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ORIGINAL PAPER

Integration Between Arbuscular Mycorrhizal Fungi, Bacterial and Fungal Bioagents for Controlling Rice Brown Spot Disease

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ABSTRACT

The main purpose of this work was to test the efficiency of Arbuscular mycorrhizal fungi (AMF) in inducing systemic acquired resistance against rice brown spot disease alone or in combinations with the bioagents, *Pseudomonas fluorescens* (Pf) and *Trichoderma viride* (T.v.). *In vitro* test showed inhibition of 52.85% and 57.14%, respectively for the two selected bioagents with deformation and lysis for the pathogen spores. Under greenhouse conditions, the mixture of AMF + Tv and AMF + Pf + Tv, showed the highest efficiency in reducing the disease incidence after the fungicide treatment, whereas they achieved 65.60 and 48.40%, respectively. Field trials at two locations showed potentiality of AMF combined with the other bioagents in decreasing the percentages of disease incidence and severity. Also, the applied treatment increased the plant growth parameters, *i.e.*, chlorophyll content, plant height and yield. Microscopic examination for rice plant roots showed colonization structures of AMF. Also, Pathogenesis related proteins had been defined.

Key words: Rice brown spot, Arbuscular mycorrhizal fungi, bioagents, induced resistance, enzymes

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INTRODUCTION

Rice brown spot disease (RBS) caused by *Cochliobolus miyabeanus* (Ito and Kuribayashi Drechsler *ex* Dastur) Shoem Anamorph: *Bipolaris oryzae* (Breda de Haan), is one of the major rice diseases and is a world- wide distributed (Devi and Chhetry, 2013) causing seedling blight when infected seeds are used. So, seedlings or older plants become weak and in case of grain infection, grain quality and weight are decreased (Iqbal *et al*., 2015). RBS occurs especially in poor fields that suffer from scarce of water supply or irrigated with mixed water (fresh water + drainage water). Also, it is often associated with imbalances in mineral nutrition of plants, especially nitrogen element (Carvalho *et al.*, 2010). Severe infection may cause losses of 26-52% (Chakrabarti, 2001). It is remarkable to point out to the great Bengal famine in 1943 as a result of RBS epidemiological, causing up to 90% loss in rice yield (Padmanabhan, 1973). Many protective and curative trials including agricultural methods or usage of bio and chemical substances have been investigated to control the disease. Seed treatment started very early by Nisikado and Miyake (1922), who found that treating rice seeds by hot water or $CuSo_4$ was useful in reducing seedlings damage. Since then many workers investigated seed treatment by many substances whether chemical or bio ones, while fungicidal field spraying has been tried to prevent secondary air-borne infection (Ou, 1985). To keep our environment less contaminated with fungicides, less toxic to living organisms and to decrease costs to growers, we performed this study to test an eco-friendly and good alternative method than chemical one for clean agriculture.

Different bioagents, *i.e.*, Arbuscular Mycorrhizae Fungi (AMF), *Trichoderma viride* and *Pseudomonas fluorescens* have been tested. AMF are known to have an indirect effect on plant disease control whether on soil borne or foliar diseases (Hodge *et al.*, 2003). They are known to present various benefits to plants, including enhanced nutrients availability to plants and their uptake (particularly P), increased water uptake, improved biotic and abiotic stress tolerance and improved soil structure, thus significantly contributing to control diseases (Addo *et al*., 2020). *Trichoderma* species are able to colonize the root surface and rhizosphere when applied as seed treatment, protecting them from fungal invasion. Also, *Pseudomonas fluorescens* has efficiency in controlling RBS disease and seed discoloration by 28.18 and 33.33 %, respectively

(Balgude *et al*., 2017). To our knowledge, there is no available report on comparing the usage of AMF and other bioagents as rice seed dressing and their potentiality against rice brown spot disease in Egypt, in addition to their effect on growth parameters. Therefore, this study was performed to evaluate the potential effect of integration between AMF with selected bioagents, *P. fluorescens* and *T. viride* against RBS disease incidence and severity.

MATERIALS AND METHODS

The phytopathogen and antagonistic isolates:

Bipolaris oryzae was isolated from naturally infected leaves showing typical symptoms of brown spot disease according to Gomathinayagam *et al*., 2012. An active strain of *P. fluorescens* and inocula of Arbuscular Mycorrhizae Fungi (AMF) were kindly supplied by Department of Agric. Microbiology, Soils, Water and Environment Research Institute (SWERI), ARC, Giza, Egypt. While an isolate of *Trichoderma viride* was obtained from previous work of Saleh, 2012. This isolate was identified at Laboratory of Mycology, Plant Pathology Institute, Agricultural Research Center, Giza, Egypt.

***In vitro*, antagonistic action of *P. fluorescens* and *T. viride* against *Bipolaris oryzae*:**

Plates of 9 cm containing PDA medium were inoculated with discs (5 mm in diameter) taken from 7 days old culture of *Bipolaris oryzae* on one side. Two days later, by a sterilized inoculation needle, a loopful of actively *P. fluorescens* growing culture (48 hrs at 28ºC) was streaked in a gentle three cm long at the opposite side of the same plate. Experiment was made in three replicates. Plates free from bacterium were left as control. Plates were incubated at 28±2°C for five days later. Then diameter of inhibition zone was recorded, and the relative power of antibiosis (RPA) was estimated through the ratio as described by Saleh (2012) as follows:

$$RPA = \frac{Z}{C}$$

Where:

Z= Diameter of inhibition zone.

C= Diameter of spotted antagonistic isolate.

For *T. viride*, Petri dishes of 9 cm containing PDA medium were inoculated with discs of 6 mm diameter taken from the edge of expanding colonies of *Bipolaris oryzae* on one side. After two days, one disc of *T. viride* growth (6 mm diameter) was cultured onto the medium on the opposite side of the same plate. Plates containing the pathogen only were included as a check treatment. Three replicates were used for each treatment. Paired cultures were incubated at 28 ±2°C till the growth of control treatment filled the whole plate surface. As a result of the antagonistic effect inhibition% of the pathogen mycelial, growth was determined according to Khalili *et al*. (2012) as follows:

$$\text{Inhibition } \% = \frac{(R2-R1)}{R2} \times 100$$

Where:

R2 = the average of growth diameter of the pathogen in control plates and

R1 = the average of mycelial growth of the pathogen in treated plates.

Mycelial growth samples (pathogen hyphae), cut from the edge of interaction region in dual culture tests (7 days), were fixed on slide glass to observe any changes in the pathogen structures under an inverted Binocular Light Microscope (Labomed, 40x)

Preparation of the pathogenic fungi:

The phytopathogen was cultured in Petri dishes containing PDA medium and incubated at 28±2°C until full growth. To enhance sporulation, plates were exposed to continuous fluorescent light for two days. To prepare inoculum of spore suspension, 10 ml sterilized water were added in each dish, mycelia mats were gently scraped by a spatula and filtered through cheese cloth. Spore suspension was adjusted to be 10^5 (Khalili *et al*., 2012).

1- Preparation of biocontrol agents:

A) Arbuscular Mycorrhizal Fungi inoculum (AMF):

Inoculum of Arbuscular Mycorrhizae Fungi (AMF) was prepared according to the method of Massoud (2005) in order to be used throughout the current study.

