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EFFECT OF ENDOPHYTIC *PSEUDOMONAS AERUGINOSA* AND *TRICHODERMA HARZIANUM* ON SOIL-BORNE DISEASES, MYCORRHIZAE AND INDUCTION OF SYSTEMIC RESISTANCE IN OKRA GROWN IN SOIL AMENDED WITH *VERNONIA ANTHELMINTICA* (L.) SEED'S POWDER

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Abstract

Biostimulants are used in agricultural practices for plant growth improvement. These fertilizers improve microbial activity and cause a negative impact on soil-borne pathogens. In recent years, stimulating plant's natural defense is considered as most promising alternative strategy for crop productivity. The present study was carried out to examine the effect of endophytic *Pseudomonas aeruginosa* and *Trichoderma harzianum* in soil amendment with *Vernonia anthelmintica* seed's powder, on root rotting fungi, plant growth, mycorrhizal population around roots, phosphorous uptake and stimulation of plant defense markers like poylphenol and antioxidant status in okra. Combine application of Vernonia with *Pseudomonas aeruginosa* and *Trichoderma harzianum* significantly ($p<0.05$) suppressed *Rhizoctonia solani* and *Fusarium oxysporum* with complete reduction of *Macrophomina phaseolina* and *Fusarium solani. Pseudomonas aeruginosa* and *T. harzianum* alone or in Vernonia amended soil significantly reduced nematode's galls on roots. Organic amendment also improved plant resistance against root diseases as evident from enhanced DPPH radical scavenging capacity and polyphenol content in treated plants as compare to control. VA Mycorrhizal spores were found significantly ($p<0.05$) higher in number around roots received *Pseudomonas aeruginosa* or *T. harzianum* alone or in Vernonia amended soil. Whereas, higher concentrations of phosphorus in okra shoots were found in plants received biocontrol agents in amended soil. Mixed application of PGPR and *T.harzianum* in amended soil produced tallest plants than other treatments. Soil amendment with Vernonia seed's powder alone or with biocontrol agents offer a non-chemical means of plant disease control.

Key words: *Vernonia anthelmintica, Pseudomonas aeruginosa, Trichoderma,* Systemic resistance, Mycorrhizae, Okra.

Introduction

The intensive use of inorganic fertilizer may cause soil acidity and nutrients imbalance resulting in reduced yield (Kang & Juo, 1980; Obi & Ebo, 1995: Ojoniyi, 2000). Incorporating the organic matters or crop residues to soil have been reported to have beneficial effect on crop plants and prodcued healther plants with improved crop yield (Abawi & Widmer. 2000; Akhtar & Malik, 2000). Due to adverse effect of chemical pesticides, scientists are looking for its replacement and organic amendment is now being receiving popularity as they are efficient in controlling the pests and diseases without affecting environment (Lazarovits, 2001). Organic amendments improve soil quality, enhanced soil suppressiveness against soil-borne pathogens and cause a positive effect on crop production and plant health (Bailey & Lazarovits, 2003). Stimulating the defense system of plants by the application of organic amendments along with microorganisms increasing popularity. The soil organic matter can increase the amount of nutrients in soil and its avaiability to plants (Dormaar *et al.,* 1988; Khalid *et al*., 2007). Several antimicrobial by-products (e.g. organic acids, hydrogen sulfide, phenols, tannins and nitrogenous compounds) are released during the decomposition of organic amendments or synthesized by microorganisms involved in such degradation (Mian, & Rodriguez-Kabana, 1982). Phenolics are phytochemicals, synthesized in plants in response to biotic or abiotic stresses (Briskin, 2000; Dai & Mumper 2010). In resistant varieties of plants phenolics are accumulated very rapidly at the site of infection after pathogen's attack, resulting in the effective isolation of the pathogen (Chérif *et al*., 1991; 1992). Furthermore organic fertilizers enhance the antioxidant content in plants and consequently improve plant defense against pests and diseases (Dumas *et al*., 2003).

