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Antagonistic potential of certain soilborne fungal bioagents against *Monosporascus* root rot and vine decline of watermelon and promotion of its growth

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Abstract

Monosporascus cannonballus responsible for cucurbits *Monosporascus* root rot and vine decline, is worldwide spread notably in Tunisia. The most appropriate strategies to suppress disease development are those able to reduce the ascospores population using eco-friendly approach treatments. Seven soilborne fungal isolates were tested *in vitro* (by dual confrontation technique) and *in vivo* in the greenhouse as potential bioagents against three virulent *M. cannonballus* isolates. *In vivo* experiments were divided into two assays, preventive and curative treatments. *Trichoderma viride* and *T. harzianum* exhibited high inhibitory activities against *M. cannonballus* mycelial growth with values more than 90%, followed by *Aspergillus niger* (87.89%) and *Paecilomyces victoriae* (80.44%). Furthermore, these two *Trichoderma* spp. when applied preventively and curatively in *in vivo* trials, reduced significantly disease incidence (8.33% and 16.67-20.83%), root disease index (0.79-0.8 and 1.25-1.17), and reduced also ascospores index (1.5-1.54 asc/g of peat) and (2.54-2.42 asc/g of peat), respectively, in comparison with control treatments. Moreover, *T. viride* and *T. harzianum* enhanced the growth development of watermelon plants treated preventively and curatively in the greenhouse. They significantly improved different horticultural measurements with mean values of plant height (76.75-79.83 cm, and 81.83-80.92 cm), root volume (2.39-2.22 cm3, and 1.84-1.88 cm3), above grounds fresh weight (16.07-16.57 g, and 12.84-14.93 g) and dry wt. (2.49-2.6 g, and 2.66-2.70 g), underground fresh wt. (0.725-0.654 g, and 0.717-0.690 g) and dry wt. (0.147-0.214 g, and 0.156-0.152 g). Based on current results, it appears that *Trichoderma* spp. could be employed in soil treatments to promote watermelon plant growth and development.

Keywords: *Monosporascus cannonballus*, Ascospores population, Biocontrol activity, *Trichoderma* spp., *in vitro* antagonism, Horticultural measurement

1. Introduction

Several fungal species cause worldwide plant lesions, rots, loss of secondary; tertiary and feeder roots. These are associated with sudden and uniform collapse of entire fields 1-2 weeks prior to harvest, resulting in total crop loss. The main species associated with these syndromes is *M. cannonballus* (Cohen *et al*., 2000). Indeed, the onset of root infection occurs during early stages of growth, followed by wilting and death of plants later in the season (Cohen *et al*., 2012). *Monosporascus* root rot and vine decline (MRRVD) is particularly severe in arid and semi-arid worldwide cucurbits production. According to Boughalleb *et al*., (2010); Ben Salem *et al*., (2013); Rhouma *et al*., (2018), this disease is prominent in several melon and watermelon-producing areas in Tunisia, and can infect and produce perithecia in different cucurbit roots (Mertely *et al*., 1993).

Investigations on the biology of *M. cannonballus* demonstrated that its ascospores function as the only known survival structures in soil (Stanghellini *et al*., 2000). Furthermore, Waugh *et al*., (2003) pointed that one melon plant infected by *M. cannonballus* could support the production of approximately 400.000 ascospores. Theses authors added that fields are considered problematic when the soil is infected with two ascospores/ g of soil which could be associated with significant crop losses, and concluded that *M. cannonballus* is a monocyclic pathogen. Consequently, this pathogen has a great potential to maintain and/or increase its inoculum build up in the cucurbits rhizosphere (Cohen *et al*., 2012). Waugh *et al*., (2003) reported that management of *M. cannonballus* can be accomplished in case of early detection and quantification of its primary inoculum. In several studies, ascospores were extracted from soils through a physical method based on a sucrose centrifugation technique (Stanghellini and Rasmussen, 1992; Boughalleb *et al*., 2010).

The most appropriate strategies used to suppress plant disease development were those able to reduce the size of pathogen population (Fry, 1982). Control of MRRVD is currently based on integrating different approaches (Cohen et al., 2012; Ben Salem *et al*., 2015a). Farmers used to apply fungicides treatments (Cohen *et al*., 2007; Ben Salem *et al*., 2015b); however, these chemical methods cause hazards to human health and increase environmental pollution. Therefore, alternatives strategies are required for plant diseases control. Biological control is the best alternative and eco-friendly approach for such treatments, defined as total or partial destruction of pathogen populations by other organisms, which occur routinely in nature (Rojo *et al*., 2007). For example, the use of *Trichoderma* spp. (Mennatoullah *et al*., 2010; Boughalleb-M'Hamdi *et al*., 2018), and *Chaetomium* spp. (Sales *et al*., 2007) against MRRVD presented high efficacy when tested under *in vitro* and *in vivo* conditions. Reda *et al*., (2008) revealed that beneficial bacteria are also able to inhibit *M. cannonballus* growth and induce resistance in melon. The objective of the present investigation was to screen certain soilborne fungal antagonist's for abilities to reduce *M. cannonballus* growth under *in vitro* and *in vivo* conditions.

2. Materials and methods

2.1. Fungal cultures

Seven antagonistic fungal isolates namely; *T. viride*, *T. harzianum*, *Penicillium purpurascens*, *Chaetomium globosum*, *Aspergillus niger*, *A. glaucus* and *Paecilomyces victoria*, were isolated from Tunisian cucurbits rhizosphere from a field located at Chott Meriem, (Sousse). Meanwhile, three highly virulent isolates of *M. cannonballus* (MT3, MT4 and MT41) were recovered from cucurbit plant in experimental field of the High Institute of Agronomy, Chott Meriem, Tunisia.