***B) Pseudomonas fluorescens* inoculum:**

A strain of *P. fluorescens* was cultured and maintained on King's B medium (King *et al*., 1954) at 28±2ºC for 2 days. The biomass of *P. fluorescens* was prepared by inoculating a disc of a pre-culture of *P. fluorescens* in a 500 ml Erlenmeyer flask containing 200 ml of King's broth medium, then incubated on a rotatory shaking incubator (120 rpm) for 72 hours at 28±2ºC. The density of bacterial cell culture was adjusted to be 10^8 CFU /ml using hemocytometer slide.

C) Preparation of *T. viride*:

Inoculum of *Trichoderma viride* was prepared by growing *T. viride* in conical flasks containing 500 ml PD broth (PDB) medium and incubated for 15 days. The fungal mass was blended, and concentration was adjusted to be 10^8 spore/ml (El-Gremi and Saleh, 2013).

2- Rice grains treatment with bioagents inoculum:

Each inoculum of the three bioagents biomass (AMF, *P. fluorescens* and *T. viride*) was applied as seed dressing according to Saleh, (2002) as follows: inoculum of AM-fungi was added to peat at the rate of 1/1 g (peat was wetted with water before mixing). While the cell suspension of the antagonistic isolates (*P. fluorescens* and *T. viride*) containing 10^8 CFU/ml of the bacterium or 10^8 spore/ml of the fungus was added to peat at the rate of 1ml/g peat, well mixed and left at room temperature for 48 hr before seed dressing. Rice grains soaked for 24h in water were wetted by 10% Arabic gum water solution and then dressed with the prepared antagonistic peat at rat of 1g/g grains. Treated grains were then ready for sowing.

Plant materials:

Rice grains cv. Giza 177 was used in this experiment. Rice grains were surface sterilized with Sodium Hypochlorite (5%) for 5 min., washed several times with sterile distilled water and then were air dried.

Greenhouse experiment:

A pot experiment was performed at the greenhouse of Rice Pathology Research Dept., Rice Research and Training Center (RRTC), Sakha Agricultural Research Station. Seed dressing method was applied according to Saleh, 2002. Pots with 15 cm diameter were filled with clay loam soil, planted with the susceptible rice cultivar (Giza177) and arranged according to the following treatments: 1. Arbuscular Mycorrhizal Fungi (AMF); 2. *P. fluorescens* (P.f) (10^8 CFU/ ml); 3. *T. viride* (T.v.) (10^8spore/ ml); 4. AMF + P.f; 5. AMF + T.V.; 6. P.f + T.V.; 7. AMF + P.f + T.V.; 8. Fungicide Del cup (Copper sulphate pentahydrate) and 9. Check treatment (control). The used fungicide Del cup ($CuSo_4.5H_2o$) 6% L/fed was sprayed at a rate equivalent to 5 cm/ L. After 21 day of sowing, seedlings were sprayed with the pathogen spore suspension (10^5spores/ml). All treated pots were kept in a moistened dark chamber with 98-100% relative humidity for 24 hours and then transferred out under sprinkler with half an hour interval. A completely randomized design (CRD) with three replicates for each treatment was conducted. Data were recorded 5 days after inoculation, a randomly 25 leaves were selected per pot to calculate disease incidence% and disease severity.

Field trail:

The same previous design of treatments was conducted at two locations, Sakha and Gemmiza farms of Agricultural Research Stations, at the same season of 2017. Grains of Giza 177 rice cultivar were directly planted after being dressed with the different bioagents (as mentioned before) according to Saleh (2002). Three plots ($1m^2$/plot) as replicates were used for each treatment. A randomized complete blocks design was conducted. Percentage of disease incidence and severity as well as some plant growth parameters including chlorophyll content, plant height and yield (g) were recorded.

Assessment of disease incidence and severity of brown spot:

Percentages of incidence and severity of brown spot were recorded under artificial and natural conditions after 30 and 70 days of cultivation, respectively. Ten hills were randomly selected from each plot and data were collected on number of tillers/hill, number of diseased tillers/hill and number of spots/hill. Percentage of disease incidence and severity were assessed according to Rashed *et al*. (2002). Disease severity was scored according to the Standard Evaluation System for rice (IRRI, 2002) as following:

1- Disease incidence (%) =

$$\frac{\text{No. of diseased leaves or tillers}}{\text{No. of total examined leaves or tillers}} \times 100$$

2 - Disease severity =

$$\frac{\text{Sum of total ratings}}{\text{Total no. of observed leaves or tillers} \times \text{Maximum grade in the scale}} \times 100$$

On the other hand, efficacy % for each bio-control agent severity was calculated according to Muhanna *et al*. (2016) as follows:

$$\text{Efficacy \%} = \frac{\text{Control} - \text{treatment}}{\text{Control}} \times 100$$

AMF microscopical examination:

A sample of Giza 177 rice cultivar roots was cut into pieces of 1cm. long. The pieces were immersed in a 100ml flask containing KOH 10% solution w/v. Then flasks containing roots were sterilized in an autoclave for 15-20 minutes at 121°C. Sterilized root pieces were then washed with distilled water several times before transferring them into the staining solution. Root pieces were stained with ink and vinegar according to Vierheilig *et al*. (1998) and examined under stereomicroscope.

Assay of enzymes activity:

To determine the defense related enzyme activity of rice plants against *Bipolaris oryzae*, activities of Peroxidase (POX), Poly phenoloxidase (PPO) and Catalase (CT) were assessed according to methods described by Allam and Hollis (1972), Maxwell and Bateman (1967) and Chandlee and Scandalios (1984), respectively. Activities were measured after 24, 48 and 72 hours (h) of the pathogen inoculation of rice plants grown under greenhouse conditions.

Statistical analysis:

Means were compared using multiple range tests according to Duncan (1955) using computer program COSTAT.

RESULTS

Effect of *Pseudomonas fluorescens* and *Trichoderma viride* on the pathogen fungal growth:

Data presented in Table (1) show the ability of *Pseudomonas fluorescens* and *Trichoderma viride* to inhibit the mycelial growth of *Bipolaris oryzae* resulting in 52.85 and 57.14% inhibition in mycelial growth, respectively compared with the check treatment. In addition, the two bioagents caused deformation and lysis to the pathogen spores compared with the healthy one (Fig 1 & 2).

Table (1): *In vitro*, antagonistic effect of *Pseudomonas fluorescens* and *Trichoderma viridi* against *Bipolaris oryzae* mycelial growth

Treatment	Mycelial growth (mm)	Growth inhibition %
Pseudomonas fluorescens	3.3 b	52.85
Trichoderma viride	3 c	57.14
Control	7 a	-

Means followed by the same letter (s) within the column are not significantly different (P≤ 0.05).