Okra [*Abelmoschus esculentus* (L.) Moench] is an important vegetable crop is grown worldwide including Pakistan (Athar & Bokhari, 2006). Various soil-borne fungi like *Macrophomina phaseolina, Rhizoctonia solani, Fusarium* spp., and *Meloidogyne* spp., the root knot nematodes have been reported to attack this crop (Afzal *et al.,* 2013; Sultana *et al.,* 2005). Various organic matters have been used to manipulate the quality of soil and achieve an economic level of diseases control through stimulation of antgonistic microorganisms. The most common group of microbes associated with organic matters and cause disease suppression are fluorescent pseudomonads (Bulluck & Ristaino 2002; Garbeva *et al.,* 2006; Larkin & Honeycutt 2006) and *Trichoderma* spp., (Bulluck & Ristaino 2002). Production of antibiotics, competition for nutrients, parasitism, hydrolytic activities, such as chitinases and glucanases and activation of plant defenses have been reported as mechanisms of plant diseases control by *Trichoderma* spp., (Afzal *et al.,* 2013; Harman *et al.,* 2004). Whereas induction of systemic resistance in plants by the fluorescent pseudomonads are well documented (De Meyer

& Hofte, 1997; Hass & Defago, 2005; Lugtenberg & Kamilova, 2009; Shahzaman *et al.,* 2015; Weller *et al*., 2007). Similarly, soil-borne fungi and root knot nematode have been reported to suppress by *Vernonia anthelmintica* soil amendemnt (Batool *et al.,* 2013; Sultana *et al.,* 2011), however, induction of systemic resistance by the *V. anthelmintica* soil amendemnt yet not investigated. The present report describes the effects of organic amendment with biocontrol agents on root rotting fungi, plant growth, phosphorous uptake, VAM population and stimulation of defense system of okra.

Materials and Methods

Biological antagonist: Cultures of endophytic *Pseudomonas aeruginosa,* a plant growth promoting bacterium and *Trichoderma harzianum,* used in this study were obtained from Karachi University Culture Collection (KUCC).

Experimental design / Screen house experiment: Dry powder of *Vernonia anthelmintica* (L.) seeds was mixed with soil (sandy loam, pH 8.0) @ 1.0% w/w and transferred to 12 cm diam clay pots at one Kg per pot. A natural infestation of 3-6 sclerotia of *Macrophomina phaseolina* g^{-1} of soil, was found (Shiekh & Ghaffar, 1975), whereas 5-10% colonization of *Rhizoctonia solani* on sorghum seeds was found used as bait (Wilhelm, 1955). The soil also had a natural infestation of *Fusarium solani* and *F. oxysporum* (3000 cfu g^{-1} of soil) as determined by a soil dilution technique (Nash & Synder, 1962). The pots were watered on alternate days to allow the decomposition of the organic substrate. After two weeks, aqueous suspensions of *Pseudomonas aeruginosa* (10^8 cfu/ml) grown on King's B broth and *Trichoderma harzianum* (10^7 cfu/ml) grown on potato dextrose broth were drenched onto each pot at 25 ml per pot. Pots without amendment/antagonists served as control, whereas aqueous suspension (100 ppm) of a fungicide, topsin-m at 25 ml per pot served as positive control. Six seeds of okra were sown in each pot and pots were kept randomized on a screen house bench of Department of Botany, University of Karachi at 50% water holding capacity with four replicates of each treatment. After germination, only four seedlings were kept in each pot and excess were removed. Each pots were then inoculated with 500 eggs/J_2 of root knot nematode. Observations were recorded after 6 weeks of nematode's inoculation on root rotting fungi, root knot nematode, plant growth, VAM population and phosphorus uptake by okra plants. Effect of biocontrol agents and soil amendment on host defense was also evaluated using polyphenols and antioxidant activity as markers.