2.2. *In vitro* antifungal potential of fungal bioagents against pathogenic *M. cannonballus*

Antifungal activities of the seven fungal antagonists on radial mycelial growth of the three pathogenic *M. cannonballus* isolates was determined by dual confrontation technique on Potato dextrose agar (PDA) according to Boughalleb-M'Hamdi *et al*., (2017).

Two discs plugs (0.5 cm diameter) of each pathogen and antagonist (4 days-old culture) were transferred separately to a single PDA plate (9 cm diameter). The antagonist plug was placed on one side of the plate (about 2 cm from the edge of the plate towards the center), while the pathogen plug was placed at the other side of the plate opposite to the antagonist plug, leaving a distance of 5 cm between the two plugs. A plug of PDA medium was used as control treatment, while the pathogen plug was placed at the other side. Three replicates (two plates / replicate) for each individual treatment were conducted and the plates were incubated at 28 ± 2°C for five days. The percent of inhibition of pathogen radial mycelial growth was evaluated according to the formula of Hmouni *et al*., (1996):

$$I (\%) = (1- Cn/C0) \times 100$$

Where: Cn is the diameter of radial growth of the pathogen in the presence of the antagonist, whereas, C0 is the diameter of growth of the pathogen in the control treatment.

2.3. *In vivo* antifungal potential of the fungal bioagents

In vivo experiments were divided into two assays of preventive and curative treatments at March, 2015 in the greenhouse. The first preventive assay was carried out by dipping roots of watermelon seedlings (cv. Crimson sweet) 15 days old, into a flask containing a conidial suspension of the different antagonists (3×10^8 cfu\ml each) for 30 min. 24 h before adding 50 ml (9×10^6 cfu\ ml) of each pathogenic isolate, separately. For curative treatments, watermelon seedlings were treated with each antagonist separately 7 days after inoculation of each pathogen, by adding 10 ml of fungal antagonist's suspension to each pot (3×10^8 cfu\ ml). Watermelon seeds were sown in nursery seed trays, with 18 plants per each treatment having 3 replicates. The soil substrate used in this *in vivo* experiment consisted of a mixture of peat and vermiculite (1:1), which was autoclaved twice at 120°C. The pots were then placed in a greenhouse for 60 days. Two controls were performed; one by inoculating the plants with the pathogen only (positive control), while the other with dist. water (negative control). The experimental design was a randomized complete block design (RCBD), and the entire experiment was repeated twice.

Inoculation of the soil substrate with *M. cannonballus* ascospores only was performed as reported by Stanghellini *et al*., (2000); Aleandri *et al*., (2017), with some modifications. *M. cannonballus* isolates were obtained from two-month-old PDA agar cultures, perithecia were washed and then ascospores were sieved (32 µm). Ascospores concentration was adjusted with dist. water (5 ascospores/g of peat). All growth parameters were measured 2 months after inoculation.

The number of symptomatic plants and the total number of plants evaluated in each treatment were used to estimate the disease incidence (DI) of MRRVD, by using the following formula: DI (%) = (Total no. of symptomatic plants/ Total no. of plants) x 100 in reference to Ben Salem *et al*., (2015a).

Watermelon plants were carefully removed after 2 months, the root system was then gently washed in tap water. Roots were inspected visually for evidence of root necrosis, and for observing roots bearing perithecia of *M. cannonballus* containing single spored asci. Each root system was rated for the severity of *M. cannonballus* lesions using a root disease index (RDI) which is an adapted scale from

Aegerter *et al*., (2000), where 0 = no symptoms; 1 = few lesions (covering <10% of root) and secondary root rot is slight; 2 = rot of secondary roots or lesions covering approximately 25% of the root; 3 = lesions covering at least 50% of the root and dead secondary roots; and 4 = general root rot where most of the root is affected.

Soil samples treated with seven fungal antagonists and inoculated with three *M. cannonballus* isolates separately, were air-dried at room temperature and sieved through a 2-mm mesh before their ascospores quantification was accomplished. *M. cannonballus* ascospores were extracted by a method adopted from Boughalleb *et al*., (2010). Initially, sub-samples were sieved through a 250 µm sieve. A 20-g subsample was placed in 200 ml of water, agitated for 5 min. and then passed through two superposed sieves (75 and 30 µm). The collected material was washed and centrifuged at 2000 g for 4 min. The supernatant was discarded and then 30-40 ml of 50% sucrose solution was added to the pellet and then centrifuged again for 2 min. at 2000 g. After centrifugation, the supernatant was passed through a mesh of 30 µm. The materials retained were distributed in Petri dishes. This suspension was stored at 4ºC until being analyzed. The ascospores characteristics and count were done under a stereomicroscope (Nikon SMZ 1000) at a magnification of ×60. After the initial (Pi = 5 asc/g peat) and final (Pf) ascospores count, the following formula was applied to determine the percentage of the ascospores index (AI) according to Ferreira, (2011):

$$AI\ (\%) = (1-Pi/Pf)\ x100$$

After determination of the fresh wt. of above ground (stem + leaves) and underground (root) portions, plant samples were placed in an oven at 60°C for 48 h to determine the dry wt. (Heitholt, 1989). The height of the plant was measured (cm) using a flat ruler. Root volume (cm3) was determined by the immersion method as described by Musick *et al*., (1965), through comparing the levels of water before and after immersing the whole root in a known volume of this water.

2.4. Statistical analysis

Data were analyzed by ANOVA using SPSS version 20.0 statistical software (SPSS, SAS Institute, USA). Differences between treatments were determined by Duncan multiple range test at 5% of significance level.

