Egypt. J. Phytopathol., Vol. **47**, No. 1, pp. 175 -197 (2019)
ISSN 1110-0230

Granular Bioactive Formulation of *Trichoderma viride* and Arbuscular Mycorrhizal Fungi for Biological Control of Cumin Wilt Disease

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Cumin wilt caused by *Fusarium oxysporum* f. sp. *cumini* is the most destructive abundant disease, limiting the cumin production in Egypt. The objective of this study was to evaluate the efficacy of granular bioactive formulation of *Trichoderma viride* (GBTV) and arbuscular mycorrhizal fungi (AM) for biological control of cumin wilt disease. Ten *Trichoderma* species were isolated from the rhizosphere of healthy cumin plants and tested in dual culture assay against the growth of *F. oxysporum*. *Trichoderma viride* TC40, *T. hamatum* and *T. koningii* recorded the highest reduction in the growth of the target pathogen. Under greenhouse conditions, use of AM+GBTV formulation minimized *F. oxysporum* incidence and increased plant survival percentages. GBTV treatment alone came the second when compared to infected control. The greatest proportional increases in cumin plant growth were elicited by GBTV formulation and/or AM treatments. The highest level of mycorrhizal root colonization was noticed in the presence or absence of the pathogen. The biochemical parameters of the infected plants with AM or by GBTV treatment showed a pronounced increase in the plant content of total phenols, peroxidase and polyphenoloxidase activities. Under field conditions, AM+GBTV treatment highly reduced disease incidence of wilt symptoms and increased plant survival as equal as chemical fungicide. It also improved plant growth parameters and yield. The suggested formula is highly recommended for Fusarium wilt management and yield production of cumin plants.

Keywords: Fusarium wilt, Trichoderma bioformulation, Arbuscular Mycorrhiza, cumin

The annual herbaceous spice medicinal plant Cumin (*Cuminum cyminum* L.) belongs to family *Apiaceae*. The plant is believed to be a native of the Mediterranean and Near Eastern regions (Deepak *et al*., 2008). Uses of cumin seeds are numerous such as cooking, desserts, cosmetics, perfumes and therapeutic medicine. The typical pleasant aroma of the seeds is due to their volatile oil content, the principal constituent of which is cuminaldehyde (Verma *et al*., 2018).

Cumin crop suffers from a number of diseases. Among the major threats to the cumin is wilt disease caused by *Fusarium oxysporum* f. sp. *cumini* Patel, Prasad

Mathur and Mathur. The disease is one of the most limiting factors for cumin production in several plant growing areas (Tawfik and Allam, 2004 and Bhatnagar *et al*., 2013). The pathogen is soil and seed-borne under severe conditions, up to 70-80% crop losses were observed in many fields. It causes under soil infection, the pathogen is able to produce fungal chlamydospores survive in soil up to 6 years even with the absence of the host plant, which complicates control (Haware *et al*., 1996). Because of their efficiencies, chemical fungicides are extensively used for the management of cumin diseases, however it may gradually developes resistance and potential hazards may also extend to non-target organisms and the surrounding environment as well as, and the human health. Therefore, the biological control using bio agents became a proper alternative option (Harman, *et al*., 2004 and Mahmoud, 2017).

The genus Trichoderma includes prevalent soil-borne fungi with well-known diverse anti-phytopathogen activities, global distribution and reproductive potentiality. Mechanisms of action include rivalry for space and/or nutrients, antibiosis, mycoparasitism, antibiosis, and host-induced systemic resistance (Ghorbanpour *et al*., 2018), synthesizing a large set of enzymes like cellulases, amylases, lipases and pectinases, as well as secondary metabolites such as siderophores (Harman *et al*., 2004). Granular formulation of *Trichoderma* is a unique among other types of formulations because of its long viability as well as easy to be used by the farmers. Therefore, it can be applied in the field (Bharti *et al*., 2017).

Arbuscular Mycorrhizal Fungi (AMF) are soil fungi, obligate endophytes that live in mutualism with roots of 80% of the vascular plants (Kehri *et al.,* 2018). AMF are found in all terrestrial ecosystems with varied extent of pH, salinity, organic matter, and environmental conditions. AMF improve the plant growth and metabolic processes, increases the resistance to drought, salinity, heavy metals, as well as enhances the immunity against various pathogenic mycobiota (Chen *et al.,* 2018). Many fungal pathogens have been extensively bio controlled by AMF. (Tanwar *et al*., 2013 and Zhang *et al*., 2018). The biocontrol mechanisms exerted by AMF comprise direct rivalry with other soil-borne pathogenic fungi for nutrients, space, and colonization sites, changing of the soil microbial composition in the rhizosphere area. AMF may indirectly decrease the losses resulting from the disease by damage compensation, growth improvement and triggering the plant immunity against the phytopathogen attack (Hafez, *et al*., 2013). Abdel-Fattah *et al*. (2011) reported triggering multiple defense-related reactions in bean plants against infection with Rhizoctonia root rot as a result of application of AMF.

Herein, a novel strategy was planned to evaluate the efficacy of AM and a granular bioactive formulation of *Trichoderma* (GBTV), individually and in combinations, for controlling Fusarium wilt disease under greenhouse and field

conditions. Also, study their role in enhancing growth, physiological activities and yield of cumin plants.

Materials and Methods

The Causal organism and Trichoderma species:

The pathogenic fungus *F. oxysporum* was isolated from naturally diseased cumin roots exhibiting typical symptoms of wilt disease obtained from the growing fields of Ismailia Agricultural Research Station, ARC, Ismailia Governorate. The fungus was isolated on potato dextrose agar (PDA) plates (Difco, USA), supplemented with chloramphenicol (5mg/L) and streptomycin sulphate (5 mg/L) and incubated at 25±2°C for 5-7 days. Hyphal tip technique was used to obtain pure cultures of the isolated fungal pathogen. The recovered isolates were maintained onto slants of potato carrot agar medium and kept at 4°C for further studies. The isolated fungi were identified according to their cultural, morphological and microscopically characteristics as described by Booth (1977); Domsch *et al*. (1980) and Watanable (2002).

