Pak. J. Bot., 52(3): 1083-1086, 2020. DOI: http://dx.doi.org/10.30848/PJB2020-3(44)

POTENTIAL OF ENDOPHYTIC FUNGUS *ASPERGILLUS TERREUS* AS POTENT PLANT GROWTH PROMOTER

AROOJ JAVED[1], AZHAR HUSSAIN SHAH[2*], ANWAR HUSSAIN[3*], ZABTA KHAN SHINWARI[4], SEEMA ALI KHAN[1], WAJIHA KHAN[5] AND SOHAIL AHMAD JAN[2]

[1]*Department of Botany, Hazara University Mansehra, Khyber Pakhtunkhwa, Pakistan*
[2]*Department of Biotechnology, Hazara University Mansehra, Khyber Pakhtunkhwa, Pakistan*
[3]*Department of Botany, Abdul Wali Khan University Mardan, Khyber Pakhtunkhwa, Pakistan*
[4]*Pakistan Academy of Sciences, Islamabad, Pakistan*
[5]*Department of Biotechnology, COMSATS University Islamabad, Abbottabad Campus, Pakistan*
**Corresponding author's email: drahshu@gmail.com; drhussain@awkum.edu.pk*

Abstract

Fungal endophytes are well-noted residents inside plant tissues to assist plant development and fitness. However, plant growth-promoting endophytes (PGPE) are less reported therefore, the objectives of present study were to isolate fungal endophytes, identify and illustrate their potential for plant growth-promotion. Endophyte *Aspergillus terreus* was selected to check its role in promoting growth of tomato seedlings. The isolate showed positive results for hydrogen cyanide and indole acetic acid (IAA) production, phenols and flavonoids production under *In vitro* conditions. Endophyte associated tomato seedlings showed higher growth than the control. The results showed that isolated and characterized microbial endophytehad a considerable impact on plant growth parameters and could be helpful as inoculants to improve a sustainable farming system without posing any adverse effect on environment.

Key words: *Aspergillus terreus,* Endophytic fungus, Isolation, Identification, Tomato.

Introduction

Plants are in continuous interaction with microbial symbionts, several noted as to be pathogen while others showed mutual relationship to the host plant. In previous studies, microbes are reported as useful group of microbes and known to be endophytes (Khan *et al*., 2016). Fungal endophytes promote the growth of host plants via production of active metabolites, helping in plant productivity, high yielding crops, induce resistance to different stresses (biotic and abiotic) and achieving a prudent characters for improving agricultural practices (Jose *et al.,* 2009; Amin, 2016). Redman *et al*., (2011) reported that endophytes promote growth of rice plants under abiotic stress condition by enhancing it biomass and yield in both *In vitro* and green house conditions.

Efficient agricultural and horticultural practices are needed for proper identification of endophytic fungi. In addition, it is important to check these endophytes for its possible applications for crop improvement (Sturz *et al*., 2000; Afzal *et al.,* 2017; Afzal *et al.,* 2018; Afzal *et al.,* 2019). Many isolated microbial strains play key role in controlling of many plant diseases (Rahman *et al.,* 2017; Urooj *et al.,* 2018). Some plant growth promoting bacteria provide salt tolerance to many important plant species (Nauman *et al.,* 2018). The microbial interaction with host as growth promoters has been recognized particularly in leguminous as well as non- leguminous families (Chanway, 1996). Endophytes can improve plant yield and other performances by increasing nutrient availability and promote its lateral root formation (Pillay & Nowak, 1997; Yates *et al*., 1997). Many of endophytes has been identified as being used as artificial inoculation for sustainable practices in agriculture, as *Fusarium moniliforme, phoma* sp. and *Penicillium* sp., which improve growth in maize roots (Yates *et al*., 1997). The endophytes associated with rice shows better growth and development (Muhammad *et al*., 2012).*Aspergillus terreus* is known to be isolated as abundant filamentous fungus from rhizospheric soil as well as aquatic places (He *et al*., 2004 and Damare *et al*., 2006) and decays of old plant and animals (Reddy & Singh, 2002). *A. terreus* is able to produce important metabolites i.e., lipases and cellulases and itaconic acid, respectively (Kuenz *et al*., 2012). Therefore, the current study was conducted to isolate, identify and characterize beneficial endophytic fungi from tomato plant and to check its activities as best plant growth promoter.

