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Mycolytic activity of *Trichodermia viride* against *Macrophomina phaseolina* associated with soybean seeds

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Abstract: The soybean seeds were subjected to health evaluation using standard blotter and agar plate techniques, for detection of seed-borne mycoflora. Thirty-one fungal species comprising nineteen genera were detected. Several pathogenic and storage fungi were observed by both methods. Among the fungal pathogens recovered, *Macrophomina phaseolina* was found predominant (up to 27.3%) and it was used as test pathogen for further studies. Antagonistic properties of seven seed-borne *Trichoderma* isolates were evaluated against *M. phaseolina* through dual culture technique. *Trichoderma* T44sn isolate markedly retarded the pathogen growth, reaching 81.23% after 5 days of incubation. Eight days later the pathogen was completely overgrown by T44sn mycelia and sclerotia formation was lacking compared to the control plates. The antagonistic effect of the cell-free extracts of *Trichoderma* isolates against *M. phaseolina* by agar well diffusion assay proved that T44sn isolate was the potent antagonistic isolate against *M. phaseolina*, which generated 2.47 cm inhibition zone of the pathogen growth, followed by T42sn 2.20 cm. *Trichoderma* sp. T44sn strain was Molecularly identified using 18S rRNA as *T. viride*. Its chitinase activity was found to be 0.20 U, this low activity on non-specific medium for the enzyme production reflecting the mycolytic potentiality of the present *T. viride*. Hence, we recommend further investigations on the use of *T. viride* as a biological agent for use as biocontrol agents against plant pathogens..

Key words: Seed-fungi, soybean, *Macrophomina phaseolina*, *Trichoderma viride*, biological control

Introduction

Soybean (*Glycine max* (L.) Merrill) is one of the most important of seed crops known for its excellent protein (~40%), oil (~20%) and starch content (~21%). It is also a good source of vitamin B complex, particularly thiamine and riboflavin. Soybean protein is rich in valuable amino acid like lysine (5%), in which most of the cereals are deficient. Soybean can substitute for meat and to some extent to milk [1]. Due to its high protein content and gross output of vegetable oil among the cultivated crops [2], it likely resembles a solution for overcoming the world's protein hunger. Additionally, soybean also improves soil fertility by adding nitrogen from the atmosphere, that makes it an attractive crop [3]. According to the General Authority of Statistics, the cultivated area under soybean in 2017 in Egypt was 15000 ha annually with a production of about 45000 metric tons with an average yield of 3.0 metric tons per hectare [4**]**. Cultivation mainly in Menia, Beni Suef and Assuit areas, being 10188, 1604 and 767.0 ha, respectively. Unfortunately, this valuable and economic crop is subjected to attack by a variety of seed and soil-borne fungal pathogens, which can affect seed quality and cause diseases that significantly impact yield or marketability of seed lots. Seed-borne pathogens may reduce germination by seed rot and increased post-emergent damping-off, that

cause severe economic losses and represent a major threat to food production [5].

In this respect [6] surveyed the seed borne fungi associated with soybean by employing standard blotter method and the result of this study indicated that *Macrophomina phaseolina* was found predominant in samples (8.5 - 28.5%), while the occurrence of *Cladosporium* sp. (0.3 - 0.5) was lower. Other seed fungi including *Aspergillus flavus*, *Aspergillus niger* and *Penicillium* spp., *Fusarium* sp., *Rhizoctonia stolonifera*, *Colletotrichum truncatum*, *Cercespora kikuuchii*, and *Pseudomonas glycine* were detected using blotter method [7].