The collected rhizospheric soil samples of healthy cumin plants were used to isolate *Trichoderma* species using a selective medium of Elad *et al*. (1991). The developed colonies were transferred onto PDA slants and identified after growing them on malt extract agar for two days at 25°C according to Bissett (1991) and Kubicek and Harman (2002). The identification was confirmed at the Mycological Center, Assiut University (AUMC), Egypt.

Antifungal activity of Trichoderma spp. In Vitro using dual culture assay:

The antagonistic potential of ten *Trichoderma* spp. isolated from rhizosphere soil of healthy cumin plants grown in Ismailia governorate was evaluated against *F. oxysporum* f.sp. *cumini* (FOC) using dual culture technique (Dhingra and Sinclair, 1995). Five-mm mycelial disc in diameter of 5-days old culture of the antagonistic fungi was paired against the same sized mycelial disc of FOC 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. All plates were incubated at 25±2ºC until FOC completely covered the PDA surface of the control treatment. The inhibition percentage of radial growth of the pathogen was calculated according to the following equation:

$$\text{Inhibition (\%)} = (R1–R2)/R1 \times 100$$

Where: R1 = radial growth of the pathogen in the control, R2 = radial growth of the pathogen in dual culture (with the antagonist).

The antagonism reaction of Trichoderma spp.:

Based on the previous screening, the antagonism potentiality of *Trichoderma* isolate was evaluated using a scale of 1 to 5 after the 8^{th} day of dual growth

according to (Bell *et al.*, 1982), where, 1=*Trichoderma* overgrowing pathogen, and 5= pathogen overgrowing *Trichoderma*. Pathogens developed from plates of dual cultures were then microscopically investigated and the changes in the mycelium of the pathogen were recorded.

The most effective isolate of *Trichoderma* was identified on malt extract agar (Kubicek and Harman, 2002) and the identification was confirmed at Assiut University Mycological Centre (AUMC), Egypt.

Greenhouse experiment:

The effect of the formulated *T. viride* and/or arbuscular mycorrhizal fungi (AM) was evaluated against *F. oxysporum* under greenhouse conditions.

Inoculum preparation

Preparation of T. viride formula

The inoculum of *T. viride* was prepared using 14 days old culture grown on potato dextrose broth under static conditions as active ingredients. The flask content was put on a blender and blended at 150 rpm for 15 min. The concentration was adjusted to 2.5×10^7 spores/ml using a haemocytometer slide. To produce one kg of formulation, 625 ml of the whole culture mixed with 161g kaolin (Merk) and 1500 g semolina, were blended well with water if needed. The dough was then rolled through a small hand-operated bread machine into sheets, which were folded and extruded 10-15 times at different roller gap settings until it became homogeneous. The dough sheets were then extruded, without refolding, at a narrow gap to yield a one mm thick sheet. The sheets were then placed on aluminum foil and air-dried at ambient laboratory conditions (25±2°C). The dried sheets were ground in a grinder into granules and sieved to specific sizes (2.30 to 2.80 mm).

Preparation of AMF inoculum

A mixture of AM, kindly provided by Prof. Dr. Gamal Abdel-Fattah, professor of Microbiology, Botany Department, Faculty of Science, Mansoura University, was used. The mixture consists of equal proportions of spores of *Glomus mosseae*, *Glomus clarum* and *Glomus aggregatum* in suspension format at 1×10 unit L^{-1}. The mass inoculum was prepared using the pot culture technique on Sudan grass as a host plant. Spores of the previous formula were inoculated on surface-sterilized (10% sodium hypochlorite for 30 min.) sudan grass seeds, which were sown in plastic pots (40 cm diameter), containing previously sterilized sandy loam soil (two successive cycles each of 121°C for 30 min). The plants were grown in the greenhouse (25-30ºC) with a transparent plastic roof and open sides. No fertilizer or any chemical was applied to soil. Thirty days after planting, the sudangrass plants were cut above the soil surface and the soil was dried in the pot, then crushed by hand and used as AM inoculum.

Preparation of F. oxysporum inoculum

Inoculum of *F. oxysporum* was prepared by growing on potato dextrose agar plates and incubated at (25±2ºC) for five days then mycelium plugs were carried on sterilized medium of sorghum: coarse sand: water (2:1:2 v/v) and incubated at room temperature for ten days; to be ready to use.

Greenhouse evaluation of GBTV and/or AM on cumin

Pots were filled with 5 kg/pot disinfected soil; clay: sand (2:1, v/v) and singly infested with the previously prepared pathogens inoculum at the rate of 0.3% (w/w), then regularly watered to near field capacity with tap water and left for one week to guarantee the spread of the fungus. AM was used for inoculation at 40 g/pot while GBTV was used at 1.5 g/pot. Chemical fungicide (F) used through the present investigation as a positive control was Rizolex-T (50% WP). Dosage, used as recommended by the Ministry of Agriculture, was 3g/kg seeds "Balady" and applied as a seed dressing. The treatments applied were;

(1) Negative control without any treatment, (2) Arbuscular mycorrhiza (AM), (3) Granular Bioactive *T. viride* formulation (GBTV), (4) *F. oxysporum* pathogen (P), (5) P + AM, (6) P + GBTV, (7) P + recommended fungicide (F), and (8) P + GBTV + AM. All pots were arranged in a randomized block design and kept in the greenhouse.

Disease assessment

Un-emerged seeds were recorded after 20 days, while percentage of dead seedlings and survived plants were recorded after 45 days from planting.

Growth parameters

Five plants of each treatment were carefully recovered after 60 days from the pathogen inoculation, rinsed with running water to remove any particles of soil and the following parameters were recorded: plant height (cm), numbers of branches and leaves, number of umbels, fresh and dry weights (g) of the plant.

