ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

Antifungal Activity of Endophytic Fungi Isolated from Date Palm Sap (*Phoenix dactylifera* L.)

Raed Abdennabi[1]*, Mohamed Ali Triki[2], Riadh Ben Salah[3], and Neji Gharsallah[1]

[1]*Laboratory of Plant Biotechnology, Faculty of Science, University of Sfax, Sfax, Tunisia*

[2]*The Olive Tree Institute, Soukra, Sfax, Tunisia*

[3]*Laboratory of Microorganisms and Biomolecules (LMB), Centre of Biotechnology of Sfax, Sfax, Tunisia*

***Corresponding Author**: Raed Abdennabi, Laboratory of Plant Biotechnology, Faculty of Science, University of Sfax, Sfax, Tunisia.

Received: November 08, 2017; **Published:** December 11, 2017

Abstract

In this study we isolated endophytic fungi from date palm sap (*Phoenix dactylifera* L.) and assessed their antifungal activity against *Fusarium oxysporum* and their potential for production of proteases. Date palm sap of different varieties yielded fungal isolates where 34.78% originated from Ameri variety, 30.44% from Aligue variety, 26.08% from Khalet variety and 8.70% from thokar variety. Out of endophytic fungal isolates tested by *in vitro* confrontation tests against *Fusarium oxysporum*, *Trichoderma sp*, *Gliocladium sp* and *Aspergillus sp*. isolates showed inhibition activity against the pathogen. *Trichoderma sp*, exhibited the highest antifungal activity against *Fusarium oxysporum* (72%) followed by *Gliocladium sp* (56%) and *Aspergillum* (7.7%). Additionally, six fungal isolates were found positive for protease enzymes production. Therefore, our results suggest that endophytic fungi associated with date palm sap are potential agents for antifungal activity and production of enzymes of biotechnological interest.

Keywords: *Date Palm Sap; Phoenix dactylifera L.; Endophytic Fungi; Fusarium oxysporum; Antifungal Activity; Protease Activity*

Introduction

In the past few decades, many studies have focused on endophyte microorganisms that live inside plant tissues. These endophytes are present in all plants and are extremely abundant and often very diverse [1-3]. They can survive in plants for all or part of their life cycle without causing any apparent damage or diseases [4]. Endophytes can be transmitted vertically as well as horizontally: vertical transmission occurs through seeds and vegetative propagation of the host and horizontal transmission occurs through spores, external to host tissues [5]. In the 1970's, endophytes were considered to be neutral: that is, they were believed to neither cause any harm nor benefit the plant. However, later on, many studies revealed that endophytes play an important role in host protection against predators and pathogens [6]. A notable example is endophytic fungi that live asymptomatically within the living tissue of the host plant and establish mutualistic symbiosis. Many are capable of synthesizing bioactive compounds that can be used by the plant for defense against pathogenic microorganisms [7-10]. These compounds are promising in medicine, agriculture and industries [11,12]. The production of secondary metabolites from endophytes is associated with environmental factors. The endophytic interaction with its host may also favor the synthesis of secondary metabolites [7,13]. Endophytes are known to produce metabolites such as alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavonoids, phenols, phenolic acids, and peptides. The endophytic fungus like Cryptosporiopsis cf. quercina isolated from a medicinal plant native to Eurasia *Tripterygium wilfordii*, was behind the production of cryptocandin A, an antifungal peptide linked to echinocandins and pneumocandins, and demonstrating activity against *Sclerotinia sclerotiorum* and *Botrytis cinerea* [14]. Cryptocandin and its derivatives are used in the study to fight against some fungal diseases of the skin and nails [14,15]. The number and nature of the products purified from the fungus dependent cultural conditions and the plant source from which it was isolated [14]. Another species *Pestalotiopsis, Pestalotiopsis jesteri* product of hydroxyjesterone jesterone and [14], the latter is one of the few products purified from endophytic where the total synthesis of a bioactive product was accomplished [14].

The sap of the palm tree, commonly called " legmi" is a white liquid syrupy widely consumed in southern Tunisia. It is a very sweet juice characterized by a high content of sugars, minerals and antioxidant molecules. Date palm Sap or "Legmi" is considered responsible for various biological activities and have been used for centuries for its pharmaceutical properties. Besides its antibacterial and antifungal properties, "Legmi" presents many other beneficial biological activities such as antioxidant anti-inflammatory, antitumor, hepatoprotective, local anesthetic, immunostimulatory and antimutagenic activities [16].

