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INDUCTION OF SYSTEMIC RESISTANCE IN COTTON BY THE NEEM CAKE AND *PSEUDOMONAS AERUGINOSA* UNDER SALINITY STRESS AND *MACROPHOMINA PHASEOLINA* INFECTION

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Abstract

Induction of systemic resistance by neem cake (*Azadirachta indica)* and endophytic *Pseudomonas aeruginosa,* a plant growth promoting bacterium, was evaluated in cotton *(Gossypium hirsutum* L.*)* under salinity stress and fungal infection. The combination of biocontrol agent and organic matter induced tolerance against fungal infection (*Macrophomina phaseolina)* and salinity stress (EC= 17.3 dS, m^{-1}) by producing salicylic acid 6.9–8.6 mg/ mL as compared to 2.8–4.6 mg/mL in control plants in field experiment, while polyphenols was found 3.1–3.7 mg/mL in neem cake + bacteria treated plants as compared 2.7–2.9 mg/mL in control plants. Cotton plants inoculated with *M. phaseolina* showed maximum infection in control plants (75%), while 37.5% plants were found infected in neem cake + *P.aeruginosa* treatment in field experiments. A significant ($p<0.05$) increase in shoot length was observed in combined treatment of neem cake + *P.aeruginosa* (30.5–31.7 cm) compared to control plants (25.7–26.7 cm) kept under biotic or salinity stresses. Similarly, free radical scavenging activity was found highest in plants received *P.aeruginosa* in neem cake amended soil in both DPPH (2, 2-diphenyl-2-picrylhydrazyl) and ABTS (2,2 –azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) methods. Similar results were also observed in screen house experiment. Application of endophytic *P.aeruginosa* in neem cake amended soil hold promise for the induction of systemic resistance in cotton.

Key words: *Pseudomonas,* Neem cake, Induced systemic resistance, Biotic stress, Abiotic stress.

Introduction

The biological control of soil-borne pathogens with organic amendments and microbial antagonists is gaining popularity in crop protection system, due to adverse effects of chemicals (Bharathi *et al*., 2004, Senthilraja *et al*., 2010). Several antimicrobial by-products (e.g. organic acids, hydrogen sulfide, phenols, tannins and nitrogenous compounds) are released during decomposition of organic matters or synthesized by microorganisms involved in such degradation (Mian & Rodriguez-Kabana, 1982; Oka, 2010). Phenolics are phytochemicals, synthesized in plants in response to biotic or abiotic stresses (Briskin, 2000, Dai & Mumper, 2010). Neem and its products has widely been reported to control insect pests (Ascher, 1993, Schmutterer, 1995), plant parasitic nematodes (Akhtar & Mahmood 1995), plant fungal diseases (Dubey *et al.,* 2009, Ehteshamul-Haque *et al.,* 1995; 1998) and act as a potential agricultural fertilizer (Gajalakshmi & Abbasi, 2004). The neem seed cake has also been reported to improve antioxidant status of plants (Ghimeray *et al*., 2009).

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). Salicylic acid (SA) is a natural phenolic compound is essential for the development systemic acquired resistance (SAR) in plants (Gaffney *et al*., 1993, Hussain *et al*., 2015). SA also play an important role in the response to salinity stress (Miura & Tada, 2014). Plant growth promoting rhizobacteria (PGPR) can induce systemic defense in plant against a variety of bacterial diseases, nematodes, pests and fungal infection (DeMeyer & Hofte, 1997, Hass & Defago, 2005). The bacteria belonging to the fluorescent *Pseudomonas* have been reported to suppressed several plant diseases (Noreen *at al*., 2015; Siddiqui & Ehteshamul-Haque, 2001). Among the fluorescent *Pseudomonas, P. aeruginosa,* isolated from rhizosphere, rhizoplane and also as endophyte were found to reduce several soilborne pathogens on several crops (Afzal *et al*., 2013; Bokhari *et al.,* 2014; Ehteshamul-Haque *et al.,* 2013; Shafique *et al.,* 2015a; 2015b). Besides, Direct suppression *P. aeruginosa* also induced systemic resistance in plants via synthesis of salicylic acid (DeMayer & Hofte, 1997, DeMayer *et al.,* 1999).

