease on royal poinciana, *S. antibioticus* was the most efficient among the tested SA isolates to inhibit *N. dimidiatum* growth *in vivo*.

In parallel to the curative treatments, the impact of the preventive application of the same BCAs on the aggressiveness of *N. dimidiatum* on royal Poinciana were evaluated. Consequently, individual BCA candidates were applied one-week before inoculation with *N. dimidiatum*. All treatments of the BCA tested suppressed the stem canker disease in varying degrees (Figure 18c). Based on the disease symptoms on seedlings, the preventive application of *S. antibioticus* one week before the pathogen inoculation (*Sa*+*Nd*) was the most effective treatment in suppressing the pathogen invasion, followed by the other two tested BCA (*Sr*+*Nd* or *Sc*+*Nd*) treatments. This was evident from the minimal damage of necrotic tissues seen on seedlings caused by *N. dimidiatum* in *S. antibioticus* treatment, compared to the other BCA candidates (Figure 19d). This clearly suggests that the application time of the BCA isolate is a critical component to be considered for disease suppression, and that the BCA treatment has to precede *N. dimidiatum* infection to achieve the best results.

In order to confirm these results, the responses of the pathogen to all chemical and biological treatments examined on the numbers of conidia and defoliated leaves were determined. It was obvious that the preventive application of BCA3 *S. antibioticus* (*Sa*+*Nd*) caused a greater reduction in the number of conidia, followed by Cidely® Top-treated plants (*Nd*+CT) and the curative treatment of the same BCA candidate (*Nd*+ *Sa*; Figure 18e). There was about two-fold reduction in total number of conidia of *N. dimidiatum* in the preventive *S. antibioticus*-treated seedlings compared with the fungicidal and the curative *S. antibioticus* treatments. That any of

S. rochei (BCA1) or *S. coelicoflavus* (BCA2) applied before the pathogen on plants was more effective than *S. rochei* or *S. coelicoflavus* applied after *N. dimidiatum* were found. This was evident in the conidia counts which were lesser in *Sr*+*Nd* and *Sc*+*Nd* treatments (Figure 18e). However, none of the curative or preventive treatments of *S. rochei* and *S. coelicoflavus* reached to the level of plant protection as found in BCA3 isolate, albeit the timing of *S. antibioticus* application.

Similarly, the number of falling leaves in seedlings inoculated with *N. dimidiatum* significantly ($P<0.05$) increased compared to the chemical-sprayed, BCA-inoculated or non-inoculated seedlings at 4 wpt (Figure 18f). *D. regia* seedlings treated with BCA3 before *N. dimidiatum* infection (*Sa*+*Nd*) significantly ($P<0.05$) reduced the number of defoliated leaves and were comparable to its corresponding control without pathogen infection. Thus, seedlings of *Nd*+*Sr*, *Nd*+*Sc* and *Sc*+*Nd* showed the lowest number of falling leaves among other treatments. No disease symptoms were noticed in Cidely® Top-sprayed, BCA-inoculated or non-inoculated seedlings (Figure 19).

