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AMELIORATING EFFECTS OF MYCORRHIZA AND PGPR ON VARIOUS PHOTOSYNTHETIC PARAMETERS UNDER ALUMINIUM STRESS IN GROUNDNUT (ARACHIS HYPOGAEA L.)SEEDLINGS

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ABSTRACT

Aluminium (Al) toxicity is one of the primary constraints for plant growth, development, yield and photosynthesis process. It has been demonstrated that mycorrhizae play an important role for survival and growth of plant in acidic soil. In the present study we hypothesized that co-inoculation of mycorrhizae and PGPR would enhance photosynthetic activities of plant in Al toxic soil. The groundnut (Arachis hypogaea L. cv. Girnar-3) seeds were grown in plastic pots filled with vermiculite irrigated regularly with or without aqueous solution of $AlCl_3$ (0, 50, 250, 500 and 1000µM at pH 4.5) in combination with mycorrhizal strain, Glomusetunicatum and Pseudomonas putida species as a PGPR. The effects of these treatments were studied on 25 day old seedlings. The chlorophyll content, carotenoid content, maximal PSII quantum yield (Fv/Fm) and leaf gas exchange measurement viz net photosynthetic rate (Pn), transpiration rate (E), intercellular CO_2 concentration (Ci) and stomatal conductance (gs) were analysed. A significant decrease in all these photosynthetic parameters was recorded in groundnut seedlings with an increase in Al concentration. Co-inoculation of mycorrhiza and PGPR showed significant positive impact to ameliorate Al stress through enhancing the chlorophyll content and efficiency of photosynthetic parameters.

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INTRODUCTION

Aluminium (Al) toxicity and acidity of soilare naturally interlinked phenomenon responsible for diminishing agricultural productivity (Hornung 2005; Kochian *et al*. 2004). Naturally and/or anthropogenic means, soil acidity is increasing day by day. It has been reported that around 40% of arable lands are acidic worldwide (Bojórquez-Quintal *et al*. 2017; Von Uexküll & Mutert 1995) . The acidic pH (below 5.5) of soils leads to wide range of stresses such as proton toxicity, nutrient deficiency, hormonal imbalance, physiological disorders, susceptibility to various diseases and metal ion toxicities. Among them Al toxicity severely affects and hampered plant's growth and productivity (Mihailovic *et al*. 2008; Mossor-Pietraszewska 2001).

Al constitutes approximately 7% of the total solid matter of earth's crust and represents as the most abundant metal (Delhaize & Ryan 1995; Gupta *et al*. 2013; Silva 2012).Al toxicity has not been reported at pH higher than 5.5, as in this pH range Al remains as harmless oxides and alumino silicates (Ma *et al*. 2001). However, when soils pH lesser than 5.5, Al is solubilized into the toxic trivalent cations Al^{3+}forms (Seguel *et al*. 2013; Watanabe & Okada 2004) and possibly affect plant growth, development, photosynthesis process, productivity(Yang *et al*. 2015). Antioxidant defence mechanisms of the plant are also affected (Latef & Chaoxing 2011; Zhang *et al*. 2007)lead to production of reactive oxygen species (Giannakoula *et al*. 2010).

Soil acidification is a natural process and has been attributed to various reasons including decomposition of organic matter that produces hydrogen ions (H^+), microbial respiration and absorption of mono and bivalent cations (K^+, Ca^{2+}, Mg^{2+}) by plant, erosion and leaching in excessive rainfall areas (Hutchinson & Whitby 1977; Rengel & Tang 2003; van Breemen *et al*. 1983). In addition to natural process, anthropogenic factors are also major reason for increasing soil acidity such as excessive use of ammonical fertilizers (Bolan & Duraisamy 2003), industrial emissions of sulphur dioxide (SO_2)

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and nitrogen oxides (NO_x) (Clair & Hindar 2005; Driscoll *et al*. 2001; Evangelou & Zhang 1995; Kopáček *et al*. 2006).

