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Combination of biopesticide formulations to manage *Meloidogyne incognita* and *Fusarium oxysporum* f. sp. *cepae* disease complex in onion

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ABSTRACT: Biopesticide formulations of *Bacillus pumilus* (BPS4) and *Pseudomonas monteilii* (PMS2) were tested in combination with *Trichoderma harzianum* (IIHR-TH2) against *Meloidogyne incognita* (Kofoid and White) Chitwood and *Fusarium oxysporum* f. sp.*cepae* Schlecht (FOC) infecting onion under nursery and field conditions. Seed and soil treatment with biopesticides was done for nursery experiment at 20 g kg^{-1} seeds and 6 g kg^{-1} coco-peat, respectively. For field trials, biopesticide enriched vermicompost was applied to the beds at 50g m^{-2}. Application of biopesticides significantly reduced the disease incidence in nursery experiment. Combination treatments of biopesticides were more effective and reduced the nematode infestation (47.6% to 71.4%) as well as disease incidence of FOC (63.5% to 72.0%), in the field experiment. There was a significant increase in the yield (26.1% to 28.8%) of the crop over control. Thus, the results outline the potential of combination treatments of *B. pumilus*, *P. monteilii* and *T. harzianum* in the production of healthy onion seedlings under nursery conditions whereas increased yield and the management of *M. incognita* and FOC complex under field conditions.

Keywords: *Bacillus pumilus*, *Fusarium oxysporum* f.sp. *cepae, Meloidogyne incognita,* Onion, *Trichoderma harzianum.*

INTRODUCTION

Onion (*Allium cepa* L.; Family: Amaryllidaceae) is the most widely cultivated species of the genus *Allium*. It is an important fresh market vegetable crop with an annual production of 88.5 million tonnes worldwide and 19.4 million tonnes in India (FAOSTAT, 2017). Onion is highly susceptible to diseases at seedling and bulb development stages. Due to the relatively slower growth rate at the early stage of development, onion seedlings are prone to diseases right from germination till the first true leaf stage. High infestation of pathogens at this stage leads to production of impaired seedlings and in severe cases cause stand losses to the crop by killing the seedlings. Infestation by root-knot nematode (RKN) *Meloidogyne incognita* (Kofoid and White) Chitwood leads to failure of crop by impaired development of seedlings causing stunting or killing of the crop; a patchy stand development is therefore characteristic of heavy nematode infestation (Noling 1997). Seedling loss up to 20-40% has been reported due to *M. incognita* infestation (Anonymous 1997). Fusarium basal rot caused by *F. oxysporum* f. sp.*cepae* (FOC) is another disease which can kill seedlings during early stages in the field (Cramer 2000; Saxena and Cramer 2009). The association of plant parasitic nematodes with pathogenic fungus is reported to be highly devastating producing greater loss than caused by either alone (Francl and Wheeler 1993; Abdel-Momen and Starr 1998; Reddy 2011).

Earlier studies on biological control in onion have reported the, use of *Bacillus subtilis*, *Pseudomonas fluorescens* and *Trichoderma* spp. individually or in combination against white rot (Utkhede and Rahe 1983; Sallam *et al.* 2009), onion wilt (Sharifi and Ramezani 2002), onion leaf spot (Kohl *et al.* 2003), leaf blight (Gent and Schwartz 2005) and basal rot (Rajendran and Ranganathan 1996; Coskuntuna and Ozer 2008). In the present study combination formulations were evaluated for the management of RKN and FOC disease complex in onion.

Use of combination bioformulations with diverse mode of action is widely recommended for controlling disease complex involving association of various phytopathogens (Akhtar and Siddiqui 2007, 2008; Rao *et al.* 2012; Sowmya *et al*. 2012, 2013, 2015; Rajinikanth *et al.* 2013). This brings down the use of chemical pesticides and the cost of crop protection. Consortia biopesticides

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have multiple and holistic applicability in protecting plant health; promoting plant growth; strengthening plant-microbe associations under stress, pollutant, or contaminant-affected regions; and protecting plants from the attack of phytopathogens through biological control (Arora *et al.* 2013). Also, synergistic effects of bioagents in combined treatments have been reported (Kerry 2000; Siddiqui and Shaukat 2004).

