Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.7081

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **6 (6):** 6-19 (2018)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Molecular and Microscopic Identification of Fungi in Micropropagation of Nodal and Shoot Tip Culture of Orange

Sharmina Yeasmin[1‡], Md. Samiul Islam[1,2‡], Tareq Sujon[1], Razia Sultana[1], Md. Shah Alam[1], Biswanath Sikdar[1,3], Md. Asadul Islam[1,3], Md. Khalekuzzaman[1,3*]

[1]Department of Genetic Engineering and Biotechnology, Faculty of Life and Earth Science, University of Rajshahi-6205, Bangladesh

[2]Department of Plant Pathology, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, P.R. China

[3]Professor Joardar DNA and Chromosome Research Laboratory, Department of Genetic Engineering and Biotechnology, Faculty of Life and Earth Science, University of Rajshahi-6205, Bangladesh

‡ Both the authors contributed equally in the paper and hence will be considered as first author

*Corresponding Author E-mail: kzaman63@gmail.com

Received: 5.11.2018 | Revised: 12.12.2018 | Accepted: 19.12.2018

ABSTRACT

The present investigation was designed to throw light on the molecular identification and characterization of specific fungi responsible for the contamination in tissue culture medium of orange to find out cost-effective control measures. Both the fungi isolate 1 and isolate 2 grew optimally at 25 ^{0} C, pH-6, and 15% sugar content. Molecular identifications of those fungi were performed through PCR and a BlastN search of GenBank 18s rDNA revealed that the isolate-1 and isolate-2 had approximately 92% identity with Alternaria alternata (accession no. MH263651) and Fusarium culmorum (accession no. MH261354). In the antimicrobial activity, a plant extract of Ocimum tenuiflorum almost inhibited the growth of isolate-1 and isolate-2 was inhibited by Ficus hispida. The antagonistic test was performed through the post-harvest fungi isolate Alternaria cucumerina (accession no. MH279485) which 18s rDNA was matched 99% identity.1. It showed respectively 33.33% and 47.13% percentage of growth inhibition (PGI) against isolated two fungal strains.

Key words: *Micropropagation, Alternaria alternate, Fusarium culmorum, Molecular analysis, Biological control.*

INTRODUCTION

Orange (*Citrus sinensis* L.) is an important fruit belonging to the family Rutaceae of the genus *Citrus*. It is one of the major commercial fruit crops that are consumed all over the world both as fresh fruit or juice due to its high vitamin C content and effective antioxidant constituents[17]. Orange is a hybrid fruit between pomelo (*Citrus maxima*) and mandarin (*Citrus reticulate*) and it has genes that are 25% pomelo and 75% mandarin[36]. The cultivation of citrus fruit obtained nearly south Asian part of the world.

Cite this article: Yeasmin, S., Islam, M.S., Sujon, T., Sultana, R., Alam, M.S., Sikdar, B., Islam, M.A., Khalekuzzaman, M., Molecular and Microscopic Identification of Fungi in Micropropagation of Nodal and Shoot Tip Culture of Orange, *Int. J. Pure App. Biosci.* **6(6):** 6-19 (2018). doi: http://dx.doi.org/10.18782/2320-7051.7081

Copyright © Nov.-Dec., 2018; IJPAB

But the seedling choice is a critical factor for the establishment of new citrus groves and also make sure that the groves productivity and health[10].

For the above facts, micropropagation method has attained many outlooks recently due to their ability in producing healthy, disease free, and authentic seedlings[14]. Likewise, genetic transformation techniques have been shown to a powerful strategy as compared to conventional breeding for cultivar improvement[20]. Micropropagation can be defined as the growing of disease-free plant cells or tissues separate from the mother or wild plant on artificial media *in vitro*. It is an important technique in the study of plant genetics, plant morphology, plant metabolism, and plant physiology. The practice of plant tissue culture has contributed towards the propagation of a large number of the plant from small pieces of stock plant in a relatively short period of time[7]. Generally, tissue culture consists of taking a piece of a plant such as sterile leaves, stem tip, meristem, node, embryo, or even a seed[29].

The nutrient media in plant tissue culture usually employed but this *in vitro* media gets contaminated by micro-organisms. Contamination of tissue culture medium the microorganism is considered to be the most important reason for losses in products during *in vitro* culture of plants. Such microorganisms include bacteria, fungi, viruses, yeast, mites, and trips[5]. Among these micro-organisms, like fungi and bacterial infections such as *Alternaria alternate, Aspergillus fumigatus, Aspergillus niger, Aspergillus flavus, Rhizopus stolonifer, Fusarium oxysporum, Fusarium culmorum, Pseudomonas flourescens, Escherichia coli, Proteus sp, Micrococcus spp, Bacillus cereus, Bacillus subtilis, Erwinia sp*., etc. are considered most harmful to the plant tissue cultures[12]. If these contaminants are not controlled properly, then it makes a great loss to the agriculture industries as well as sectors dependent on them including the loss of expensive culture, time and production of infected plants used for the plantation that collectively hamper growth and yield[33].

Every step of plant tissue culture method must be considered in order to a contamination free. These steps encompass the type of handling of explants, preparation of media, subculturing, incubation and finally storage of sterile culture explants. Tissue culture media contaminants can be prevented through protected growth conditions of tissue cultures, irrigating explants with filtered water rather than standard city water and sub-culturing the plant cultures under laboratory cleanliness and an air source[23]. To prevent tissue culture medium by using medicinal plant extracts have been showing a remarkable observation. This is the first report to finding the *in vitro* efficacy of different plant extracts in contaminated tissue culture medium.

The main objectives of this study were to investigate and identify two most common fungal contaminant of the *in vitro* tissue culture medium with the hope of ingenious means of preventing contamination of the cultures and their *in vitro* control system by using plant extracts which may help to conduct a successful gene transfer program.

MATERIAL AND METHODS

Source of explant and preparation of tissue

Orange shoot tip was used as an explant in this study. Murashige and Skoog (MS, Sigma) media were used for this purposes[27]. The MS media was sterilized by autoclaving at 121°C for 20 minutes. The explants were excised (2-3 cm) and surface sterilized with sodium hypochlorite (NaOCl) (Sigma-Aldrich Cheniere) solution for 2-3 minutes[38]. Then explants were rinsed with 3-4 successive changes of sterile distilled water (SDW). Finally, explants were aseptically transferred to the MS culture medium, labeled and incubated at 25°C ± 2 for 2-3 weeks.

Isolation of microbial contaminants

From the contaminated culture tubes, contaminated tissue was removed from the tissue culture unit in an aseptic condition and visually examined (Fig 1a). Then contaminants microbes which are emerging in tubes were isolated by inoculating them on solidified Potato Dextrose Agar (PDA) media and

incubated for 7-10 days at 25°C (Fig 1c). The pure isolated fungal colony was obtained from repeated sub-culturing of the isolate in PDA media and store to preserve at 4°C in a refrigerator[6].