Determination of fungal infection and growth parameter: Roots were washed, cut into 1 cm long pieces (5 from each plant) and surface sterilized with 1% Ca $(OCl)_2$. The surface sterilized root pieces were transferred onto potato dextrose agar plates supplemented with penicillin (100,000 units/litre) and streptomycin (0.2 g/litre). Incidence of root infecting fungi was recorded after incubation for 5days at 28°C. The infection percentage for each pathogen was calculated using the formula:

$$\text{Infection \% of a pathogen} = \frac{\text{Number of plants infected by a pathogen}}{\text{Total number of plants}} \text{ X } 100$$

Plant growth parameters, such as plant height and fresh weight of shoot, root length and root weight were also recorded. Whereas number of galls per root system was recorded for nematode's infection.

Determination of polyphenol: Okra leaves were oven-dried at 80°C for 24 hours. Dried leaves were ground into fine powder using a clean pestle mortar and finally crushed samples were suspended in ethanol. Samples were collected in screw capped centrifuge tubes. The extracts were centrifuged for 20 minutes at 3,000 rpm. The supernatants were collected and used for analyzing phenolic content and antioxidant activity.

The estimation of polyphenol was done by Folin-Ciocalteu phenol reagent as describe by (Chandini *et al*., 2008). Where 100 µl aliquot of ethanolic leaves extract were mixed with 2 ml of 2% Na_2CO_3 and allowed to stand for 2 minutes at room temperature. After incubation 100 µl Folin-Ciocalteu phenol reagent was added and mixture was mixed thoroughly and allowed to stand for 30 minutes at room temperature in dark. Absorbance of samples was recorded at 720 nm using spectrophotometer and phenolic content was expressed as gallic acid equivalents.

DPPH radical scavenging activity: Antioxidant activity in okra was determined using DPPH (2, 2-Diphenyl-1-picrylhydrazyl) assay (Zubia *et al.,* 2007) with some modification. An aliquot of 200 µl of ethanolic leaves extract (0.2 mg/ml of ethanol) was mixed with 800 µl of 100 mM Tris-HCl buffer (PH 7.4). The mixture was added to 30 µM DPPH (dissolved in DMSO) and vortex, then left to stand at room temperature in the dark. The absorbance was measured at 517 nm after 1 minute and 30 minute of incubation, using UV-visible spectrophotometer against ethanol, used as blank. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{\% of inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \text{ X } 100$$

where the $A_{control}$ is the absorbance of the control (DPPH solution without sample), the A_{sample} is the absorbance of the test sample (DPPH solution plus test sample).

Determination of VAM spores: Wet sieving and decanting technique was used to isolate VAM spore from soil (Gerdemann & Nicolson, 1963). Briefly, 100g soil sample from each replicate of each treatment was placed in a 1000 ml graduated cylinder and volume was makeup up to 1000ml with distilled water. The cylinder was vigorously shaken for 10 minutes and then left for 20-30 minutes to allow heavy soil particles to settle down. The suspension was then decanted through the stack on sieves of various pore sized 710, 350, 150 & 53 µm (arranged in decreasing order of pore size from top to bottom). Same process was repeated 2-3 times and the residue from sieves (150 & 53 µm) were collected into Petri plates with little distilled water. Intact VAM fungal spores were examined and counted under stereomicroscope (Nikon,

Japan). The number of spores were counted and expressed as number of spores/100g of soil sample.

