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Diversity of fungal communities in the soil of three nurseries in Tlemcen (North-West of Algeria)

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Abstract:

Fungi play a central role in most ecosystems and seem to dominate the microbial biomass in soil habitats, where they are important decomposers and occupy a notable position in the natural carbon, nitrogen and phosphorus cycles. Despite the fact that fungal species have been studied in several contexts, the diversity of some soil fungal species in nurseries is still unknown. This study aimed to explore the diversity of fungal species occurring in nursery soil of Tlemcen province (North-west of Algeria) and to test the antagonist effect of some isolates of Trichoderma sp. on species of the genus Pythium and those of *Diplodia*. Soil and root samples from young seedlings showing symptoms of oomycete infection and other fungal were collected in three nurseries of Tlemcen region. Following a soil baiting using fresh ornamental leaves, three Phytophthora species were isolated and identified based on morphology and microscopic observation. About the others groups, the dilution method using physiological water, revealed the presence of significant fungal biodiversity on the PDA medium. Twelve fungal species and five oomycetes have been isolated and identified, namely: *D. sapinea, Lasio. exigua, F. oxysporum, Aspergillus sp., Penicellium sp., Mucor sp., Trichoderma sp., Alternaria sp. P. cinnamomi, P. gonapodyides, P. ramorum, Py. ultimum* and *Py. Polare*. Significant antagonist activity was recorded for isolates of *Trichoderma sp.* against *Pythium sp*. and *Diplodia sp.*

Keywords: Fungi; Soil; Oomycetes; Nursery; Tlemcen

الملخص

تلعب الفطريات دورًا مركزيًا في معظم النظم البيئية ويبدو أنها تهيمن على الكتلة الحيوية الميكروبية في موائل التربة ، حيث تعتبر عوامل تحلل مهمة وتحتل مكانة بارزة في دورات الكربون والنيتروجين والفوسفور الطبيعية. على الرغم من حقيقة أن الأنواع الفطرية قد تمت دراستها في عدة سياقات ، إلا أن تنوع بعض أنواع فطريات التربة في المشاتل لا يزال غير معروف. هدفت هذه الدراسة إلى استكشاف تنوع الأنواع الفطرية الموجودة في تربة المشتل في ولاية تلمسان (شمال غرب الجزائر) واختبار التأثير المضاد لبعض عزلات Trichoderma sp. على أنواع جنس Pythium و تلك الخاصة ب Diplodia . جمعت عينات التربة والجذور من الشتلات الصغيرة التي تظهر عليها أعراض الإصابة بالفطريات الفطرية في ثلاث مشاتل بمنطقة تلمسان. بعد تطبيق تقنية و تحديدها بناءا على الشكل المورفولوجي Phytophthoraاصطياد التربة باستخدام أوراق نباتات الزينة الطازجة، و بالتالي تم عزل ثلاثة أنواع من جنس تم عزل و تحديد PDA.حول المجموعات الأخرى ، كشفت طريقة التخفيف باستخدام المياه الفسيولوجية عن وجود تنوع بيولوجي فطري كبير على وسط و هي: Oomycetes اثني عشر نوعا من الفطريات وخمسة من نوع D. sapinea ،Lasio. exigua ،F. oxysporum ،Aspergillus sp. ،Penicellium sp. ،Mucor sp. ،Trichoderma sp. ،Alternaria sp. P.cinnamomi ،P. gonapodyides ،P. ramorum ،Py. Ultimumو Py.polare. كما تم تسجيل نشاط مضاد فى عزلات Diplodia sp ضد Pythium sp. Trichoderma sp.

الكلمات الرئيسية: الفطريات؛ تربة؛ Oomycetes، مشتلة، تلمسان

Introduction

Soil is not simply the medium in which plants take root and draw the nutrients essential for their development, but also a fantastic reservoir of Microorganisms (bacteria and fungi), in terms of diversity and density. In fact, one gram of vegetated soil contains about 1 billion bacteria divided into 5 to 25 000 species, most of which are not yet known or even cultivable in the laboratory (Curtis *et al.,* 2002). Thus, as much as 200 meters of hyphae per gram of dry soil (Leake *et al.,* 2004).

