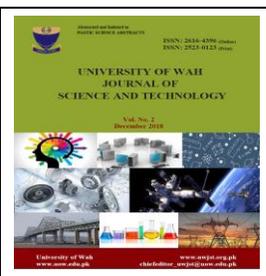




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Physiological Responses of Plant Growth Promoting Rhizobacteria, Biochar and Chemical Fertilizer Under Salinity Stress

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Abstract—This research work is conducted to evaluate the physiological responses of phosphate solubilizing rhizobacteria (*Pseudomonas* sp.), biochar and N-fertilizer under salt stress. Biochar and fertilizer are mixed in soil (5:1) while *Pseudomonas* sp. is applied as seed soaking at 10^6 cells/ml prior to seed sowing. Salt stress with 150mM NaCl is applied for three days (3d) at three (3) leaf stage. The obtained results depicted significant effect of *Pseudomonas* sp. on root fresh weight and leaf area both under unstressed and salt stress conditions followed by biochar. Treatment with biochar and *Pseudomonas* sp. resulted in increased root fresh weight, leaf area, chlorophyll fluorescence and decreased osmotic potential by 60% particularly under salt stress. On the contrary, fertilizer treatment is found to be ineffective on seed germination (results not presented here), however increased chlorophyll content by 77%. Under salt stress, fertilizer treatment increased the osmotic potential of leaves. The combined treatments of fertilizer with *Pseudomonas* and biochar significantly increased root fresh weight, chlorophyll content and leaf area under salt stress. It is inferred that combined application of biochar and *Pseudomonas* can augment the effects of N-fertilizer on plants.

Index Terms—Stomatal index, Leaf area, Salt stress, Inoculation, Trichomes, Plant growth promoting rhizobacteria (PGPR).

I. INTRODUCTION

SALINITY is detrimental for several processes occurring in plant like photosynthesis, lipid metabolism and synthesis of protein. The initial response of plant to salinity is decreased in leaf surface area followed by inhibition in the expansion of leaf.

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Plant photosynthesis is badly affected when plant is exposed to salinity [1]. Salinity stress causes significant reduction in water uptake that ultimately results in reduced growth rate due to excessive buildup of salts in the plant causing premature senescence [2]. However, plants have adopted certain biochemical pathways that offer salt tolerance to plants. These pathways are associated with preservation and uptake of water, protection of protoplast, maintaining ion homeostasis, synthesis of osmolytes, specific proteins and certain oxidant scavenging enzymes that protect the plant from detrimental effects of free radicals [1].

Fertilizers both organic and inorganic add fertility to soil [3]. However in tropical conditions, inorganic fertilizers are less competent in weathered and highly degraded soils and there is less accessibility to resource-pool in using inorganic fertilizers [4]. Therefore, organic fertilizers are preferable use as they enhance efficiency of nutrients because of rapid rate of mineralization in the soil [1, 3, 5-20].

Biochar addition seems to be an efficient technique only if it is applied to permanently farmed soils [5]. Enhancement in yield generally occurs when soil is treated with hardwood biochars and chars possessing high N content [21].

Many plant growth promoting rhizobacteria (PGPR) facilitate plant growth indirectly by reducing plant pathogens or directly by facilitating the uptake of nutrients from environment. In addition, PGPR can also affect plant growth by increasing germination rate, root and shoot growth and weight, yield, leaf area, chlorophyll content, K and N uptake, protein content and delayed leaf senescence [22]. In [7], it is stated that combined inoculation of *Azotobacter*, *Azospirillum*, *Pseudomonas*, and *Mesorhizobium* increases grain yield and biomass in chickpea. Whereas it increases biomass of maize by 99 % and 96 % respectively, when seeds are inoculated with two P-solubilizing bacteria (*Serratiamarcescens* EB-67 and *Pseudomonas* spp. CDB-35) [11].