Materials and Methods

Isolation and identification of endophytic fungi

Isolation of endophytic fungi from Date palm sap "Legmi" was carried out using the protocol described by Strobel., *et al*. [17] with slight modifications. Fresh "Legmi" samples were collected from different Date Palm plantations in Tozeur, Tunisia. The samples of saps were placed on plates containing potato dextrose agar (PDA) media supplemented with 200 mg/L Streptomycin to suppress bacterial contamination and therefore promote fungal growth. The parafilm wrapped petri dishes were incubated at 25 ± 2°C till the fungal mycelia starts growing from the samples. Single hyphae endophytic fungi were transferred into a new agar slants and stored at 4°C for further studies. Collected fungi were identified on the basis of their morphological features [18-26].

Preliminary-Antifungal assay

Antifungal activities of isolated endophytic fungi were tested based on the protocol of Zhang., *et al.* [27] with slight modifications. The petri dishes containing media for fungal growth were prepared and 1 ml of liquid fungus was spread over the surface of the agar media using sterile cotton swab. Nine millimeter diameter of actively growing fungal culture discs from PDA plates were cut using a sterile cork borer and placed on the surface of the respective agar media seeded with test fungi. These plates were sealed with parafilm and kept in refrigerator at 4°C for 12 hours for complete diffusion of antifungal compounds if any, thereafter they were incubated at room temperature for next 5 days for organisms such as *Fusarium oxysporum*. After incubation the diameter of the inhibition zone was measured in millimeter by using scale.

Antagonistic activity of endophytes

The antagonistic activity of endophytic fungi was carried out using an isolate of *Fusarium oxysporum*, collected from diseased olive plantations in Sfax, Tunisia. Three strains namely *Trichoderma sp*, *Gliocladium sp* and *Aspergillus sp*., isolated from the date palm *Phoenix dactylifera* L. saps were used in the direct confrontation method in order to investigate their putative antagonistic activity against *F. oxysporum*. Mycelium discs (diameter, 5 mm) of 4-day-old *F. oxysporum* were placed at the right side of a potato dextrose agar (PDA) plate. The endophytic fungi were then inoculated at the left side of each *F. oxysporum* inoculated PDA plate. The plates were incubated at 25°C for 5 days. The percentage of inhibition was calculated using the following equation: Inhibition (%) = [(growth diameter in the control sample – growth diameter in the sample with treated endophytes) × 100]/growth diameter in the control sample.

Protease assay

The production of enzymes by fungal endophytes was qualitatively determined by using Hankin and Anagnostakis method [28]. Protease assay was performed by growing fungi on Gelose agar medium amended with 0.4% gelatin and having a pH adjusted to 6.0. After 5 days of incubation, plates were flooded with saturated aqueous ammonium sulphate. The undigested gelatin precipitates with ammonium sulphate and digested areas around the fungal colony would appear as clear zones.

Results and Discussion

Morphological identification of endophytic fungi isolated from date palm sap

The results for the enumeration of fungi isolated from different date palm saps are given in figure 1. These results showed a variable frequency of fungi isolated from different date palm cultivars saps. Indeed, the rates are 34.78% in the sap of variety Amerie, 30.44 of

Aligue, 26.08 for Khalt and 8.7% in that of Dhokar. The morphological identification of fungi isolated was performed on PDA medium for the determination of the macroscopic characteristics of the culture (color and appearance of the fungal colonies). On the other hand, the microscopic observations were made on young crops by determining the morphological characters of seed heads of each fungi based on key identifications of Nasraoui., *et al* [29].

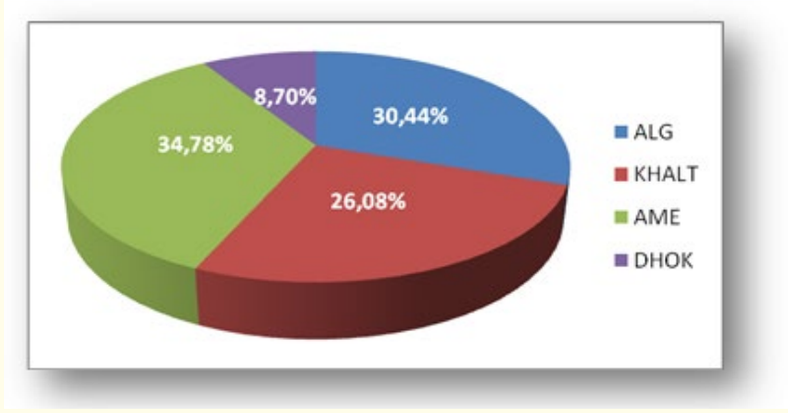

Figure 1: *Frequency of isolated fungi per variety.*

ALG: Aligue; KHALT: Khalt; AME: Ameri and DHOK: Dohkar.