Fungi have many different functions in soils, which include either active roles, such as the degradation of dead plant material, or negative roles by degrading plant tissues (Roots, leaves, vascular tissues… (Yan *et al.,* 2019).

The complex interactions between these microbial populations and the soil determine the health of soil. The impact of these microorganisms on plant growth and health is still poorly understood. The rhizospheric microflora is naturally made up of a complex assembly of prokaryotic and eukaryotic microorganisms (Cardon and Gage, 2006; Kent and Triplett, 2002). Among these microorganisms, some are present in the rhizosphere without their influence on plant development, such as commensal fungi, some are favorable to plants, such as mycorhize fungi. While, others have deleterious effects on plants. They are known as parasitic or phytopathogenic fungi (Lepinay, 2013).

The majority of plant diseases are caused by this genus of soil-based fungi, widely distributed in the soil; causing crop rots damaging many plants, in forests, agricultural fields or nurseries which do not respect health measures. As other countries, diseases caused by soil fungi are encountered in Algeria. These fungal species growing in contact with earthy agglomerates or in cavernous interstices in the soil are, morphologically or biologically structured depending on the nature of the environment to which they are subjected. The survival of parasitic fungal species in the soil is also manifested by the production of particularly adapted organs such as mycelial encystments or clamydospore, mycelial nodules or sclerotic, cords or rhizomorphs originating from the collection of hyphae (Viennot-Bourgin *et al.,* 1970). According to Tschen *et al.* (1985), this is one reason why these telluric species are difficult to control. In addition, their infection process is rapid, either through the soil by root, irrigation water.

In order to deepen our knowledge on soil-based fungi associated with the mortality of young seedlings in North-west Algerian nurseries, a study was carried out, and aimed to: (i) Inventory the fungal mycoflora associated with the three nurseries of Tlemcen province; (ii) Identify and characterize isolated fungal species; and finally (iii) to demonstrate the antagonistic properties of some isolates of Trichoderma sp. on species of the genus *Pythium* and those of *Diplodia*.

Material and methods

Description of prospected sites

The study was conducted in Tlemcen province (North-west of Algeria) between January and March 2021. Three nurseries were selected in this region: Nursery 1 “The Growing Tree” (34°53’27”N ; 1°20’35’’W), it is a nursery type in which soil and its activity are based on intensive methods for growing different forest, fruit and ornamental species. Its area exceeds 5 ha. Nursery 2 “My garden” (34°54’16’’N; 1°19’56’’W). It is a nursery cultivating on soil, forest, fruit and ornamental trees, and exotic plants on the soil. The irrigation system is based on sprinkling with fresh water and precipitation. It is located at "Aboutachfine" -Tlemcen, and its area is more than 1 ha. Nursery 3: “La Rocade” (34°53’ 49’’N ; 1°21’01’’W). It is specialized in growing fruit trees and some forest trees, with an area exceeding 3 ha. The irrigation system is based on simple watering.

Sampling

The choice of these nurseries is based on the health status of the seedlings that exists, as well as on the presence of symptoms of *Phytophthora* and *Pythium* infections, as described by several authors (Brasier 1996; Scanu *et al.*, 2013 and Smahi *et al.*, 2017a), namely the thinning of the aerial part, discoloration or yellowing of the leaves, the presence of reduced leaves, and the progressive necrosis of the fine nourishing roots.

To inventory the fungal biodiversity involved in this state of degradation of young seedlings, we carried out random sampling, based on the visualization of diseased seedlings. A total of 42 samples were collected from the forest, fruit, and ornamental plants: (15 samples from the first nursery, 15 from the second nursery, and 12 from the third). Our sampling procedure was based on the method described by Scanu *et al.* (2013) and Smahi *et al*. (2017a):

For each seedling, roots and 500 g of rhizosphere soil were collected in opposite directions. Roots samples were cut from each seedling and placed in plastic bags, labeled and transported to the laboratory stored at 5 °C until use.

