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Alleviation of transplantation shock of tissue cultured raised blackgram (*Vigna mungo* L. Hepper) by inoculation with Arbuscular Mycorrhizal fungi and rhizobium

[1] Chandrama Prakash Upadhyaya, [2] Nishakant Pandey, [3] Vishnu Bhat, [4] Neera Bhalla-Sarin

[1, 2, 4] School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi, India
[1, 3] Department of Botany, University of Delhi (North Campus), New Delhi, India
[1] Present address: Department of Biotechnology, Dr Harisingh Gour Central University, Sagar, Madhya Pradesh, India

Abstract
In spite of few successful reports of blackgram (*Vigna mungo* L. Hepper) regeneration, their survival rate is low on transplantation as these *in vitro* raised plantlets are usually sensitive to hardening as well as transplantation to soil. The survival rate during hardening and transplantation has been reported to be 65-70%. This study evaluated the response of tissue culture raised blackgram to arbuscular mycorrhizal (AM) fungi, *Fasciculatum* and to the nitrogen fixing bacteria *Rhizobium leguminosarum*, recovers during hardening process and transplantation shock, growth, nodulation and nitrogen fixation activity. The overall survival rate of plants increased up to 90-95% in the *in vitro* raised tissue cultured plants. The results also demonstrated that the dual inoculation of *in vitro* grown blackgram plants with Rhizobium and AM fungi significantly increased the percentage of root colonization, plant biomass, nodulation, and nitrogen fixation activity in comparison with the plantlets inoculated with either *Rhizobium* or AM fungi alone.

Keywords: blackgram (*Vigna mungo* L. Hepper), Arbuscular Mycorrhizal, transplantation shock

1. Introduction
Blackgram (*Vigna mungo* L. Hepper) is an important grain legume with its origin and center of genetic diversity in India (Lukoki *et al.* 1980; Saini & Jaiwal 2002) [18]. Because of its high protein content and perfect combination of all nutrients, including proteins, carbohydrates, fat, minerals, amino acids and vitamins (Karamany 2006) [14], it is extensively used in Asia especially in India and now also grown in the Southern United States, West Indies, South east Asia and other tropics and subtropics (Delic *et al.* 2009) [6]. *Vigna* species which had been earlier recalcitrant to regeneration have now responded favorably via direct or indirect organogenesis or somatic embryogenesis. We have earlier reported an efficient, rapid and direct multiple shoot regeneration system amenable to *Agrobacterium*-mediated transformation using coteledonary explants of blackgram (*Vigna mungo*) established in our laboratory. The transgenic blackgram overexpressing the glyoxalase I was tolerant to different abiotic stresses (Bhomkar *et al.* 2008) [4]. The effect of the explant type and its age, type and concentration of cytokinin and auxin either alone or in combination, the genotype usedon multiple shoot regeneration efficiency and frequency was optimized. The coteledonary explants excised from 24 hr water soaked seedlings directly developed multiple shoots (an average of 4-5 shoots/ explant) from the cut ends of the node in the cultures on MSB (MS salts and B_5 vitamins) medium containing 1.0 µM 6-benzylaminopurine. The regenerated plants were morphologically similar to seed-raised plants and required 8 weeks' time from initiation of culture to establish them in soil. However, the hardening and establishing of the tissue cultured plants to soil is still a major bottleneck in case of this important crop.

The success of micropropogation at commercial scale depends on cost effective large scale ability of transferring the plants out of culture medium with high survival rate. During the field transfer, the *in vitro* raised plantlets are unable to cope with the environmental changes as well as compete with soil microbes (Chandra *et al.* 2010) [5] as plants cultivated *in vitro* behave differently from field grown plants. High mortality is observed upon transfer of shoots to *ex vitro* conditions as the cultured plants have nonfunctional stomata, weak root system and poorly developed cuticle (Mathur *et al.* 2008) [20]. Their guard cells have thinner cell walls and contain more starch and chloroplasts (Marin *et al.* 1988). In various plant species, the leaves formed *in vitro* are unable to develop further under *ex vitro* conditions and be replaced by newly formed leaves (Preece and Sutter 1991; Diettrich *et al.* 1992) [26, 8]. *In vitro* grown plantlets have large stomata with altered shape and structure. During acclimatization to *ex vitro* conditions, leaf thickness generally increases; leaf mesophyll progresses in differentiation into palisade and spongy parenchyma; stomatal density decreases and stomatal form changes from circular to elliptical. Development of cuticle, epicuticular waxes, and effective stomatal regulation of transpiration occurs leading to stabilization of water potential of field transferred plantlets. (Pospısilova *et al.* 1999) [25].