The results of the morphological identification revealed the presence of five kinds of frequently isolated fungi in four saps analyzed. This is *Penicillium* sp, *Fusarium* sp, *Trichoderma* sp, *Aspergillus* sp, and *Gliocladium* sp.

Fusarium species

The Isolations have proved that *Fusarium* (Figure 2) are present only in the saps of Ammeri and Kalet varieties and not in those of Dhokar and Aligue. This can be explained by the susceptibility of these first two varieties to this fungus in its non-pathogenic form. The fungus crosses through the ligno-cellulose tissue and then passes into the flow of the xylem which contains the sap.

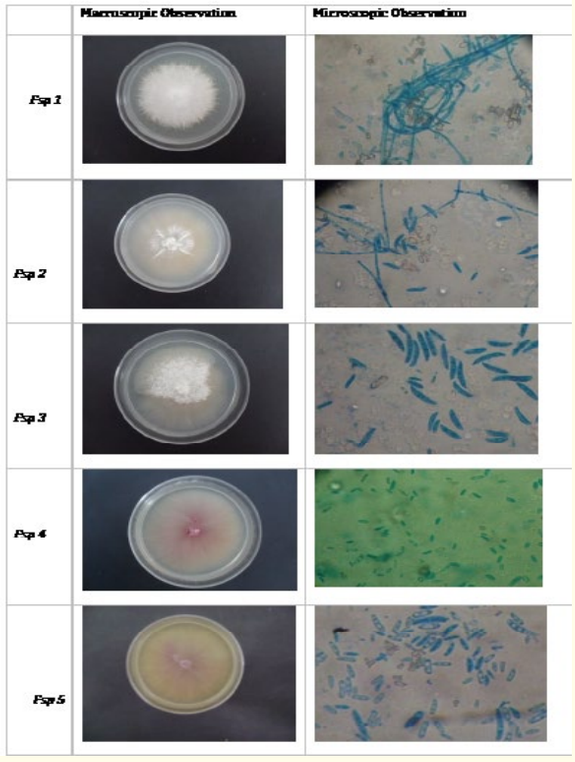

Figure 2: *Macroscopic and microscopic observation of Fusarium sp.*

Aspergillus species

Aspergillus sp. (Figure 3) is often present as a saprophyte on various plant materials, but can, however, sometimes acquire a parasitic character. This species is isolated from the Aligue sap variety. It has the following characteristics: slow-growing white culture initially and becomes gray and then black out after sporulation, which is the type called "black mold".

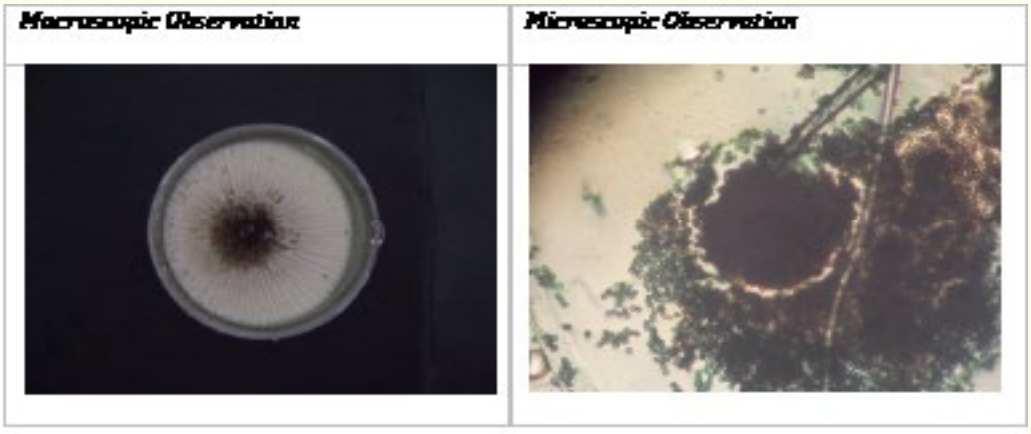

Figure 3: *Macroscopic and microscopic observation of Aspergillus sp.*

Penicillium species

The Isolations performed revealed the presence of two species of *Penicillium* (Figure 4). These species are isolated from Ammeri sap variety and Dhokar variety. The two *Penicillium* sp. exhibit rapid growth and invade the Petri dishes in about 4 days.

