PHOSPHATE SOLUBILIZING AND BIOCONTROL POTENTIAL OF AN *Aspergillus niger* ISOLATE FROM EGYPTIAN SOIL

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Phosphorus (P) is one of the most essential plant nutrients that significantly influence overall plant growth (Wang *et al.,* 2009). It has an influence on different key metabolic processes, like development and division of plant cells, energy transportation, signal transduction, biosynthesis of macromolecular, photosynthesis, and plant respiration (Shenoy and Kalagudi, 2005; Ahemad *et al.,* 2009; Khan *et al.,* 2009). The environment does not supply plants with soluble P. Primary and secondary minerals and/or organic compounds are therefore mainly the source of P. The concentration of P in the soil solution is much lower compared to other nutrients and ranges from 0,001 to 1 mg / l (Brady and Weil, 2002). P-mineral compounds generally contain aluminum (Al), iron (Fe), manganese (Mn), and calcium (Ca) and differ from soil to soil. For example, P forms a complex in acid soils with Al, Fe, and Mn, while it reacts very strongly with Ca in alkaline soils. In all conditions, however, the types of P soil compounds are determined primarily through soil pH and soil mineral type and concentrations (Khan *et al.,* 2014).

The P of phosphatic fertilizers or manure reacts extremely strongly with soil components and then becomes unavailable to plants. The insoluble kinds of P like tricalcium phosphate (Ca_3PO_4), aluminum phosphate (Al_3PO_4), iron phosphate (Fe_3PO_4), etc. these forms can be converted into soluble Phosphate by Phosphate solubilizing organisms in different soil types (Gupta *et al.,* 2007; Song *et al.,* 2008; Khan *et al.,* 2013; Sharma *et al.,* 2013). Generally, soil microorganisms are more efficient in supplying P by solubilization to plants from both inorganic and organic sources. (Toro, 2007; Wani *et al.,* 2007) and mineralization of complex Phosphate compounds (Ponmurugan and Gopi, 2006).

Plants encounter multiple biotic threats and adverse environmental factors. Pests, parasites, and pathogens have been identified since ancient times as the cause of biotic stress in plants. Two types of

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fungal parasites occur: Biotrophic fungi are certain fungi that require nutritional supplies from living plants and nectrotrophs which kill the plant using toxins (Glazebrook, 2005). In combination with bacteria, they can cause, among other symptoms, vascular wilts, leaf spots, and cankers and infect various parts of a plant (Gimenez *et al.,* 2018). Treating plant fungal infection with chemical pesticides harms human health and the environment. Therefore, an environment-friendly and cost-effective alternative are always required. Using fungi as biological control agents (BCAs) is an attractive treatment to substitute dangerous pesticides and deliver a wide field application potential (Das and Abdulhameed, 2020).

Built on their wide scope of disease prevention and increase in yield, fungal biological control agents are gaining much acceptance (Kaur and Reddy, 2017). Non-pathogenic and saprophytic fungi that inhabit soil can support several crops as they not only encourage development but also guard them against diseases (Pandya and Saraf, 2010) examples of such fungi include the genera *Aspergillus*, *Trichoderma*, *Penicillium Verticillium,* and others. Isolates of *Aspergillus*, *Paecilomyces,* and *Penicillium* are known to have an antagonistic effect on soil-borne pathogens in the rhizosphere of crops (Noveriza and Quimio, 2004).

Our research aims to help crop producers to increase their production by the introduction of novel fungal treatment with dual function to solubilize phosphate in the soil and to protect plants from soil-borne pathogens. The research will focus on screening soil for phosphate solubilizing fungi and the evaluation of their abilities. The obtained fungi could be used in the production of ecofriendly biofertilizers. Using such fertilizers could increase the profits of crop growers, reduce the use of harmful chemical fertilizers, reduce greenhouse gases emission, improving soil quality, and reduce production costs.

MATERIALS AND METHODS

Collection of rhizosphere soil sample

The soil sample was collected from the Agricultural Genetic Engineering Research Institute field near the sorghum rhizosphere. The sample comprised of soil adhering to the roots of sorghum. The sample was carefully shaken in plastic bags to separate the soil from the sorghum roots, and immediately transferred to a cooler until arrival at the laboratory. The sample was stored at 4ºC for further analysis.

