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A Study on Root Exudation Pattern and Effect of Plant Growth Promoting Fungi during Biotic and Abiotic Stress in Pigeonpea

Aashif Iqubal Khan[1], Rishi Ram Bhandari[2,*], Ambika Pokhrel[2], Ram Nandan Yadav[1]

[1]Institute of Agricultural sciences, Banaras Hindu University, Varanasi, U.P. India
[2]Institute of Agriculture and Animal Science, Tribhuvan University, Paklihawa Campus, Paklihawa, Nepal
*Corresponding author: rishi.bhandari63@gmail.com

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Abstract An experiment was conducted to observe the interaction of *Fusarium udum* and *Macrophomina phaseolina* with a rhizospheric microbe *Pseudomonas* [AKC-O11] to see their impact on pigeonpea under biotic and abiotic conditions. Both biotic [*Fusarium udum* and *Macrophomina phaseolina*] and abiotic stress (NaCl) were applied and performances of these microbes were evaluated. The strain was used individually and in combination with the stresses and applied as seed bacterization of pigeonpea (Var. MA-3) seeds to see the impact on total phenol content in plant root exudates. The bacterized seeds were grown under invitro conditions and after three days of germination the seedlings were exposed to biotic stress due to challenge of the pathogens [*Fusarium udum* and *Macrophomina phaseolina*] and abiotic stress due to irrigation with salt solution of 100 mM. Root exudates were collected at 48 h, 96 h and 144 h after the application of stresses. The collected root exudates were processed for total phenolic content and High Pressure/Performance Liquid Chromatography (HPLC) analysis. It was observed that total phenol content was low in seeds bacterized with *Pseudomonas* strain but the concentration increased when the plants were challenged with the pathogen particularly *Macrophomina phaseolina* and NaCl. Similarly, a similar trend was also observed in gallic acid accumulation. The above results indicates that *Pseudomonas* strain (AKC-O11) have potential to be used as biocontrol agent that can help pigeonpea plants to combat attack of *Macrophomina phaseolina* and *Fusarium udum* as well as salinity.

Keywords: *pigeonpea, plant growth promoting fungi, biotic, abiotic, total phenolic content*

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1. Introduction

Pigeonpea (*Cajanus cajan* L Millsp.) is grown as a pulse crop in many parts of the Indian subcontinent [1], a cross-pollinated, annual legume crop belongs to Family: Fabaceae and also known as dal. It is an important annual legume crop of rainfed agriculture in the semiarid tropics originated from eastern part of peninsular India. The cultivation started since 3,500 years ago in all tropical and semitropical regions of the world. Pigeonpea is cultivated in more than 25 tropical and subtropical countries, in the form of sole crop or intermixed with cereals, such as sorghum (*Sorghum bicolor*), pearl millet (*Pennisetium glaucum)*, or maize (*Zea mays)* and with other legumes, such as peanuts (*Arachis hypogaea)*. Pigeonpea can grow with temperature variation from 26 to 30°C in the rainy season (June to October) and 17 to 22°C in the post rainy season (November to March) [2]. Pigeonpea is highly sensitive to temperature at germination, flowering and pod development. In world, pulses or grain legumes (solely harvested for dry grains) are grown in 69.29 million hectares with production of 64.0 million Metric ton and productivity of 924 Kilogram hectare^{-1} during 2009. India is the largest grower (30% share in area), producer (23% share in production) and consumer. Nepal contributes about 0.4% of world pulse area and production [3]. Diverse climate and environmental conditions of Nepal offer opportunities for growing many species of food legumes. Grain legumes research received relatively little attention in Nepal as the primary need is on assuring food supply for the increasing population. In Nepal, pulses (includes soybean) occupies 10.08 % of total cultivated land, ranking fourth in area after rice, wheat and maize. During 2015-16 the total production of pulses was 363,693 Metric ton in an area of 327,321 hectares in Nepal [4]. The productivity of Pigeonpea has been low and stagnant for last four decades due to various diseases. More than 50 diseases have been reported to affect Pigeonpea however only few of them are responsible for economic loss [5,6]. The wilt disease of pigeonpea was

first reported from India by [7] and gave detailed account of the pathogen in 1918. *Fusarium* attacks at early stage of plant growth but causes severe problem at flowering and podding stages [6,8]. On potato dextrose agar (PDA) medium, mycelium is hyaline, slender, whitish to pale pink or grayish purple in color and branched with little aerial growth or slimy growth. It produces both types of micro as well as macro conidia and are unicellular or septate. Macro conidia are hyaline 3-5 septa and at later stage chlaymydospores are produced, which are usually intercalary, in pairs, globose and sub globose type [9]. In the susceptible plant at rhizospheric hypha and germ tubes of spores penetrate seedlings through root tips, wounds or point of formation of lateral roots. The mycelium damages the xylem cell and ultimately wilting of plants occurs. The growth of *Fusarium* pathogen is maximum at 28°C. Mycotoxins are secondary metabolites produced by *Fusarium* species which affect plant and animal health's [10,11]. The metabolites secreted by the pathogen are enzymes [12], toxins [13] and polysaccharides [14]. Mycotoxins produced by *Fusarium* species affect about 25% of the world food crops [15]. Toxins help pathogen in rapid and extensive invasion of the plants. The *Fusarium* wilt of pigeonpea is directly proportional to fusaric acid toxicity [13,16]. The occurrence of *Fusarium* wilt diseases is influenced by soil temperature [17], soil pH (like pH 6 is most suitable) [18] and high dose of nitrogenous fertilizer [19,20]. The disease is soil and seed borne and difficult to control with the help of fungicide. Inter and mixed cropping of sorghum reduces wilt in pigeonpea [21]. Seed treatment with *Bacillus subtilis* significantly reduced the incidence of pigeonpea wilt [22].

