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Full Length Research Paper

Chlorophyll *a* fluorescence determines the drought resistance capabilities in two varieties of mycorrhized and non-mycorrhized *Glycine max* Linn

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Arbuscular mycorrhizal fungi, namely, *Glomus mosseae* and *Glomus intraradices,* and an endophyte, *Piriformospora indica* was used to assess the growth of two varieties (JS 335 and TAMS 98-21) of soybean (*Glycine max* L.) subjected to drought stress. Chlorophyll a fluorescence was measured by Handy PEA (plant efficiency analyzer) and analyzed by Biolyzer programme. The JIP-test (Chlorophyll a fluorescence rise at J I and P steps) was applied to evaluate *in vivo* vitality based on *in vivo* biospectroscopy, which supplied images, spectra and kinetics.We report that in JS-335 variety of soybean, *G. intraradices* induced the highest drought resistance followed by G. *mosseae* and *P. indica,* whereas in TAMS 98-21 variety, *G. mosseae* induced highest drought resistance followed by *G. intraradices and P. indica.*

Key words: *Glomus mosseae, Glomus intraradices, Piriformospora indica, Glycine max* L., arbuscular mycorrhizal.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are common soil microbes whose association with roots can have wide-range effects on growth of the host plant (Klironomos et al., 2001). AM symbiosis can also alter plant water relations and responses to drought (Auge, 2001). In many instances, these AM-induced changes have been interpreted as improved drought resistance (Davies et al., 1993). AMF are now well known as growth promoting fungi and 80% plants possess this symbiotic association including angiosperms, gymnosperms, pteridophytes and bryophytes. These are essential components of the soil microflora in ecosystem restoration. The mycorrhiza plays a significant role in the mycorrhizosphere by changing the mineral nutrient composition, hormonal balance, carbon allocation patterns, and other aspects of physiology (Fisher and Jayachandran, 2005; Andersen and Andersen, 2006; Barroetavena et al., 2006; Burrows and Fennell, 2006; Copetta et al., 2006; Druege et al., 2006). When AM plant and non-AM control have similar size and phosphorus concentration, mycorrhizal plants often show higher stomata conductance and transpiration, whether plants are adequately watered or subjected to drought. AMF inoculation improved tomato growth under salt or saltless condition and reduced cell membrane osmosis, MDA (malonaldehyde) content in salinity. The salt tolerance of tomato was enhanced by AMF activity in roots. The mycorrhized plants showed higher potential than corresponding non-AM plants in salinity (Zhong-Qun et al., 2007). AM colonization enhances osmotic solute accumulation of trifoliate orange

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seedlings, thus providing better osmotic adjustment in AM seedlings. Glucose and sucrose contents of well-watered and drought-stressed roots, fructose contents of well-watered roots and sucrose contents of drought-stressed leaves were notably higher in AM than in non-AM seedlings (Qiang-Sheng et al., 2007a, b).

An induced drought stress tolerance involves several physiological processes: (i) modification of foliar water relation parameters, such as gas exchange, leaf water potential, leaf-tissue elasticity and stomatal behaviour and alterations of root turgor and the root to shoot signals. At severe drought, fertilisation and *Pseudomonas mendocina* inoculation, alone or in combination with either of the selected AM fungi, increased significantly phosphatase activity in lettuce roots and proline accumulation in leaves (Kohler et al., 2008). The water stress caused a decrement of 33% for the colonization of *Glomus versiforme* on *Citrus tangerine* roots reported (Qiangsheng et al., 2007b). Under well-watered and water-stressed conditions, *G. versiforme* inoculation increased the leaf phosphorus (P) content by 45 and 27%, and decreased the leaf malondialdehyde and hydrogen peroxide contents by 25 and 21, and 16 and 16%, respectively, compared with the control.

Piriformospora indica, a new plant growth promoter was discovered by Verma et al. (1998). It is a member of Basidiomycotina and is characterized by the formation of typical pyriform chlamydospores. A perusal of literature shows that *P. indica* has enormous potential for growth promotion of plants by colonization of roots (Rai et al., 2001). *P. indica* is similar to arbuscular mycorrhizal fungi in many respects (Rai and Varma, 2005; Deshmukh et al., 2006). But, unlike arbuscular mycorrhizal fungi, it can be cultured in artificial medium. Later, *P. indica* was assessed for its growth stimulation in timber-yielding and medicinal plants. Taking into consideration the growth promoting potential of *P. indica*, *Glycine max* plants were used for evaluation of its efficacy by using Chlorophyll a as a biomarker. Moreover, some results showed that creeping bent grass inoculated with the AM fungus *G. intraradices* tolerated drought conditions for longer periods and recovered more quickly from wilting than did non-mycorrhizal turf.

Soybean (*G. max* L.) Merrill is a major source of protein and edible oil. It is widely grown on a large scale in the semiarid and tropical region and occupies the first place in world edible oil production. Much evidence indicates that drought stress decreases soybean leaf area and leaf weight (Pandey et al., 1984; De Costa and Shanmugathasan, 2002). Drought stress affects physiological processes as translocation at the whole plant level, leaf expansion and gas exchange at the organ level, and photosynthesis at the sub-cellular level and at the end, it reduces growth and yield. Due to drought reduction in photosynthetic activity takes place.