Isolation of phosphate-solubilizing fungi (PSF)

Nearly 10 g of soil sample was moved to a sterilized Erlenmeyer flask having 90 mL of sterile distilled water. The sample was shaken at 120 rpm for 60 min. Then, a series of 10-fold dilutions of the suspension were prepared and 300 µL was plated on Pikovskaya's agar from each dilution. Pikovskaya's agar was made from 10 g glucose, 0.2 g NaCl, 0.2 g KCl, 0.5 g $(NH_4)_2SO_4$, 0.1 g

$MgSO_4 \cdot 7H_2O$, 0.0001g $FeSO_4 \cdot 7H_2O$, 0.0001g $MnSO_4 \cdot 7H_2O$, 0.5 g yeast extract, and 18 g agar in 1000 mL of distilled water, and 0.5% tricalcium phosphate as recalcitrant P source (Pikovskaya, 1948). The phosphate solubilizing fungi (PSF) was identified after eight days of incubation at 25ºC by the existence of a clear halo zone around fungal colonies. The experiment was repeated three times. Identified PSF was purified by repeated culturing on potato dextrose agar (PDA) at 25ºC. Microscopic examination was performed with an Olympus-FSX100 Inverted Microscope.

DNA extraction from fungi

DNA extraction of fungi was performed following the method of Aamir *et al.,* (2015). The genomic DNA was extracted from fungal cultures grown in liquid PDA for five days at 25ºC. Fungal mass was obtained by filtering the culture broth by 10 ml syringes encompassing glass wool to retain the fungal mass and let the broth pass through. The fungal mass was put in a 2ml tube containing sterile glass beads and lysis buffer (50mM EDTA, 100 mM Tris HCl [pH8.0], 3% SDS). Homogenization of fungal mass was accomplished using the Tissue Lyser II homogenizer (Qiagen, Germany). The fungal homogenate was centrifuged at 13,000 rpm for 10 min and the supernatant was transferred to a new microcentrifuge tube. 2 of RNase A (10mg/ml) was added to the supernatant and incubated at 37ºC for 15 min. After that, an equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1) was added followed by centrifugation at 13,000 rpm for 10 min. The upper layer was transferred to a fresh microcentrifuge tube. Next, an equal volume of 100% ethanol was added. After precipitation at -20ºC for 30 min, the tube was centrifuged at 13,000 rpm for 10 min to pellet down the DNA. The DNA pellet was washed with 70% ethanol followed by centrifugation at 12,000 rpm for 5 min. The DNA pellets were air-dried and dissolved in sterilized ddH_2O.

Amplification of fungi Internal Transcribed Spacer region (ITS)

The ITS region of the *nuc* rRNA gene was amplified with primers ITS1: 5′TCCGTAGGTGAACCTTGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ (Wang *et al.,* 2018). Primers obtained in the lyophilized state were centrifuged at 1000 rpm for 5 min, deionized nuclease-free ddH_2O was added by recommended volume described in the primers sheet to form a concentration of 100 µM, vortexed thoroughly until complete resuspension then primers dilution applied 1:10 to from a regular PCR working concentration of 10 µM. Polymerase chain reactions (PCR) were conducted using 20-µL reaction mixture containing 0.5 µL of each primer (10 pmol/µL), 1.0 µL of genomic DNA (10 ng/µL), 8 µL of 2 × PCR Master Mix buffer (0.05 µg/µL Taq polymerase, 4 mM $MgCl_2$, and 0.4 mM dNTPs), and 10 µL of ultrapure sterile water. PCR cycling conditions were as follows: one initial cycle of denaturation at 94ºC for 3 min, followed by 34 cycles of 94ºC for 30 s, 50ºC

for 30 s, and extension at 72ºC for 45 s, and a final extension for 5 min at 72ºC.