Therefore, the objective of this investigation was to standardize a method of producing bio-agent colonized seedlings of onion using bioagents *viz. Bacillus pumilus*, *P. monteilii* and *T. harzianum*, which would help in the management of RKN and FOC disease complex in onion both in the nursery and field. It was hypothesized that the seedlings with roots colonized by the bioagents would carry the bioagents to the field, which, in turn, would enrich the field soil with bioagent propagules and subsequently develop a disease suppressive soil. The effect of bioagent formulations individually and in combination on the crop yield and disease complex incidence was also assessed through the field experiments.

MATERIALS AND METHODS

Nematode culture maintenance

The culture of root knot nematode *M. incognita* host race 2 was maintained at Nematology lab, ICAR-Indian Institute of Horticultural Research, Bengaluru in tomato CV Arka Samrat. The identity of females of *M. incognita* was confirmed through perineal cuticular pattern (Sasser and Carter 1982). Eggs of *M. incognita* were extracted from the culture plants from infected tomato plants maintained at Nematology lab, ICAR-IIHR by agitating the roots in 0.05% NaOCl for 2–3 min (Hussey and Barker 1973). The eggs were then collected and rinsed with sterile water on nested 150 and 25 μm pore sieves. The egg masses were incubated and second stage juveniles (J2) that emerged from the eggs on a sieve were collected daily for 5 days (Southey 1986) and used for further experiments.

Bioformulations

Talc based formulations of *B. pumilus* (BPS4), *P. monteilii* (PMS2), *T. harzianum* (IIHR-TH2 ITCC No. 222) – 1% W.P. ; combination formulations of *B. pumilus* (BPS4) + *T. harzianum* (IIHR-TH2 ITCC No. 222) – 1% W.P., and *P. monteilii*(PMS2) + *T. harzianum* (IIHR-TH2 ITCC No. 222) – 1% W.P. were developed at Nematology laboratory, ICAR-IIHR, Bengaluru.

Nursery experiment

Onion seeds (cv. Nasik Red) were sown in seedling protrays (98 wells, 35 x 33 x 22 mm, Velkan Engineering Pvt, Ltd.) filled with sterilized cocopeat (UAS- GKVK, Bengaluru). Biopesticide treatments and inoculation of pathogens were done prior to sowing. Three individual biopesticide formulations *viz. B. pumilus* (BPS4), *P. monteilii* (PMS2) and *T. harzianum* (IIHR TH2) and two combination formulations *P. monteilii*(PMS2) +*T. harzianum* (IIHR TH2) and *B. pumilus* (BPS4) + *T. harzianum* (IIHR TH2) were evaluated for plant growth promotion and disease complex incidence. Five replications were maintained in completely randomized design for the following treatments- T1: PMS2, T2: IIHR-TH2, T3: BPS4, T4: PMS2 + IIHR-TH2, T5: BPS4 + IIHR-TH2, T6: Chemical and T7: untreated Control. Seed treatment was done by mixing 20 g talc formulation of PMS2, IIHR-TH2, BPS4, PMS2 + IIHR-TH2 and BPS4 + IIHR-TH2 per kg of onion seeds. Substrate treatment was done by treating coco-peat with the respective talc formulations at 6 g Kg^{-1} of coco-peat. Chemical treatment was done by applying carbofuran (FMC India Private Ltd., Bengaluru) and carbendazim (Dow Agro Sciences India Private Ltd., Mumbai) at 3g kg^{-1} cocopeat. Untreated cocopeat served as control. Pathogens were inoculated at 50 infective juveniles of RKN and 5 x 10^4 conidia of FOC per well. Data on plant growth parameters *viz.* shoot length (cm) and weight (g); root length (cm) and weight (mg) were recorded 30 days after sowing (DAS).The root galling index (RGI) was recorded on a scale of 0 to 5 as described by Taylor and Sasser (1978) scoring 0 for no galls, 1 for 1 - 2 galls, 2 for 3 - 10 galls, 3 for 11 - 30 galls, 4 for 31 - 100 galls and 5 for more than100 galls. Percent disease incidence in nursery was recorded as seedling mortality using formula, Per cent disease induced mortality = (n /N) X 100 [n = number of plants killed, N= is the total number of plants raised] as described by Singh *et al*. (2004).