Materials and Methods

Isolation of endophytic fungi: Isolation of endophytic fungi from tomato plants was isolated by following method of Ezra *et al.,* (2004).The collected samples were sterilized by washing with running tap water. The plant parts (stem, leaves and roots) were separated after surface sterilization. Each plant part was cut into 2 to 5 mm and then inoculates on autoclaved hagem media (minimal nutrient media) contained in Petri plates. After incubation of 7 days at 28^{o}C, the strains growing out from plant segments was shifted to PDA (potato dextrose agar) media for obtaining pure cultures of isolates. The production of secondary metabolites was checked on Czapek media.The fungal isolates were observed under light microscope. The identification of isolates was confirmed by species description used by Klich (2002).

Genomic DNA extraction and fungal identification: DNA was extracted by following method of Khan *et al.,* (2012) and identification of isolated strain was confirmed by sequencing the ITS of 18S rDNA. The universal primer NS1 5' (GTA GTC ATA TGC TTG TCT C) 3'and NS24 5' (AAA CCT TGT TAC GAC TTT TA) 3' were used for polymerase chain reaction. For homology identification,

Basic Local Alignment Search Tool (BLASTn) was used (http//www.ncbi. nlm.nih.gov/BLAST/). The resultant sequences was aligned as ClustalW by using MEGA 7 software and the phylogenetic tree was constructed and the evolutionary history was inferred using the parsimony tree y using same software (Tamura *et al.,* 2007).

Phytochemicals screening: The extracts of endophytes were checked for the existence of the active constituents with little amendments from culture filtrate.

Production of indole acetic acid (IAA): 1 ml of centrifuge liquid culture was added to 2ml ofSalkowski's reagent using the methodology of Gordon & Weber (1951), and kept indark for 20 mins. Appearance of of pink colour shows the presence of IAA.The spectrophotometer at 540 nm was used for quantification of active metabolites. The final concentration of IAA was estimated using a standarad curve of IAA.

Determination of total flavonoid: 0.5 ml of fungal culture was taken in glass tube and mixed with 0.1 ml of $AlCl_3$ (10%) folllowed by addition of 0.1 ml CH_3CO_2K (1M).The final volume was adjusted by addition of 4.3 ml of 80% methanol to the liquid mix and incubted for 30 minutes. The absorbnace was checked at 415 nm by following method of Eom *et al.,* (2007).

Determination of total phenols: Phenol contents was observed using reagent (Folin-Ciocalteau) according to method described by Malik & Singh (1980) with few modification. 0.5 ml culture filtrate was taken and water was added to make final volume of 3 ml. The mixture was added with 0.5 ml reagent followed by addition of 2 ml 0f 20% sodium carbonate. Appearance of blue color indicated phenol presence. Samples were kept in boiled water for exactly one minute, cooled and absorbance was checked at 650 nm.

HCN production: To check the ability of isolates for optimum HCN production, a piece of isolate was inoculate on potato dextrose agar slant media with addition of 4.4g glycine/liter. Whatman filter paper No. 1 was saturated in sodium carbonate 2% in picric acid (0.5%) solution and kept in the test tube. Completely sealed the test tubes with parafilm and incubated for 7 days at 28ºC. Discoloration of filtr paper from yellow after incubation shows the presence of HCN production (Lorck, 2004).

Screening of endophytes for plant growth promotion: To study the effect endophytes, the tomato (*Solanum lycopersicum* L.) genotype Riogrande was treated with fungal biomass. Ten seeds were sown in 100 gm soil. The experiment was arranged in triplicate. For plant microbe interaction one gram biomass of fungal strain was applied to every pod. Autoclaved distilled water was used as control. The morpho-biochemical data (estimation of chlorophyll content, shoot and root length, fresh and dry weight) was recorded after one month seedlings were harvested. For getting dry weight, seedlings were oven dried at 50ºC for 8 days.

Statistical analysis: Software SPSS for Window version 12.0 was used for calculating the values for different tests such as one-way analysis of variance (ANOVA), Duncan's Multiple Range Test (DMRT).