Purification of the PCR product

ITS PCR product was purified using the AxyPrep™ DNA Gel extraction kit (Cat. # AP-GX-50) according to manufactures instructions. PCR product was transferred into a 1.5 ml microfuge tube, then a three X sample volume of buffer DE-A was added. Subsequently, Half X buffer DE-A volume of buffer DE-B was added and mixed well. An AxyPrep column was placed into a 2 ml microfuge tube. Then, the mix was transferred into the column, centrifuged at 12,000 xg for 1 min. The filtrate was discarded from the 2 ml microfuge tube and the AxyPrep column was returned to the 2 ml microfuge tube and 500 µl of bufferW1 was added. The mix was centrifuged at 12,000 xg for 30 sec. The filtrate was discarded from the 2 ml microfuge tube. The AxyPrep column was returned to the 2 ml microfuge tube and 700 µl of buffer W2 was added, centrifuged at 12,000 ×g for 30 sec, then the filtrate was discarded from 2 ml microfuge tube. The AxyPrep column was placed back into the 2 ml microfuge tube, centrifuged at 12,000 xg for 1 min to remove residuals of previous solutions. The AxyPrep column was transferred into a clean 1.5 ml microfuge tube. 30 µl of prewarmed deionized water (65ºC) was added to the center of the membrane and incubated for 5 min at room temperature for the elution of DNA followed by centrifugation at 12,000 xg for 1 min. DNA was quantified using the absorption of light at 260 and 280 nm (A260/280) by Thermo Scientific™ NanoDrop 2000. The sample was sequenced in the forward direction by Macrogen Inc. (Korea).

Bioinformatic analysis of DNA sequence

The raw sequence was proofread and edited manually with BioEdit 7.0.9 (Hall, 1999). A homology search was performed using The Basic Local Alignment Search Tool (BLAST) against the National Center for Biotechnology Information (NCBI) database, USA (http://www.ncbi.nlm.nih.gov) on the DNA level. The edited sequence was aligned using Clustal W (Thompson *et al.,* 1994) and adjusted manually as required. Finally, a neighbor-joining (NJ) phylogenetic tree was constructed with the Kimura 2-parameter model to calculate sequence divergence and subjected to 1000 bootstrap replications using MEGA 6.0 (Tamura *et al.,* 2013), with gaps treated as complete deletions.

Antagonistic effect of phosphate solubilizing fungi

Phosphate solubilizing fungi isolate was checked for antagonistic effect against two pathogens belonging to the genus Fusarium (*Fusarium solani* and *Fusarium verticillioides*). The pathogens used in the present research were obtained from the Molecular Microbiology Lab, Agricultural Genetic Engineering Research Institute (AGERI)/ARC, Egypt. Two-disc plugs (0.5-cm diameter) of pathogen and antagonist (4 days old cul-

ture) were transferred respectively to a single potato dextrose agar (PDA) plate (9-cm diameter). The antagonist plug was placed on the one side of the plate (about 2 cm from the edge of the plate towards the center), while the pathogen plug was placed at the other side of the plate opposite to the antagonist plug leaving 5 cm between the two plugs. A plug of the PDA medium was used as a control treatment while the pathogen plug was placed on the other side. Three replications (two plates/replicate) for each treatment were made, and the plates were incubated at 28 ± 2ºC for 5 days.

RESULTS AND DISCUSSION

Isolation of phosphate solubilizing fungi (PSF)

The formation of halo zones around colonies on Pikovskaya's medium is an indication that the microorganism has the phosphate-solubilizing capability. In the present study, six fungal isolates were isolated from a sorghum field, only one showed phosphate solubilizing capacity by displaying a clear zone of dissolved phosphate in solid Pikovskaya's medium, which indicated that this isolate is phosphate solubilizing fungi (PSF) exhibited the desired P-solubilizing ability (Fig. 1). The fungal isolate was named AG-A01. Microscopic examination of fungi showed that it tends to form filaments (hyphae) and conidial heads of the organism to be globose and dark brown and the spores are carbon black/dark brown color.

Phosphate solubilizing fungi was reported recently as an alternative strategy for using rock phosphate (RP) in soil fertilization (Wang *et al.,* 2018). Rock phosphate has a direct application for applying phosphate to the soil, but its effectiveness is highly dependent on the soil type which soil PH is the most important factor (Sulbarán *et al.,* 2009). An alternative was the use of microorganisms capable of solubilizing rock phosphate and of releasing soluble phosphorous through organic acid production, sugar chelating, pH reduction, and enzyme production. Several reports have shown that certain microorganisms can solubilize insoluble phosphate rock and release soluble phosphate. Fungi Aspergillus was an effective phosphate solubilizer among the microbes (Wang *et al.,* 2018; Adhikari and Pandey, 2019).

