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MICROBIOLOGY

Phosphate solubilizing efficiency of Mycopesticides

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Abstract

In the present investigation, plant growth promoting activities of mycopesticides (beneficial mycopesticides or biological control fungi) *viz. Trichoderma, Paecilomyces, Beauveria* and *Metarhizium* were assessed. Their efficacy for the growth enhancement of Groundnut was investigated through laboratory as well as by pot studies. Results of *In vitro* studies have shown their higher potential of P-solubilization these fungi. In the experiment, two native cultures and five fungal isolates (procured from MTCC) were used. P solubilization ability of the test mycopesticides have shown good solubilization zones by the PlA isolate on PKVK agar. Not only this, TvA and ThM has also shown good P solubilizing zone on PKVK medium - supplemented with rose bengal dye. In liquid culture medium, ThM showed the highest P solubilizing ability (309.33 µg/ ml). HPLC analysis was carried out to determine the presence of organic acids in the culture supernatant of promising cultures. TvM showed presence of pyruvic acid, formic acid, orotic acid, citric acid and butyric acid. Efficacy of mycopesticides in pots study as PGP has revealed seed treatment as the best one, but recommended fertilizer was found significantly superior than all mycopesticides treatments. Amongst mycopesticides, *Trichoderma* spp. proved to be the best followed by *Paecilomyces*. *Beauveria* in most cases remained at par with *Paecilomyces* but was inferior to *Trichoderma*. *Metarhizium* was better than control but was inferior to other fungal treatments. Efforts clearly indicated that mycopesticides can significantly enhance groundnut growth and production as a plant growth promoting fungi (PGPF) proving their dual ability.

Highlights

- *Trichoderma viride* (Pers) (AAU1, TvA) and *Paecilomyces lilacinus* (Thom) Samson (AAU2, PlA) were used for the study.
- *In vitro* study of phosphate solubilization activity of mycopesticides were determined
- Efficacy of mycopesticides for growth enhancement of groundnut in pots were done

Keywords: Mycopesticides, P solubilization, plant growth promotion, HPLC, groundnut

Beneficial mycopesticides belonging to the genus *Trichoderma* and *Paecilomyces* are amongst the most commonly used soil biocontrol fungi; useful to control soil borne fungal and nematode diseases. Entomopathogenic fungi (mycopesticides of insects) *Beauveria* and *Metarhizium* are broad-spectrum bioagents to control soil and foliage insects damaging groundnut crop. Due to their ability to protect plants under different soil conditions, these fungi have been widely used and commercially available world over. Such fungi also produce numerous biologically active antagonists' compounds, including cell wall degrading enzymes and secondary metabolites useful to them for quicker action and better virulence (Sandeep and Jisha, 2013). *Trichoderma* species are among the most commonly studied biocontrol microbes and they exhibit plant-growth-promoting activity (Chet, 1987; Duffy *et al*., 1996). Other mechanisms including production of hormone, metabolites and release of nutrients from soil or organic matter have been predicted (Kleifeld and Chet, 1992). Mycopesticides have ability to produce and exude organic acids, proteins and other metabolites, which make them important biological weathering agents of natural rocks and minerals (Burford *et al.*, 2003; Fomina and Gadd, 2003; Gadd, 1993). Thus, in present investigation, beneficial mycopesticides *viz. Trichoderma*, *Paecilomyces*, *Beauveria* and *Metarhizium* useful for control of plant diseases and insects are chosen for studies on phosphate solubilizing ability to prove their additional role as plant growth promoting rhizosphere fungal inoculants (PGPF) and their impact on groundnut growth enhancement in pot conditions.

Table 1. Inorganic P release by different mycopesticides in PKVK broth

Fungal Isolates	Phosphorus concentration (µg/ ml)		
	3rd Day	5th Day	10th Day
TvM	209.67[b]	140.00[a]	58.00[a]
TvA	145.33[c]	80.00[b]	47.00[ab]
ThM	309.33[a]	59.00[bc]	45.33[b]
PlA	10.00[e]	44.67[c]	10.00[e]
PfM	80.67[d]	49.33[c]	32.67[cd]
BbM	27.00[e]	53.00[bc]	36.00[bc]
MaM	23.33[e]	125.00[a]	23.00[d]
S.Em.	10.70	8.15	3.55
CD at 5%	32.47	24.72	10.78
CV %	16.11	17.93	17.11

Materials and Methods

Microbes: *Trichoderma viride* (Pers) (AAU1, TvA) and *Paecilomyces lilacinus* (Thom) Samson (AAU2, PlA) were provided from Department of Microbiology and Biofertilizer Project, BACA, AAU, Anand, whereas *Trichoderma viride* (Pers) (MTCC 793), *Trichoderma harzianum* (Rifai) (MTCC 792), *Paecilomyces fumosoroseus* (Wize) Brown and Smith (MTCC 6292), *Beauveria bassiana* (MTCC 7690) and *Metarhizium anisopliae* (MTCC 6060) were procured from MTCC (Microbial Type Culture Collection), Chandigarh.

