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RESPONSE OF N_2-FIXING ACTIVITY IN SOYBEAN (*GLYCINE MAX* (L.) MERR.) INOCULATED WITH PLANT GROWTH PROMOTING RHIZOBACTERIA TO WATER DEFICIT

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Abstract

Effects of water deficit stress under un-inoculated and inoculated soybeans with UPMB10, UPMB12, UPMR19, UPMB10 + UPMR19, UPMB12 + UPMR19 growth promoting rhizo-bacteria were studied in a glasshouse. Acetylene reduction assay (ARA) and N redistribution response to a 7-day drought period at onset of pod (OP) and onset of seed (OS) stages were evaluated. Drought stressed at OS stage had higher nitrogenase activity than OP stage. It appears that during OS stage, fixed nitrogen is redistributed in soybean tissues to support seed N demand rapidly than OP stage under stress condition. Root length and root dry weight were significantly decreased by increasing drought stress, and reduction percentages were varied in the un-inoculated soybeans compared to inoculated and co-inoculated soybean. Co-inoculated and inoculated soybeans minimized the effects of water stress applied at OP and OS stages. Decrease of N redistribution in response to drought stress during OP was associated with the inability to recover N_2-fixation. Drought stress at OP stage inhibited N_2-fixation, and shortened the pod-filling period resulting in decreased seed biomass. On the other hand, malondialdehyde (MDA) and proline contents at OP stage were higher than at OS stage indicating the N_2-fixing activity can recover at OS stage and reduced detrimental effect of water stress.

Introduction

Water deficit stress is the most prominent limiting factor at the initial phase of plant growth and establishment (Jaleel *et al*. 2009). Soybean is considered as a sensitive plant to several abiotic stresses (Van Heerden and Krüger 2000), particularly lack of water during onset of pod (R3), full pod (R4), onset of seed (R5) and seed-filling (R6) processes (Sionit *et al.* 1987, Doss *et al*. 1974). Mederski *et al*. (1973) claim that drought stress during processes was responsible for a pod abortion. However, the seed size was decreased by the stress during R5 and R6 processes (growth stages R5 and R6) (Krivosudska and Filova 2013). Therefore, onset of pod, full pod and onset of seed are 3 prominent stages for successful soybean pod-filling.

Since it is known that, the initial three reproductive growth stages of soybean are sensitive to water stress. An experiment was carried out with indigenous PGPR and *Bradyrhizobium* isolates: (i) determine the most sensitive growth stage (R3 or R5) to water stress condition, (ii) determine the severity of the stages onto soybean pod-filling and (iii) estimate the N_2 fixing activity of soybean with PGPRs as the mechanism to increase tolerance against water stress conditions.

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Materials and Methods

Indigenous UPMB10 and UPMB12 strains were isolated from oil palm roots and *Bradyrhizobium japonicum* (UMPR19) from soybean roots. Seeds of soybean (*Glycine max* (L.) Merr. cv. Williams) were surface sterilized by soaking in 95% ethanol for 10 sec, followed by 3% sodium hypochlorite for 1 min and then 6 times washed with sterile water (Bhuvaneswari *et al.* 1980). Sterilized soybean seeds with bio-inoculants (UPMB10, UPMB12, UPMR19) and co-inoculated with UPMB10 + UPMR19 and UPMB12 + UPMR19) were sown in polyethylene bag containing approximately 8 kg soil samples of clay loam Serdang series that was air dried and sieved. The treatments were replicated three times in a factorial based on Randomized Complete Block Design (RCBD).

Seventy five per cent evapotranspiration (ER) were imposed at the onset of pod stage (42 DAI) for 7 days and at the onset of seed stage (54 DAI) for 7 days. The plants were watered manually considering the method of evapotranspiration replacement (ER) (Klapwijk and De Lint 1974). There were two levels of ER, namely: 100% ER (control) and 75% ER (high water stress). All bags initially received equal volumes of water to maintain them near to the predetermined pots capacity (1.2 liter per 1 kg pot) and moisture lost by ER was replaced daily. Calibration was done weekly to take care of changing water demands of the plants with age.

A total of 162 plants in each replication were uprooted and root length was measured device named Win RHIZO Pro 2007 (Regent Instrument Inc. Co.). For root dry weight (DW) all samples weighted by digital balance after oven drying at 70^{o}C for at least 48 hrs. Nitrogenase activity was measured by Acetylene reduction assay (ARA) (Hardy *et al*. 1968) at the onset of pod, onset of seed and pod-filling stages after 42, 54 and 65 DAI, respectively of soybean inoculated and co-inoculated with UPMB10, UPMB12 and UPMR19.