Macrophomina phaseolina (TASSI) G. GOIDANICH, is a soil borne fungus which causes seedling blight, root rot and charcoal rot of more than 500 crop species [23]. The disease symptoms are characterized by the presence of numerous black microsclerotia varying from 100 µm to 1 mm in stems, leaves, roots and 50-300 µm in culture [24]. Pycnidia may also sometimes be seen. These are black and globose varying from 100-250 µm in length with a truncate ostiole [25,26].

Pseudomonas are rod shaped, gram negative bacteria bearing flagella, aerobic in nature and contains high G + C (59.68 %) [27]. Fluorescent pseudomonads are called so as they produce a soluble fluorescent pigment called Pyoveridin formally Fluorescin, it is believed to be a siderophore. This large and heterogeneous group of fluorescent pseudomonads is comprised of species *P. putida*, *P. flurescens*, *P. syringe* and *P. aeruginosa* [28]. *Pseudomonas* spp. are used as biocontrol agents in agricultural crops as they have very high adoptive potential [29]. *Pseudomonas* protects plant from pathogens by colonizing roots of various crops like cereals, pulses, oilseeds, vegetables and promotes their growth [30]. *Pseudomonas fluorescens* is an effective biocontrol agent for different fungal pathogens [31].

Phenolic compounds are important secondary metabolites, synthesized and polymerized in plants cell for defense against infection and play important role in mechanism of plant resistance [32]. *Sclerotium rolfsii* infection was controlled by increasing the phenolic compounds in the host tissue [33]. At the site of pathogen invasion papillae deposition takes place [34]. The papillae constitutes of lignin, callose, cellulose, chitin, gums, silicon, suberin, proteins and phenols [35,36,37]. The phenol changes the composition of microflora in any environment [38]. The phenols have antimicrobial activities and have the capacity to denature proteins [39]. Production of phenolics with antimicrobial activities gives rise to resistance in plants [40]. The main roles of phenolics in plant protection are through contributing to structural integrity, photosynthesis and nutrient uptake in vascular plants [41]. Phenols like gallic and tannic acids have antimicrobial activities against various microorganisms and obtained from cascalote (*Caesalpinia cacalaco*) plant. The seed treated with plant growth promoting rhizobacteria (PGPR) elicits phenolic compounds in crops [42,43]. Polyphenol oxidases (PPO) are copper containing enzymes which are ubiquitous in nature and are capable of oxidizing ortho diphenolic compounds like caffeic acid and catechol to their respective quinones. These PPO generated quinones are highly reactive and they cross link with proteins. The cross-linking leads to the production of brown pigments in damaged plant tissues [44]. The PPO activity is found both in dicotyledonous as well as monocotyledonous plants [45].

The PGPR colonizes on plant roots, promote plant growth and reduce disease or insect damage [46] with beneficial effects. They help in plant growth by production and release of secondary metabolites like plant growth regulators /phytohormones /biologically active substances that will reduce deleterious effects of phytopathogenic organisms in the rhizosphere facilitating the availability and uptake of certain nutrients from the root environment [47]. The positive effects of PGPR on growth and yield of cultivated plants have been repeatedly reported [48-53].

The major constraints associated with PGPR are Natural variation, artificial multiplication and viability e.g., Rhizobia; PGPR bacteria will not live forever in a soil and over time growers will need to re-inoculate seeds to bring back populations. Bacteria produces some volatile organic compounds which are bacterial determinants involved in induced systemic resistance (ISR) in plants. The saline soil is limiting factor in arid region which have adverse effects on agricultural practice. This salinity can be eliminated by the application of biofertilizers, which stimulate the plant defense mechanism and allow crop cultivation in that area. Plant inoculated with PGPR increases the adaptability to salt and drought stress [54]. Some PGPR trigger ISR [55] and this ISR suppresses disease resistance in both green house and field conditions [56,57].

When PGPRs are inoculated under salt stress conditions then ACC-deaminase activity mitigates the inhibitory effects of salt stress on root growth by lowering the ethylene concentration in the plant, which results in prolific growth. The enhanced yield was recorded in wheat due to seed treatment of PGPR in salt stressed condition and similar result was found in sunflower plants when they treated with *Pseudomonas flourescens* biotype F and *Pseudomonas flourescens* CECT 378T in saline sand condition (100 mM (milimolar) NaCl) and increase in fresh weight (10%) also recorded [58].

2. Materials and Methods

2.1. Layout of Experiment

In this experiment uniform pigeonpea seeds (Variety MA-3) were used and sown in test tubes of (20cm x 3.5cm) followed by filling them with sand. The experiment was laid using completely randomized design with 12 different treatments. The combination of different treatments is given in the Table 1.