Field experiment

Field trials were carried out in the Rabi season of the year 2014 and 2015 at the Block-VI, Nematology experimental plots, ICAR-Indian Institute of Horticultural Research, Bengaluru (Latitude: 13º58' N; Longitude: 78º E; Altitude: 890 M). Rabi season of the year 2014 had an average maximum temperature of 28.8°C and a minimum temperature of 14.9°C with an average rainfall of 23.6 mm. Rabi season of the year 2015 had an average maximum temperature of 30.4°C and a minimum temperature of 18.6°C with an average rainfall of 106.3 mm. (Annual report, ICAR-IIHR, 2014; 2015). The initial nematode population and propagules of FOC was determined before starting the experiment (Cobb 1918; Komada 1975).

Thirty days old seedlings were transplanted at a spacing of 20cm in bed size of 1 x 2 sq m. Prior to transplantation, biopesticide-enriched vermicompost was applied on the beds at 50 gm^{-2} and mixed with the soil. Individual and combination treatments were enriched at 1 kg talc formulation per 500 kg vermicompost (UAS-GKVK, Bengaluru) as described by Rao *et al*. (2012).

Chemical treatment was done by applying carbofuran and carbendazim at 3g m^{-2}. Untreated beds served as control. Data on disease incidence (%) and yield (tons/ha) was recorded at 120 DAS. Damage due to *M. incognita* was determined by counting the number of root galls and the root galling index (RGI) was recorded on a scale of 0 to 5 as per Taylor and Sasser (1978); scoring 0 for no galls, 1 for 1 - 2 galls, 2 for 3 - 10 galls, 3 for 11 - 30 galls, 4 for 31 - 100 galls and 5 for more than 100 galls. Disease incidence by FOC was determined by counting the number of both healthy and diseased onion plants and per cent incidence was calculated as per Wheeler (1969).

Statistical analysis

Experimental data were subject to Analysis of Variance procedures of SPSS (Statistical Package for Social Sciences, Inc., 2001, Model 11.0. Chicago). Treatment means were compared using the Duncan's multiple range test at $P < 0.05$.

RESULTS

Nursery experiment

All biopesticide treatments proved to be better than untreated control and chemical treatment. There was a significant difference between treatments as determined by one-way ANOVA ($p < 0.05$). Duncan post-hoc test divided the means for groups in homogeneous subsets with a Harmonic Mean Sample Size = 5.0 for plant growth parameters *viz.* shoot length and weight; root length and weight RGI and disease incidence (Table 1). At 30 DAS combination formulations proved to be the most effective in increasing the plant growth parameters and reducing the disease complex incidence. T4: PMS2 + IIHR-TH2, and T5: BPS4+IIHR-TH2 recorded maximum shoot length (40.1 cm, 38.0 cm) and weight (4.9 g, 4.6 g); root length (16.0 cm, 16.2 cm) and weight (5.7 mg, 5.3 mg), respectively and were at par with each other. Root length at 30 DAS was significantly higher in combination treatments and all the other treatments were at par. However, a significant difference was also recorded for the root weight where the individual treatments T1 and T3 were at par and followed the combination formulations; indicating the development of better root system with the application of biopesticides. A similar trend was recorded for the shoot length where the individual treatments were at par with each other and followed the combination formulations. There was not much effect on plant growth promotion by chemical treatment and it was at par with control for shoot length (35.8 cm, 35.0 cm); root length (12.6 cm, 12.1 cm) and weight (2.0 mg, 2.0 mg), respectively.

Biopesticides reduced the nematode infestation (50.0% to 80.0 %) as well as disease incidence of *F. oxysporum*f. sp. *cepae* (73.3% to 88.4%) over control. The least RGI and disease incidence in T5: BPS4+IIHR-TH2 (0.8, 10.6 %), were at par with T4: PMS2 + IIHR-TH2 (1.2, 12.6%). Chemical treatment recorded RGI and disease incidence of 2.6 and 52.0%, respectively which were significantly lesser than control (Table 1; Figure 1).