Results and Discussion

Fresh and contamination free parts of tomato plants were used for isolation of potent endophytes. The surface sterilization procedure for isolation showed standard result for control plate. Different fungal strains was emerged in the low nutrient medium, based on distinct colony characteristic a tan brown colony ends with white corner was selected for further study to check its potential for growth promotion. For initial identification of chosen strains morphological study was done on PDA plate after 3 days of inoculation, on surface initially white and then pyriform vesicle, lanose, tan to brown color appear and on reverse it showed golden exudates (Fig. 1-a,b) and after 7 days maximum growth was observed on plants (Fig. 1-c). On microscopic study it showed unbranched conidiophore, appearance of columnar conidial arrangement and biseriate conidial heads (Fig. 1-d, e). Our macroscopic and microscopic observed characteristics of *A. terreus* showed similarities with findings of Klich (2002) and Afzal *et al.,* (2013).

Molecular identification of the isolated fungal strain S4 was carried out by phylogenetic tree analysis using the parsimony tree constructed method from 17 selected taxa and aligned ITS sequences. Molecular proof of identity of *A. terreus* showed 100% match with reference strain KF660536. The 18S rDNA sequence data was checked with sequences data available on Gene-Bank (Fig. 2). Tested strain *Aspergillus terreus* (*A. terreus*) was previously reported as endophytefrom a healthy old leaf of *Swieteniam acrophylla* leaf (Darah *et al*., 2014) and *A. terreus*identified as efficient endophyte from stem of *Achyranthus aspera*, an herb of medicinal importance (Goutam *et al*., 2016).

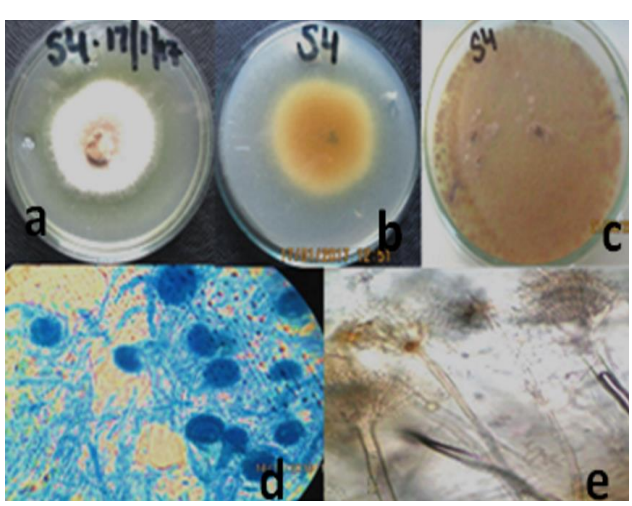

Fig. 1. Isolation and morphological examination of S4. a. The obverse on PDA growth after 3 days, b. reverse on PDA after 3 day releasing metabolites, c. on PDA after 7 days fully matured fungus growth, d and e. branching and conidiophores under light microscope.

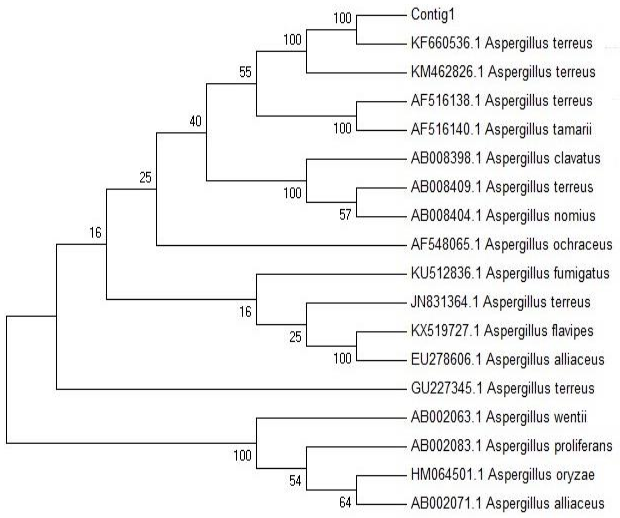

Fig. 2. Identification of endophytic fungal strain S4 as *Aspergillus terreus* by phylogenetic analysis of the ITS region of 18S rDNA gene.

Table 1. Phytochemical screening of isolated fungal extract.