To minimize effect of Al toxicity in acidic soil, several strategies have been employed to improve agricultural productivity. Traditionally, farmers apply lime to the soil to increase pH of the soil and minimize Al toxicity. However, buffing capacity of soil slow down the effects of lime leading to practically unsustainable and economically unfeasible (Seguel *et al*. 2012; Xu *et al*. 2012). Development of Al tolerant/resistant genotypes also have been reported but the process is very complex and transgenic plants are also prone to other diseases(Ma *et al*. 2000; Seguel *et al*. 2013).Application various synthetic chemicals also have been employed to enhance productivity of plant in Al toxicity areas, but it disturbs ecological balance of the soil, groundwater contamination, development of variants pathogens (Seguel *et al*. 2013).

Thus, there is an urgent need to develop simple, cost effective and eco-friendly biological methods for management of Al stress. Plants in their natural habitat are colonized both by intracellular and endocellular microorganisms(Gray & Smith 2005; Grover *et al*. 2011). Microorganisms, especially mycorrhizal fungi (Baum *et al*. 2015; Qin *et al*. 2015; Sharifi *et al*. 2007)and plant growth promoting rhizobacteria (PGPR) could be a promising tool in these aspects(Barea *et al*. 2002; Carrenho *et al*. 2007; Clark 1997; Glick 2012; Roesti *et al*. 2006) for their beneficial characteristics including production of plant growth promoting substances, detoxification of Al toxicity (Aguilera *et al*. 2017; Antoun & Prévost 2005). It has also been well documented that interaction of rhizobacteria and fungi with plants are very useful for improving plant health condition under various stress and enhances productivity by both directly and/or indirectly(Dimkpa *et al*. 2009; Latef & Chaoxing 2011; Roesti *et al*. 2006). Many plant growth promoting rhizobacteria (PGPR) directly stimulate plant growth and development by providing plants with fixed nitrogen, phytohormones, iron and soluble phosphate (Hayat *et al*. 2010). The present experiment focuses on the enhancement of plant health and increased resistance of plants against Al ion stress by application of plant growth promoting bacteria and mycorrhiza. In the present study, we used Groundnut (*Arachis hypogeal* L.) as our experimental model as it is most prevalent worldwide in more than 100 countries (Leff *et al*. 2004; Upadhyaya *et al*. 2006) and having great nutritional, medicinal and commercial importance (Pandey *et al*. 2012; Pasupuleti *et al*. 2013).

MATERIALS AND METHODS

Plant material, Mycorrhizae and PGPR strain

The seeds of Groundnut (*Arachis hypogaea* L.cv. Girnar-3) were collected from ICAR-Directorate of Groundnut Research, Junagadh, Gujart. The *Glomus etunicatum* an arbuscular mycorrhiza (AM) fungi is obtained from TERI, New Delhi and strain of *Pseudomonas putida* is provided by MTCC, Chandigarh, Punjab.

Growth conditions and Al treatments

The groundnut seeds were first surface sterilized with 0.5% sodium hypoclorite (NaOCl) for 15 min and then soaked in distilled water at room temperature (25°C) overnight. The healthy imbibed seeds were selected and germinated in plastic boxes (25cm x 15cm x 5cm) on 4 layers of moist germination papers in the dark for 4 days. After germination, the uniform seedlings were transferred in plastic pots filled with equal amount of vermiculite and grown at two different growth conditions (i)at only acidic pH (4.5)and (ii)at acidic pH (4.5)+PM (PGPR + Mycorrhiza). The plastic pots were irrigated at regular interval with or without aqueous solution of $AlCl_3$ (0, 50, 250, 500 and 1000µMat pH 4.5) in combination with mycorrhizal strain, *Glomus etunicatum* and *Pseudomonas putida* species as a PGPR. The acidic pH of vermiculite is maintained through diluted HCl solution. The whole experiment was conducted according to a simple randomized complete block design using different Al concentrations. Each treatment was replicated three times and 12 hour (day/night) photoperiod was maintained. Plants were harvested for experiments after 25 days.