Morphology of two fungal isolates

The fungal isolates were identified using cultural characteristics, morphology, and microscopic comparison with standards by Barnett and Hunters[3] procedure. Morphology analysis of this two isolated fungal strains was done using PDA (Potato Dextrose Agar), SDA (Sabouraud Dextrose Agar), and CDA (CzapekDox Agar) media. A pure culture of both isolates growing culture on PDA, a mycelial disc of 5 mm in diameter was cut and placed at the center of three new different media. Then Petri-plates were kept for incubation at room temperature (25°C) for 7-10 days. Based on their colony diameter, shape, size, color, texture, form, margin, reverse color, elevation, dry weight etc. compared to each medium morphology. The morphological analysis was subjected under a light microscope at 40X magnification ((LABOMED, LX400, USA).

Growth profiling of two fungal isolates

The growth profiling test of fungi gives an idea about the ability of fungal growth, making easier to discriminate different strains of same or different species. For this purposes, different growth profiling such as temperature, pH, different sugar concentrations, and carbohydrates test was done against the two isolated fungal strains. Solidified PDA media was prepared with around 100 ml for each isolates growth profiling tests and autoclaved at 121°C for 20 minutes and the media was placed in a Petri-plates to allowed it solidify. Four different temperature (4^0 C, 25^0 C, 30^0 C, and 37^0 C), three different pH (3, 6 and 9), three different sugar concentration (5%, 10%, and 15%) containing 20gm/L value and four different sugar has been used for optimized this analysis. 6 mm diameter discs of mycelium were cut from a pure culture on PDA media and inoculated in the different growth profiling culture plates. Then the plates were incubated at 25°C for 7 days. After 7 days the dry weight is measured using electrical balance by collecting the fungal mycelium and record physiological and morphological characteristic[21,9].

PCR amplification with ITS primer and sequencing

The extraction of genomic DNA isolation was done by fungal DNA/RNA/Protein Kit (QIAGEN) followed by their 'Quickstart' protocol. The quantity and quality of the isolated fungal DNA were confirmed by spectrophotometrically and electrophoretically. After that isolated both DNA was subjected to the PCR amplification using the internal transcribed spacers primers 'ITS-2F' and 'ITS-2R' (Sigma, USA) and Thermo Fisher Dream Taq Blue PCR master mix (2X). PCR amplification was performed in a 50 µl (one isolate) of the reaction mixture which contained 25 µl Taq blue PCR master mix (2X), 1 µl of forward and 1 µl of reverse primer, 1 µl of genomic DNA and rest of the DNase free PCR water. PCR conditions (Q-cycler 96, Hain Life Science) were as follows: initial denaturation at 95^0 C for 2 min, followed by 35 cycles of denaturation at 94^0 C for 1 min, primer annealing 51.5^0 C for 30 seconds, primer extension 72^0 C for 2 min each with a final elongation at 72^0 C for 10 min and hold at 4^0 C. The amplicons were amplified by 1% agarose gel in 0.5 Tris/Borate/EDTA buffer and the amplified bands were detected against 1 kb ladder (DL5000 DNA Marker, TSINGKE). Products of both sequencing reactions were analyzed in Malaysia Ltd. via Invent Biotech, Dhaka, Bangladesh. Both the sequence analysis was performed using a BlastN search of NCBI database and phylogenetic relationship obtained CLUSTAL W and MEGA 6.0 software[35]. Both the fungal nucleotide sequences were deposited in the GenBank database.

Cellulolytic activity of two fungal isolates

For this study, 5cm/1cm cellulose strip was placed in the liquid PDA culture of the fungal strains before incubation at room temperature (25^0 C). After 7-10 days, if the cellulose strip degrades then it showed positive result against the fungal isolates[22].

Control measurement with different NaCl concentrations

Different NaCl concentrations were used as a criterion to study fungal growth. The PDA media was prepared according to the manufacturer's instructions and autoclaved at 121^0 C for 20 minutes. For each isolate, three different NaCl concentrations were used such as 0.5%, 1%, and 2% respectively. After that 25 ml of PDA media was transferred to Petri-plates with different NaCl concentrations and 5cm of mycelium disc were placed at the center of the Petri-plates and incubated at 25^0 C for 7-10 days. After the incubation period, the growth was observed and the dry weights were recorded.

***In vitro* efficacy of different plant extracts**

Plant extracts are highly effective against microbial infections. In this study, we used four different plant extracts to inhibit the growth of fungi as biological control measures. Table 1 shows plant materials used for this study with their local name, family name, and usable parts.

Table1. List of plants for *in vitro* efficacy test

Scientific name	English name	Family name	Usable parts
Coccinia grandis	Ivy gourd	Cucurbitaceae	Roots
Azadirachta indica	Neem	Meliaceae	Leaves
Ficus carica	Common Fig	Moraceae	Leaves
Ocimum tenuiflorum	Holy basil	Lamiaceae	Leaves

Preparation of plant extracts and fungal growth control measurement

At first, the collected plant parts were washed with running tap water and sterilized with 70% ethanol. Then they were dried in the drier at 37^0 C. The dried parts were grinded using mortar and pestle. Then the dust was dissolved at 100g/L concentration in methanol solution. After 24 hours the mixture was firstly filtered through the filter paper. 1 ml of each methanol plant extracts were added on 25 ml of PDA media plates to control the fungal strain using the 'Poisoned food technique'. After that, seven-day-old culture fungi isolates (5 mm disc) were inoculated in the center of the media plates and incubated for 7 days at 25^0 C[31,2]. For direct analysis of efficacy of plant extracts into tissue culture medium, we added methanolic extracts of plant extracts in three different concentrations. After autoclaving the culture medium carefully added plant extracts at 0.10%, 0.01% and 0.05% concentrations by using sterile micro-pipette.

Antagonistic test

The antagonistic test was performed through the post-harvest fungi isolate *Alternaria cucumerina* (accession no. MH279485). Dual culture plate method was used to determine the percentage inhibition of radial growth (PIRG). In this method, 5 ml of mycelia discs of the test fungal isolates was placed 2 cm away from the edge of the Petri-plates from the periphery of Petri plates and a single antagonist mycelia disc were placed similarly on the opposite end of a test sample of the test fungi[16]. As a control, isolated test fungi were placed in a similar manner on a fresh PDA plate. All pairings were carried out in quadruplicate and incubated at 25^0 C. Antagonistic activity was tested 7 days after incubation by measuring the radius of the test fungal colony. The two readings were transformed into percentage inhibition of radial growth (PIRG) using the formula developed by Skidmore and Dickinson[34]. The following formula is given below:

$$\textbf{PGI (\%) = KR-R1/KR x 100}$$

KR- Direction of the antagonist colony

R1- The radius of the test fungal colony in the control plate

Statistical analysis tools

All the analysis of the present study were conducted in triplicate for the tune of exact results and statistical analysis. All the data were expressed as a mean and standard error (M±SE) using Microsoft Excel software 2013.