Isolation of pathogen

To detect oomycetes and other soil fungi from samples, two methods were used; the first was the baiting method, and the second was dilution with different concentration.

1. Rhizosphere soil and root pieces were water-flooded with fresh oak leaves acting as baits for Phytophthora zoospore infection (Jung *et al.*, 1996). According to our experience, the rose petals have been excellent bait for *Phytophthora* spores (Fig.1). After 3 to 5 days of incubation at room temperature, leaves showing necrotic lesions were carefully dried on sterile filter paper, cut into small squares (0.5x0.5 cm), and placed on a selective medium (Synthetic Mucor Agar) (Scanu *et al.*, 2014). Then, plates were incubated at 20 °C in the dark and monitored daily for any growth. All colonies representing typical morphology of *Phytophthora* spp. and *Pythium* spp. were subcultured separately on Potatoes dextrose Agar (PDA) for morphological and microscopic identification. The pure cultures were incubated at 20 °C in the dark.

2. The serial dilution method has been used to reliably quantify the bacterial, fungal, or viral load (Koch, 1883 ; Ben-David and Davidson, 2014). The dilution was performed with sterile physiological water as follows:

From each sample, 1g of soil is added to 9 ml of physiological water, which corresponds to the 10-1 dilution. Then, 1 ml of this dilution is added to 9 ml of sterile water to have the 10-2 dilution until reaching the 10-5 dilution. After, 1 ml of each dilution was placed on PDA (Potatoes Dextrose Agar). The plates were incubated for 5-7 days at 25 ° C in the dark.

Identification of isolates

The pure cultures obtained were grouped according to their macroscopic characteristics to choose only representatives of each group for identification. These morphological characteristics are: The texture and macroscopic aspect of the thallus, its color (face and reverse), its elevation, the smell and the shape of the margin.

Figure 1. (Left) The appearance of blackish spots on the rose petals, (Right) The development of the mycelium surrounding the squares of the rose petal on PDA.

For some oomycetes, a sporulation technique has been implemented to produce sporangia: for each isolate, 4 to 5 squares of 1 cm2 were taken and placed in a petri dish containing about 10 ml of non-sterile distilled water as used by several authors (Scanu *et al.*, 2014; Smahi *et al.*, 2017b). The plats are incubated at laboratory temperature under permanent light to accelerate the appearance of sporangia, otherwise daylight. The small squares of mycelium were checked daily under a microscope for the presence of sporangia. The shape of sporangia, chlamydospores, hyphal swellings and pedicels were recorded using "Oxion Euromex" to identify each isolate.

The other fungal species were identified, based on microscopic criteria (The shape of the vesicle, the disposition and the shape of the spores and conidia, septation of conidia, the partitioning of the mycelium, etc). Guides used are: Barnett and Hunter (1975), Champion (1997); and Botton *et al.* (1990), Erwin and Ribeiro (1996), Phillips *et al.* (2012) and (2013), Jung *et al.* (2017), Smahi *et al.* (2017a, b),

Antagonist test

Two isolate of *Trichoderma* species (*Tri. Harzianum* and *Tri. Viride*) present in the three nurseries were evaluated for their ability to control *Pythium sp*. and *Dilplodia sp.* using the method described by Benhanou and Chet (1996): 5 mm discs of each culture of Trichoderma (5 days old culture) and the same of another agar disc containing tested fungi placed opposite to each other and close to the periphery of 90 mm Petri plates containing PDA. All pairing was carried out in two replicates and incubated at 25°C.

Data analysis

The statistical analysis was performed using the MINITAB.19. The number of each fungal species was evaluated through standard error (SE). Then, a parametric test (One way ANOVA) was performed to check differences among species. Fisher test was used for multiple comparisons (<0.05).

Results and discussion

Symptomatology

During the field survey, seedlings showing symptoms of dieback and root rot disease were observed in the three nurseries (Fig.2): The most common symptoms recorded in all sites prospected are wilting and leaf yellowing, typical of root rot disease.