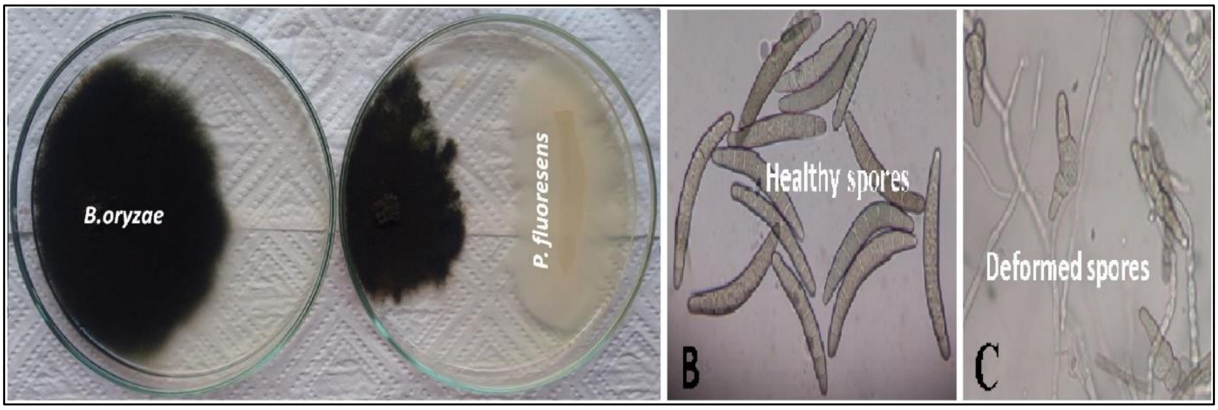

Fig (1): Effect of *Pseudomonas fluorescens* against *B. oryzae in vitro* (B & C, 40X).

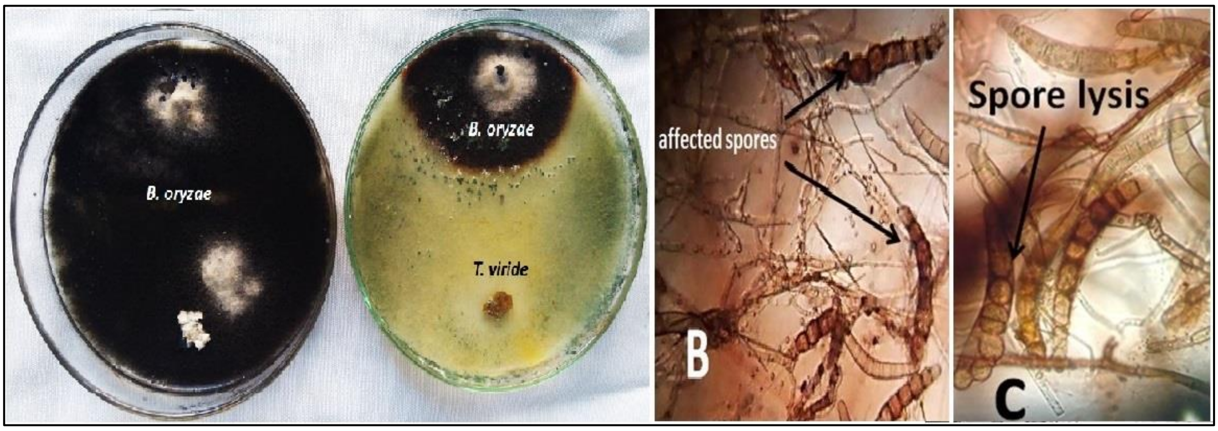

Fig (2): Effect of *Trichoderma viride* against *B. oryzae in vitro* (B & C, 40X).

Greenhouse experiment:

The results of rice seed dressed with Arbuscular Mycorrhizal Fungi (AMF), *P. fluorescens* (P.f) and *T. viride* (T.v) treatments alone or in combination as well as fungicidal treatment and the untreated one are presented in Table (2). The recorded data showed that all the treatments had different efficiency for reducing the brown spot disease incidence compared with the untreated one. The chemical fungicide Del cup 6% revealed the lowest disease incidence %, disease severity and the highest efficiency. The corresponding percentages are 3.33, 10.66 and 79.62%, respectively. While significant decreases in both disease incidence and severity, being 6% and 18% with increase in efficiency (65.60%) when seeds were coated with the mixture of the two bioagents, AMF + T. *viride*, followed by the two same significant treatments; AMF alone, that recorded low disease incidence, severity and high efficiency being 8.33, 29 and 44.58%, respectively and the mixture of AMF, *P. fluorescens* and *T. viride* which recorded 7.66% 27 and 48.40%, respectively. Whereas, the treatment of *T. viride* alone achieved disease incidence, severity and efficiency of 9.33, 31.33 and 40.12 %, respectively. The bioagent and fungicide treatments achieved lower disease severity and higher efficiency than the check which recorded the highest disease incidence and severity, being 13.33 and 52.33%, respectively.

Table (2): Effect of seed dressing with Arbuscular mycorrhizal fungi (AMF), *P. fluorescens* (P.f.) and *T. viride* (T.v.) as bioagents on decreasing rice brown spot disease under greenhouse conditions

No.	Treatment	disease incidence %	disease severity %	% Efficacy
1	AMF	8.33 d	29.00 e	44.58
2	P.f.	11 b	40.00 b	23.56
3	T.v.	9.33 c	31.33 d	40.12
4	AMF+ P.f.	11.00 b	41.00 b	21.65
5	AMF+ T.v.	6.00 e	18.00 g	65.60
6	Pf+ T.v.	10.33 b	35.33 c	32.48
7	AMF+ P.f.+ T.v.	7.66 d	27.00 f	48.40
8	Del cup	3.33 f	10.66 h	79.62
9	Control	13.33 a	52.33 a	-

Means followed by the same letter (s) within the column are not significantly different (P≤ 0.05).

Field trails:

Data presented in Tables (3 and 4) show that all bioagent treatments or their combinations were efficient in reducing disease incidence and severity compared with check treatment and the fungicide treatment. The bioagent treatments exceeded Del cup fungicide in reducing disease incidence and severity % at Sakha location in contrary to Gemmiza location, where the fungicide was the best in achieving the least disease incidence and disease severity %. At Sakha location, Table (3) show that dressing rice seeds with AMF + T.v. treatment showed the lowest disease incidence and severity (13.33% & 21.33%, respectively), followed by the treatment of T.v. only (18.66% & 38.66%, respectively) and the AMF + P.f. treatment (18.66% & 46.66%, respectively). Whereas the fungicide treatment recorded the highest disease incidence % and severity (30.66 and 69.33 %, respectively). Data also showed that there were no significant differences among all treatments in chlorophyll content. Del cup fungicide treatment gave the highest plant height (111.44cm) followed by treatments with AMF only (100.44cm), P.f.+ T.v. (98.77cm) and the mixture of AMF + P.f. + T.v. (99.44cm), respectively, the last two treatments were not significantly different. Also, the fungicide treatment achieved the highest yield (958.33g/m^2) followed by treatents with AMF (886.66 g/m^2), AMF + P.f. + T.v. (846.66 g/m^2) without significant difference than T.v. (843.33 g/m^2).

At Gemmiza location, data presented in Table (4) show the efficiency of all the bioagent treatments or their combinations after Del cup fungicide treatment. As Del cup fungicide recorded the least disease incidence and severity (18% & 22%, respectively), followed by AMF treatment (24% & 52%, respectively) and AMF + P.f. (30.66% & 50.66%, respectively). Significant differences were found between all treatments in chlorophyll content. Dressing seeds with the mixture of AMF + P.f. + T.v. and treatment of P.f. only gave the highest chlorophyll content being, 39.93 & 39.73, respectively followed by the fungicide (38.83). Also, treatment with AMF treatment was equal with Del cup fungicide in plant height as they recorded the highest length (97.22cm &96.33cm, respectively) followed by AMF + T.v. (93.11cm) and AMF + P.f. (91.22cm), respectively. All the treatments were effective than the check in plant yield and significant differences were found. The treatment of T.v. was the best achieving 982g/m^2 followed by AMF + P.f. +T.v. (900g/m^2) and P.f. + T.v. (865g/m^2). Del cup fungicide showed no significant differences with AMF + T.v. treatment recording 830 &825 g/m^2, respectively.