3. Results

3.1. *In vitro* antifungal efficacy of bioagents against *M. cannonballus* isolates on PDA

The seven antagonistic fungal isolates exerted high significant reduction (<0.01) on radial mycelial growth of *M. cannonballus* isolates after five days of incubation. The linear decrease of growth of all the pathogenic isolates ranged from 95.16% (MT41/*T. harzianum*) to 47.25% (MT3/ *P. purpurascens*) (Table 1). Statistical analysis revealed high significant interactions between *M. cannonballus* isolates and the antagonists (<0.01).

The two *Trichoderma* spp. showed a good ability to limit the mycelial growth of all *M. cannonballus* isolates *in vitro*. In fact, the mycelial growth of the three *M. cannonballus* isolates decreased in presence of *T. viride* and *T. harzianum* with values ranging between 91.93 and 92.11%, respectively (Table 1, Fig. 1). Moreover, *in vitro* assay revealed that *A. niger* possessed a good antifungal potency with mycelial inhibition rate between 88.7% (MT3) and 87.89% (MT4), followed by *Paecilomyces victoriae* (80.44%) and *P. purpurascens* (48.15%) (Table 1). This antagonistic potency was not only on the mycelial growth reduction, but also on the microscopic hyphal aspect. Compared to controls, *M. cannonballus* isolates treated with *Trichoderma* spp. and *A. niger* exhibited a mycelium with strong lyses, induction of mycelial cords via anastomosis between hyphal filaments and mycelium winding (Fig. 1).

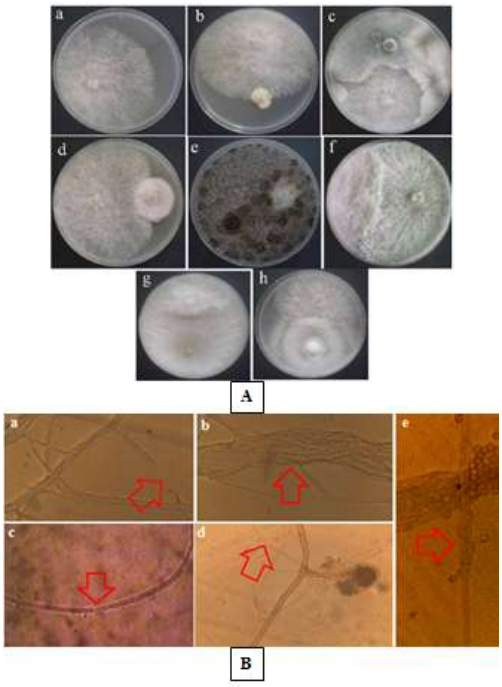

Fig. 1: A: Dual confrontation of *M. cannonballus* after incubation at 28°C for five days on PDA. a) Control. b) *P. purpurascens*. c) *A. glaucus*. d) *C. globosum*. e) *A. niger*. f) *T. harzianum*. g) *T. viride*. h) *Paecilomyces victoriae*; **B**: *In vitro* mycelial interaction between *M. cannonballus* and antagonists (*T. viride*, *T. harzianum* and *A. niger*) (a, b, c, d and e) after 5 days of incubation at 28°C on PDA medium (Gr x 40) (one plate per treatment was illustrated), revealing (a) lyses of pathogen fungal mycelia in the presence of *T. viride*, (c) *T. harzianum* and (d) *A. niger*; (b) transformation into cords and (e) mycelium rolling up.

Table 1: Effect of direct confrontation of seven fungal antagonists on mycelial growth inhibition of three *M. cannonballus* isolates (MT3, MT4 and MT41) after five days of incubation at 28°C

Antagonists spp.	**Mycelial growth inhibition percentage (%)[a]**				**P-value [d]**
	MT3	**MT4**	**MT41**	**Mean**	
T. viride	89.23±0.20a[b]C[c]	91.93±0.24aB	94.62±0.19aA	91.93	<0.01
T. harzianum	89.23±0.29aB	91.93±0.40aB	95.16±0.40aA	92.11	<0.01
P. purpurascens	47.25±0.46eB	48.33±0.24eAB	48.86±0.21fA	48.15	<0.05
C. globosum	56.94±0.37dB	65.82±0.46dA	51.02±0.30eC	57.93	<0.01
A. niger	88.7±0.30a	87.89±0.40b	87.08±0.23b	87.89	≥0.05
A. glaucus	75.24±0.28cA	68.78±0.23dB	69.32±0.17dB	71.11	<0.01
Paecilomyces victoriae	80.08±0.29bB	82.78±0.25cA	78.47±0.25cB	80.44	<0.01
Mean	75.24	76.78	74.93	nd	nd
P-value	<0.01	<0.01	<0.01	nd	nd

[a] Inhibition percent of mycelial growth formula: I (%) = (1- Cn/Co) x 100 where Cn is the mean diameter of the colonies in the presence of the antagonist, and Co the mean diameter of the control colonies, means of nine Petri dishes (three plates per replicate). Duncan's Multiple Range Test, values followed by different letters are significantly different at P≤0.05. [b]Duncan's Multiple Range Test is for comparison of mycelial growth inhibition means among fungal antagonists for the same isolate. Small letters are for means of comparison in the same row. [c] Duncan's Multiple Range Test is for comparison of mycelial growth inhibition means among isolates for the same fungal antagonist. Capital letters are for means comparison in the same column. [d] Probabilities associated with individual F tests. nd: not determined.

3.2. *In vivo* antifungal potential of the fungal bioagents on watermelon plants infested with *M. cannonballus* in the greenhouse

Statistical analysis indicated that plants inoculated with *M. cannonballus* isolates and treated preventively and curatively by the seven antagonistic fungi was highly significant (<0.01). However, no difference was detected between *M. cannonballus* isolates (≥0.05). Watermelon plants seemed healthy with no symptoms of *M. cannonballus* infection (disease incidence = 0%), when treated with *T. viride*, *T. harzianum*, *C. globosum* and *A. glaucus* for MT3 isolate; *T. viride*, *T. harzianum*, *P. purpurascens* and A. niger for MT4, and *P. purpurascens*, *C. globosum* and *A. niger* for MT41 isolate, when used as preventive treatment (positive control = 100%; negative control = 0%).