Figure 4: *Macroscopic and microscopic observation of Penicillium sp.*

The surface appearance of the two fungal colonies is different between the two isolates suggesting that they actually represent two different species:

Penicillium digitatum: Has a green tint surface, color of the colony is green with a white edge.

***Penicillium* species:** Has a velvety surface green tint.

Gliocladium species

Gliocladium (Figure 5) have been isolated from three saps; Khalet, Ameri and Aligue. It has a slow growth rate, maturing within (5 - 7 days). The colour of the surface is white cream at first, but quickly develops various shades of green. The outer fringe can remain white. Some strains may also have a pink color on the surface. Color changes seem to depend on the medium used (powder or traditional PDA).

Figure 5: *Macroscopic and microscopic observation of Gliocladium sp.*

***Trichoderma* species**

The *Trichoderma* sp. isolated from Aligue variety, is a growing mold which expires after 3 days of growth. It begins with white tufts which then become compact. Green tufts may develop in the colony generally from the border. And this seems to be related to production of conidia. These often appear as concentric rings.

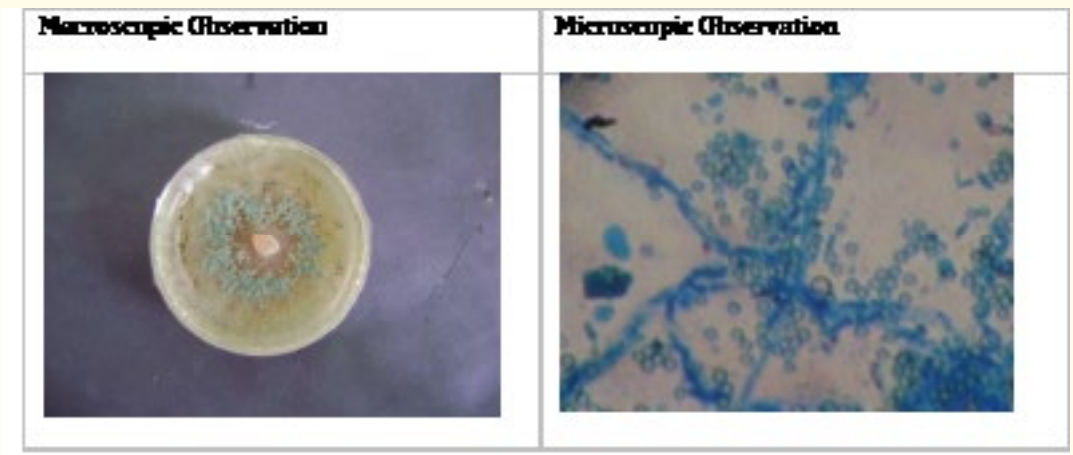

Figure 6: *Macroscopic and microscopic observation of Trichoderma sp.*

Antagonistic activity of endophytes

The three fungal antagonists isolated from four studied varieties of date palm sap (Figure 7): *Trichoderma sp. Aspergillus sp*. and *Gliocladium sp*. showed a significant reduction in mycelial growth of the tested pathogen *Fusarium oxysporum*. Indeed *sporum* this result confirms several researches that showed that *Trichoderma* is a well significant reduction in *Fusarium oxysporum* growth rate are recorded after a week of incubation at 25°C. These reductions are in the order of 72% and 56% by *Trichoderma sp*. and *Gliocladium sp*., respectively, compared to the control. However the confrontation with *Aspergillum sp*. allowed a small reduction of 7.7% growth rate of *Fusarium oxysporum* when compared to the control (Figure 7).

Our results clearly show that *Trichoderma* sp. lead to slow the growth of the phytopathogenic fungus, *Fusarium oxy* known fungus by its antifungal properties against several phytopathogenic fungi [29,30].