Table (3): Effect of seed dressing with Arbuscular mycorrhizal fungi (AMF), *P. fluorescens* (P.f.) and *T. viride* (T.v.) as bioagents on decreasing rice brown spot disease at Sakha location

No.	Treatment	Disease incidence %	Disease severity %	Chlorophill (SPAD)	Plant height (cm)	Yield (g)
1	AMF	25.33 e	40 f	45.8 ab	100.44 b	886.66 b
2	P.f.	25.33 e	46.66 d	43.2 b	95.99 de	760.00 f
3	T.v.	18.66 f	38.66 g	45.23 ab	95.11 e	843.33 c
4	AMF + P.f.	18.66 f	46.66 d	46.2 ab	98.11 bcd	790 e
5	AMF + T.v.	13.33 g	21.33 h	44.3 ab	97.22 cde	820.00 d
6	P.f. + T.v.	28 d	42.66 e	43.2 b	98.77 bc	820.00 d
7	AMF + P.f.+ T.v.	29.33 c	52 c	46.7 a	99.44 bc	846.66 c
8	Del cup	30.66 b	69.33 b	46.53 ab	111.44 a	958.33 a
9	Control	37.33 a	78.66 a	43.13 b	92.11 f	756.66 f

Means followed by the same letter (s) within the column are not significantly different (P≤ 0.05).

Table (4): Effect of seed dressing with Arbuscular mycorrhizal fungi (AMF), *P. fluorescens* (P.f.) and *T. viride* (T.v.) as bioagents on decreasing rice brown spot disease at Gemmiza location

No.	Treatment	Infection %	Severity %	Chlorophill (SPAD)	Plant height (cm)	Yield (g)
1	AMF	24 h	52 g	38.63 c	97.22 a	801 e
2	P.f.	40 e	102.66 e	39.73 ab	89.67 d	805 e
3	T.v.	36 f	61.33 f	31.50 e	85 e	982 a
4	AMF + P.f.	30.66 g	50.66 h	38.63 c	91.22 c	771 f
5	AMF + T.v.	52 c	110.66 c	36.00 d	93.11 b	825 d
6	P.f. + T.v.	72 b	197.33 a	28.56 f	83.44 f	865 c
7	AMF + P.f. + T.v.	48 d	109.33 d	39.93 a	89.33 d	900 b
8	Del cup	18 i	22 i	38.83 bc	96.33 a	830 d
9	Control	76 a	181.33 b	36.15 d	92.11 bc	710 g

Means followed by the same letter (s) within the column are not significantly different (P≤ 0.05).

AMF microscopical examination:

Staining rice roots of Giza 177 cv. showed the colonization of Arbuscular mycorrhizal fungi to them. Figure (3) illustrates the presence of arbuscule, longitudinal hyphae and vesicles.

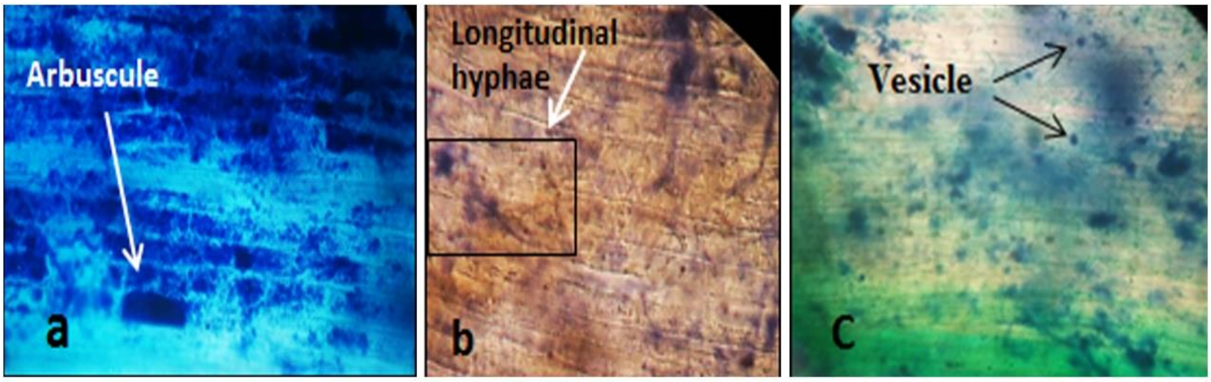

Fig (3): Light micrographs of arbuscular mycorrhizal fungi (AMF) in Giza 177 rice cultivar roots, (a) arbuscule, (b) longitudinal hyphae and (c) vesicle.

Assay of enzymes:

Data illustrated in Figure (4) show the changes in activity of peroxidase (POX) when rice plants were artificially inoculated with *Bipolaris oryzae* under greenhouse conditions. In all treatments POX activity was higher than in the check. Among all treatments, AMF + P.f. + T.v, fungicide and P.f. + Tv treatments were the highest in POX activity (3.35, 3.05 and 2.28, respectively) after 24h of inoculation. However, POX activity was decreased due to these treatments after 48h (0.97, 2.71 and 0.81, respectively) and then increased again after 72h (3.32, 4.9 and 3.32). Similar results were also observed at the treatment of AMF + Tv. Other treatments showed an increase in POX activity after 48h, then reduced after 72h of inoculation. Activity of polyphenoloxidase (PPO) recorded the best after 24h of inoculation at P.f. + Tv, AMF + P.f. + Tv, AMF and fungicide treatments (0.49, 0.17, 0.14 and 0.14, respectively) where AMF was similar with the fungicide (Fig. 5). After 48h and 72h, PPO activity fluctuated in all treatments, being higher than in the check. The fungicide was the only treatment that maintained stable till 72h for PPO activity. Data presented in Figure (6) show that the treatments of the fungicide, AMF + P.f. + Tv, P.f. and AMF + T.v. showed the highest after 24h of infection in catalase activity (160.49, 148.16, 116.79 and 104.34, respectively). While, after 48h catalase activity was increased due to treatments, *i.e.*, AMF + T.v.; T.v; AMF + P.f. + Tv and AMF + P.f. which recorded 129.22; 122.61; 97.42 and 81.25, respectively. Also, catalase activity increased after 72h in plants treated with AMF + Pf; P.f.; AMF + T.v. and AMF only, respectively.