The malondialdehyde (MDA) concentration was calculated as a measure of lipid peroxidation which was estimated following the method of Heath and Packer (1968). Approximately 1 g of roots with nodule samples were collected at the onset of pod, onset of seed and pod-filling stages (42, 54 and 65 DAI), were ground in liquid nitrogen. Samples were homogenized with the addition of 300 µl water, 35 µl BHT (Butylatedhydroxytoluene), 165 µl SDS (Sodium dodecyl sulphate) and 2 ml TBA (Thiobarbituric acid) and heated for 60 min at 95^{o}C. The tubes were transferred into an ice bath, added 3 mln-butanolwas and vortex for 60 s. Centrifuged at 5000 rpm for 10 min. Aabsorbance of butanol layer was recorded at 532 nm against pure butanol. 1, 1, 3, 3-tetraethoxypropane (TEP) was used in calibration curve of Heath and Packer (1968).

The proline concentration in soybean roots and leaves was measured spectrophotometrically using the method of Bates *et al.* (1973). 0.5 g roots and leaves samples collected at the onset of pod, onset of seed and pod-filling stages (42, 54 and 65 DAI), were homogenized with 5 ml of sulfosalicylic acid (3%) using a mortar and pestle and filtered. The filtrate was made up to 10 ml with sulfosalicylic acid and 2.0 ml of filtrate was incubated with 2.0 ml of glacial acetic acid and 2.0 ml ninhydrin and boiled in a water bath at 100^{o}C for 30 min. Then, the mixture was then cooled and 6.0 ml of toluene was added. The absorbance was recorded at 570 nm.

The collected data were analyzed statistically using the Statistical Analysis System. The data were computed by CRD (Experiment 1) and double factorial (Experiment 2) analyses. Following the ANOVA, differences among treatment means were determined using DMRT comparison method (whenever applicable) at 5% level of significance.

Results and Discussion

Root length and dry weight of soybean un-inoculated, inoculated and co-inoculated at 75% ER (OS) were higher than 75% ER (OP) (Table 1), because, generally high rates of N_2-fixation

have found in OS (R5) compared to OP (R3) growth stages under stress condition. Water stress induced maximum reduction of N_2-fixation at OP stage than OS stage. This decrease was significantly reduced by less activity of UPMB10, UPMB12 and UPMR19 with inoculation or co-inoculation (PGPR + *Bradyrhizobium)*, leading to a loss of fixed N. Consequently, the enhancement of N by fixing nitrogen with UPMB10, UPMB12 and UPMR19 in inoculated and co-inoculated with UPMB10 + UPMR19 and UPMB12 + UPMR19 under water stress, due to enhanced N in shoots at both stages, but, the abilities of fixed nitrogen under stress at OS stage was more than OP stage. Because, during OS stage, biological nitrogen fixation redistributed in soybean tissues to support seed N demand rapidly than OP stage under stress condition (Mastrodomenico and Purcell 2012).

Table 1. Effect of water treatments on root length, root dry weight, N_2-fixing activity of soybean un-inoculated, inoculated and co-inoculated with PGPRs and *Bradyrhizobium* at onset of pod (PO) and onset of seed (OS) stages.

Bacterial inocula	Water treatments (ER%)	Root length (cm)	Root dry weight (g/plant)	N_2-fixing activity (nmol C_2H_4/plant/h)
Un-inoculated	100	395.2d	39.91g	37.61c
Un-inoculated	(75) OP	225.4e	23.24h	22.42d
Un-inoculated	(75) OS	260.8e	33.96g	25.67d
UPMB10	100	618.4a	71.63c	48.27b
UPMB10	(75) OP	364.8d	48.34f	29.90d
UPMB10	(75) OS	470.5c	51.90e	39.70c
UPMB12	100	581.1b	62.37d	45.71b
UPMB12	(75) OP	379.3d	29.95h	19.52e
UPMB12	(75) OS	460.5c	51.17e	39.68c
UPMR19	100	544.5b	51.90e	45.80b
UPMR19	(75) OP	427.9c	28.67h	28.79e
UPMR19	(75) OS	471.9c	48.52f	38.78c
UPMB10+UPMR19	100	692.2a	90.83a	50.68a
UPMB10+UPMR19	(75) OP	385.5d	23.55h	22.46d
UPMB10+UPMR19	(75) OS	578.0b	71.06c	41.83b
UPMB12+UPMR19	100	687.0a	82.88b	51.38a
UPMB12+UPMR19	(75%) OP	477.2c	33.96g	33.68c
UPMB12+UPMR19	(75) OS	593.6b	69.39d	43.49b

Means with the same letters in the column are not significantly different.