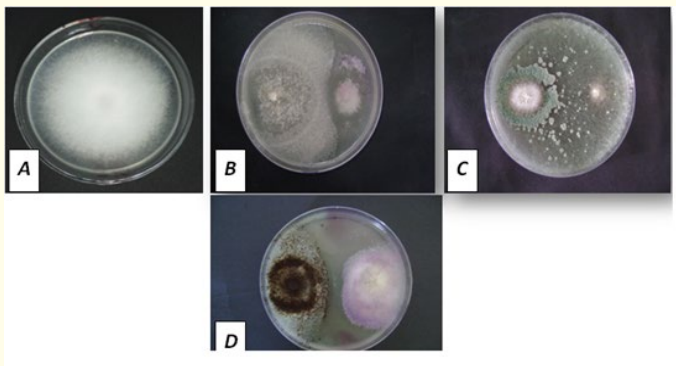

Figure 7: *Representative petri plates showing A: Negative control Fusarium oxysporum, B: Confrontation of Gliocladium spp. in left with the Fusarium oxysporum at right, C: Confrontation of Trichoderma in left with the Fusarium oxysporum at right and D: Confrontation between Aspergillus sp. in left with Fusarium oxysporum at right*

Proteolytic activity

Proteases are used in clinical applications especially in the treatments of diseases like diabetes. It is known from historical records that extracts of Tulsi plant being used for the diabetic control (REF). Proteases are one of the enzymes involved in controlling diabetes as reported by Ladenburger., *et al* [31]. Its administration delays Insulin-dependent diabetes mellitus (IDDM) onset in an animal model for autoimmune diabetes, in the non-obese diabetic mice.

Five of the endophytic fungi isolate showed positive reaction for protease activity. Figure 8 shows representative petri plates indicating the presence of enzymes from isolated endophytic fungi by qualitative test. In the figure 8, plate (A) shows a clear zone around the fungal colony indicating degradation of gelatin due to protease activity by 3 strains of *Fusarium spp.* Respectively (34, 36, 28 mm, Table 1). Plate B in figure 8 represents the production of protease enzyme by *Gliocladium spp* (strain 8) with 35 mm halo diameter (Table 1). Plate B in figure 8 tested for *Aspergillus sp*. protease activity. We showed that this strain possessed 34 mm with strain 9 and 26 mm with strain 10.

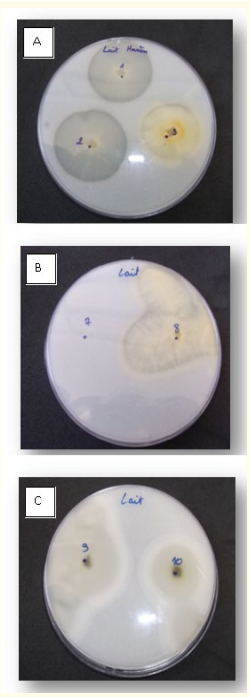

Figure 8: *Representative petri plates showing clear zone of degradation of gelatin by the protease enzyme. A: strains 1, 2 and 3 Fusarium sp, B: strains 7 and 8 Gliocladium sp and C: strains 9 and 10 Aspergillus sp*

N° Strain fungi	Diameter (mm)
1	34
2	36
3	28
8	35
9	34
10	26

Table 1: *Diameter of the proteolytic activity of the fungal strains.*

Conclusion

The present study leads to the need of further in depth studies on these isolated bioactive endophytic fungal isolates. Many are able to produce quite a good amount of antifungal compounds tested in preliminary test Further growing those in large scale, modifying culture conditions like changing pH, changing growth media and supplying some stimulants might help in getting better production of the particular bioactive compound and enzyme. Further, the best proved active isolates should be identified using available methods to place these fungi in the fungal kingdom. There are no reported studies on bioactive compounds and protease enzymes to the present knowledge.

Acknowledgements

Financial support of the Tunisian Ministry of Higher Education and Scientific Research is gratefully acknowledged.

Conflict of Interest

None of the authors had a conflict of interest.

Bibliography

1. Stone J K and Petrini O. "Endophytes of forest trees: a model for fungus-plant interactions". In: The Mycota. (Eds.): G.C. Carroll and P. Tudzynski. V. Part B. Plant relationships, Springer- Verlag, Berlin (1997): 129-142.

2. Schulthess F M and Faeth S H. "Distribution, abundances, and associations of the endophytic fungal community of Arizona fescue (Festuca arizonica)". *Mycologia* 90.4 (1998): 569-578.

3. Arnold A E Z*., et al.* "Are tropical fungal endophytes hyperdiverse?" *Ecology Letters* 3 (2000): 267-274.

4. Petrini O., *et al.* "Ecology, metabolite production and substrate utilization in endophytic fungi". *Journal of natural toxins* 1.3 (1992): 185-196.