The cotton (*Gossypium hirsutum* L.) is one of the most important crops of Pakistan is affected by *Macrophomina phaseolina,* besides, cotton leaf curl virus (Anjum *et al.,* 2014; Parveen, 2011, Watkins, 1981). *Macrophomina phaseolina* has wide host range and is responsible for causing losses on more than 500 cultivated and wild plant (Ijaz *et al.*, 2012, Khan, 2007). Suppression of parasitic nematodes and soil borne fungi have been reported by organic amendments (Oka, 2010). However, induction of systemic resistance in plants by the neem cakes soil amendment is poorly investigated (Bhuvaneswari *et al.,* 2012). The present report describes the effects of organic amendment with neem cake alone or with *P. aeruginosa* on root rotting fungi, plant growth, and stimulation of defense system in cotton.

Materials and Methods

Experimental design: The experiment was conducted in randomized complete block design with four replicates to evaluate the induction of systemic resistance in cotton by the neem cake and *P. aeruginosa* under *M. phaseolina* infection and salinity stress. The experiments were conducted under in screen house and also in field plots.

Bacterial culture: An endophytic isolate of *P. aeruginosa* (ABPL-251), a plant growth promoting bacterium (PGPB), obtained from Karachi University Culture Collection was used in these experiments. The bacterial isolates was originally isolated from root of a healthy okra plant and have shown significant biocontrol activity against soilborne diseases in our previous studies (Afzal *et al*., 2013; Shafique *et al*., 2015b).

Fungal inoculum: *Macrophomina phaseolina,* isolated from diseased roots of cotton and multiplied on potato dextrose agar (PDA) was used in these experiments.

Screen house experiment: Neemex powder (neem cake) purchased from Sigma Energy (pvt) Ltd, Karachi was mixed with sandy loam soil (pH 8.0) at 1% w/w. The soil had a natural infestation of *M. phaseolina* (2-8 sclerotia g^{-1} of soil) as determined by wet sieving and dilution plating (Sheikh & Ghaffar, 1975), 3-10% colonization of *Rhizoctonia solani* on sorghum seeds used as baits (Wilhelm, 1955) and 3000 cfu g^{-1} of soil of a mixed population of *Fusarium oxysporum* and *F. solani* as determined by soil dilution technique (Nash & Snyder, 1962). Earthen pots (15 cm diameter) were filled with 1 kg amended soil and kept at 50% water holding capacity (WHC) by the daily adjustment of water (Keen & Raczkowiski, 1921). The pots were watered for 7 days to allow complete decomposition of organic matter. Cotton seeds, purchased from Tassco Seed Corporation (FH-1000) were sown in each pot at 6 seeds per pot and 25mL aqueous suspension of *P.aeruginosa* (8×10^8cfu/mL) grown in KB broth was drenched onto each pot. After germination four seedlings were kept in each pot and excess were removed. Plants grown in unamended or un-inoculated soil served as control. Five day old culture of *M. phaseolina* was macerated in one liter distilled water and 25 mL of this suspension (mycelial/sclerotial) containing 1g fungus was inoculated around the roots by careful removing of the top layer of soil and after inoculation the soil was spread over inoculum. Non-infested soil served as control.

Salinity stress: For salinity stress 1% sea salt solution (EC= 17.3 dS, mL^{-1}) was applied daily to plants after planting in earthen pots lined with polythene sheets to avoid salt leaching. Seedlings not received salt but received equal amount of water served as control. The experiment was terminated after 30 days and cotton seedlings were carefully uprooted and roots were washed under tap water. Observation on plant growth, such as plant height and fresh weight of shoot, root length, fresh root weight, fungal infection and plant stress resistant markers like phenolic content and salicylic acid were determined.

