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INDUCTION OF SYSTEMIC RESISTANCE IN COTTON BY THE PLANT GROWTH PROMOTING RHIZOBACTERIUM AND SEAWEED AGAINST CHARCOAL ROT DISEASE

AFSHAN RAHMAN[1*], FARZANA KOREJO[1,4], VIQAR SULTANA[2], JAHAN ARA[3] AND SYED EHTESHAMUL-HAQUE[1]**

[1] Agricultural Biotechnology & Phytopathology Laboratory, Department of Botany, University of Karachi, Karachi 75270, Pakistan
[2] Biotechnology & Drug Development Laboratory, Department of Biochemistry, University of Karachi, Karachi 75270, Pakistan
[3] Postharvest & Food Biochemistry Laboratory, Department of Food Science & Technology, University of Karachi, Karachi 75270, Pakistan
[4]Department of Plant Science, University of Sindh, Jamshoro, Sindh, Pakistan
[*]Corresponding author's e-mail: afshan.rahman48@yahoo.com

Abstract

Combined effect of *Pseudomonas aeruginosa* and *Sargassum ilicifolium* was evaluated in inducing resistance in cotton against charcoal rot disease caused by *Macrophomina phaseolina* under screen house conditions. In this study, plants inoculated with *P. aeruginosa* in *S. ilicifolium* amended soil showed the highest antioxidant activity both in DPPH (2, 2-diphenyl-2-picrylhydrazyl) and ABTS (2,2 -azinobis (3-ethylbenzthiazoline-6-sulphonicacid) test. Infection percentage of *M. phaseolina* was significantly suppressed in *P. aeruginosa* used alone or with *S. ilicifolium* amended soil. Proline is a potent osmolyte which greatly elevated in *M. phaseolina* infested plant that was found to reduce in *P. aeruginosa* + seaweed treatment. Increased level of salicylic acid in *S. ilicifolium* +*P. aeruginosa* treatment under charcoal rot stress indicated the induction of systemic resistance. In another signaling molecule, polyphenol was significantly enhanced by mixed application of *P. aeruginosa* + brown seaweed *S. ilicifolium*. Our results suggested that elevated level of phenolic contents positively correlated with highest antioxidant activities that may provide a protection via induction of resistance in cotton plant against charcoal rot disease.

Keywords: cotton, charcoal rot disease, induced systemic resistance, plant growth promoting rhizobacteria (PGPR), seaweed.

Introduction

The cotton (*Gossypium hirsutum* L.) is one of the most important crop of Pakistan, about 60% of the total foreign exchange of Pakistan (Iqbal *et al.,* 2005; Ali & Sultan, 2007). Besides Cotton Leaf Curl Virus, several soil borne diseases affect cotton production in Pakistan (Parveen, 2011). *Macrophomina phaseolina* (Tassi) Goid is a soilborne fungus which causes charcoal root rot disease on many important crops, including cotton ((Ijaz *et al.,* 2012; Magyarosy *et al.,* 1985). Control of *M. phaseolina* by convential method is difficult, since it produced sclerotia which survive in soil in adverse condition (Khanzada *et al*., 2012). However induction of systemic resistance in plant against plant pathogens is gaining popularity as alternate method of disease control (Shafique *et al.,* 2015ab).

In recent years, the inoculation of plant growth promoting rhizobacteria **(**PGPR) in agriculture is a new approach to develop systemic resistance against soil borne pathogens (Viswanathan & Samiyappan, 1999). Plant growth promoting rhizobacteria **(**PGPR) induced systemic resistance (ISR) indirectly by increasing plant nutrition, production of antibiotics in the rhizosphere and enhanced plant defense responses by altering the host's signaling pathways through jasmonic acid (JA) and salicylic acid (SA) (Pieterse *et al.*, 2000). It has been reported that root colonization of *Pseudomonas* strains induce ISR which defend the plants against different types of pathogen such as *Pseudomonas syringae* pv. tomato and *Xanthomonas campestris* pv. armoraciae, the fungal root pathogen *Fusarium oxysporum* and the fungal leaf pathogen *Peronospora parasitica* (Pieterse *et al.,* 1996). Besides' rhizobacteria compete with soil pathogen for nutrients and space and increased plant growth and yield (Elad & Chet, 1987), secrete antibiotics (pyrrolnitrin, pyocyanine, and 2,4-diacetyl phloroglucinol) (Pierson & Thomashow, 1992) and siderophores which bounds the accessibility of Fe required for the growth of pathogens (Kloepper *et al*., 1980; Lemanceau *et al*., 1992).

