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FULL-LENGTH RESEARCH ARTICLE

# Effect of Native Soybean Rhizobia and AM Fungi in the Improvement of Nodulation, Growth, Soil Enzymes and Physiological Status of Soybean Under Microcosm Conditions

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**Abstract** The arbuscular mycorrhizal (AM) fungi-rhizobia synergism is a promising approach for improving the growth and nutrition of soybean. It is, therefore, imperative to evaluate potential soybean rhizobia and AM fungi singly to identify their stress protectant physiological traits, enhance growth and nodulation of soybean and improve soil health. The efficacy of five root nodulating soybean rhizobia and an indigenous AM fungus, *Glomus intraradices,* was evaluated on soybean (cv JS 93-05) under microcosm conditions. In general, all the inoculated plants showed higher fresh shoot and root weight, and nodule number as compared to uninoculated control plants. The plants inoculated with *Bradyrhizobium japonicum* (strain USDA 110), *B. liaoningense* 17c (MTCC 10753) and AM fungus showed higher growth and nodulation. However, the plants inoculated with rhizobia 12c (unidentified), *B. japonicum* DE2-5a (MTCC 10751) and USDA 205 did enhance nodulation but found at par with the other inoculated plants. Interestingly, these inoculated plants found to have comparatively higher nitrogen and phosphorus uptake. *B. japonicum* (strain USDA 110), native slow growing rhizobia isolate DE2-5a and AM fungi were also found to stimulate proline content in shoots, and trehalase and fluorescein diacetate activities in the rhizosphere soil. Considering the growth and physiological responses of AM fungi and rhizobial strains (DE2-5a and 17c) to soybean, there is a need for further testing the synergistic responses to evolve better survival candidates under drought-stress conditions.

**Keywords** Native AM fungi · Soybean rhizobia · Trehalase activity · Physiological parameters

## Introduction

Soybean [*Glycine max* (L.) Merril], also called as 'Golden Bean', is an important oilseed and pulse crop contains about 20 % edible oil, 40 % protein and ample amount of essential amino acids along with other bioactive molecules. The production of soybean in 2010 was 10.47 million ton from 9.3 million hectare [1] and currently India has surpassed China in terms of area. Major producers of soybean are USA, Brazil, Argentina, China and India. In India, it is mainly concentrated in central India (Madhya Pradesh, Maharashtra and Rajasthan) grown under rainfed conditions. When compared to other soybean growing countries soybean productivity is low, which is mainly due to poor utilization of soil resource base and recurrent drought. The soybean rhizobial symbiont are classified into slow-grower, *Bradyrhizobium* spp. and fast-grower, *Sinorhizobium* spp. [29]. Effective rhizobial inoculation is a common practice in agricultural legume production which requires survival and establishment of inoculated rhizobia in the soil environment [8]. Since, mycorrhizal fungi as well as rhizobia are commonly found inhabiting the common rhizosphere and colonizing the roots of crop plants including soybean. These two groups of microbes exert positive effects on plant growth by improving phosphorus and nitrogen. Recent studies have corroborated a positive effect of the interactions between arbuscular mycorrhizal (AM) fungi and

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rhizobia under drought conditions [25] and it was found that inoculation with AM fungi protect soybean plants against the detrimental effects of drought and helped them cope with the premature nodule senescence induced by drought stress [25]. It has been widely studied and established fact that the inoculation of nitrogen fixing root nodulating rhizobia (*Bradyrhizobium* sp.) in soybean has been found to enhance the growth and contribute nitrogen to the soybean plants [12]. However, the effectiveness of rhizobia and nodulation may be affected under high doses of applied nitrogenous fertilizers [32]. The high N requirement of the crop is fulfilled mainly by establishing N-fixing symbiosis with rhizobia. Soybean inoculation is a common practice worldwide for a century when effective rhizobia were absent or in insufficient numbers in soil. However, selection of niche-based strains adapted to local environmental conditions and to newly developed plant varieties stays a need of an hour. In the current study, the response of native AM fungi and rhizobia on induction of stress protectant physiological traits in host were identified besides studying rhizosphere soil enzyme activities including trehalase activity and growth attributes of soybean. The trehalose, a non-reducing disaccharide, has been found in a wide variety of organisms including fungi and bacteria [14]. It has been shown that mycorrhizal fungi synthesizes trehalose in the extraradical mycelium, which serves as the main storage carbohydrate and also as an abiotic-stress protectant in plants colonized by AM fungi and nitrogen fixing micro-organisms [20, 22]. Magnitude of hydrolysis of trehalose by trehalase through microbes in the root zone soil may help plants to survive under stress and can be included as potential soil quality indicator [14].