However, when plants were treated curatively, the antagonists showed varied antifungal activity with mean value of disease incidence 34.72% (ranging between 0 (MT4/ *Paecilomyces victoriae*) and 100% (MT4/ *A. glaucus*), and 40.28% (ranging between 0 (MT3/ *T. viride*) and 75% (MT3/ *Paecilomyces victoriae*; MT3/ *P. purpurascens*)). These obtained results indicated that *T. viride* and *T. harzianum* applied curatively reduced significantly disease incidence, recording the lowest value of 25% compared with positive control (100%) and negative control (0%) (Table 2). These findings were confirmed after 2 months by above ground symptoms on watermelon plants infested with *M. cannonballus* isolates (Fig. 2).

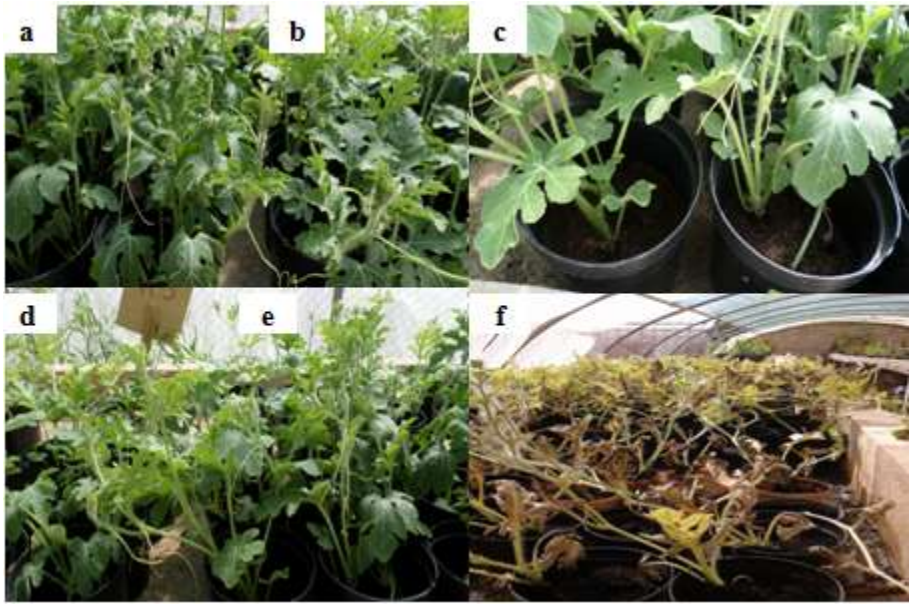

Fig. 2: Above-ground symptoms observed on watermelon plants inoculated with three *M. cannonballus* isolates and treated preventively and curatively by seven fungal antagonists after 2 months in the greenhouse. a: watermelon treated preventively by *T. harzianum* (no collapse); b: watermelon treated preventively by *T. viride* (no collapse); c: negative control (no collapse); d: watermelon treated curatively by *T. harzianum* (initial wilting and reversible turgor loss); e: watermelon treated curatively by *T. viride* (initial wilting and reversible turgor loss); f: positive control (total collapse of all plants).

Table 2: Disease incidence of *Monosporascus* root rot and vine decline (MRRVD) recorded by watermelon seedlings inoculated with three *M. cannonballus* isolates (MT3, MT4 and MT41) and treated preventively and curatively with seven antagonist's *in vivo* assay. Positive and negative controls were performed by inoculating the plant with the pathogen only, and with dist. water, respectively.

Treatments	Preventive treatments				
	MT3	**MT4**	**MT41**	**Mean**	**P-value[b]**
Positive control	100±0a[a]	100±0a	100±0a	100	nd
Negative control	0±0b	0±0c	0±0b	0	nd
T. viride	0±0b	0±0c	25±1.77b	8.33	≥0.05
T. harzianum	0±0b	0±0c	25±1.77b	8.33	≥0.05
P. purpurascens	25±1.77b	0±0c	0±0b	8.33	≥0.05
C. globosum	0±0b	25±1.77bc	0±0b	8.33	≥0.05
A. niger	12.5±1.25b	0±0c	0±0b	4.17	≥0.05
A. glaucus	0±0b	25±1.34bc	25±1.77b	16.67	≥0.05
Paecilomyces victoriae	12.5±1.25b	37.5±1.25b	0±0b	16.67	≥0.05
Mean	16.67	20.83	19.44	nd	nd
P-value	<0.01	<0.01	<0.01	nd	nd
Treatments	**Curative treatments**				
	MT3	**MT4**	**MT41**	**Mean**	**P-value**
Positive control	100±0a	100±0a	100±0a	100	nd
Negative control	0±0c	0±0c	0±0b	0	nd
T. viride	0±0c	25±1.77bc	25±1.77ab	16.67	≥0.05
T. harzianum	12.5±1.25bc	25±1.77bc	25±1.77ab	20.83	≥0.05
P. purpurascens	75±1.77ab	75±1.77ab	37.5±1.73ab	62.50	≥0.05
C. globosum	25±1.77bc	50±1.9abc	50±1.9ab	41.67	≥0.05
A. niger	25±1.77bc	37.5±1.73bc	50±1.9ab	37.50	≥0.05
A. glaucus	50±1.9abc	100±0a	75±1.77ab	75	≥0.05
Paecilomyces victoriae	75±1.77ab	0±0c	75±1.9ab	50	≥0.05
Mean	40.28	34.72	37.50	nd	nd
P-value	<0.01	<0.01	<0.01	nd	nd

[a] Duncan's Multiple Range Test is for disease incidence of MRRVD mean values showing comparison among the three *M. cannonballus* isolates (Means of 6 plants per each of three replicates). [b] Probabilities associated with individual F tests. nd: not determined. The disease incidence of MRRVD was calculated by using the following formula: DI (%) = (Total no. of symptomatic plants/ Total .o. of plants) x 100.