Determination of phosphorous in okra leaves: Dry ashing method as suggested by Rayan *et al.* (2001) was used for the estimation of total phosphorus in plant shoot. Where leave samples were dried in an oven at 120°C for 24 hours. The sample was dissolved in 2N HCl and 10 ml of the filtrate was transferred in 100 ml of volumetric flask. 10 ml of Ammonium Vanadomolybdate reagent was added and diluted with 80 ml distilled water. After 30 minutes of incubation absorbance was recorded at 410 nm wavelength on UV-visible spectrophotometer. Potassium dihydrogen phosphate (KH_2PO_4) was used to prepare standard curve for the calculation of phosphorous in ppm. Concentration of phosphorus was calculated using the formula:

$$\% P = \text{ppm P (from calibration curve)} \times \frac{R}{Wt} \times \frac{100}{10000}$$

where: R= Ratio between total volume of the digest and volume of the digest used for measurement

Wt = Weight of the dry plant (g)

Statistical analysis: For fungal infection two way ANOVA was used to compare the means among the treatments and also among different fungal pathogens. The follow up of ANOVA include least significant difference (LSD) at ($p<0.05$) to compare the means. Whereas for plant growth parameters, VAM population and phosphorus concentration one way ANOVA was used and LSD at ($p<0.05$) was calculated (Gomez & Gomez, 1984).

Results

Effect of soil amendment and biocontrol agents on root rotting fungi, root knot nematode and plant growth of okra: Less infection of *F. solani* was found in all the treatments including control plants. However, *P. aeruginosa* and *T. harzianum* treated plants showed no infection of *F. solani* (Table 1). Soil amendment with Vernonia seeds caused a significant suppressive effect on *F. oxysporum, R.* solani and *M. phaseolina. Pseudomonas aeruginosa* and *T. harzianum* were also found effective against *F. oxysporum* in soil amended with Vernonia. Application of *P. aeruginosa* and *T.harzianum* alone or in Vernonia amended soil significantly ($p<0.05$) suppressed *R. solani* and *M. phaseolina* (Table 1). Soil amendment with Vernonia alone or application of *P. aeruginosa*, *T. harzianum* or topsin-m in Vernonia amended soil produced taller plants than control plants. Mixed application of *P. aeruginosa* with *T. harzianum* in Vernonia amended soil produced greater shoot length and fresh shoot weight (Table 2). Application of *P. aeruginosa* and *T. harzianum* alone or in Vernonia amended soil caused a suppressive effect on root knot nematode by reducing the numbers of galls on plant roots (Table 2).

VAM population around roots and phosphorus uptake by plants: Number of VAM spores were found significantly ($p<0.05$) higher in *P.aeruginosa* or *T.harzianum* treatments, but they did not show improvement in phosphorus uptake by okra plant. Higher concentrations of phosphorus in shoots were found in plants grown in Vernonia amended soil used alone or with *P. aeruginosa*, *T. harzianum* or topsin-m (Table 3).

Polyphenol and antioxidant activity: Polyphenol contents were found highest in plants grown in Vernonia amended soil (72.2 mg %) as compared to control plants (47 mg %). *Pseudomonas aeruginosa* treated plants also showed slightly higher concentration of polyphenols, while topsin-m caused a reduction in polyphenol contents in okra shoots (Table 4). Antioxidant activity at 1st minute was found highest in plants grown in Vernonia amended soil (33.5%) followed by *P. aeruginosa* treatment (31.0%) as compared to control plants (6.2%). Antioxidant activity was increased in all treatments with time but *P.aeruginosa* or Vernonia treatments still showed higher activity than control plants (Table 4). However highest activity at 30 minutes was found in mixed application of *P. aeruginosa* with *T. harzianum* in Vernonia amended soil (Table 4). These finding indicates the role of *P. aeruginosa* and organic soil amendment in host defense against pathogen via increasing polyphenols content and antioxidant activity.

Table 1. Effects of *Pseudomonas aeruginosa* and *Trichoderma harzianum* on infection of *Fusarium solani, F. oxysporum, Rhizoctonia solani* and *Macrophomina phaseolina* on okra in soil amended with *Vernonia* seeds powder.