Field experiment

The initial nematode population was 130 ± 12 infective juveniles/ 100 cc soil and initial disease propagules of FOC was 10^5 cfu/g soil. There was a statistically significant difference between treatments as determined by ANOVA ($p < 0.05$). Duncan post-hoc test divided the means for groups in homogeneous subsets with a Harmonic Mean Sample Size = 5.0 for yield, RGI and disease incidence (Table 2). A significant increase in the yield (26.1% to 28.8%) was recorded over control by the biopesticide treatments. The highest yield was recorded by the combination formulationsT5: BPS4+ IIHR-TH2 (22.9 tons/ha) and T4: PMS2+ IIHR-TH2 (22.7 tons/ha) which were at par. The individual formulations T3: BPS4 (22.6 tons/ha), T1: PMS2 (22.5 tons/ha) and T2: IIHR-TH2 (22.4 tons/ha) followed the combination formulationsand were at par with each other. Higher yield by combination formulationsindicated the synergism of BPS4 and PMS2 with IIHR-TH2 in onion. Chemical treatment did not increase the yield significantly but was better than control. Biopesticides reduced the nematode infestation (47.6% to 71.4%) as well as disease incidence of *F. oxysporum* f. sp. *cepae* (63.5% to 72.0%) over control. The least RGI and disease incidence were recorded by T5: BPS4 + IIHR-TH2 (1.2, 11.8 %), which was at par with T4: PMS2 + IIHR-TH2 (1.4, 12.4%), followed by T2: IIHR-TH2 (1.6, 13.8%). Chemical treatment recorded RGI and disease incidence

Table 1. Effect of biopesticides- *B. pumilus* (BPS4), *P. monteilii* (PMS2) and *T. harzianum* (IIHR-TH2 ITCC No. 222) on onion seedlings at 30 DAS under nursery conditions.

Treatment	Root length (cm)	Shoot length (cm)	Root weight (mg)	Shoot weight (g)	Root Gall Index (scale of 1-5) *	*Disease incidence (%)
P. monteilii (PMS2)	13.6 ± 0.89^{b}	36.8 ± 0.84^{bc}	3.0 ± 0.02^{b}	4.1 ± 0.45^{c}	1.6± 0.55^{ab}	23.8 (29.20)^{e}
T. harzianum (IIHR-TH2)	13.1 ± 0.65^{b}	35.9 ± 0.84^{bc}	2.5± 0.04^{cd}	4.3 ± 0.30^{bc}	1.4± 0.55^{ab}	18.8 (25.70)^{c}
B. pumilus (BPS4)	13.4 ± 0.55^{b}	36.1 ± 1.25^{bc}	2.9 ± 0.04^{bc}	3.7 ± 0.17^{d}	2.0± 0.71^{b}	21.8 (27.83)^{d}
PMS2 + IIHR-TH2	16.0 ± 0.71^{a}	40.1 ± 0.22^{a}	5.7 ± 0.03^{a}	4.9 ± 0.19^{a}	1.2± 0.45^{ab}	12.6 (20.79)^{b}
BPS4 + IIHR-TH2	16.2 ± 0.91^{a}	38.0 ± 1.00^{ab}	5.3 ± 0.02^{a}	4.6 ± 0.18^{ab}	0.8± 0.84^{a}	10.6 (19.00)^{a}
Chemical	12.6± 1.14^{b}	35.8 ± 0.84^{bc}	2.3 ± 0.02^{de}	3.3 ± 0.28^{e}	2.6± 0.55^{c}	52.0 (46.15)^{f}
Control	12.1 ± 3.17^{b}	35.0 ± 3.67^{c}	2.0 ± 0.06^{e}	2.9 ± 0.25^{f}	4.0 ± 0.71^{d}	89.6 (71.19)^{g}
CD(0.05)	1.9164	2.0574	0.0472	0.3557	0.1419	3.4898
SEd	0.9285	0.9969	0.0229	0.1724	0.0687	1.6908

Note:*value in parenthesis are angular transformed; means followed by the same letter in each column are not significantly different ($P < 0.05$) according to Duncan's multiple range test

Table 2. Evaluation of biopesticides- *B. pumilus* (BPS4), *P. monteilii* (PMS2) and *T. harzianum* (IIHR-TH2 ITCC No. 222) on *M. incognita* and *F. oxysporum* f. sp. *cepae* in onion under field conditions.