From past decades, seaweed extract was applied as bio-fertilizer to improve crop yield and productivity and suppressed soilborne pathogens (Raghavendra *et al.,* 2007; Khan *et al.,* 2009; Ehteshamul-Haque *et al*., 2013). Marine brown algae contain large number of naturally occurring source of potential elicitors which induced systemic resistance in plants against biotic and abiotic stresses (Ganapathi *et al.,* 2013). Raghavendra *et al.,* (2007) utilized extract of *Sargassum wightii*, a brown alga which induces resistance in cotton against *Xanthomonas campestris* pv. *malvacearum.* Klarzynski *et al.,* (2000) reported that *Laminaria digitata* contain linear *β*-1,3 glucans which induced several defense responses in tobacco in cell suspension cultures. Similarly, Aziz *et al.,* (2003) observed that *Laminaria* spp. provide protection and induced defense responses against *Botrytis cinerea* and *Plasmopara viticola* in grapevine. In our studies, *Sargassum* spp., have shown significant suppressive effect on root rotting fungi and root knot nematode (Ara *et al*., 1996; 1997). The present report describes the suppression of *M. phaseolina* on cotton via induction of systemic resistance by the *S. ilicifolium* and *P. aeruginosa.*

Materials and Methods

Plant growth promoting rhizobacterium (PGPR): Highly effective plant growth promoting rhizobacterial strain (PGPR) *Pseudomonas aeruginosa* (ABPL-251) was obtained from Karachi University Culture Collection (KUCC). Concentration of bacterial cells ($8x10^8$cells/mL) in KB broth was achieved by dilution to maintain uniform cell density.

Isolation of *M. phaseolina*: *M. phaseolina* was isolated from diseased cotton plant and cultured on potato dextrose agar medium.

Experimental design / Screen house experiment: Dry powder of brown seaweed *S. ilicifolium* at 1% w/w was mixed in sandy loam soil having pH 8.0. The soil had a natural infestation of 3-7 sclerotia g^{-1} of soil of *M.phaseolina* as determined by the method described by Sheikh & Ghaffar, (1975), 3000 cfu of mixed population of *F.oxysporum* and *F.solani* (Nash & Sanyder, 1962) and 3-6 % colonization of sorghum seeds used as baits (Wilhelm, 1955). One kg of un-amended and amended soil was filled in 15 cm diameter of earthen pots and kept at 50% water holding capacity by the daily adjustment of water. The pots were watered 2-3 days to allow complete decomposition of organic matter. Six sterilized seeds of cotton were sown in each pot and (25 mL) liquid suspension (10^8 cfu/mL) of *P. aeruginosa* (ABPL-251) was drenched onto each pot for soil inoculation. After germination four seedlings per pot were kept and excess were removed. After 15 days of seedling emergence, plants were artificially infested with 25 mL suspension of *M. phaseolina* @ 1.2×10^3 sclerotia/ g of soil. Unamended / uninoculated plants served as control. The experiment was conducted with Complete Randomized Block design with four replicates on a screen house bench at the Department of Botany. The plants were uprooted after 30 days of *M. phaseolina* treatment. Data on plant growth, infection of *M.phaseolina* and plant disease resistance markers were determined.

Determination of *M.phaseolina* infection: Infection % of diseased plants was calculated by method of Narayanasamy (2011). After washing with distilled water, the infected tap root tissues were cut into 1 cm long pieces and surface sterilized with 1% $Ca(OCl)_2$ for about 3 minutes. The sterilized root tissues were aseptically transferred to Petri dishes containing potato dextrose agar (PDA) with addition of penicillin, (100,000 units $litre^{-1}$) and streptomycin (0.2 g $litre^{-1}$) antibiotics to prevent the bacterial contamination. Petri dishes were incubated at room temperatures (25 –27°C) for 5 days for the growth of pathogen. Infection % of pathogen was calculated by using following formula:

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

Induction of defense mechanism: Cotton leaves were homogenized in EtOH (96% v/v) at the concentration of 10mg mL^{-1} and centrifuged at 1600×g for 15 minutes and supernatant was collected for biochemical analysis.