Table 1. Combination of different treatments in the experiment

S.No.	Treatment (T)	Combinations
1	T1	P1+S
2	T2	P2+S
3	T3	P1+ P3+S
4	T4	P2+ P3+S
5	T5	P1+ P3
6	T6	P2+ P3
7	T7	P3+S
8	T8	P3
9	T9	P1
10	T10	P2
11	T11	S
12	T12	C

Note: P1 = *Fusarium udum*, P2 = *Macrophomina phaseolina*, P3 = *Pseudomonas* (AKC-O11), S= Salt and C = Control.

2.2. Media Preparation

The King's B (Table 2) and PDA medium (Table 3) was prepared by mixing all the ingredients of medium and sterilized at 15 PSI pressure at 121°C for 30 minutes.

Table 2. Composition of King's B Medium [59]

S.N.	Components	Amount in gram for 1 Liter of medium
1	K2HPO4	20.0 g
2	Peptone	2.0 g
3	MgSO4.7H2O	1.5 g
4	Glycerol	15 ml
5	Agar	15 g
6	Distilled water	1000 ml
pH 7.2		

Table 3. Composition of PDA Medium [60]

S.N.	Components	Amount in gram for 1 Litre of medium
1	Potato	200 g
2	Dextrose	20 g
3	Agar	20 g
4	Distilled Water	1000 ml
pH 6.8 ± 0.2		

2.3. Sand Culture

The principle behind the sand culture is the same as that of liquid culture, except that sand is used only for plant support. The sand was washed carefully to remove impurities from sand and washed with distilled water until the pH of wash water is the same as that of distilled water [61]. The washed sand was sun dried and filled up to 4 cm in test tube of (20cm x 3.5cm). The test was then plugged with non-absorbent cotton plug and then sterilized in autoclave at 15 PSI pressure and 121°C temperature for half an hour.

2.4. Materials Used

Two fungal isolates *Fusarium udum*, *Macrophomina phaseolina* and one bacterial strain fluorescent *Pseudomonas* (AKC-O11) were obtained from culture pool of "Hoffmann Laboratory". Pigeonpea seed (Var. MA-3) was used in the experiments. The fluorescent *Pseudomonas* (AKC-O11) were revived on King's B medium by streaking with the help of an inoculation loop in previously poured petri plate containing King's B medium. The plates were incubated at 28 ± 2°C in biochemical oxygen demand (BOD) incubator. The culture was preserved in slants of King's B medium for further use. The control of *Fusarium udum* was re-cultured on PDA medium by transferring the mycelia with the help of an inoculation needle in previously poured petri plate containing about 25 ml PDA medium. These plates were incubated at 25±2°C temperature for few days till the mycelia grew actively. The culture was preserved by placing mycelia blocks in PDA slants, taken from the growing edges of developing culture with the help of a cork borer.

The control of *Macrophomina phaseolina* was re-cultured on PDA medium by transferring the mycelia with the help of an inoculation needle in previously poured petri plate containing about 25 ml PDA medium. These plates were incubated at 25±2°C temperature for few days till the mycelia grew actively. The culture was preserved by placing mycelia blocks in PDA slants, taken from the growing edges of developing culture with the help of a cork borer. *Macrophomina phaseolina* was mass cultured on Richard's liquid medium (Table 4) [62] for 15 days in BOD incubator at 25°C. After incubation the mat of fungal mycelium was washed in distilled water and collected on sterilized blotting paper to remove the excess moisture from the fungal mat. The suspension of pathogen was prepared by mixing 5g fungal mycelium in 50 ml of distilled water and blended it in mortar and pestle.

The 10 ml of this suspension containing 1g fungus was used as inoculums [62].

Table 4. Composition of Richard's liquid medium

S.N.	Components	Amount in g for 1 L of medium
1	Peptone	10 g
2	Potassium dihydrogen phosphate	5 g
3	Magnesium sulphate	2.5 g
4	Ferric Chloride	0.02 g
5	Sucrose	50 g
6	Distilled water	1000 ml

2.5. Seed Sterilization and Seed Sowing

The pigeonpea seeds (Var. MA-3) were surface sterilized with 0.1 % sodium hypochlorite for two minutes and then washed three times with distilled water [62]. These sterilized seeds were then transferred into sterilized moist chamber and incubated in growth chamber at 28°C for 4 days to get uniform germination (sprouting). The germinated seeds were transferred in test tube containing sand followed by drenching with sterilized distilled water. The culture tubes were incubated in growth chamber at 28°C [63] for better germination of pigeonpea seeds.

2.6. Antagonistic Test

Antagonistic activity of the bacterial strain (AKC-O11) was tested against the soil borne pathogens *Fusarium udum* and *Macrophomina phaseolina* by using the dual culture technique [64]. PDA (about 25 ml) was poured in sterilized petri plates and media was allowed for solidification. Both the bacterial and fungal cultures were inoculated in petri plate keeping the distance 5cm from each other. The whole procedure was performed in aseptic environment of laminar air flow.