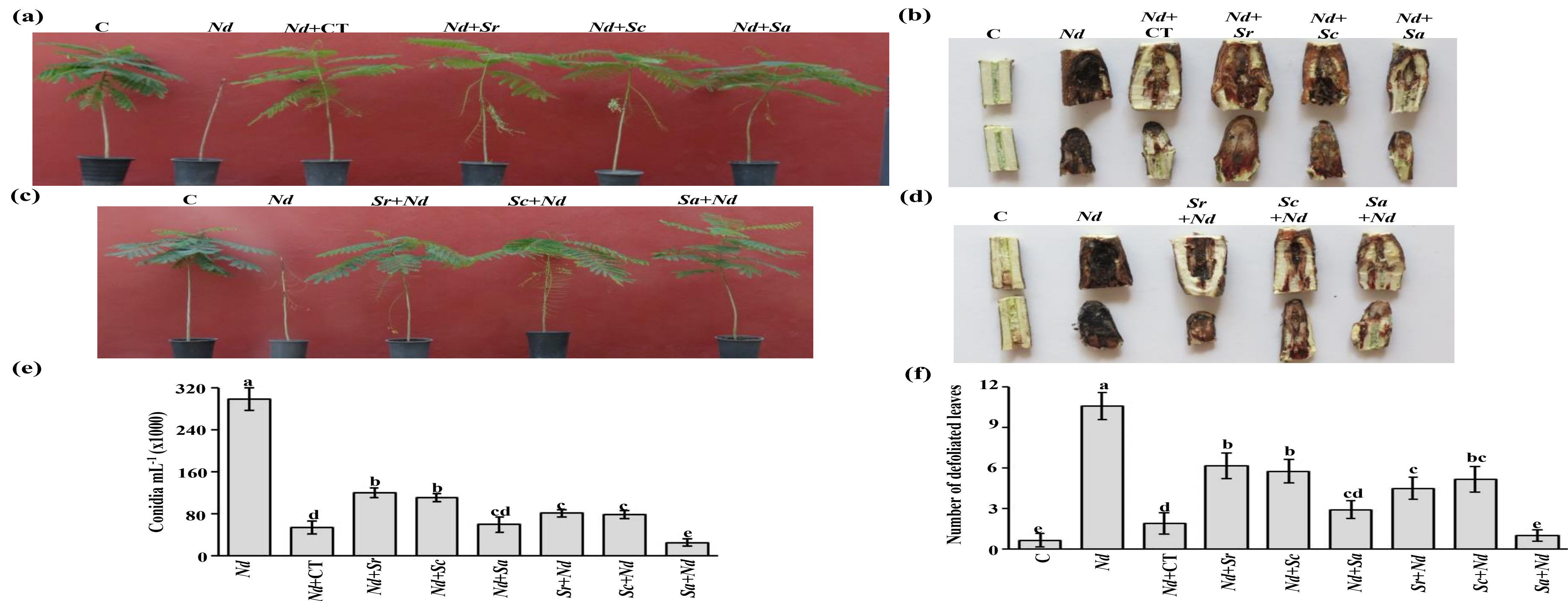


Figure 18: Antagonistic effect of biological control agents candidates against stem canker disease on *Delonix regia* under greenhouse conditions

Effect of (a) curative and c preventive biocontrol treatment of *Streptomyces rochei* UAE2 (BCA1; #25), *S. coelicoflavus* UAE1 (BCA2; #23) and *S. antibioticus* UAE1 (BCA3; #17); and (b) & (d) symptoms of inoculated regions with or without *Neoscytalidium dimidiatum*. In e, number of conidia after the recovery of the pathogen from affected stem tissues; and f number of defoliated leaves of inoculated seedlings after 4 wpt. In (e) & (f), values with different letters are significantly different from each other at $P<0.05$ according to Duncan's multiple range test. C, non-inoculated control seedlings, *Nd*, seedlings inoculated with *N. dimidiatum* only; *Nd*+BCA, seedlings inoculated with *N. dimidiatum* one week prior to treatment with the individual BCA; BCA+*Nd*, seedlings inoculated with the individual BCA one week prior to *N. dimidiatum* inoculation; BCA, biological control agent; CT, Cidely® Top; wpt, weeks post treatment.

Figure 19: Effect of chemical and biological control treatments on *Delonix regia*

Photos were taken at 4 wpt with either Cideyl Top or inoculated BCA. C, non-inoculated control seedlings, *Nd*, seedlings inoculated with *N. dimidiatum* only; CT, Cidely® Top; BCA, biological control agent; *Sr*, *Streptomyces rochei* UAE2 (BCA1); *Sc*, *S. coelicoflavus* UAE1 (BCA2); *Sa*, *S. antibioticus* UAE1 (BCA3); wpt, weeks post treatment.

In general, the pathogen appeared to mimic disease progression in the presence of *S. rochei* or *S. coelicoflavus*, while a strong inhibitory effect on the pathogen in case of *S. antibioticus* treatment was exhibited. This suggests that the preventive treatment with a BCA candidate a week before inoculation with *N. dimidiatum* effectively suppresses the pathogen invasion. In addition, *S. antibioticus* that possesses multiple mode of actions successfully manage stem canker disease on royal poinciana, particularly when applied as a preventive treatment.