RESULTS AND DISCUSSION

Morphology and microscopic characterization

The bold visualization of colonies developed on different media (PDA, SDA, CDA) recommended for this particular fungal genus is considerable importance in identification. All three culture media supported the growth of test fungi to various degrees. Out of them, the isolate-1 showed maximum mycelial growth (6.8±1.5) on PDA media after 7-10 days of the incubation period (Figure 1b). The difference in surface coloration of fungal colonies was distinct on three growth media as observed in case of PDA media it was brown on the surface; in case of SDA, Brownish to white on the surface. Similarly, in CDA media, it looked Pinkish white on the surface. Isolate-2 also showed maximum mycelial growth on PDA media after 7 days of the incubation period (Figure 1d). In PDA, it was white on the surface and attained white color also in SDA and CDA media. In PDA, fungi were characterized with distinct radial furrows on the reverse. On the other hand, no radial furrows exhibit in SDA and CDA. Both the isolates showed maximum growth at 25-28°C temperatures and pH 6.

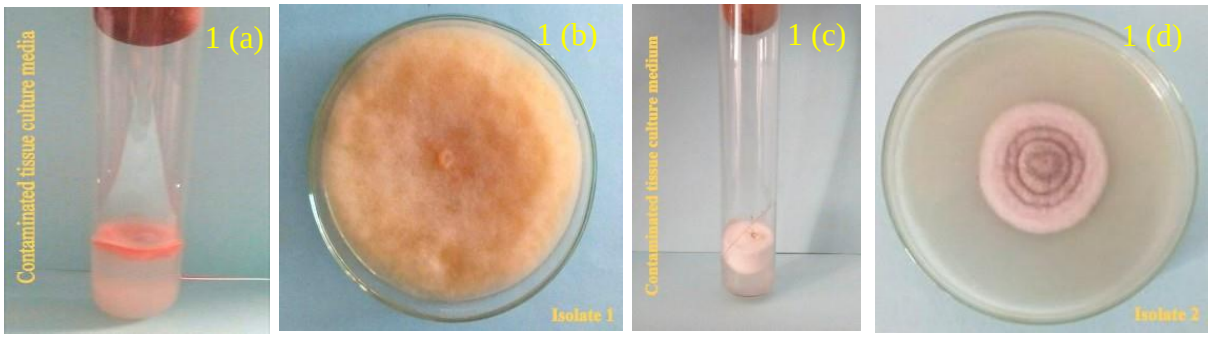


Figure 1. Figure shows the contaminated orange tissue culture nodal shoot and tip culture medium (1a, and 1c) and figures 1b and 1d show the morphology of this two isolated fungi.

These findings are similar to Misaghi *et al*.[26] and Kang *et al*.[19]; indicating the observed temperature ranges 25-30°C are the most favorable for the majority of *Alternaria* and *Fusarium* sp, that contaminates the tissue culture medium. Aimanianda *et al*.[1] reported that the diameter of the fungal conidia and spore ranged usually 4-6 x 2-6 µm. The microscopic view of both isolates in different media showed in figure 2 and 3.

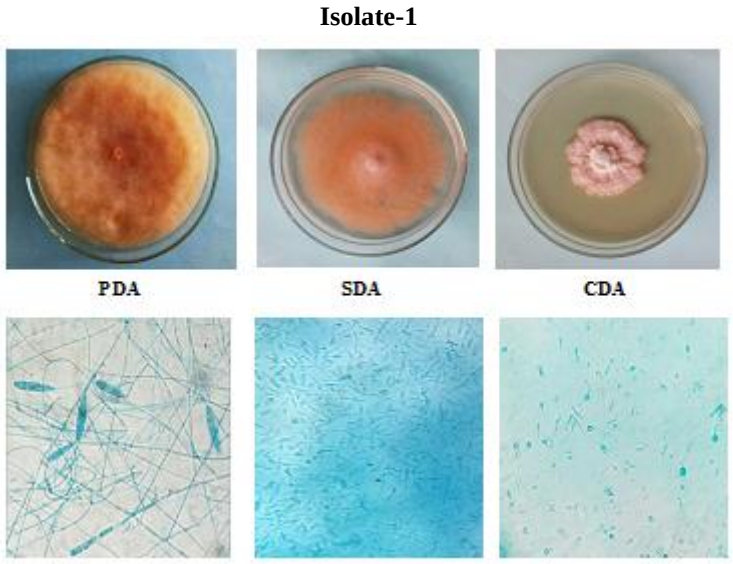


Figure 2. Microscopic view of isolate-1 on different growth medium and their colony characterization. Hyphae of isolate 1 showed thin spread like structure, conidia show branched chain of spores in SDA and CDA media shows single micro-conidia.

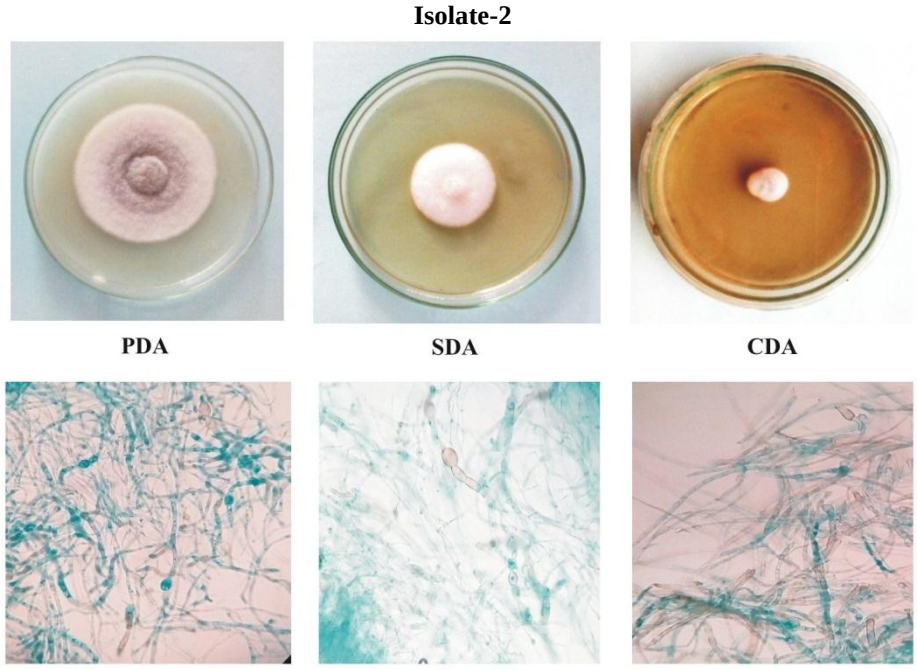


Figure 3. Microscopic view of isolate-2 on different growth medium and their colony characterization. Mycelium of isolate 2 showed thread like structure with true hyphae, colonies are very first growing but the spore is not visualized.