At the leaf level, different symptoms have been observed. First, the leaves begin to dry out from the margin and progress to the leaf blade, as well as total or partial yellowing affecting only one side of the leaf blade in some plants. Then, wilting of the leaves and inflorescences occur with necrosis on the margins of the leaves.

At the root level, different symptoms were observed. Indeed, rot and necrosis were reported in most of the seedlings. Sometimes, this root necrosis of a dark brown color tending to black, can go up to the aerial part of the plant and invade in the xylem tissues of the crown or those of the trunk.

Figure 2. Symptoms observed on infected seedlings in prospected nurseries: (A) spots on infected leaves; (B) Dieback and wilting of plants; (C) Death of young seedlings; (D) necrosis of the collar.

Fungal isolation and identification

Isolation carried out from 42 infected seedlings yielded a total of 12 fungal colonies belonging to different groups, and 5 Oomycetes (Table 1). Based on morphological features and microscopic characteristics, the species have been identified as: *Fusarium oxysporum, Alternaria solani, Alt. alternate, Trichoderma harzianum, Tri. viride, Aspergillus niger, Asp. fumigatus, Penicellium solitum, Phytophthora gonapodyides, P. cinnamomi, P. ramorum, Pythium ultimum, Py. polare, Mucor racemosus, Rhizopus stolonifer, Diplodia sapinea* and *Lasiodiplodia exigua* (Figure 3).

Table 1. Isolation of fungal species from young seedlings from the three nurseries