Co-inoculation of PGPR and Mycorhiza

The culture of *Pseudomonas putida* was maintained in LB (Luria Bertani) media. The powder form of mycorrhizal strain of *Glomus etunicatum* contains 10 spores per gram. The test bacteria *Pseudomonas putida* was inoculated in 10 ml Luria Bertani (LB) broth and grown overnight at 37ºC at 130 RPM(Sambrook *et al*. 1989).Next day it was washed twice with 10 ml normal saline and then mix with 0.5 gm of mycorrhizal strain into this solution and seedlings were inoculated with this mixed washed culture(Ballesteros-Almanza *et al*. 2010).

Determination of the photosynthetic parameters of leaves

Chlorophyll Content

Chlorophyll was extracted bydimethylformamide (DMF) method(Inskeep & Bloom 1985; Suzuki & Ishimaru 1990).The fresh leaf samples (0.5g) were cut into small pieces and incubated in 5ml of DMF solution and then kept in fridge at 4˚C for 24 hrsin dark covered with aluminium foil. The absorbance was recorded at 663, 647 and 480 nm with using Perkin Elmer UV/Vis spectrophotometer. The amounts of Chlorophyll a, Chlorophyll b, total Chlorophyll, and carotenoid were determined using equation of (Wellburn 1994)and expressed as mg g^{-1} FW.

Chlorophyll fluorescence measurement

F_v/F_m is the most used chlorophyll fluorescence measuring parameter which reflects resultant of plant stress on PS II. The test allows the maximum amount of the light energy to take the fluorescence pathway. It compares between dark-adapted fluorescent state, otherwise known as minimum fluorescence (F_0) and maximum fluorescence (F_m). Maximum fluorescence is the maximum number of reaction centers reduced or closed by a saturating light pulse. In stress condition, fewer open reaction centers available, and the F_v/F_m ratio remains lowered.

The value of F_v/F_m was measured by using PAM fluorimeter (DUAL-PAM-100, Heinz Walz GmbH, Effeltrich, Germany) as described by (Misra *et al*. 2012) and(Kalaji *et al*. 2014).

Leaf gas exchange measurements

The net photosynthetic rate (P_n), transpiration rate (E), intercellular CO_2 concentration (C_i) and stomatal conductance (g_s) were measured using a portable photosynthesis system CI-340 (CID Bio-Science) according to manufacturer's protocol. The measurements were performed for one randomly selected plant per treatment. Data were automatically recorded in each 5 second. The conditions of the environment during the experiment were: air flow rate of 500 µmol s^{-1}, leaf and block temperature 25 ˚C and relative humidity 35%.

Statistical analysis

All experiments were carried out in three replicates having five different treatment and dual growth conditions. The data were analyzed by Graph pad prism software (GraphPad prism 5.01 version, La Jolla, Ca, USA). Data represent the mean ± standard errors of mean and were calculated from three replications for each treatment. Variations of means in different Al concentrations were compared by analysis of variance (ANOVA) followed by Tukey's post-test. A P value less than 0.05 was considered as significant.