For the evaluation of the impact of any stress and, similarly, of mycorrhizosphere activity on plants, we apply the JIP-test, which provides a quantitative analysis of the *in vivo* vitality-behaviour, performance-of pigment system II (PSII), that is, a quantitative description of the biophysical phenotype-macrostate, by accessing the different microstate-functional building blocks. The JIP test is an analysis of the fast fluorescence kinetics O-J-I-P exhibited by all oxygenic photosynthetic organism upon illumination, based on a simple model and the theory of energy fluxes in Biomembranes. It is well documented that biophysical tools, not only in the recognition and evaluation of the beneficial role of mycorrhiza symbiosis on PSII activity (Tsimilli-Michael and Strasser, 2000) but it is more in biophysical phynotyping of the photosynthetic apparatus of a plant under any stress caused by changes in different environmental conditions like temperature, drought light intensity. The JIP-test analyzes the *in vivo* vitality, based on *in vivo* biospectroscopy, which supplies the images, spectra and kinetics (Strasser et al., 2007). The present method is nondestructive and rapid.

The present study was aimed to (i) analyse and evaluate the drought stress tolerance of soybean after colonization of roots by *P. indica* (Varma et al., 1998), *Glomus mosseae* and *Glomus intraradices,* (ii) compare the performance and efficiency of two soybean varieties, and (iii) analyze the *in vivo* performance of pigment system II.

MATERIALS AND METHODS

Plant material

Two varieties of Soybean (JS-335 and TAMS-9821) were obtained from PKV (Punjabrao Deshmukh Krishi Vidyapeeth), Akola, Maharashtra state, India**.** We selected these two varieties of Soybean (JS-335 and TAMS-9821) because of higher yield, and thus local farmers use them.

Mycosymbionts

P. indica, G. mosseae and *G. intraradices.*

Sterilization of seeds of soybean

The seeds of soybean *(Glycine max)* were surface disinfected (0.1% $HgCl_2$ and water for 2 min). Then seeds were incubated over night at room temperature in sterile distilled water in dark and then transferred in earthen pots diameter 41.5 cm).

Experimental

Inoculation procedure

The seeds of *G. max* were sown in the sterilized soil with sand having 3:1 ratio respectively. Then 10 g soil culture of *G. mosseae* (Gm), *G. intraradices* (Gi) and *P. indica* (Pi) was inoculated to *G. max* seeds except control in triplicates. Plants were watered daily and fertilized once at weak 1 ml to each pot with Hewitt solution (Hewitt, 1966).

The host plants (*G. max*) were treated as follows:

Before stress (From 0 to 21 days):
1. Non-inoculated (control)
2. Inoculated with *P. indica*
3. Inoculated with *G. mosseae*
4. Inoculated with *G. intraradices*

Drought stress (From 22 to 45 days): The irrigation was stopped after 21 days. The measurements of Chlorophyll a fluorescence of the plants were recorded as per the following treatment:

1. Non-inoculated (control)
2. Non-inoculated with water stress
3. Inoculated with *P. indica*
4. Inoculated with *G. mosseae*
5. Inoculated with *G. intraradices*

Drought recovery (From 46-65 days): The stressed plants were irrigated from 46 days after inoculation and the chl a fluorescence was measured.

Measurement of Chlorophyll a fluorescence

Chlorophyll a fluorescence was measured by Handy PEA (Hansatech Instrument Pvt. Ltd, UK), which is a fluorimeter having high resolutions 12 bit/s, (22) (Strasser et al., 2007). The samples were dark adapted for 15 min before the measurements and then they were illuminated with continuous light (650 nm peak wavelength, 3000 µmol photons^{-2} S^{-1} maximum light intensity) provided by an array of three light emitting diodes focused on a circle of 5 mm diameter of sample surface. The first observations were recorded after seven days of germination of seeds of *G. max.* Subsequently, three more observations were recorded at the interval of seven days. After three successive measurements, the plants were put under drought stress conditions (irrigation was stopped) and the observations were noted with the interval of 4 days. On every week, the height was measured.

This first stage of measurements with Handy PEA was to ensure that the sample to be measured is fully dark-adapted. While the sample is dark adapted, the different measurement options may be manually configured. Files of measurement had been transferred to a computer and analyzed the vitality and efficiency of the plants, along with the photosynthetic activities (performance index, Specific fluxes and yields) by using the software Biolyzer (Rodriguez et al., 2003; Repkova et al., 2008; Zivcak et al., 2008). The formulae pertaining to photosynthetic activities or quantification of Pigment system II are given in Table 1.

The plants were irrigated every alternate day. The Hewitt solution was added after one-week interval with 1 ml to each pot (Hewitt, 1966).

Staining of root samples and assessment of root colonization

The standard method was used for clearing and staining of roots to observe the AMF spores and their colonization in symbiosis with roots (Giovannetti and Mosse, 1980). To assess the colonization, randomly selected stained root samples were focused under compound microscope, 10 to 100× magnifications for quantification and qualification. The method, proposed by Giovannetti and Mosse (1980) was used for assessment of root colonization.

Statistical analysis

To analyze whether there is any significant difference between the performances of each culture with the control, we used 'one way ANOVA' for the relative variability within the separate classes of the experiment.

RESULTS

The measurements of Chl a fluorescence have been used to calculate the specific energy fluxes (ABS/RC, ETo/RC, TRo/RC and DIo/ RC), phenomenological fluxes (ABS/CS, ETo/Cs TRo/CS, DIo/CS, RC/CSo, ABS/CSo) and PIabs.

After exposing both varieties of soybean (JS-335 and TAMS-9821) to drought stress, no significant changes were observed into the fluorescence transients until Day 8 after watering was stopped. But, after 8 days the effects on the absorption per reaction center (ABS/RC) and performance index per absorption (PIabs) showed a considerable deviation from the control sample. In this condition both varieties behaved similarly. In order to simplify data representation, the data were pooled to give three periods.

Before drought: Measurements of the Day 0 to 21.
Drought stress: Measurements of the day from 22 to 45 included watercontrols and unwatered drought stressed plants.
Drought recovery: Measurements from day 46 to 65 included watered control, unwatered drought stressed and rewatered drought recovery plants.