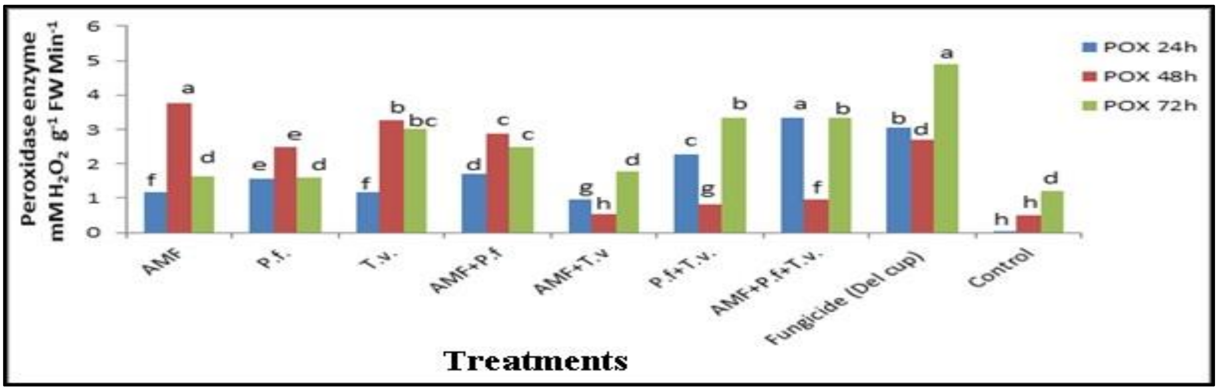

Fig (4): Changes in peroxidase activity in Giza 177 rice cultivar treated with three bio-control agents (AMF, P.f. and T.v.) and the fungicide Del-cup.

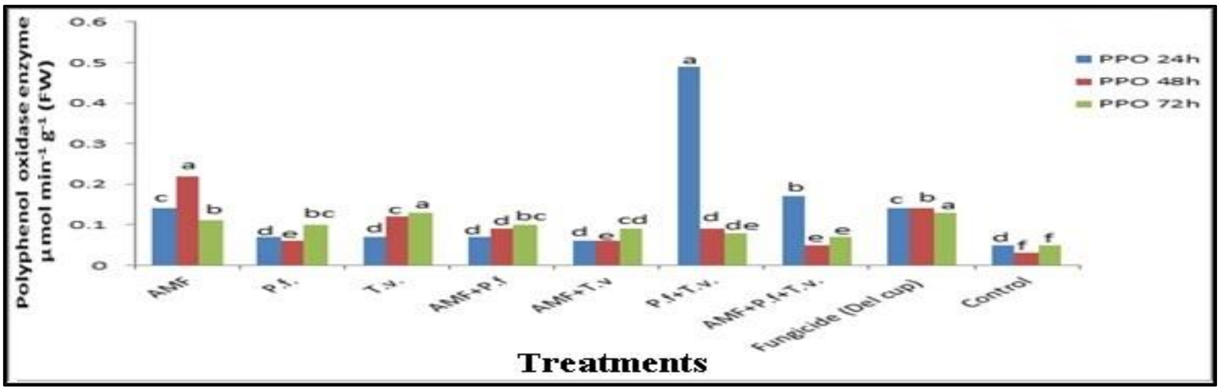

Fig (5): Changes in polyphenoloxidase activity in Giza 177 rice cultivar treated with three bio-control agents (AMF, P.f. and T.v.) and the fungicide Del-cup

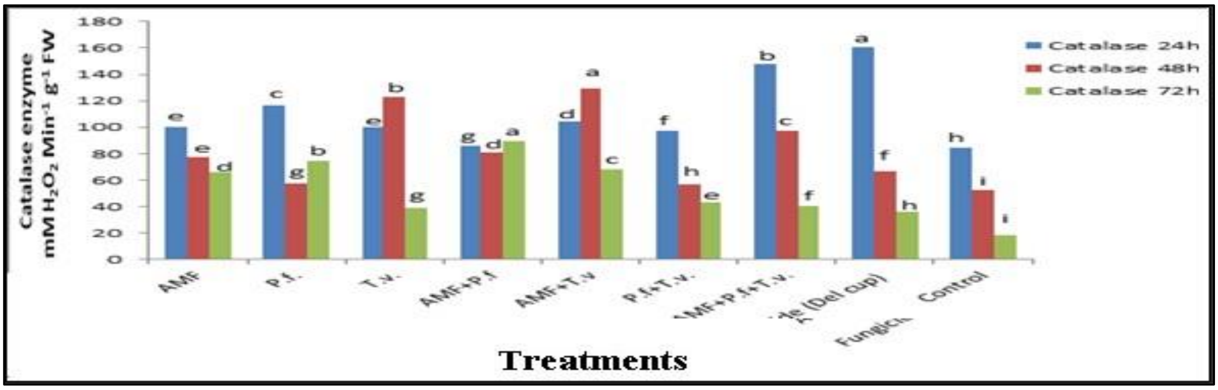

Fig (6): Changes in catalase activity in Giza 177 rice cultivar treated with three bio-control agents (AMF, P.f. and T.v.) and the fungicide Del-cup

DISCUSSION

Farmers used to apply intensive chemical fertilizers and pesticides to get a good yield and quality of crops. Nowadays, peoples view towards using pesticides in agriculture has been changed as they are harmful to the environmental purity and human health (Pal and Gardener, 2006). Thus, number of carcinogenic pesticides has been removed from markets and the need to alternative solution becomes a necessity. One of these solutions is biological control, which has proved efficiency against many plant diseases (Abohatem *et al*., 2011 and Kumar *et al*., 2014). Many efficient bio- control agents with various mechanisms in plant diseases management have been applied. One of these mechanisms used to reduce the incidence of plant diseases is the induction of a systemic acquired resistance (Abo-Elyousr *et al*., 2008). As accumulation of pathogenesis-related PR proteins such as chitinase, â-13-glucanes, phenylalanine ammonia lyase, peroxidase, phenolics and phytoalexins, results in defense reaction (Kloepper *et al*., 2004).

To manage rice brown spot (RBS) disease in this study we used AMF in comparative study with *Pseudomonas fluorescens* and *Trichoderma viride* aiming to enlarge our biological base in controlling rice diseases. The *in vitro* antagonism test showed the efficiency of *Pseudomonas fluorescens* and *Trichoderma viride* in suppressing the pathogen growth of *Bipolaris oryzae*. *T. viride* showed faster growth rate than *Bipolaris oryzae* for space and nutrients. Thus, the pathogen growth is inhibited (Khalili *et al*., 2012). Angelica *et al.*, 2001 assumed the ability of *T. viride* to produce amylase which aids in faster growth on PDA medium. Besides, producing extracellular cellulose and pectinase enzymes that hydrolyze other fungal cell walls. While P. *fluorescens* has antifungal secondary metabolites that are capable of lysing chitin, the most important component in fungal cell wall (Pandey and Chandel, 2014).

Under greenhouse conditions, the obtained results showed that after fungicide treatment, dressing seeds with the combinations of AMF + T.v. and AMF + P.f. + T.v., respectively were more efficient in RBS control followed by AMF alone and T.v. alone. These findings are in agreement with Morandi (1990) and Siqueira *et al*. (2002) who pointed out that the role of AMF in controlling plant diseases doesn't enclosed in increasing nutrient absorption only. But one of the factors that contribute to enhancing their role in controlling plant diseases is production of phenolic or secondary inhibitory compounds during their interaction with plants. Mart´ınez-Medina *et al.*, 2009 found that combined inoculation of AMF with *T. harzianum* resulted in a general synergistic effect on disease control. Also, Gomathinayagam *et al*. (2012); Gaikwad and Balgude (2016) and Kumar *et al*. (2017) stated that *Trichoderma* spp. and *Pseudomonas fluorescens* are promising bioagents in rice diseases management (*i.e*., blast and brown spot). While Navaneetha *et al*., 2015 showed that *Trichoderma* spp. colonize and penetrate plant root tissues resulting in morphological and biochemical changes in the plant leading to Induced Systemic Resistance (ISR) responses to a wide range of pathogens and different environmental conditions. Also, (Heydari and Pessarakli, 2010 and Balgude *et al*., 2017) reported the effectiveness of *P. fluorescens* in controlling the rice brown spot and its ability to produce the antibiotic 2, 4-diacetylphloroglucinol (DAPG) which may induce host defenses.