Field experiment: The experiment was repeated under field condition in 2×2 meter field pots (sandy loam soil) at Crop Disease Research Institute, Pakistan Agricultural Research Council, Karachi University Campus, Karachi. The soil had natural infestation of root rotting fungi as mentioned above. The neem cake, 70gm was mixed in 2 meter row and watered at one day interval for 7 days to allow decomposition of organic matter. Thirty seeds of cotton were sown in each row. Each treatment was replicated four times and randomized in complete block design. The experimental treatments and plant parameters were same as used in the pot experiment except that the aqueous suspension of *P. aeruginosa* (8×10^8 cfu. mL^{-1}) and mycelial suspension of *M. phaseolina* were used at 250 mL per row. Plants irrigated with equal amount of water or saline water thrice in a week. Observations were recorded after 30 days of growth.

Determination of fungal infection: To assess the efficacy of soil amendment and *P. aeruginosa* under *M. phaseolina* infection and salinity stress the roots were washed thoroughly with sterilized water and the causal fungi were isolated, where tap roots were cut into small pieces (1 cm long), surface sterilize with sodium hypochlorite (1%) for 3 minute. The sterilized root pieces were aseptically transferred onto Petri dishes containing potato dextrose agar (PDA) supplemented with penicillin (100,000 units $litre^{-1}$) and streptomycin (0.2 g $litre^{-1}$) and incubated at room temperatures (25–30°C) for 5 days. Fungi emerged from root pieces were identified and infection percentage were calculated (Shafique *et al.,* 2015a).

Sample preparation for biochemical analysis: The leaves of cotton were placed in small paper bags and dried in oven at 70°C.The dried leaves were extracted with EtOH (96% v/v) at the concentration of 10 mg mL^{-1} at room temperature. The samples were centrifuged at 1600×g for 15 minutes and supernatant was separated for biochemical analysis.

Antioxidant activity

DPPH -free radical scavenging activity: The DPPH-radical scavenging activity of sample was determined using DPPH (2, 2-Diphenyl-1-picrylhydrazyl) assay (Tariq *et al.,* 2011). An aliquot of 200µL of extract was mixed with 800µL of 10 mMTris-HCl buffer (PH 7.4). In the mixture 30µM DPPH (dissolved in DMSO) was added and vortex. Control was made by 1mL of aqueous ethanol with 1 mL of DPPH. The absorbance was measured at 517 nm on UV-visible spectrophotometer at 0 minute against aqueous ethanol as blank. The antioxidant activity was calculated by using the formula:

$$\text{Antioxidant activity} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

ABTS assay: ABTS (2,2 –azino-bis (3-ethylbenzthiazoline -6-sulphonic acid), radical scavenging activity was measured by the ABTS cation decolorization assay as described by Re *et al.* (1999) with some modifications. According to this method 10 mL of 7.0 mM ABTS was reacted with 176µL (140mM) potas-

sium persulphate. The solution was kept overnight in the dark to yield the $ABTS^+$ radical cation. After 24 hours incubation ABTS radical cation solution was diluted with 50% ethanol and 100µL of ABTS reagent mixed with 1µL of sample aliquot. Butylated hydroxytoluene (BHT) was used as standard at the same concentrations as sample. A micro plate reader (BioRad) was used to read the absorbance at 415 nm at 0 minnute. Ethanol was used as a blank. Control consists of 100 µL ABTS reagent and 1µL ethanol solvent. The inhibition percentage was calculated by the following formula:

$$\text{Scavenging activity (\%)} = \frac{\text{1-absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Total polyphenol analysis: For estimation of polyphenol, 100 µL aliquots were mixed with 2 mL of (2% w/v) Na_2CO_3 and allowed to stand for 2 minutes at room temperature. After incubation 100 µL of 50% Folin-Ciocalteu Phenol reagent was added and reaction mixture was mixed thoroughly and allowed to incubate for 30 minutes at room temperature in dark. Absorbance of samples was recorded at 720 nm on spectrophotometer (Chandini *et al.,* 2008). Gallic acid was used to prepare standard curve for the calculation of phenolic content in µg mL^{-1}.