Macrophomina phaseolina (Tassi) Goid was reported as the most destructive soil and seed-borne fungal pathogen affecting soybean in Egypt and worldwide. It causes charcoal rot disease and affects nearly 500 species in more than 100 plant families throughout the world [8,9]. Being seed-borne [10] in nature, it is located both on the seed coat and cotyledons [11] and causes charcoal rot by infecting the roots due to the adherence of microsclerotia to the seed coat during germination and emergence [12]. In this respect, positive correlations have been reported between the inoculum level of *M. phaseolina* in the seedbed and disease severity [13]. The use of chemical control against this pathogen has sometimes given good results. However, improper use of fungicides leads mostly to the phenomena of resistance that in addition to the adverse effects on the development of the antagonistic microflora. To overcome the difficulties previously stated, it is clear that an alternative strategy against the disease of the soybean is needed. Biological control has in recent years many advances in the fight against many pathogens of crops. *Trichoderma* is a hyperparasite genus that uses various mechanisms of biological control, which include: parasitism, antibiosis, competition, induction of resistance and growth promotion **[**14, 15]. *Trichoderma* spp. produce a wide spectrum of secondary metabolites that are known to serve as triggers of systemic resistance against a broad range of plant pathogens [6]. Besides, the fungus can colonize plant roots leading to induce growth and nutrient adsorption for the plant [16]. There are numerous reports of various *Trichoderma* spp. induced resistance against different plant pathogens [15].

In the present study, *Macrophomina phaseolina* was the most aggressive pathogen so we coincide it appropriate to make our contribution through the *in-vitro* study on seven antagonist species of *Trichoderma* against *Macrophomina phaseolina* that causing charcoal rot disease in soybean.

MATERIALS AND METHODS

1. Collection of samples

Thirty soybean seed samples collected from growing fields in different regions of Dakahlia and Damietta governorates during the growing season of 2016 - 2017 were used in this study. The samples were collected in a 50×50 m area from each sampling site in a random zigzag pattern. Fully mature soybean fruits were collected in paper bags, labeled in the field, kept cooled until reached the laboratory. The extracted seeds were then spread out to dry on a porcelain plate at room temperature (25 ±2 ℃) for a few days. The seeds were then placed in a labeled envelope until testing.

2. Detection of seed borne-fungi of soybean

Detection of seed-borne fungi was done using seed health testing techniques recommended by the International Seed Testing Association [17] namely, Standard moist blotter and agar plate methods. Each of the collected seed samples of soybean was surface sterilized by putting in 1% aqueous sodium hypochlorite solution (NaOCl) for 5 minutes followed by 3 successive rinses in sterilized tap water. The excess water was removed and the sterilized seeds were placed between sterilized tissue paper until dryness.

2.1. Standard blotter method (SBM):

Surface sterilized seeds were plated on 9 cm diameter sterile Petri dishes containing sterile blotter moistened with sterilized tap water at 10 seeds per Petri dish. The dishes were incubated at 20 ± 2 ℃ for 7 days under cool white fluorescent light with alternating cycles of 12h light and 12h darkness. After incubation, the seeds were examined for recording the seed-borne fungal infections grown on the incubated seeds.

2.2. Agar plate method

Seeds were placed in Petri dishes containing potato dextrose agar (PDA) media at 10 seeds per Petri dishes, and incubated and examined as in the SBM.

3. Isolation and identification of seed-borne fungi

Samples of soybean seeds prepared according to ISTA by the preceding two methods were examined under a stereoscopic binocular microscope (6-50 x) for the presence of seed-borne fungi and study their habit characters. The light microscope was used for confirmation. By the aiding of stereoscopic binoculars, hyphal tips from the colonized fungi on seeds were picked up by the tips of sterilized stretched capillary tubes and then transferred to plates containing PDA. A pure culture of each of the detected fungus was obtained and all isolates were maintained on potato carrot agar medium (PCA) for further studies. The pure isolates were identified by means of comparison with the description sheet of Commonwealth Mycological Institute, kew, surrey, England (CMI), Danish Government Institute of Seed Pathology [18-23]**.**

4.1. Dual culture assay

The antagonistic potential of seven *Trichoderma* spp. isolated from soybean seeds was evaluated against *M. phaseolina* using dual culture technique [24]. Five-millimeter diameter mycelial disc of each test antagonist fungus taken from 5-day old culture was paired against the same sized mycelial disc of *M. phaseoliana* at the opposite end on 9 cm diameter PDA Petri- plates. The pathogen and antagonist discs were placed at equal distances from the periphery of the Petri plate. The PDA plates inoculated only with either antagonists or phytopathogen served as control. The plates were incubated at 25±2ºC. The growth of the pathogen and the control were recorded. The percent inhibition of radial growth = (R1–R2)/R1 × 100. Where R1 = radial growth of the pathogen in control. R2 = radial growth of the pathogen in dual culture with antagonists.