In vivo, at Sakha location, although the biocontrol agents alone or in combinations exceeded the fungicide treatment in reducing RBS disease incidence %, the treatments of AMF, AMF + P.f. +T.v. and T.v., respectively achieved the highest yield after the fungicide treatments. Similar results were found by Carvalho *et al*. (2015) when they suppressed rice leaf blast severity *in vivo* by three mycorrhizal extracts of *Waitea circinata*. They suggested the presence of phenolic compounds *i.e*., benzophenones. Besides a wide range of biological properties, including antifungal, antimicrobial, antioxidant and cytotoxic activities were found in the crude extract of *Waitea circinata* En07 (Wu *et al*. 2014). The treatments of AMF only, AMF + P.f. + T.v. and P.f. + T.v. followed the effect of Del cup fungicide in plant height. Sanni, 1976 also, observed better growth in mycorrhizal rice plants (*Gigaspora gigentia*) than the control. Similar results were obtained by Jangde (2013) who found that mycorrhizal rice plants were higher than the nonmycorrizal ones, achieving high productivity. Al-Taweil *et al*. (2009) stated that *Trichoderma* genus has a biocontrol activity as it has an ability to synthesize antagonistic compounds (proteins, enzymes and antibiotics) and growth promoting substances (vitamins, hormones and minerals). On the other hand, Voisard *et al*. (1989) reported that *Pseudomonas fluorescens* is known as a growth promoting rhizobacteria (PGPR) and plays a major role in inducing systemic resistance and control of plant pathogens.

At Gemmiza location, the treatments of AMF, AMF + P.f. and T.v., respectively followed the fungicide treatment in reducing RBS disease incidence % with good effect on other plant parameters. This is in accordance with Mousavi *et al*. (2014) who tested the ability of mycorrhizal fungus, *Piriformospora indica*, in inducing rice plant resistance against the blast disease and noticed an increase in the related defense genes, *i.e*., PR1b, LOX, NPR1 and WRKY85 in the treated plants comparing with the control. However, the treatments of T.v.; AMF + P.f. + T.v. and P.f. + T.v. exceeded the fungicide treatment in yield, Similar results were found by Gupta and Ali (1993) and Secilia and Bagya-raj (1994b) who reported a significant increase in the grain yield, by AMF colonization (*Glomus intraradices & G. fasciculatum*) in wet land rice under both pot and field conditions. Also, Nielsen *et al*. (2002) and Picard (2008) mentioned that the antagonistic activity of *Pseudomonas flouresens* is due to the production of biosurfactant antibiotics and a number of antimicrobial compounds such as 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines (PHZ), pyrrolnitrin (PRN), pyoluteorin (PLT), hydrogen cyanide (HCN). They all encourage the occurrence of defense reaction as a result of accumulation of PR proteins such as chitinase, â-13-glucanes, phenylalanine ammonia lyase, peroxidase, phenolics and phytoalexins (Kloepper *et al*., 1992). Data also was in accordance with Harish *et al*. (2007) who found *Trichoderma* spp. are effective in controlling rice brown spot disease and increasing rice plant growth. Also, Baker (1988) mentioned that *Trichoderma* species are able to colonize the root surface and rhizosphere from the treated seeds, protecting them from fungal diseases and stimulating plant growth and productivity.

Also, Bodker *et al*. (1998) and Cordier *et al*. (1998) reported that mycorrhizal fungi induced systemic resistance in pea and tomato plants infected with *Aphanomyces euteiches* and *Phytophthora parasitics*, respectively.

Pathogenesis-Related Proteins *i.e*., peroxidase (POX), polyphenoloxidase (PPO) and catalase were found to be induced in rice seedlings due to interactions between the tested bioagents and *B. oryzae* under the greenhouse. These enzymes were found to be involved in plant defenses against many pathogens (Abohatem *et al*., 2011; Mousavi *et al*., 2014). Peroxidase is known to have a role in the deposition of lignin and suberin in plant cell-walls (Baaziz and Saaidi, 1988 and Vieira *et al*., 2003). In this study, POX activity increased in AMF + P.f. + T.v., fungicide and P.f. + T.v. treatments after 24h of inoculation then decreased after 48h and raised again after 72h as it occurred in AMF + T.v also. While, the other treatments raised in POX activity after 48h, then decreased after 72h of infection. Thus, these results agreed with those obtained by Massoud and Kamel (2015) who showed an increase in POX activity in aliginated *Macrophomina phaseolina* with both of AMF and *T. viride*. Also, Vidhyasekaran, *et al*. (2001) observed increasing in lignification and peroxidase, PAL and 4CL activities in *P. fluorescens* treated plants in rice against *Xanthomonas oryzae* pv. *oryzae*.

Similar trends also were observed by Jaiti *et al*. (2007) as AMF induced plant protection against *Fusarium oxysporum* f.sp. *albedinis* by increasing peroxidase and polyphenol oxidase activities. These results were also was noticed in our work as PPO activity recorded the best in P.f. + T.v., AMF + P.f. + T.v., AMF and the fungicide treatments, after 24h of infection. Then the activity was fluctuated in all treatments being higher than the check after 48h and 72h. The fungicide was the only treatment that maintained stable till 72h in PPO activity. Ozgonen *et al*., 2009 identified 12 different phenolic compounds in pepper plants by mycorrhiza fungus and *Phytophthora capsici Leonian* pathosystem, which were higher after 6 days after inoculation, but decreased on the 3rd and 9th days later. Catalases are one of the few enzymes that exhibit dual enzyme activity, they play a role of specific peroxidase and their function is to protect cells from toxic effects of hydrogen peroxide (Luhova, *et al*., 2003). Treatments of the fungicide, AMF + P.f. + T.v., P.f. and AMF + T.v. exhibited high values of catalase activity after 24h of infection. Thus, induction of these enzymes inside rice seedlings showed the efficiency of the different bio-control agents and interpreted their role in increasing the resistance. The present results encourage further trails of deep study to use AMF in the future as a new bio- control agent in rice ecosystem alone or in combinations with other common bio- control agents in controlling brown spot disease or other rice diseases.

CONCLUSION

Rice brown spot disease is a world- wide distributed. It affects the grain quality and causes losses in yield. To provide a clean environment, bio- control agents were used in the form of seed dressing as alternative to fungicides. AMF is a promising bio-control agent in rice diseases management. AMF colonized rice roots, also *P. fluorescens* and *T. viride* inhibited the mycelial growth of *Bipolaris oryzae* resulting in deformation and lysis of the pathogen spores. AMF proved efficiency under greenhouse or field trails in disease management weather alone or in combinations with *P. fluorescens* and *T. viride,* in addition to increasing plant height and yield. The bioagent treatments exceeded Del cup fungicide in disease management at Sakha location in contrary to Gemmiza location. Pathogenesis related proteins (PR proteins) *i.e*., POX, PPO and catalase were induced so, plant resistance was enhanced against the pathogen.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

Abo-Elyousr A.M.K.; Hussein M.A.M.; Allam A.D.A. and Hassan A.H.M. 2008. Enhanced onion resistance against stemphylium leaf blight disease, caused by *Stemphylium vesicarium*, by di-potassium phosphate and benzothiadiazole treatments. Plant Pathol. J. 24 (2): 171–177.