2.7. Application of Stress

In this experiment biotic stress was applied through two different plant pathogenic fungi *Fusarium udum* and *Macrophomina phaseolina* and abiotic was stress applied by using salt (NaCl). Four days old pigeonpea plants were selected for the inoculation of *Macrophomina phaseolina* inoculums suspension [1 %] which was prepared by blending 5g fungal mycelium in 50 ml of sterilized distilled water with the help of mortar and pestle [62]. The culture suspension of *Macrophomina phaseolina* was poured at the rate of 1ml around the roots of plant in each culture tube with the help of micropipette, thereafter the roots were covered with sand [62]. Similarly, four days old pigeonpea plants were selected for the inoculation of *Fusarium udum*, the inoculums suspension of 2 x 10^6 spores per ml was prepared in sterilized distilled water [65]. One ml of inoculums suspension of *Fusarium udum* was drenched around the roots of plant in the culture tube with the help of micropipette and then the roots were covered with sand under aseptic environment of laminar air flow.

For the application of abiotic stress 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mM of NaCl solution was applied by irrigating the plant roots in the test tube in aseptic environment. 100 mM salt concentration was selected as final solution because plants were not grown beyond 100 mM NaCl salt solution applied at the time of seed sowing. Sterilized distilled water was applied to the plants with the help of micropipette whenever necessary in the experimental period. Sampling was done three times at the interval of 48 h.

2.8. Seed Bacterization by *Pseudomonas* (AKC-O11)

The healthy and uniform seeds were selected and surface sterilized and washed with distilled water. King's B Broth medium was prepared, sterilized and inoculated with bacterial strain (AKC-O11). The inoculated flasks were incubated in an orbital shaker at 28±2°C for two days. After two days these cells were harvested by centrifuging at 1000 rpm for 5 minutes. These cells were used to prepare the bacterial strain suspension having OD (Optical Density) 0.347 (10^7 cfu/ml) and carboxyl methyl cellulose (CMC) was added at the rate of 1% as sticker to adhere the bacterial cells on the surface of seeds. Surface-sterilized pigeonpea seeds were then bacterized by soaking the seeds into the bacterial suspension for 4 hours followed by air drying at room temperature in aseptic conditions. Seeds coated with only suspension of CMC without bacteria served as control [66].

2.9. Sampling of Root Exudates

After inoculation with mycelial suspension of *Macrophomina phaseolina* the sampling of Root-exudates was done at 48 h interval (3 times) by adding 10-12 ml of ethyl acetate into the growing plant test tube and it was mixed properly with the sand. Test tubes were kept as such for 30 minutes so that it dissolves the root exudates completely. The exudate-ethlyacetate solution was filtered with sterilized filter paper, the filtrate was collected in conical flasks and the flasks were kept as such for complete evaporation of ethyl acetate. Methanol was added in the flasks for proper dissolution of the root-exudates, thereafter root exudates were collected in culture vials for high pressure/performance liquid chromatography (HPLC). These samples were filtered with the help of syringe (5 ml) through 0.22 µm membrane filter. 20 µl filtered samples was loaded in HPLC. The chromatograms developed by HPLC were used for further analysis.

2.10. Estimation of Total Phenolic Content (TPC)

TPC was assayed according to [67]. Plant root exudates were mixed properly in 50% methanol. 50 µl of sample was taken in a test tube and 950 µl of distilled water was added to it. 500 µl of folin reagent (1:1; folin reagent: distilled water) was added along with 1 ml of 20% of sodium carbonate and mixed thoroughly and allowed the color of the mixture to be changed to blue. To the reaction mixture, 10 ml of distilled water was added. The reaction mixture was incubated for 20 minutes at room temperature. After the end of incubation period optical density of samples was measured at 725 nm wavelength and the concentration was determined against a standard curve prepared by gallic acid.

2.11. High Performance Liquid Chromatographic (HPLC) Analysis

High performance liquid chromatography of fractionated material was performed in HPLC system equipped with two shimadzu LC-10 AT VP reciprocating pumps, a variable UV-VIS detector, an integrator and Winchrom software for data recording and processing (Winchom, Spinco Biotech, Pvt. Ltd., Chennai, India) [68]. Running conditions included a mobile phase of acetonitril and

water (60: 40, v/v), and flow rate 1.0 ml/min, an injection volume of 20 µl and detection at 290 nm and 254 nm. Fractionated material (1 mg/ml) and phenolic acids dissolve in HPLC-grade methanol were injected into the sample loop and the means of peak areas of individual compounds were taken for quantification. Tannic, caffeic, vanillic, chlorogenic, ferulic, cinnamic and salicylic acids were used as internal and external standards. Phenolic compounds present in the sample were identified by comparing retention time (Rt) of standards of ferulic acid (3.622 min), tannic acid (3.096 min), gallic acid (3.592 min), p-cinnamic acid (2.599 min), shikimic acid (3.625 min), syringic acid (3.623 min) and t-chlorogenic acid (2.968 min). Amount of individual compounds were calculated by comparing peak areas of reference compounds with those in the samples run under the similar conditions.

2.12. Statistical Analysis

Experiments were performed using completely randomized design. The one-way variance analysis was performed to test the significance of the significance of the observed differences using SPSS version 16. The differences between the parameters were evaluated by means of the Duncan's test and P values ≤0.01 were considered as statistically significant.