Watermelon plants treated preventively with *P. purpurascens*, *C. globosum*, A. niger and *A. glaucus* showed disease symptoms on roots with relatively high disease severity index values of 1.55, 2.04, 1.88 and 1.84, respectively. However, the lowest value was noted on plants treated with *T. viride* (0.79) and *T. harzianum* (0.80) (positive control = 3.71; negative control = 0) (Table 3). Perithecia of *M. cannonballus* were not observed on watermelon roots treated preventively with *T. viride*, *T. harzianum* and *A. niger*.

Observing the infested roots treated curatively, *T. viride* and *T. harzianum* showed the most significant reduction of disease severity index with values ranged between 1.25 (0.63 (MT4) -2 (MT41)), and 1.17 (0.88 (MT3) -1.5 (MT41)), respectively, whereas, positive control value = 3.71 (Table 3). In addition, perithecia of this pathogen were not observed on roots treated curatively by these two *Trichoderma* spp. However, *M. cannonballus* isolates were pathogenic on watermelon plants in the presence of some antagonists such as; *C. globosum*, *A. niger*, *A. glaucus* and *P. victoriae* with root severity mean values of 2.33, 2.54, 2.17 and 2.42, respectively. These infested watermelon plants showed roots with typical symptoms of MRRVD including; lesions, rots, loss of secondary, tertiary and feeder roots, in

addition to production of perithecia (Fig. 3).

Fig. 3: Roots inoculated with three *M. cannonballus* isolates and treated preventively and curatively with seven antagonist's showing *in vivo* symptoms in the greenhouse. a: *M. cannonballus* alone (positive control); b: uninoculated plants (negative control); c: *T. viride* + *M. cannonballus* after a preventive treatment; d: *T. viride* + *M. cannonballus* after a curative treatment; e: *T. harzianum* + *M. cannonballus* after a curative treatment; f: *T. harzianum* + *M. cannonballus* after a preventive treatment.

Table 3: Root disease index recorded by watermelon seedlings inoculated with three *M. cannonballus* isolates (MT3, MT4 and MT41) and treated preventively and curatively with seven antagonistic fungal species in the greenhouse. Positive and negative controls were performed by inoculating the plants only with the pathogen and with dist. water, respectively.

Treatments	**Preventive treatments**				
	MT3	**MT4**	**MT41**	**Mean**	**P-value[b]**
Positive control	3.58±0.25a[a]	3.54±0.29a	3.24±0.58a	3.45	≥0.05
Negative control	0±0e	0±0f	0±0e	0	nd
T. viride	0.75±0.29d	0.75±0.5e	0.88±0.25d	0.79	≥0.05
T. harzianum	0.63±0.25d	0.88±0.25e	0.88±0.48d	0.80	≥0.05
P. purpurascens	1.88±0.48b	1.38±0.48de	1.38±0.48cd	1.55	≥0.05
C. globosum	1.5±0.71bc	2.5±0.41b	2.13±0.75b	2.04	≥0.05
A. niger	1.25±0.65bcd	2.25±0.65bc	2.13±0.63b	1.88	≥0.05
A. glaucus	1.88±0.25b	1.75±0.25cd	1.88±0.25bc	1.84	≥0.05
Paecilomyces victoriae	1±0.41cd	1.38±0.25de	1.25±0.29cd	1.21	≥0.05
Mean	1.39	1.61	1.53	nd	nd
P-value	<0.01	<0.01	<0.01	nd	nd
Treatments	**Curative treatments**				
	MT3	**MT4**	**MT41**	**Mean**	**P-value**
Positive control	3.88±0.25a	3.75±0.29a	3.5±0.58a	3.71	≥0.05
Negative control	0±0e	0±0f	0±0d	0	nd
T. viride	1.13±0.25d	0.63±0.25ef	2±0.71bc	1.25	≥0.05
T. harzianum	0.88±0.25d	1.13±0.48de	1.5±1c	1.17	≥0.05
P. purpurascens	1.88±0.48c	2.88±1.03b	1.5±0.41c	2.09	≥0.05
C. globosum	2.5±0.71bc	2.25±0.5bc	2.25±0.65bc	2.33	≥0.05
A. niger	3.13±0.25b	1.75±0.5cd	2.75±1.04ab	2.54	≥0.05
A. glaucus	2.88±1.03b	1.5±0.41cd	2.13±0.85bc	2.17	≥0.05
Paecilomyces victoriae	2.13±0.25c	2.13±0.85bc	3±0.41ab	2.42	≥0.05
Mean	2.05	1.78	2.07	nd	nd
P-value	<0.01	<0.01	<0.01	nd	nd

[a] Duncan's Multiple Range Test for root disease index mean values showing comparison among the three *M. cannonballus* isolates (Means of 6 plants per each of three replicates). [b] Probabilities associated with individual F tests. nd: not determined. Root disease index scale of 1- 4; where 0 = no symptoms; 1 = few lesions (covering <10% of root), secondary root rot slight; 2 = rot of secondary roots or lesions covering approximately 25% of the root; 3 = lesions covering at least 50% of the root and dead secondary roots; and 4 = general root rot, most of the root affected.