N° Sample	Nursery	Host plant	Sampling part	+/-	Identified species
ZR 1	Nursery 1	*Psidium guajava*	Soil	+	*Alternaria sp., Py.ultimum, Aspergillus sp., Mucor sp., Penicellium sp., Trichoderma sp.*
ZR 2	Nursery 1	*Cupressus sp.*	Collar+soil	+	*Py.polare, Mucor sp., Penicellium sp., Alternaria sp., Aspergillus sp.,*
ZR 3	Nursery 1	*Leucanthemum sp.*	Soil	+	*Py.ultimum, Trichoderma sp., Aspergillus sp.,*
ZR 4	Nursery 1	*Ficus carica*	Soil	+	*Penicellium sp., Trichoderma sp.*
ZR 5	Nursery 1	*Rosa sp.*	Root	+	*Py.polare, Mucor sp., Aspergillus sp., Trichoderma sp.,*
ZR 6	Nursery 1	*Cupressus sp.*	Collar	+	*D. sapinea, Alternaria sp.*
ZR 7	Nursery 1	*Abies sp.*	Soil	+	*D. sapinea, P. cinnamomi, Penicellium sp.*
ZR 8	Nursery 1	*Pteridium aquilinum*	Soil	-	-
ZR 9	Nursery 1	*Cupressus sp.*	Collar	-	-
ZR 10	Nursery 1	*Pteridium aquilinum*	Root	+	*Py.ultimum, Trichoderma sp., Mucor sp.,*
ZR 11	Nursery 1	*Quercus suber L.*	Soil	+	*Lasio.exigua, Mucor sp.,*
ZR 12	Nursery 1	*Quercus suber L.*	Soil	+	*Py.polare, Penicellium sp.,*
ZR 13	Nursery 1	*Quercus suber L.*	Soil	+	*Mucor sp., Aspergillus sp.,*
ZR 14	Nursery 1	*Citrus limon*	Soil	+	*Mucor sp., Aspergillus sp.,*
ZR 15	Nursery 1	*Ricinus communis*	Collar	+	*Alternaria sp., Aspergillus sp., Fusarium sp. Penicellium sp.,*
ZR 16	Nursery 2	*Cupressus sp.*	Collar	+	*Mucor sp., Aspergillus sp., Penicellium sp., Py.ultimum,*
ZR 17	Nursery 2	*Pteridium aquilinum*	Root	+	*F. oxysporum, Aspergillus sp., Penicellium sp.,*
ZR 18	Nursery 2	*Prunus dulcis*	Soil	+	*Py.ultimum, F. oxysporum*
ZR 19	Nursery 2	*Citrus limon*	Soil	+	*P. cinnamomi, Aspergillus sp., Trichoderma sp.,*
ZR 20	Nursery 2	*Quercus suber L.*	Collar	+	*Lasio.exigua, F. oxysporum, Trichoderma sp., Penicellium sp.*
ZR 21	Nursery 2	*Ficus carica*	Soil	-	-
ZR 22	Nursery 2	*Eucalyptus globulus*	Soil	+	*P. gonapodyides, Mucor sp., Alternaria sp.*
ZR 23	Nursery 2	*Prunus armeniaca*	Soil	+	*P. ramorum, Trichoderma sp., Alternaria sp.*
ZR 24	Nursery 2	*Abies sp.*	Collar	+	*D. sapinea, Alternaria sp., Penicellium sp.*
ZR 25	Nursery 2	*Abies sp.*	Collar	+	*D. sapinea, Mucor sp., Trichoderma sp.,*
ZR 26	Nursery 2	*Citrus limon*	Soil	+	*Py.polare, Penicellium sp.,*
ZR 27	Nursery 2	*Olea europea*	Soil	-	-
ZR 28	Nursery 3	*Ficus carica*	Soil	+	*Py.ultimum, Aspergillus sp., P. cinnamomi, Mucor sp.*
ZR 29	Nursery 3	*Pteridium aquilinum*	Root	+	*Penicellium sp., Mucor sp., Trichoderma sp.*
ZR 30	Nursery 3	*Olea europea*	Soil	-	-
ZR 31	Nursery 3	*Quercus suber L.*	Collar	+	*Lasio.exigua, Alternaria sp., P. gonapodyides, P. cinnamomi, Alternaria sp.*
ZR 32	Nursery 3	*Prunus armeniaca*	Root	+	*Alternaria sp., P. cinnamomi, F. oxysporum, Aspergillus sp.,*
ZR 33	Nursery 3	*Prunus dulcis*	Root	+	*Alternaria sp., Mucor sp., Aspergillus sp.*
ZR 34	Nursery 3	*Abies sp.*	Soil	+	*D. sapinea, P. gonapodyidesdes, F. oxysporum,*
ZR 35	Nursery 3	*Eucalyptus globulus*	Soil	+	*Py.polare, P. cinnamomi*
ZR 36	Nursery 3	*Eucalyptus globulus*	Soil	-	-
ZR 37	Nursery 3	*Abies sp.*	Soil	+	*D. sapinea, Alternaria sp., Aspergillus sp., Penicellium sp.*
ZR 38	Nursery 3	*Citrus limon*	Soil	+	*Alternaria sp., Mucor sp., P. ramorum, Aspergillus sp.,*
ZR 39	Nursery 3	*Cupressus sp.*	Collar	-	-
ZR 40	Nursery 3	*Cupressus sp.*	Collar	+	*Alternaria sp., P. gonapodyides, Trichoderma sp.*
ZR 41	Nursery 3	*Cupressus sp.*	Collar	+	*P. ramorum, Aspergillus sp., Mucor sp.*
ZR 42	Nursery 3	*Citrus limon*	Soil	+	*Py.ultimum, F. oxysporum, Aspergillus sp., Mucor sp.*

In the first nursery: Analysis of variance (one-way ANOVA), using Fisher's test, showed many significant differences in the frequency of isolation of fungal species with a 95% confidence level. The average difference (ANOVA) showed that the species belonging to the genera *Aspergillus* and *Mucor* present with any significant difference between them. In contrast, they were highly significant compared to the other species. In addition, the two species of *Pythium* (*Py. ultimum* and *Py. polare*) showed a slight significant difference compared to the other species. On the other hand, the species with an important economic value as *Lasio. exigua* and *P. cinnamomi* were significantly different than those caused by saprophytic species, using Fisher's test ($p < 0.05$).