Abohatem, M.; Chakrafi, F.; Jaiti, F.; Dihazi, A. and Baaziz, M. 2011. Arbuscular Mycorrhizal fungi limit incidence of *Fusarium oxysporum* f. sp. *albedinis* on Date palm seedlings by increasing nutrient contents, total phenols and peroxidase activities. The Open Horticulture Journal, 4: 10-16.

Addo, E.S.; Yasuda, M.; Lee, C.G.; Kanasugi, M.; Fujii, Y.; Omari, R.A.; Abebrese, S.O.; Bam, R.; Brempong, S.A.; Dastogeer, K.M.G. and Okazaki, S. 2020. Arbuscular mycorrhizal fungi associated with rice (*Oryza sativa* L.) in Ghana: Effect of regional locations and soil factors on diversity and community assembly. Agronomy, 10(4): 559.

Allam, A. I. and Hollis, J. P. 1972. Sulphide inhibition of oxidase in rice roots. Phytopathology, 62(3): 634-639.

Al-Taweil, H. I.; Bin Osman, M.; Abdul Hamid, A. and Yussof., W.M.W. 2009. Optimizing of *Trichoderma viride* cultivation in sub merged state fermentation can be a novel strategy in plant disease management. American Journal of Applied Sciences, 6 (7): 1284-1288.

Angelica, M.; Barbosa, G.; Rehn, K.G.; Menzes, M. and Mariano, R.R. 2001. Antagonism of *Trichoderma sp* on *Cladosporium herbarum* and their enzymatic characterization. Braz. J. Microbiol., 32:98-104.

Baaziz, M. and Saaidi, M. 1988. Preliminary identification of date palm cultivars by esterase isoenzymes and peroxidase activities. Can. J. Bot. 66: 89-93.

Baker, R. 1988. *Trichoderma* spp. as plant-growth stimulants. Biotechnol. 7(2): 97–106.

Balgude Y.S.; Gaikwad A.P. and Kshirsagar C.R. 2017. *Pseudomonas fluorescens*, a potential bioagent for effective management of diseases in organic rice production. J. Rice Res. 10 (2):80-84.

Bodker, L.; Kjoller, R. and Rosendahl, S. 1998 Effect of phosphate and the arbuscular mycorrhizal fungus *Glomus intraradices* on disease severity of root rot in peas (*Pisum sativum*) caused by *Aphanomyces euteiches*. Mycorrhiza 8: 169±174.

Carvalho, M.P.; Rodrigues, F.A.; Silveira, P.R.; Andrade, C.C.L.; Baroni, J.C.P.; Sa´ Paye, H. and Junior, J.E.L. 2010. Rice resistance to brown spot mediated by nitrogen and potassium. J. Phytopathol. 158(3):160-166.

Carvalho, J.C.B.; Sousa, K.C.I.; Brito, D.C.; Chaibub, A.A.; Luzini, A.P.; Côrtes, M.V.C.B.; Filippi, M.C.C.; Kato, L.; Vaz, B.G.; Costa, H.B.; Romão, W. and Araújo, L.G. 2015. Biocontrol potential of *Waitea circinata*, an orchid mycorrhizal fungus, against the rice blast fungus *Tropical Plant Pathology* 40:151–159.

Chakrabarti, N.K. 2001. Epidemiology and disease management of brown spot of rice in

India. In: Major fungal disease of rice: Recent Advances. Kluwer Academic Publishers. pp. 293–306.

Chandlee, M. and Scandalios, J.G. 1984. Analysis of variants affecting the catalase developmental program in maize scutellum. Theoretical and Applied Genetics, 9: 71-77.

Cordier, C.; Pozo, M.J.; Barea, J.M.; Gianinazzi, S. and Gianinazzi-Pearson, V. 1998. Cell defense responses associated with localized and systemic resistance to *Phytophthora parasitica* induced in tomato by an arbuscular mycorrhizal fungus. Mol Plant Microbe Interact 11: 1017-1028.

Devi, O.J. and Chhetry, G.K.N. 2013. Evaluation of antifungal properties of certain plants against *Drechslera Oryzae* causing brown leaf spot of rice in Manipur valley. Inter. J. Sci. and Res. 3 (5):1-3.

Duncan, B.D 1955. Multiple range and Multiple F-test. Biometrics, 11:1-42.

El-Gremi Sh.M.A. and Saleh, M.M. 2013. Controlling *Sarocladium oryzae* by antagonistic microorganisms on rice plants in Nile Delta- Egypt. J.Biol.Chem.Environ.Sci., 8 (4):63-74.

Gaikwad, A.P. and Balgude, Y.S. 2016. Induction of systemic resistance in rice against blast disease by bioagents and chemicals, J. Rice Research, 9 (2): 63-66.

Gomathinayagam, S.; Persaud, S.A. and Rekha, M. 2012. Comparative study of biological agents, *Trichoderma harzianum* and *Trichoderma viride* for controlling brown spot disease in rice. J. Biopest., 5: 28-32.

Gupta, N. and Ali, S.S. 1993. VAM inoculation for wetland rice. Mycorrhiza News 5:5-6.

Harish, S.; Saravavakumar, D.; Radjacommar, R.; Ebenezar, E.G. and Seetharaman, K. 2007. Use of plant extracts and biocontrol agents for the management of brown spot disease in rice. Biocontrol 53(3): 555-567.

Heydari, A. and Pessarakli, M. 2010. A review on biological control of fungal plant pathogens using microbial antagonists. J. Biol. Sci., 10: 273-290.

Hodge, A.; Gosling, P.; Goodlass, G. and Bending, G. 2003. Arbuscular Mycorrhizal Fungi (AMF) in organic farming, Defra Project OF 0333: 1-70.

Iqbal, M.F.; Hussain, M. and Waqar, M.Q. 2015. Evaluation of best fungicide for controlling brown leaf spot in transplanted rice. Int. J. Adv. Res. Biol.Sci. 2(7): 44–48.

IRRI. 2002. Standard Evaluation System for Rice (SES). International Rice Research Institute. Manila, Philippines. 56p.

Jaiti, F.; Meddich, A. and El Hadrami, I. 2007. Effectiveness of arbuscular mycorrhizal fungi in the protection of date palm (*Phoenix dactylifera* L.) against Bayoud disease. Physiol Mol Plant Pathol., 71 (4-6): 166-73.

Jangde, N. 2013. Mycorrhizal Study in Selected Cultivars of Rice. M.Sc. Thesis, The Rajendra Agricultural University, Bihar, Pusa. 111pp.

Khalili, Elham; Sadravi, M.; Naeimi, S. and Khosravi, V. 2012. Biological control of rice brown spot with naïve isolates of three *Trichoderma* species. Brazilian Journal of Microbiology: 297-305.

King, E.O.; Ward, M.K. and Raney, D.E. 1954. Two simple media for the demonstration of phycocyanin and fluorescin. J. Lab. Clin. Med. 44: 301–307.

Kloepper, J.W.; Ryu, C.-M. and Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology, 94:1259-1266.

