ALTERNATIVE CULTURE MEDIA FOR GROWTH AND SPORULATION OF TRICHODERMA HARIZIANUM

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Article received 15.10.2017, Revised 25.11.2017, Accepted 9.12.2017

ABSTRACT
Microorganisms need nutrients for their biological activities and reproduction. Culture media such as PDA, used for growing fungi in laboratories. Because of high cost and don’t available at all time. The present study was conducted to supersede Potato in PDA medium with powdered leaves of either Moringa oleifera or mint and to examine their effect on mycelial, conidial and biomass production of bioagent fungus Trichoderma harzianum and pathogenic fungi: Fusarium graminearum and F. oxysporum. The patterns of T. harzianum growth was substantially affected by Moringa Dextrose Agar (MoDA) and Mint Dextrose Agar (MiDA). Both the media were found to magnify the mycelial growth of bioagent fungus as compared to pathogenic fungus F. oxysporum. MoDA characterized by enhancing spore production in T. harzianum (19.6×10⁸), in comparison to Fusarium graminearum (7.13×10⁹) and F. oxysporum (8.73×10⁸) after 9 days of inoculation. Dry weights of T. harzianum (0.75 mg) and F. oxysporum (0.35 mg) mycelia were also sharply incremented in MoDB compared to PDB (0.2 and 0.15gm) respectively in which the dry weight not inhibited by modified media.

It was concluded that the utilization of M. oleifera or Mint leaves as a component of culture media in laboratories is a feasible and cheap source as compared to commercially prepared PDA. Furthermore, the present formulated media could be subsidiary act as a selective medium for the magnification and sporulation of T. harzianum. In integration, the leaf powders of both sources can be stored for longer time periods in comparison to potato.

Keywords: Trichoderma harzianum, alternative culture, Moringa oleifera, mint.

INTRODUCTION
The choosing of medium is found to be essential and paramount for the production and manufacturing of valuable metabolites at laboratory scale as well as pilot scale. Further, it has been well documented that fungi required opportune nutrients for their magnification and reproduction in a set environmental conditions (Ravimannan et al., 2014; Pathmanathan et al., 2016). These requisites are generally met by utilizing felicitous commercially available culture medium which not only enhance the magnification of desired microorganisms but also suppress the outgrowth of competing organisms (Deivanayaki and Iruthayaraj, 2012).

Due to financial limitations of laboratories, there are circumscribed utilization of high cost ready-made microbial culture media that further stimulate the researchers to come forward for the investigation of alternative media by utilizing locally available cheap raw materials (Pathmanathan et al., 2016).

For instance, Potato dextrose agar (PDA) is one among the sundry fungus growing media which is commercially expensive than manually prepared PDA. However, the manual methods of PDA preparations are further tedious and time consuming. Therefore, it is essential to design incipient medium for the growth of biologically valuable fungus such as Trichoderma harzianum, a natural fungicide. The large scale utilization of T. harzianum will not only reduces the cost of chemical fungicides but additionally, transmutes the soil conditions as propitious for plants growth (Panahian et al., 2012).

Aforetime, various research groups have reported the effects of culture medium in fungus magnification. In a study, Pandey et al. (2006) investigated the consequences of various culture media in the growth, sporulation and morphological features of Alternaria alternata, a causative agent of leaf spot/ rust and anthracnose diseases on Dolichos lablab. Similarly, Deivanayaki and Iruthayaraj (2012), utilized vegetables predicated alternative source to prepare culture media for the cultivation of fungi and bacteria. Furthermore, Ravimannan et al., (2014) studied the feasibility of legume seeds based culture media to grow sundry fungi including Aspergillus, Trichoderma, Fusarium, Sclerotium and Penicillium sp. Their investigation suggested that legume seeds act as a promising source of proteins and may be utilized as an alternative media. Therefore, in present study we proposed an alternative plant predicated media to supersede PDA. The selected plants were Moringa oleifera and mint that are distributed in many tropics and subtropical countries. The leaves of both plants are not only kenned to possess medicinal value against a broad range of human diseases but also have a potent alimantal value (Rodriguez-Fragoso et al., 2008; Rockwood et al., 2013).
On the other hand, *T. harzianum* is considered as an auspicious biological control agent against various plant pathogenic fungi. The magnification capability and inoculum potential are two crucial parameters for the activity of biocontrol agent. Consequently, the present study was conducted to replace potato from PDA medium with other facilely and frugally available natural nutrient sources such as *M. oleifera* and Mint leaves, for the magnification and reproduction of bioagent fungus (*T. harzianum*). In addition, growth inhibitory effect of formulated media was also investigated against plant pathogenic fungi (*Fusarium graminearum* and *F. oxysporum*).