The effects of drought on photosynthetic activity of plants have been analyzed by normalizing the experimental signals along with the comparison among the performance indices (PIabs) between two varieties. The changes in quantum efficiencies and absorption per reaction center (ABS/RC) have a significant effect on the performance index (PIabs) of both varieties. This is confirmed by strong analytical change in PIabs in both the varieties during drought recovery phase. The continued drought caused a severe diminishing effect in performance (PIabs) and increase in dissipation DIo/RC in both the varieties. It can be observed that both varieties have been recovered during the rewatering period (Table 1)

We observed that the PI abs/PI abs, control undergoes wide changes during the course of the stress. Though drought condition is shown to affect both non-inoculated and inoculated plants, the beneficial role of the symbiont concerning the tolerance to drought stress is clearly revealed. φ po/ φpo control appears much less sensitive than PI abs/PI abs, control and much less appropriate in detecting vitality changes (Figure 1). However, magnify-cation of the changes it undergoes reveals that it exhibits a trend similar to that of PI abs/PI abs, control for both non-inoculated and inoculated plants.

Apparently, the experimental signals were transformed into the normalized signals by using JIP-test and con-comitantly calculated by applying biophysical parameters and plotted. The plots illustrate the comparison

Table 1. Percentage colonization and height of drought stress plants after inoculation with *P. indica*, *G. mosseae* and *G. intraradices*.

Soybean (JS-335 variety)		
Treatment	**Height (cm)**	**% colonization**
Non-inoculated (control)	15.6(± 1.15)	0
Non inoculated with water stress (control)	15.5(± 0.70)	0
G. intraradices	17.43(± 0.92)	90 (±5.00)
G. mosseae	16.33(± 0.76)	95(± 2.88)
P. indica	17.23 (± 0.92)	85(± 7.63)

Soybean (TAMS-21 variety)		
Treatment	**Height (cm)**	**% Colonization**
Non-inoculated (Control)	18.5(± 1.32)	0
Non inoculated with water stress (control)	17.25(± 0.35)	0
G. intraradices	18.33 (± 0.28)	95(± 7.63)
G. mosseae	17.66(± 1.04)	100(± 2.88)
P. indica	17.33(± 1.60)	90(± 5.77)

All values are means ± S.D. Mean value are significantly differently at P 0.05. No. of replicates 15.

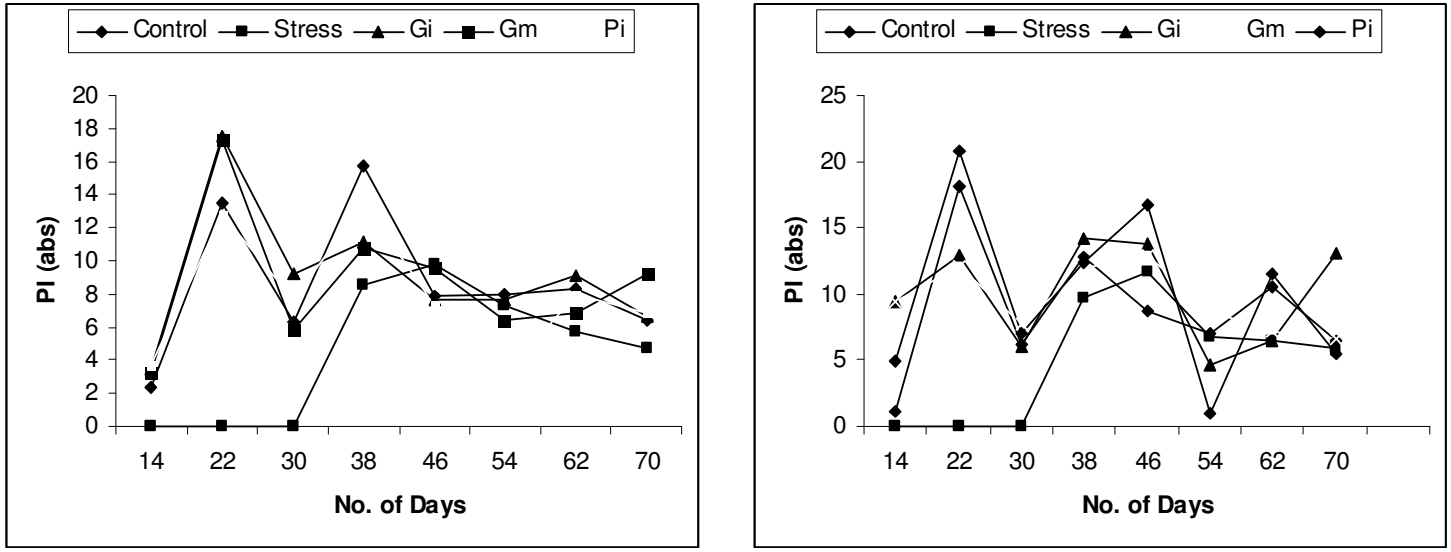

Figure 1. The relative performance indices of JS-335 ($PIabs_{,stress,Gm+stress}$ /$PIabs_{,control}$) and TAMS 98-21 ($PIabs_{,stress,Gi+stress}$ /$PIabs_{,control}$), for 70 days treatment after inoculation of Germplasms with mycorrhiza and endophytes (*G. mosseae, G. intraradices* and *P. indica*).

between relative biophysical constraints of two varieties (JS-335 and TAMS-9821). They divulge discern in maximum quantum yield of primary photochemistry (Φ_{Po}), maximum yield of electron transport (Φ_{Eo}), Quantum efficiency of the microstate reactions (ψ_o) and the performance indices (PI_{abs}) of two varieties, which are extremely suitable indices for the *in vivo* vitality analysis of the photosynthetic system (Figure 2) (Strasser et al., 2007). These performance indices express the driving force of the photosynthetic reaction, held in the system microstates, which act as the representatives of the system macro state (biophysical phenotyping).

