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Fungal diversity and the occurrence of antagonistic fungi in organic and conventional farming systems in Oman

Elham Ghasemi Kazerooni

A thesis submitted in partial fulfillment

of the requirements for the degree

Doctor of Philosophy

in

Crop Sciences

Department of Crop Sciences

College of Agricultural and Marine Sciences

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Dedication

To my parents and brothers, without them none of my success would be possible and those who will never give up on their dreams no matter how unrealistic they might seem to others

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Fungal diversity and the occurrence of antagonistic fungi in organic and conventional farming systems in Oman

Abstract

In Oman, the farming system in the majority of farms follows a conventional system, which is characterized by growing multiple crops mainly for home consumption, but also for local market and sometimes for export. In addition, some farms have started recently adopting organic farming. This study was conducted to assess fungal diversity in the rhizosphere of crops under conventional and organic farming systems in Oman and also to investigate for the potential presence of antagonistic fungal species that can be used in future biocontrol programs.

The first part of the study dealt with evaluating the efficiency of direct plating in comparison to pyrosequencing in estimating fungal diversity in soil. Analysis of 10 soil samples collected from two farms in Oman showed that pyrosequencing detected significantly more fungal phyla, classes and genera compared to direct plating. Pyrosequencing detected five unique fungal classes that were not recovered by direct plating. This could be related to the ability of pyrosequencing to detect uncultivable and slow growing fungal species.

The second part of the study analyzed fungal diversity in conventional and organic farms growing cucumbers and tomatoes using pyrosequencing. Pyrosequencing results revealed that fungal diversity varied between the different cultivation systems. Richness estimates indicated that soils from the organic farms have higher fungal diversity compared to soil from conventional farms. *Ascomycota* and *Microsporidia* were the most dominant fungal phyla in most of the samples. Other dominant phyla included *Chytridiomycota* and *Basidiomycota*. *Microsporidetes*, *Dothideomycetes*,

Eurotiomycetes and *Leotiomycetes* were the common classes in most soil samples. Five and four unique classes were detected in the rhizospheres of cucumber and tomato grown organically, respectively. The differential level of fungal diversity within and among farms could be related to the variation in the cultural practices employed. It also shows that organic farming favors higher levels of fungal diversity.

The last part of the study examined the biocontrol potential of 36 fungal isolates obtained from the rhizospheres of tomato and cucumber in Oman against *Pythium aphanidermatum* and *Rhizoctonia solani*, the causal agents of damping-off of cucumber. *In vitro* screening of the 36 fungal isolates against *P. aphanidermatum* and *R. solani* showed that isolates TO144 and TT266 were the most effective in inhibiting the mycelial growth of *P. aphanidermatum* and *R. solani*. Inoculation of cucumber seedlings with the antagonistic isolates TO144 and TT266 did not have any negative effects on the survival of cucumber seedlings. Isolate TT266 significantly improved dry weight of cucumber seedlings compared to the control ($P \leq 0.05$). The two antagonistic fungal isolates significantly increased the survival of cucumber seedlings inoculated with *P. aphanidermatum* from 7% to 62% when T0144 was used and to 38% when TT266 was used. In addition, they also significantly increased the survival of cucumber seedlings inoculated with *R. solani* from 15% to 31% when T0144 was used and to 69% when TT266 was used. Identification of TO144 and TT266 to the species level using sequences of the ITS region showed that they are *Trichoderma asperellum* and *Talaromyces pinophilus*, respectively. This is the first report of the potential of *Talaromyces pinophilus* as a biocontrol agent for *Pythium* and *Rhizoctonia* damping-off of cucumber. The study also shows that fungal isolates present in the rhizosphere of vegetable crops can be potential biocontrol agents for soil borne diseases.

تنوع الفطريات ووجود الفطريات ذات خاصية المكافحة الحيوية في نظم الزراعة العضوية والتقليدية في سلطنة عمان

الملخص

يتبع النظام الزراعي في معظم المزارع في سلطنة عمان نظاما تقليديا يتميز بزراعة محاصيل متعددة تستخدم للاستهلاك المنزلي، وللأسوق المحلية وأحيانا للتصدير. وبالإضافة إلى ذلك، بدأت بعض المزارع مؤخرا في تبني الزراعة العضوية، وقد أجريت هذه الدراسة لتقييم التنوع الفطري في المنطقة المحيطة بجذور المحاصيل في إطار نظم الزراعة التقليدية والعضوية في عمان، وكذلك للتحقق من وجود أنواع فطرية يمكن استخدامها في برامج المكافحة الحيوية.