Present investigation is aimed to evaluate the effects of biochar, Pseudomonas and N-fertilizer alone and in combination on some physiological traits of maize under control and induced salt stress.

II. MATERIALS AND METHODS

A. Plant material and growing conditions

Maize seeds are surface sterilized by shaking in 95% ethanol for 2 to 3 min followed by shaking in 10% chlorox for 2-3 min. Thereafter, seeds are thoroughly washed with autoclaved distilled water. Pot experiment is conducted and seeds are sown in plastic pots (width 7cm, length 11cm) and grown in growth chamber with 14h photoperiod at 22/26°C. The biochar (derived from Poplar sawdust) is autoclaved prior to mixing in soil with urea (5:1). N-Fertilizer (1.20 g/pot), is put immediately after sowing and then immediately irrigated [8].

B. Inocula preparation

Inoculum is prepared by inoculating LB broth with 24h old fresh culture of Pseudomonas sp. (Acc No KF307196) the culture is incubated on a rotary shaker for 48h followed by centrifugation on a centrifuge (Labofuge 400e) for 10 min at 10,000 rpm. The supernatant is discarded while pellet is suspended in distilled water to adjust the optical density 1 at OD 660nm to get bacterial count of 10^6 cells/ml. Sterilized seeds are then soaked in the inocula thus prepared for 2 to 4h. Seeds are sown in plastic pots (6 plants per pot) containing biochar @ 5g/Kg in autoclaved soil and sand mixed in 3:1 ratio.

C. Induction of salt stress

Salt stress is induced after 2 weeks of sowing by adding aqueous solution of NaCl (150mM) for 3d.

D. Osmotic potential

Cell sap (after 17 days of sowing) from the leaves of control and treated plants is extracted to measure osmotic potential with the help of freezing point osmometer using the technique developed by Capell and Doerffling [10].

E. Root fresh weight

Fresh weight of leaf and root is measured using electric balance.

F. Leaf area

After 17 days of sowing, plants are harvested and their leaf area is calculated using equation (1), by taking average height (cm) and width (cm) of three plants from all the treatments [23].

$$\text{Leaf area (cm}^2\text{)} = \text{height} \times \text{width} \quad (1)$$

G. Measurement of stomata and epidermal appendages

Dehydrated leaves are randomly taken from the plant and boiled in lactic acid. The adaxial surface of leaf is peeled and observed in a light microscope at 20x, and the total number of stomata and other epidermal cells are counted.

Stomatal Index (SI) is calculated using equation (2), according to Ogaya et al. [24].

$$\text{SI}(\%) = \frac{\text{No. of stomata}}{\text{No. of stomata} + \text{No. of epidermal cells}} \times 100 \quad (2)$$

H. Chlorophyll fluorescence

Chlorophyll fluorescence is measured with a portable Chlorophyll Fluorometer after 20 min of dark adaptation. Chlorophyll fluorescence is expressed as $\frac{F_v}{F_m}$ ratio. It is calculated as:

$$\frac{F_v}{F_m} = \frac{F_m - F_0}{F_m} \quad (3)$$

Where F_m and F_0 are maximal and minimal fluorescence of dark adopted leaves respectively and F_v is variable fluorescence [25].

I. Chlorophyll content

Chlorophyll content of fully expanded young maize leaves is measured using Soil-Plant Analyses Development (SPAD) instrument [26].

J. Statistical Analysis

Analysis of variance (ANOVA) is performed using factorial randomized complete plot design to assess significant variation. Significant differences between treatment means is determined through (LSD) tests. Least significant difference is measured at $P < 0.05$ and MS Excel software is used to illustrate and compare data on figures [27].

III. RESULTS

A. Osmotic potential

The maximum increase (150%) in the osmotic potential is due to fertilizer treatment which is decreased in combined treatment with Pseudomonas sp. under salt stress as shown in Fig. 1. Fertilizer addition to biochar and Pseudomonas sp. increased the osmotic potential of leaves over biochar used alone. The combined treatment of Pseudomonas sp. with biochar had no significant effect as compared to Pseudomonas sp. inoculation alone but the combined treatment of fertilizer with biochar and Pseudomonas sp. are 21% and 46% lower over that of salt stress respectively.