3. Results

The TPC in pigeonpea plant root exudates differed in various combinations of treatments (biotic and abiotic stress both) observed at the different time intervals (48, 96 and 144 h). TPC was increased in the treatments comprising of the pathogens compared to salt stress at 48 h. However, highest concentration of TPC was observed in the treatment where the pathogen *Fusarium udum* was applied along with the salt and *Pseudomonas* (AKC-O11). Between the two pathogens TPC content in root exudates of plants challenged with the pathogen *Macrophomina phaseolina* was high as compared to *Fusarium udum*. TPC in individual application of (AKC-O11), *Fusarium udum* and NaCl was even lower than the control plants but its content was high when the treatments were combined (Figure 1).

TPC also increased in the treatments comprising of the pathogens compared to salt stress at 96 h. However, highest concentration of TPC was observed in the treatment where the pathogen *Macrophomina phaseolina* was applied individually. Between the two pathogens TPC content in root exudates of plants challenged with the pathogen *Macrophomina phaseolina* was high as compared to *Fusarium udum.* In individual treatment of *Pseudomonas* (AKC-O11), *Fusarium udum*, *Macrophomina phaseolina* and NaCl TPC was higher in pathogen challenged plants but lower in *Pseudomonas* (AKC-O11) and NaCl treated plants compared to control plants. It was also observed that higher concentration of TPC accumulated in the treatments where the pathogens were applied along with salt then with the strain *Pseudomonas* (AKC-O11) (Figure 2).

TPC was increased in the treatments comprising of the pathogens compared to salt stress at 144 h. However, highest concentration of TPC was observed in the treatment where the pathogen *Macrophomina phaseolina* was applied along with *Pseudomonas* (AKC-O11) and lowest in control plants. Between the two pathogens TPC content in root exudates of plants challenged with the pathogen *Macrophomina phaseolina* was high as compared to *Fusarium udum*. TPC in individual application of (AKC-O11), *Fusarium udum*, *Macrophomina phaseolina* and NaCl was even higher than the control plants. TPC content was higher where the pathogen *Macrophomina phaseolina* was applied compared to *Fusarium udum* when the treatments were combined. TPC content was high when the treatments were combined with salt in compared to individual applications (Figure 3).

In general, TPC was increased and found maximum at 96 h. Thereafter it was declined at 144 h. When the plants were subjected to only salt stress TPC was increased up to 96 h and found maximum and then declined in 144 h but when the plants were subjected to only (AKC-O11), TPC was increased up to 144 h in an increasing order. When plants were subjected to both the pathogenic fungi (*Fusarium udum* and *Macrophomina phaseolina*) the trend of TPC was increased up to 96 h then drastically decreased in 144 h.

When the pigeonpea seeds were bacterized with the *Pseudomonas* strain and the plants were challenged with the pathogens, TPC was increased and found maximum in 96 h and then it declined in 144 h. However, when these bacterized plants were subjected to salt stress, TPC was increased up to 144 h. When the seeds bacterized plants were exposed to *Fusarium udum* and salt stress simultaneously, TPC was maximum in 48 h and decreased in 96 h. However, TPC in most of the treatments were high compared to the control plants particularly at 96 and 144 h.

HPLC analysis of pigeonpea root exudates under various combinations of treatments (biotic and abiotic stress) at the different time intervals 48 and 96 h varied. Analysis of root exudates for both (AKC-O11) inoculated and un-inoculated plants under pathogen challenge at 48 h showed that gallic acid content in the treatments comprising *Macrophomina phaseolina* were high compared to the *Fusarium udum* treated plants. Gallic acid was not detected in several treatments comprising *Fusarium udum,* Gallic acid content in the salt stressed plants were also low compared to *Macrophomina phaseolina* treated plants. However, gallic acid content increased in the same treatments when the seeds were bacterized with the *Pseudomonas* strain (AKC-O11) (Figure 4).

Similarly, analysis of root exudates for both (AKC-O11) inoculated and un-inoculated plants under pathogen challenge at 96 h showed that gallic acid content in the treatments comprising *Macrophomina phaseolina* were high compared to the *Pseudomonas* strain (AKC-O11) treated plants. Gallic acid was detected in all treatments but it was lower when applied in combination with *Pseudomonas* strain (AKC-O11). Gallic acid content in salt stressed plants along with *Fusarium udum* were high compared to *Macrophomina phaseolina* treated plants.

However, gallic acid content decreased in the same treatments when the seeds were bacterized with the *Pseudomonas* strain (AKC-O11) (Figure 5). The lowering down of gallic acid concentration may be attributed to its conversion to other forms like gallotannins in such treatments.