Determination of phenolic content: Total phenolic content was determined by the method of Chandini *et al.* (2008). Briefly, 100µL supernatant was mixed in 2 mL of (2% w/v) Na_2CO_3 and allowed to stand for 2 minutes at room temperature. After incubation 100µL (50%) of freshly prepared 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 developed blue color was measured at 720 nm on spectrophotometer. The content of the total soluble phenols was calculated according to standard curve obtained from a Folin-Ciocalteau reaction with gallic acid and expressed as phenol equivalents in mg gallic acid equivalents (GAE) per g of dried sample.

Determination of salicylic acid: Salicylic acid was determined by the spectrophotometric method of Warrier *et al.* (2013). Aliquots 100 µL was mixed with 3 mL (0.1%) freshly prepared ferric chloride. Absorbance of reaction mixture was measured at 540nm. One hundred mg of salicylic acid was dissolved in 100 mL of ethanol for preparation of standard curve and amount of SA (µg mL^{-1}) was calculated and expressed in mg/g^{-1} dried sample.

Determination of proline: Slightly modified method of Bates *et al.* (1973) was used to determine free proline content in leaves. Dried powder of leaves (0.5g) was homogenized in 5mL (3%) sulpho-salicylic acid. Filtered through Whatman No. 1 filter paper and filtrate was used for the estimation of proline content. The filtrate (2.0 mL) was reacted with 2.0 mL glacial acetic acid and 2.0 mL ninhydrin (1.25 g ninhydrin was dissolve in 30 mL of glacial acetic acid with 20 mL of 6N orthophosphoric acid). The sample was then heated on a boiling water bath for about 1 hour at 100 °C. Brick red color appeared in reaction mixture. After cooling the reaction mixture, 3 mL toluene was added and shaked thoroughly. Separate the color solution (chromospheres) containing toluene was separated by using separating funnel and absorbance was recorded at 520 nm on spectrophotometer against reagent blank (toluene). Standard curve of proline was prepared in (µg mL^{-1}) concentration. The concentration of proline was expressed as µmol g^{-1} DM by using following formula:

"µmol proline /g of DM = [(µg proline/mL) x 3mL toluene)/115.5 µg/µmole] / [(g sample)/5]"

Antioxidant activities

Determination of DPPH -free radical scavenging activity: The DPPH -free radical scavenging activity was measured by method of Tariq *et al.* (2011). The DPPH (30µM) working solution was prepared in DMSO and

diluted with ethanol to attain absorbance of about 0.9 ± 0.02 at 517 nm using the spectrophotometer. Aliquot 0.2mL was mixed in (0.8mL) of 100mM Tris-HCl buffer (pH 7.4). In this reaction mixture, 1mL of 30µM DPPH was mixed and vortex. 1mL aqueous ethanol with 1 mL of DPPH served as control. Absorbance of developed violet color of sample was measured at 0 min and incubated for 30 min in the dark, after 30 minutes optical density was recorded at 517 nm using UV-visible spectrophotometer, against aqueous ethanol, used as blank. The antioxidant activity was calculated by using the following formula:

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

Determination of ABTS -free radical scavenging activity: ABTS assay was used purposed by Re *et al.* (1999). To estimate ABTS free radical scavenging activity of plant extract, 10mL of 7.0mM ABTS was mixed with 176µL (140mM) potassium persulphate and kept overnight in the dark to develop $ABTS^{+}$ radical cation. After 24 hours incubation ABTS radical cation solution was diluted with 50% ethanol for an initial absorbance ≈ 0.700 (± 0.02) at 415nm on spectrophotometer. One µL of sample aliquot was mixed with 100µL of ABTS. A micro plate reader was used to read the absorbance at 415nm at 0, 1, 2, 3, 4 and 5 minute intervals. BHT was used as standard solution at the same concentrations as sample while, ethanol was used as blank. 100µL ABTS reagent and 1µL ethanol solvent used as control. The inhibition % calculated by
Scavenging activity (%) = {1-abs of sample / abs of control} x100

Statistical analysis: Data were statistically analyzed according to standard procedures for analysis of variance and mean separation (least significant difference). Plant growth, phenolic content and salicylic acid were analyzed using One-way ANOVA and means were separated using least significant difference (LSD). Fungal infection and antioxidant activities were analyzed by Two-way ANOVA (Gomez and Gomez, 1984).