The spider plot of (Figure 3) presents the impact of drought stress on different parameters derived by the JIP test from the fluorescence transient, for non-inoculated and inoculated (with Pi, Gi and Gm

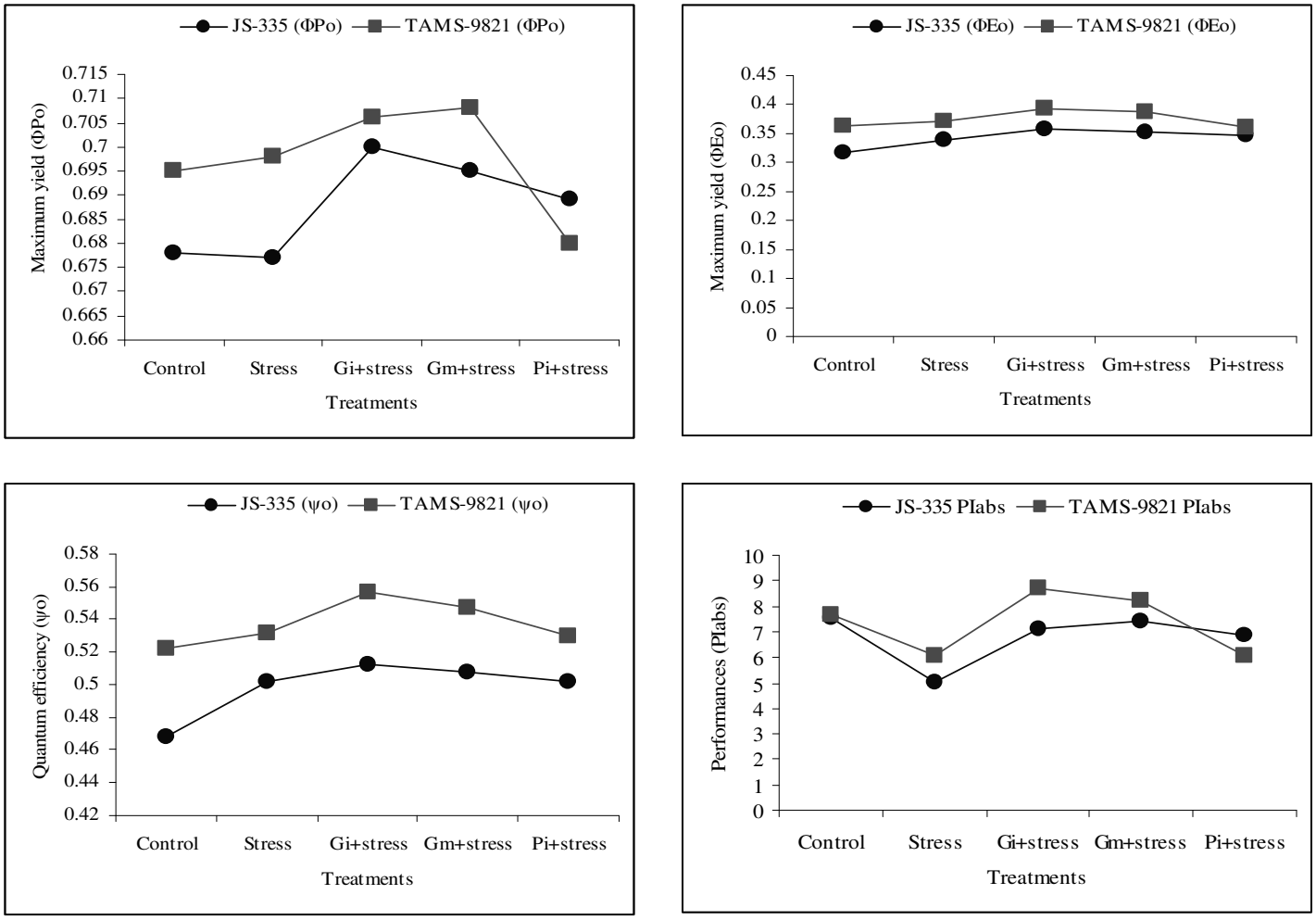

Figure 2. Comparison between relative biophysical parameters of two varieties (JS-335 and TAMS-9821), which are calculated by JIP-test, reveals discern in (*a*) maximum quantum yield of primary photochemistry (Φ_{Po}), (*b*) maximum yield of electron transport (Φ_{Eo}), (*c*) Quantum efficiency of the microstate reactions (ψ_o) and (*d*) the performance indices (PI_{abs}) of two varieties.

symbiont) plants. The parameters are, PIabs, φPo, φEo, ψo ABS/RC, ETo/RC, TRo/RC and DIo/ RC), phenomenological fluxes (ABS/CS, ETo/Cs TRo/CS, DIo/CS, RC/CSo, and ABS/CSo) (Table 2, Figure 3). The figure refers to the 45 day after inoculation with *P. indica, G. mosseae* and *.G. intraradices.* The spider plot has the advantages of providing an easy recognition of stress effects.

Correlation between performance per absorption (PIabs) and dissipation per reaction center (DIo/RC)

The effect of drought reduced performance per absorption (PIabs) values for stressed plants, whereas non- inoculated (control) and plants inoculated with *P. indica*, *G. mosseae* and *G. intraradices* maintained higher performance per absorption (PIabs) levels (Figure 4A and B). After re-watering, PI values of stress plants continued to decrease, whereas plants inoculated with *P. indica*, *G. mosseae* and *G. intraradices* started to recover.