4.2. The antagonism reaction of *Trichoderma* spp.

Based on the previous screening, the antagonism reaction of the most potent *Trichoderma* isolate was evaluated. The growth of the fungi and interaction between dual mycelia were scored for degree of antagonism reactions using a scale of 1 to 5 after the 5^{th} day of dual growth; where 1=*Trichoderma* overgrowing pathogen and 5= pathogen overgrowing *Trichoderma* [25]. Pathogens developed from plates of dual cultures were then microscopically investigated and the changes in the mycelium of the pathogen were recorded.

The growth of the pathogen (%) = (Radius growth of the pathogen in the direction of *Trichoderma* / Radius of growth in absence of *Trichoderma*) × 100.

4.3. Screening of *Trichoderma* spp. metabolites as a biocontrol agent

The antifungal activity of seven *Trichoderma* spp. isolated from the seed health test were evaluated against *M. phaseolina* on PDA plates, by the agar well diffusion method.

Under septic conditions, the hyphal growth of *M. phaseolina* was scrapped from 7 days old plates by adding 10 ml of sterilized water, the hyphal suspension was aggregated in sterile flasks, then agitated for 10 min at 150 rpm. 1 ml of the hyphal suspension was used to inoculate the agar plate surface by spreading over the entire agar surface. Then, a hole with a diameter of 5 mm was punched aseptically with a sterile cork borer and a volume of 0.2 ml of each *Trichoderma* spp. metabolite was introduced into the well. Then, agar plates were left in the refrigerator for 2 h to allow the even diffusion of *Trichoderma* metabolites in the agar medium, followed by incubated at 23±2 ℃ for 4 days. The clear zone of each *Trichoderma* species was measured.

5. Molecular identification

The selected fungus was molecularly identified, applying the molecular biological protocol of DNA isolation, amplification *via* polymerase chain reaction using 18S rRNA primers and sequencing of the ITS region. [26] The primers ITS2 (GCTGCGTTCTTCATCGATGC) and ITS3 (GCATCGATGAAGAACGCAGC) were utilized at the PCR, while ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used in the sequencing. Purification was performed to eliminate unincorporated PCR primers and dNTPs from PCR products by using Montage

PCR Clean-up kit (Millipore). Sequencing was performed using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA), then resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The sequence (700 bp) was deposited into the Gen Bank database (http://www.ncbi.nlm.nih.gov/BLAST) to get similarities with the related fungal sequences, the software package MEGA7 was used for multiple alignment and phylogenetic analysis.

6. Chitinase assay of *T. viride*

Chitinase production was determined in the filtrate of *T. viride* after growing on broth medium for 14 days. Chitinase was incubated with 3 mg/ml chitin azure in 0.2 M potassium phosphate (pH 7) buffer at 30℃, after 30 min. incubation, enzyme activity was terminated by boiling for 5 min., the reaction mixture was centrifuged at 5.000 rpm for 5 min. then, absorbance was measured at 575nm. One unit of chitinase was the amount of enzyme that produced an increase of 0.01 per minute in the absorbance under the assay conditions [27].

7. Statistical analysis

The experiments were arranged in one-way completely randomized, means comparison was performed based on the Duncan's Multiple Range Test at P value of $\leq$ 0.05. All the statistical analysis was performed using CoStat (CoHort Software, U.S.A) version 6.4 was used.