Therefore, current study was carried out to assess the comparative efficacy of both native rhizobia along with some reference strains and AM fungi towards induction of stress protectant physiological traits in host, higher nodulation, growth, physiological parameters and enzyme activities in rhizosphere soil of soybean grown under microcosm conditions.

## Materials and Methods

### Procurement and Purification of Soybean Rhizobial Cultures

Three native soybean root nodulating rhizobia viz., DE2-5a (*Bradyrhizobium japonicum*, MTCC 10751); 17c (*B. liaoningense*, MTCC 10753, an extra slow growing strain); and 12c (unidentified isolate) recovered from Malwa region of central India [29] and maintained at Directorate of Soybean Research (ICAR), Indore and two reference strains viz., USDA 110 (*B. japonicum*) and USDA 205 (*Sinorhizobium fredii*, fast growing) procured from curator, USDA/ARS Beltsville, MD, USA were used in the study. Pure creamy and white/hyaline colonies were picked up from yeast extract mannitol agar medium-congo red and streaked on the same medium followed by incubation at 28 °C for 2–7 days or until appearance of pure colonies.

### AM Fungi Inoculum

Mixed native AM fungal culture (consisting of spores dominant in *G. intraradices*, hyphal/mycelial biomass) was procured from Microbiology Section, DSR, Indore, India and used for the trial after assessing the infectious propagules (IP) density by the method of [28]. Infectious propagules density was expressed as IP number per g soil inoculum.

### Potting Mixture, Inoculations and Experimental Details

Microcosm trial using unsterilized soil (pH 7.6 (1:2.5, soil water ratio); organic carbon (%) 0.51; Olsen P(mg/kg) 6.88; Mineral N (mg/kg) 6.84; DTPA extractable Zn (mg/kg) 1.72) consisted of seven treatments which comprises three native rhizobia, two reference rhizobial strains, one AM fungus and uninoculated control was conducted in UV-protectant black polythene bags (8 kg capacity, 20 cm wide and 30 cm high, 1 cm deep gusseted type) on soybean (cultivar, JS 93-05) using completely randomized design in five replications.

AM inoculation @ 50 g crude soil inoculum consisting of 500 IP (*G. intraradices* as dominant AM species; containing roots bits and hyphal and mycelial mass) was inoculated by layering method just below the seeds. Rhizobial suspension (yeast extract mannitol broth (1 $OD_{620nm}$) @ 0.5 ml per seed was inoculated around the seeds during the sowing.

### Growing Conditions and Measurements

Plants were grown (mid February to mid April) under ambient atmosphere conditions for 60 days after germination with a temperature range of 24–36 °C and relative humidity from 10 to 15 %. Plants were watered regularly as and when required. At harvest, plants were examined for agronomic and nutritional parameters (number of nodules per plant, fresh and dry weight of shoots and nitrogen content in nodules and P uptake in shoots) and physiological parameters (total chlorophyll and proline content in leaves). Root samples were observed for AMF colonization.

The shoots were dried at 70 °C for 96 h until constant weight attained. Dried shoots were used for P analysis by the method of Kitson and Mellon [15]. Total nitrogen in roots along with nodule samples was analyzed by Micro-Kjeldahl's apparatus method.

Total chlorophyll was extracted from leaf tissues with 80 % acetone and determined according to age old method of Arnon [3], where 200 mg leaf material was extracted in 80 % acetone solution, homogenized and centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant was taken at 663 and 645 nm. The proline content in fresh leaves was determined by the method of Bates [5], where 500 mg of leaf extract was used, homogenized in 10 ml of 3 % aqueous sulphosalicylic acid. Four milliliters of the filtrate and equal volume of glacial acetic acid and ninhydrin was subjected to heating in a boiling water bath for 1 h. After terminating the reaction, 8 ml of toluene was added to the reaction mixture. Toluene layer was separated and intensity of red colour of supernatant was determined colorimetrically at 520 nm. Proline was calculated after running a series of standard proline and expressed as µg/mg. Soil enzyme activities of fluorescein diacetate (FDA) hydrolysis, soil dehydrogenase, acid- and alkali- phosphatase and trehalase were estimated as per standard methods.

In brief, acid and alkaline phosphatase activity was determined by the method of [33] using acetate buffer for acid phosphatases and borax-NaOH buffer for alkaline phosphatases. Fluorescein diacetate hydrolysis activity in soil was determined by hydrolyzing the FDA [4] using 60 mM potassium phosphate buffer of pH 7.6. The soil dehydrogenase activity was determined by the method of Cassida [7]. Trehalase activity (hydrolysis of trehalose) in soil was determined by glucose oxidase peroxidase method [16].