Analysis of salicylic acid: Estimation of salicylic acid (SA) was done by spectrophotometric method described by Warrier *et al.* (2013). SA measurements were carried out by using 100 µL of the cooled aliquots of leaves mixed with 0.1% freshly prepared ferric chloride. The volume of the reaction mixture was made up to 3.0 ml with ferric chloride and absorbance was recorded at 540nm by spectrophotometer. The standard curve was prepared, where 100 mg of SA was dissolved in 100 mL of ethanol and amount of SA (µg.mL^{-1}) was calculated and expressed in mg/gm^{-1} dried sample.

Statistical analysis: Data were analyzed using two way ANOVA for plant growth, resistance markers and antioxidant activity, whereas, for fungal infection three way ANOVA was used. Means were separated using least significant difference (LSD) according to Gomez & Gomez (1984).

Results

Screen house experiment

Salinity stress: Neem cake amendment of soil reduced the adverse effect of salinity on plant growth and produced significantly ($p<0.05$) taller plants (23.7 cm) as compared 18.1 cm in salinity control plants, while 3.8 g/ plant fresh weight of shoots were found in neem cake + *P. aeruginosa* treatment under salinity stress compared to 1.4 g / per plant in salinity control plants (Table 1). In non- stressed condition, *P. aeruginosa* in neem caked amended soil significantly reduced *M. phaseolina* (6.2%) as compared to 56.2% in control plants. Whereas under salinity stress only 25% control plants were found infected with *M. phaseolina* (Table 2). Neem cake and *P. aeruginosa* alone or mixed significantly suppressed *F. solani* in non-stressed plants. Antioxidant activity at 0 minutes was found highest in neem cake + *P. aeruginosa* treatment (36.7%), which was slightly reduced due to salinity (25.2%), but was found significantly higher as compared to plant received only salinity (8.6%) (Table 3). Similarly neem cake and *P. aeruginosa* also induced higher amount of polyphenols (4.0-5.5 mg/mL) and salicylic acid (8.8–9.9 mg/mL) in plants as compared to plants grown in un-amended soil whether received salinity or not (polyphenol 2.2–2.8 mg/mL) and (salicylic acid 3.7-5.6 mg/mL) (Table 3). However, in ABTS method antioxidant activity was found increased due to salinity than control plants (Table 3).

Table 1. Effect of neem cake and *Pseudomonas aeruginosa* on the growth of cotton under salinity and biotic stresses (*Macrophomina phaseolina*) in screen house experiment.

Treatments	Shoot length (cm)			Shoot weight (g)			Root length (cm)			Root weight (g)		
	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress
Control	19	18.1	18.2	1.65	1.49	1.65	15.1	14.8	14.6	0.35	0.24	0.32
Neem cake	27.7	23.7	20.4	4.94	2.81	2.51	19.6	18	18.8	0.66	0.42	0.40
P. aeruginosa	25.8	21	22.2	4.0	2.12	2.12	21.3	18.6	19.3	0.68	0.46	0.46
Neem cake + *P. aeruginosa*	28.9	22.6	26.6	5.44	3.84	5.08	23.1	21	22.4	0.77	0.44	0.64
$LSD_{0.05}$	Treatments = 2.2[1] Stress = 1.10[2]			Treatments = 1.39[1] Stress = 0.69[2]			Treatments= 1.6[1] Stress=0.83[2]			Treatments= 0.11[1] Stress=0.05[2]		

[1]Mean values in column for each parameter showing differences greater than LSD values are significantly different at $p<0.05$

[2]Mean values in row for each parameter showing differences greater than LSD values are significantly different at $p<0.05$

Table 2. Effect of neem cake and *Pseudomonas aeruginosa* on the infection of *Macrophomina phaseolina* and *Fusarium solani* in cotton under salinity and biotic stresses (*Macrophomina phaseolina*) in screen house experiment.