JS-335, *G. intraradices* shows high performance indices (PIabs) and low dissipation at its concerned reaction center DIo/RC as compared to *G. mosseae* and *P. indica* (Figure 4a, Table 3), that means *G. intraradices* promotes better growth than *G. mosseae* and *P. indica* even in drought stress condition. The recovery of *G. intraradices* inoculated plants was more rapid after irrigation followed by *G. mosseae* and *P. indica*. The stressed plants of both varieties depict increase in dissipation at their concerned reaction center (DIo/RC) abreast decreasing in performance index (PIabs), during drought stressed conditions.

In TAMS-9821 *G. mosseae* shows best performance index (PIabs) with low dissipation per reaction center followed by *Glomus intraradices* and *P. indica* (Figure 4b, Table 3). *G. mosseae* recovered rapidly after irrigation followed by *G. intraradices* and *P. indica*. In over all

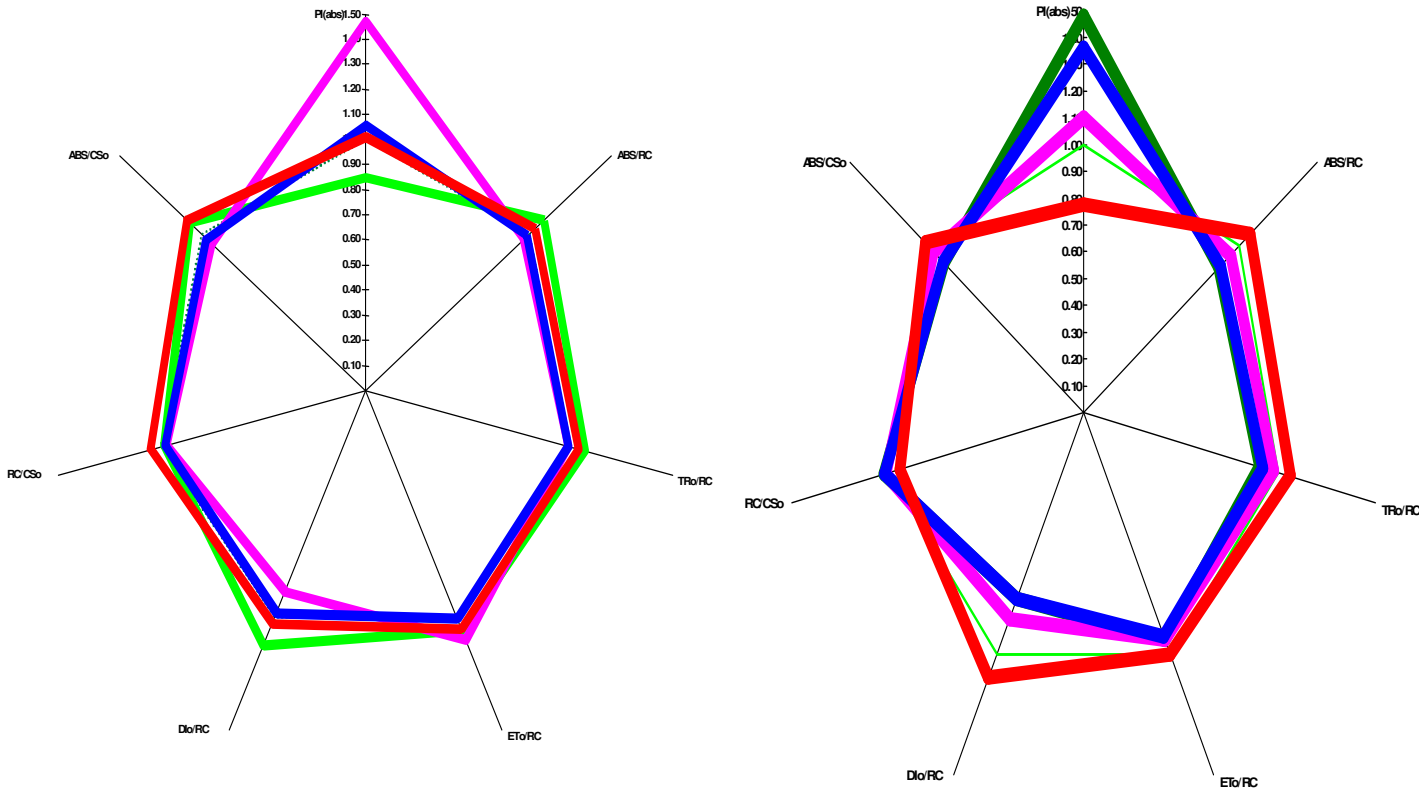

Figure 3. Soybean (JS-335 variety): a, Radar plot after 45 days after stress condition (water irrigation was stopped), (TAMS-9821 variety): b, Radar plot after 45 days after stress condition (water irrigation was stopped), (*Piriformospora indica* (Pi)-Red, *Glomus intraradices* (Gi)-Pink, *Glomus mosseae* (Gm)- Blue, Control-Dark-green, Stress-Light-green.

Table 2. Formulae for the calculation of the specific fluxes, phenomenological fluxes and yields from the fluorescence data, as per JIP-test.