The endomycorrhizal fungi infect and produce extremely branched hyphae within the plant cell. This infection creates an absorptive structure with a very high surface area for transfer of nutrients between the plant and the fungus. It is a well-established fact that the mycorrhizal fungal hyphae secrete alkaline phosphatases into the rhizosphere which result in the increase in root growth and strength even under mineral stress (Woolhouse 1975; Janos 1980) [38, 13]. Therefore, the regulation of these enzymes is critical to the survival of the plants in soils with limited mineral phosphorus (P) in

particular (Duff *et al*. 1994). The effectiveness of the tripartite symbiosis – AM fungi, *Rhizobium* and legume plant, depends on the competition of the three symbionts for carbon (Jakobsen and Rosendahl, 1990) [12]. Roots with AM fungi receive about 4.0 – 20.0% more photosynthates as compared with non-mycorrhizal roots (Kormanik *et al.* 1981; Smith and Smith 1996; Saxena *et al*. 1997) [15, 35, 33]. The synchronization between the two symbiotic systems needs an optimal P level in the nutrient medium to stimulate the nodulation and nitrogen fixation and not to slow down the formation of effective mycorrhizal associates. This association enhances the root health and also establishment of the plants in soil.

In this study we evaluated the response of tissue culture raised blackgram to arbuscular mycorrhizal fungi (AM) species *Glomus* and the nitrogen fixing bacteria *Rhizobium leguminosarum*, to transplantation shock. We also evaluated the effectiveness of triple symbioses i.e. between tissue cultured raised blackgram plants, AM fungi (*Glomus sp.*) and *R. leguminosarum* on the plant growth, nodulation and nitrogen fixation capability.

2. Materials and methods

Raising plantlets

Procedure standardized in our laboratory was followed for raising the in vitro plantlets as reported by Bhomkar *et al*. 2008 [4]. Rooted plantlets were taken out gently from the medium and the roots were washed in tap water to remove remaining media and transferred to autoclaved peat soil in plastic pots (diameter 12 cm) for hardening. The plants were covered with polybags and watered with modified Hoagland's medium devoid of nitrogen sources such as $Ca(NO3)_2$. $4H_2O$ and KNO_3. These pots were maintained in plant growth chamber at 28± 2ºC under 16h photoperiod (fluorescence density of 50 µmol $m^{-2}s^{-1}$) and 70% humidity. The *Rhizobium* or mycorrhiza was applied at stages of hardening and transplanting to the soil.

Application of *Rhizobium* strain

Five different strains of *Rhizobium* were collected from the Division of Microbiology, Indian Agriculture Research Institute, New Delhi. Four *Rhizobium* strains *viz* JC-3169, S-3154, JC-24,S-60; specific to nodulation in cowpea roots were screened for their nodulation ability in blackgram roots before applying them to micropageted plantlets at hardening stage or during transplanting and establishment in soil. A soybean specific *Rhizobium* strain, S-119 was used as the negative control.

The *in vitro* raised rooted plantlets were transferred to sterile autoclaved peat soil in plastic pots (12 cm). The plantlets were inoculated with suitable *Rhizobium* strain by addition of 5.0 ml of liquid broth culture of *Rhizobium* (cultured in YEM medium) near the roots of the plantlets up to 2 - 3 times a week for a period of 10-12 days, or till the plantlets were hardened (maximum of two weeks). After two weeks of hardening, the plantlets were transplanted to sterile garden soil (autoclaved 2-3 times) with or without *Rhizobium* and maintained in the glass house with 16 h photoperiod, 70 % humidity and at temperature of 28 ± 2˚C. These plants were uprooted after 10-12 weeks and the degree of nodulation, plant length, number of leaves and yield was recorded from each plant. Screening was also done to find out the stage at which the *Rhizobium* induced better nodulation on the roots of *in vitro* raised blackgram plantlets. The experiments were designed in such a way that the *Rhizobium* strains were applied at the time of hardening or at the time of transplanting the hardened plants to the soil or during both the stages. This screening also helped in finding out the suitable *Rhizobium* strain for developing maximum number of nodules at a specific stage of its application. Six *in vitro* raised plantlets per *Rhizobium* strain were used in the experiment.