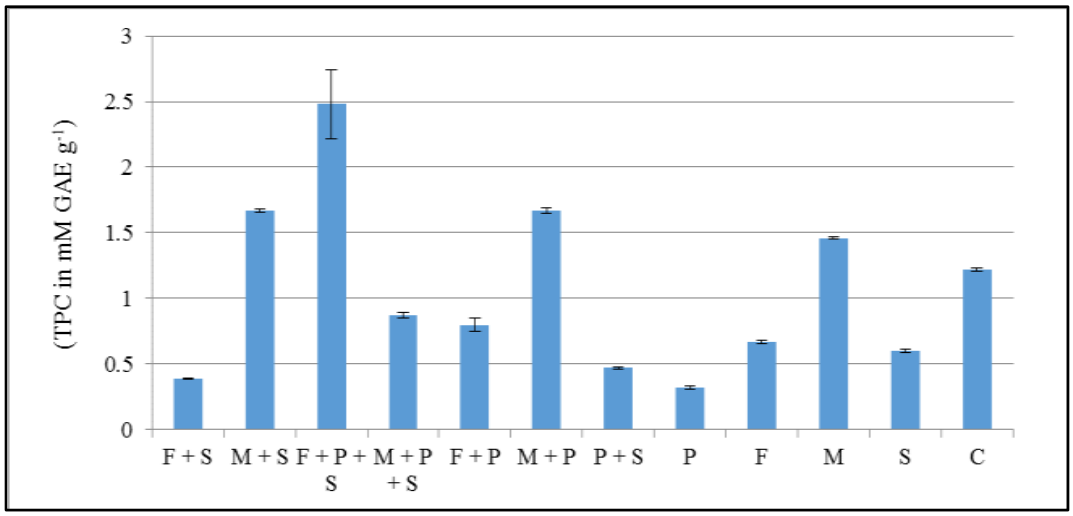

Figure 1. Total phenolic content (TPC) in pigeonpea root exudates at 48 h due to different treatments (*Pseudomonas* strain (AKC-O11) bacterized seed and unbacterized seed) under the challenge of the pathogens *Macrophomina phaseolina, Fusarium udum* and salinity

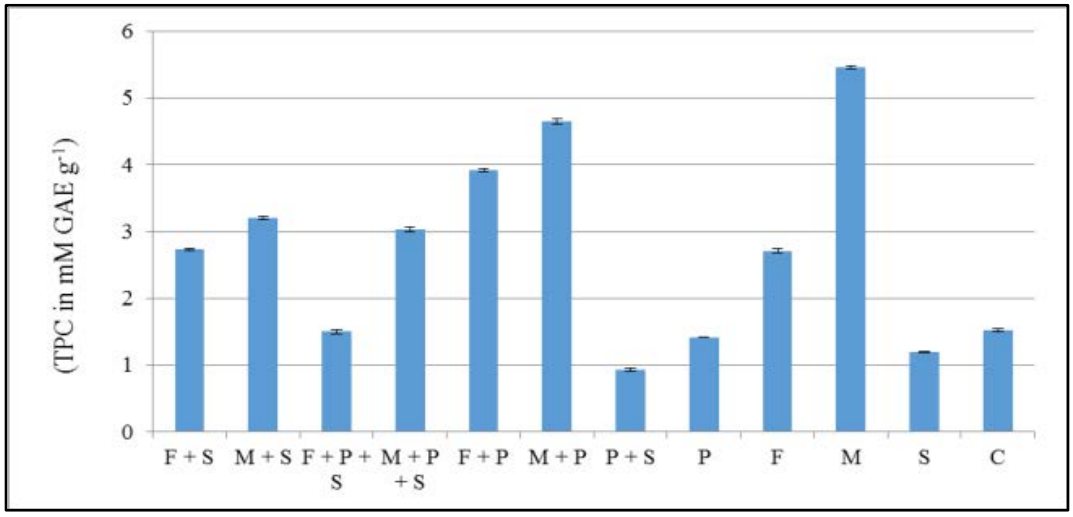

Figure 2. Total phenolic content (TPC) in pigeonpea root exudates at 96 h due to different treatments (*Pseudomonas* strain (AKC-O11) bacterized seed and unbacterized seed) under the challenge of the pathogens *Macrophomina phaseolina, Fusarium udum* and salinity

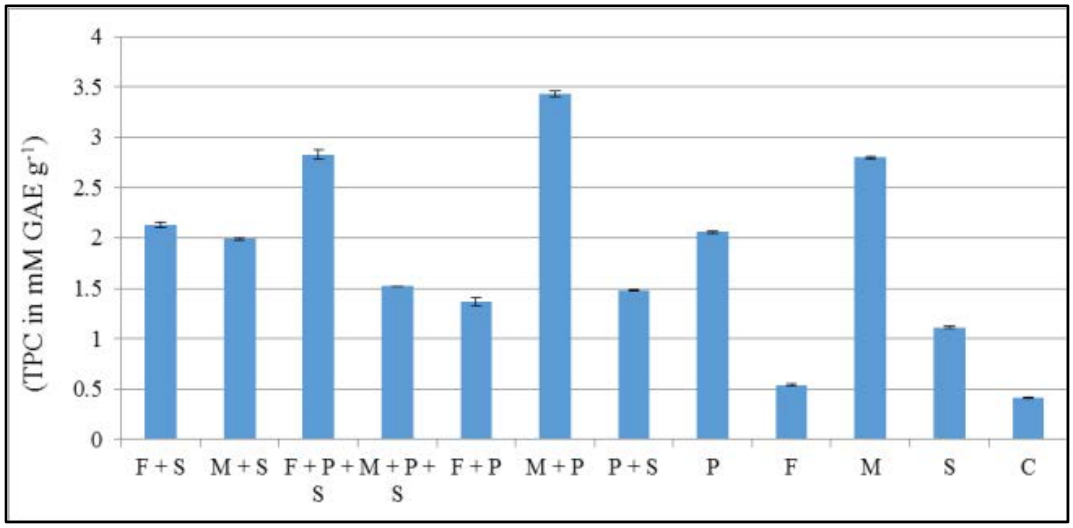

Figure 3. Total phenolic content (TPC) in pigeonpea root exudates at 144 h due to different treatments (*Pseudomonas* strain (AKC-O11) bacterized seed and unbacterized seed) under the challenge of the pathogens *Macrophomina phaseolina, Fusarium udum* and salinity