In the second nursery, analysis of variance (one-way ANOVA) using Fisher's test showed some significant differences in the frequency of species isolated with a confidence level of 95% (Fig. 4). Indeed, the three oomycetes identified as *P. cinnamomi, P. gonapodyides* and *P. ramorum* and the species *Lasiodiplodia exigua* have the lowest rate and they are significant with the saprophytes of the genera *Penicellium* and *Trichoderma.*

The two species identified as *Py. ultimum* and *D. sapinea* recorded an occurrence rate of around 17%. Those species show a slightly significant difference from other isolated species.

In the third nursery, species of the genus Aspergillus and Alternaria are the most present. Analysis of variance (one-way ANOVA) using Fisher's test ($p < 0.05$), showed highly significant differences between these species and others such as *Py. polare* and *Lasiodiplodia exigua*. The two species identified as *Py. ultimum* and *D. sapinea* recorded an occurrence rate of around 17%. Those species show a slightly significant difference from other isolated species.

The inhibitory activity of the two isolates of Trichoderma (*Tri. Viride* and *Tri. Harzianum*) showed that it affects against all the fungal species tested (*Diplodia sp.* and *Pythium sp*.).
After 5 days of incubation, the two strains of *Tri. Viride* and *Tri. Harzianum* showed good inhibitory activity against pathogenic tested strains, with the appearance of a zone of inhibition by dispersing certain metabolites which will stop their growth.

Trichoderma strain shows an antagonistic effect on some phytopathogenic fungi by the ability to suppress the disease (Sundaramoorthy and Balabaskar, 2013). In fact, it produces lytic enzymes wich increase its antagonist action by acting synergistically (Benitez *et al.*, 2004). The antagonistic action of Trichoderma species against phytopathogenic fungi either by the secretion of cell wall hydrolytic enzymes or by the production of antibiotics (Elad, 2000).