Table 2. Detection of organic acids by HPLC

Acids	Retention time of standard organic acids[#] (RT, min[a])	Organic acids (RT, min)	
		TvM	PlA
Formic	6.86	5.01	-
Pyruvic	8.13	8.14	-
Lactic	9.01	-	-
Orotic	9.88	9.90	-
Acetic	10.97	-	10.36
Oxalic*	11.28	-	11.57
Citric	11.99	12.67	-
Uric	14.06	-	-
Propionic	15.51	-	-
Butyric	31.58	32.18	-

[a] Mean values calculated from five different standard mixture solution containing each organic acid.

[#] **Dinkci *et al.* (2007)**

In vitro study of phosphate solubilization activity of mycopesticides:

Agar plate assay

Phosphate solubilization activity of mycopesticides was checked on Pikovskaya's and NBRIP medium by the method described by Azouni (2008). Sterilized PKVK agar medium was prepared and plated. Fungal strains were spot inoculated on PKVK agar plates. Plates were incubated at 28+20 C for seven days. Zones of clearance were observed around the colonies. Solubilization index was evaluated according to the ratio of total diameter (colony + halo zone) to the colony diameter. The same experiment was carried out using NBRIP medium and PKVK-supplemented with 0.003% w/v rose bengal dye for further confirmation.

Broth assay

PKVK broth (100 ml) was prepared in 250 ml flasks and inoculated with 1 ml (10^9 spores/ ml) of respective fungal cultures keeping an uninoculated flask as control. Inoculated flasks were incubated for three days on shaker at 27±2° C temperature. Finally, 9 ml of clear suspension from broth was taken in a test tube. One ml of trichloro acetic acid (TCA) was (added to the tube and centrifuged at 3000 rpm for 30 min. Vanado - molybdate method (APHA, 1995) for phosphate estimation was followed than to check the ability of fungal cultures for solubilization of phosphate in broth. The same experiment was carried out for evaluating P solubilization after 5 and 10 days of incubation.

Table 3. Effect of mycopesticides on plant height of groundnut at 30 Days

Treatments	Plant height at 30 DAS (cm)
	Seed application
TvM	26.22^{a}
TvA	25.78^{a}
ThM	26.44^{a}
PlA	24.44^{a}
PfM	24.22^{a}
BbM	24.11^{a}
MaM	24.00^{a}
Kalisena	25.33^{a}
RD	27.66^{a}
Control	19.67^{b}
S.Em.	1.22
CV%	8.59

Detection of organic acids by HPLC

The culture filtrates were analysed for the presence of organic acids by reverse-phase high performance liquid chromatography (HPLC) at Sophisticated Instrumentation Centre for Applied Research and Testing (SICART), Vallabh Vidyanagar. A gradient programme using a mobile phase of aqueous 0.5% (w/v) $(NH_4)_2HPO_4$ – 0.4% (v/v) acetonitrile at pH 2.25 was used to separate organic acids on a C_{18} column (Dinkci, *et al*., 2007). The best flow rate time program was applied to take the chromatogram in 35 min.

Efficacy of mycopesticides for growth enhancement of groundnut in pots:

Pot experiment was carried out in Kharif, 2008 using completely randomized design. Number of treatments was 10 and all treatments were triplicated. Various treatments given were TvM, TvA, ThM, PlA, PfM, BbM, MaM, *A. niger*- Kalisena (Bioagent treated check, Marketed 'P' solubilizers fungi) and recommended fertigation (treated check, chemical fertilizers). Untreated check was also kept for comparison. Variety of groundnut used was GG-2. Basal fertilizer dose for N: P: K was kept as 25:50:0. Twenty five kg N/ ha in the form of urea was applied in all the treatments except T_{10}. Fifty kg P_2O_5 was applied in the form of rock phosphate containing 35% P_2O_5 in treatments T_1 toT_8. In treatment T_9, SSP was used. Crop was harvested at 90 DAS of sowing. Pot size was 30 cm diameter (10 kg soil).