Parameter	Formulae
Specific fluxes	$ABS/RC=(Mo/V_1)/[1-(Fo/Fm)]$
Trapping per reaction center	$Tro/RC= (Mo/ V_1)$
Dissipation per reaction center	Dlo/RC= (ABS/RC) (TRo/RC)
Electron transport per reaction center	$Eto/RC=(Mo/ V_1)(l- V_1)$
Yield as ratios of fluxes	φ Po= (TRo/RC)/(ABS/RC)
Maximum yield of electron transport	φ Eo= (ETo/RC)/(ABS/RC)
Efficiency of a trapped exciton to move an electron into the electron transport chain	ψo= (ETo/RC)/(TRo/RC)
Relative variable fluorescence at the I-step	$V1=(F_1-Fo)/(Fm-Fo)$
Initial slope of the V= f (t) transient	$Mo=4(F_{300\mu s}-Fo)/(Fm-Fo)$

experiments, *G. mosseae* treated plants recovered rapidly followed by *G. intraradices* and *P. indica* (Figure 5). And the *G. mosseae* show better performance followed by *G. intraradices* and *P. indica* because in the *G. intraradices* show maximum height and but low PI abs than *G. mosseae*, also the *P. indica* show maximum height with low Piabs than G. *mosseae* and hence in overall experiment *G. mosseae* play a very important role to resist soybean plant in drought stress conditions followed by *G. intraradices* and *P. indica*.

Correlation between performance per absorption (Plabs) and height (cm)

By proving the high sensitivity of the performance index, we also checked whether and how it is related with physiological parameters, commonly used for the evaluation of the impact of symbiosis on the vitality of the plants (Figure 5) shows indeed a striking correlation between the height of the plant and the performance index PI abs. The data presented come from non-inoculated

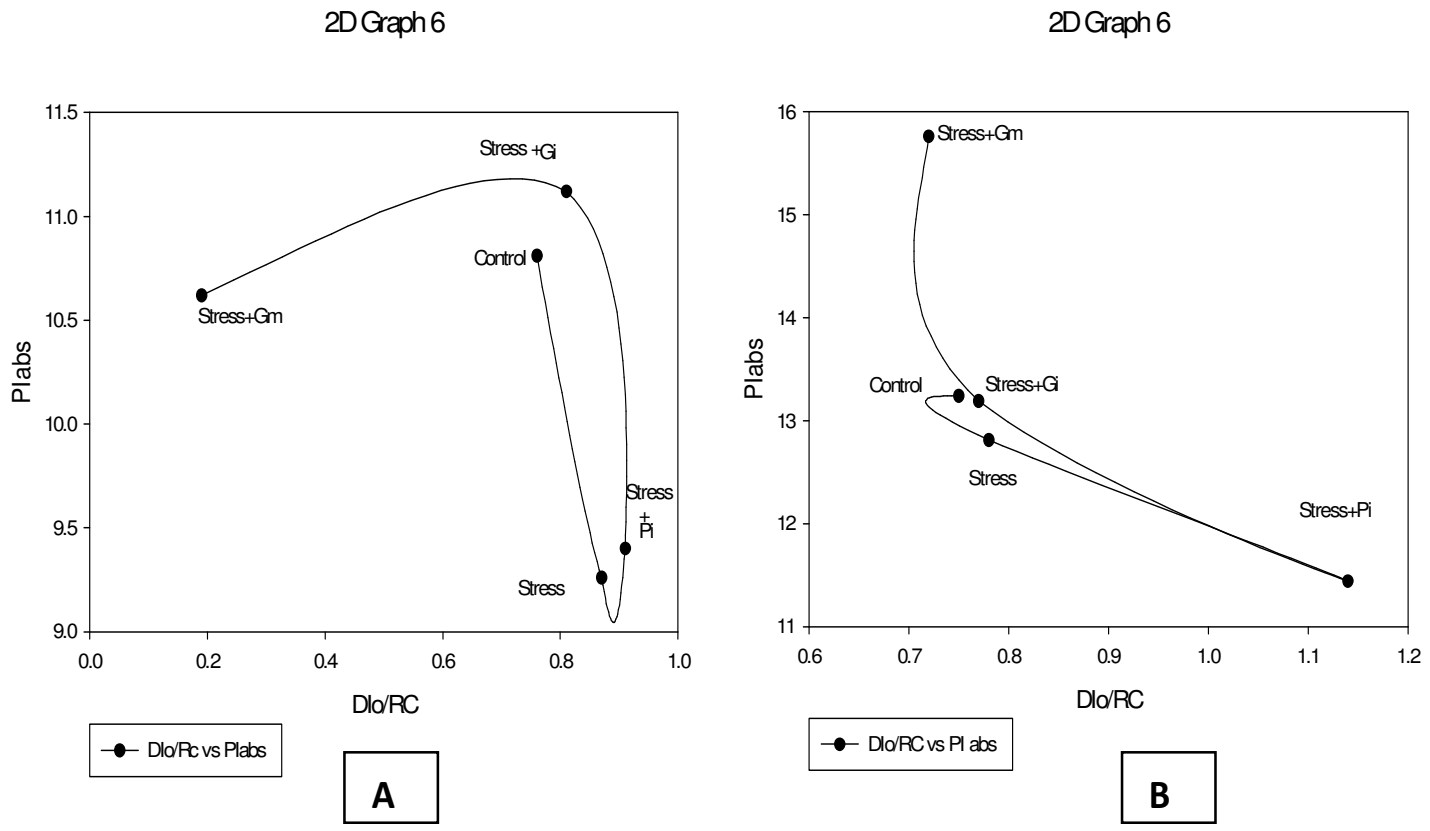

Figure 4. (A) Effect of drought stress condition on Performance per absorption (PIabs) and Dissipation per reaction center (DIo/RC) of JS-335 (B) and TAMS-9821 (Soybean variety). (Control = with water, Stress= without water, Stress+ *Piriformospora indica*, (Pi), Stress+ *Glomus mosseae*, (Gm), Stress+ *Glomus intraradices* (Gi). (Stress + Pi, Gm and Gi water irrigation was stopped).