RESULTS AND DISCUSSION

Occurrence of soybean seed-borne mycoflora

Altogether thirty-one fungal species comprising nineteen genera were isolated from the collected soybean seed samples following standard blotter (SB) and agar plate (AP) methods. No differences were observed between the SB (17 genera and 29 species) and AP (18 genera and 28 species) techniques with regard to the frequency of the recovered seed-borne fungi (Table 1). *Aspergillus flavus* (100%), *A. niger* (86%), *A. ochraceus* (80%) and *Penicillium* species (50%) were most predominant. Similar, to the present study, these fungi were reported to be seed borne in soybean by a number of other workers [28-30] on soybean seeds. *Cladosporium* spp. (43.3%), *Alternaria alternata* (37.3%), *Fusarium acuminatum* (30%) and *Trichoderma* spp. (30%) were recorded at moderate percentages. These species have been reported to reduce the germination of seed and damage the seeds in storage. Fungal toxins of most concern are produced by species within the genera of *Aspergillus*, *Fusarium* and *Penicillium* that frequently occur in major food crops in the field and continue to contaminate them during storage, including cereals, spices, medicinal and oilseeds [31-33]. Among these mycotoxins, aflatoxin B1 (AFB1), fumonisin B1 (FB1) and ochratoxin A (OTA) are the most toxic to mammals, causing a variety of toxic effects including hepatotoxicity, teratogenicity and mutagenicity, resulting in diseases such as toxic hepatitis, hemorrhage, oedema, immunosuppression, hepatic carcinoma, equine leukoencephalomalacia (LEM), esophageal cancer and kidney failure [34-36]. AP technique succeeded to recover some fungi that absent in SB e.g., *Alternaria chlamydospora*, *Aspergillus terreus*, *Mucor* spp. and *Neurospora crassa*. This may be due to that these fungi may need an external supply of nutrient that is not present in the seeds **[**37**]**. On the other hand, the SB method enhanced the recovery of *Aspergillus albicans*, *A. flavipes* and *Stachybotrys chartarum*. The absence of these mycoflora in AP technique may be attributed to the antagonistic activities of the fast-growing saprophytes which were dominantly recovered in this technique. *Fusarium acuminatum* was the most abundant species among all *Fusarium* species (30 and 15% in AP and SB techniques, respectively) followed by *F. equiseti* (10 and 8% in AP and SB techniques, respectively) were, *F. proliferatum* was the least dominant among *Fusarium* species (6.7% for each method).

Results of the present study showed that soybean seeds infected with several pathogenic fungi such as *Fusarium* spp., *Colletotrichum truncatum*, *M. phaseolina* and *Rhizoctonia solani*. Among these fungi, *M. phaseolina* was found predominant (up to 27.3%) and it was used as test pathogen for further studies. The pathogen appeared as greyish mycelial growth on incubated soybean seeds in different detection methods. Among the seed-borne

diseases of soybean, charcoal rot caused by *M. phaseolina*, is one of the most serious fungal disease which cause severe damage by reducing seed yield and quality in warmer, tropical and sub-tropical regions of the world [38, 6]. Soybean diseases reduce the yield on an average of 10 to 30% in most production area [39].

Table 1. The occurrence of soybean seed-borne fungi using standard blotter and agar plate methods.

Fungus	Agar plate			Standard blotter		
	Frequency (%)	Range (%)	Mean ± SD (%)	Frequency (%)	Range (%)	Mean ± SD (%)
***Absidia* spp.**	3.33	0	0.07±0.07	13.3	1-4	0.27±0.15
Alternaria alternata	36.7	1-8	1.33±0.41	37.3	1-5	0.93±0.28
Alternaria chlamydospora	3.33	0	0.03±0.03	0	0	0
Aspergillus albicans	0	0	0	23.3	0.5-3	0.47±0.16
Aspergillus flavipes	0	0	0	3.33	0	0.03±0.03
Aspergillus flavus	80	2-90	20.6±4.01	100	12-88	47.97±4.44
Aspergillus fumigatus	10	1-6	0.27±0.20	3.33	0	0.07±0.07
Aspergillus glaucus	6.67	1-2	0.1±0.07	56.7	1-16	2.0±0.53
Aspergillus nidulans	3.33	0	0.03±0.03	17	0.5-1	0.17±0.07
Aspergillus niger	66.7	1-23	7.60±1.54	86	1-34	12.7±1.56
Aspergillus ochraceus	63.3	1-25	6.7±1.37	80	2-31	16.47±1.67
Aspergillus tamarii	30	4-20	2.3±0.87	13.3	1-30	0.3±0.15
Aspergillus terreus	3.33	0	0.03±0.03	0	0	0
Cephalosporium acremonium	6.67	0.5-3	0.2±0.14	13.3	1-4	0.27±0.15
***Cladosporium* sp.**	43.3	1-17	1.93±0.67	40	1-12	2.13±0.63
Colletotrichum truncatum	6.67	0.5-1	0.07±0.046	6.67	0.5-1	0.067±0.047
Epicoccum nigrum	6.67	0.5-1	0.07±0.047	6.67	1-2	0.1±0.07
Fusarium equiseti	10	0.5-5	0.17±0.03	8	0.5-3	0
Fusarium proliferatum	6.7	0.5-1	0.03±0.03	6.7	0.5-1	0.0±0.0
Fusarium acuminatum	30	0.5-10	1.4±0.40	15	0.5-8	1.2±0.2
Macrophomina phaseolina	27.3	1-3	1.4±0.43	13	0.5-2	0.87±0.30
***Mucor* sp.**	3.33	0	0.03±0.03	0	0	0
Nigrospora oryzae	33.3	1-21	2.67±0.96	63.3	1-5	1.27±0.27
Neurospora crassa	3.33	0	0.73±0.73	0	0	0
***Penicillium* spp.**	50	1-7	1.17±0.33	3	1-2	0.33±0.10
Rhizoctonia solani	3.33	0	0.4±0.14	3.33	0	0.17±0.08
Rhizopus stolonifer	43.3	1-10	1.4±0.43	37	1-8	0.87±0.30
Stachybotrys chartarum	0	0	0	3.33	0	0.03±0.03
***Stemphylium* sp.**	6.7	0.5-1	0.07±0.05	6.7	0.5-1	0.03±0.03
Trichoderma viride	30	1-35	2.23±1.20	10	1-3	0.20±0.12
Trichothecium roseum	2	0.5-1	0.067±0.046	16.7	1.3	0.27±0.13