Treatment	Yield (tons/ha)*	Root Gall Index (scale of 1-5) *	Disease incidence (%)*
P. monteilii (PMS2)	22.5 ± 0.11[bc]	2.0 ± 0.45[bc]	15.2 (22.95)[c]
T. harzianum (IIHR-TH2)	22.4 ± 0.12[c]	1.6± 0.55[abc]	13.8 (21.81)[b]
B. pumilus (BPS4)	22.6 ± 0.09[b]	2.2± 0.45[c]	15.4 (23.11)[c]
PMS2 + IIHR-TH2	22.7± 0.13[a]	1.4± 0.45[ab]	12.4 (20.62)[a]
BPS4 + IIHR-TH2	22.9 ± 0.13[a]	1.2 ± 0.55[a]	11.8 (20.09)[a]
Chemical	18.5 ± 0.05[d]	2.8 ± 0.45[d]	31.0 (33.83)[d]
Control	17.8 ± 0.04[e]	4.2± 0.45[e]	42.2 (40.51)[e]
CD(0.05)	0.1127	0.6527	0.9511
SEd	0.0546	0.3162	0.4608

Note:*value in parenthesis are angular transformed; means followed by the same letter in each column are not significantly different (P < 0.05) according to Duncan's multiple range test

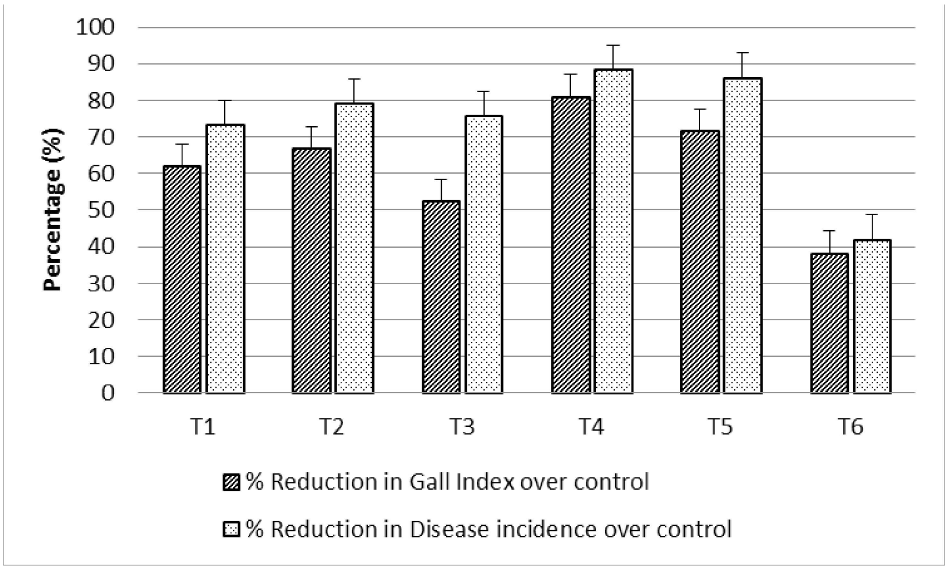


Figure 1. Effect of biopesticides on disease incidence under nursery conditions in onion. [T1: *P. monteilii* (PMS2), T2: *T. harzianum* (IIHR-TH2), T3: *B. pumilus* (BPS4), T4: PMS2 + IIHR-TH2, T5: BPS4 + IIHR-TH2, T6: Chemical, T7: Control]

of 3.2 and 31.0%, respectively which were better than control (Table 2; Figure 2, 3).