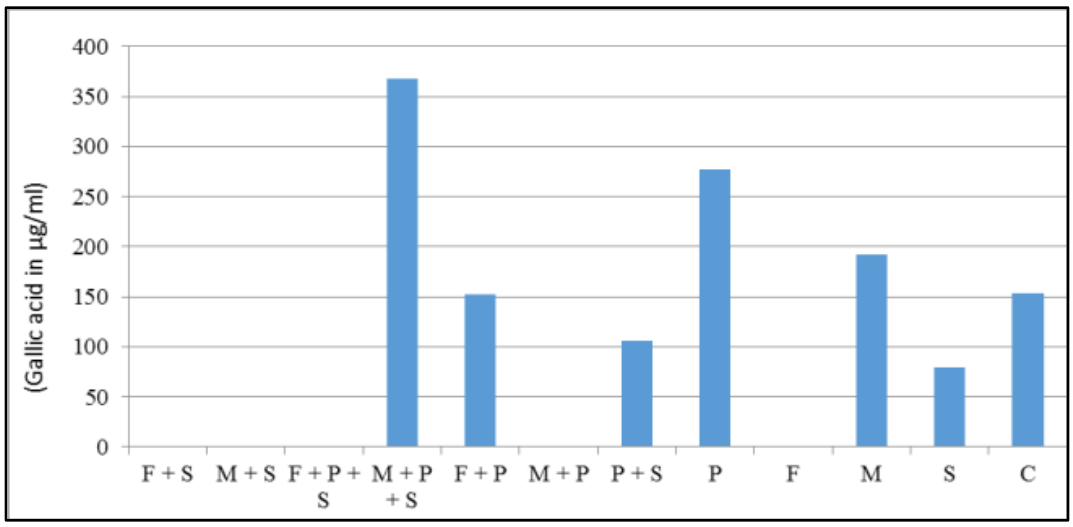

Figure 4. Concentration of Gallic Acid in pigeonpea root exudates at 48 h due to different treatments (*Pseudomonas* strain (AKC-O11) bacterized seed and unbacterized seed) under the challenge of the pathogen *Macrophomina phaseolina*, *Fusarium udum* and salinity

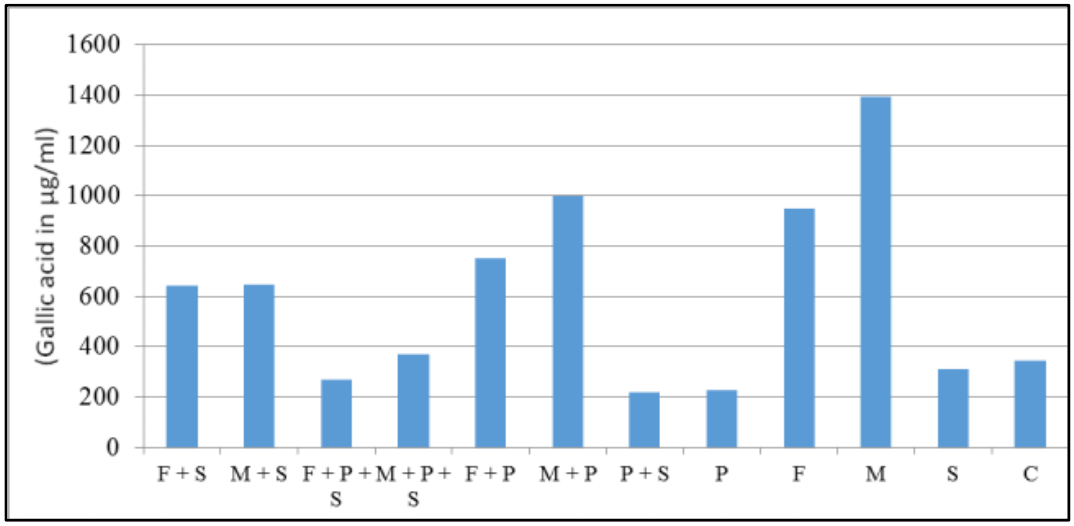

Figure 5. Concentration of Gallic Acid in pigeonpea root exudates at 96 h due to different treatments (*Pseudomonas* strain (AKC-O11) bacterized seed and unbacterized seed) under the challenge of the pathogen *Macrophomina phaseolina*, *Fusarium udum* and salinity

4. Result and Discussion

In the present experiment interactions of pathogens namely *Fusarium udum* and *Macrophomina phaseolina* with a rhizospheric microbe *Pseudomonas* (AKC-O11) were studied to see their impact on pigeonpea under abiotically stressed (NaCl) conditions. Both biotic (*Fusarium udum* and *Macrophomina phaseolina*) and abiotic stress were applied and performances of these microbes were evaluated in vitro conditions. The abiotic stress was applied by the application of 100 mM NaCl solution and biotic stress was applied by preparing cell/spore suspension of these microbes. Root exudates consist of important small molecular weight compounds secreted in the rhizosphere by plant through physical, chemical and biological interaction. The root exudates have the ability to extend defense response in plants against biotic stressed due to the presence of antimicrobial, phytotoxic, nematicidal and insecticidal compounds [69]. Root exudates at times also serve as rich source of energy and nutrients for some bacteria [70]. Secretion of root exudates depends on the presence of microorganism in the rhizosphere [71,72]. The root exudates are classified into two groups one of them is low molecular weight compounds which are amino acids, organic acids, sugars, phenolics and the second is high molecular weight of compounds which are polysaccharides and proteins [73]. PGPR have ability to modify the chemicals present in rhizosphere [74]. Plant root exudates contain enzymes, free oxygen, ions, mucilage and carbon containing primary and secondary metabolites [75,76].