تناول الجزء الأول من الدراسة تقييم كفاءة العزل المباشر بالمقارنة مع طريقة تفاعل سلسلة البايرو (pyrosequencing) في تقدير التنوع الفطري في التربة، حيث أظهر تحليل عشرة عينات من التربة تم جمعها من مزرعتين في عمان أن طريقة pyrosequencing أكثر كفائة بكثير في الكشف عن الفطريات مقارنة بالعزل المباشر. كشفت طريقة pyrosequencing خمسة طوائف فريدة من نوعها لم تكتشف بالعزل المباشر. ويمكن أن يكون هذا مرتبطا بقدرة pyrosequencing الكشف عن الأنواع الفطرية الغير القابلة للزراعة وكذلك الأنواع بطيئة النمو.

أما الجزء الثاني من الدراسة فقد ركز على تحليل التنوع الفطري في المزارع التقليدية والعضوية التي تزرع الخيار والطماطم (البندورة) باستخدام تقنية pyrosequencing. كشفت النتائج أن التنوع الفطري يختلف بين أنظمة الزراعة المختلفة. حيث وجد أن التربة من المزارع العضوية تمتلك تنوع فطري أعلى بالمقارنة مع التربة من المزارع التقليدية. كانت أسكوميكوتا و ميكروسبوريديا (Ascomycota and Microsporidia) أكثر شعب الفطريات السائدة في معظم العينات، كما وجدت كذلك شعب اخرى مثل تشيتريديوميكوتا وباسيديوميكوتا. ميكروسبوريديتس، دوثيدوميستس، وروتيوميستس و ليوتيوميستس. (Chytridiomycota and Basidiomycota. Microsporidetes, Dothideomycetes, Eurotiomycetes and Leotiomycetes). تم الكشف عن ستة وأربع فئات فريدة من نوعها في المنطقة المحيطة بجذور الخيار والطماطم المزروع عضويا، على التوالي. ويمكن أن يرتبط المستوى التفاضلي للتنوع الفطري داخل المزارع وفيما بينها بالتفاوت في الممارسات الزراعية المستخدمة، كما تشير الدراسة الى أن الزراعة العضوية تحتوي على مستويات أعلى من التنوع الفطري.

أما الجزء الأخير من الدراسة فقد ركز على فعالية المكافحة الحيوية ل 36 عزلة فطرية تم الحصول عليها من المنطقة المحيطة بجذور الطماطم والخيار في عمان ضد فطري االبيثوم أفانيدرماتوم وريزوكتونيا سولاني، واللذان يسببان موت بادرات الخيار. أظهر الفحص المختبري للعزلات الفطرية ال 36 ضد االبيثوم أفانيدرماتوم والريزوكتونيا سولاني قدرة سلالتين (TO144 وTT266) على التاثير في نمو االبيثوم أفانيدرماتوم والريزوكتونيا سولاني. لم لم تؤثر سلالات (TO144 و TT266) سلبا على شتلات الخيار، وفي المقابل حسنت

السلالة TT266 بشكل ملحوظ من الوزن الجاف لشتلات الخيار مقارنة مع الشاهد ($P \leq 0.05$). عززت السلالات بشكل كبير من بقاء الخيار الملقح باالبيثوم أفانيدرماتوم من 7٪ إلى 62٪ عندما تم استخدام T0144 وإلى 38٪ عندما تم استخدام TT266. وبالإضافة إلى ذلك، زادت بشكل ملحوظ من بقاء الخيار الملحق بالريزوكتونيا سولاني من 15٪ إلى 31٪ عندما تم استخدام T0144 وإلى 69٪ عندما تم استخدام TT266. اظهر التشخيص باستخدتم الحمض النووي انتماء السلالتين TO144 و TT266 الى تريكوديرما أسبريلوم و تالاروميسز بينوفيلوس، على التوالي. هذا هو التقرير الأول عن إمكانية استخدام تالاروميسز بينوفيلوس(*Talaromyces pinophilus*) كعامل مكافحة حيوية للبيثوم والريزوكتونيا المسببين لموت بادرات الخيار. وتبين الدراسة أيضا أن العزلات الفطرية الموجودة في منطقة الجذور في محاصيل الخضروات يمكن أن تكون عوامل مكافحة حيوية محتملة للأمراض التي تنتقل عن طريق التربة.