Kloepper, J.W.; Tuzun, S. and Kuc, J.A. 1992. Proposed definitions related to induced disease resistance. Biocontrol Science and Technology 2: 349-351.

Kumar, S.; Abhilasha, A.L.; Kumar, N.; Jaiswal, S.; Kumar, H.; Kumar, A. and Kumar, M. 2017. Effect of bio control agents and botanicals against Blast of Paddy caused by *Pyricularia oryzae*. International Journal of Chemical Studies; 5(1): 314-318.

Kumar, R.; Kumari, A.; Zacharia, S. and Tiwari, S. 2014. Efficacy of *Trichoderma viride* and *Pseudomonas fluorescens* against Paddy Brown Spot In- Situ. Trends in Biosciences 7(14): 1712-1716.

Luhova, L.; Lebeda, A.; Hedererova, D. and Pec, P. 2003. Activities of amine oxidase, peroxidase and catalase in seedlings of *Pisum sativum* L. under different light conditions. Plant soil and Environ., 49(4):151-157.

Mart´ınez-Medina, A.; Pascual, J.A.; Lloret, E. and Roldan, A. 2009. Interactions between arbuscular mycorrhizal fungi and *Trichoderma harzianum* and their effects on Fusarium wilt in melon plants grown in seedling nurseries. J. Sci. Food Agric. (89): 1843-1850.

Massoud, O.N. 2005. Microbiological and chemical evaluation of compost and its application in organic farming. Ph.D. thesis, Dep. of Biology, Fac. Sci. Minufiya University, pp 179.

Massoud, O. N. and Kamel, S. M. 2015. The inhibitory effects of free and encapsulated arbuscular mycorrhizal fungi and *Trichoderma viride* against charcoal rot (*Macrophomina phaseolina*) on common bean (Phaseolus vulgaris L.). Egyptian Journal of Biological Pest Control, 25(2): 489-497.

Maxwell, D.P. and Bateman, D.F. 1967. Change in the activities of some oxidases in extracts of Rhizoctonia infected bean hypocotyls in relation to lesion maturation. Phytopathology, 57: 132-136.

Morandi, D. 1990. Effect of endomycorrhizal infection and biocides on phytoalexin accumulation in soybean roots. Agric Ecosyst Environ 29: 303-305.

Mousavi, S.H.; Babaeezad, V.; Sharifnabi, B.; Tajic Ghanbari, M. A.; Massah, A. and Alavi, S. M. 2014. Induction of blast disease resistance in rice plants by endophyte fungus *Piriformospora indica* Iran. J. Plant Path., 50(3): 127-129.

Muhanna, N.A.S.; Essa, T.A.; El-Gamal, M.A.H. and Kamel, S.M. 2016. Efficacy of free and formulated arbuscular mycorrhiza, *Trichoderma viride* and *Pseudomonas fluorescens* on controlling tomato root rot diseases. Egyptian Journal of Biological Pest Control, 26(3): 477-486.

Navaneetha, T.; Prasad, R.D. and Venkateswar, R. L. 2015. Liquid Formulation of *Trichoderma* species for management of Gray mold in Castor (*Ricinus communis* L.) and Alternariaster leaf blight in Sunflower (*Helianthus annuus* L.). J Biofertil. Biopestici, 6:1.

Nielsen, T.H.; Sorensen, D.; Tobiasen, C.; Andersen, J.B.; Christophersen, C.; Givskov, M.; Sorensen, J. 2002. Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas spp.* from the sugar beet rhizosphere. Appl. Environ. Microb. 68:3416–3423.

Nisikado, Y.; Miyake, C. (1922). Studies on the Helminthosporiose of the Rice plant. Berichte des Ohara Instituts fur Landwirtschaftliche Biologie, Okayama Universitat 1922. 2(2): 133-194.

Ou, S.H. 1985. Rice disease (2nd ed.). Kew: Commonwealth Mycological Institute. 380 pp.

Ozgonen, H.; Yardimci, N. and Kiliç, H.C. 2009. Induction of phenolic compounds and pathogenesis-related proteins by mycorrhizal fungal inoculations against *Phytophthora capsici* Leonian in Pepper. Pakistan Journal of Biological Sciences 12(17):1181-1187.

Padmanabhan, S. Y. 1973. The Great Bengal Famine. Annu. Rev. of Phytopathol., 11: 11-26.

Pal, K.K. and Gardener, B.M. 2006. Biological Control of Plant Pathogens. The Plant Health Instructor DOI: 10.1094/PHI-A-2006-1117-02.

Pandey, A.K. and Chandel, S.C.R. 2014. Efficacy of Pseudomonas as biocontrol agent against plant pathogenic fungi. Int. J. Curr. Microbiol. App. Sci., 3(11): 493-500.

Picard, C. 2008. Genotypic and phenotypic diversity in populations of plant-probiotic *Pseudomonas spp*. colonizing roots. Naturwisenschaften, 95: 1-16.

Rashed, R.; Hossain, M.; Islam, M. R.; Akter, N; Mazumder, A.R. and Zakaria, M. 2002. Effect of brown spot on the yield and yield contributing characters of different hybrid varieties/ lines of Boro rice. Pakistan, J.Plant Pathology 1(2-4):58-60.

Saleh, M.M. 2002. Biological control of some soil borne diseases on corn. M.Sc. Thesis. Fac. of Agric., Tanta Univ., Egypt, pp 119.

Saleh, M.M. 2012. Studies on the sheath rot disease of rice. Ph.D. Thesis. Fac. of Agric., Kafrelsheikh Univ., Egypt, pp 147.

Sanni, S.O. 1976. Vescular-arbuscular mycorrhiza in some nigerian soils: The effect of *Gigaspora gigantean* on the growth of rice. New Phytol. 77: 673-674.

Secilia, J. and Bagyaraj, D.J. 1994. Evaluation and first-year field testing of efficient vesicular arbuscular mycorrhizal fungi for inoculation of wetland rice seedlings. World Journal of Microbiology and Biotechnology 10: 381-384.

Siqueira, J.O.; Lambais, M.R. and Sturrmer, S.L. 2002. Fungos micorrízicos arbusculares: características, associação simbiótica e aplicação na agricultura. Biotecnol Cienc Desenv 25: 12-21.

Vidhyasekaran, R.; Kamala, N.; Ramanathan, A.; Rajappan, K.; Paranidharan, V. and Velazhahan, R. 2001. Induction of systemic resistance by *Pseudomonas fluorescens* Pfl against *Xanthomonas oryzae* pv. *oryzae* in rice leaves. Phytoparasitica 29(2):155-166.

Vieira, Dos Santos C.; Letousey, P.; Delavault, P. and Thalouarn, P. 2003. Defense gene expression analysis of *Arabidopsis thaliana* parasitized by *Orobanche ramosa*. Phytopathol., 93: 451-7.

Vierheilig, H.; Coughlan, A.P.; Wyss, U. and Piche, Y. 1998. Ink and vinegar, a simple staining technique for Arbuscular-Mycorrhizal Fungi. Applied and Environmental Microbiology, 64 (12): 5004-5007.

Voisard, C.; Keel, C.; Haas, D. and Defago, G. 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J.; 8:351-358.

Wu, S.B.; Long, C. and Kennelly, E.J. 2014. Structural diversity and bioactivities of natural benzophenones. Nat Prod Rep 31:1158–1174.