Identification and phylogenetic analysis of the AG-A01 isolate

DNA was extracted from Fungi. Then, the amplification of the selected Internal Transcribed Spacer (ITS) region resulted in a band with approximately 600 bp (Fig. 2) which is the expected size according to the designed primers. THE purified PCR product was sent to sequencing and the resulted sequence was compared to sequences in the gene bank using The Basic Local Alignment Search Tool (BLAST) on the NCBI website. The isolate AG-A01 was identified as *Aspergillus niger* with similarity 100% with *Aspergillus niger* isolate WA-TKA small subunit ribosomal RNA gene. Phylogenetic tree analyses with bootstrapping of the partial sequence (398 bp) of the fungi ITS region

suggested that this isolate was more closely related to *Aspergillus niger* than any *Aspergillus* group (Fig. 3). The AG-A01 isolate groups with *Aspergillus niger* with strong statistical support and shares 100% nucleotide sequence identity.

Antagonism effect of AG-A01 strain

The antagonism test showed clearly that there was a significant reduction in mycelial growth of tested pathogens after the confrontation with *Aspergillus niger*-isolate AG-A01 (Fig. 4). The pathogens *Fusarium solani* and *Fusarium verticillioides* exhibited a similar reduction of mycelium which reached nearly 99%. This reduction could be explained for the ability of *Aspergillus niger* to grow faster than the two fungi and preventing even their spores to grow and multiple. The results of this test agreed with the findings of many reports illustrated the role of *Aspergillus* species in the biocontrol of plant pathogens. For instance, Anwer *et al.,* 2017 who obtained an isolate from *Aspergillus niger* that can soluble phosphate and act as a biocontrol agent. Ruangsanka, (2014) stated that *Aspergillus niger* isolate had the highest pathogen inhibition percentage (64%) against the devastating soil-borne pathogen *F. oxysporum*. *Aspergillus niger* was also reported as a potential biocontrol agent for controlling fusarium wilt of tomato (Sharma *et al.,* 2011). In conclusion, in the current study, we isolated an efficient phosphate solubilizing fungus which is also a strong biocontrol agent. Due to its several positive effects, the *Aspergillus niger* isolate has the potential to be developed as a commercial product which can increase crop nutrient uptake and, hence increasing their yield.

SUMMARY

Phosphorus (P) is one of the most important plant nutrients that greatly affect overall plant growth. P-mineral always presents in soil with the insoluble condition. Phosphate compounds generally contain aluminum (Al), iron (Fe), manganese (Mn), and calcium (Ca) which vary according to soil type. Phosphate solubilizing fungi play a major role in rising soil phosphate bioavailability for plants by realizing phosphate from its compounds. The present study was aimed to isolate and characterize phosphate solubilizing fungi from Egyptian soil using a solid Pikovskaya (PVK) medium. In total, 6 fungal isolates were able to grow on Pikovskaya (PVK) medium but only one isolate (AG-A01) showed phosphate-solubilizing capacity. DNA was extracted from the isolate followed by amplification of selected Internal Transcribed Spacer (ITS) region and DNA sequencing. After analyzing the DNA sequence, the isolate AG-A01 was identified as *Aspergillus niger*. The fungal isolate also displayed an antagonism effect against two plant pathogens (*Fusarium solani* and *Fusarium verticillioides*). Thus, we consider this fungal isolate as a promising tool for the development of an efficient bio-fertilizer for the plant which has the potential to protect plants from pathogens especially fusarium species.

REFERENCES

Aamir S., Sutar S., Singh S. K., and Baghela A., (2015). A rapid and efficient method of fungal genomic DNA extraction, suitable for PCR based molecular methods. Plant Pathol. Quar, 5: 74-81.

Adhikari P., and Pandey A., (2019). Phosphate solubilization potential of endophytic fungi isolated from Taxus *wallichiana Zucc*. roots. Rhizosphere, *9*: 2-9.