Treatments	*F. solani*	*F. oxysporum*	*R. solani*	*M. phaseolina*
	Infection %			
Control	18.7	37.5	25	31.2
Topsin-M	6.2	25	31.2	50
P. aeruginosa (PA)	0	31.2	18.7	12.5
T. harzianum (TH)	0	50	18.7	12.5
P. aeruginosa+ TH	0	25	18.7	12.5
Vernonia @ 1%	6.2	12.5	6.2	18.7
Vernonia @ 1% + Topsin-M	0	18.7	25	12.5
Vernonia @ 1% + PA	0	25	18.7	25
Vernonia @ 1% + TH	6.2	18.7	12.5	12.5
Vernonia + PA + TH	0	6.2	0	6.2

L.S.D ($p<0.05$) Treatments = 13.5[1], Pathogens = 8.5[2]

[1]Mean values in the column showing difference greater than LSD value are significantly different at $p<0.05$

[2]Mean values in the row showing difference greater than LSD value are significantly different at $p<0.05$

Table 2. Effects of *Pseudomonas aeruginosa* and *Trichoderma harzianum* on infection of root knot nematode and growth of okra in soil amended with *Vernonia* seeds powder.

Treatments	Shoot length (cm)	Fresh shoot weight (g)	Root length (c)	Root weight (g)	No. of knots
Control	30.1	2.2	5.3	0.2	7.0
Topsin-M	33.0	2.5	6.7	0.2	5.5
P. aeruginosa (PA)	34.0	2.4	6.5	0.2	3.3
Trichoderma harzianum (TH)	31.2	2.5	5.0	0.3	2.5
P. aeruginosa+ TH	29.3	2.4	6.8	0.4	1.9
Vernonia @ 1%	40.2	4.1	6.9	0.4	1.8
Vernonia @ 1% + Topsin-M	38.3	3.9	7.4	0.3	1.2
Vernonia @ 1% + PA	37.6	4.5	6.4	0.3	0.9
Vernonia @ 1% + TH	38.2	4.1	6.8	0.2	1.5
Vernonia + PA + TH	44.2	4.6	7.0	0.5	0.0
L.S.D ($p<0.05$)	6.1[1]	0.6[1]	ns	0.2[1]	1.7[1]

[1]Mean values in the column showing difference greater than LSD value are significantly different at $p<0.05$

Table 3. Effects of *Pseudomonas aeruginosa* and *Trichoderma harzianum* on mycorrhizal population and phosphorus uptake by okra plants in soil amended with *Vernonia* seeds powder.

Treatments	No. of VAM/ g soil	Phosphorus (ppm)
Control	9.1	1.20
Topsin-M	14.0	1.39
P. aeruginosa (PA)	19.3	1.22
Trichoderma harzianum (TH)	15.4	1.03
P. aeruginosa+ TH	17.0	2.13
Vernonia @ 1%	8.8	3.74
Vernonia @ 1% + Topsin-M	11.6	4.89
Vernonia @ 1% + PA	14.5	4.21
Vernonia @ 1% + TH	16.4	4.74
Vernonia + PA + TH	10.8	4.36
L.S.D ($p<0.05$)	5.3[1]	0.89[1]

[1]Mean values in the column showing difference greater than LSD value are significantly different at $p<0.05$