DISCUSSION

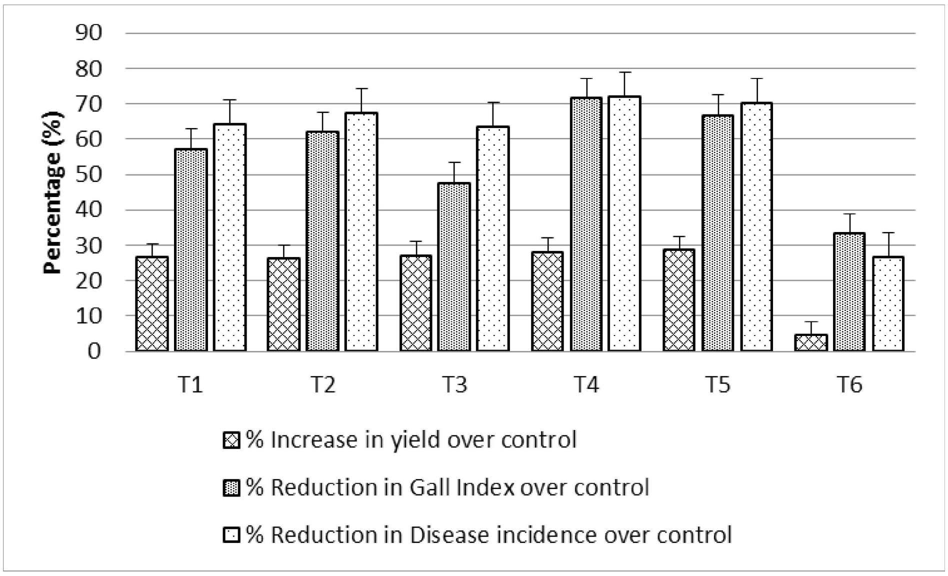
In the present study, biopesticides promoted plant growth in nursery conditions; showed reduced incidence of disease complex caused by *M. incognita* and FOC in onion seedlings. Similar findings on the use of biopesticides in nurseries for raising up healthy pathogen free seedlings have been reported by earlier researchers. Sharma *et al.* (2007) reported *Pseudomonas* spp. to effectively control the damping-off diseases in vegetable nurseries. The bacterial treatments significantly increased the activity of peroxidase and phenylalanine ammonia-lyase in chilli and tomato plant tissues and enhance seedling stand and yield. Aziz (2012) showed that *B. cereus* helped in the early development of shallot (*Allium ascalonicum*) by the production of IAA. *P. montelii* (MBL K22) showed plant growth promotion *via* IAA production and also antagonism against fungal phytopathogen *E. turcicum* causing Turcicum Leaf Blight and responsible for a severe loss of yield in maize (Bhati and Singh, 2012). These studies highlighted the benefits of using bioagents to protect seedlings by development of early induced systemic resistance. Bio-inoculants also act as stimulants for early emergence of seedlings. Rao (2007) reported the production of bio-agent colonized seedlings of papaya using *P. fluorescens* and *T. harzianum*, which effectively led to the management of RKN in papaya both in the nursery and in the field as well. Rao *et al.* (2009) reported that treatment of nursery bed with *P. fluorescens* and *T. harzianum* each at 20g/m^2 and subsequent application of 5 tons of farm yard manure enriched with 5 kg each of *P. fluorescens* and *P. lilacinus* per hectare managed *M. incognita* and *Ralstonia solanacearum* complex in tomato.

Combination formulation of PMS2 and IIHR-TH2 proved to effectively reduce the incidence of RKN and FOC in onion under nursery and field conditions. The siderophores producing strain, PMS2 (unpublished data) helped in the suppression of *M. incognita* and FOC and promotion of plant growth of onion in the present study. In addition, the nematicidal effect of IIHR-TH2 via *pra1* and *β-tubulin* genes (Kumar 2014) considerably reduced RKN infestation. The synergistic effects of bioagents PMS2, BPS4 and IIHR-TH2 in combination clearly showed their potential in production of healthy onion seedlings.

Similar findings of biological control using fungal and bacterial antagonists have been suggested in onion by Rajendran and Ranganathan (1996) where fungal antagonists, *T. viride*, *T. harzianum*, *T. hamatum*, *T. koningii* and *T. pseudokoningii* and bacterial antagonists, *P. fluorescens* and *Bacillus subtilis* were effective against FOC. A combination of *T. viride* and *P. fluorescens* were more effective in reducing Fusarium basal rot incidence under pot and field conditions. In another experiment, Patil (2012) reported the reduction in basal rot disease by seed treatment with *T. harzianum*, followed by soil application of *T. harzianum*. Coskuntuna and Ozer (2008) reported that based on seed treatment with *T. harzianum*, a significant reduction in basal rot incidence on onion sets under pot and field conditions was observed. *T. harzianum* has the ability to stimulate a chemical response in onion. They also showed that *T. harzianum* treatment of onion seeds induced the accumulation of antifungal compounds absorbing UV light in onion, which may be involved in the control of FOC during set development under pot and field conditions. Rajendran and Ranganathan (1996) reported that combined seed treatment of *T. viride* was the most effective in reducing disease incidence under pot and field conditions. Srivastava and Tiwari (2003) reported that seed treatment with *T. viride*, followed by its soil application reduced damping-off disease in onion seedlings. Combination treatment of *T. harzianum* and *B. amyloliquefaciens* were found to be more effective against onion bulb rot on field and in storage transplanting or at harvesting. When the antagonists were applied to the topping areas of onion bulbs at harvest *T. harzianum* was the most effective biological control agent against basal rot, with the number of rotten bulbs recorded at 4%, while that of the control was 16% (Lee *et al.* 2000).