Ahemad M., Zaidi A., Khan M. S., and Oves M., (2009). Biological importance of phosphorus and phosphate solubilizing microorganisms—an overview. Phosphate solubilizing microbes for crop improvement. Nova, New York, 1-4.

Anwer M. A., Singh K., and Singh R. N., (2017). *Aspergillus niger*: A phosphate solubilizing fungus as biocontrol agent. In Biopesticides and Bioagents (p. 43-75). Apple Academic Press.

Brady N. C., and Weil R. R. (2002). The nature and properties of soils, 13th edn. Prentice Hall of India, New Delhi, 960.

Das M., and Abdulhameed S., (2020). Agro-processing Residues for the Production of Fungal Bio-control Agents. In Valorisation of Agro-industrial Residues,Vol. II: Non-Biological Approaches, (p. 107-126). Springer, Cham.

Gimenez E., Salinas M., and Manzano-Agugliaro F., (2018). Worldwide research on plant defense against biotic stresses as improvement for sustainable agriculture. Sustainability, 10*:* 391.

Glazebrook J., (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol., 43: 205-227.

Gupta N., Sabat J., Parida R., and Kerkatta D., (2007). Solubilization of tricalcium phosphate and rock phosphate by microbes isolated from chromite, iron and manganese mines. Acta Botanica Croatica, 66: 197-204.

Hall T. A., (1999. January). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In Nucleic acids symposium series (Vol. 41: 95-98). [London]: Information Retrieval Ltd., c1979-c2000.

Kaur G., and Reddy M. S., (2017). Role of phosphate-solubilizing fungi in sustainable agriculture. In Developments in Fungal Biology and Applied Mycology (p. 391-412). Springer, Singapore.

Khan M. S., Zaidi A., Wani P. A., Ahemad M., and Oves M., (2009). Functional diversity among plant growth-promoting *rhizobacteria*: current status. In Microbial strategies for crop improvement (p. 105-132). Springer, Berlin, Heidelberg.

Khan M. S., Ahmad E., Zaidi A., and Oves M., (2013). Functional aspect of phosphate-solubilizing bacteria: importance in crop production. In Bacteria in agrobiology: Crop productivity (p. 237-263). Springer, Berlin, Heidelberg.

Khan M. S., Zaidi A., and Ahmad E., (2014). Mechanism of phosphate solubilization and physiological functions of phosphate-solubilizing microorganisms. In Phosphate solubilizing microorganisms (p. 31-62). Springer, Cham.

Noveriza R., and Quimio T. H., (2004). Soil Mycoflora of Black Pepper Rhizosphere in the Philippines and Their *in Vitro* Antagonism against *Phytophthora Capsici* L. Indonesian Journal of Agricultural Science, *5:* 1-10.

Pandya U., and Saraf M., (2010). Application of fungi as a biocontrol agent and their biofertilizer potential in agriculture. J. Adv. Dev. Res., 1*:* 90-99.

Pikovskaya R. I., (1948). Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Mikrobiologiya, 17: 362-370.

Ponmurugan P., and Gopi C., (2006). In vitro production of growth regulators and phosphatase activity by phosphate solubilizing bacteria. African Journal of biotechnology, 5: 348-350.

Ruangsanka S., (2014). Identification of phosphate-solubilizing fungi from the asparagus rhizosphere as antagonists of the root and crown rot pathogen *Fusarium oxysporum*. Science Asia, 40: 16-20.

Sharma B. K., Loganathan M., Singh R. P., Bag T. K., Rai R. K., Rai A. B., and Rai M., (2011). *Aspergillus niger*, a potential biocontrol agent for controlling fusarial wilt of tomato. Journal of Mycopathological Research, 49: 115-118.

Sharma S. B., Sayyed R. Z., Trivedi M. H., and Gobi T. A., (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. Springer Plus, 2: 587.

Shenoy V. V., and Kalagudi G. M., (2005). Enhancing plant phosphorus use efficiency for sustainable cropping. Biotechnology Advances, 23: 501 -513.