Strain	IAA	Flavonoid	Phenol	HCN
S4	+	+	+	+

Table 2. Estimation of secondary metabolites of isolated fungal filtrate.

Strain	IAA (ug/ml)	Total flavonoid (ug/ml)	Total phenols (ug/ml)
S4	0.67±.10	1.83±.03	4.16±.05

The culture supernatant of the *A. terreus* was checked for different active secondary substances such as, production of indole acetic acid, total flavonoids and phenols calorimetrically. The strain showed effective results which can be helpful to enhance plant growth parameters. Results showed positive for presence of active metabolites such as IAA, flavonoids, phenols and HCN in tested samples (Table 1). The quantitative estimation of IAA, phenolic and flavonoid content were calculated by using the values of standard curves. The concentrations of IAA, flavonoids and phenolic in S4 liquid culture were 0.67±10, 1.83±.03 and 4.16±.05 respectively (Table 2). Our results are supported by Huang *et al.*, (2007) who reported 42 fungal endophytes from *Nerium oleander* and found important bioactive constituents as phenolics and their derivatives, flavonoids. Similarly, endophytic *Xylaria* sp. Produces key secondary metabolites (phenolics and flavonoids) (Liu *et al*., 2007). Accoroding to Shi *et al*., (2009), plants treated with certain endophytes have potential to increased growth attributes of plants due to production of active IAA compounds.

The bioassay experimental results showed a significant difference among fungal treated and control plants. Statistical analysis of the data clearly indicated that endophyte inoculated plants increased shoot length upto 79% than untreated control. Root length showed twenty seven fold increased than untreated seedlings, fresh and dry weight of tomato seedlings recorded a significant higher weight (3 and 3.5 respectively) folds than control plants. Total chlorophyll content was also highest in endophyte inoculated plants than control (Table 3). Overall plant growth parameters were increased by inoculating *A. sterreus* as plant growth promoter. Our results are in line with the conclusions of Sumera *et al*., (2008) who reported 37 different isolated endophytic fungi as plant growth promoters that increased the plant shoot length upto 75.5% for waito-c rice variety. Our results showed that *A. terreus* significantly enhanced plant growth traits *In vivo* experiment due to active constituents present in high amount. Our results are also supported by Dai *et al.*, (2003) and Lu *et al.*, (2000), who observed that filamentous fungi can produce *In vitro* active IAA metabolites and helps to enhance plant growth attributes. Similarly, inoculation of two endophytic fungi *P. citrinum* and *A. terreus* on sunflower plants exhibited resistance to disease caused by pathogen *Sclerotium rolfsii* and increase biomass production and yield of sunflower (Waqas *et al*., 2015).

Table 3. *In vivo* growth promotion experiment.

Sample	SL (cm)	RL (cm)	FW (g)	DW (g)	CHL
Control	5.69 ± 0.76^a	1.26 ± 0.22^a	0.14 ± 0.04^a	0.02 ± 0.00^a	20.2 ± 1.56^a
S4	10.2 ± 2.27^b	3.88 ± 1.32^b	0.47 ± 0.30^a	0.07 ± 0.04^a	32.5 ± 2.20^b

The results shows mean ± standard deviation for n = 3 pots and different letters represent statistical difference at $p<0.05$

Conclusion

The identification of *A. terreus* as plant growth promoting endophytic fungal isolate via its potent ability to produce active secondary metabolites IAA, phenols, flavonoids and fungal treated tomato seedlings showed effective growth attributes significantly than control. This important endophyte could be useful for improving production of crop plants and can be used as good biofertilizer.

References

Afzal, H., S. Shazad, S. Qamar and S.Q.U. Nisa. 2013. Morphological identification of *Aspergillus* species from the soil of Larkana District (Sindh, Pakistan). *Asian J. Agric. Sci.*, 1: 105-117.

Afzal, I., I. Iqrar, Z.K. Shinwari and A. Yasmin. 2017. Plant growth-promoting potential of endophytic bacteria isolated from roots of wild *Dodonaea viscosa* L. *Plant Growth Regul.*, 81(3): 399-408.

Afzal, I., Z.K. Shinwari and I. Iqrar. 2015. Selective Isolation and Characterization of Agriculturally Beneficial

Endophytic Bacteria from Wild Hemp using Canola. *Pak. J. Bot.,* 47(5): 1999-2008.