Mycorrhizal colonization percentage in roots of all the plants (inoculated and uninoculated) were assessed after clearing and staining roots [23] and quantified by frequency distribution method [6].

### Statistical Analysis of Data

The data was analyzed using the analysis of variance, JMP Software; SAS Institute Inc. [27]. The least significant differences were used to separate the treatment means using DMRT test (Costat statistical software, Cohort Berkeley, Calif.).

## Results

The data presented in Table 1 showed that plants inoculated with rhizobia and AM fungi produced higher shoot and root fresh weight, and nodule number over uninoculated plants. Among the inoculated plants, plants inoculated with USDA110 and AM fungi showed higher growth and nodulation although, the nodulation was lower in USDA110 inoculated plants. On the other hand, inoculated plants showed significantly higher N and P uptake, when compared to uninoculated plants. Interestingly, the plants inoculated with AM fungi and strain DE 2-5a isolated from Dhar district and USDA 205 found to have higher nitrogen and phosphorus uptake, but statistically at par (Table 1).

Strain DE 2-5a selected from a pool of potential rhizobia as efficient nitrogen-fixer showed best performance under microcosms (in vivo) conditions in previous kharif season. Physiological parameters like total chlorophyll content and proline content in the fresh leaves of soybean were analyzed and depicted in Fig. 1. All inoculated plants were found to have higher proline content than the uninoculated plants. Among the inoculated plants, significantly higher proline content was recorded in the plants inoculated with DE 2-5a strain. On the other hand, the chlorophyll content did not differ significantly among the inoculated plants. However, all the inoculated plants did show higher chlorophyll content over uninoculated plants (Fig. 1). The plants inoculated with AM fungi were found to have higher total chlorophyll as compared to other treatments.

Soil enzyme activities like dehydrogenase, FDA hydrolysis, acid and alkaline phosphatase activity, were analyzed in the rhizosphere soils obtained from soybean and data presented in Fig. 2. Among the inoculated plants, alkaline

**Table 1** Evaluation of soybean rhizobial isolates and AM fungi on growth and nutrient uptake of soybean (cultivar 93-05) grown in unsterilized soil for 60 days

| Treatments | Shoot fresh weight (g/plant) | Root fresh weight(g/plant) | Shoot dry weight (g/plant) | Nodule number (nos./plant) | N content in nodules (%) | Percent AM colonization | Shoot P uptake (mg/kg) |
|---|---|---|---|---|---|---|---|
| Uninoculated | 3.77d | 1.33ab | 0.78b | 8.33c | 1.54e | 1.66 | 632.0c |
| USDA-110 | 9.92a | 2.36a | 2.23ab | 24c | 2.14b | 2.33 | 726.3b |
| 12c | 8.55ab | 1.22ab | 1.86ab | 56b | 1.63de | 2.99 | 702.3b |
| 17c | 4.15cd | 2.52ab | 0.60b | 47.33b | 2.04b | 4.66 | 738b |
| DE 2-5a | 7.69ab | 0.71b | 0.85b | 22.66c | 1.86c | 5.99 | 980.3a |
| AMF | 9.15a | 1.61ab | 3.28a | 86a | 2.37a | 15.33 | 945.3a |
| USDA-205 | 6.45bc | 1.29ab | 2.19ab | 25.66c | 1.72 cd | 2.66 | 963a |
| LSD (0.05) | 2.42 | 1.65 | 1.82 | 16.77 | 0.14 | ND | 54.38 |

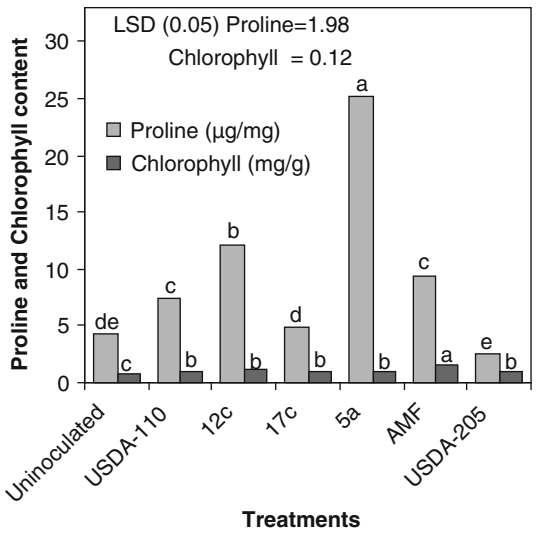


**Fig. 1** Influence of soybean rhizobia and AM fungus on proline and chlorophyll content in leaves of soybean (cultivar JS93-05) grown in pots. The bars of treatment followed by same letter did not differ significantly by Duncan's multiple range test (DMRT; $P = 0.05$); LSD, least significant difference by ANOVA