(with Pi, Gi, Gm) *G. max* plants in the absence (control) and plants inoculated with *P. indica, G. mosseae* and *G. intraradices*.

After exposing the soybean plants to drought stress the maximum height was recorded in plants inoculated with *G. mosseae* followed by *P. indica* and *G. intraradices* as compared to non-inoculated with water (control) and non-inoculated with water stress (stress). The highest percentage of colonization of the soybean plants was shown by *G. mosseae* followed by *P. indica* and *G. intraradices* as compared to inoculated with water (control) and non-inoculated with water stress (Figure 6).

DISCUSSION

As compared to controls, arbuscular mycorrhizal fungi not only improve height of the plants but also protect them from drought stress condition (Figure 4a, b). Similarly, Marulanda et al. (2003) reported that AMF increases tolerance to drought stress in plants. It must be due to the fact that AMF absorbs more water from the soil than non-AM plants under water deficit conditions (Marulanda et al., 2003; Khalvati et al., 2005). The excess water absorption is because of increased mycelial network.

G. mosseae, P. indica and *G. intraradices* increased growth and drought resistance in soybean (Figure 5.). The maximum height and percentage of colonization was found in plants inoculated with *G. mosseae* (Table 1). Porcel et al. (2006) reported that under water deficit conditions, *G. mosseae* accelerated the decrease of PIP gene expression in roots of *G. max.*

In general, stressed plants cope with suboptimal physical conditions by adjusting thermo dynamical stability (Tsimilli-Michael and Strasser, 2002). The parameters RC/ABS and ETo/ABS increased in mycorrhizal plants. This might be due to less deactivation of RC in the presence of mycorrhiza under water stress conditions, which cause perturbation in electron transfer (ETo), so that the number of active RC controlled the intensity of the photosynthetic reactions.

Drought tolerance of mycorrhizal plants can be influenced by altered physical parameters of the soil: an increased root conductance and an altered relation of substrate water capacity to water tension led to an improved plant performance in soybean (*G. max* L.) under stress. In addition, a desiccating mycorrhizal soil

Table 3. Statistical analysis between JS-335 and TAMS-9821 calculated with the JIP test.

Reactions	JS-335					TAMS-9821				
	Control	Stress	Pi	Gi	Gm	Control	Stress	Pi	Gi	Gm
ABS/RC	2.63 (±0.11)	2.77 (±0.08)	2.84 (±0.12)	2.69(±0.12)	2.64 (±0.09)	2.66 (±0.19)	2.68 (±0.13)	3.10 (±0.91)	2.67 (±0.16)	2.54 (±0.25)
TRo/RC	1.78 (±0.28)	1.90 (±0.07)	2.02 (±0.25)	1.88 (±0.07)	1.84 (±0.07)	1.90 (±0.10)	1.90 (±0.07)	1.96 (±0.10)	1.90 (±0.05)	1.83 (±0.13)
DIo/RC	0.76 (±0.06)	0.87 (±0.06)	0.91 (±0.26)	0.81 (±0.11)	0.19 (±0.07)	0.75 (±0.11)	0.78 (±0.11)	1.14 (±0.87)	0.77 (±0.14)	0.72 (±0.13)
ETo/RC	0.99 (±0.14)	1.02 (±0.08)	1.02 (±0.10)	1.03 (±0.09)	0.99 (±0.11)	1.05 (±0.14)	1.08 (±0.11)	1.10 (±0.11)	1.07 (±0.14)	1.62 (±0.12)
PI(abs)	10.81 (±2.91)	9.26 (±1.39)	9.4 (±3.58)	11.12 (±3.27)	10.62 (±2.78)	13.24 (±6.41)	12.81 (±4.67)	11.44 (±6.60)	13.19 (±5.00)	15.76 (±7.54)
Dfabs	1.033	0.966	0.973	1.046	1.026	1.121	1.107	1.058	1.120	1.197

All values are means ± S. D. mean value are significantly differently at P 0.05. No. of replicates 15.

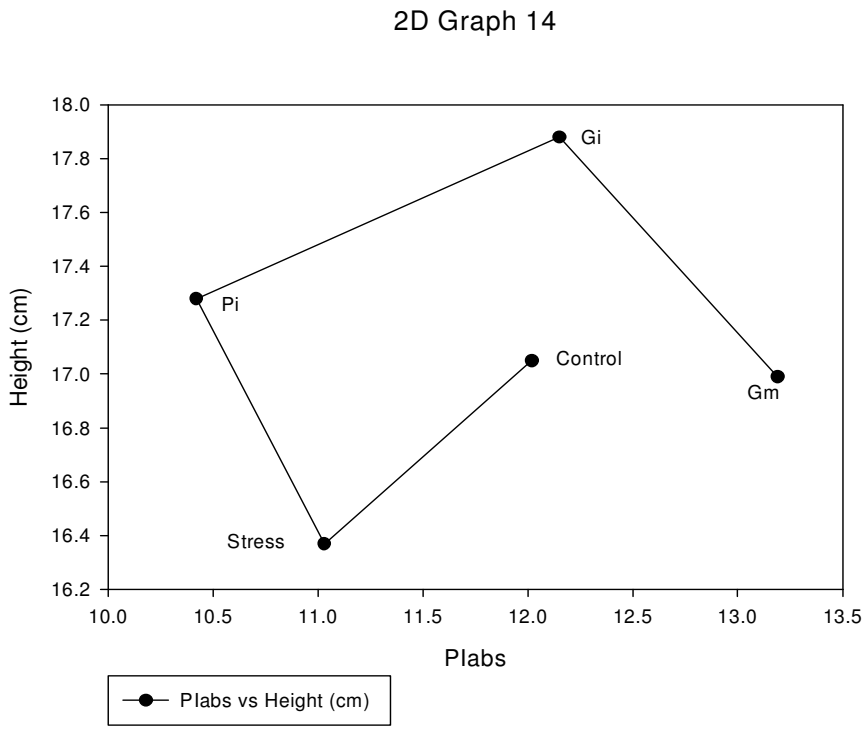

Figure 5. Effect of drought stress condition on Performance per absorption (Plabs) and Height (cm) of soybean variety (Control = with water, Stress -without water, Stress+ *Piriformospora indica*, (Pi), Stress+ *Glomus mosseae*, (Gm), Stress+Gi- *Glomus intraradices* (Gi). (Stress + Pi, Gm and Gi water irrigation was stopped).