Application of Mycorrhiza

The identified spores of *Glomus Fasciculatum* were obtained from the Division of Microbiology, IARI, New Delhi, as the rhizospheric soil, which was used as the inoculum for these AM fungal species. Each gram of this inoculum consisted of 80–100 spores besides other components (viz. hyphae, arbuscules and vesicles) of fungus, root segments of a natural host (fodder *Sorghum*) and soil particles. Mycorrhiza was inoculated at the time of hardening of plantlets in sterile peat soil or during transplantation of the hardened plants to the autoclaved garden soil in plastic pots or during both hardening and transplantation. For application of mycorrhizal inoculum, the *in vitro* grown plantlets were carefully placed in such a way that their roots extended up to 3 to 4 cm deep in pits made in sterile peat soil or garden soil and 1.0 gm of mycorrhizal inoculant was applied near the roots. This was done to allow as many spores as possible to be in close proximity to the root epidermis. The plantlets transferred to sterilized peat soil or garden soil without mycorrhizal inoculum served as the control (non-mycorrhizal plantlets). These plantlets were transferred to the glass house with 16 h photoperiod, 70 % humidity and a temperature of 28 ± 2˚C. The performance of the plantlets was evaluated at regular intervals by measuring various parameters, viz. percentage survival during hardening and after transplantation to soil, plant length, and number of leaves, yield per plant and the establishment of mycorrhizal association with roots.

Four sets of experiments were established: 1) *In vitro* raised plantlets without *Rhizobium* or AM fungi treatment 2) *Rhizobium* treated plantlets during hardening and transplantation 3) AM fungi treated plantlets during hardening and transplantation and 4) *Rhizobium* treatment during hardening and AMF treatment during transplantation

Assessment of root colonization by AM fungi

In order to assess the root colonization by AM fungi, a portion of rootlets from mycorrhiza treated and untreated plants were harvested and stained with 0.05% trypan blue in lactophenol (Phillips and Hayman, 1970) and mounted in lactic acid: glycerol (1:1) on a slide for observation under a microscope.

Measurement of Nitrogenase activity of nodules

Gas chromatography apparatus (Nucon GC, India) was used to measure the nitrogen fixation based on the principle of the acetylene reduction. The basis of this assay is that in the presence of 0.03 to 0.10 atmospheres of acetylene, virtually all electron flow through nitrogenase is used to reduce acetylene (C_2H_2) to ethylene (C_2H_4) because the nitrogenase has a higher affinity for acetylene than other substrates. The ability of nitrogenase to reduce acetylene to ethylene was first recognized by Dilworth (1966) [3]. A gas chromatograph is used to determine the amount of ethylene formed. Nitrogenase not only catalyzes the reduction of atmospheric N_2 to NH_3, but

can also reduce acetylene (C_2H_2). This is also referred to as "Closed Acetylene Reduction Assay" and has been used as a measure of nitrogenase activity and an indicator of N_2 fixation in Rhizobium/legume symbiosis (Hardey *et al.* 1968; Turner and Gibson 1980). The acetylene reduction assay provides an instant measure of nitrogenase activity (Frank *et al.* 1994). The plants were uprooted and the roots with the nodules were cut from the plants. The roots were packed in 60 ml glass tubes which were covered with air tight lids. Six ml of air was removed from the tube with the help of a syringe and the same amount of C_2H_2 was added to replace the air. The tubes were incubated for 45-60 min at 37C. The air samples were loaded on the tube with the help of syringe to the burner of the instrument and the reading was recorded on the attached computer. The standard formula was used to calculate the production of C_2H_4 by the nitrogenase activity (reduction of acetylene) of nodules. Data are expressed as nanomoles or micromoles of ethylene produced per hour per plant or per unit weight of nodules.