Type of culture media and their nutrient compositions significantly affected the mycelia growth rate and conidial production of fungal isolates. PDA is one of the most commonly used culture media because of its simple formulation and ability to support the mycelial growth of a wide range of fungi. Several workers stated PDA to be the best media for mycelial growth[37,25,32]. Hence, fungi are recognized and identified basically by their phenotypes[39]. The overall morphological characterization of two isolated fungal strains showed in table 2 and 3.

Table 2. Colony morphology of fungal isolate-1 on different media

Characteristics	PDA	SDA	CDA
Diameter(cm)	6.8±2.1	6.1±2.1	4.4±1.5
Form	Circular	Filamentous	Filamentous
Margin	Entire	Filiform	Undulate
Elevation	Convex	Umbonate	Convex
Surface	Smooth	Rough	Rough
Front color	Brown	Brownish to white	Pinkish white
Dry weight(gm)	0.234±0.03	0.201±0.03	0.132±0.04

Table 3. Colony morphology of fungal isolate-2 on different media

Characteristics	PDA	SDA	CDA
Diameter(cm)	5.1±2.1	3.9±2.1	1.6±1.5
Form	Circular	Circular	Irregular
Margin	Entire	Filiform	Entire
Elevation	Raised	Umbonate	Raised
Surface	Smooth	Rough	Rough
Color	White	Brownish to white	White
Dry weight(gm)	0.196±0.03	0.137±0.03	0.058±0.04

Growth profiling test

Growth profiling in temperature

The present study revealed differences in temperature optima for mycelial growth of *Alternaria alternta* and *Fusarium culmorum*. Mycelial growth occurred over a range of 4°C to 30°C, with maximum growth at 25°C (Fig 4a). The growth of fungi decreased when the temperature increased more than 30°C. The fungus showed maximum mycelial growth, 6.6cm (isolate-1) and 4.8 cm (isolate-2) at 25°C. And the dry weight of the fungi at 25°C was 0.195gm and 0.130 gm. According to this profiling both the fungal strain belong to Mesophiles group. In accordance with the review of Daniel and Danson[8]; Freidrich and Wingtgen[13], increased temperature led to increasing inactivity but that there was a limit to the increase in activity because higher temperatures led to a sharp decrease in activity.

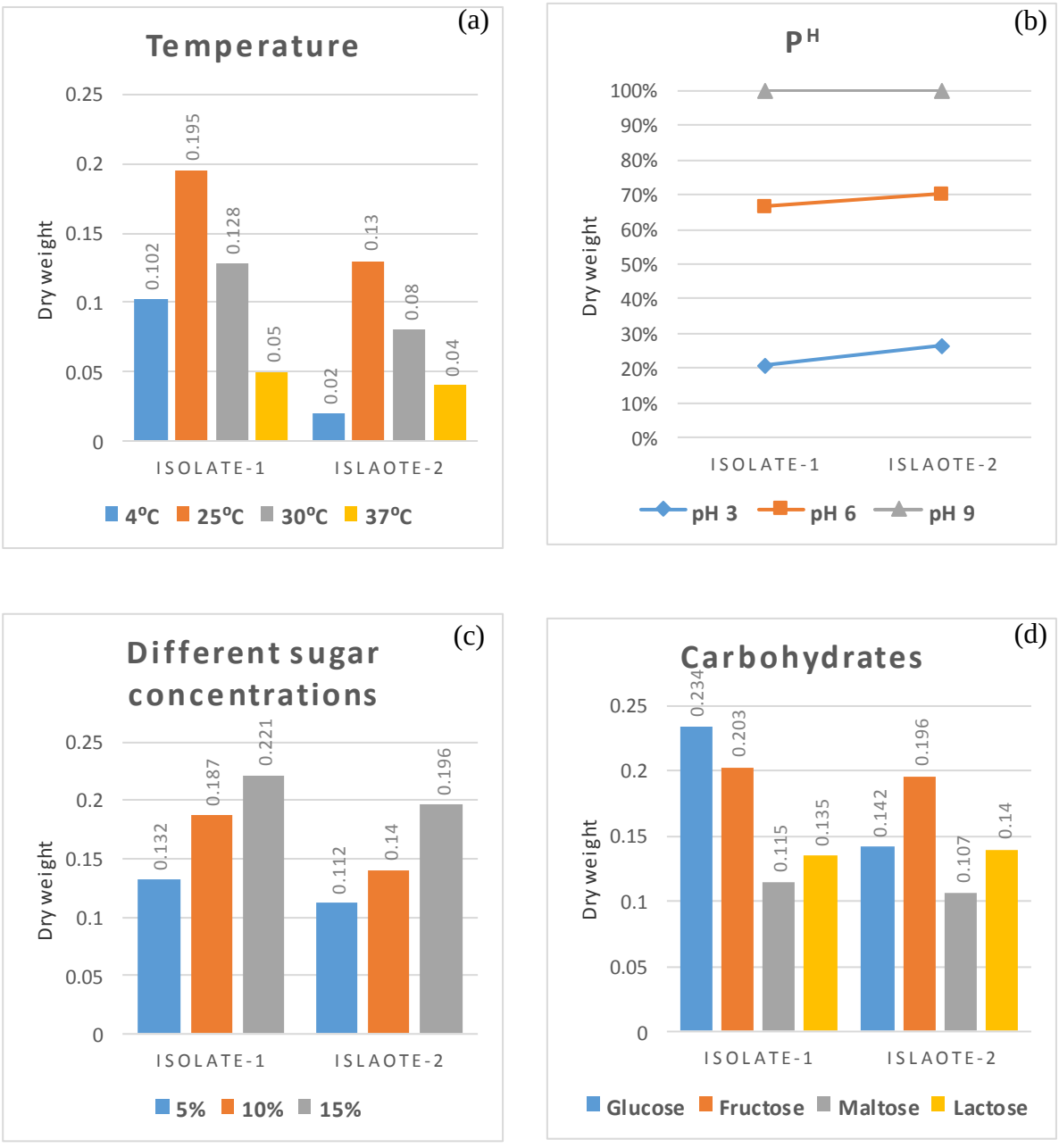


Figure 4. The graphical presentation shows the growth profiling test against two fungi isolates in different temperature (a); different pH (b); different sugar concentrations (c); and different carbohydrates (d).