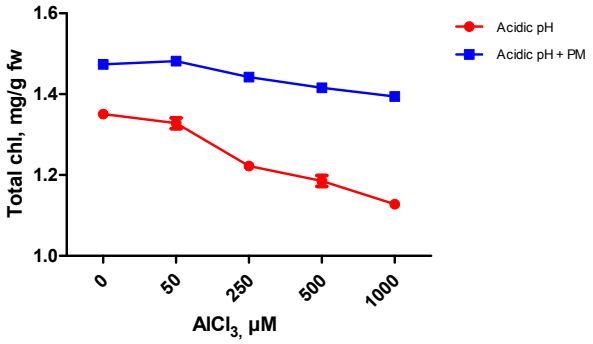
RESULTS

Photosynthetic parameters

Chlorophyll and carotenoid content

Chlorophyll and carotenoid content were significantly decreased with increasing concentration of Al stress in acidic soil condition.[Fig 1(a)& (c)]. Lowest concentration of chlorophyll (16.50%) and carotenoid (37.68 %) was observed in plantlets those subjected to 1000µM concentration of Al at acidic pH. Interestingly, when groundnut seedlings were grown with co-inoculation of *Glomus etunicatum* and *Pseudomonas putida* at acidic pH(4.5) under different concentrations of Al, the chlorophyll and carotenoid content improved to 23.8% and 61.63%, respectively at maximum Al stress condition.

Chlorophyll a/b ratio increased (2.20 to 3.32) with increasing concentration of Al toxicity [Fig.1(b)]. In contrast, application of *Glomus etunicatum* and *Pseudomonas putida* to the seedling with highest dose of Al significantly declined Chlorophyll a/b ratio (1.768 to 2.122).



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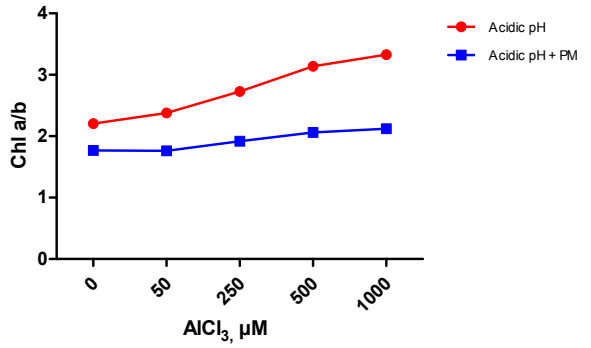

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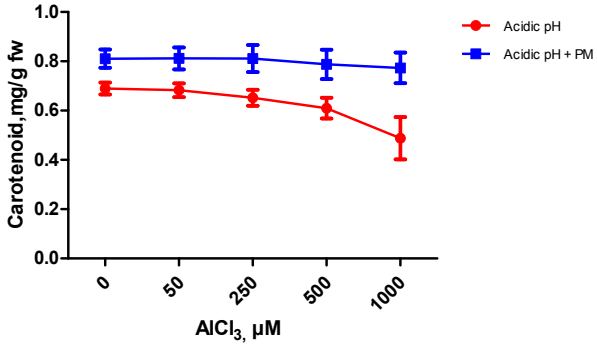


(c)

Fig. 1 (a) Total chlorophyll content (b) Chl a/b ratio and (c) Carotenoid content of groundnut seedlings grown at different concentration of $AlCl_3$ (0, 50, 250, 500 and 1000µM at pH 4.5) in combination with mycorrhizal strain, *Glomusetunicatum* and *Pseudomonas putida* species as a PGPR.Values are means ± SE of triplicates.PM=(PGPR + Mycorrhiza).

Maximal PSII quantum yield (Fv/Fm)

The value of Fv/Fm also decreased from 0.759 to 0.657 when dose of Al stress increased but application of mycorrhiza and PGPR strain revealed a positive impact on Fv/Fm value (0.790-0.749) even at 1000µM concentration of Al (Fig.2a).

Net photosynthetic rate (Pn)

Under acidic condition, increased aluminiumtoxicity significantly diminished photosynthetic rates. The net photosynthetic rate was gradually decreased with increasing concentration of $AlCl_3$.The maximum decreased in net photosynthetic rate (19.2%) was observed at 1000µM concentration of Al, when compared with those of control atacidic pH. But the application of *Glomus etunicatum* and *Pseudomonas putida* showed strong positive impact on net photosynthetic rate. Maximum photosynthetic rate (51.85%) was observed when compared to non-treated seedlings (Fig 2b).