Table 4. Efficacy of mycopesticides by seed application on groundnut (Kharif)

Treatments	Shoot Length	Root Length	Dry Shoot Weight	Dry Root Weight
	(cm)	(cm)	(g)	(g)
TvM	41.11^{abc}	29.89^{abc}	3.00^{abc}	1.81^{ab}
TvA	38.89^{bc}	28.45^{abc}	2.96^{abc}	1.48^{bc}
ThM	43.33^{ab}	30.85^{abc}	3.00^{abc}	2.00^{a}
PlA	38.11^{bc}	28.14^{abc}	2.78^{bc}	1.16^{cd}
PfM	35.67^{bc}	27.48^{bc}	2.67^{c}	1.07^{cd}
BbM	35.56^{bc}	26.89^{bc}	2.56^{c}	0.99^{de}
MaM	33.56^{c}	25.78^{bc}	2.04^{d}	0.87^{de}
Kalisena	44.22^{ab}	33.22^{ab}	3.22^{ab}	2.11^{a}
RD	48.44^{a}	35.56^{a}	3.33^{a}	2.22^{a}
Control	33.44^{c}	24.00^{c}	1.89^{d}	0.62^{e}
S.Em.	2.71	2.23	0.29	0.14
CD at 5%	8.01	6.60	0.87	0.42
CV%	11.99	13.35	18.68	17.20

Seed, soil and foliar applications were applied for all treatments. In soil and seed treatment, 1 ml of mycopesticides consisting of 10^9 spores/ pot was applied. Foliar spray of cell free extract was done at 30 DAS @ 1ml 1:10 diluted metabolites/pot. Three seeds were sown in pots. Watering was done in the pots as and when required. Observations were recorded as mentioned below.

Observations recorded

Seed Germination at 5 DAYS (%), Plant height (cm), fresh shoot and root weight (g) and Shoot and Root length (cm) at harvest.

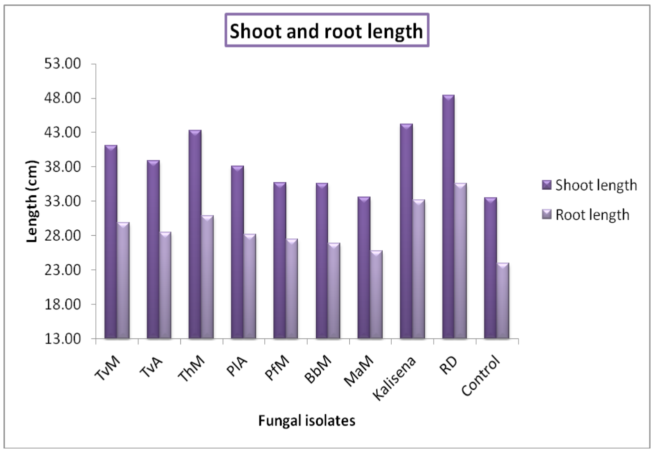

Figure 1. Efficacy of mycopesticides by seed application on shoots and root length of groundnut in pot.

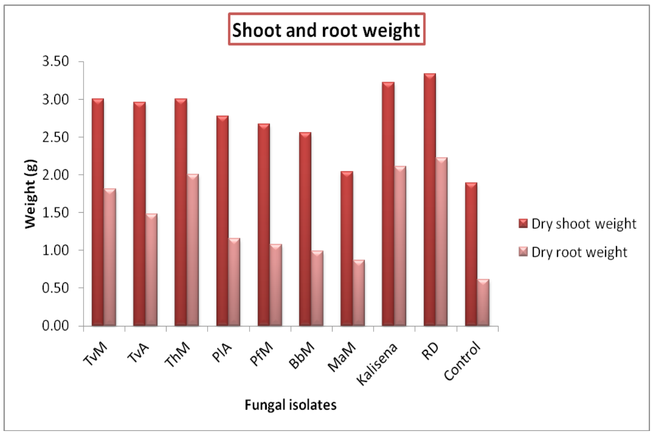

Figure 2. Efficacy of mycopesticides by seed application on dry shoot and root weight of groundnut in pot.

Results and Discussion

Microbially mediated solubilization of insoluble phosphates through release of organic acids is often combined with production of other metabolites. *In vitro* studies show the potential of P-solubilization of entomopathogenic fungus.