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Abbreviation and Symbols

%	Percentage
Bp	Base pair
^{o}C	Degree Celsius
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
µl	Microlitre
NCBI	National Center for Biotechnology Information
P	Phosphorus
K	Potassium
N	Nitrogen
EC	Electrical conductivity
TIC	Total inorganic carbon
TOC	Total organic carbon
mS	Millisiemens
Mg	Milligram
Kg	Kilogram
OR	Organic farm
CN	Conventional farm
TO144	*Trichoderma asperellum* isolate TO144
TT266	*Talaromyces pinophilus* isolate TT266

1. Introduction

Oman is situated at an arid region in the eastern part of the Arabian Peninsula and in summer, the temperature can rise up to 50 °C. Most farms in Oman use traditional methods (conventional) to promote biodiversity by cultivating several crops in the same field. The majority of farms in the northern part of the country grow date palms and citrus (mainly acid lime). In addition, vegetable crops such as tomatoes and cucumbers are cultivated almost all over the country (Al-Sadi et al., 2013; Kazeeroni and Al-Sadi, 2016). Tomato is considered the top vegetable crop in terms of production, with an annual production of 111,000 tons in 2016. Cucumbers are also produced widely in the country, with an annual production of 28,000 tons (FAO, 2017). Tomatoes are usually produced in open fields, while cucumber production is mainly in greenhouses (Al-Sadi et al., 2013; Al-Sadi et al., 2014; Al-Sadi et al., 2015a; Al-Sadi et al., 2015c).

Animal manures are commonly used in farms in Oman, especially for date palms and citrus (Al-Sadi et al., 2011a; Al-Azizi et al., 2013; Al-Sadi et al., 2015b). However, potting media, composts and several organic and inorganic fertilizers are introduced and used for vegetable crops, mainly cucumbers and tomatoes (Al-Mazroui and Al-Sadi, 2015). In addition, the infection of cucumbers by pathogenic fungi necessitates frequent applications of systemic and contact fungicides (Al-Sadi, 2012; Al-Sadi et al., 2012a; Al-Sadi et al., 2015a). On the other hand, there is a shift towards organic farming in some farms in the country, but the growth rate of this sector is very slow.

Soil is a precious and complex natural resource that represents a huge reservoir of biodiversity with several billion prokaryotic and eukaryotic microorganisms (Abed et al., 2013; Al-Sadi et al., 2015c; Thomson et al., 2015; Kazeeroni and Al-Sadi, 2016; Wang et al., 2017). These microbes significantly share biomass and ecosystem functions in both natural and managed agricultural soils (Sidorenko et al., 1978). Intensive agricultural production system degrades soil quality and function and over the time causes a decline in crop yields which endanger food security and production (Eldridge et al., 2014). Fungi are the most dominant eukaryotic species in terms of biomass in soil. Fungi play important roles as decomposers, nutrient cyclers, soil aggregators, pathogens and mycorrhizal symbionts (Guo et al., 2015; Thomson et al., 2015; Stott and Taylor, 2016).

Changes in land use and agricultural practices have resulted in reduction in soil quality, fertility and productivity (Cherubin et al., 2015; Price et al., 2015). The productivity and health of soils rely to some extent on the processes of soil microbial communities (Guo et al., 2015; Heilmann-Clausen et al., 2015; Stott and Taylor, 2016). Excessive use of inorganic fertilizers and pesticides can affect soil microbial populations and result in reduction of microbial diversity or changes in microbial communities (Esmaeili Taheri et al., 2015; Filimon et al., 2015; Pose-Juan et al., 2015; Rangel et al., 2015). Microbial abundance, diversity and activity largely have implications on sustainable productivity of agricultural land and production systems. Information on the microbial communities associated with rhizospheres and their complex interrelationship is essential in the selection of sustainable crop rotations and management practices (Lenc et al., 2015; Chen et al., 2017).