Treatments	*M. phaseolina*			*F. solani*		
	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress
	Infection %					
Control	56.25	25	68.7	100	87.5	93.7
Neem cake	18.7	18.7	31.2	62.5	81.2	75
P. aeruginosa	12.5	187	50	62.5	81.2	68.7
Neem cake + *P. aeruginosa*	6.25	12.5	12.5	50	75	62.5
$LSD_{0.05}$	Treatments= 17.4[1], Stress=8.7[2], Pathogen=8.7[3]					

[1]Mean values in column for each fungal pathogen showing differences greater than LSD values are significantly different at $p<0.05$

[2]Mean values in row for each stress showing differences greater than LSD values are significantly different at $p<0.05$

[3]Mean values in row for each fungal pathogen under different stress showing differences greater than LSD values are significantly different at $p<0.05$

Biotic stress: Application of *P. aeruginoa* in neem cake amended soil produced tallest plant (26.6 cm) with highest fresh shoot weight (5.0 g) and root length (22.4 cm) as compared to respective control plants, which showed a plant height (18.2 cm), fresh shoot weight (1.6 g) and root length (14.6 cm) (Table 1). Plant grown in neem cake amended soil with *P. aeruginosa* showed significantly ($p<0.05$) less infection (12.5%) of *M. phaseolina* as compared to plants inoculated with *M. phaseolina* (68.7%) and grown in un-amended soil (Table 2). Highest antioxidant activity by DPPH method at 0 minutes was found in plants grown in neem cake amended soil and inoculated with *P. aerginosa* (31.7%), as compared to control plants (9.3%). The same pattern was found when antioxidant activity was examined by ABTS method (Table 3). The concentration of polyphenols was noted significantly ($p<0.05$) higher in neem cake+ *P. aeruginosa* treatment (Table 3). *Pseudomonas aeruginosa* and neem cake alone or combined induced synthesis of higher amount of salicylic acid (7.7–8.9 mg/mL) as compared to 4.7 mg/mL in control plants (Table 3).

Field plot experiment

Salinity stress: In field experiment, neem cake with *P.aeruginosa* showed a positive impact on plant growth in both normal and salinity stressed plants, with improvement in plant height (31.7– 34.0 cm) and fresh weight of shoot (5.0- 10.6 g) as compared to 25.5-26.7 cm of plant height and 3.9–4.7 g fresh weight in control plants (Table 4). Protective effect of neemcake + *P. aeruginosa* (25%) against *M.phaseolina* was also found in plants stressed with salinity as compared 68.7% control plants were found infected (Table 5). Neem cake + *P. aeruginosa* improved antioxidant activity of cotton plants, both normal and salinity stressed plants as compared to control and plants given only salinity stress (Table 6). Plant resistance markers like phenolic contents (3.1–4.0 mg/mL) and salicylic acid (8.6-9.1 mg/mL) were also induced in higher amount than control plants, 2.4–2.9 and 3.8–4.6 mg/mL respectively (Table 6).