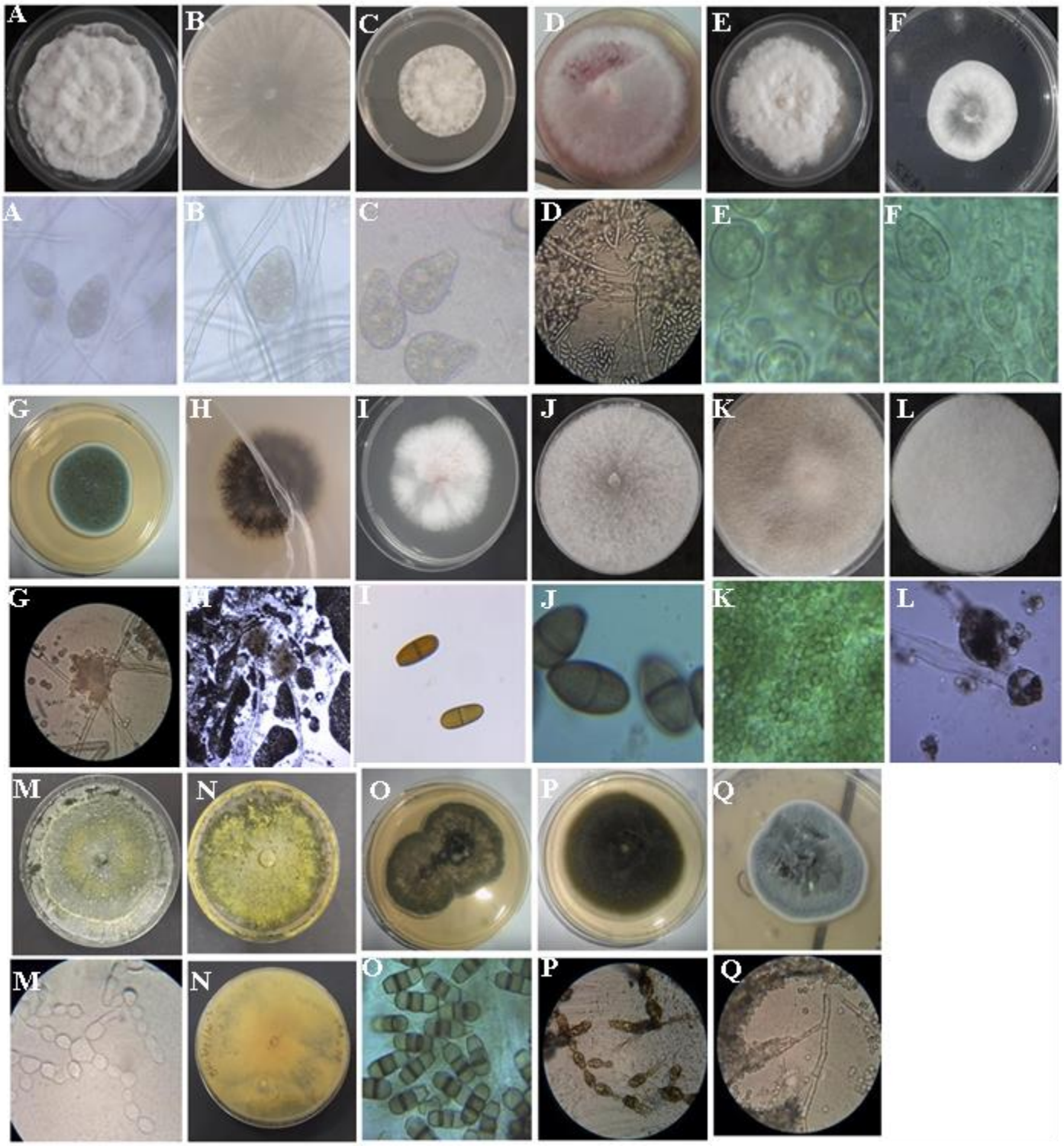

Figure 3. Colony morphology and microscopic features of the fungi isolated on PDA: (A) *Phytophthora gonapodyides*, (B) *P. cinnamomi*, (C) *P. ramorum*, (D) *Fusarium oxysporum* (E) *Pythium ultimum*, (F) *Py. polare*, (G) *Aspergillus fumigatus*, (H) *Asp. niger*, (I) *Dilplodia sapinea*, (J) *Lasiodiplodia exigua*, (K) *Mucor racemosus*, (L) *Rhizopus stolonifer*, (M) *Trichoderma harzianum* (N) *Tri. viride*, (O) *Alternaria solani*, (P) *Alt. alternate*, (Q) *Penicellium solitum*.