3. Results and Discussion

Effect of Rhizobium strain

Hardened plantlets in sterile peat soil maintained turgidity and showed signs of adaptation by increase in shoot height and emergence of new leaves. These plantlets were transplanted to autoclaved sterile garden soil in bigger plastic pots (diameter 30cm).

The degree of nodulation was maximum with the *Rhizobium* strain S-3154 followed by strain JC3169 (Fig. 1 A). The untreated control plantlets were unable to develop visible nodules even after 10-12 weeks of growing in soil. The soybean specific *Rhizobium* strain S-119 was also unable to induce any visible nodule on the roots of *in vitro* grown *Vigna mungo* plantlets. The growth parameters such as shoot length, number of leaves and yield per plant were better with the S-3154 strain of *Rhizobium*. On the basis of these observations, the S-3154 strain of *Rhizobium* was subsequently used for application during the hardening of *Vigna mungo* plantlets.

The *Rhizobium* (strain S-3154) was applied at different stages, viz. during hardening of plantlets or at the time of transplanting the hardened plantlets in the soil or at both the stages. The nodules of size 2-3 mm could be identified only after 6-8 weeks on the roots of the plantlets (Fig.1, C1). Application of *Rhizobium* in the soil during transplantation did not increase the number or the size of nodules on the roots. Therefore, the application of *Rhizobium* inoculant was sufficient only at the time of hardening in the sterile peat soil. Over 90% of the *in vitro* grown plantlets were able to survive after treatment with *Rhizobium* during hardening and the plantlets showed better growth in terms of number of leaves and shoot growth (Fig. 1, C) than untreated plants (Fig. 1, B).

Effect of Mycorrhiza

Our results showed that the roots of *in vitro* grown *Vigna mungo* plantlets inoculated with *G. fasciculatum* established a good mycorrhizal association (Fig 2). The roots of even one-month-old plantlets showed intracellular hyphae with well-developed arbuscules, intercellular hyphae and vesicles (Fig 2 B, C & D). A large number of intercellular vesicles developed in arbuscular mycorrhizal fungi within five to six weeks (Fig 2 A). No evidence of mycorrhizal fungus was seen in the control non-mycorrhizal treated plantlets. It is important to mention that the application of AM fungi in the hardening stage of the plantlets did not have any significant positive effect (Table 1). However, the survival rate of plantlets increased when the AM fungi inoculant was applied during the transplanting stage. Association of mycorrhiza with the roots of legumes has been reported earlier by Robert *et al.* 1987) [29, 30] in cowpea, Sankaranarayanan *et al.* (2000) [32] in blackgram and Rabie (2005) in mungbean. In general, low rate of survival of *in vitro* grown plantlets in soil unexposed to mycorrhiza could be due to factors such as, weak root system or unfavorable nutritional and environmental conditions, (Pierik 1988; Schubert *et al.* 1990) [23, 34] poorly developed cuticle (Wetzstein and Sommer, 1982) [37] and/or non-functional stomata (Lee and Wetzstein, 1988) [17] distinct from *in vitro* conditions. Better establishment of the hardened plantlets in soil in the presence of mycorrhizal inoculants can be attributed to symbiotic association of plant roots with arbuscular mycorrhizal fungi. This result in enhanced growth and yield probably because of increased acquisition of important mineral nutrients as reported by Dela-Cruz (1988) [7] and Kwapata *et al.* (1985) in different legumes.