3.3. *In vivo* potency of fungal biocontrol agents on ascopsores populations of *M. cannonballus*

Application of antagonists preventively and curatively reduced the ascospores population levels (<0.01) (Tables 4 and 5). Results revealed and confirmed the efficiency of both *T. harzianum* and *T. viride* isolates by decreasing significantly the ascospores densities which varied from 1.42 (MT41) - 1.69 (MT3) asc/g of peat, and between 1.29 (MT41) - 1.73 (MT3) asc/g peat, respectively (positive control = 5.5 asc/g of peat). The lowest reduction of ascospores number was registered on plants treated by *T. harzianum* and *T. viride* with means of -260.68, and -243.47%, respectively. The effect of the other antagonists varied between 3.28 (*Paecilomyces victoriae*) - 4.23 (*P. purpurascens*) asc/g peat. The decrease of ascospores index ranged from -52.54 to -18.17%, respectively (positive control = 8.5%).

When plants were curatively treated with *T. harzianum* and *T. viride*, they reduced significantly the ascospores population with values of 2.42 (IA= -109.55) asc/g of peat, and 2.45 (IA= -110.24) asc/g of peat, respectively. However, the other antagonists expressed less efficiency with respect to ascospores densities varying between 3.76 (*P. purpurascens*) asc/g of peat - 4.61 (*Paecilomyces victoriae*) asc/g of peat. The reduction percent of *M. cannonballus* ascospores ranged from -33.3 and -8.46% for *P. purpurascens* and *Paecilomyces victoriae*, respectively (positive control = 5.42 asc/g of peat; IA of positive control = 7.25%) Tables (4 and 5).

3.4. *In vivo* potency of fungal biocontrol agents in promoting growth parameters of watermelon plants infested with *M. cannonballus*

The interaction between *M. cannonballus* and the seven antagonists was significant (p<0.05). However, there were no significant differences between *M. cannonballus* isolates. Growth promotion results for watermelon plants treated preventively are presented in (Tables 4 and 5). All treatments differed significantly from positive control. The best treatment was the combination of *T. harzianum* and *T. viride* with *M. cannonballus* isolate (MT4), which showed significant increase in growth parameters of aboveground parts, compared with the other treatments. The values of the aboveground fresh wt. were about 18.138 and 16.5 g, plant height were of 79 and 83 cm, however, aboveground dry wt. with MT3 were (2.65 and 6.52 g). After 9 weeks in the greenhouse, the root system was collected from all treatments and checked. Both MT3 and MT4 isolates produced fewer roots of infested plants. The underground fresh, dry wt. and root volume values for plants treated with *T. viride* ranged between 0.688 g (MT41) and 0.758 g (MT3), from 0.138 g (MT3) to 0.125 g (MT4 and MT41), and from 2.025 cm3 (MT3) to 2.825 cm3 (MT41), for the three growth parameters, respectively. For *T. harzianum*, results revealed low difference compared with the previous values. The improvement rates of the three growth parameters compared with the negative and positive controls were 98% - 260% for underground fresh wt., 87.5 % - 400% for underground dry wt., and 102% - 400 % for root volume, respectively. Watermelon plants treated curatively showed an increase of the above and underground growth parameters compared with the positive control, however, there were a slight difference for plants treated preventively.

Table (4): Ascospores population dynamics recorded by watermelon seedlings inoculated with three *M. cannonballus* isolates (MT3, MT4 and MT41), and treated preventively and curatively with seven fungal biocontrol agents *in vivo* assay. Positive control was inoculated with the pathogen only.

Treatments	Preventive treatments				
	MT3	**MT4**	**MT41**	**Mean**	**P-value[c]**
Positive control	5.29±0.23a[a]	5.96±0.51a	5.25±0.6a	5.5	≥0.05
T. viride	1.73±0.06eA[b]	1.48±0.32dAB	1.29±0.06eB	1.5	<0.05
T. harzianum	1.69±0.28e	1.5±0.61d	1.42±0.29e	1.54	≥0.05
P. purpurascens	4.23±0.09b	4.26±0.08b	4.21±0.14b	4.23	≥0.05
C. globosum	3.83±0.1c	3.79±0.13bc	3.76±0.13c	3.79	≥0.05
A. niger	3.79±0.21c	3.79±0.45bc	3.68±0.13c	3.75	≥0.05
A. glaucus	3.78±0.13c	3.75±0.09bc	3.71±0.19c	3.75	≥0.05
Paecilomyces victoriae	3.21±0.13dB	3.43±0.03A	3.21±0.13dB	3.28	<0.05
Mean	3.44	3.5	3.32	nd	nd
P-value	<0.01	<0.01	<0.01	nd	nd
Treatments	**Curative treatments**				
	MT3	**MT4**	**MT41**	**Mean**	**P-value**
Positive control	5.41±0.23a	5.66±0.51a	5.20±0.53a	5.42	≥0.05
T. viride	2.58±0.23e	2.66±0.25e	2.1±0.46c	2.45	≥0.05
T. harzianum	2.7±0.14eA	2.26±0.19fB	2.29±0.26cB	2.42	<0.05
P. purpurascens	3.71±0.11d	3.83±0.1d	3.73±0.13b	3.76	≥0.05
C. globosum	4.3±0.13c	4.35±0.37bc	4.09±0.68b	4.25	≥0.05
A. niger	4.08±0.22c	4.16±0.05cd	4.26±0.06b	4.17	≥0.05
A. glaucus	4.28±0.09c	4.29±0.05c	3.83±0.95b	4.13	≥0.05
Paecilomyces victoriae	4.65±0.11bA	4.69±0.09bA	4.5±0.07abB	4.61	<0.05
Mean	3.96	3.99	3.75	nd	nd
P-value	<0.01	<0.01	<0.01	nd	nd

[a] Duncan's Multiple Range Test for ascospores population means in comparison among the three *M. cannonballus* isolates treated with different antagonistic fungal spp.; Small letters are for means of comparison of the different antagonists in the same column. [b] Duncan's Multiple Range Test is for ascospores population means in comparison among the seven antagonistic fungal spp. for the different *M. cannonballus* isolates; Capital letters are for comparison of means in the same row (Means of 6 plants per each of three replicates). [c] Probabilities associated with individual F tests. nd: not determined.