Transpiration rate (E)

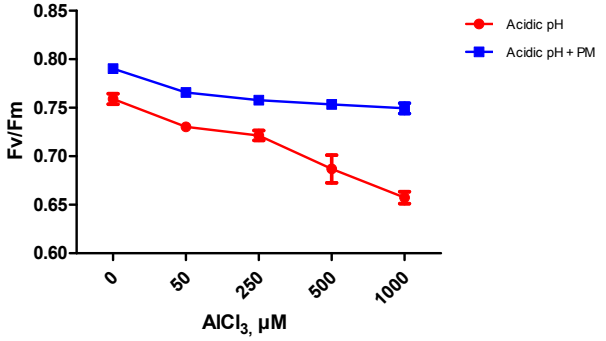
Rate of transpiration has been linked to Al toxicity. In the present study we also observed on increased concentrations of Al the net transpiration rate was drastically reduced. However, application of mycorrhiza and PGPR strain, significantly increased transpiration rate when compared to those of Al stress at acidic pH (Fig.2c).

Water use efficiency (WUE)

Water use efficiency value is an important indicator of plant growth which equal to ratio of water used for plant metabolism to amount of water lost due to transpiration. As shown in Fig-2d, WUE value declined on increased concentration of Al. However, application of *Glomus etunicatum* and *Pseudomonas putida* in acidic soil had significantly enhanced WUE value, indicating importance role of mycorrhiza and PGPR on ameliorating effect of Al toxicity.

Intercellular CO_2 Concentration (Ci) and Stomatal conductance (gS)

As reduced photosynthetic activity was observed in the acid aluminium treated seedlings that showed damage to the leaf tissues. Hence we investigated both intercellular CO_2 concentration and stomatal conductance in the plant seedling and observed significant reduction in both parameters respectively [13.5% (Fig.2e) and 21.27 % (Fig.2f)] at the highest dose of Al (1000µM) compared to non-treated plants. Furthermore, co-inoculation of mycorrhizae and PGPR significantly elevated intercellular CO_2 concentration and stomatal conductance, 10.15% and 27.02% respectively when compared with non-treated seedlings (Fig.2e & 2f).



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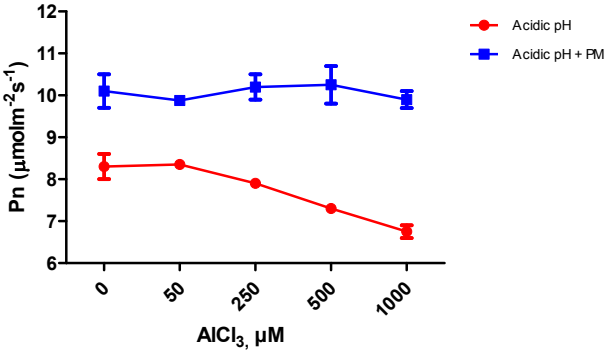

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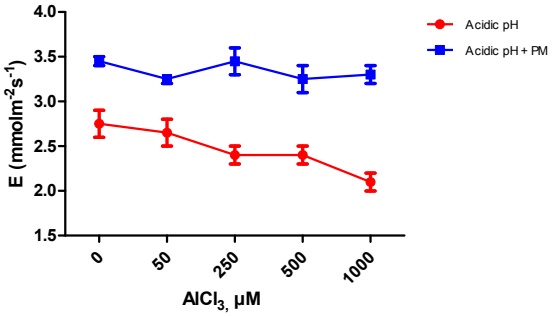

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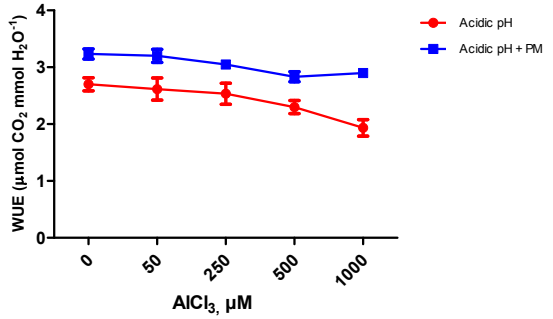