3.5 Discussion

In agriculture, a great progress has been made to substitute the use of chemical fungicides, enhance growth and boost defense of plants by microorganisms (Ab Rahman *et al.,* 2018; Mathew *et al.,* 2020; Vinchira-Villarraga *et al.,* 2021). Actinobacteria, are widely distributed in terrestrial ecosystems (e.g., soil) where they can play an essential role in recycling of dead materials and potential BCAs for pathogen attacks (Barka *et al.,* 2016). *Streptomyces* spp. are biotechnologically valuable actinobacteria that are well-recognized for their production of bioactive

secondary metabolites yielding a plethora of antibiotics and antifungal agents. To prevent damages and losses caused by plant diseases, SA isolates can be employed as a major component of IDM (Saeed *et al.,* 2017b; Ab Rahman *et al.,* 2018). The study hypothesized that the antifungal compounds and/or CWDEs produced by the SA strains living in their natural habitats can be deployed to combat the causal agent of *D. regia* stem canker disease.

In the present study, screened actinobacterial communities (SA and NSA) from local soils of the UAE for their properties as BCAs capable to restrict the negative effects of *N. dimidiatum* on *D.regia* were done. Forty-seven actinobacterial strains (31 SA and 16 NSA) were obtained from the rhizosphere of healthy *D. regia* trees. The genus *Streptomyces* has been reported to be the most dominant among the isolates (Palaniyandi *et al.,* 2013); whereas NSA strains have been rarely isolated and are generally less abundant is soils (Jose & Jebakumar, 2013). Along with that, 65.9% and 34.1% of the isolates in this study were considered as SA and NSA groups, respectively. The isolation of NSA (e.g, *Actinoplanes*, *Microbispora*, *Micromonospora,* and *Streptosporangium*) was facilitated by the use of polyvalent *Streptomyces* phages. Earlier studies have indicated the potential value of using such *Streptomyces* phages to eliminate *Streptomyces* colonies from isolation plates (Kurtböke *et al.,* 1992). Isolates of NSA have previously been reported to manage cavity spot disease of carrots caused by *Pythium coloratum* (El-Tarabily *et al.,* 1997), post-emergence damping-off of cucumber caused by *Pythium aphanidermatum* (El-Tarabily, 2006), and dieback disease on mango caused by *Lasiodiplodia theobromae* (Kamil *et al.,* 2018). This, however, was not the case with those affiliated to this group in the present study; where none were able to adequately control stem canker disease.

For a proper assessment of BCA candidates against *N. dimidiatum*, a number of screening steps were followed developed by Köhl *et al* (2011). First, primary *in vitro* screening assays were performed to identify the diffusible antifungal metabolites- and/or CWDEs-producing actinobacterial isolates. Second, *in vivo* apple fruit bioassays were carried out to select the potential SA antagonists to *N. dimidiatum*. After the identification of promising BCA candidates to the species level, a greenhouse experiment was carried out to evaluate them in managing stem canker disease on *D. regia* seedlings. Consequently, 10 antagonists were considered as highly diffusible antifungal metabolite-producing isolates on fish meal extract agar plates; while 11 possessed CWDEs to hydrolyze cell-wall of *N. dimidiatum* on colloidal chitin broth. Many potent BCA isolates also showed large inhibition of *N. dimidiatum* on agar plates containing mycelial fragment of *N. dimidiatum* and colloidal chitin agar plates. This indicates that these antagonists may have the ability to secrete chitinase and ß-1,3-glucanases to lyse the viable hyphal cells of *N. dimidiatum*. Previously, several reports have used similar techniques to isolate glucanolytic and chitinolytic BCAs against *Phytophthora fragariae*, *Pythium aphanidermatum* and *L. theobromae* (Valois *et al.,* 1996; El-Tarabily, 2006; Kamil *et al.,* 2018). The data obtained from the *in vitro* studies, in the current study, highlight the importance of SA in serving as potential BCAs against *N. dimidiatum*.

It is understood that greenhouse and field research will ascertain the effectiveness and efficacy of the tested indigenous BCA isolates. To eliminate extensive labor and reduce wasted time generated from these controlled studies, in addition to the high number of potential BCA isolates to be tested, the antagonistic candidates using the “novel” apple fruit bioassay were screened. For that reason, the pathogenicity of *N. dimidiatum* was demonstrated on mature apple fruits in the absence