**MATERIAL AND METHODS**

**Plant Sampling:** Leaves of *Moringa oleifera* were obtained and amassed according to (Ojiako et al., 2011; Obaid et al., 2017). Whereas the mint leaves were collected from private fields of Hilla countryside, Iraq. The accumulated leaves were washed initially under running tap water followed by distilled water. Leaves were air dried at room temperature in darkness, grinded to fine powder by utilizing an electrical blender. The obtained powder was stored discretely in sterile containers until further use (Umechuruba and Elenwo, 1999).

**Source of fungal isolates:** *T. harzianum*, *F. graminearum* and *F. oxysporum* isolates were obtained from the Unit of Advanced Mycology, Department of Biology, College of Science, University of Babylon.

**Growth and maintenance of fungal isolates:** The isolates of *T. harzianum*, *F. graminearum* and *F. oxysporum* were inoculated on PDA containing Petri dishes and incubated for 5 days at 26±2°C. PDA slants were prepared by pouring 20 ml of PDA in 50 ml of glass tubes and allowed to solidify. The pure cultures of *T. harzianum*, *F. graminearum* and *F. oxysporum* were taken from the edge of recently engendered colonies and inoculated separately into each tube under aseptic conditions followed by 5 days of incubation at 26±2°C. The prepared slants were regularly maintained and stored in refrigerator at 5°C for later use (Alnuaimy et al., 2017).

**Growth diameter and sporulation:** Two types of culture media, i.e. *M. oleifera* Dextrose Agar (MoDA) and Mint Dextrose Agar (MiDA) were acclimated to study the growth patterns of fungi. Briefly, 2gm of previously described leaves powder of each plant source was taken and thoroughly dissolved (separately) in 100 ml of distilled water using vortex and mixer stirred until it becomes homogenous. The prepared content was further supplemented with dextrose and agar to a same concentration as used in PDA. The pH of the culture medium was adjusted. Commercially available PDA was prepared by dissolving 3.9 g of PDA in 100ml distilled water and used as a control medium. Chloramphenicol at a concentration of 250 mg/L was added to the medium to obviate bacterial growth. The growth medium was autoclaved for 15 minutes at 15psi (121°C) in 250 ml conical flasks. After autoclaving, medium was allowed to cool to 45-50°C and poured into sterile petri dishes. Using sterile cork borer, 4 mm diameter discs of recently growing mycelia tissue of each isolates (*T. harzianum*, *F. graminearum* and *F. oxysporum*) were cut and placed to the center of prepared petri plates of relevant culture media. The inoculated plates were incubated at 26±2°C for 4 days. At regular intervals of 24 h, the growth diameters of fungal isolates from intersecting lines of petri plates were examined up to four days. Furthermore, the quantity of sporulation of these isolates at 5, 7 and 9 days of incubations were calculated by using McFarland methods. Briefly, 10 ml of 0.85 % sterile saline containing 0.1 tween 80 (v/v) was used to suspend the spores from colony surface of each tested fungi as described by Obaid et al., (2017). The optical density at 530 nm of diluted spore suspensions were measured by using spectrophotometer (Sharma and Sharma, 2011). All the experiments were carried out in three replicates.