Frequency (%) = (No. of infected samples) / (Total No. of tested samples) ×100

Mean of sample infection = (∑fungus incidence in all examined samples)/ (Total No. of examined samples)

Dual culture test

Seven *Trichoderma* isolates were tested against *Macrophomina phaseolina* and there was a significant difference between these isolates but the most active isolate of the antagonistic fungi against the pathogen and showed high degree of antagonism reaction after five days of dual culturing was T44sn (81.23) followed by it T23sn and T11sn (75.97, 73.73). The lowest growth of *M. phaseolina* (61, 63.03, 63.7) was recorded by T42sn, T21sn, respectively. The best antagonism reaction, being 1 was recorded by all isolates except T22sn was 2 **(Table 2)**. The high degree of antagonism reaction means the occurrence of strong mycoparasitism. *Trichoderma* species were found to produce inhibition halos and sporulated over the colonies of the pathogen, with different degrees. However, mycoparasitism has been proposed as the major antagonistic mechanism displayed by *Trichoderma* spp. can attach to and coil around the pathogen, in some cases, from appressoria on the host surface, wherein *Trichoderma* spp. produce several cell wall degrading enzymes

and probably also antibiotics, the combined activities of these compounds result in parasitism and dissolution of the cell walls forming walls from holes, which act as direct entry of *Trichoderma* hyphae into the target fungus **[**40,41**]**

These results were in confirmed with the finding of **[**42], who reported that *T. koningi* and *T. harzianum* were found to be effective in reducing radial growth of *R. solani*, *T. koningi* strains produced toxic metabolites with strong activity against *R. solani*, inhibiting the mycelial growth. [43] also documented that *T. harzianum* significantly inhibited the growth of *M. phaseolina*. *T. viride* and *T. harzianum* and a greater inhibition on *M. phaseolina* than *T. hamatum*. There are several mechanisms such as hyperparasitism, inhibition and antibiosis. [44] screened seventeen *Trichoderma* strains against *R. solani in vitro*. All strains including *T. harzianum*, *T. viride* and *T. aureovride* have inhibited the growth of *R. solani*. Mycoparasitism involved morphological changes such as coiling and formation of appressorium-like-structures, which serves to penetrate the host [45]. The first contact between *Trichoderma* spp. and pathogen *M. phaseolina* occurred after 2 to 3 days of inoculations, followed by growth inhibition. Differential antagonistic activity has even been observed for various *Trichoderma* spp. which demonstrate semi specificity in the interaction of *Trichoderma* with the host [46]. [47] reported that *T. harzianum* ALL42 were capable of overgrowing and degrading *R. solani* and *M. phaseolina* mycelia, coiling around the hyphae with the formation of appressoria and hook-like structures. The interaction between fifteen isolates of *T. harzianum* and the soil-borne pathogen.