Dual application of Rhizobium and Mycorrrhiza

The experiments involving the application of *Rhizobium* and mycorrhiza at the stages of hardening and transplantation were repeated thrice. The results showed that *Rhizobium* inoculant was sufficient for causing nodulation and promoted hardening of *in vitro* grown blackgram plantlets when applied close to the roots in the sterile peat soil. However, the mycorrhizal inoculants proved better when applied during the transplanting stage in soil and helped in the establishment of the hardened plantlets in the soil. Similar observation has been reported in the micropropagated *Leucaena leucocephala* and *Sesbania sesban* plantlets (Puthur *et al.* 1998; Subhan *et al.* 1998) [27, 36]. In our experiments, the plantlets treated during hardening with *Rhizobium* and transplanted to soil with mycorrhiza showed more number of nodules as compared to the non-mycorrhizal or only *Rhizobium* treated plantlets (Table 1 and Fig. 3). Therefore, it is concluded that AM fungal species enhance the degree of nodulation in the case of all *in vitro* grown plantlets of blackgram. Mineral phosphorus (P) has a key role in the energy metabolism of all plant cells including in the nitrogen fixation (Dilworth 1974) [10], and it was established by Al-Niemi *et al.* (1997) [1] that nodulating legumes require more mineral P than legumes growing on mineral nitrogen. The AM fungi coupled with legumes are an essential link for effective phosphorus nutrition, leading to enhanced nitrogen fixation that in turn promotes root and mycorrhizal growth (Yasmeen *et al.* 2012) [40]. In addition, the synergistic outcome of twin colonization of roots with AM fungi and *Rhizobium* on growth, nutrient uptake and nitrogen fixation in soybean (Bethlenfalvay *et al.* 1990) [3] and pea (Xavier and Germida 2003) [39] has also been reported. We have also analyzed the nitrogenase activity of the nodules formed due to dual association of the microbes with blackgram. The non-nodulated plants did not show significant nitrogenase activity. In the case of *Rhizobium* treated plants the nodules which developed were enough to show nitrogenase activity and the formation of C_2H_4 was detected. The nitrogenase activity of *Rhizobium* treated plants was *ca*. 8000 nM. The plants which were treated with both *Rhizobium* and mycorrhiza showed enhanced nitrogenase activity (*ca.* 11000 nM per hour per g

weight of the nodules) (Table 2). This data also supports the statement that the application of mycorrhiza to the *Rhizobium* treated plants shows enhancement in nodulation on the plant roots. Results have been reported in the case of seeds grown soybean (Robert *et al.* 1987) [29, 30] and cowpea (Arshad *et al.* 2002) where mycorrhizal inoculation in legume crops was found to increase their vegetative growth and yield, in addition to improving nodulation on their root system. Manjunath *et al.* (1991) reported that the dual inoculation with arbuscular mycorrhiza and *Rhizobium* is beneficial to seeds grown *Leucaena* as it improved nodulation, mycorrhizal colonization, dry weight, nitrogen and phosphorus content of the treated plants compared to single inoculation with either organism.