Staining of AM infection root

Samples of 56-days of cumin roots were gently pulled at the end of the greenhouse experiment. Roots were washed several times with tap water to remove all the remaining soil particles, then cut into small segments (0.5-1.0 cm) and heated at 90 ºC for 45 min. in 10% KOH to remove host cells cytoplasm and nuclei. After that, root segments were rinsed in tap water and stained with 0.05% trypan blue (Sigma) in lactophenol (Phillips and Hayman, 1970) for 15 min. at 90 ºC. Then washed by tap water to remove the excess stain. Forty randomly selected root pieces were mounted on slides in lactoglycerol and examined microscopically for the determination of the degree of AM root colonization (Trouvelot *et al.*, 1986).

Evaluation of some physiological activities in cumin plants:

At 28 days of plant growth, extraction and activity of both polyphenoloxidase and peroxidase enzymes were determined using a spectrophotometric method

according to Seleim *et al*. (2014). Total phenolic contents of fresh leaves were determined by using the Folin-Ciocalteu reagent method according to Blainski *et al*. (2013). After 60 days, total chlorophyll, chlorophyll a, chlorophyll b and carotene in cumin leaves were determined according to Robinson and Britiz (2000).

Field experiment:

The experiment was carried out under field conditions at Ismailia Agricultural Research Station, ARC, Ismailia Governorate, Egypt during the summer season (2018). The same treatments previously carried out in the greenhouse were re-applied under field conditions under natural infection to study some growth parameters and yield components of cumin plants. Plots (each 3×6 m^2) were plowed well, all weeds were removed and soil was leveled, dissected to lines. Ammonia nitrate (15.5%, P_2O_3) and potassium sulfate (48%, K_2O) were applied at recommended doses. For soil treatment, GBTV formulation and/or AM mixture at levels of 67.5 g and 1800 g/18 m^2, were applied. The inocula of bio-agents were ground and finally mixed with soil at the time of seed sowing. Cumin seeds "Balady" were planted in hills 5 cm apart on one side of row ridge. After planting, the soil was ridged up around the plants, either along rows or around individual plants as hills. All plots with three replicates were arranged in a randomized block design.

Evaluation of growth parameters and yield components of cumin:

During the flowering stage, ten cumin plants were randomly selected and plant height, number of branches and dry weight of the plants were measured. During the harvest stage, umbels number, weight of seeds per plant and weight of 1000 seed were determined as yield parameters.

Statistical analysis

The statistical analysis software; CoStat version 6.4 (CoHort Software) was used for the analysis of variance (ANOVA) of the data, comparison among means was carried out using Duncan's new multiple range test at probability (P) level ≤ 0.05 (CoStat, 2005).

Results and Discussion

Dual culture assay:

Ten *Trichoderma* isolates obtained from the rhizosphere of healthy cumin plants were tested in dual culture assay against growth of FOC. All *Trichoderma* isolates strongly inhibited the growth of the target pathogen, with different degrees. *T. viride* isolate TC40, *T. hamatum* and *T. koningii* recorded the highest reduction of FOC pathogen (66.76, 65.0 and 61.67%, respectively). All isolates had an antagonism reaction of (1) except *T. atroviride*. The best antagonism reaction (1) was recorded of *T. viride* isolate TC40 on *F. oxysporum*, which was sporulated over the colonies of the target pathogen (Table 1 and Fig. 1). So, it was selected for further studies. Similar trends of the results were observed in the findings of Aghnoom *et al.* (2002)

and Deepak *et al.* (2008) who reported *T. harzianum* as a potential antibiosis and hyper-parasitism against Fusarium wilt of cumin. Also, Abhiram and Masih (2018) screened the antagonistic ability of *Trichoderma viride* against *F. oxysporum* strains under *in vitro* conditions. They revealed that, *T. viride* strongly retarded the growth of target pathogen strains, with different degrees ranging from 14.72 up to 71.00% in sealing agar plate method.

High degree of antagonism reaction observed in Table 1 & Fig. 1 means the occurrence of strong mycoparasitism. *Trichoderma* species were found to produce inhibition zones and cover the colonies with the sporulation of it by different degrees. Mycoparasitism is the major antagonistic mechanism displayed by *Trichoderma* spp., which can attach to and surround by the pathogen, in some cases, form appressoria on the host surface, wherein, *Trichoderma* spp. produce many cell wall degrading enzymes and may be antibiotics that result in parasitism and dissolve of the cell walls, which act as a direct entry of *Trichoderma* hyphae into the pathogen (Vinale *et al*., 2014; Mohamed, 2017 and El-Gazzar, *et al*., 2018). In this respect, Monteiro *et al*. (2010) showed that *T. harzianum* ALL42 was able to completely inhibit *R. solani* and *M. phaseolina* mycelia, by surrounding around the hyphae with the formation of appressoria and hook-like structures. Similar result was obtained by Gajera *et al.* (2012) who indicated that *T. koningi* MTCC 796 and *T. harzianum* NABII were capable of overgrowing and degrading *M. phaseolina* mycelia, coiling around the hyphae with apressoria and hook-like structures. as well as secreat a cell wall degrading enzymes like chitinase, β-1, 3 glucanase, protease and cellulase.

Table (1): Growth of cumin *F. oxysporum* as affected by *Trichoderma* spp. in a dual culture test

Trichoderma isolates	Growth reduction (%)	Antagonism reaction
T. asperellum	57.50 bc	1
T. atroviride	55.83 c	2
T. hamatum	65.00 a	1
T. harzianum TC1	43.33 e	1
T. harzianum TC30	59.17 bc	1
T. harzianum TC5	42.50 e	1
T. koningii	61.67 ab	1
T. viride TC18	49.17 d	1
T. viride TC20	54.17 cd	1
T. viride TC40	66.67 a	1