Table (2): Growth of *M. phaseoliana* as affected by *Trichoderma* spp. in a dual culture test

***Trichoderma* isolate**	**Growth reduction (%) of *M. phaseoliana* after**			**Antagonism reaction**
	24 hours	**72 hours**	**120 hours**	
T44sn	45.80 e	69.13 b	81.23 a	1
T42sn	31.13 g	58.40 e	63.70 d	1
T21sn	56.70 c	74.27 a	75.97 b	1
T22sn	68.30 a	69.10 b	70.80 c	1
T23sn	42.80 f	59.23 d	63.03 d	2
T11sn	48.80 d	58.13 e	61.00 d	1
T12sn	62.43 b	68.17 c	73.73 bc	1

Means followed by the different letter within each column are significantly different using Duncan's Multiple Range Test at P value of $\leq .0.05$

Antifungal activity of *Trichoderma* cell-free extracts

Antibiosis is the mechanism by which certain microorganisms respond to the presence of others, secreting compounds or metabolites capable of inhibiting or impeding their development [47]. The crude extract of *Trichoderma* contains a mixture of secondary compounds that exhibit different biological functions, that is, phytohormones, phytotoxins, mycotoxins, pigments and antibiotics [4.0,48]. This study was aimed to evaluate the best antagonistic effect of cell-free extracts of seven *Trichoderma* isolates against *M. phaseolina* by agar well diffusion by measuring clear zone to each isolate. Data in **Fig. (1)** indicated considerable differences among these isolates. In this connection, T44sn isolate was the best antagonistic isolate against the pathogen, which recorded a 2.47 cm inhibition zone of the pathogen growth, followed by it T42sn 2.20 cm. The rest of *Trichoderma* isolates had lower inhibition effect. Since T44sn isolate produce active secondary metabolites (SMs) against *M. phaseolina*, the results verified with those obtained in previously performed dual culture tests and a maximum inhibition was observed with it. This fact suggests that the additional main mechanism of action of this isolate is antibiosis, that is, the production of secondary compounds with antibiotic properties. The same obtained result was confirmed with the other plant pathogenic fungi confronted. In this respect, [49] reported the production of *T. viride* and *T. harzianum* metabolites as those with the highest antifungal potential of different species of *Colletotrichum*, among several other species tested. **[**50, 51**]** reported a maximum inhibition rate of *F. oxysporum* pathogen using filtrates from *T. viride* and *T. harzianum*. **[**41**]**

recorded the antifungal activity of the *T. viride* crude mycelia extract against the growth of different pathogens e.g., *Fusarium solani*, *Rhizoctonia solani* and *Sclerotium rolfesii*. The above results were an attitude by the work of **[**48**]** who reported a broad-spectrum inhibition of non-volatile metabolites of *T. brevicompactum* stains against *Sclerotium rolfsii*, *Colletotrichum gloesporioides*, *Verticillium dahliae*, *Fusarium oxysporum* and *Cylindrocladium* sp. pathogens. At the present time, several SMs have been reported, and these are the main groups found in *Trichoderma* isolates: polyketides, peptaibols, terpenoids/steroids; pyrones and daucanes [52]. The SMs may play an antibiotic function against plant pathogens [40], act as a plant growth regulator i.e., gibberellic acid and indole acetic acid **[**53,54**]**. It acts as a defense system and plays an important role in the communication of the fungus with the other microorganisms, insects and plants [55]. Although these molecules are not essential, in some cases they are important for the selective survival of organisms under certain conditions **[**56**]**, eliminating microbial competition and, consequently, leading to greater availability of scarce food sources in the soil [57]. This may explain the obtained growth reduction of *M. phaseolina*

Molecular identification of *Trichoderma* sp. T44sn

The selected fungal strain (*Trichoderma* sp. T44sn) was identified using the molecular technique (18S rRNA) as a perfect tool for identification. From Blast results, strain T44sn exhibited 99% similarity with the previously identified *T. viride* Fig. (2) represents the constructed phylogenetic tree of *Trichoderma* sp. T44sn, which comes in line with the previous morphological identification. Because of high sensitivity and specificity, molecular identification is widely used for rapid identification of filamentous fungi at various taxonomic levels. The technique is set up for the comparison of the sequence coding for 18S rRNA gene after PCR amplification, through the internal transcribed spacer (ITS), which fragment size is uniform in numerous groups of fungi, making nucleotide sequencing of ITS fractions prerequisite for revealing interspecific, and in some cases, intraspecific variation [58].