Results and Discussion

Induce resistance against *M. phaseolina*: Growth parameters of plant significantly ($p<0.05$) enhanced by amendment of *S. ilicifolium* and *P. aeruginosa* in individual and combined treatment. They also protected the plant from adverse effect of *M. phaseolina.* The plant height, shoot weight, root length and root weight were significantly ($p<0.05$) increased by the combined application of *S. ilicifolium* and *P. aeruginosa* (Fig.1). Pise & Sabale (2010) found that *S. ilicifolium* significantly enhanced plant growth and development by increasing the total chlorophyll content, carotenoid, protein, amino acid, total carbohydrate content, total polyphenol, total nitrogen content and total ash content of leaves. There are several reports that plant growth promoting rhizobacteria (PGPR) used as a biofertilizer increased nutrient uptake by solubilization of organic matters (Rahman *et al.,* 2016; Cattelan *et al.,* 1999), induce synthesis of phytohormone (Egamberdiyeva, 2007) and also produce antibiotics, siderophores, antifungal compounds to inhibit the growth of pathogens (Jeun *et al.,* 2004).

Highest infection of *M. phaseolina* was found in plants artificially inoculated with *M. phaseolina* followed by untreated control plants, whereas highest infection of *R. solani* and *F.* solani was found in control plants i.e. only exposed to natural population of soilborne pathogens (Table 1). Treatment of *P. aeruginosa* (PGPR) alone or in *Sargassum* amended soil significantly reduced the infection % of soilborne pathogenic fungi (Table 1) by producing by inducing resistance through phytoalexin and antibiotic compounds production**.** Plant growth promoting rhizobacteria (PGPR) has been known to induce systemic resistance (ISR) in various crops against wide range of soilborne pathogenic micro-organism and fungal diseases (Zhang *et al.,* 2004; Jetiyanon *et al.,* 2003; Shafique *et al.,* 2015ab).

Table 1. Effect of *S. ilicifolium* amendment and *P. aeruginosa* on infection % of *M. phaseolina, R. solani* and *F. solani* on cotton plant.

	Infection%		
Treatments	***M. phaseolina***	***R. solani***	***F. solani***
Control	56.25	100	56.25
Sargassum ilicifolium	25	31.25	50
P. aeruginosa	18.75	50	18.75
S. ilicifolium + *P. aeruginosa*	12.5	31.25	12.5
M. phaseolina	68.75	37.5	62.5
S. ilicifolium + *M. phaseolina*	31.25	31.25	43.75
M. phaseolina + *P. aeruginosa*	37.5	12.5	37.5
S. ilicifolium + *M. phaseolina* + *P. aeruginosa*	18.75	6.25	18.75
$LSD_{0.05}$ = Treatments[1] = 14.6[1], Pathogens[2] = 8.9 [2]			

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

[2]Mean values in rows showing differences greater than LSD values are significantly different at $p<0.05$.