The present findings reveal that soybean inoculated with UPMB10, UPMB12, UPMR19 and co-inoculated of UPMB10 + UPMR19 and UPMB12 + UPMR19 subjected to well-watered and high stress level at OP and OS stages had significant effect on MDA and proline contents of roots and shoots (Table 2). Un-inoculated soybean imposed to water stress had highest MDA content and proline content in roots and shoots compared to inoculated and co-inoculated soybean.

With regard to this hypothesis, generally, interaction between PGPRs and *Bradyrhizobium* decreased the deleterious effect of drought stress on soybeans plant helps them to survive and continue their growth under harsh environmental condition (Zhang *et al*. 1996).

Table 2. Effect of water treatments on MDA and proline contents in root and leaf of soybean un-inoculated, inoculated and co-inoculated with PGPRs and *Bradyrhizobium* at onset of pod (OP) and onset of seed (OS) stages.

Bacterial inocula	Water treatments (ER%)	MDA (µg/ groot)	MDA (µg/ gleaf)	Proline (mg/g root)	Proline (mg/ gleaf)
Un-inoculated	100	39.15^{e}	58.62^{e}	15.13^{g}	9.95^{f}
Un-inoculated	(75) OP	59.9^{a}	90.66^{a}	20.23^{a}	13.03^{a}
Un-inoculated	(75) OS	46.8^{c}	78.31^{c}	17.87^{d}	10.87^{d}
UPMB10	100	24.98^{g}	56.52^{e}	15.7^{f}	9.61^{f}
UPMB10	(75) OP	55.67^{a}	86.73^{b}	19.87^{b}	10.65^{d}
UPMB10	(75) OS	58.6^{a}	73.13^{c}	17.5^{e}	10.5^{e}
UPMB12	100	35.62^{e}	56.46^{e}	15.77^{f}	9.7^{f}
UPMB12	(75) OP	56.59^{a}	67.8^{d}	19.92^{b}	12.73^{a}
UPMB12	(75) OS	42.9^{c}	73.69^{c}	17.62^{d}	10.62^{e}
UPMR19	100	36.59^{e}	57.53^{e}	15.91^{f}	9.75^{f}
UPMR19	(75) OP	57.5^{a}	55.37^{e}	19.01^{b}	11.85^{c}
UPMR19	(75) OS	44.6^{c}	74.53^{c}	17.71^{d}	10.71^{d}
UPMB10+UPMR19	100	32.71^{f}	53.05^{f}	15.54^{g}	9.35^{g}
UPMB10+UPMR19	(75) OP	54.55^{b}	84.55^{b}	18.52^{c}	12.52^{b}
UPMB10+UPMR19	(75) OS	40.5^{d}	41.55^{g}	19.37^{a}	10.37^{e}
UPMB12+UPMR19	100	33.4^{f}	53.35^{f}	15.5^{g}	9.4^{g}
UPMB12+UPMR19	(75) OP	54.66^{b}	85.65^{b}	18.61^{c}	12.49^{b}
UPMB12+UPMR19	(75) OS	41.7^{d}	71.63^{c}	17.41^{e}	10.41^{e}

Means with the same letters in the column are not significantly different.

Furthermore, MDA and proline contents in roots were more than shoots at both growth stages, which mean sensitivity of soybean's roots was more than shoots under water deficit stress. On the other side, MDA and proline contents of soybean at OP stage was more than OS stage because drought had the largest and prominent effects on recovery of N_2-fixation and N redistribution at OS stage compared to OP stage. That means N_2-fixing activity can recover at OS stage and reduced detrimental effect of water stress. Kheradmand *et al*. (2014), Naveed *et al*. (2014) and Pruden and Salon (2014) found the same results in different crops.

In conclusion, the overall results of present research indicated that some trait like N_2-fixing activity could have an effective role at different soybean growth stages (especially at onset of pod and onset of seed) under high degree of water stress level. The most sensitive stages of plant growth and development was onset of pod compared to onset of seed because, fixed biological nitrogen redistributed in soybean tissues to support seed N demand rapidly at OS (R5) stage compared to OP (R3) stage under stress condition. It seems co-inoculation and inoculation of PGPR *Bacillus* spp. isolates UPMB10 and UPMB12, and also *Bradyrhizobium japonicum* isolate UPMR19 influenced biochemical and physiological parameters of soybean and helped plant tolerate drought stress to a higher level as compared to un-inoculated soybean.

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