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

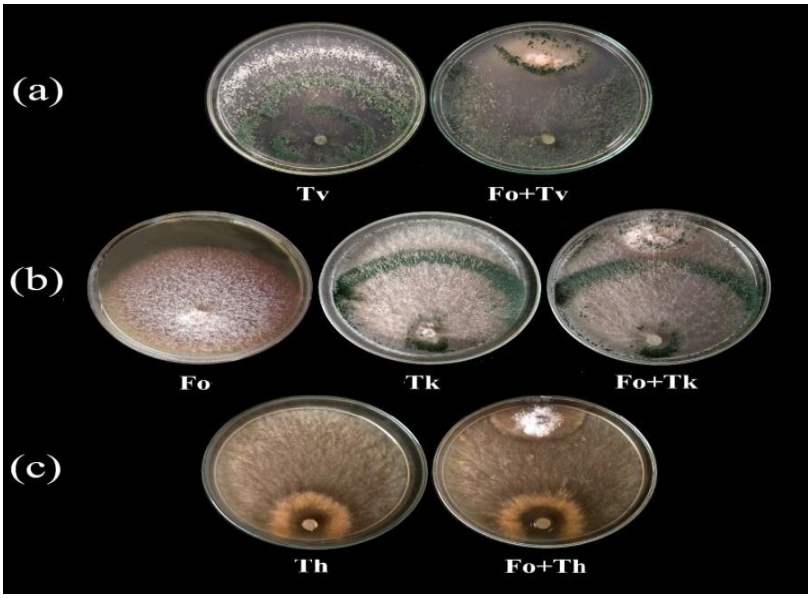

Fig. 1: Dual culture test of three individual *Trichoderma* spp. against *Fusarium oxysprum* f. sp. *cumini* showing overgrowth of *Trichoderma* spp. after 8 days incubation, (a) *T. viride* TC40, (b) *T. koningii,* and (c) *T. hamatum*. Where, Fo: *F. oxysprum,* Tv: *T. viride,* Fo+Tv: *F. oxysprum* and *T. viride,* Tk: *T. koningii,* Fo+Tk: *F. oxysprum* and *T. koningii,* Th: *T. hamatum,* and Fo+Th: *F. oxysprum* and *T. hamatum.*

Greenhouse experiment

Data presented in Table (2) show a significant (P≤.0.05) reduction in percentage of FOC infection either on seeds or at seedling stage as a result of (GBTV) and/or (AM) treatment. Moreover, such disease reduction, however was greatly marked when GBTV combined with AM (10.5 & 8.7%), followed by GBTV (13.2 & 8.7%) and AM (15.5 & 11.7%) and P+F (17.0 & 12.0%), respectively.

In return, the obtained results clearly indicate that the percentage of cumin plants was increased significantly in dual (GBTV+AM), followed by sole treatment (GBTV, AM, F) either in cumin plant infected or uninfected with FOC pathogen.

Effect on growth parameters:

Biotreatments with GBTV and AM enhanced growth parameters of cumin plants over the control either infected or uninfected plants with wilt disease caused by FOC (Table 3). Dual application with GBTV plus AM exceeded that of single application GBTV or AM in this respect. On the other side, it was noted that increases in most growth features of cumin plant did not reach to the significant level especially in the number of umbles (the most economical part of cumin crop).

The obtained results are in agreement with Tanwar *et al*. (2013) who reported appreciable results in increasing growth parameters of tomato plants due to the combined inoculation of AM and *T. viride* under infection stress of Fusarium wilt pathogen. These results were confirmed also by the work of Al-Askar *et al*. (2014) and Ezzat *et al*. (2015). They reported a significant improvement in almost all vegetative growth and tuber yield parameters of Jerusalem artichoke plants due to the use of a mixture of AM and the *Trichoderma* species (in mixture) or antioxidant hydroquinone under natural conditions. The pronounced positive effects on the vegetative growth parameters of plants may be attributed to the fact that plants under inoculation with AM increased utilization of water and nutrients, particularly phosphorus, and that in turn, enhanced the vegetative growth. AM takes up a significant fraction of all plant photosynthetically fixed carbon, while the mycorrhizal plant obtains nutrients, such as inorganic phosphate via the AM hyphae (Pérez-de-Luque *et al.,* 2017; Chen *et al.*, 2018). The inoculation with AM can improve plant growth and biomass accumulation of bioenergy crops (*Galega orientalis* and *Helianthus tuberosus)* even in non-sterile soil containing naturally occurring AM, that is why more than 80% of vascular plant families are capable of forming the AM symbiosis (Van der Heijden and Sanders, 2002).

Table (2): Influence of GBTV, AM treatments or their combinations on the development of *F. oxysporum* wilt of cumin under greenhouse conditions

	Treatment	Seed rot%	Infected seedling%	Plant survival %
Non-Infected	C	7.250 e	2.500 c	90.25 b
	AM	3.500 f	1.750 c	94.75 a
	GBTV	3.500 f	2.000 c	94.50 a
	AM + GBTV	3.500 f	1.250 c	95.25 a
Infected	P	25.00 a	13.50 a	61.50 f
	P + AM	15.50 bc	11.75 ab	72.75 e
	P + GBTV	13.25 cd	8.750 b	78.00 d
	P + F	17.00 b	12.00 ab	71.00 e
	P+AM+ GBTV	10.50 de	8.750 b	80.75 c

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

Where; C = negative control, AM = arbuscular mycorrhiza, GBTV = granular formulated *T. viride*, P = *Fusarium oxysporum* pathogen and F = Recommended fungicide

Table (3): Cumin growth as influenced by GBTV and/or AM treatments under greenhouse conditions

	Treatment	Shoot length (cm)	Root length (cm)	No. of branches plant^{-1}	No. of leaves plant^{-1}	No. of umbels plant^{-1}	Shoot fresh weight g^{-1}	Shoot dry weight g^{-1}
Non-Infected	C	20.00 d	7.250 de	4.00 ab	7.50 a	3.75 ab	1.600 a	0.5120 b
	AM	24.00 ab	9.625 b	4.25 a	7.25 a	3.75 ab	1.683 a	0.5552 ab
	GBTV	23.75 ab	8.750 bc	4.00 ab	7.50 a	3.75 ab	1.743 a	0.5750 ab
	AM + GBTV	24.50 a	11.00 a	4.25 a	7.50 a	4.00 a	1.750 a	0.5950 a
Infected	P	17.30 e	5.550 f	2.50 c	6.25 b	2.50 b	0.800 d	0.2240 d
	P + AM	22.25 bc	8.125 cde	3.25 bc	7.50 a	3.00 ab	1.045 c	0.3135 c
	P + GBTV	22.45 abc	8.700 bcd	4.00 ab	7.75 a	3.50 ab	1.083 c	0.3248 c
	P + F	20.75 cd	7.000 e	3.25 bc	7.00 ab	3.00 ab	0.985 cd	0.3054 c
	P + AM + GBTV	23.00 ab	8.000 cde	3.50 ab	7.75 a	3.25 ab	1.345 b	0.3821 c

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

Where; C=negative control, AM= arbuscular mycorrhiza, GBTV = granular formulated *T. viride*, P = *Fusarium oxysporum* pathogen and F = Recommended fungicide.