In vitro study of phosphate solubilization activity of mycopesticides

Agar plate assay

PlA showed the highest P solubilization zone with solubilization index 5.87. MaM showed second best P solubilization zone. TvA and ThM showed fine P solubilizing zone when inoculated in PKVK-supplemented with 0.003% w/v rose bengal medium. For TvA and ThM, solubilization index was 2.5 and 2.14 respectively. However, PKVK medium reported better for P solubilization in comparison to other media. In the present study, species of two fungus *Paecilomyces* and *Trichoderma* clearly showed halo zones of P-solubilization on agar plates.

Broth assay

ThM showed highest solubilization of phosphate (309.33 µg/ ml) in PKVK broth (Table 1). TvM and TvA also showed the next best efficiency of P solubilization on 3rd day of inoculation. However, other test fungus did not show efficient solubilization. Overall P concentration decreased as time of incubation increased. PlA, BbM and MaM showed an increase in available P concentration up to 5 days of incubation and then it started decreasing, which may be due to the consumption of free P as substrate by fungi.

Detection of organic acids by HPLC

Culture filtrates of two mycopesticides, *T. viride* and *P. lilacinus* were analysed for the presence of key organic acids by HPLC at SICART. Peak identity was confirmed by comparing peaks of standard organic acids (Dinkci *et al.*, 2007 and oxalic acid standard run). TvM culture filtrate showed presence of formic acid, orotic acid, citric acid and butyric acid (Table 2), moreover a close peak to pyruvic acid was also noticed. Similar types of peaks were also observed in ThM culture filtrate. In PlA, predominantly acetic and oxalic acids were noticed. Presence of organic acids in the culture filtrate supported the P solubilizing capacity of these mycopesticides and their usefulness in controlling soil diseases.

Efficacy of mycopesticides for growth enhancement of groundnut in pots.

Mycopesticides were tested for their ability of plant growth promotion of groundnut in pots in Kharif 2009. PGPR ability was assessed by seed, soil and foliar treatments in groundnut, among which seed treatment proved best.

Effect of mycopesticides on germination at 5 days

Germination count revealed 80% germination of groundnut in various mycopesticides treatments, indicating no inhibitory effect of mycopesticides on seed. After 30 days of sowing of groundnut, plant height was measured, which revealed that all treatments were at par with each other in case of seed treatment (Table 3). In seed application, seeds were treated directly with spore as well as culture filtrate having secondary metabolites of mycopesticides and observations were recorded (up to 90 days) at regular interval as mentioned.

Shoot and root length

Recommended N+P showed significantly superior shoot length (48.4 cm) over all treatments. ThM treatment remained at par with Kalisena for shoot length and showed 29.5% higher shoot length compared to control. TvM treatment also showed good crop response and shoot length was 22.9% higher than control. For root length, recommended dose was highly significant followed by Kalisena. Treatments TvM, TvA, ThM and PlA were found at par with each other. Treatments of PfM, BbM and MaM were found inferior and remained at par with each other for root length (Figure 1).

Shoot and root weight

For dry shoot weight, again recommended dose was best followed by Kalisena. TvM, TvA and ThM treatments were at par with each other for dry shoot weight. TvM and ThM showed 58.9% higher dry shoot weight compared to control (Figure 2). For dry root weight, ThM found at par with Kalisena and recommended dose. ThM showed 224.8% higher dry root weight, whereas TvM showed 194.05% higher dry root weight compared to control. TvA was at third position for dry root weight. PlA and PfM were at par with each other for dry root weight. BbM and MaM treatments were also at par with each other for dry weight (Table 4). Figures 1 and 2 indicating common letters do not differ significantly from each other at 5% level significance according to DNMRT.

Thus, three pot experiments indicated that *Trichoderma* spp. was superior among all mycopesticides followed by *Paecilomyces* spp. for improvement of plant growth in respect of height, fodder and grain yield of groundnut. *Trichoderma* spp. are having P solubilization ability by producing many organic acids *viz.* Butyric, Citric, Formic, Pyruvic, Orotic acids besides their transmissible biocontrol characteristics and found to have dual role. However, insect controlling fungus *Beauveria,* which is also having P solubilizing ability showed marginal increase in growth parameters and yield. It is evident that biocontrol fungi (mycopesticides) have P solubilizing ability proving them to be plant growth promoting fungi (PGPF) for groundnut.

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