B. Fresh weight of roots

All the treatments significantly increased (26%-30%) fresh weight of leaves under both unstressed and salt stressed conditions respectively (results not presented).

The maximum significant increase (28%) in root fresh weight is in plants inoculated with Pseudomonas sp. under unstressed condition as shown in Fig. 2. The biochar and fertilizer treatments has similar magnitude of increase and their combined treatment do not differ significantly. Pseudomonas sp. has significant (20%) increase over salt stress. Biochar treatment under salt stress is less stimulatory but its effectively is enhanced when combined application is made with Pseudomonas sp. and fertilizer increasing the value by 10% and 12% over control

respectively. Combined application of *Pseudomonas* sp. and fertilizer under salt stress also proved effective resulting in 11% significant increase in root weight over control.

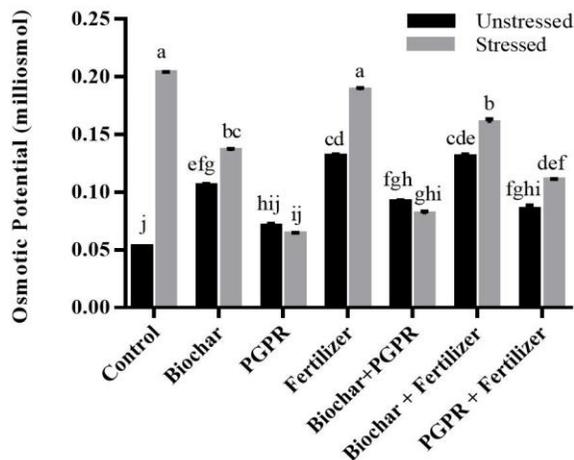


Fig. 1. Effect of Biochar, PGPR (*Pseudomonas* sp.) and N-fertilizer on osmotic potential of maize (*Zea mays* L.) leaves under salt stress. Biochar and fertilizer are mixed in soil (5:1) while *Pseudomonas* sp. is applied as seed soaking prior to seed sowing. Salt stress is induced for 3d after 2 weeks of sowing. The bars containing the same English letters differ non-significantly from each other at $P < 0.05$.

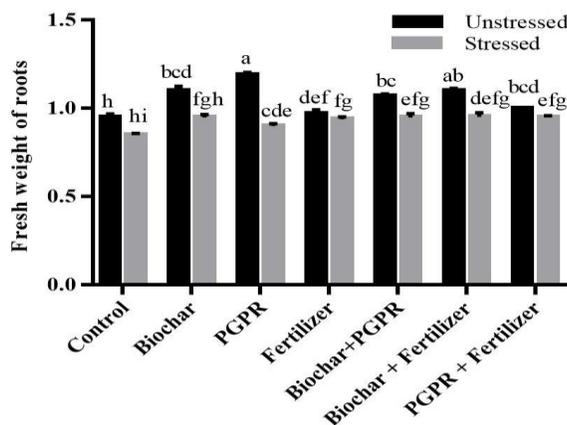


Fig. 2. Effect of Biochar, PGPR (*Pseudomonas* sp.) and N-fertilizer on fresh weight of maize (*Zea mays* L.) roots under salt stress. (Treatments detail shown in Fig. 1).

C. Leaf area (cm²)

All the treatments under unstressed condition shows significant increase in leaf area over untreated control as shown in Fig. 3. Biochar treatment is less stimulatory but its effectivity is enhanced when combined application is made with fertilizer and *Pseudomonas* sp. and shows significant increase of 76% and 68% in leaf area as compared to control respectively. Similarly, the effect of *Pseudomonas* sp. is enhanced by 25% when combined application is made with fertilizer. Biochar in combination with *Pseudomonas* sp. showed 42% and 53% increases in maize leaf area as compared to *Pseudomonas* sp. and biochar treatments applied singly. Combined

application of biochar with fertilizer shows maximum increase (60%) over salt stress.