The efficacy of *Trichoderma* species as biofertilizers has gained support from multiple reports indicating that when applied to soil, seeds or plant surfaces, it increases the solubility of nutrients as well as the nutrient uptake capacity of the root. This benficial effect was explained by the capacity of Trichoderma fungus to

modulate root architecture and/or through the production of compounds that increase nutrients availability, such as siderophores and organic acids (Singh, *et al*., 2014 and Contreras-Cornejo, *et al*., 2015). Phytohormones *i.e*., Auxins, cytokinins, gibberellins, ethylene and abscisic acid produced by *Trichoderma* spp. have been shown to play important roles in bioagent fungus-plant interactions including plant root architecture, augmenting plant biomass and enhancing of plant resistance to biotic and abiotic stress (Guzmán-Guzmán *et al*., 2019). In this connection, Martínez-Medina *et al.* (2014) reported the positive relation between the bio-control ability of *Trichoderma* spp. against *Fusarium oxysporum* in melon plants and their induction of the phytohormones.

Mycorrhizal root colonization

Effect of the tested treatments on mycorrhizal colonization on the cumin plant was investigated. Data in Figs. (2 and 3) indicate considerable differences among treatments in the mycorrhizal colonization of cumin roots. The single inoculation with AM was the most effective treatment in increasing level of mycorrhizal root colonization under infection conditions by FOC pathogen (73.50%). However, treatments with AM+GBTV mixture came at the second rank (60%). The same pronounced increase in the level of mycorrhizal root colonization compared with non-infected treatments was recorded by a sole AM application, being as 90%, followed by the dual inoculation with AMF+GBTV (66.67%).

Colonized cumin roots in the presence and absence of Fusarium infection were found to have different mycorrhizal structures i.e., hyhae, spores, vesicles and arbuscules. AM established faster, then enhanced growth of cumin roots. Cumin plants may have a rhizosphere that stimulates colonization and subsequent functioning of AMF species by production of exudates. In this connections, physico-chemical soil factors e.g., clay content, total nitrogen and electrical conductivity, as well as phytobiont species, and seasonal climate were reported to have a positive correlation with AMF spore density (Silva-Flores *et al.* 2019). These above results are in agreement with the finding of Mwangi *et al.* (2009) who reported that colonization by AMF and spore density were reduced in combinations of *T. viride* + AMF and *T. virens* + AMF treated pots as compared to AMF alone treated pots. Similar results were obtained by Tanwar *et al.*, (2013) who reported a higher AM colonization and spore number of tomato root by sole inoculation of AMF treatment, compared to its dual inoculation with *T. viride*. But, this decrease has no effect on biocontrol efficiency of bioagents. According to Rousseau *et al.* (1996), antagonistic activity of Trichoderma against AMF is a complex mechanism involving production of antibiotic substances and cell wall degrading enzymes followed by AMF spore wall penetration. These results are also in accordance with Wyss *et al.* (1992) who reported that Trichoderma decreased colonization of soybean roots by *G. mosseae* due to elevated levels of the plant defence compound glyceollin, which is antimicrobial phytoalexin.

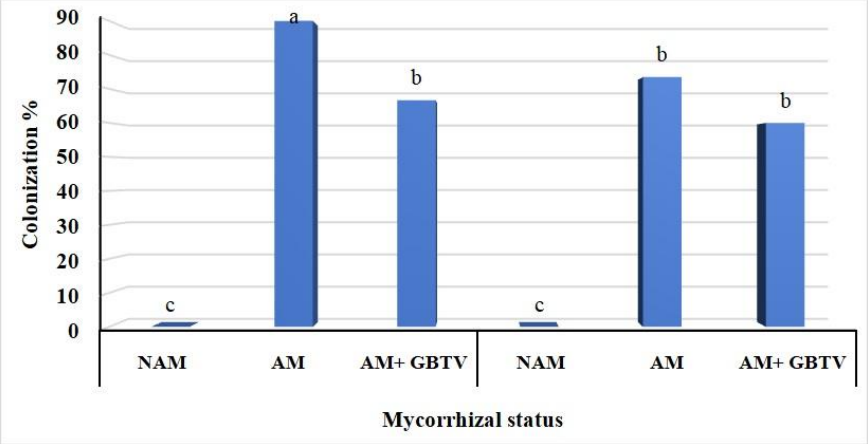

Fig. (2): Mycorrhizal colonization of cumin roots as affected by GBTV and/or AM treatments under greenhouse and *F. oxysporum* infection.

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

Where; C=negative control, NAM= non-mycorrhiza, AM= arbuscular mycorrhiza, GBTV = granular formulated *T. viride* and P=*Fusarium oxysporum* pathogen