Phenolics are plant low molecular compounds which are synthesized during the activation of phenylpropanoid pathways and it helps in PGPR mediated ISR pathway [66] and having antifungal activity also [37]. The concentration of phenolics is indirectly proportional to plant mortality during the pathogen attack [67]. In the present study we investigated that the changes in the phenolic content and profile in the root exudates of pigeonpea under the challenges of biotic and abiotic stresses mediated by a rhizospheric bacterial species. The results showed that the concentration of phenolic compounds increases in root exudates in the plants treated with (AKC-O11) under both biotically and abiotically challenged plants. This shows the importance of the microbe in modulating at root exudation pattern under challenged conditions. Moreover,

the highest TPC at 96 h further showed that the TPC concentration in the exudates increased over time and sustained for a longer. Pulses are highly sensitive to salinity [77] and under saline condition pigeonpea germination was greatly affected [78]. High concentration of salt in the root zone reduces soil water potential and availability of water and thereby reduces seed germinations [79]. In the present experiment, we observed a similar effect as germination was affected at 100 mM NaCl and above [80]. Some Pseudomonads have the ability to degrade toxins produced by pathogens [81]. *Fusarium* infection was lowered by *Pseudomonas* (AKC-O11) due to antagonistic activity. Wilt of pigeonpea can be controlled by seed treatment with antagonist because they produce extracellular antagonistic substances effective against the pathogen [82]. Similarly, biological control of *Fusarium udum* and *Heterodera cajani* was achieved by some bacterial strains in pigeonpea fields [83].

Between the two pathogens, TPC content was high in the *Macrophomina phaseolina* challenged plant root exudates as compared to the *Fusarium udum* treated plants which further shows that the plants have a better chance to suppress *Macrophomina phaseolina* infection due to high concentration of antimicrobial phenolic compounds. It is reported that seed bacterized with PGPR results higher concentration of phenolics accumulation in plants [84,85,86] but our results showed that the minimum concentration of phenolics were secreted when seeds were bacterized with PGPR. It is probably due to the fact that the effect of PGPR in more prominent when the plants are challenged by any stresses. Gallic acid is a phenolic compound which was important role as antioxidant and antimicrobial compounds [87]. Our experiment showed that plants under treatment with biotic (*Macrophomina phaseolina* and *Fusarium udum*) and abiotic stressess (NaCl 100 mM) and their combination, concentration of gallic acid was low at the initial period but it was enhanced with time. It demonstrates a constant activation of the phenylpropinoid pathway over the time. The higher concentrations of secondary metabolites in the plant host suppress the growth and development of plant pathogenic microorganisms and help the plant to release their stress [66].

For the current experiment, it can be concluded that *Pseudomonas* strain (AKC-O11) have potential to be used as biocontrol agent that can help pigeonpea plants to combat attack of *Macrophomina phaseolina* and *Fusarium udum* as well as salinity caused by higher concentration of NaCl. Moreover, the results also indicates a common pattern of defense response as observed in pigeonpea plants against both the biotic and abitic stresses when they are bacterized by the *Pseudomonas* strain (AKC-O11). Relatively low TPC in the salt treated plants at the initial period further claims that plant respond to the biotic stress via the phenylpropanoid pathway where as it response to the abiotic stress is not through the same pathway at least in the initial period.

5. Summary and Conclusion

Plant growth-promoting rhizobacteria (PGPR) not only helps in plant growth and development but also protects from various biotic (pathogens) and abiotic stresses. Here we are tried to know that how *Pseudomonas* strain (AKC-O11) help to protects plants from biotic (*Fusarium udum* and *Macrophomina phaseolina*) as well as abiotic (NaCl) stresses. The strain was used individually and in combination with the stresses and applied as seed bacterization of pigeonpea (Var. MA-3) seeds to see the impact on total phenol content in plant root exudates.

The bacterized seeds were grown under in-vitro conditions and after three days of germination the seedlings were exposed to biotic stress due to challenge of the pathogens (*Fusarium udum* and *Macrophomina phaseolina*) and abiotic stress due to irrigation with salt solution of 100 mM. Root exudates were collected at 48 h, 96 h and 144 h after the application of stresses (biotic and abiotic). The collected root exudates were processed for total phenolic content and HPLC analysis. It was observed that total phenol content was low in seeds bacterized with *Pseudomonas* strain but the concentration increased when the plants were challenged with the pathogen particularly *Macrophomina phaseolina*. Similar trend was also observed in gallic acid accumulation. The above results indicates that *Pseudomonas* strain (AKC-O11) have potential to be used as biocontrol agent that can help pigeonpea plants to combat attack of *Macrophomina phaseolina* and *Fusarium udum* as well as salinity.

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