phosphatase was found at par. However, the inoculated plants showed significantly higher activity when compared to uninoculated plants. Acid phosphatase activity in all the inoculated plants except, AM fungi and DE 2-5a did not influence by the inoculations. In general DE 2-5a and AM fungi was found to maintain higher acid and alkaline phosphatase activity. Fluorescein diacetate hydrolysis and soil dehydrogenase activities data are presented in Fig. 2. The plants inoculated with DE 2-5a strain and AM fungi followed by strain 17c showed higher FDA activity when compared to other inoculations. Moreover, the activity was significantly higher than the uninoculated plants. Soil dehydrogenase activity did not influence by the inoculations. Trehalase activity (Fig. 3) observed in the rhizosphere soil of soybean showed that plants inoculated with AM fungi, rhizobia (DE 2-5a and 17c and USDA110) showed comparatively higher activity and differed significantly over the control plants. The mycorrhizal colonization assessed in the roots of all the inoculated plants did not show any trend. However, the colonization level in the mycorrhizal plants was 15 % higher as compared to rest of the treatments. The colonization level in other inoculated plants including control plants varied from 2 to 5 %.

## Discussion

In general, soybean forms symbiotic association with both rhizobia and AM fungi in nature to gain maximum resources from environment for proper growth and development of plants [2]. In the present study, inoculation of

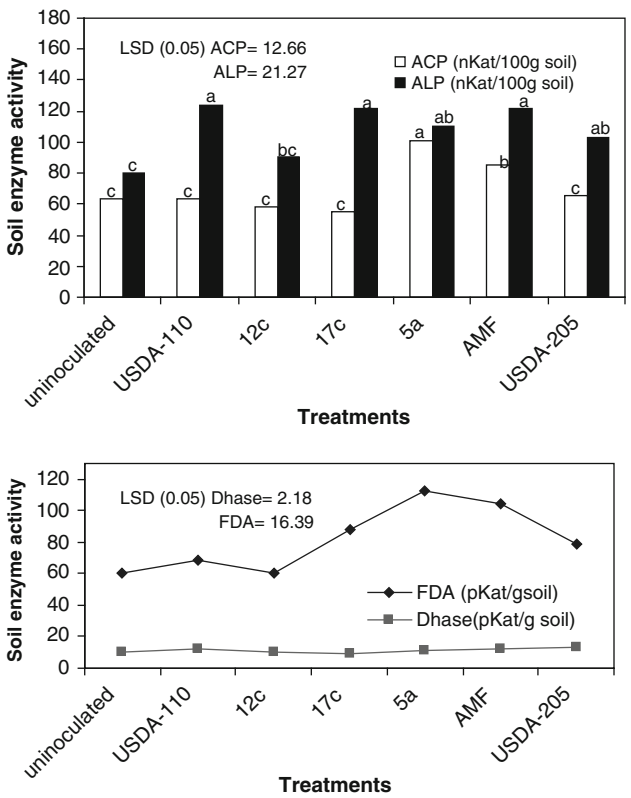


**Fig. 2** Influence of soybean rhizobia and AM fungus on acid and alkaline phosphatases (ACP/ALP) (top) and soil dehydrogenase (DHA) and fluorescein diacetate (FDA) (bottom) in rhizosphere soil of soybean (cultivar JS93-05) grown in pots. The bars (top) of treatment means followed by same letter did not differ significantly by Duncan's multiple range test (DMRT; $P = 0.05$); LSD, least significant difference by ANOVA; nKat, nano Katal; pKat, pico Katal