Growth profiling in different sugar concentrations

The experiment was conducted to examine the effect of sugar concentrations on the growth of fungi. Mycelial growth occurred over a range of 5%-15% sugar concentrations. It was observed that increasing sugar concentrations had a stimulatory effect on the growth of fungi (Fig 4c). At 15% sugar concentration the maximum mycelial growth was 7 cm and 6.4 cm with dry weight 0.221gm and 0.196gm respectively. At 15% sugar concentration, it gave maximum biomass production which was achieved within 7 days of incubation periods. Our study confirmed by Fang *et al*.[11].

Growth profiling in different carbohydrates

For the growth profiling of the fungi different carbohydrates: glucose, lactose, fructose, and maltose were used at 20gm/L concentration in PDA media. The maximum growth of the isolate-1 was observed in glucose with a 7.1cm diameter of mycelium growth with 0.234gm of dry weight. In case of isolate-2 fructose showed the highest growth on fructose with 6.3cm diameter and 0.196gm of dry weight (Fig 4d).

Cellulolytic activity test

According to the study, the isolated fungal strains didn't degrade the cellulose strip. So there was no cellulolytic activity present in the isolated fungal strains. Similar findings were obtained by Nilsson *et al*.[28], where *Alternaria*, *Fusarium* sp., showed negative cellulolytic activity results.

PCR amplification and sequencing

In gel electrophoresis, isolated two fungal genomic DNA was high molecular weight and it was seen approximately on the top of the 11000 bp DNA marker. The 'ITS' region of ribosomal DNA was amplified for future sequencing to confirm the identity of the fungal isolates. Amplified isolated fungal DNA was approximately 1300 bp and 1400 bp which compared with 1kb+ DNA marker (Fig 5a, b).

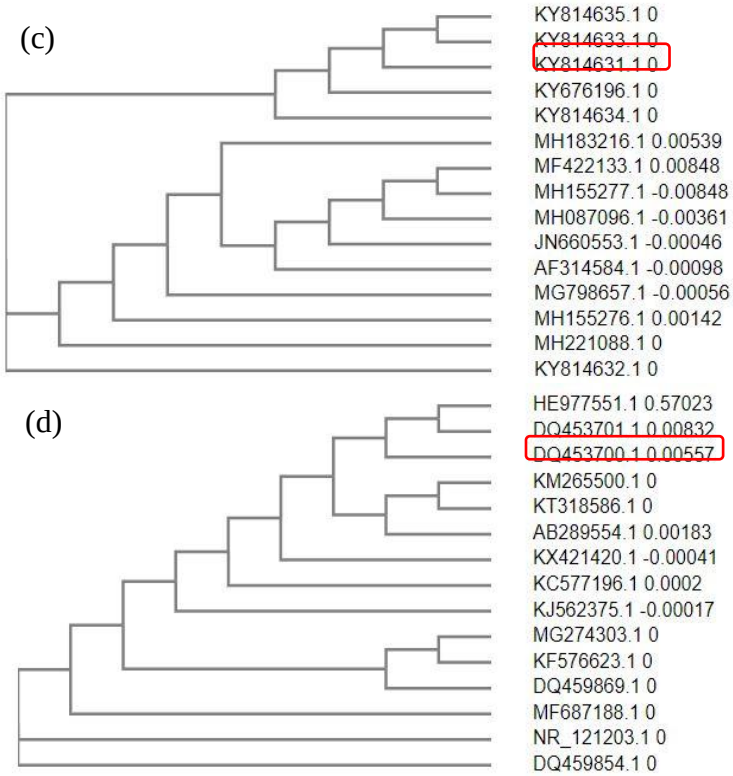


Figure 5. Figure 5 (a, b) shows the isolated two fungal strain was amplified ITS region approximately in 1300 bp and 1400 bp with ITS-IIF and ITS-IIR primer. Phylogenetic relationship was obtained by the Neighbor-Joining method (5c, d). The evolutionary relation was obtained through the CLUSTAL OMEGA and MEGA 6 software.

Sequencing reactions performed with both types of templates using PCR-amplified fragments and cloned DNA fragments showed the same nucleotide sequences in size, allowing a continuous stretch of bases to be determined. A BlastN search of GenBank revealed that the fungal isolate-1 had approximately 92% identity with *Alterneria alternata* and isolate-2 had approximately 92% identity with *Fusarium culmorum* (Fig 5c, d). Both nucleotide sequences were deposited in the GenBank and have the accession no. MH263651 and MH261354 respectively. These findings of *Alternaria* and *Fusarium* groups based on morphology and growth profiling which is poorly supported by ITS rDNA sequence data. But effective identification may help further control management of this two diseases. Our results are congruent with the Gilbertson[15] and Lievens *et al*.[24].

Control measurement with different NaCl concentrations:

Along with the experiment on control measurement, NaCl proved as a great antifungal compound. NaCl inhibited the fungal growth to a great extent (Fig 6a and 6b). The growth inhibition rate was determined based on the equation [(ΔC – ΔT)/ΔC] x 100 Broekaert *et al*.[4] where ΔC is the diameter of control fungal colony and ΔT is the diameter of treatment fungal colony. We observed that 2% NaCl was able to inhibit 55% fungal growth (Fig 6), which can be a great promising factor for successful future control practice. Different concentration of NaCl in the PDA media was used from 0.5-1.5% concentrations. At 2% concentration, the growth of the two fungi was 2.2 cm and 1.1 cm in diameter. It was observed that increasing NaCl concentration decreased the growth of isolated fungi.

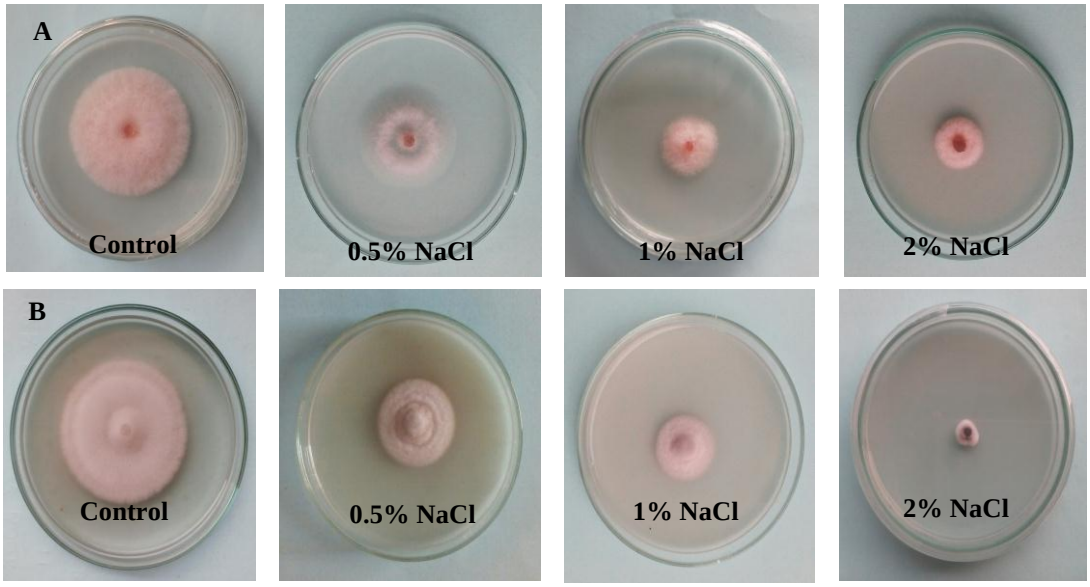


Figure 6. Showing inhibitory growth pattern of two fungal isolates (A, isolate-1, and B, isolate-2) in different NaCl concentrations.