Song O. R., Lee S. J., Lee, Y. S., Lee S. C., Kim K. K., and Choi Y. L., (2008). Solubilization of insoluble

inorganic phosphate by *Burkholderia cepacia* DA23 isolated from cultivated soil. Brazilian Journal of Microbiology, 39: 151-156.

Sulbarán M., Pérez E., Ball M. M., Bahsas A., and Yarzábal L. A., (2009). Characterization of the mineral phosphate-solubilizing activity of *Pantoea aglomerans* MMB051 isolated from an iron-rich soil in South Eastern Venezuela (Bolívar State). Current Microbiology, *58*: 378-383.

Tamura K., Stecher G., Peterson D., Filipski A., and Kumar S., (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and Evolution, 30: 2725-2729.

Thompson J. D., Higgins D. G., and Gibson T. J., (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22: 4673-4680.

Toro M., (2007). Phosphate solubilizing microorganisms in the rhizosphere of native plants from tropical savannas: An adaptive strategy to acid soils? In 1st International Meeting on Microbial Phosphate Solubilization (p. 249-252). Springer, Dordrecht.

Wang X., Wang Y., Tian J., Lim B. L., Yan X., and Liao H., (2009). Overexpressing AtPAP15 enhances phosphorus efficiency in soybean. Plant Physiology, 151: 233-240.

Wang X., Wang C., Sui J., Liu Z., Li Q., Ji, Song X., Hu Y., Wang C., Sa R., Zhang J., Du J., and Liu X., (2018). Isolation and characterization of phosphofungi, and screening of their plant growth-promoting activities. Amb Express, 8: 1-12.

Wani P. A., Khan M. S., and Zaidi A., (2007). Synergistic effects of the inoculation with nitrogen-fixing and phosphate-solubilizing rhizobacteria on the performance of field-grown chickpea. Journal of Plant Nutrition and Soil Science, 170: 283-287.

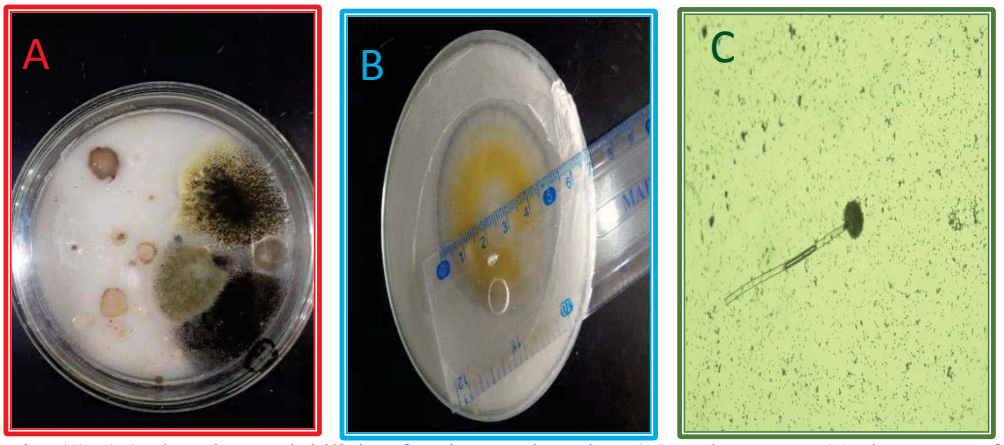

Fig. (1): (A) phosphate solubilizing fungi screening plate (B) Isolate AG-A01 clear zone of dissolved phosphate in solid Pikovskaya's medium (Picture from the back of the plate), (C) Microscopic Examination of fungal isolate.

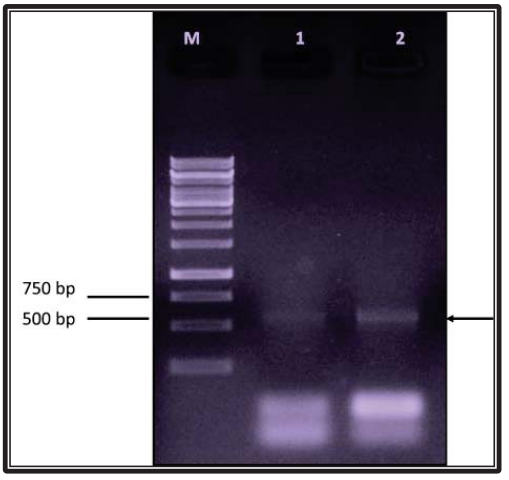

Fig. (2): PCR product of Fungi ITS region Lane 1 and 2 are the same.