5. Carroll G. "Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont". *Ecology* 69.1 (1988): 2-9.

6. Azevedo J L., *et al.* "Endophytic microorganisms: a review on insect control and recent advances on tropical plants". *Electronic Journal of Biotechnology* 3.1 (2000): 40-65.

7. Schulz B., *et al.* "Endophytic fungi: a source of novel biologically active secondary metabolites". *Mycological Research* 106.9 (2002): 996-1004.

8. Corrado M and Rodrigues K F. "Antimicrobial evaluation of fungal extracts produced by endophytic strains of Phomopsis sp". *Journal of Basic Microbiology* 44.2 (2004): 157-160.

9. Owen N L and Hundley N. "Endophytes-the chemical synthesizers inside plants". *Science Progress* 87.2 (2004): 79-99.

10. Giménez C R., *et al.* "Fungal endophytes and their role in Plant Protection". *Current Organic Chemistry* 11.8 (2007): 707-720.

11. Tan R X., *et al.* "Endophytes: a rich source of functional metabolites". *Natural Product Reports* 18 (2001): 448-459.

12. Maria G L., *et al.* "Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India". *Journal of Agricultural Technology* 1 (2005): 67-80.

13. Petrini O. "Microbial Ecology of Leaves". New York: Springer Verlag (1991): 179-197.

14. Strobel G., *et al.* "Natural products from endophytic microorganisms". *Journal of Natural Products* 67.2 (2004): 257-268.

15. Strobel G A. "Microbial gifts from rain forests". *Canadian Journal of Plant Pathology* 24 (2002): 14-20.

16. Ben Thabet I., *et al.* "Physicochemical characteristics of Date Sap ''Lagmi'' from Deglet Nour palm (Phoenix dactylifera L.)". *International Journal of Food Properties* 12.3 (2009): 659-670.

17. Strobel G., *et al.* "Taxol from Pestalotiopsis microspora, an endophytic fungus of Taxuswallachiana". *Microbiology* 142 (1996): 435-440.

18. Raper K B and Thom C A. "A manual of the Penicillium". Williams and Wilkins, Baltimore (1949): 875.

19. Raper K B and Fennell D I. "The genus Aspergillus". Williams and Wilkins, Baltimore (1965): 686.

20. Ellis M B. "Dematiaceous Hyphomycetes". *Commonwealth Mycological Institute, Kew, Surrey* (1971): 608.

21. Ellis MB. "More Dematiaceous Hyphomycetes". *Commonwealth Mycological Institute, Kew, Surrey* (1976): 507.

22. Booth C Fusarium. "Laboratory guide to the identification of major species". Commonwealth Mycological Institute, Kew, Surrey (1977): 58.

23. Christensen M and Raber K B. "Synoptic key to Aspergillus nidulans group species and related Emericella species". *Transactions of the British Mycological Society* 71.2 (1978): 177-191.

24. Pitt J I. "The genus Penicillium and its telemorphic stato Eupenicillium and Talaromyces". Academic Press, London (1979).

25. Pitt J I. "A laboratory guide to common Penicillium species". Commonwealth scientific and Industrial Research Organization, Division of food research, North Ryde (1985).

26. Moubasher A H. "Soil fungi in Qatar and other Arab countries". *The Scientific and Applied Research Center, University of Qatar, Doha* (1993): 566.

27. Zhang Y., *et al.* "Broad-Spectrum Antimicrobial Epiphytic and Endophytic fungi from Marine Organisms: Isolation Bioassay and Taxonomy". *Marine Drugs* 7.2 (2009): 97-112.

28. Hankin L and Anagnostakis S L. "The use of solid media for detection of enzyme production by fungi". *Mycologia* 67 (1975): 597-607.

29. Bouzid N. "Les champignons parasites des plantes cultivées". *Biologie, systématique, pathologie, maladies. Ed. centre de publication universitaire* (2006): 79, 222, 286, 288, 289, 292.

30. Triki M A., *et al.* "Recrudescence du dépérissement de l'olivier causé par les champignons telluriques en Tunisie". Proceeding of Olive bioteq Sfax (2009).

31. Ladenburger WU., *et al.* "Protease treatment delays diabetes onset in diabetesprone nonobese diabetic (nod) mice". *International Journal of Immunotherapy* 13.3-4 (1997): 75-78.

Volume 13 Issue 4 December 2017