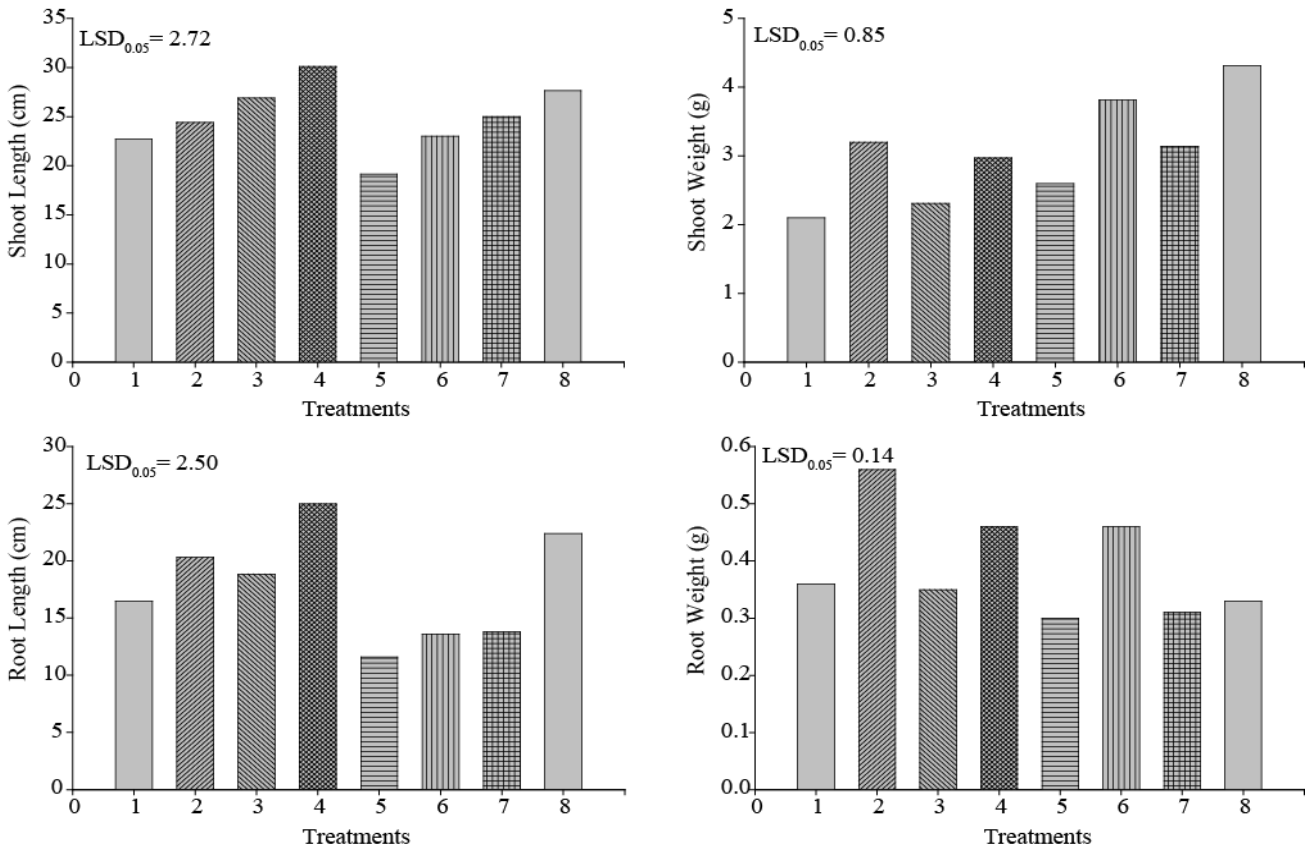

Fig 1. Effect of *Sargassum ilicifolium* amendment and *Pseudomonas aeruginosa* on growth parameters of cotton under biotic stress. (1= control, 2= *S. ilicifolium*, 3= *P. aeruginosa*, 4= *S. ilicifolium*+*P. aeruginosa*, 5= *M. phaseolina*, 6= *S. ilicifolium*+*M. phaseolina*, 7= *P. aeruginosa* +*M. phaseolina*, *8= S. ilicifolium*+*P. aeruginosa*+*M. phaseolina).*

The synthesis of phenolic contents was found highest in mixed treatment of *S. ilicifolium* + *P. aeruginosa* (Fig. 2). Singh *et al.*, (2002) studied the role of phenolic compounds in disease resistance and reported that they are accumulated against pathogen attack in host plant and served as a part of active defense response. However, in our study highest accumulation of phenolic content in cotton plant was found in combined application of *P. aeruginosa* and *S. ilicifolium* in natural soil followed by in plants artificially inoculated with *M. phaseolina*, but received *P. aeruginosa* in *Sargassum* amended soil (Fig. 2). This study clearly indicates that phenolic content did not synthesized in enough quantity in plants only inoculated with *M. phaseolia***.** *Pseudomonas aeruginosa* induced systemic resistance in cotton more efficiently in *Sargassum* amended soil than used alone.