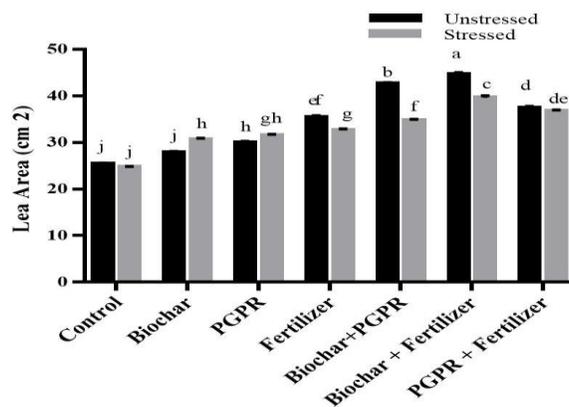


Fig. 3. Effect of Biochar, PGPR (*Pseudomonas* sp.) and N-fertilizer on leaf area of maize (*Zea mays* L.) under salt stress. (Treatments detail shown in Fig. 1).

D. Stomatal index (%)

Significant increases in stomatal index is observed in all the treatments as compared to control as shown in Fig. 4. The maximum increase (60%) is due to *Pseudomonas* sp. Fertilizer treatment showed least (34%) increase in stomatal index over control. The addition of fertilizer to biochar and *Pseudomonas* sp. decreased the stomatal index by 10% and 21% over biochar and *Pseudomonas* sp. under unstressed condition. Under salt stress, the stomatal index is maximum in plants inoculated with *Pseudomonas* sp.

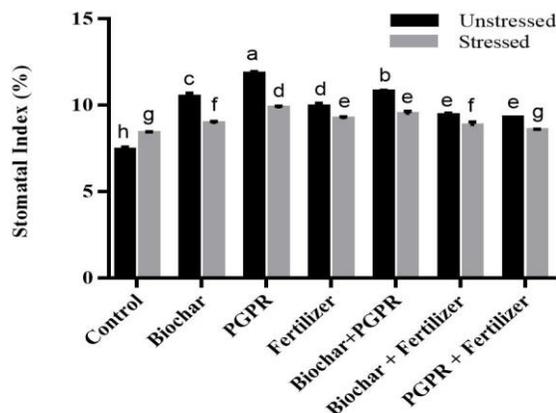


Fig. 4. Effect of Biochar, PGPR (*Pseudomonas* sp.) and N-fertilizer on stomatal index of maize (*Zea mays* L.) leaves under salt stress. (Treatments detail shown in Fig. 1).

E. Chlorophyll fluorescence (F_v/F_m)

All the treatments under unstressed condition display significant increase in chlorophyll fluorescence over uninoculated, untreated control as shown in Fig. 5 Under unstressed condition, fertilizer, biochar and *Pseudomonas* sp. equally stimulates chlorophyll fluorescence of leaves by 11% over control. However biochar in combination with *Pseudomonas* sp. further

augmented its stimulatory effect by 10% as compared to *Pseudomonas* treatment made alone. Under salt stress, fertilizer treatment shows decrease in chlorophyll fluorescence value as compared to control, nevertheless this decrease is compensated by the combined treatments of fertilizer with biochar and *Pseudomonas* which efficiently increased chlorophyll fluorescence.