Afzal, I., Z.K. Shinwari, S. Sikandar and S. Shahzad. 2019. Plant beneficial endophytic bacteria: Mechanisms, diversity, host range and genetic determinants. *Microbiol. Res*., 221: 36-49.

Amin, N. 2016. Endophytic fungi to control of Cocoa Pod Borer (*Conopomorpha cramerella*) on Cocoa plantation. *Res. J. Pharm. Biol. Chem. Sci*., 6: 1496.

Chanway, C.P. 1996. Endophytes: they're not just fungi. *Can. J. Bot.,* 74: 321-322.

Dai, C., B. Yu, Z. Xu and S. Yuan. 2003. Effect of environmental factors on the growth and fatty acid composition of five endophytic fungi from *Sapium sebiferum*. *Chin. J. Appl. Ecol.,* 14: 1525-1528.

Damare, S., C. Raghukumar and S. Raghukumar. 2006. Fungi in deep-sea sediments of the Central Indian Basin. *Deep-Sea Res. Oceanogr*., 11: 14-27.

Darah, I., C.L. Chong and S.H. Lim. 2014. Antimicrobial activity of endophytic fungi isolated from *Swietenia macrophylla* leaves. *Nat. Product Commun*., 9(2): 247-250.

Eom, S.H., W.J. Cheng, J.P. Hyoung, E.H. Kim, M.I. Chung, M.J. Kim, C. Yu andD.H. Cho. 2007. Far infrared ray irradiation stimulates antioxidant activity in *Vitis flexuosa* Thunb. Berries. *Kor. J. Med. Crop Sci*., 15: 319-323.

Ezra, D., W.H. Hess and G.A. Strobel. 2004. New endophytic isolates of *M. albus*, a volatile antibiotic-producing fungus. *Microbiol.,* 150: 4023-4031.

Gordon, S.A. and R.P. Weber. 1951. Colorimetric estimation of indoleacetic acid. *Plant Physiol*., 26: 192.

Goutam, J., S. Singh., R.N. Kharwar and V. Ramaraj. 2016. *In vitro* potential of endophytic fungus *Aspergillus terreus* (JAS-2) associated with *Achyranthus aspera* and study on its culture conditions. *Biol. Med. (Aligarh).,* 8: 349.

He., J., E.M. Wijeratne, B.P. Bashyal, J. Zhan, C.J. Seliga, M.X. Liu, E.E. Pierson, L.S. Pierson, H.D. VanEtten and A.A. Gunatilaka. 2004. Cytotoxic and other metabolites of *Aspergillus* inhabiting the rhizosphere of Sonoran desert plants. *J. Nat. Prod*., 67: 1985-1991.

Huang, W.Y., Y.Z. Cai, K.D. Hyde, H, Corke and M. Sun. 2007. Endophytic fungi from *Nerium oleander* L. (Apocynaceae): main constituents and antioxidant activity. *World J. boilobiol. Biotechnol.,* 23: 1253-1263.

Jose, G.M.V., B.J. Hans and V.L.L. Luis. 2009. Assessing fungal root colonization for plant improvement. *Plant Signal Behav*., 4: 445-447.

Khan, A.L., A. Al-Harrasi, A. Al-Rawahi, Z. Al-Farsi, A. AlMamar and M. Waqas. 2016. Endophytic fungi from frankincense tree improves host growth and produces extracellular enzymes and indole acetic acid. *PLoS ONE.,* 11: e0158207.

Khan, S.A., M. Hamayun, A.L. Khan, I. Lee, Z.K. Shinwari and J. Kim. 2012. Isolation of plant growth promoting endophytic fungi from dicots coastal sand dunes of Korea. *Pak. J. Bot.,* 44(4): 1453-1460.

Klich, M.A. 2002. Identification of common *Aspergillus* species. *Centraalbureau voor* Schimmel cultures, *Utrecht, The Netherlands.* pp. 116. ISBN 90-70-351-46-3.

Kuenz, A., Y. Gallenmüller, T. Willke and K.D. Vorlop. 2012. Microbial production of itaconic acid: developing a stable platform for high product concentrations. *Appl. Microbiol. Biotechnol*., 96: 1209-1216.