**Fungal biomass:** Fungal biomass production was determined in two culture media i.e. *M. oleifera* dextrose broth (MoDB) and potato dextrose broth (PDB). Under sterile conditions, culture media were prepared individually in conical flasks (250 ml) containing 100 ml of commercially available PD for PDB medium and 2% of powdered leaves of *M. oleifera* supplemented with dextrose for MoDB medium. Media were inoculated discretely with three mycelial plugs (1cm in diameter) of seven days old cultures of *T. harzianum* and *F. oxysporum*. The inoculated flasks were incubated at 26±2°C for 28 days under aseptic conditions. After incubation, mycelium from each flask was harvested by filtration in Buchner funnel using a dry sterile filter paper (Whatman paper No.1). The mycelia masses were washed three times with sterilized distilled water to remove the remaining media. The harvested mycelia were dried at 60°C for 6 h followed by an overnight drying at 40°C. Then the fungal dry weight was measured by using an electronic weighing balance.

Statistical analysis was carried out according to the instructions of Snedecor and Cochran, (1969). The means were compared by LSD at probability 0.01. All the experiments were conducted in triplicate.
using Randomized Complete Block Design (RCBD).

**RESULTS**

**Radial growth:** Both the formulated media i.e. MoDA and MiDA were able to enhance the growth of *T. harzianum*, a valuable natural bio control agent. On the other hand, these media have shown inhibitory effects against the magnification of pathogenic fungus (*F. graminearum* and *F. oxysporum*). The growth patterns of *T. harzianum* were substantially affected by MoDA and MiDA (Figure 1). After 4 days of inoculation the green colored conidia were found to cover the whole surface of the medium. But, constrained mycelial growth of *F. graminearum* and *F. oxysporum* was observed in both the media. Furthermore, both of *F. graminearum* and *F. oxysporum* produced cottony type of mycelial texture in MoDA medium but the colonies of *F. graminearum* in MIDA medium were mostly flat with low mycelial density.

In terms of colony diameters, results (Figure 2), revealed that *T. harzianum* has shown significantly higher mycelial growth on MoDA (2.54, 4.8, 6.84 and 8.0 cm) followed by MiDA (2.4, 4.4, 6.6 and 7.9 cm) after 1, 2, 3 and 4 days of inoculation respectively in comparison to growth diameters on PDA (2.14, 4.3, 6.1 and 6.9 cm) medium. In contrast, the growth of pathogenic fungi (*F. graminearum* and *F. oxysporum*) was found to be significantly lesser than *T. harzianum* in all of three culture media. The growth diameters of *F. graminearum* after 1, 2, 3 and 4 days of incubation were as following: MoDA medium (1.2, 2.02, 2.16 and 3.8 cm), MiDA medium (1.1, 1.9, 2.36 and 3.5 cm) and PDA medium (1.3, 2.55, 4.83 and 5.8 cm) respectively. Whereas the radial growths of *F. oxysporum* in various medium were as following: MoDA (2.03, 3.06, 4.73 and 6.03 cm), MiDA (1.8, 2.83, 4.2 and 5.34 cm) and PDA (1.9, 3.23, 5.44 and 6.4 cm) after four days of incubation.

![Figure 1](https://example.com/figure1.png)

**Figure 1:** Colony appearance of *F. graminearum* (F.g), *F. oxysporum* (F.o) and *T. harzianum* (T.h), isolates after 4 days of incubation on MoDA (Mo), MiDA (Mi) and PDA at 26±2 °C.

![Figure 2](https://example.com/figure2.png)

**Figure 2:** Effect of different culture media: A- MoDA (L.S. D<sub>0.05</sub> = 0.45), B- MiDA (L.S. D<sub>0.05</sub> = 0.35) and C- PDA (L.S. D<sub>0.05</sub> = 0.60) on radial growth of *T. harzianum*, *F. graminearum* and *F. oxysporum* after 4 days of incubation at 26±2°C.