Biotic stress: Under *M. phaseolina* stress, highest plant height was found in neem cake + *P. aeruginosa* (30.5 cm) or *P. aeruginosa* (30.0 cm) treatments as compared to 25.7 cm in control plants, while 14 cm root length was found in *P. aeruginosa* + neem cake treatment as compared to 9.0 cm in respective control plants (Table 4). Plant grown in neem cake amended soil showed significantly ($p<0.05$) less infection of *M. phaseolina* (43.7%) as compared to plants inoculated with *M. phaseolina* (75%) and grown in un-amended soil (Table 5). The efficacy of neem cake increased when *M. phaseolina* infested plants received *P. aeruginoa* (Table 5). Neem cake and *P. aeruginosa* also significantly ($p<0.05$) suppressed *M. phaseolina, R. solani* and *F. solani* both in naturally infested and *M. phaseolina* inoculated soil (Table 5). Plants grown in neem caked amended soil and received *P. aeruginosa* showed highest antioxidant activity as compared to *M. phaseolina* inoculated and control plants in both test methods i.e., DPPH and ABTS, with enhanced production of phenolic contents (3.7 mg/mL) and salicylic acid (6.9 mg/mL) as compared to respective control plants i.e. 2.7 and 2.8 mg/mL respectively (Table 6).

Discussion

Among the various stress of plants, salinity and fungal infection are two most important factors limiting crop production worldwide. The global annual losses in agricultural production from salt affected lands are more than US$ 12 billion (Flowers *et al.,* 2010). Plant resistance against abiotic and biotic stresses is a physiological state that improved defensive capability of plant. Phenolic content and salicylic acid served as a defense molecules under various stress. In this study, phenolic content (4.0 mg mL^{-1}gallic acid equivalents) and salicylic acid (9.1 mg mL^{-1}) were found significantly higher in plants treated with *P. aeruginosa* and grown in neem cake amended soil as compared to 2.41 mg mL^{-1} of polyphenols and 3.86 mg mL^{-1} of salicylic acid in control plants. The accumulation of both molecules actually decides the level of tolerance of a plant against stresses (Khan *et al.,* 2015). Phenolic compounds are well known secondary plant metabolites and total phenolic content of plant is associated with their antioxidant activities due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. Naseer *et al.* (2014) reported that neem leaves posses higher values of total phenolic content than other medicinal plants. Similarly, salicylic acid (SA) is a natural phenolic compound that influences a variety of biochemical and molecular events related with induction of disease resistance. SA has been reported to play a significant role in expression of local resistance controlled by major genes and systemic induced resistance extended after an early pathogen infection (Hammerschmidt & Smith-Becker, 2000). The antioxidant activity in DPPH-free radical scavenging activity was found increased in neemcake + *P. aeruginosa* treatment (34.0%) than control plants (12.5%) in field experiment**.** Different organic matters have been investigated for their antioxidant activity and neem cake amendment has been found to be more effective with regard to natural antioxidants (Ghimeray *et al.,* 2009). Reactive oxygen species (ROS) are potentially capable of altering normal plant cellular metabolism and was found increased in numerous plant species under salinity stress. Plants are protected by number of enzymatic and non- enzymatic antioxidants in relation to the scavenge free radicals (Bagheri *et al.,* 2013). Ghimire *et al*. (2011) and Naseer *et al*. (2014) reported that *Azadirachta indica* (neem) posses a highest scavenging activity as compare to other medicinal plants. The increased concentration of polyphenol with antioxidant activity is also supported by the study of Ghimeray *et al*. (2009) and Kumar *et al*. (2008). The polyphenol accumulation and enhanced antioxidant ability in salt tolerant plants have been reported (Ksouri *et al*., 2012a, 2012b; Ozgur *et al*., 2013). The total polyphenol content was found twice high in halophytes (Ksouri *et al*., 2012b).

Table 3. Effect of neem cake and *Pseudomonas aeruginosa* on free radical scavenging activity, phenolic content and salicylic acid in cotton under salinity and biotic stresses (*Macrophomina phaseolina*) in screen house experiment.