Discussion

Due to increasing concern over the pesticides and fertilizers, consumers are buying an increasing percentage of organic products, despite rising costs. The recent growth in sales of organic products have encouraged the growers to transit to organic production systems (Klonsky 2004). The biological control of soil-borne pathogens with organic amendments, microbial antagonists and micronutrients is gaining popularity in crop protection system to reduce the disease in economically important crops (Bharathi *et al.,* 2004; Ikram & Dawar, 2015; Senthilraja *et al*., 2010). In this study soil amendment with Vernonia seeds powder caused significant control of root rotting fungi, root knot nematode and improved growth of okra. Use of organic amendments for improving crops, increasing agricultural productivity and suppressing soil-borne diseases is well documented (Ehtesamul-Haque *et al.,* 1996; Ikram & Dawar, 2015; Lazarovits, 2001; Stone *et al.,* 2003; Sultana *et al.,* 2011). Application of Vernonia seeds has been reported to caused nematode's mortality and suppressed root rotting fungi (Batool *et al.,* 2013; Sultana *et al.,* 2011). In this study application of an endophytic fluorescent *Pseudomonas* and *T. harzianum* alone or in Vernonia amended soil showed significant suppression of root rotting fungi, produced healthier plants and improved antioxidant status of the okra plants. Antagonistic potential of fluorescent *Pseudomonas* against soilborne plant pathogens have been reported (Afzal *et al.,* 2013; Bokhari *et al.,* 2013; 2014; Siddiqui *et al*., 2000; Siddiqui & Ehteshamul-Haque, 2001). *Pseudomonas* spp., present in decomposing organic matter may enhance growth and yield of crops (Sylvia, 2004) by producing plant growth hormones and chemical compounds (e.g. siderophores, tannins, phenols) which are antagonistic to various soilborne pathogens (Antonio *et al*., 2008). Similarly *Trichoderma* spp., are most common soil fungi, present in plant root ecosystems and well known for their biocontrol potential against soilborne plant pathogens (Afzal *et al.,* 2013; Harman *et al*., 2004; Lugtenberg & Kamilova, 2009; Weller *et al*., 2007).

In this study plants received *P. aeruginosa* or grown in amended soil showed maximum amount of polyneols as compared to other treatments with improved antioxidant status of okra plants. The phenolic metabolites in plants are synthesized in response to biotic or abiotic stresses and protect the plants against these stresses (Briskin, 2000). These compounds are synthesized very rapidly and accumulated at the infection site in resistant varieties resulting in the effective isolation of the pathogen (Chérif *et al*., 1991). Toor *et al.* (2006) reported that organic fertilizers increased the content of ascorbic acid and total phenolics in tomato. Similarly Dumas *et al.* (2003) reported that inorganic fertilizer reduce the antioxidants while organic fertilizer was proven to enhance the antioxidant content in plants. It is also known that phenolic compounds are potential antioxidants and free radical-scavengers. Kumar *et al*. (2008) reported that there should be a close relation between the content of phenolic compounds and antioxidant activity.

In this study soil amendment with Vernonia seeds showed no effect on VAM population around roots, while *P. aeruginosa* and *T. harzianum* increased the population, however, this increased VAM population did not show the improved status of phosphorus in shoots. While, soil amendment with Vernonia caused improvement in phosphorus uptake which was further improved when PGPR or *T. harzianum* was used in amended soil. Bokhari *et al.* (2013) found higher number of VAM spores around roots of mungbean treated with mycorrhizospheric fluorescent *Pseudomonas* with improved phosphorus status in leaves. Soil amendment with Vernonia seeds alone or with *P. aeruginosa* or *T. harzianum* offer a non-chemical means of plant disease control.

Table 4. Effects of *Pseudomonas aeruginosa* and *Trichoderma harzianum* on polyphenol content and antioxidant activity in okra in soil amended with *Vernonia* seeds powder.

Treatments	Polyphenol content (mg % gallic acid)	Antioxidant activity (%)	
		1 minute	30 minutes
Control	47	6.3	24.8
Topsin-M	34.7	10.7	29.7
P. aeruginosa (PA)	51.5	31.0	34.0
Trichoderma harzianum (TH)	42.6	1.2	16.5
P. aeruginosa+ TH	53.4	6.7	18.4
Vernonia @ 1%	72.2	33.5	37.8
Vernonia @ 1% + Topsin-M	60.6	24.7	35.6
Vernonia @ 1% + PGPR	56.7	8.3	37.9
Vernonia @ 1% + TH	54.4	25.8	29.4
Vernonia + PGPR + TH	52.8	25.5	40.8
L.S.D ($p<0.05$)	7.1[1]	2.5[1]	2.3[1]

[1]Mean values in the column showing difference greater than LSD value are significantly different at $p<0.05$

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