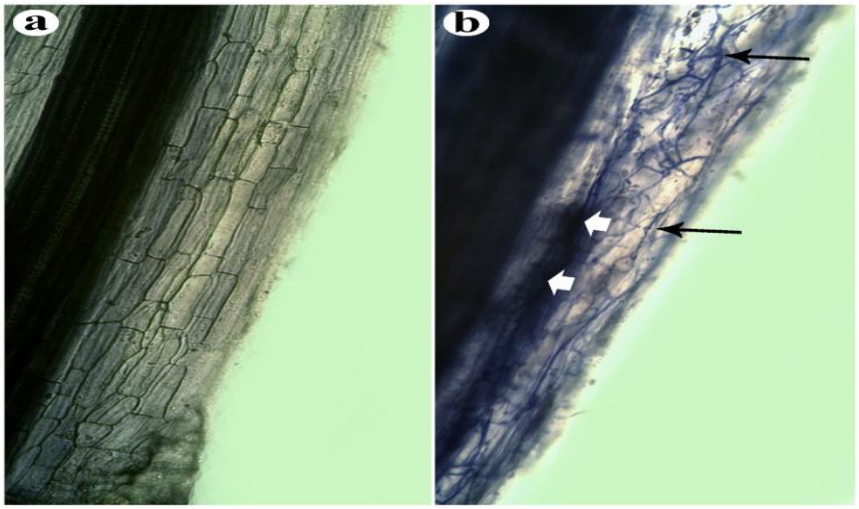

Fig. 3: Light micrographs of cumin roots colonized with AM (×400), (a) non-mycorrhizal root, and (b) colonized root displaying the typical mycorrhizal structures intraradical hyphae (arrows) and arbuscules (arrowheads).

Physiological parameters of cumin as affected by GBTV and/or AM treatments

The physiological response of cumin plants in terms of total polyphenols, polyphenoloxidase, peroxidase enzymes and photosynthetic pigments contents in all investigated GBTV and/or AM treatments is displayed in Table (4). In the presence of FOC, a significant increase was recorded in total phenol contents as response to sole GBTV treatment (197.0 mg/ catechol $100g^{-1}$ FW). Treatment of single AM came after (187.40 mg catechol $100g^{-1}$ FW), in comparison to untreated-infected control (170.91 mg catechol $100g^{-1}$ FW). On the other hand, the peroxidase activity was sharply increased to eight-fold by a single AM application (81.00 Unit. min^{-1} g^{-1}) as compared to untreated-infected control (10.2 Unit. min^{-1} g^{-1}) Polyphenoloxidase increased also by AM+GBTV combination or AM application alone (2.84 and 3.08 Unit. min^{-1} g^{-1}, respectively) as compared to fungicide treatment (0.27 Unit. min^{-1} g^{-1}) and infected control (0.40 Unit. min^{-1} g^{-1}). Without infection stress, similar increases were recorded in both peroxidase and polyphenoloxidase activities (211.6 and 6.44 Unit. Min^{-1} g^{-1}, respectively) by a single AM application as compared to negative control (36.00 and 1.92 Unit. Min^{-1} g^{-1}, respectively). It is well known that total phenol, peroxidase (POD) and polyphenoloxidase (PPO) are physiological parameters reflecting the health condition of the plant. During symbiosis interactions between plant roots and mycorrhizal fungi, many reports emphasized that an induced resistance mechanism rather than increased tolerance or other effects plays a major role in plant defense against a broad spectrum of pathogens (Abdel-Fattah *et al.* 2011; Pérez-de-Luque *et al.* 2017; Mauch-Mani *et al.* 2017; Zhang *et al.* 2018). In this connection, Abdel-Fattah *et al.* (2011) investigated the biocontrol activity of a mixture of AM fungi against Rhizoctonia root rot of common bean, caused by *Rhizoctonia solani*, under greenhouse assay. Plant root colonization by AM fungi minimized both disease severity and disease incidence of the target pathogen as well as significantly increased growth parameters, yield parameters and mineral nutrient concentrations. Also, it exhibited different physical and biochemical changes including improvement plant nutrition, improvement plant growth, increase in cell wall thickening, cytoplasmic granulation, and accumulation of total phenols and induction of the defense-related enzymes (phenylalanine ammonia-lyase, POD and PPO) in plant root. Also, Zhang *et al.* (2018) evaluated the symbiotic efficiency of *Rhizophagus irregularis* CD1 on plant growth promotion and Verticillium wilt disease over a range of 3 to 94% in 17 cotton varieties. In this respect, *Rhizophagus irregularis* colonization sharply inhibited the symptom development of *Verticillium dahliae* pathogen associated with the highest-symbiotic efficiency variety (Lumian 1) and more strongly elevated the expression of pathogenesis-related genes and lignin synthesis-related genes.

Concerning photosynthetic pigments, generally, the presence of the pathogen markedly reduced the content of photosynthetic pigments of cumin leaves. Under infection stress, soil treated with all biotreatments alleviated the harmful effect of the pathogen, in which the highest significant increase of total Chls (38.90 and 46.03%,

respectively) were recorded by sole AM and GBTV applications. Concerning carotenoids, the majority of biotreatments showed in significant increase compared to infected control and recommended fungicide treatments. Without infection stress, the mixture of AM+GBTV presented the highest significant increase of total Chls (45.4%), followed by a single AM or GBTV treatments (21.23 and 24.97%, respectively) as compared to the negative control. These results complies with Tanwar *et al.* (2013) who reported a significant increase of photosynthesis, chlorophyll content and nutrient content in tomato plants as a response to *T. viride* and/or AM treatments.

It is well established that the chlorophyll content is a good parameter reflecting the health condition of the plant, it enhances the efficacy of photosynthetic with a better potential for disease resistance and decrease in photophosphorylation rate usually occurring after infection (Amaresh and Bhatt, 1998). The acquisition of carbon is strongly modulated by the surface area of photosynthesizing leaves; hence, understanding leaf area development is germane to the efforts to increase yield (Kays and Nottingham, 2008).