Treatments	Antioxidant activity at 0 minutes (% inhibition)						Phenolic contents			Salicylic acid		
	DPPH method			ABTS method			mg mL^{-1} gallic acid			mg mL^{-1}		
	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress
Standard (BHT)	70.4	70.4	70.4	70.4	70.4	70.4	--	--	--	--	--	--
Control	12.7	8.6	9.3	42.4	38.4	48.7	2.29	2.85	2.85	3.73	5.6	4.7
Neem cake	34.3	18.2	28.1	55.1	51.3	50	2.70	2.97	3.63	7.26	8.76	7.66
P. aeruginosa	31.7	15	20.1	50	50	50	3.35	4.2	3.33	8.23	8.9	8.6
Neem cake+ *P. aeruginosa*	36.7	25.2	31.7	53.4	51.3	50	4.0	5.5	3.78	8.8	9.9	8.9
$LSD_{0.05}$	Treatments= 4.3[1] Stress=2.0[2]			Treatments= 2.9[1] Stress=1.39[2]			Treatments= 0.21[1] Stress=0.10[2]			Treatments=0.08[1] Stress=0.04[2]		

[1]Mean values in column for each parameter showing differences greater than LSD values are significantly different at $p<0.05$

[2]Mean values in row for each parameter showing differences greater than LSD values are significantly different at $p<0.05$

Table 4. Effect of neem cake and *Pseudomonas aeruginosa* on the growth of cotton under salinity and biotic stresses (*Macrophomina phaseolina*) in field experiment.

Treatments	Shoot length (cm)			Shoot weight (g)			Root length (cm)			Root weight (g)		
	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress
Control	25.5	26.7	25.7	3.91	4.79	3.04	11.6	9.25	9.0	1.03	0.47	0.48
Neem cake	31.4	30.6	28.8	6.64	6.67	3.58	13.6	11.0	11.4	1.68	0.61	0.58
P. aeruginosa	29.7	29	30.0	5.33	5.37	4.99	11.3	12.5	10.5	1.63	0.57	0.55
Neem cake+ *P. aeruginosa*	34.0	31.7	30.5	10.6	5.09	5.88	18.3	14.9	14	2.19	0.66	0.64
$LSD_{0.05}$	Treatments=1.48[1] Stress=0.74[2]			Treatments=0.98[1] Stress=0.49[2]			Treatments=1.94[1] Stress=0.97[2]			Treatments= 0.31[1] Stress=0.51[2]		

[1]Mean values in column for each parameter showing differences greater than LSD values are significantly different at $p<0.05$

[2]Mean values in row for each parameter showing differences greater than LSD values are significantly different at $p<0.05$

Table 5. Effect of neem cake and *Pseudomonas aeruginosa* on the infection of *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium solani* in cotton under salinity and biotic stresses (*Macrophomina phaseolina*) in field experiment.

Treatments	*M. phaseolina*			*R. solani*			*F. solani*		
	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress
	Infection %								
Control	62.5	68.7	75	56.2	50	68.7	68.7	75	81.2
Neem cake	25	37.5	43.7	18.7	25	43.7	25	31.2	56.2
P. aeruginosa	18.7	31.2	50	6.2	18.7	31.2	31.2	37.5	62.5
Neem cake+ *P. aeruginosa*	6.2	25	37.5	0	6.2	6.2	18.7	43.7	50
$LSD_{0.05}$	Treatments= 13.1[1], Stress=6.5[2], Pathogen=8.0[3]								

[1]Mean values in column for each fungal pathogen showing differences greater than LSD values are significantly different at $p<0.05$
[2]Mean values in row for each stress showing differences greater than LSD values are significantly different at $p<0.05$
[3]Mean values in row for each fungal pathogen under different stress showing differences greater than LSD values are significantly different at $p<0$

Table 6. Effect of neem cake and *Pseudomonas aeruginosa* on free radical scavenging activity, phenolic content and salicylic acid in cotton under salinity and biotic stresses (*Macrophomina phaseolina*) in field experiment..