**The sporulation of *T. harzianum* and *F. oxysporum***: Quantity of spores were calculated after 5, 7 and 9 days of inoculation of *T. harzianum*, *F. graminearum* and *F. oxysporum* in MoDA, MiDA and PDA media.
The results revealed that MoDA medium significantly increased the spore production ability of *T. harzainum* after 3, 5 and 7 days of inoculation (Figure 3). As shown in Figure 3, the rate of sporulation after 9 days of inoculation were found in the following order: *T. harzainum* (19.6×10^8) > *F. oxysporum* (8.73×10^8) > *F. graminearum* (7.13×10^8). It has been clearly observed that the type of formulated medium significantly affects the process of sporulation. For instance, the number of spores in MoDA medium (multiplied by 10^8) were as: *F. graminearum* (2.5, 5, 7.13), *F. oxysporum* (3, 6, 8.73) and in *T. harzianum* (9, 12.06 and 19.6) after 5, 7 and 9 days of incubation respectively. Whereas, the sporulation in MiDA medium (multiplied by 10^8) was: *F. graminearum* (2.01, 4.50 and 6.80), *F. oxysporum* (3.8, 6 and 8) and in *T. harzianum* (6.01, 10 and 17.0) after 5, 7 and 9 days of incubation respectively. In contrast, the spore count in PDA medium (× 10^8) was: *F. graminearum* (3.80, 6.90 and 8.3), *F. oxysporum* (4.1, 7.20 and 9.67) and in *T. harzianum* (5.23, 9.13 and 11.96) after 5, 7 and 9 days of incubation respectively. Results shown that MoDA and MiDA media significantly increased sporulation of *T. harzianum* while the spore production competency was found to reduce significantly in *F. graminearum* and *F. oxysporum* in both culture media in comparison to control media (PDA).

**Figure 3:** Effect of different culture media: A- MoDA (L.S.D 0.05 = 1.6), B- MiDA (L.S. D 0.05 = 1.05) and C- PDA (L.S. D 0.05 = 1.4) on sporulation of *T. harzainum, F. graminearum* and *F. oxysporum* after 5, 7 and 9 after incubation at 26±2 °C.

**Fungal biomass -Dry weight:** Dry weight of *F. oxysporum* and *T. harzainum* were examined after 28 days of incubation on MoDB and PDB growth medium. The results (Figure 4) revealed that dry weight of *T. harzainum* (0.75 mg) and *F. oxysporum* (0.35 mg) were higher in MoDB medium in comparison to PDB (*T. harzianum* 10^6) was: *F. graminearum* (2.01, 4.50 and 6.80), *F. oxysporum* (3.8, 6 and 8) and in *T. harzianum* (6.01, 10 and 17.0) after 5, 7 and 9 days of incubation respectively. In contrast, the spore count in PDA medium (× 10^8) was: *F. graminearum* (3.80, 6.90 and 8.3), *F. oxysporum* (4.1, 7.20 and 9.67) and in *T. harzianum* (5.23, 9.13 and 11.96) after 5, 7 and 9 days of incubation respectively. Results shown that MoDA and MiDA media significantly increased sporulation of *T. harzianum* while the spore production competency was found to reduce significantly in *F. graminearum* and *F. oxysporum* in both culture media in comparison to control media (PDA).

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(0.2 mg) and *F. oxysporum* (0.15 mg). Dry weight of *T. harzianum* in MoDB was more than three and a half-fold higher than that of PDB. While the dry weight of *F. oxysporum* in MoDB was more than two-fold high in comparison to PDB.