and presence of individual BCAs previously identified from the *in vitro* tests. Laboratory screening bioassays are of great merit, because they provide a rapid detection of antagonistic candidates and a relative potency of their products. For example, *in vivo* assays on carrots showed that the tested BCAs had the ability to inhibit growth of the soil-borne pathogen *P. coloratum* (El-Tarabily *et al.,* 1997). Similarly, BCA candidates were found to reduce the lesion size of the foliar fungal pathogen *L. theobromae* on mango fruits (Kamil *et al.,* 2018). Al Raish *et al.* (2020) have used apple fruit bioassay to determine the effect of chemical fungicides on *N. dimidiatum* in the laboratory prior the "real-life" experiments in the field. In general, *in vivo* fruit bioassays may precisely speculate the results to be obtained in the greenhouse/field trials. It is believed that the reduced pathogen population detected *in vitro* could potentially support the cell-wall integrity of apple fruits against *N. dimidiatum* infection. *In vitro* tests, it was consistently found that many isolates completely killed the pathogen; whereas others showed minimum or even no effect on disease lesion development on apple fruits (Table 8). Thus, this suggests that the ability to produce antifungal compounds in agar plates does not necessarily reproduce the same performance on plants (El-Tarabily *et al.,* 1997; Kamil *et al.,* 2018). This data indicate that both *in vitro* and *in vivo* bioassays are essential pre-screening methods to assess the effect of potential antagonists on disease development before proceeding with the pot experiments.

Actinobacteria have long been a beneficial biocontrol against plant pathogens and a remarkable source in suppressing plant diseases. Such examples of BCAs employing antibiosis include, but no limited to, *L. theobromae*, *Pythium ultimum*, *P. coloratum*, *Rhizoctonia solani*, *Thielaviopsis punctulata* and *Fusarium oxysporum f. sp. lycopersici* (Yuan & Crawford, 1995; El-Tarabily *et al.,* 1997; Saeed *et al.,* 2017b;

Kamil *et al.,* 2018; Vinchira-Villarraga *et al.,* 2021). In the present study, three isolates representing 6.4% of the total isolated actinobacteria were further identified according to their promising *in vitro* and *in vivo* results; thus, belonging to *Streptomyces* spp. The data confirm previous findings that SA are predominantly isolated from soils and are distinct for the production of bioactive secondary compounds (Barka *et al.,* 2016). Based on their 16S rRNA sequence comparisons, cultural and morphological characteristics, isolates #25 (BCA1), #23 (BCA2) and #17 (BCA3) were identified as *S. rochei* UAE2, *S. coelicoflavus* UAE1 and *S. antibioticus* UAE1, respectively. The adverse effect of *S. rochei* and *S. antibioticus* UAE1 on *N. dimidiatum* was associated with the production of diffusible antifungal metabolites; and volatile compounds in case of *S. rochei* only. On the other hand, *S. coelicoflavus* and *S. antibioticus* were considered as CWDEs-producing isolates.

Fungal cell-wall is mainly composed of chitin and ß-glucans (Osherov & Yarden, 2010). Chitinase and ß-1,3-glucanase activities have been related to the degradation of the cell-walls and suppression of populations of plant fungal pathogens. Therefore, CWDEs synthesized by BCAs are responsible for effective disintegration of fungal mycelial or conidial walls (Berini *et al.,* 2018). In the current study, one of the criteria for the selection of potential BCAs was based on the production of chitinase and ß-1,3-glucanase against *N. dimidiatum*. Several SA, such as *Streptomyces viridicans S. viridodiasticus* and *Streptomyces* spp., have previously been tested for their abilities to produce chitinases against phytopathogens (Gupta *et al.,* 1995; Singh *et al.,* 1999; El-Tarabily *et al.,* 2000). Moreover, ß-glucanase-producing SA isolates have been reported to hydrolyze fungal cell-wall glucans, resulting in the suppression of root rot of raspberry and *Fusarium* wilt of cucumber caused by *Phytophthora*

fragariae and *Fusarium oxysporum f. sp. cucumerinum*, respectively (Valois *et al.,* 1996; Singh *et al.,* 1999).

In many cases where a single mode of action has intensively been studied for a single biocontrol strain, antagonistic interactions driven by more than one mode of action in individual or a group of BCAs may lead to better results. In comparison to *S. rochei* (BCA1) that produced diffusible and volatile inhibitory antifungal compounds and *S. coelicoflavus* (BCA2) that produced CWDEs, *S. antibioticus* (BCA3) possessing synergistic mechanisms of antagonism had additive effect of suppression on stem canker disease. This is in agreement with a previous study when pre-inoculated mango plants with *Streptomyces samsunensis* resulted in higher levels of disease protection in mango against *L. theobromae* than those pre-inoculated with *Streptomyces cavourensis* UAE1 or *Micromonospora tulbaghiae*, producing diffusible inhibitory antifungal metabolites or CWDEs, respectively (Kamil *et al.,* 2018). A mixture of *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* possessing different modes of action has been reported effective in controlling root rot and crown rot of cucumber caused by *P. aphanidermatum* compared to *S. spiralis* (El-Tarabily *et al.,* 2009).