Detection and quantification of fungi in soil is very important. Several methods can be used for the estimation of fungal diversity in soils. Most studies in the past focused on the use of direct culture of microorganisms (Al-Sadi et al., 2015b; Thomson et al., 2015; Kazeeroni and Al-Sadi, 2016). With the advent of next generation sequencing technologies, 454 pyrosequencing has been used for assessing fungal diversity in some studies (Esmaeili Taheri et al., 2015; Kazeeroni and Al-Sadi, 2016). It helped uncover the presence of several fungal populations, including many of the taxa that are not cultivable on synthetic media. In addition, the method helps quantify the presence of each fungal taxa.

Previous studies have shown that the population size and structure of soil flora and fauna can be affected by several factors, including the cultivation techniques, plant species, and the application of organic and inorganic fertilizers and pesticides (DeAngelis et al., 2015; Matsushita et al., 2015; Tardy et al., 2015; Van Geel et al., 2015; Coleman-Derr et al., 2016). However, little information is available concerning the effect of cultivation systems on fungal diversity in this part of the world. Information is lacking concerning the level of fungal diversity in organic farms and conventional farms in Oman.

Tomatoes and cucumbers face several challenges, with damping-off and vine decline diseases being two of the most limiting diseases, especially to cucumbers. Losses due to these diseases can reach as high as 75% of cucumber seedlings, especially because of damping-off (Al-Sadi et al., 2011c). The diseases are caused by several fungal species, the most important of which are *Pythium* and *Rhizoctonia* species. In Oman, *Pythium aphanidermatum*, *P. spinosum* and *R. solani* are the common fungal pathogens causing damping-off disease, while *Pythium* spp., *Rhizoctonia solani*, *Monosporascus* spp. and *Fusarium solani* are associated with vine decline (Al-Sadi et al., 2011c).

Management of damping-off can be attained by using a number of methods. Mefenoxam fungicide has been proven to be an effective fungicide for Pythium damping-off (Moorman and Kim, 2004; Al-Sadi et al., 2012a). Crop rotation with a non-host plant or field sanitation can be considered as alternative methods for controlling *Pythium* populations (Davis and Nunez, 1999). Although applying chemicals may help and give some degree of control, their side effects on humans and the environment are detrimental. The use of biocontrol agents is a safe alternative to the use of chemicals. Several biocontrol agents have been found effective in managing damping-off disease of cucumber. These include the use of *Trichoderma harzianum*, *Pythium oligandrum* and *Pseudomonas aeruginosa* (Al-Rawahi and Hancock, 1998; Punja and Yip, 2003; Al-Hinai et al., 2010; Blaya et al., 2013; Yu et al., 2014).

The isolation and identification of antagonistic microorganisms as potential and suppressive biocontrol strains from different sources such as soil and plant is becoming more popular (Huang et al., 2016; Siala et al., 2016; Raza et al., 2017). Biocontrol agents may suppress diseases in various ways. Their mode of action may involve the production of antimicrobial compounds, release of plant growth promoting compounds, induction of defense mechanisms in plants, and nutrient competition with pathogens (Ting et al., 2014; Arroyave-Toro et al., 2017; Collazo et al., 2017; Culebro-Ricaldi et al., 2017; Zhang et al., 2017). In Oman, little is known whether fungi present in the rhizosphere of crops could be used as potential biocontrol agents against major soil borne pathogens.

This study was conducted to investigate fungal diversity in rhizosphere soils from conventional and organic farms in Oman and to isolate potential antagonistic fungal species.

The study objectives to:

1- Compare the efficacy of direct plating and pyrosequencing in estimating fungal diversity in soil.
2- Investigate the level of fungal diversity in conventional vs organic farming systems growing cucumbers and tomatoes.
3- Investigate the potential presence of antagonistic fungal species in the rhizosphere of cucumbers and tomatoes that can be used for the management of damping-off disease caused by *Pythium* and *Rhizoctonia*.

Microbial abundance, diversity and activity largely have implications on sustainable productivity of agricultural land and production systems. Information on the microbial communities associated with rhizospheres and their complex interrelationship is essential in the selection of sustainable crop rotations and management practices (Lenc et al., 2015; Chen et al., 2017). Improving soil quality can be considered as potential way for improving food security and production.