***In vitro* efficacy of plant extracts against two fungal isolates**

Though all four plant extracts, used in this antifungal activity, the mycelium growth of the fungal isolates-1 was less inhibited (15%) by ivy gourd. The other three extracts can inhibit the fungal growth around 65%, 69%, and 73% respectively, which was really a good result for effective control practice. On the other hand, the four plant extracts showed a positive result for fungal isolates-2. The four extracts inhibited the fungal growth around 55%, 63%, 60%, and 68% respectively (Fig 7a). This study concluded that the plant extract *of Ocimum tenuiflorum* (Holy Basil*)* almost inhibited the growth of isolate-1 and isolate-2 was inhibited by *Ficus carica* (Common Fig). Beside the ivy gourd, all three plant extracts

showed significant antifungal activity and our results were obtained in the previous study conducted by Hammer *et al*.[18]. When *Ocimum tenuiflorum* (Holy basil) plant extracts was added into the isolate-1 culture medium in different concentrations, it anxiously inhibit the mycelim growth. On the other side, methanolic extracts of *Ficus carica* (Common Fig) showed significant inhibition of isolates-2 mycelium growth (Fig 7b). This *in vitro* control measures is the first findings of this two fungal isolates. Further studies need to isolate this operative compound from the plant extracts which help to produce potential and eco-friendly remedy for tissue culture fungal contaminants.

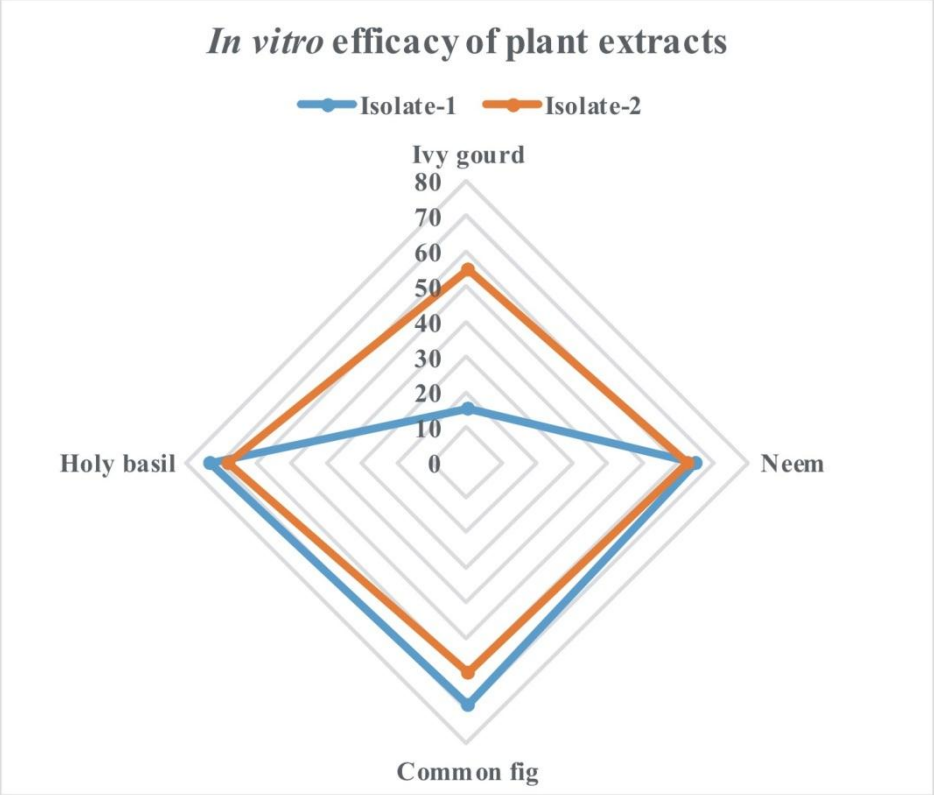
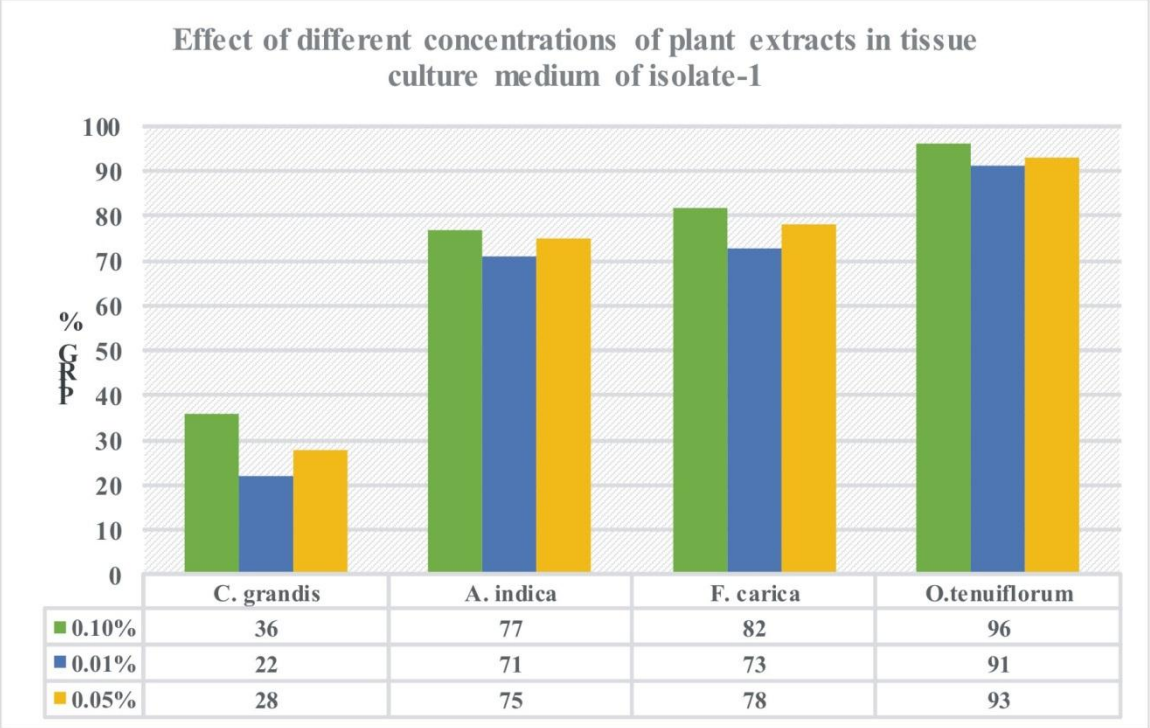


Figure 7a. The radial chart shows the reduction of the percentage of inhibition growth rate (PIRG) by using different plant extracts against two fungal isolates.