Preventive plant protection has become particularly important to control plant disease incidence and severity (Juroszek & von Tiedemann, 2011). This was evident when any of the BCAs was applied prior to inoculation with the pathogen (Figure 7). The results obtained from the reductions in conidial numbers of *N. dimidiatum in planta* and falling leaves of *D. regia* indicated that *S. rochei*, *S. coelicoflavus* or *S. antibioticus* showed considerable potential as BCAs against *N. dimidiatum*; thus *S. antibioticus* was the most effective. This could be attributed to the establishment of total biomass of the BCA population needed to provide systemic suppression of *N.*

dimidiatum without damaging the host plant. Curative applications of individual BCAs also displayed significant reduction in disease symptoms, of which the best treatment using *S. antibioticus* was comparable to the effective chemical treatment, Cidely® Top. In general, preventive activities were more effective than curative actions of BCAs. It was concluded that *S. antibioticus* was the most efficient BCA among the tested strains, and it can serve as a preventive biofungicide on *D. regia* trees to control *N. dimidiatum*.

The development of stable populations of BCAs adapted to the harsh environment is a key factor to increase the productivity of commercial BCA products (Palaniyandi *et al.,* 2013). In the UAE, *Streptomyces globosus* UAE1 and *S. samsunensis* UAE1 have been reported as successful BCAs against diseases on date palm and mango, respectively (Saeed *et al.,* 2017b; Kamil *et al.,* 2018). The actinobacterial BCAs, identified in this study, are capable of producing spores resistant to extremely hot temperatures and well-adapted to the local dry soils and arid environments found in the UAE (Goodfellow & Williams, 1983). This enables them to be as a main component in IDM programs. As a result, the use of BCAs, as natural enemies to *N. dimidiatum* and as a safer strategy, can contribute to the protection in agriculture, considering health and ecological risks in the UAE and elsewhere.

This study argues that the significant success of these selected strains as BCAs might probably be due to the mechanism of actions. Also, it should be note that other factors by these BCAs, such as ISR, may contribute to enhance disease resistance of the host plant (Martínez-Hidalgo *et al.,* 2015). Further investigation in this field of ISR and/or other protective mechanisms developed by BCAs is on top of priority. Here, in this study, it was report SA, for the first time, as microbial antagonists to manage diseases caused by *Neoscytalidium* species. The present study is also the first to report

the use of BCA to control stem canker of *D. regia* caused by *N. dimidiatum* under greenhouse conditions. Testing these treatments on a large-scale (example: field) to determine the feasibility of such recommendations is on top of priorities. Future research will focus on the development of biotechnological tools to improve BCA mechanisms to manage stem canker disease for a sustainable agriculture.

Chapter 4: General Conclusion

In conclusion, under greenhouse and field conditions, Cidely® Top proved to be the most effective fungicide against *N. dimidiatum* among all tested chemical treatments. This data suggest that the causal agent of stem canker disease on *D. regia* in the UAE was *N. dimidiatum*. On the other hand, the multiple mode of actions in *S. antibioticus* can be particularly effective to produce synergistic actions against the fungus. This study is the first to explore the potential to use both biological control and fungicides to further develop an integrated disease management strategy against stem canker disease in *D. regia*.

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List of Publications

Al-Raish, S. M., Saeed, E. E., Sham, A., Al-Blooshi, K., El-Tarabily, K. A., & AbuQamar, S. F. (2020). Molecular Characterization and Disease Control of Stem Canker on Royal Poinciana (*Delonix regia*) Caused by *Neoscytalidium dimidiatum* in the United Arab Emirates. *International Journal of Molecular Sciences,* 21(3), 1033. https://doi.org/10.3390/ijms21031033

Appendix

List of Supplementary Data

Used media

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

1. **In order to increase the populations of SA and decrease other non-actinobacterial isolates, pre-treatments of soils were followed (Hayakawa & Nonomura, 1987).**

   Briefly, the soil pre-treatments involved preparing serial dilutions of the soil suspension by suspending the sample in yeast extract (YE) (BBL, Becton Dickinson, Cockeysville, M.D., U.S.A.) and sodium dodecyl sulfate (SDS) (Sigma) (YE 6% + SDS 0.05%) for 20 min at 40°C to remove other factors promoting bacterial growth or injurious to germinating actinobacteria spores (Nonomura and Ohara, 1969*a*) and diluting with water. YE and SDS were included to increase and decrease the numbers of actinobacteria s and bacteria, respectively (Nonomura and Hayakawa, 1988). HVA was used since it is superior to other media currently used for the isolation and enumeration of soil actinobacteria . It permits the growth of large numbers of soil actinobacteria and limits the growth of soil bacteria more than other media (Hayakawa and Nonomura, 1987).