Table (4): Physiological characteristics of cumin as affected by GBTV and/or AM treatments under greenhouse and *F. oxysporum* infection

Treatment	Total phenols (100mg GAG g^{-1} F.W.)	Peroxidase (Unit. Min^{-1} g^{-1})	Polyphenol oxidase (Unit. Min^{-1} g^{-1})	Photosynthetic pigments (mg g^{-1} F.W)			
				Chl a	Chl b	Chls	Carotenoids
C	168.32de	36.00f	1.92bcd	0.8961d	0.4234ab	1.320c	0.3328ab
AM	173.75cd	211.6a	6.44a	1.1311bc	0.4692a	1.6003b	0.3333ab
GBTV	170.92cde	120.4c	1.40cde	1.2361ab	0.4135abc	1.6496b	0.3439a
AM + GBTV	166.67e	141.2b	1.96bcd	1.3793a	0.5380a	1.9172a	0.3119abc
P	170.91cde	10.20h	0.40e	0.5526e	0.2839bcd	0.8364e	0.3140abc
P + AM	187.40b	81.00d	3.08b	0.8810d	0.2808bcd	1.1618cd	0.2975abc
P + GBTV	197.01a	47.60e	1.04de	0.9376cd	0.2838bcd	1.2214cd	0.3441a
P + F	172.00cde	43.00e	0.27e	0.7889de	0.2069d	0.9458cde	0.2674c
P + AM + GBTV	175.23c	20.00g	2.84bc	0.8481d	0.2544cd	1.1025cde	0.2836bc

Means followed by the different letters within each column are significantly different using Duncan's Multiple Range Test at *P* value of ≤.0.05

Where; C = negative control, AM = arbuscular mycorrhiza, GBTV = granular formulated *T. viride*, P = *Fusarium oxysporum* pathogen and F = Recommended fungicide

Greenhouse evaluation of GBTV and/or AM treatments on the disease development of cumin

Evaluation of GBTV and/or AM treatments under epiphytic conditions for their action on FOC indicates a considerable difference in the disease rating of cumin plants (Fig. 4). In this connection, control treatment showed obvious disease symptoms of seed rot, being 17.75%, with infected plants being 28.0%. Soil treated with the dual inoculation of AM+GBTV showed the highest protection levels and reduced the disease mortality symptoms, to be 60.83% without significant differences between the chemical fungicides (62.67%). Treatments of a sole GBTV came next in this respect, recording 55.76%, followed by a single AM application, being 47.0% compared to infected control treatment.

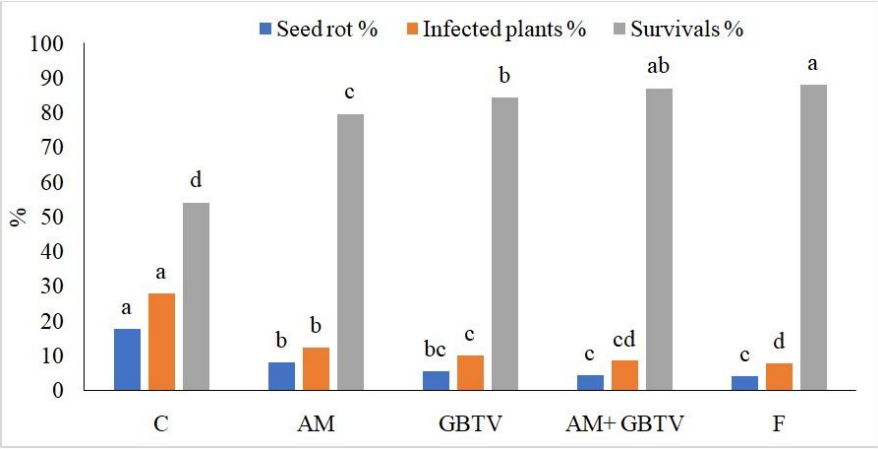

Fig. (4): Disease rating of cumin wilt affected by GBTV and/or AM treatments under greenhouse conditions

Means followed by the different letter within each column are significantly different using Duncan's Multiple Range Test at Pvalue of ≤.0.05

Where; C= control, AM= arbuscular mycorrhiza, GBTV = granular formulated *T. viride* and F= Recommended fungicide

Singh *et al.* (2007) found that seed treatment with *T. harzianum* formulation resulted in good control of wilt and gave higher yield. Deepak *et al.* (2008) indicated that when the bioagents (*T. viride* and *T. harzianum*) were applied in combination with NPK and plant leaves powder (*Datura stramonium*, Azadrichta *indica* and Lantana *camera*) at different quantities, it resulted in better control of cumin wilt incidence over the individual treatments. Sharma *et al.* (2011) observed the effect of bio-priming with antagonistic microflora on wilt incidence of cumin. They found that talc-based formulation of *T. viride* was the best, followed by *Aspergillus versicolor*, *T. harzianum* and *Pseudomonas fluorescens*. Numerous Trichoderma

isolates have been isolated from cumin rhizosphere soils which have shown antagonistic activity against cumin wilt pathogen *F. oxysporum* f.sp. *cumini* (Sharma *et al.* 2012). The isolates of *T. koningiopsis*, *T. asperllum* and *T. harzianum* were identified as potential bioagents in reducing wilt incidence under field conditions. Seed dressing and soil application with *Trichoderma viride*, *Trichoderma harzianum*, and *Pseudomonas fluorescens* lowered wilt disease incidence in cumin. Chawla and Gangopadhyay (2013) found that seed treatment with *Psuedomonas fluorescens* + *T. harzianum* resulted in least disease incidence and better growth, yield contributing parameters and higher seed yield. Also, Bhatnagar *et al.* (2013) found that seed treatment with a combination of carbendazim, *T. viride* and neem seed kernel extract (10%) resulted in minimum wilt incidence (7.2%). The above results are in agreement with Mwangi *et al.* (2009) who reported a pronounced increase in growth of tomato, napier and tea plants due to roots colonization by individual and co-inoculation of *T. harzianum* PS2+AMF. These findings also comply with Dehariya *et al.* (2015) who asserted that co-inoculation of T. *harzianum* + AMF gave significant wilt disease reduction and growth promoted of pigeon pea plant. In this connection, the dual inoculation by *T. harzianum* + AMF, followed by single AMF treatments showed the maximum significant increase in P uptake as compared to untreated infected control with *Fusarium udum* pathogen.

Field evaluation of GBTV and/or AM treatments on the performance of cumin plants

Data (Table 5) indicate that significant variations were recorded in growth parameters on cumin plants as a response to GBTV and/or AM treatments. Soil treated by AM+GBTV combination or a single AM exhibited the highest increase of all plant growth characters (plant height, branches and numbers of umbels plant^{-1} and dry weight plant^{-1}), compared to control treatment. GBTV treatment ranked the third, followed by that treated with chemical fungicide compared with the control. In respect to yield component, no significant differences were observed among all biotreatments in increasing number of umbels plant^{-1}. However, combined treatment of AM+GBTV or a single AM showed the maximum significant increase in weight of seed plant^{-1} (2.146 and 2.063g plant^{-1}, respectively) and weight of 1000 seeds (3.074 and 2.839g plant^{-1}, respectively), followed by fungicide treatment and a single GBTV as compared to control.