**DISCUSSION**

Previously, Sobitasimon (2011) reported the utilization of organic waste including potato peel, brinjal, banana, papaya, guava, spinach and sugar cane for the growth of *T. harzianum* and *T. viride* isolates. In a study, Yadav et al., 2012 observed higher growth and spore production (2.0x10⁶ cfu g⁻¹) rate of *T. viride* using Maize husk as a substrate. In present investigation, two cheap and easily available culture media (MoDA and MiDA) were formulated for the enhanced growth of *T. harzianum*. Results revealed that the higher mycelial growth of *T. harzianum* was obtained in MoDA medium than MiDA medium. The sporulation ability of *T. harzianum* was also found to be altered among the formulated media in following order: 19.6x10⁶ in MoDA > 17.0x10⁶ in MiDA > 11.96x10⁶ in PDA. While the sporulation of pathogenic fungi *F. graminearum* and *F. oxysporum* were higher in PDA (8.3x10⁶ and 9.67x10⁶) followed by MoDa (7.14x10⁶ and 8.4x10⁶) and MiDa (6.8x10⁶ and 8.0x10⁶) respectively. Therefore, MoDA was the most suitable culture media for *T. harzianum*. This might be due to the presence of a variety of essential phytochemicals in *M. oleifera* leaves. Studies substantiated that *M. oleifera* leaves contains 7 times more vitamin C than oranges; 10 times more vitamin A than carrots; 17 times more calcium than milk; 9 times more protein than yoghurt; 15 times more potassium than bananas; 25 times more iron than spinach and considered to be valuable source of highly digestible proteins, Ca, Fe, vitamin C and carotenoids (Rockwood et al., 2013; Abatneh et al., 2014).

Mint leaves have also been known to contain significant amount of micronutrients, vitamins, antioxidants, polyphenols and fiber content (Kodandaramreddy and Kavita, 2013) with potent antioxidant, antimicrobial, antiviral, anti-inflammatory, and anti-carcinogenic activities (Rodriguez-Fragoso, et al., 2008; Mallick et al., 2016; Fatima and Anjum, 2016). Furthermore, the inhibitory effect of *M. oleifera* and mint against magnification of pathogenic fungi *F. oxysporum* and *F. graminearum* could be cognate to their antifungal bioactive metabolites (Yasmeen et al., 2011; Marwal et al., 2013; Obaid et al., 2017).

In terms of fungal dry weight, the nutritional value of Moringa leaf extract was found to enhance *T. harzianum* biomass in comparison to other media (MiDB and PDB). Whereas the magnification of pathogenic fungi (*F. oxysporum* and *F. graminearum*) biomass were not restricted by MoDB and MiDB media compared with that in radial growth and sporulation. In contrast, Mustafa et al., 2009, studied the effect of five semi synthetic media including PDA on biomass of *Trichoderma* and reported PDA as a promising medium for mycelia growth.

Though PDA is kenne to utilize as a prevalent culture medium to grow a variety of fungi. But due to its higher cost (1kg of PDA≈ $85), scientific communities are looking forward to developing frugal and facil available media. There are a number of studies concentrated to screen alternative culture media to supersede PDA. Preliminary screening of alternative culture media for the growth of some selective fungi were performed by Tharmila et al., 2011. Another group of researchers
investigated the maximum growth of *T. viride* and *T. harzianum* on sugarcane bagasse and wheat medium respectively (Chanchal, and Gupta, 2014). Similarly, the formulated MoDA medium in present study was not only found to enhance the growth of *T. harzianum*, a bio-control agent but also exhibited lower cost (1 Kg of MoDA= $40) as compared to PDA. Thus, the utilization of MoDA as culture media in laboratories for the growth of *T. harzianum* would be a feasible and more frugal affair in comparison to commercially available PDA. In addition, the present formulation could be utilized as a selective medium for the growth and sporulation of *T. harzianum*. Furthermore, the suggested formulation can be stored at room temperature for about three months. Previously also, Doerr and Cameron, 2005 reported that Leaf powder of *M. oleifera* found to be stable up to 6 months by keeping temperature below 24°C and preventing light, air and humidity exposure (Doerr and Cameron, 2005).

**Conclusions**

There have been a variety of valuable microbes are present in our circumventions. But their mass production requires a well-defined composition of commercially available high cost nutrient media. The present study provides an alternative and cheap source of culture medium for the mass production of a valuable bio-control fungus *T. harzianum* using either *M. oleifera* or Mint leaves powder. These findings will not only be useful in laboratory conditions but may also be implemented on large scale for sustainable crop production and to maintain green environment.

**ACKNOWLEDGEMENTS:**

The authors are thankful to Department of Biology, College of Science, and University of Babylon, Iraq for providing the requisite facilities and to Dr. Ali. Jalil Obaid for technical assistance.

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