2. **Mycelial Fragment Agar**

   The aim of this experiment was to screen the actinobacteria isolates (31 streptomycete and 16 non-streptomycete) for their ability to produce clear zones on *N. dimidiatum* mycelial fragment agar as described by Valois *et al.* (1996. *N. dimidiatum* was grown in 30 mL of potato dextrose broth (PDB) (Difco Laboratories, Detroit, MI, USA) at 28°C for 7 d in the dark. The cultures were then autoclaved and centrifuged (7500 x *g*, 10 min), and the pellet was rinsed four times with sterile deionized water. The mycelial mats were then resuspended in 30 mL of sterile deionized water and homogenized using an Omni-mixer (Omni International, Inc., GA, USA) at 4000 rpm for 20 min. Three volumes of sterile water and agar at a concentration of 2% were then added to the fragmented mycelium suspensions and the resulting mycelial fragment agar was sterilized by autoclaving at 121°C for 20 min (Valois *et al.* 1996). Seven-d-old isolates grown on OMYEA were inoculated onto the mycelial fragment agar medium and incubated at 28°C for 15 d in the dark until zones of clearing were seen around and beneath the colonies. Five replicate plates were used for each isolate. Clear zone diameters were measured (mm) and used as an indicator of glucanolytic activity.

3. Tryptic Soy Agar Medium

Tryptic Soy Agar (1/10 strength) TSA made up from:

Tryptic Soy Broth	3.0 g
Bacteriological agar	15.0 g
Distilled water	1000.0 mL
Ampicillin	50.0 mg mL^{-1}
Cycloheximide (Sigma)	75.0 mg mL^{-1}
Chloramphenicol (Sigma)	12.5 mg mL^{-1}

(a) A 250 mg capsule of ampicillin plus 400 mg cycloheximide were added in 10 mL of 70% ethanol. This was shaken and allowed to stand for 30 min, before adding to 200 mL of sterile distilled water. Eight mL of this solution is used per 200 mL of TSA.

(b) A 250 mg capsule of chloramphenicol is added to 10 mL of 70% ethanol, and allowed to stand for approximately half an hour, before adding to 200 mL of sterile distilled water. Two mL of this solution is used per 200 mL of TSA. The agar medium is autoclaved at 121°C for 20 min and the antibiotics are added to the molten agar (45°C) immediately before pouring plates.

4. Colloidal chitin broth

Colloidal chitin (Dry weight)	2 g
Calcium carbonate	0.02 g
Ferrous sulphate	0.01 g
Magnesium sulphate	0.05 g
Potassium chloride	1.71 g
Disodium hydrogen phosphate	1.63 g
Distilled water	1000.0 mL
pH	7.2

5. Potato dextrose agar (PDA)

Distilled water	1000.0 mL
PDA (Gibco, BRL, U.K.)	39.0 g

6. Potato dextrose Broth (PDB) (Lab M Limited, Lancashire, UK).

Distilled water	1000.0 mL
PDB (Gibco, BRL, U.K.)	39.0 g

7. Colloidal chitin agar

Preparation of colloidal chitin

Crude chitin (from crab shells, Sigma) was washed alternately in 1 N NaOH and 1 N HCl for 24 h periods each, on five occasions. Then, it was washed four times with 95% (v/v) ethanol. Fifteen grams of the purified white chitin is then dissolved with 100 mL of concentrated HCl and stirred in an ice bath for 20 min. The mixture is then filtered through glass wool, and the solution is poured into cold distilled water to precipitate the chitin. The insoluble chitin on the glass wool is treated again with HCl, and the process is repeated until no more precipitate is obtained when the filtrate is added to cold water. The colloidal chitin is allowed to settle overnight and the supernatant is decanted. The remaining suspension is neutralised to pH 7.0 with NaOH. The precipitated chitin is centrifuged, washed with sterile distilled water, and stored as a paste at 4°C.