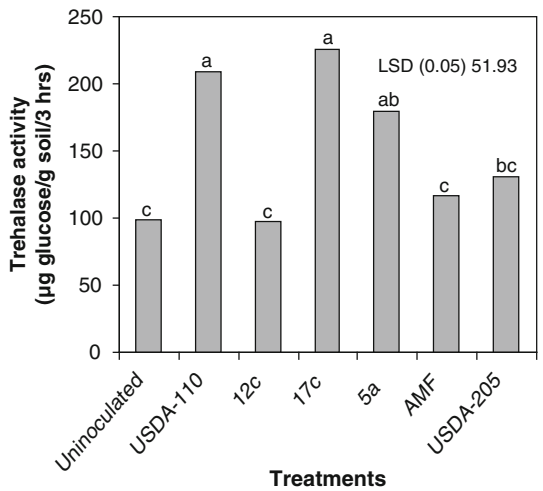


**Fig. 3** Influence of soybean rhizobia and AM fungus on trehalase (hydrolysis of trehalose) activity (µg glucose/g soil/3 h) in rhizosphere soil of soybean (cultivar JS 93-05) grown in pots. The bars of treatment followed by same letter did not differ significantly by Duncan's multiple range test (DMRT; $P = 0.05$); LSD, least significant difference by ANOVA

rhizobia and AMF individually improved fresh shoot and root weight, nodulation of soybean and the results are in the concurrence with results reported by many researchers [21, 34]. Similarly, inoculation of AM fungi and rhizobia significantly improved N and P uptake in soybean. Present findings are in agreement with the work of Rahmani et al. [24], where they showed that effective and thermotolerant isolates were able to fix nitrogen and increase soybean dry weight and nitrogen content of the shoots as well as in the seed yield. Previous studies carried out proposed that nitrogen fixation in soybean are sensitive to high temperatures [13]. Significantly higher AM colonization and proline content in AM-inoculated plants over uninoculated plants can be explained as osmotic adjustment in AM-mediated plants where active accumulation of various ions, amino acids, cellular expansion, sugars etc., takes place [18]. Thus, a high proline level may help plants to survive temporal drought and recover from stress [26]. On the other hand, when compared to rhizobial inoculated plants, proline content decreased in AM plants suggest reduced drought-stress injury in AM plants as a result of drought avoidance, while higher proline levels suggest superior osmotic adjustment and thus drought tolerance in colonized hosts [25, 26]. Wu and Xia [35] indicated that AM colonization decreased the proline accumulation of AM-seedlings leaves and roots. They suggested that AM colonization enhanced host plant drought tolerance, which did not correlate with proline but with non-structural carbohydrates (NSC), $K^+$, $Ca^{2+}$ and $Mg^{2+}$.

Crowe group reported that all organisms where trehalose is present play an important physiological role as protectant against abiotic stress [9]. Furthermore, trehalose found to protect the cells by stabilizing cell structures and enables proteins to maintain their native conformation under stress conditions [30]. Interestingly, higher trehalase activity (enzyme responsible for hydrolysis of trehalose) in the rhizosphere of rhizobial strain DE 2-5a may play critical role at higher temperature during nodule senescence due to the presence of highly metabolically active microbial community. Although, no scientific evidence is available in relation to trehalase activity supporting stress-protectant properties. However, higher trehalase activity in soil is related to soil microbial community in terms of better soil quality [14] and supports higher population of symbiotic organisms (AM fungi and rhizobia) [16]. The higher trehalase activity due to rhizobial inoculation also supports higher FDA activity than DHA as observed in the current study. Kumar and Tarafdar [17] found that dehydrogenase activity is mainly contribution of bacteria and actinomycetes in arid soils and only 5 % by the fungal activity. It is clear that DHA is an indicator of bacterial activity in soil, whereas FDA hydrolysis is the indicator for measuring overall microbial activity as it is govern by lipase, protease and esterase enzymes originating from all microbial sources [4]. From the results of FDA and ALP, it seems that there is some relationship between FDA and ALP that might be due to more fungal population in black soil (Vertisols) as compared to arid soil [4, 17], where they reported a significant relationship between DHA and ALP might be due to lower fungal population. Hence in the present study, the higher FDA could be due to higher fungal population and activity in black soil.

The mycorrhizal colonization assessed in the roots of all the inoculated plants did not show any trend. However, the colonization level in the mycorrhizal and non- AM-rhizobial plants was higher as compared to uninoculated plants. According to a report, the AM fungi associated with legumes are an essential link for effective phosphorus nutrition, leading to enhanced nitrogen fixation that in turn promotes root and mycorrhizal growth [11]. Even though mycorrhizae can greatly enhance the acquisition of mineral nutrients [19], but in return scavenge lots of carbon from the host plants [10]. Therefore, if the host plants have the ability to take up enough nutrients, such as P, they might not harbour many mycorrhizae due to the balance of the C and P budgets [31]. In conclusion, better growth and physiological responses of AM-fungi and -rhizobial strains (DE 2-5a and 17c) on to soybean suggests that they are candidates to be tested synergistically to evolve as potential bio-inoculants for soybean that would perform better under stress conditions at field level.

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