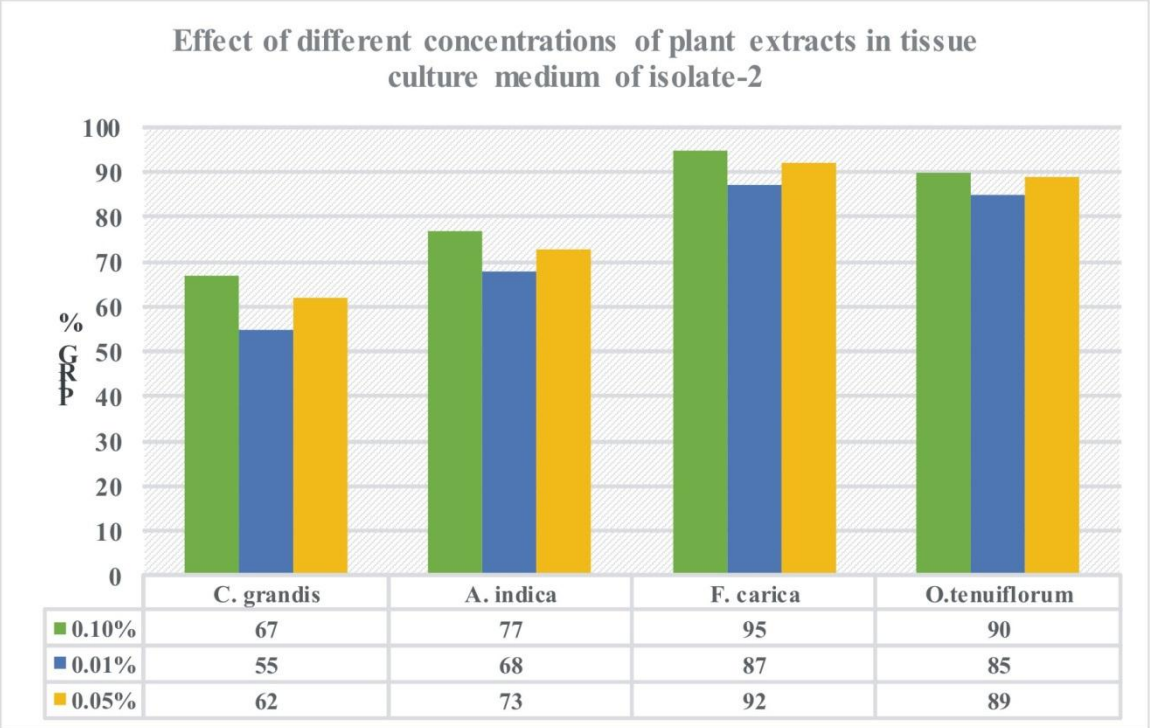


Figure 7b. Graph shows inhibition pattern of different methanolic plant extracts on both fungal isolates.

Antagonistic activity assays *in vitro*

The assay for antagonism was performed on PDA on Petri dishes by the dual culture method. After that, the inhibitory effect was measured by manual measurement using millimeter scale. To test antagonism of *Alternaria cucumerina* fungi, 5 mm of mycelia agar disc from fungi isolates cultures was placed on the one side of a Petri dish containing PDA medium and another 5 mm mycelia agar disc was also placed 3 cm away from the disc of fungal isolate on the same dish. Paired cultures were incubated at 25^{o}C. Results from the dual culture assay showed that antagonistic microorganisms inhibited the mycelial growth of isolated fungal strain, with

varying efficiencies and the result of PGI was 33.33% and 47.13% respectively (Fig 8). This result is confirmed by Pandey *et al*.[30], where they found bacterial strain *Pseudomonas* and fungal strain *Alternaria* showed significant antagonistic activity against the *Alternaria alternata* and *Fusarium culmorum*.

Figure 8. Antagonistic activity of other fungi against isolated fungi.

Acknowledgments

This research is carried out as a part of grant supported by Ministry of Science and Technology (MoST) - Government of the People's Republic of Bangladesh and Ministry of Education (MoE), Bangladesh. The authors also thank Professor Joarder DNA & Chromosome Research Lab, Department of Genetic Engineering and Biotechnology, the University of Rajshahi for providing lab facilities.

Conflict of interest

The authors declared that there is no conflict of interest regarding this publication.

REFERENCES

1. Aimanianda, V., Bayry, J., Bozza, S., Kniemeyer, O., Perruccio, K., Elluru, S. R., Romani, L., Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature*. 460 **(7259):** 1117-1120 (2009).
2. Ali- Shtayeh, M. S., Abu Ghdeib, S. I., Antifungal activity of plant extracts against dermatophytes. *Mycoses*. **42(11-12):** 665-672 (1999).
3. Barnett, H. L., Hunter, B. B., Illustrated genera of imperfect fungi. USA: American Phytopathological Society (APS Press) (1998).
4. Broekaert, I., Lee, H. I., Kush, A., Chua, N. H., Raikhel, N., Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). *Proc Nat Acad Sci*. **87(19):** 7633-7637 (1990).
5. Cassels, A. C., Production of healthy plants. In proceedings of the Institute of Horticultural Symposium: Micropropagation in culture. USA: Dullforce, WM (1996).
6. Choi, Y. W., Hyde, K. D., Ho, W. H., Single spore isolation of fungi. *Fungal div*. **3(1):** 29-38 (1999).
7. Daniel, R. L., The many dimension of plant tissue culture research. *Web Aggie Horticul*. **22(1):** 201-210 (1998).
8. Daniel, R. M., Danson, M. J., A new understanding of how temperature affects the catalytic activity of enzymes. *Trends Bio Sci*. **35(10):** 584-591 (2010).
9. Dhandhukia, P. C., Thakkar, V. R., Standardization of growth and fermentation criteria of *Lasiodiplodia theobromae* for production of jasmonic acid. *Afri J Biotechnol*. **6(1):** 707-712 (2007).
10. Esmaeilnia, E., Dehestani, A., *In vitro* plant regeneration from mature tissues of Thomson navel sweet orange (*Citrus sinensis* L. *Osbeck*.). *Biharean Biol*. **9(1):** 9-14 (2015).
11. Fang, Q. H., Zhong, J. J., Submerged fermentation of higher fungus *Ganoderma lucidum* for production of valuable bioactive metabolites—ganoderic acid and

polysaccharide. *Bio Eng J*. **10(1):** 61-65 (2002).