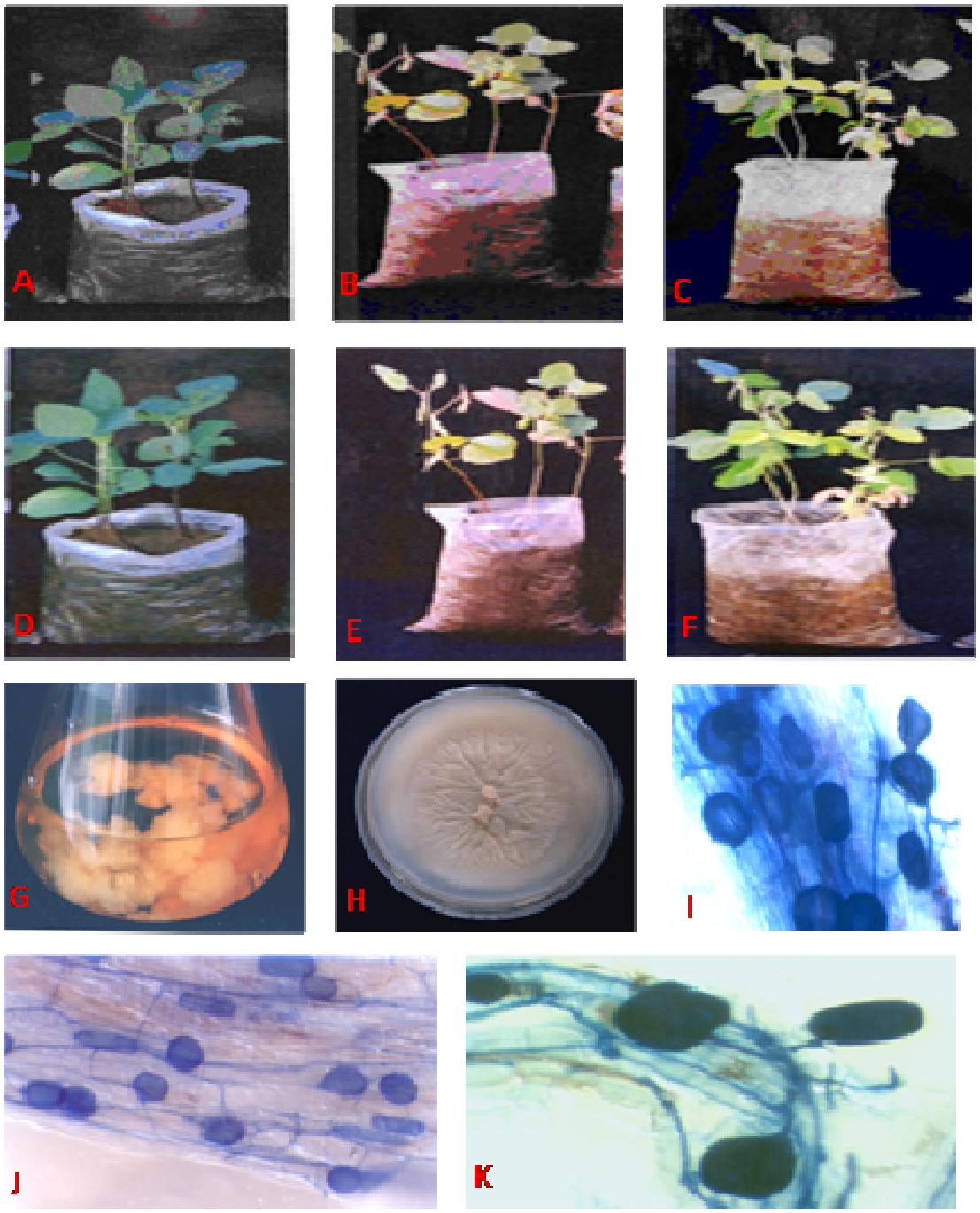

Figure 6. JS-335 variety: A, Before water stress, B, after water stress (water irrigation was stopped), C, after recovery; TAMS-9821 variety: D, before water stress, E. after water stress, F, after recovery; G, Culture of *Piriformospora indica* in Kaefer's Broth, H, Plate culture of *P. indica* culture in Kaefer's agar medium, I, spores of *G. intraradices*, J, spores of *G. mosseae*, K, Spores of *P. indica*.

can maintain substantially higher water contents as compare to a non mycorrhizal soil because of the aggregating out come of mycorrhizal hyphae on soil structure (Auge, 2001).

In the present study, it was found that the *G. mosseae* increased percentage of colonization, height and drought stress tolerance capacity of soybean plants followed by *G. intraradices* and *P. indica*.

Conclusion

From the discussion (Figures 3, 4 and 5) it is concluded that *G. mosseae* promotes growth of soybean in drought

stress condition followed by *G. intraradices* and *P. indica.* In addition, JIP-test is a rapid and non-destructive technique and can be applied for evaluation of mycorrhized plants subjected to draught stress conditions.

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