Indeed, *T. harzianum* presented a good improvement of the above (14.93 g) and underground (0.717 g) fresh wt., and above and underground dry wt. (2.7 and 0.156 g, respectively). *Trichoderma* treatments were very effective against *M. cannonballus* infested watermelon plants; the severity of infection was reduced and the growth parameters of these plants improved as well.

4. Discussion

The control of soilborne pathogens was difficult as they produce viable structures such as ascospores which were resistant to adverse environmental conditions (Cohen *et al*., 2000). However, Rojo *et al*., (2007) pointed that the misuse of fungicides to manage these pathogens caused enormous problems to ecosystem and human's health.

Table 5: Percentage of *M. cannonballus* ascospores index recorded by watermelon seedlings inoculated with three *M. cannonballus* isolates (MT3, MT4 and MT41), and treated preventively and curatively with seven fungal biocontrol agents *in vivo* assay. Positive controls were inoculated with the pathogen only.

Treatments	Preventive treatments				
	MT3	**MT4**	**MT41**	**Mean**	**P-value[c]**
Positive control	5.48±0.5a[a]	16.11±0.74a	3.91±0.79a	8.5	≥0.05
T. viride	-190.16±0.82dA[b]	-251.19±2.17bAB	-289.07±1.11cB	-243.47	<0.05
T. harzianum	-202.51±1.77d	-317.39±1.17b	-262.15±2.15c	-260.68	≥0.05
P. purpurascens	-18.38±0.39ab	-17.33±0.36a	-18.8±0.51ab	-18.17	≥0.05
C. globosum	-30.79±0.47bc	-32.12±0.52a	-33.01±0.54ab	-31.97	≥0.05
A. niger	-32.34±0.69bc	-33.32±0.95a	-36.19±0.56ab	-33.95	≥0.05
A. glaucus	-32.57±0.54bc	-33.39±0.45a	-34.95±0.67ab	-33.64	≥0.05
Paecilomyces victoriae	-55.83±0.62cB	-45.99±0.28aA	-55.81±0.60bB	-52.54	<0.05
Mean	-61.9	-79.40	-80.67	nd	nd
P-value	<0.01	<0.01	<0.01	nd	nd
Treatments	**Curative treatments**				
	MT3	**MT4**	**MT41**	**Mean**	**P-value**
Positive control	7.49±0.5a	11.11±0.74a	3.15±0.75a	7.25	≥0.05
T. viride	-95.23±1.00e	-89.06±1.05d	-146.44±1.79b	-110.24	≥0.05
T. harzianum	-85.52±0.75e	-122.15±1.08e	-120.98±1.33b	-109.55	≥0.05
P. purpurascens	-34.77±0.5d	-30.78±0.45c	-34.35±0.54a	-33.3	≥0.05
C. globosum	-16.36±0.47bc	-15.52±0.75bc	-24.89±1.14a	-18.92	≥0.05
A. niger	-22.95±0.63c	-20.13±0.29bc	-17.32±0.33a	-20.13	≥0.05
A. glaucus	-16.99±0.38bc	-16.63±0.28bc	-39.32±1.7a	-24.31	≥0.05
Paecilomyces victoriae	-7.57±0.4bA	-6.69±0.35bA	-11.13±0.33aB	-8.46	<0.05
Mean	-30.21	-32.21	-43.48	nd	nd
P-value	<0.01	<0.01	<0.01	nd	nd

[a] Duncan's Multiple Range Test for percentage of *M. cannonballus* ascospores index mean in comparison among the three *M. cannonballus* isolates treated with three different antagonistic fungal spp.; small letters are for means comparison of the different antagonist's in the same column. [b] Duncan's Multiple Range Test is for percentage of *M. cannonballus* ascospores index mean in comparison among the seven antagonistic spp. for the different *M. cannonballus* isolates; capital letters are for comparison of means in the same row (Means of 6 plants per each replicates of three). [c] Probabilities associated with individual F tests. nd: not determined. After the initial (Pi = 5asc/g peat) and final (Pf) ascospores count, the following formula was applied to determine the percentage of the ascospore index (AI): AI (%) = (1-Pi/Pf) x100

Biological control involves the use of one living organism to control another, and this management technology has received much attention in recent times. The number of biocontrol agents (BCAs) registered for use is relatively low, although their application was successful and proved to cause enhancement in crop growth (Ben Salem *et al*., 2016).

In the current study, in dual culture *in vitro* assays using several fungal genera such as; *Trichoderma*, *Penicillium*, *Chaetomium*, *Aspergillus* and Paecilomyces spp. as BCAs against the tested *M. cannonballus* isolates, revealed that *Trichoderma* spp. inhibited the growth of all these pathogenic isolates. In fact, the radial mycelial growth of the three *M. cannonballus* isolates decreased in presence of *T. viride* and *T. harzianum* with values ranging between 91.93 and 92.11%, respectively. These *in vitro* results on the efficacy of BCAs were similar to those reported by other authors such as Medeiros *et*

al., (2006). Zhang *et al*., (1999) reported that *T. virens* exhibited *in vitro* antifungal activity by inhibiting mycelial growth of *M. cannonballus* and other soilborne pathogens such as *Didymella bryoniae*, *Macrophomina phaseolina* and *Phomopsis cucurbitae*. *T. album* isolates significantly suppressed the growth of *M. cannonballus* and it subsequently overgrew the pathogen (Zhang *et al*., 1999), while, *Bacillus megaterium* was less inhibitive (Mennatoullah *et al*., 2010).