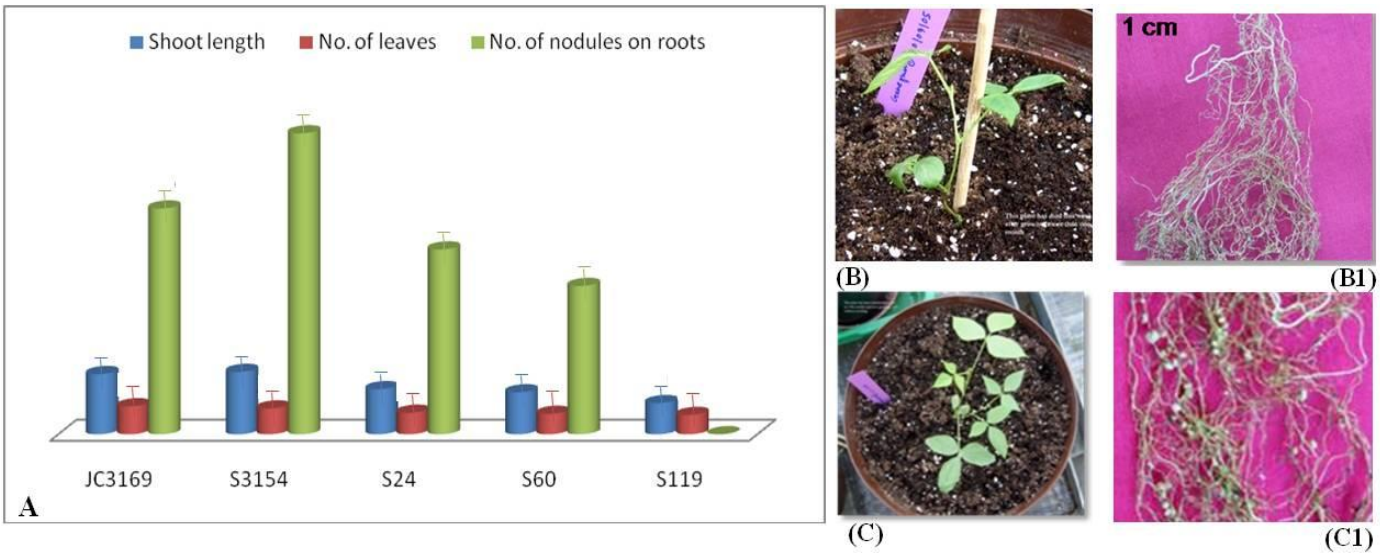

Fig 1: Application and Screening of *Rhizobium*

(A) Histogram showing screening of suitable strain of *Rhizobium* for nodulation and further plant growth. Growth parameters of *in vitro* grown plantlets were recorded 10-12 weeks after transplantation to the soil. While the strains JC 3168, S-3154, S24, S60 of *Rhizobium* are cowpea specific, S-119 is soybean specific used as the control. The application of *Rhizobium* strain S-3154 resulted in maximum number of nodules on the roots of *V. mungo* plantlets and better growth followed by the *Rhizobium* strain JC3169.

(B) Hardening of *in vitro* raised *Vigna mungo* plantletss without *Rhizobium*. **(B1)** The roots of the plants without any nodule formation.

(C) Hardening of *Vigna mungo* plantletss in the presence of *Rhozobium.* **(C1)** Nodules on the roots of *V. mungo*