12. Felise, H. B., Nguyen, H. V., Pfuetzner, R. A., Barry, K. C., Jackson, S. R., Blanc, M. P., Bronstein, P. A., Kline, T., Miller, S. I., An inhibitor of gram-negative bacterial virulence protein secretion. *Cell Host Microbe*. **4(4):** 325-336 (2008).
13. Friedrich, H., Wintgen, H., The hydrogen atom in a uniform magnetic field—an example of chaos. *Phy Rep*. **183(2):** 37-79 (1989).
14. George, E. F., Hall, M. A., De Klerk, G. J., Plant propagation by tissue culture. Netherlands: Springer (2008).
15. Gilbertson, R. L., Molecular phylogenetic relationships among *Altemaria* species and related fungi based on analysis of nuclear ITS and mtSSU rDNA sequences. *Mycol Res*. **104(1):** 1312-1321 (2000).
16. Gomathi, S., Ambikapathy, V., Antagonistic activity of fungi against *Pythium debaryanum* (Hesse) isolated from Chilli field soil. *Adv Appl Sci Res*. **2(4):** 291-297 (2011).
17. Gorinstein, S., Martın-Belloso, O., Park, Y. S., Haruenkit, R., Lojek, A., Ciz, M., Caspi, A., Libman, I., Trakhtenberg, S., Comparison of some biochemical characteristics of different citrus fruits. *Food Chem*. **74(3):** 309-315 (2001).
18. Hammer, K. A., Carson, C. F., Riley, T. V., Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol*. **86(6):** 985-990 (1999).
19. Kang, Z., Huang, L., Krieg, U., Mauler-Machnik, A., Buchenauer, H., Effects of tebuconazole on morphology, structure, cell wall components and trichothecene production of *Fusarium culmorum in vitro*. *Pest Manag Sci*. **57(6):** 491-500 (2001).
20. Khan, E. U., Fu, X. Z., Wang, J., Fan, Q. J., Huang, X. S., Zhang, G. N., Shi, J., Liu, J. H., Regeneration and characterization of plants derived from leaf in vitro culture of two sweet orange (*Citrus sinensis* (L.) *Osbeck*) cultivars. *Scie Hort*. **120(1):** 70-76 (2009).
21. Kour, A., Shawl, A. S., Rehman, S., Sultan, P., Qazi, P. H., Suden, P., Verma, V., Isolation and identification of an endophytic strain of *Fusarium oxysporum* producing podophyllotoxin from *Juniperus recurva*. *World J Microbiol Biotechnol*. **24(7):** 1115-1121 (2008).
22. Lee, S. F., Forsberg, C. W., Gibbins, L. N., Cellulolytic activity of *Clostridium acetobutylicum*. *Appl Env Microbiol*. **50(2):** 220-228 (1985).
23. Leifert, C., Waites, W. M., Dealing with microbial contaminants in plant tissue and cell culture: hazard analysis and critical control points. Growth and Development of Plants in Culture, Springer, Dordrecht (1994).
24. Lievens, B., Rep, M., Thomma, B. P., Recent developments in the molecular discrimination of formae speciales of *Fusarium oxysporum*. *Pest Manag Sci*. **64(8):** 781-788 (2008).
25. Maheswari, S. K., Singh, D. V., Sahu, A. K., Effect of several nutrient media on the growth and sporulation of *Alternaria alternata*. *J Mycopathol Res*. **37(1):** 21-24 (1999).
26. Misaghi, I. J., Grogan, R. G., Duniway, J. M., Kimble, K. A., Influence of environment and culture media on spore morphology of *Alternaria alternata*. *Phytopathol*. **68(1):** 29-34 (1978).
27. Murashige, T., Skoog, F., A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant*. **15(3):** 473-497 (1962).
28. Nilsson, T., Ginns, J., Cellulolytic activity and the taxonomic position of selected brown-rot fungi. *Mycol*. **1(1):** 170-177 (1979).
29. Ogunsanwo, Y. R., Sources of microbial contamination in tissue culture laboratories in southwestern Nigeria. *Afr J Agri Res*. **2(3):** 067-072 (2007).
30. Pandey, A., Trivedi, P., Kumar, B., Palni, L. M. S., Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a

sub-alpine location in the Indian Central Himalaya. *Current Microbiol*. **53(2):** 102-107 (2006).

31. Ravikumar, M. C., Garampalli, R. H., Antifungal activity of plants extracts against *Alternaria solani*, the causal agent of early blight of tomato. *Arch Phytopathol Plant Prot*. **46(16):** 1897-1903 (2013).
32. Saha, A., Mandal, P., Dasgupta, S., Saha, D., Influence of culture media and environmental factors on mycelial growth and sporulation of *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. *J Env Biol*. **29(3):** 407-412 (2008).
33. Sattigeri, V. J., Soni, A., Singhal, S., Khan, S., Pandya, M., Bhateja, P., Mehta, A., Synthesis and antimicrobial activity of novel thiazolidinones. *Arkivoc*. **2(1):** 46-59 (2005).
34. Skidmore, A. M., Dickinson, C. H., Colony interactions and hyphal interference between *Septoria nodorum* and phylloplane fungi. *Trans British Mycol Soc*. **66(1):** 57-64 (1976).
35. Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*. **30(12):** 2725-2729 (2013).
36. Tan, M. L., Song, J. K., Deng, X. X., Production of two mandarin× trifoliate orange hybrid populations via embryo rescue with verification by SSR analysis. *Euphytica*. **157(1-2):** 155-160 (2007).
37. Xu, S. O., Yuan, S. Z., Chen, X. C., Studies on pathogenic fungus (*Alternaria tennuis*) of poplar leaf blight. *J North East For Inst*. **12(1):** 56-64 (1984).
38. Yedidia, I., Benhamou, N., Chet, I., Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Appl Envi Microbiol*. **65(3):** 1061-1070 (1999).
39. Zhae, S., Simon, F. S., Effect of culture media, temperature, pH and bio-herbicidal efficacy of *Phoma exigua*, a potential biological control for salal (*Gaultheria shallon*). *Biocontrol Sci Technol* **6(1):** 1043-1055 (2006).