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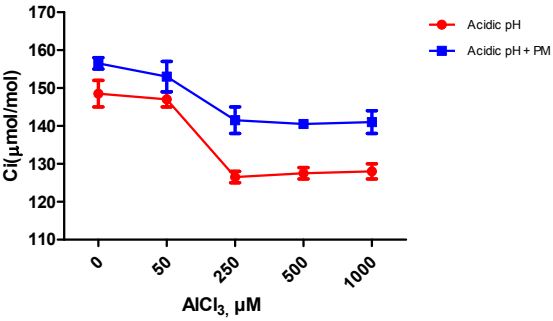

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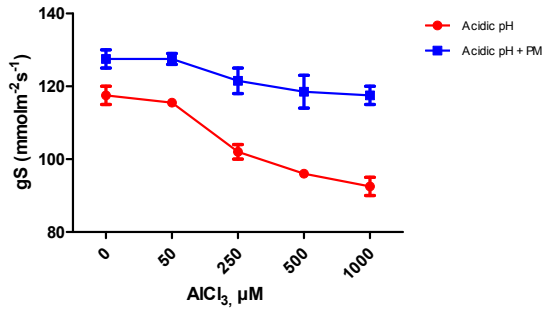

(f)

Fig. 2 Effects of PGPR and mycorrhiza on photosynthetic parameters (a) Fv/Fm (b)Pn (c) E (d) WUE (e) Ci and (f) gS ,when groundnut seedlings grown under different concentration of of $AlCl_3$at pH 4.5.Values are means ± SE of triplicates. PM= (PGPR + Mycorrhiza).

DISCUSSION

In this report, we demonstrated adverse effect of low pH and aluminium stress in chlorophyll content, carotenoid content, maximal PSII quantum yield, net photosynthetic rates, transpiration rates, water use efficiency, stomatal conductance and internal CO_2 concentration. In addition, application of PGPR and mycorrhizae significantly improved condition of plants, suggesting exogenous application of PGPR and mycorrhizae in acidic soil with Al toxicity could improve plant productivity.

Chlorophyll 'a' 'b' and total chlorophyll contents are indicator of photosynthetic and metabolic activity of plants(Hartmann *et al*. 2009; Wright & Jones 2006). Various metal toxicity such as lead,copper, cadmium and mercury has been linked with lower chlorophyll content of plant (Zengin & Munzuroglu 2005). Oncel *et al*., 2000demonstrated that treatments of Cd and Pb in wheat decreased total chlorophyll content to 50-70% (Öncel *et al*. 2000).The exact mechanism how heavy metal reduced chlorophyll quantity is poorly understood. It is believed that heavy metals inhibit metabolic processes of crops by inhibition of important enzymes those are responsible for chlorophyll biosynthesis. Role of cadmium toxicity in chlorophyll biosynthesis has been revealed earlier: it hampers biosynthesis of chlorophyll by inhibiting proto-chlorophyll reductase and synthesis of aminolevulinic acid (ALA) (Stobart *et al*. 1985). A recent study showed application of various PGPR species such as *Azospirillum*, *Azotobacter* and *Pseudomonas*) increased Chlorophyll 'a', Chlorophyll 'b' and total chlorophyll content(Al-Erwy *et al*. 2016).In strawberry plant (Karlidag *et al*. 2013) and Catharanthus roseus (Lenin & Jayanthi 2012), PGPR inoculations significantly increased the chlorophyll contents. Furthermore, combined application of *Pseudomonas sp.*, *Bacillus lentus* and *Azospirillum brasilense* significantly increased chlorophyll content in *Ociumum basilicum* plant (Heidari *et al*. 2011). In addition, importance of PGPR and mycorrhiza inoculation on growth of wheat plant has been demonstrated in green house (Gaur *et al*. 2004) as well as field conditions (Roesti *et al*. 2006). Mycorrhizae are believed to improve uptake of many essentials nutrients such as phosphorus, potassium, nitrogen and micronutrients zinc and copper (Smith & Read 2010) facilitate accumulation of plant nutrition and enhances photosynthesis (Bashan *et al*. 1990) leading to increased biomass and growth.