Liu, X.L., M.S. Dong, X.H. Chen, M. Jiang, X. Lv and G.J. Yan. 2007. Antioxidant activity and phenolics of an endophytic *Xylaria* sp. from *Ginkgo biloba*. *Food Chem.,* 105: 548-554.

Lorck, H. 2004. Production of hydrocyanic acid by bacteria. *Plant Physiol*., 1: 142-146.

Lu, H., W.X. Zou, J.C. Meng, J. Hu and R.X. Tan. 2000. New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia Annua. Plant Sci*., 151: 67-73.

Malick, C.P. and M.B. Singh. 1980. In: *Plant Enzymology and Histo Enzymology kalyani Publishers New Dehli*p. 286.

Muhammad, W., L.K. Abdul, K. Muhammad, H. Muhammad, K. Sang-Mo and K. Yoon-Ha. 2012. Endophytic fungi produce gibberellins and indoleacetic acid and promotes host-plant growth during stress. *Molecules.,* 17: 10754-10107.

Numan, M., S. Bashir, Y. Khan, R. Mumtaz, Z.K. Shinwari, A.L. Khan and A.H. Ahmed. 2018. Plant growth promoting bacteria as an alternative strategy for salt tolerance in plants: a review. *Microbiol Res.,* 209: 21-32.

Pillay, V.K. and J. Nowak. 1997. Inoculum density, temperature and genotype effects on *In vitro* growth promotion and epiphytic and endophytic colonization of tomato (*Lycopersicum esculentum* L.) seedlings inoculated with a pseudomonad bacterium. *Can. J. Microbiol*., 43: 354-361.

Rahman, A., F. Korejo, V. Sultana, J. Ara and S. Ehteshamul-Haque. 2017. Induction of systemic resistance in cotton by the plant growth promoting rhizobacterium and seaweed against charcoal rot disease. *Pak. J. Bot.,* 49: 347-353.

Reddy, C.S and R.P. Singh. 2002. Enhanced production of itaconic acid from corn starch and market refuse fruits by genetically manipulated *Aspergillus terreus* SKR10 *Bioresour. Technol*., 85: 69-71.

Redman, R.S., Y.O. Kim, C.J.D.A. Woodward, C. Greer and L. Espino. 2011. Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. *PLoS ONE.,* 6: e14823.

Shi, Y., K. Lou and C. Li. 2009. Isolation, quantity distribution and characterization of endophytic microorganisms within sugar beet. *Afr. J. Biotechnol.,* 8: 835-840.

Sturz, A.V., B.R. Christie and J. Nowak. 2000. Bacterial endophytes: potential role in developing sustainable systems of crop production. *Crit. Rev. Plant Sci*., 19: 1-30.

Sumera, A.K., M. Hamayun, S.O. Rim, I.J. Lee, J.C. Seu, Y.S. Choo, I.N. Jin, S.D. Kim, I.K. Lee and J. Kim. 2008. Isolation of endophytic fungi capable of plant growth promotion from monocots inhabited in the coastal sand dunes of Korea. *J. Life Sci.,* 18(10): 1355-1359.

Tamura, K., J. Dudley, M. Nei and S. Kumar. 2007. Molecular evolutionarygenetics analysis (MEGA) software version 4.0. *Mol. Bio. Evo*., 1596-1599.

Urooj, F., H. Farhat, S. Ali, M. Ahmed, V. Sultana, Z.I. Shams and S. Ehteshamul-Haque. 2018. Role of endophyticPenicillium species in suppressing the root rotting fungi of sunflower. *Pak. J. Bot.,* 50(4): 1621-1628.

Waqas, M., A.L. Khan, M. Hamayun, R. Shahzad, S.M. Kang and J. Kim. 2015. Endophytic fungi promote plant growth and mitigate the adverse effects of stem rot: an example of *Penicillium citrinum* and *Aspergillus terreus*. *J. Plant Interact.,* 10: 280-287.

Yates, I.E., C.W. Bacon and D.M. Hinton. 1997. Effects of endophytic infection by *Fusarium moniliforme* on corn growth and cellular morphology. *Plant Dis.,* 81: 723-728.

(Received for publication 15 November 2018)