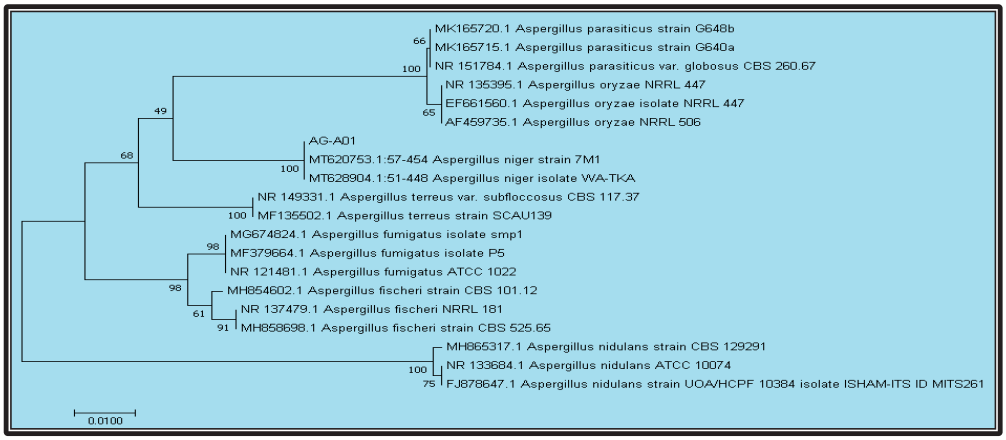

Fig. (3): Phylogenetic tree for ITS sequence of isolate AG-A01. The NJ phylogram was inferred from partial ITS sequence data. Bootstrap percentages of >70% derived from 1000 replicates are indicated at the nodes. Bar = 0.0050 substitutions per nucleotide position.

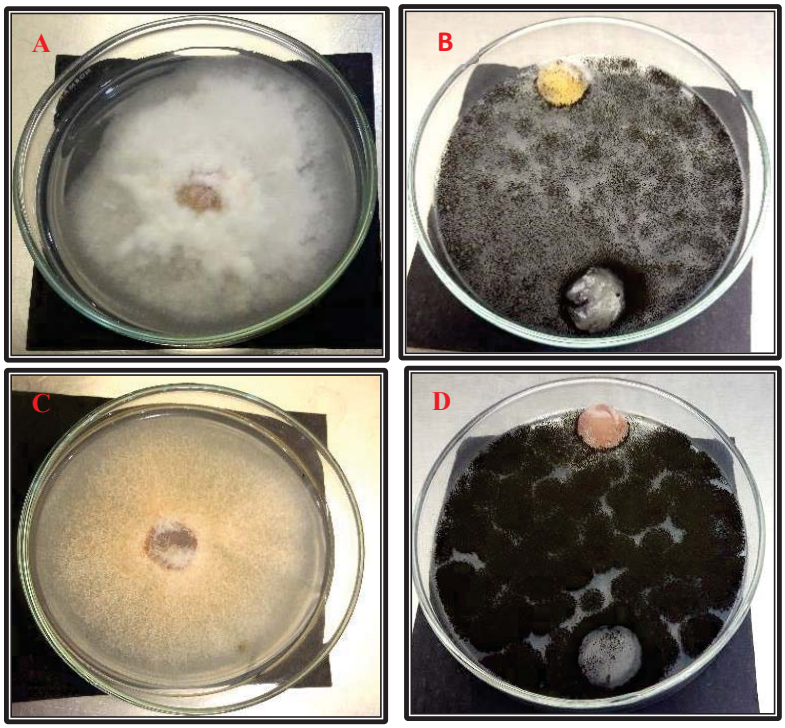

Fig. (4): Dual culture assay. (A) *Fusarium solani* growing on PDA (B) *Fusarium solani* and *Aspergillus niger* growing on PDA (C) *Fusarium verticillioides* growing on PDA (D) *Fusarium solani* and *Aspergillus niger* growing on PDA