The application of GBTV and AM singly or combined significantly lowered seed rot, infected plants and increased survival cumin plants under field condtions (Fig 4). Field results confirmed the greenhouse results. Similar trends of these results were observed in the findings of Tawfik and Allam (2004), they reported that the use of *T. harzianum* alone or in combination with water priming tend to produce a high cumin seed yield per plant, which was associated with a decreased percentage of infection. In this respect, a significant positive correlation coefficient was found between the seed yield and each of the number of main and secondary branches, the number of umbels and the weight of the mature dry plants. These results are also in accordance with Al-Askar *et al.* (2014) and Ezzat *et al.* (2015) who reported that the

combination of AM+hydroquinone (HQ) or AM+ *Tichoderma* spp. (T) gave significant disease reduction in incidence, improved plant growth and yield and enhanced the tuber quality more than the individual agent alone of Jerusalem artichoke plant, under greenhouse and field conditions.

The important component of this investigation was to use the sole and combinations of locally available bioagents: *Trichoderma viride* formulation (GBTV) and arbuscular mycorrhizal fungi (AM) for biological control of cumin wilt. These pronounced positive effects on the vegetative growth parameters of cumin plants may be returned to the fact that, AMF provide soil mineral nutrients (mainly phosphorus and nitrogen), water, and enhance the plant immunity against the attack of Fusarium wilt disease as alternative control instead of fungicide.

Table (5): Growth and yield of cumin as affected by the tested GBTV and/or AM treatments under field conditions

Treatment	Shoot length (cm)	Root length (cm)	Branches number plant^{-1}	Dry weight (g^{-1})	Number of umbels plant^{-1}	Weight of seeds plant^{-1}	Weight of 1000 seeds (g^{-1})
C	29.38 d	17.25 c	4.75 b	3.025 d	16.75 c	1.256 d	1.254 c
AM	37.00 b	19.63 ab	6.00 a	3.813 a	27.50 a	2.063 ab	2.839 ab
GBTV	35.00 c	18.75 b	5.50 ab	3.570 b	28.00 a	1.960 bc	2.722 b
AM+GBTV	39.00 a	21.00 a	6.25 a	3.745 a	29.00 a	2.146 a	3.074 a
F	37.50 b	17.00 c	5.75 ab	3.403 c	22.75 b	1.866 c	2.528 b

Means followed by the different letters within each column are significantly different using Duncan's Multiple Range Test at P value of ≤.0.05

Where; C= control, AM= arbuscular mycorrhiza, GBTV = granular formulated *T. viride* and F= Recommended fungicide

Conclusions

Our results verified the ability of AMF + GBTV formulation as safe, environment-friendly and effective means to fight *F. oxysporum* f. sp. *cumini*, the causal agent of wilt in cumin plant, as well as improve cumin plant growth and its productivity. On the other side, they can be beneficial for reducing the production cost of cumin crops and lowering excessive using of synthetic fungicides as well as biofertilizers, and that helps for decreasing of environmental pollution.

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(Received 31/03/2019;
in revised form 21/04/2019)

تركيبة حبيبية ذات نشاط حيوى من *Trichoderma viride* وفطريات الميكوريزا الشجيرية للمقاومة البيولوجية لمرض الذبول فى الكمون

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قسم بحوث أمراض البذور ، معهد بحوث أمراض النباتات ، مركز البحوث الزراعية ، الجيزه ، مصر

يعد ذبول الكمون الناجم عن الفطر *Fusarium oxysporum* f.sp. *cumini* هو المرض الأكثر تدميرا وانتشارا ، المحدد لانتاج الكمون في مصر .وتهدف هذه الدراسة الى تقييم فعالية تركيبة حبيبية ذات نشطة حيوى من *Trichoderma viride* (GBTV) وفطريات الميكوريزا الشجيرية (AM) للمقاومة البيولوجية لمرض ذبول الكمون. تم عزل عشرة أنواع فطريات *Trichoderma* من منطقة الريزوسفير الخاصة بنباتات الكمون السليمة ،واختبارها ضد نمو المسبب المرضى *Fusarium oxysporum*. حيث سجلت عزلات *T. viride* TC40 ، *T. hamatum* ،و *T. koningii* أعلى انخفاض في نمو المسبب المرض المستهدف. وتحت ظروف الصوبة أدى استخدام تركيبة من AM+GBTV الى خفض تواجد فطر *F. oxysporum* وزيادة عدد النباتات الحية ، بينما جاءت المعاملة المنفردة GBTV فى المرتبة الثانية وذلك بمقارنتها بالكونترول المصاب. وقد سجلت أكبر الزيادات النسبية في نمو النبات بواسطة تركيبة GBTV و /أو معاملة AM. كما لوحظ أعلى مستوى من الاستعمار الجذري بالفطر الشجيرى ، فى حال المعاملة المنفردة AM ، سواء في حالة تواجد أو غياب المسبب المرضى. كما أظهرت القياسات الكيميائية الحيوية فى النباتات المصابة والمعاملة بكل من AM أو GBTV زيادة واضحة في محتواها من الفينولات الكلية ونشاط انزيمى البيروكسيديز والبوليفينول أوكسيديز. وتحت ظروف الحقل أدت المعاملة AM + GBTV إلى انخفاض كبير في حالات الإصابة بأعراض الذبول وزيادة النبات الحية مشابة فى ذلك لنفس مستوى تأثير المبيد الكيماوى وذلك بالمقارنة مع الكونترول ، كما أدت أيضا إلى تحسين نمو وانتاج النباتات. ويوصى بشدة باستخدام التركيبة المقترحة لمقاومة فطر *Fusarium* وإنتاج البذور فى نباتات الكمون .