Salicylic acid (SA) is also a phenolic compound that concern with physiological and biochemical changes in plant linked with induction of disease resistance subsequent to an early pathogen attack (Hammerschmidt & Smith-Becker, 2000). The highest amount of salicylic acid was observed in plants inoculated with *M. phaseolina*, but also received *S. ilicifolium* + *P. aeruginosa* treatment (Fig. 2). Levine *et al*. (1994) and Kauss & Jeblick (1996) explained that minute accumulation of salicylic acid (10 to 100 μM) found to be efficient for pathogen-induced defense gene expression. The increased amount of SA may be locally produced or systemically moved from plant growth promoting bacteria as free SA through plant phloem (Metraux *et al*., 1990; Yalpani *et al*., 1991).

Proline accumulation is a positive corelation during salinity stress (Yoshiba *et al*., 1995), drought stress (Barnett & Naylor, 1966), heavy metals ions (Chen *et al*., 2001), oxidative (Yang *et al*., 2009) and pathogen stress (Fabro *et al*., 2004). In this research, highest accumulation of proline was found under *M. phaseolina* stress but that was significantly reduced by combined application of *S. ilicifolium* and *P. aeruginosa* under normal and stress condition (Fig. 2). According to Szabados and Savoure (2010) proline is a multifunctional amino acid and served as an important energy source after revival of stress. It was studied by Handa *et al*., (1986) that accumulated proline rapidly degraded during stress recovery in cultured tomato cells (*Lycopersicon esculentum* cv VFNT-Cherry).

Maximum DPPH-scavenging activity of free radical was recorded in plant amended with *S. ilicifolium*. The *S. ilicifolium* amendment was found effective in enhancing the free radical scavenging activity and plant showed better survival against *M. phaseolina* (Fig. 2). ABTS radical scavenging activity of plant significantly ($p<0.05$) improved by increasing reaction time from 0-5 minutes. Highest inhibition % of free radicals was recorded in plant received combined treatment of *S. ilicifolium* and *P. aeruginosa* (Table 2). Meenakshi *et al.*, (2009) and Boonchum *et al.* (2011) have been reported strong antioxidant activity in aqueous and ethanolic extracts of *Sargassum* and concluded that may be due to presence of higher amount of phenolic compound. The highest antioxidant activity in PGPR + *S. ilicifolium* treated plants indicates that antioxidant molecules also play a significant role in inducing the systemic resistance in cotton against soilborne pathogens.