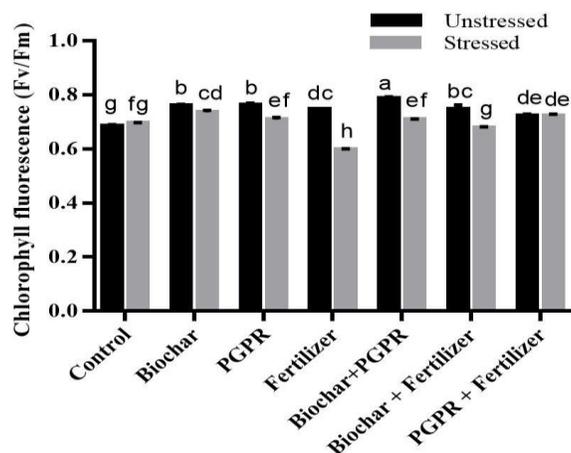


Fig. 5. Effect of Biochar, *Pseudomonas* sp. and N-fertilizer on chlorophyll fluorescence of maize (*Zea mays* L.) Leaves under salt stress. (Treatments detail shown in Fig. 1).

F. Chlorophyll content

Under unstressed condition, the increase in chlorophyll content as shown in Fig. 6 is maximum in the fertilizer treatment made alone and the combined treatment of fertilizer with *Pseudomonas* sp. The *Pseudomonas* sp. and biochar treatment had 13% and 41% increases in chlorophyll content over control. Addition of biochar to *Pseudomonas* sp. inoculated plants assist the *Pseudomonas* sp. to increase the chlorophyll content by 24% higher than that of *Pseudomonas* sp. applied alone under salt stress.

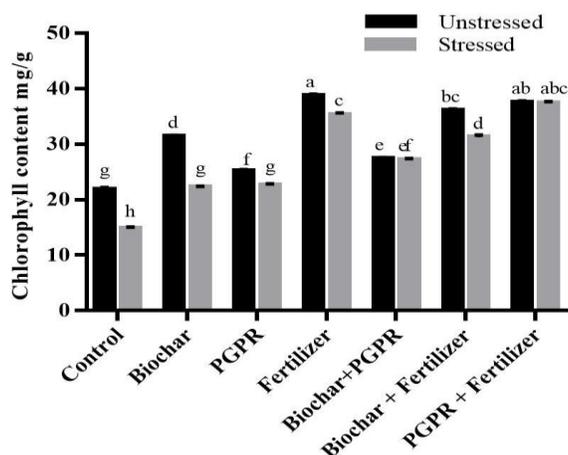


Fig. 6. Effect of Biochar, PGPR (*Pseudomonas* sp.) and N-fertilizer on chlorophyll content of maize (*Zea mays* L.) leaves under salt stress. (Treatments detail shown in Fig. 1).

IV. DISCUSSION

It is evident from the results that both *Pseudomonas* sp. and biochar improves physiological traits of maize plant studied during the present investigation. Salt stress results in increased osmotic potential due to buildup of solutes that gives rise to a secondary stress called osmotic stress thus causing cellular dehydration. Production of compatible solutes and proteins have been reported that function in abiotic stress tolerance [17-18]. It is observed that *Pseudomonas* sp. and also biochar exhibits the highest osmotic tolerance and results in significant increase in proline production [28] that is involved in cellular osmotic adaptation. Hence, the osmotic balance caused due to salinity is encountered by biochar and PGPR but is not addressed by fertilizer treatment. Fertilizer induces increase in leaf area and growth but don't keep pace with osmoregulation as a result, electrolyte leakage is also higher in fertilizer treatment [28]. The osmoregulation appears to be controlled more efficiently by PGPR and biochar applied separately. However, combined treatments of fertilizer with *Pseudomonas* and biochar proved effective in augmenting physiological traits of maize plant. Previous studies have reported that *P. putida* and *B. megaterium* exhibit the highest osmotic tolerance and show increased proline content that is involved in osmotic cellular adaptation thus suggesting that bacteria have developed mechanisms that can alleviate stresses in crop plants [6-21, 29, 30].