2. Review of Literature

2.1 Fungi

Fungi are considered as one of the major clades with eukaryotic life. They have a wider geographical distribution compared to plants and other organisms (Hernández-Restrepo et al., 2017). This hyper diverse group of microorganisms spread in various terrestrial and aquatic ecosystems by employing different strategies such as pathogenic, saprobic or symbiotic and are able to adapt themselves or escape from changing environmental conditions. The number of species for this remarkable and diverse group has been estimated to be between 1.5 and 5.1 million since many habitats have remained unexplored (Hawksworth, 1991; Blackwell, 2011). Fungal mode of growth varies and can range from unicellular to multicellular forms with the ability of producing fruiting bodies. Based on phylogenetic analysis, fungi are divided into four main groups including: Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota and the majority of plant pathogenic fungal species belong to these groups (Hawksworth, 1991; Agrios, 2005). Among soil microbial community, fungi take place after bacteria as the second most abundant group of soil microbiota. However, fungi sometimes exist as the most dominant microbial biomass in a specific soil. Some of them are plant pathogens and can cause severe economic losses. Serious economic losses on vegetables and crops can happen due to soil borne diseases. Some of the important pathogenic fungal genera are *Fusarium*, *Pythium*, *Rhizoctonia* and *Sclerotinia*. Handling and controlling soil borne disease is normally difficult because pathogens can survive and build up their populations through their persistent structures for several years (Lumsden and Ayers, 1975; Hancock, 1977; Peethambaran and Singh, 1977).

Some fungi are responsible for decomposing plant residues (Zhang et al., 2014b; Hernández-Restrepo et al., 2017). The filamentous hyphae of fungi enable them to explore and exploit more environments for food and translocate and store deficient nutrients to distant parts of the soil where nutrients may be lacking, allowing reproduction and growth to continue. Moreover, fungal hyphal filaments decompose plant residues and organic materials by releasing enzymes which breakdown all these material to the absorbable form for fungi (Zhang et al., 2014b). Some fungi play a role as parasites of other fungi, plants, nematodes and insects. Improving soil structure and aeration is another role of the soil fungi. Some of them may form mutualistic relationship with plant and help the plant to uptake more nutrients (Win et al., 2012).

2.2 Role of fungi in soil

Soils are the foundation of all terrestrial ecosystems and are colonized by a variety of microorganisms such as bacteria and fungi, with an estimated 10^7–10^9 distinct bacterial species and 1.5-5 million fungal taxa worldwide (Blackwell, 2011). Soil microorganisms certainly play a central role in soil processes including recycling, transformation and making nutrients (Jackson et al., 2013; Tardy et al., 2015; Huang et al., 2016). Soil fertility and available nutrients for plant growth play an important role and provide the optimum conditions for increasing crop yields. Soil with higher fertility has more diverse mass of microbes, which lead to less availability of niches for pathogens to compete and less availability of nutrients for pathogens to utilize and proliferate (Martin and Hancock, 1986; Manjunath et al., 2017). Similarly, plant nutrition impacts environmental sustainability, cropping systems and human health (El-Ramady et al., 2014). Soil fertility depends on the proper balance of available nutrients during a particular time of a season for crop growth. However, sufficient quantities of these nutrients cannot guarantee their availability for plant as the other factors such as presence of salt or toxic elements or soil pH may restrict plant growth (El-Ramady et al., 2014). Availability of nutrients and acquisition by plants can be influenced by soil, plant and microbes. These factors vary from one ecological region to the other or even from one field to the other field in the same area. Plant diversity can be mediated through negative or positive feedback mechanisms of soil microbial communities.

Fungi along with bacteria are one of the most important groups of organisms that play critically important and different ecological roles in maintaining sustainability, vitality and health of ecosystem. They perform important services and different activities related to water dynamics and holding capacity, soil nutrient transformation and provision, soil structure stabilization and quality, organic matter decomposition and formation, recycling of wastes and detoxification, converting plant-derived compounds into usable forms, contributing to disease suppression and plant protection against phytopathogens, promoting plant health and growth, bioremediation, filtering of contaminants, carbon storage and regulation of greenhouse gas emissions and other ecosystem biochemical processes (De Gannes et al., 2013; Peay et al., 2013; Acosta-Martínez et al., 2014; Huang et al., 2015). However in some cases, they are responsible for plant diseases and devastation of crops. Soil fungi are normally endemic to a specific region and their community dispersal varies in nature and are influenced by several factors such as climate (Al-Azizi et al., 2013; Bernard et al., 2014; Esmaeili Taheri et al., 2017).