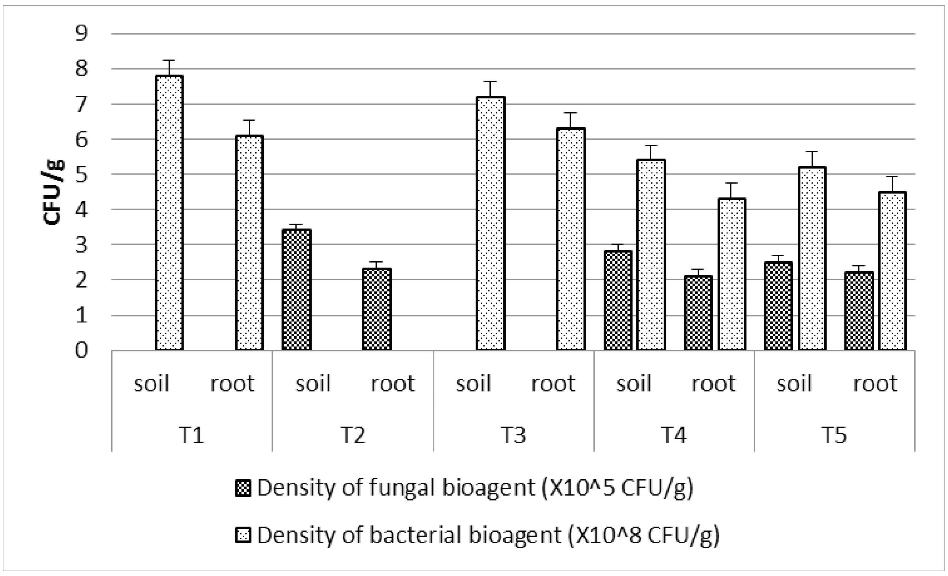
CONCLUSION

The use of biopesticides for integrated management of pathogens is an ecologically friendly alternative to chemical pesticides, which must be repeatedly used for the control of plant diseases (Elad 2000; El-Hassni *et al.* 2007). The present study indicates the effectiveness of the biopesticides in nursery and field conditions. Apparently, the combination treatments proved their efficacy, producing a cumulative effect and significantly increasing the plant growth promotion and yield while inhibiting the disease incidence drastically. As the use of chemical nematicides can be hazardous, the use of formulations of biocontrol agents, such as *T. harzianum* with *B. pumilus* or *P. monteilii*, would be an alternative for the production of healthy seedlings of onion and subsequently increase crop yield. Biopesticides, *T. harzianum* with *B. pumilus* or *P. monteilii* evaluated in these investigations are unique strains with fungicidal as well as nematicidal properties. There is a tremendous



[T1: *P. monteilii* (PMS2), T2: *T. harzianum* (IIHR-TH2), T3: *B. pumilus* (BPS4), T4: PMS2 + IIHR-TH2, T5: BPS4 + IIHR-TH2, T6: Chemical, T7: Control]

Figure 2. Effect of biopesticides on yield and disease incidence under field conditions in onion.



[T1: *P. monteilii* (PMS2), T2: *T. harzianum* (IIHR-TH2), T3: *B. pumilus* (BPS4), T4: PMS2 + IIHR-TH2, T5: BPS4 + IIHR-TH2, T6: Chemical, T7: Control]

Figure 3. Density of bioagent (CFU/g) under field conditions in onion at 120 DAS.

potential for a combination formulation of BPS4, PMS2 and IIHR-TH2 with bio-nematicidal and bio-fungicidal properties towards sustainable agriculture.

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