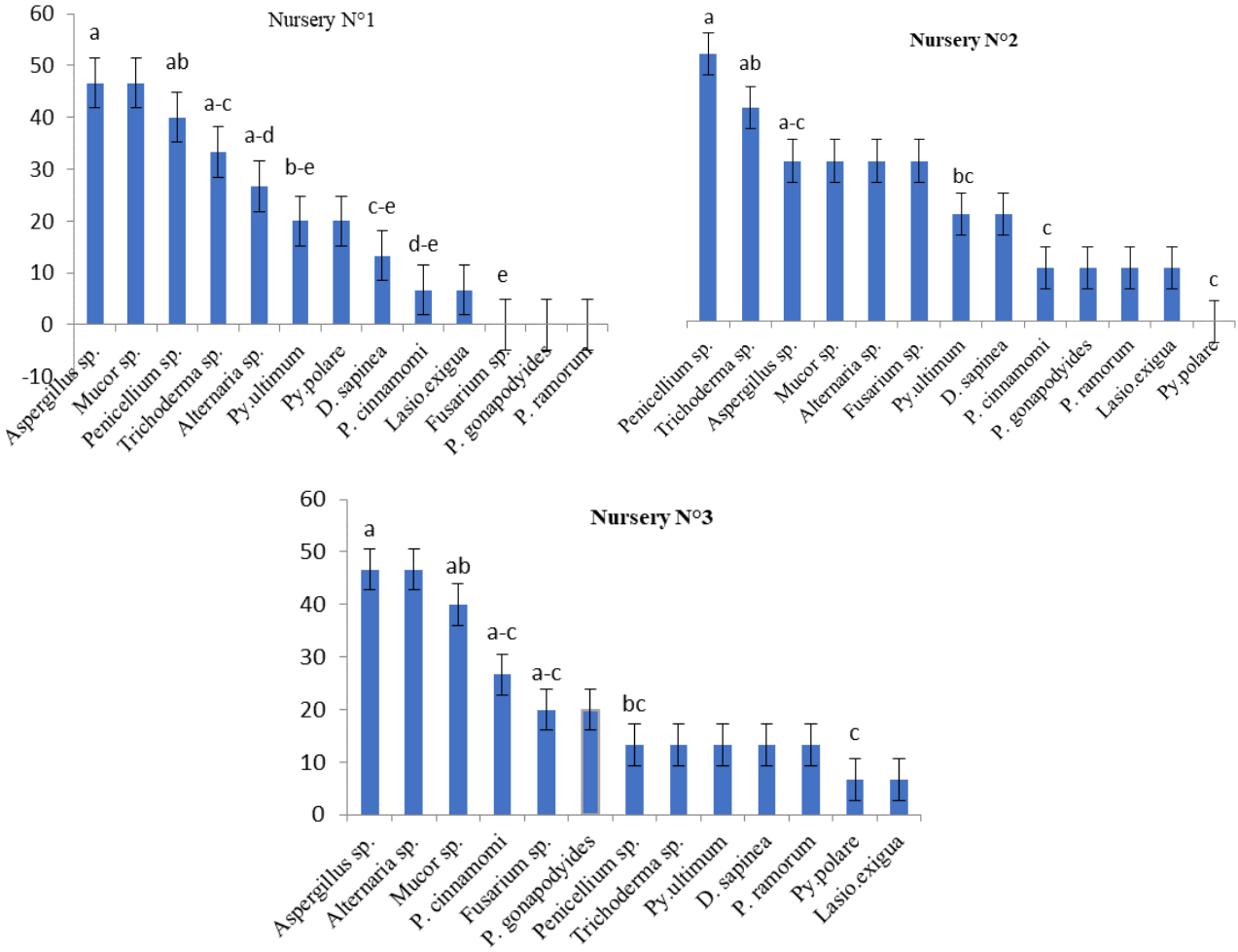

Figure 4. The average frequency of isolation from young seedlings from the three nurseries surveyed; Mean ± standard error. Different letters indicate significantly different means (Fisher's test, P <0.05). *Antagonist test*

Conclusion

According to this study, the rhizosphere of the three prospected nurseries showed the presence of a great qualitative and quantitative diversity of fungal and oomycetes species,

Macroscopic and microscopic identification showed the presence of seventeen different species, belonging to the genera *Phytophthora*, *Pythium*, *Fusarium*, *Diplodia*, *Lasiodiplodia*, *Alternaria*, *Mucor*, *Trichoderma*, *Aspergillus* and *Penicillium*. Some of them are widely studied because of their economic importance. Indeed, they can cause very significant damage to plants, and they can be dreaded parasites of nursery crops, such as *Phytophthora* species, *Pythium sp. Diplodia sp.* and *Fusarium sp*. These species are known for their variable host range on both cultivated and forest plants.

Among them, the most common in nurseries, those belonging to the genus Fusarium are the most frequent and the most damaging to crops (Nelson *et al.,* 1983). Further, listed as one of the 100 worst invasive alien species, *P. cinnamomi* is considered one of the most devastating plant pathogens worldwide (Lowe *et al.*,

2000). It causes the most severe outbreaks in topographically depressed areas poorly drained or periodically waterlogged (Brasier et al., 2004; Serrano *et al*., 2012).

Plant trade represents the major threat for the introduction and spread of alien diseases and pests in natural ecosystems through the movement of infected plants, or infested substrate and water (Brasier, 2008). The cultivation techniques in the nursery and the continuous movement of species and varieties of different plants have facilitated new associations between pathogens and hosts within nurseries (Brasier *et al.*, 2004; Burgess, 2015).

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