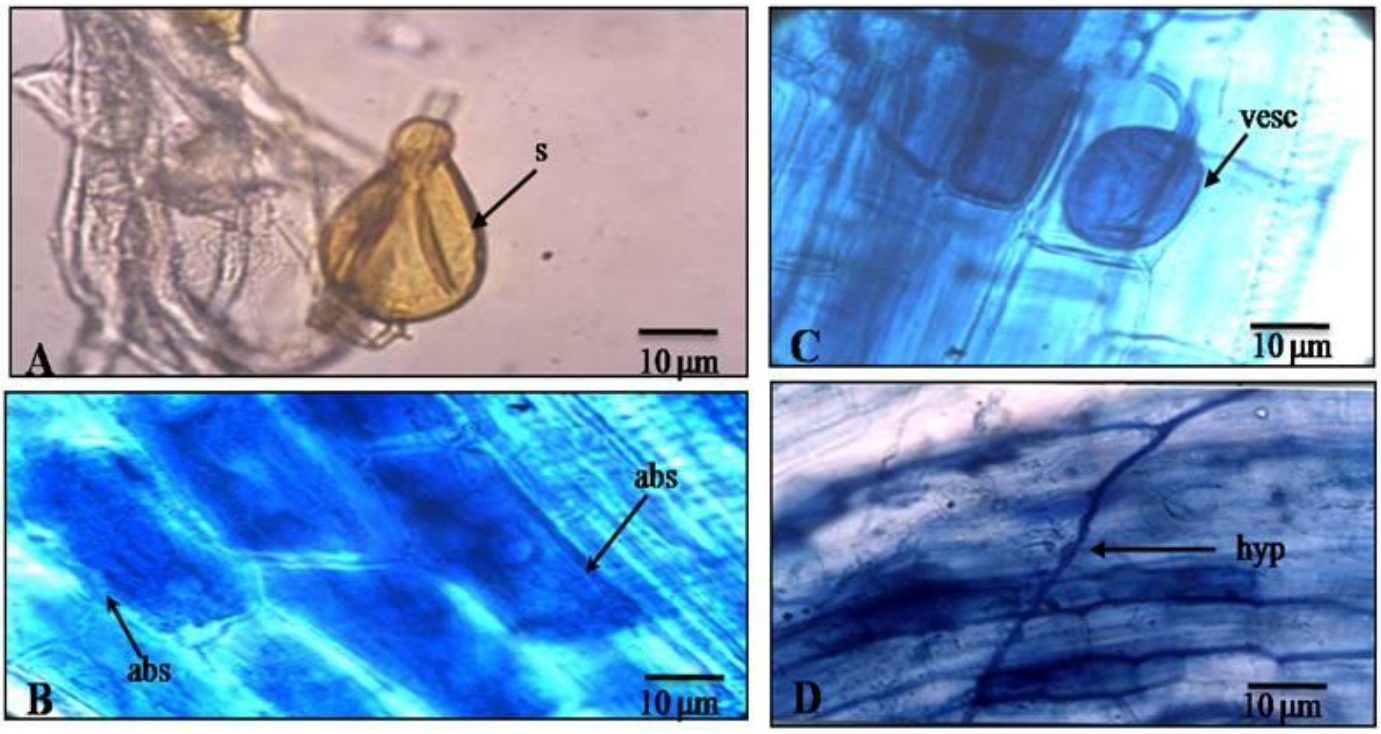

Fig 2: Trypan blue staining of the mycorrhizal association with *Vigna mungo* roots

A: germinating spores (s), **B:** deeply stained arbuscules (abs), **C:** well developed intercellular vesicles (vesc), **D:** ramifying intercellular hyphae (hyp)

Fig 3: Effect of application of *Rhizobium* and mycorrhiza on *in vitro* grown *Vigna mungo* plantlets
A) The *in vitro* grown plantlets show enhanced number of branches, leaves, height and nodulation by dual application of *Rhizobium* and mycorrhiza **(B).** No visible nodules were detected on the untreated plant roots

Table 1: Percentage survival and growth characteristics of *in vitro* grown *Vigna mungo* plants after application of *Rhizobium* and/or mycorrhiza.

Treatments C/R/ M/R+M	% survival	Length of shoot (cm)	Length of roots (cm)	No. of leaves	No. of pods per plant	No. of seeds per pod	Grain yield/ plant (gm)	No. of nodules per plant
Untreated Control (C)	70± 4.5%	35±2.6	21.4± 2	28±2	10±1	5±2	21±2.0	None
Rhizobium (R)Treated	80±3.2%	51±2.4	23±2	29±2	14±1	5±1	26±2.7	> 171
Mycorrhiza (M) treated	74± 2.8%	47±3.3	24±2	26±2	13±2	5±1	25±2.2	None
Treatment with both Rhizobium & Mycorrhiza (R + M)	95±2.6%	68±4.7	26±2	33±1	17±3	6±1	36±2	ca. 342

Data represent the mean ± SE from three independent experiments (each with 6 replicates). C= Untreated control, R=*Rhizobium* treated plants, M= mycorrhiza treated plants, R+M= Plants treated with both *Rhizobium* and mycorrhiza. All the parameters of the plant growth were recorded 90 days after treatment of *Rhizobium* or mycorrhiza.

Table 2: Nitrogenase activity (nanomoles of C_2H_4 produced/ h/ g nodules) of nodules of transgenic and the untransformed control plants as measured by Gas Chromatography.

Samples	Nitrogenase activity (nanomoles of C_2H_4 produced per hour per g of nodules)
In vitro grown plant (C)	76.05
Rhizobium treated plants (+R)	8043
Mycorrhiza treated plants (M)	85.02
Dual treatment (+R+M)	11041

Untreated and Mycorrhiza treated plants were unable to form nodules; hence, a minimum value of C_2H_4 present in the air was detected.
R= *Rhizobium* treated, M= Mycorrhiza treated, R+M= *Rhizobium* and mycorrhiza treated invitro grown *Vigna mungo* plantlets

4. Conclusion

The *in vitro* grown plants are usually sensitive to hardening process as well as transplantation. In the case of blackgram, the survival rate during hardening and the transplantation was ca. 65-70%. To improve the percentage survival of plants, plant growth promoting *Rhizobium* and AM fungi were applied during the hardening and transplantation stage respectively. This resulted in overall survival rate of plants up to 90% in both in tissue culture *in vitro* grown plants. The dual application of *Rhizobium* and mycorrhiza could further enhance establishment of *in vitro* grown plants in soil and resulted in enhanced growth, yield and nodulation in the plants. These treatments helped the plantlets in alleviating transplantation shock resulting in better survival of the *in vitro* raised plantlets. To the best of our knowledge, this is the first report of the use of *Rhizobium* and AM fungi application to the *in vitro* grown blackgram plantlets.

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