The medium contained

Colloidal chitin (Dry weight)	2 g
Calcium carbonate	0.02 g
Ferrous sulphate	0.01 g
Magnesium sulphate	0.05 g
Potassium chloride	1.71 g
Disodium hydrogen phosphate	1.63 g
Distilled water	1000.0 mL
Agar	20.0 g
pH	7.2

8. Fishmeal extract agar (El-Tarabily *et al.*, 1996).

Fishmeal extract	20.0 g
Glucose	20.0 g
Peptone	5.0 g

Sodium chloride	0.5 g
Calcium carbonate	3.0 g
Distilled water	1000.0 mL
Agar	20.0 g

9. Fishmeal extract Broth (El-Tarabily *et al.*, 1996).

Fishmeal extract	20.0 g
Glucose	20.0 g
Peptone	5.0 g
Sodium chloride	0.5 g
Calcium carbonate	3.0 g
Distilled water	1000.0 mL

10. Dialysis membrane overlay technique to assay diffusible antifungal metabolites

The effect of diffusible metabolites produced by the seven actinobacteria isolates on the growth of *N. dimidiatum in vitro* was tested using the dialysis membrane overlay technique (Gibbs, 1967). Briefly, single thickness dialysis membrane (type 45311; Union Carbide Corporation, U.S.A.) was cut into circles approximately 80 mm x 80 mm, boiled in 0.1 mM ethylenediaminetetra-acetic acid (EDTA), rinsed thoroughly and then autoclaved in deionised water at 12°C for 20 min. Each membrane circle was then placed onto the surface of HFMEA, PCA and 1/5 M32 in a Petri-dish (86 mm diameter). The surface of the agar was allowed to dry for 30 min before and after addition of the membrane in a laminar flow cabinet. The dialysis membrane was inoculated with the actinobacteria by evenly streaking spores from a 7 day-old culture of each isolate on the whole surface of the cellophane circle. Care was taken not to spread colonies beyond the circle margins. There were five replicates for each actinobacteria isolate and the plates were incubated at 28 ± 2°C in the dark for 10-14 days depending on the growth rate of the actinobacteria isolate. The

dialysis membrane and the adhering actinobacteria colony were then carefully removed from the agar plate and the center of each plate was inoculated with a disc (5 mm diameter, colonised surface down) of *N. dimidiatum* cut from the margin of a 4 day-old culture grown on PCA. The Petri-plates were incubated in the dark at 25 ± 2°C for 4 days. Colony diameter of *N. dimidiatum* was measured daily for 4 days and compared to the diameter of control plates where the pathogen was grown as above, but in the absence of the antagonist metabolites. If after 4 days of incubation, the pathogen had not grown from the agar plugs, the plugs were removed and placed onto a fresh plate of PCA and incubated for 5 days at 25 ± 2°C. This was in order to determine whether the antifungal metabolites were fungicidal or fungistatic. Significant differences between means were determined by Duncan's New Multiple Range Test at $P = 0.05$ using Superanova (Abacus Concepts, Inc., Berkeley, California, U.S.A.) .

11. Inorganic salt-starch agar (starch nitrate agar) (SNA)

Soluble starch	10 g
Potassium nitrate	2 g
Di-potassium hydrogen phosphate	1 g
Magnesium sulfate	0.5 g
Sodium chloride	0.5 g
Calcium carbonate	3 g
Ferrous sulfate	0.01 g
*Trace salt solution	1 mL
Cycloheximide (Sigma)	50 µg mL^{-1}
Nystatin (Sigma)	50 µg mL^{-1}
Distilled water	1 L
Agar	20 g

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

12. Oat-meal yeast extract agar (OMYEA)

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

13. Modified chrome azurol agar (CAS agar)

CAS agar was prepared by mixing four separately sterilized solutions. Solution 1 which is the Fe-Chrome azurol S indicator solution was prepared by mixing 10 mL of 1 mM FeCl3 (in 10 mM HCl) with 50 mL of an aqueous solution of Chrome azurol S (CAS) (1.21 mg mL^{-1}) (Sigma). The resulting dark purple mixture was slowly added to 40 mL of an aqueous solution of hexadecyltrimethylammonium bromide (HDTMA) (1.82 mg mL^{-1}) (Sigma) with continues with stirring. The resulting solution was then autoclaved separately and cooled down to 50°C.

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

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

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

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