In the present research work, the effect of PGPR is more pronounced on root growth and both PGPR and biochar are equally effective to promote shoot growth. In most of the growth parameters, PGPR override the effects of biochar but for leaf area, biochar more effective and fertilizer addition further augments its effect. Spokas et al. [13] reported significant increase in root density and crop productivity following biochar addition while Carlier et al. [14] revealed that inoculation with PGPR produced a substantial increase in plant height and root length in early growth stages of wheat. Dobbelaere et al. [31] studied the physiological responses of the plant roots to inoculation with *Azospirillum* and observed that inoculation leads to an improvement in root development and an increase in the rate of water and mineral uptake. Gholami et al. [32] observed that maize seeds inoculated with *Azospirillum*, *Pseudomonas* and *Azotobacter* strains enhanced seed germination and seedling vigour of maize.

PGPR have also been reported to increase cell division and cell elongation due to production of plant growth promoting hormones [15-37]. The growth promoting property of biochar, *Pseudomonas* sp. and fertilizer is apparent on leaf area of maize under salinity stress which appears to be related with the nutrient content of biochar and fertilizer and growth hormones produced by *Pseudomonas* sp. The combined effect being more stimulatory due to synergism between biochar and *Pseudomonas* sp. can possible be attributed to the fact that biochar served as a source of nutrients for better proliferation of *Pseudomonas* sp. Busscher et al. [38], Lashari et al. [34] also demonstrated the effects of biochar on soil properties as well as leaf area index, maize grain

yield and observed an increase in overall productivity and performance of plant.

The stomatal density and stomatal index (SI) are indicators of paleomorphic CO₂ concentration. The application of biochar and *Pseudomonas* sp. inoculation maintained higher stomatal index (SI). This might help to keep the turgidity higher and facilitate the gaseous exchange efficiently for greater assimilation of leaves but under salt stress the SI is reduced. Varela et al. [4] studied comparative effects of biochar on plant growth parameters and observed higher mean leaf width, leaf length, chlorophyll content, stem size and root size. Increased chlorophyll content can be used as an index of photosynthetic potential as well as an index of plant productivity. Furthermore, trichome has been observed in *Pseudomonas* sp. inoculated plant leaves under unstressed (non-saline) condition [32]. Trichomes are stalked protuberances that contribute to plant resistance against herbivory by physical and chemical deterrents [13]. Moreover, *Pseudomonas* sp. in combination with biochar resulted in 45% (under unstressed) and 13% (under stress) significant increase in stomatal index value over uninoculated control. Vivaset al. [9] demonstrated that PGPR inoculation resulted in an increase in overall plant physiological values including photosynthetic rate, water use efficiency (WUE) and stomatal conductance of lettuce plants.

Increasing salinity in soil decreases chlorophyll content which ultimately decreases plant growth causing a shift in many physiological activities like photosynthesis, stomatal conductance and antioxidant activity [16]. However PGPR inoculation helps ameliorating such deleterious effects of salinity stress. An experiment conducted by Fazal and Bano [1] reported that inoculation with *Pseudomonas* sp. enhanced chlorophyll production resulting in significant increase in chlorophyll content over control under unstressed condition. Similar results are observed by Heidari and Golpayegani [23], where PGPR significantly increased the catalase activity and chlorophyll content of leaves under water stress.

The amount of chlorophyll fluorescence (Fv/Fm) indicates thylakoid membrane integrity and the relative efficiency of electron transport from PSII to PSI [19]. Additionally, the flow of electrons through PSII is indicative, under many conditions, of the overall rate of photosynthesis [29]. As the aforementioned results showed increased chlorophyll fluorescence values when combined treatment of biochar and *Pseudomonas* sp. is made, so it could easily be implied that the nutrients present in biochar, and the growth promoting hormones produced by *Pseudomonas* sp. [33] can possibly attribute to increased photosynthetic activity of maize plant.

V. CONCLUSION

In the light of the present results, it is inferred that biochar and *Pseudomonas* sp. are equally effective as fertilizer and can be used to minimize the use of chemical fertilizer. The combined treatment of PGPR and Biochar may enhance the effect of N-fertilizer on leaf area and root growth.

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