Magnesium is an important molecule in biosynthesis of chlorophyll and availability of Mg molecules directly linked with concentration of chlorophyll. In the present study we observed inverse link between Al toxicity and chlorophyll content of the plant: with increase in Al toxicity quantity of chlorophyll molecules significantly reduced. The decrease in chlorophyll content is possibly due to competition between Al and Mg uptake and transport (Ma 2000; Vitorello & Haug 1996; Yang *et al*. 2015). The chlorophyll ratio has been used a stress indicator and various researchers reported higher chlorophyll ratio during biotic and abiotic stress conditions (Delfine *et al*. 1999; Monni *et al*. 2001). In line with earlier observations, the present report also observed elevated chlorophyll ratio in increase Al toxic conditions. Furthermore, during stress conditions a high ratio of chla/b also indicates the change in the PSII/PSI ratio(Anderson 1986).

F_v/F_m method is the most common tool used for investigating chlorophyll fluorescence. It shows effects of stress on PSII in dark adapted state: greater the plant stress, minimal open reaction centers available and the F_v/F_m ratio is lowered (Genty & Harbinson 1996; Llorens *et al*. 2003). The significant decrease in photosynthetic rate due to Al stress can be explained by disturbance in PSI and PSII. Al^{3+}, soluble form of aluminium has been reported to stimulate PSII which catalyse electron flow and O_2release but hampers the PSI mediated electron transport(Roy *et al*. 1988; Wavare *et al*. 1983), that leads to oxidative stress and damage to photosynthetic apparatus (Asada 2006; Peixoto *et al*. 2002). In addition, Al stress induce stomatal closure (Li *et al*. 2011; Smirnov *et al*. 2014; Vitorello & Haug 1996) and reduced transpiration rates, these observations is in line with results of our study. Furthermore, WUE remained lower in AI toxic acidic soil plants possibly transpiration competes with photosynthesis for water under Al toxicity and lead to scarcity of water (Ali *et al*. 2008; Ying & Liu 2005).

The mechanism how PGPR and mycorrhizae promote growth of plants is ill defined. PGPR believes to endrosedevelopment through production of growth promoting molecules such as phytohormones, phosphate solubilizers, production of siderophoreand various molecules that enhances nutrient supply (Phosphorous, Nitrogen, Potassium, other minerals)(Antoun *et al*. 1998; Berg 2009; Bhattacharyya & Jha 2012). Mycorrhizae impart beneficial effects in different ways such as nutrient recycling and absorption (Shokri & Maadi 2009), Soil aggregation (Aguilera *et al*. 2011; George *et al*. 2012; Wehner *et al*. 2010), production of phytohormones/amino acids (Meddad-Hamza *et al*. 2010; Smith & Read 2010; Smith *et al*. 2003), secondary metabolites production, maintaining osmotic balance, increase photosynthesis rate and tolerance of biotic andabiotic stresses (Barea *et al*. 2002; Bedini *et al*. 2013; Cumming & Ning 2003; Janos 1980; Lux & Cumming 2001; Pagano & Cabello 2013; Ruiz-Lozano 2003; Selvakumar & Thamizhiniyan 2011; Smith & Read 2010; Vierheilig *et al*. 2008).

CONCLUSION AND FUTURE PROSPECTS

The findings of this experiment showed that the co-inoculation of mycorrhizal strain, *Glomus etunicatum* and *Pseudomonas putida* species as a PGPR have positive impact on photosynthetic parameters and it can be used for amelioration of the toxic effect of aluminium under acidic soils condition. The further future investigation will be of great significance in amelioration of aluminium toxicity and formulation of soil amendment techniques. This may offer an alternative environment-friendly strategy for better plant establishment, growth, development and productivity under stressful environment.

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