El-Kolaly and Abdel-Sattar, (2013) revealed that all the tested *Trichoderma* spp. inhibited the growth of *Fusarium solani*, *M. cannonballus*, *Pythium aphanidermatum* and *Rhizoctonia solani*. *T. ressei* inhibited fungal growth significantly more than the rest of isolates including; *T. pseudokoningii*, *T. viride* and *T. harizanum*. Recently, Rhouma *et al*., (2015) added that many microbial antagonists (i.e. *T. viride*, *T. harzianum*, *P. purpurascens*, *P. digitatum*, *A. flavus*, *A. niger*, *A. brevipes*, *A. glaucus*, *Gliocladium catenulatum* and *G. virens*) were prevalent in Tunisian cucurbit rhizosphere soil, and possessed biocontrol activities against *M. cannonballus* with mycelial inhibition rate above 70%. Results of our study demonstrated that the tested fungal bioagent's modes of action in confrontation with *M. cannonballus* include; antibiosis, lysis of fungal cell wall, competition and hyperparasitism in accordance with previous results of Zhang *et al*., (1999).

Compared with the other BCA's applied preventively in the presence of *M. cannonballus*; *T. harzianum* and *T. viride* exerted highly significant antagonistic potency, showing the lowest values of disease incidence (8.33%), root disease index (0.80 and 0.79), ascospores dynamics population (1.54 and 1.5 asc/g of peat), and reduction of percent of *M. cannonballus* ascospores (-260.68 and -243.47%), respectively. Moreover, with their presence the agronomic measurements have been enhanced with respective values of plants height (76.75 and 79.83 cm), root volume (2.39 and 2.22 cm3), above ground portions fresh wt. (16.57 and 16.07 g) and dry wt. (2.49 and 2.6 g), underground portions fresh wt. (0.654 and 0.725 g) and dry wt. (0.147 and 0.214 g), respectively, compared with control treatments.

Current *in vivo* assay results were in accordance with other studies such as Sanz *et al*., (1998), who reported that *Trichoderma* spp. exhibited high antifungal activity against *Monosporascus* sp. and *Acremonium cucurbitacearum*. According to Zhang *et al*., (1999), *T. virens* colonized the root systems of muskmelon plants, significantly reduced *M. cannonballus* colonization of roots, and suppressed disease severity of seedlings by seed treatments. In another study of El-Kolaly and Abdel-Sattar, (2013), treatments with *T. harzianum* and *T. ressei* have not only reduced the incidence of MRRVD, but also reduced the *M. cannonballus* root invasion, suggesting that BCAs were limiting the pathogen infection. Ben Salem *et al*., (2016) pointed that among six antagonists evaluated for biocontrol potential against *M. cannonballus*, only *T. viride* and *T. harzianum* significantly reduced disease incidence and severity index after a preventive treatment through soil drenching. Indeed, success of the preventive applications could be attributed to the hyperparasitism of the BCAs in the plant rhizosphere, which inhibited the root infection by soilborne pathogens and reduced their inoculums build up (Rini and Sulochana, 2007). *Trichoderma* spp. was demonstrated to have potential in *M. cannonballus* disease management for *in vitro* and *in vivo* assays (Pastrana *et al*., 2016; Boughalleb-M'Hamdi *et al*., 2018).

The increasing numbers of studies have contributed to unveiling the molecular basis of the plant-*Trichoderma* interaction, and the beneficial effects of *Trichoderma* spp. to plants. Some selected *Trichoderma* strains were shown to have direct positive effects on plants such as; increasing their growth potential and nutrient uptake, fertilizer use efficiency, percentage and rate of seed germination, in addition to stimulation of plant defences against biotic and abiotic stresses. It has been reported by Segarra *et al*., (2009) that *Trichoderma* spp. can

activate induced systemic resistance (ISR) in plants, a mechanism triggered after root colonization by nonpathogenic rhizobacteria or fungi and is regulated by a specific signal transduction cascade.

Trichoderma spp. are also known to produce a large number of antibiotics including; trichodermin, trichodermol, polyketides, peptaiboils, sesquiterpenes, and steroids, all these active compounds are known to promote plant growth besides having biocontrol potential (Harman *et al*., 2004). Later, Müller *et al*., (2013) added that these fungi are prolific producers of a number of secondary metabolites with pharmaceutical and biotechnological significance that involve; non-ribosomal peptides, peptaibols, poliketides, pyrones, siderophores, beside volatile and non-volatile terpenes. For these reasons, they are major sources of many biofungicides and biofertilizers (Kaewchai *et al*., 2009). The germination of *M. cannonballus* ascospores and subsequent attachment to roots, occurs exclusively only in the cucurbits rhizosphere. According to Stanghellini *et al*., (2010), the interaction of *M. cannonballus* with susceptible cucurbits roots appears to be strongly related to the microbial composition in the rhizosphere.

Conclusion

Trichoderma spp. applied preventively and curatively showed significant effect on watermelon plants infested with *M. cannonballus*, and could be recommended for biocontrol use. *T. viride* and *T. harzianum* allowed not only the protection of plants, but also the improvement of the agronomic parameters including better axial growth and greater root biomass. Based on the current results, it is deduced that tested *T. viride* and *T. harzianum* could be employed in soil treatments as BCA's to induce cucurbits systemic resistance, through a specific signal transduction cascade. The systemic resistance induction of cucurbits by *Trichoderma* spp. against *M. cannonballus* is a subject of future research.

Conflict of interests

The authors declare no conflict of interests regarding this article.

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