2.3 Type of fungi

2.3.1 Beneficial fungi

The ecological relationships and interactions with the surrounding environment can lead to beneficial to detrimental outcomes under particular environment and conditions (Pérez-Brocal et al., 2013). Symbiosis is defined as association among partners namely a host and symbiont. The host is the producer of resource while the symbiont is considered as the consumer of resource and may provide services (Pérez-Brocal et al., 2013). Also, switching between symbiosis and parasitism is possible. A variety of beneficial fungi may live on plant parts as contributor to provide plant nutrition or protect plants against pathogens (Chandna et al., 2014; Yurnaliza et al., 2014; Vasumathi et al., 2017).

Fungi play indispensable and critical roles in terrestrial ecosystems as decomposers, biogeochemical nutrient cyclers, soil aggregators, pathogens, and mycorrhizal symbionts (Barea et al., 2011; Bagyaraj, 2014; Guo et al., 2015; Heilmann-Clausen et al., 2015; Kohler et al., 2015). Three functional groups of fungi exist in soil ecosystems are: the saprotrophs, the mycorrhizas, and the lichens (Otten et al., 2004; Abed et al., 2013; Heilmann-Clausen et al., 2015). Saprophytic fungi can be named as the largest group of fungi with the ability to grow on dead organic matter (Al-Mazroui and Al-Sadi, 2016; Hernández-Restrepo et al., 2017). Saprotrophic fungi often dominate the surface layers of the soil profile, where they decompose shed plant litter, fallen trees and debris of animals or insects.

The term mycorrhiza (meaning fungus roots) was introduced by Albert Bernard Frank in 1885 (Siddiqui and Pichtel, 2008). Mycorrhizae are classified based on the arrangement of their hyphae in or around plant roots (Smith and Read, 2008). Currently, ectomycorrhizae and arbuscular mycorrhizae are described as the most abundant types of mycorrhizae, which occupy a majority of plant species except Brassicaceae and Chenopodaceae families (Smith and Read, 2008; Bagyaraj, 2014). Arbuscular mycorrhizal fungi (AMF) account for a frequency up to 50% of soil microbial biomass. Additionally, they can be found among the other plant species such as gymnosperms, bryophytes, and ferns (Lipson and Kelley, 2014). They enhance and modulate plant growth and tissue nutrient content which lead to an increase in their survival rates under various stressful conditions (Barea et al., 2011; Bagyaraj, 2014; Lipson and Kelley, 2014; Van Geel et al., 2015). In contrast, just 3% of vascular plants are associated with ectomycorrhizal fungi but they are dominant in forest ecosystems (Lipson and Kelley, 2014). Arbuscular mycorrhizae are classified as obligate biotrophs while ectomycorrhizae are considered as saprotrophs and they can access more to soil nutrients compared to AM fungi. Mycorrhizal symbioses have been accepted universally as fundamental potent for soil health and quality restoration which result in better nutrient capture for plant (Smith and Read, 2008; Barea et al., 2011). Mycorrhizal hyphae significantly enhance the surface area for plant roots by changing their physiology and architecture. Mycorrhizal fungi produce several times more hyphae which efficiently acquit mineral nutrients under even nutrient limiting conditions, activate dissolvable nutrients and transport them back to the plant (Siddiqui and Pichtel, 2008; Smith and Read, 2008; Bagyaraj, 2014; Cavagnaro, 2014; Kohler et al., 2015; Van Geel et al., 2015). Numerous findings provided insight onto the propensity of mycorrhizal fungi in bioprotection against biotic and abiotic stresses such as pathogens, pests, drought, salinity (Smith and Read, 2008; Barea et al., 2011; Heilmann-Clausen et al., 2015).

The term endophytic fungi was coined by de Bary in 1886 that refers to systemic symbiotic fungi that occupy living plant tissues (root, leaf or transmitted to their host seed) without causing any pathogenic effect (Araújo et al., 2002; Sudha et al., 2016).