Treatments	Antioxidant activity at 0 minutes (% inhibition)						Phenolic contents			Salicylic acid		
	DPPH method			ABTS method			mg mL^{-1} gallic acid			mg mL^{-1}		
	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress	No stress	Salinity stress	Biotic stress
Standard (BHT)	70.4	70.4	70.4	70.4	70.4	70.4	--	--	--	--	--	--
Control	12.8	12.5	11.8	37.2	26.8	30.1	2.41	2.92	2.7	3.86	4.66	2.8
Neem cake	32.6	28.1	28.6	46.2	35.5	36.3	3.65	2.8	3.69	5.2	5.76	4.9
P. aeruginosa	24.7	16.8	23.1	41.4	33.5	39.7	3.48	3.78	3.04	7.5	7.8	6.23
Neem cake + *P. aeruginosa*	39.4	34.0	31.7	53.9	33.2	45.9	4.0	3.12	3.7	9.1	8.6	6.96
$LSD_{0.05}$	Treatments= 2.6[1] Stress=1.2[2]			Treatments= 3.0[1] Stress=1.4[2]			Treatments= 0.29[1] Stress=0.14[2]			Treatments=0.59[1] Stress=0.29[2]		

[1]Mean values in column for each parameter showing differences greater than LSD values are significantly different at $p<0.05$
[2]Mean values in row for each parameter showing differences greater than LSD values are significantly different at $p<0.05$

In this study application of neem cake as soil amendment alone or with *P. aeruginosa* significantly reduced infection of *M. phaseolina.* Ghimeray *et al.* (2009) reported that significant amount of azadirachtin and nimbin are present in all parts of neem tree which provides a defense role against molds, nematodes, leaf-eating insects. Similarly, Abbasi *et al*. (2005) reported 67%–90% reduction in the number of lesion (*Pratylenchus penetrans*) and root-knot nematode (*Meloidogyne hapla*) in tomato roots and suppression of root rotting fungi by the neem cake soil amendment and suggested that killing of microsclerotia of *Verticillium dahlia* may be due to the generation of ammonia during decomposition of neem cake or due to creation of diseases suppressive climate. The neem cake also improved soil nutrition status which increased plant height and weight (Kumar & Khanna, 2006). It was observed that maximum induction of resistance marker compounds like polyphenol and salicylic acid were produced by neem cake. SA affects the phytohormone level of plants and could be the one of the mechanisms of plant protection under stress condition (Shakirova *et al.,* 2003).

It was observed that plants grown in neem cake amended soil showed better plant growth and had higher concentrations of plant resistance markers like polyphenol and salicylic acid which were further increased when *P.aeruginosa* was added in amended soil. There are reports that numerous strain of fluorescent *Pseudomonas* induced systemic resistance in plants and have a practical application in natural agriculture practices (Vallad & Goodman, 2004). *Pseudomonas aeruginosa* is a beneficial soil bacteria that promote the plant growth and development through production of a variety of regulatory compounds (IAA, ammonia, exo-polysaccharides and phosphate solubilization) in the rhizosphere directly or indirectly (Ahemad & Kibret, 2014; Siddiqui & Ehteshamul-Haque, 2001). Usually, *P. aeruginosa* facilitate the plant growth and development acting as a biocontrol agents directly by assisting in resource attainment (nitrogen, phosphorus and essential minerals) and alter plant hormone levels, or reduce the potential of different plant pathogens. Many researchers revealed that under normal and stressful conditions plant vigor and productivity enhanced by the application of plant growth promoting bacteria (PGPB) like *Pseudomonas* (Glick, 2012). *Pseudomonas* was also found effective biocontrol agents for many crops grown in coastal saline soils (Paul & Nair, 2008).

Conclusion

It has been concluded that neem cake suppressed soil borne pathogen and improved plant growth via induction of resistance compounds like polyphenol and salicylic acid which improve antioxidant status of plant. The induction of systemic resistance in cotton by the neem cake was further improved by the application of *P. aeruginosa,* a plant growth promoting bactrium. Whereas, stress caused by *M. phaseolina* and salinity slightly increased polyphenol and salicylic acid with less effect on antioxidant status.

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