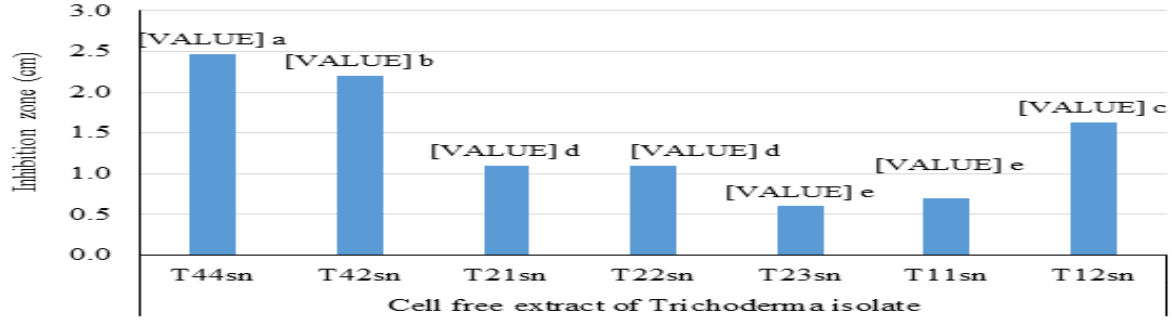

Fig. 1: Antagonistic effect of cell-free extracts of *Trichoderma* isolates against *M. phaseolina***.**

Columns designated with different letter are significantly different using Duncan's Multiple Range Test at P value of ≤.0.05.

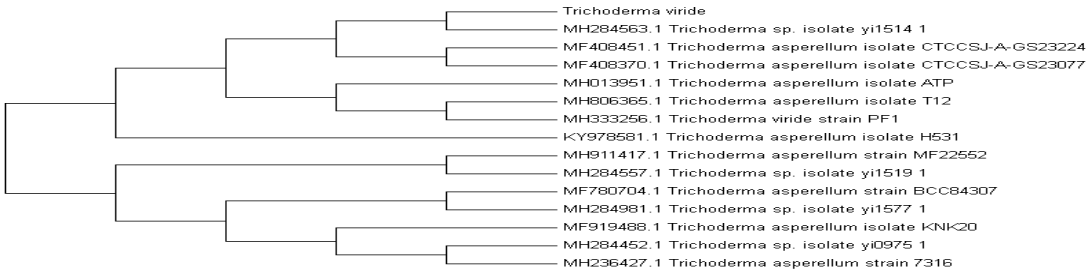

Fig. 2: Molecular phylogenetic tree of the partial sequence of 18S rRNA showing the position of *Trichoderma* strain T44sn within the respect to the closely related sequences in GenBank**.**

Chitinolytic activity:

Chitinase activity by *T. viride* was found to be 0.20 U, this low activity on non- specific medium for the enzyme production reflecting the constitutive nature of the enzyme. The present *Trichoderma* isolate was found to be a reasonable producer of chitinase. Chitinases are

important enzymes in various fields, catalyzing the degradation of chitin into N-acetyl-D-glucosamine residues [59]. Chitinolytic enzymes have been considered important in the biological control of soil-borne pathogens because of their ability to degrade fungal cell wall, which its major component is chitin, thus, reducing greatly the growth of phytopathogenic fungi. *T. viride* was reported as a most dominant chitinolytic fungal species isolated from soil that achieved the strongest activity [60]. [61] reported a crescent growth inhibition of phytopathogenic fungi e.g., *Aspergillus flavus, A. niger, M. phaseolina* and *F. moniliforme* due to treatment by purified *T. viride* chitinase enzyme. Their antifungal activity is attributed to the hydrolytic effects on fungal chitin **[**59,62**]**. The presence of chitinolytic activity in the present *Trichoderma* isolate may be suggested as a possible mechanism the its interaction with the pathogen.

In conclusion, *T. viride* showed several evidences as a biocontrol candidate against *M. phaseoliana* associated with soybean seeds. This strain has antagonistic activity against the phytopathogen as well as its filtrate have reasonable chitinolytic activity, which could synergistically act against other various phytopathogens.

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