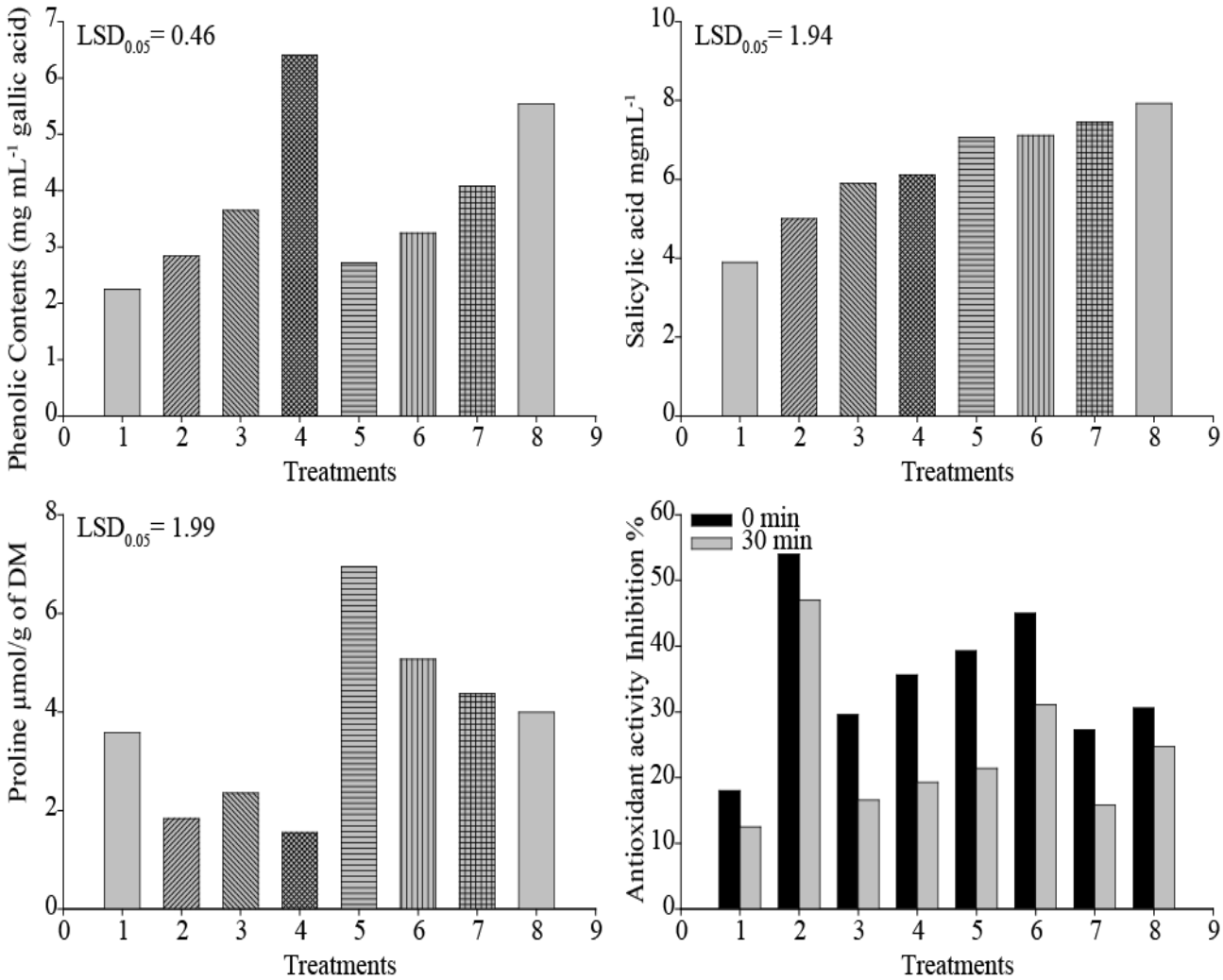

Fig 2. Effect of *Sargassum ilicifolium* amendment and *Pseudomonas aeruginosa* on phenolic contents, salicylic acid, proline contents and DPPH free radical scavenging activity.
(1= control, 2= *S. ilicifolium*, 3= *P. aeruginosa*, 4= *S. ilicifolium+P. aeruginosa*, 5= *M. phaseolina*, 6= *S. ilicifolium+M. phaseolina*, 7= *P. aeruginosa+M. phaseolina*, *8= S. ilicifolium+P. aeruginosa+M. phaseolina).*

Table 2. ***ABTS radical scavenging activity of cotton plant with combined effect of* S. ilicifolium *and* P. aeruginosa.**

Treatments	ABTS Assay %					
	0 min	1 min	2 min	3 min	4 min	5 min
Standard (BHT)	70.4	72.4	73	73.6	74.4	74.7
Control	50.3	55.6	66.5	69.0	70	70
Sargassum ilicifolium	57	62.2	68.3	70.3	70.3	71.6
P. aeruginosa	70.6	74	78.6	81	81.3	84
S. ilicifolium + *P. aeruginosa*	80	82.3	84	84	86.3	85
M. phaseolina	50.2	52.4	59.1	62.5	63.9	63.8
S. ilicifolium + *M. phaseolina*	55.6	59.1	64	66.6	67.5	71.6
M. phaseolina + *P. aeruginosa*	55.1	58.2	58.6	60.8	64	68
S. ilicifolium + *M. phaseolina* + *P. aeruginosa*	60	60.8	65.6	66.6	70	75.3
$LSD_{0.05}$= Treatments[1] = 2.89[1], Time[2] = 2.36[2]						

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

[2]Mean values in rows showing differences greater than LSD values are significantly different at $p<0.05$.

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