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VIGNA RADIATA ROOT ASSOCIATED MYCORRHIZAE AND THEIR HELPING BACTERIA FOR IMPROVING CROP PRODUCTIVITY

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Abstract

Arbuscualr mycorrhizal (AM) fungi and symbiotic bacteria have a pervasive effect upon plant form and function. We investigated the possibility of enhanced grain yield in *Vigna radiata*, by applying *Bradyrhizobium* and *Agrobacterium* in combination with mycorrhizae as inoculum. A field experiment was conducted to study the effect of bacterial and arbuscular mycorrhizal inoculants on biomass, nodulation, total grain yield, nitrogen and phosphorus contents in grain of *Vigna radiata*. A significant increase in the biomass, nodulation, total grain yield, nitrogen and phosphorus contents was observed with the application of bacterial and arbuscular mycorrhizal consortium, reflecting the existence of synergistic relationships among the inoculants. Another set of experiment was carried out under controlled environmental conditions of growth room, to localize the bacterial cells in roots and nodules, through ultrastructure studies. Co-occupancy of mycorrhizae with *Bradyrhizobium* and *Agrobacterium* in *Vigna radiata* plant roots and root nodules was also evaluated in this study. This study illustrates that arbuscular mycorrhizae with bacterial inoculants as well as bacterial inoculants alone led to a significant increase in biochemical and physiological characteristic and this tripartite association can be exploited to be used as mix inoculum for enhanced legume crop production.

Introduction

Several mechanisms working together results, stimulation and regulation of plant growth, simultaneously. Improvement of plant nutrition, production and regulation of phytohormones, suppression of disease causing organisms (Martínez-Viveros *et al*., 2010), metabolism and signaling are the important mechanisms stimulated by rhizobacteria. Multidimensional comprehension of these interactive mechanisms is worth studying to understand the symbiotic relationships (Kawaguchi & Minamisawa, 2010). However, ecological factors are also imperative to consider for potential utility and successful applications of the rhizobacteria as plant growth promoters.

Most of the plant roots have capacity to form important ecological relationships with majority of unseen soil microbes for the acquisition of limiting macro and micronutrients especially in the nutrient poor ecosystems (Marcel *et al*., 2008). The ability of forming mutual symbiotic relationship with rhizobacteria for atmospheric nitrogen fixation (Samac & Graham, 2007) and arbuscular mycorrhizal (AM) fungi for nutrient especially phosphorus uptake (Joachim *et al*., 2009; Aslam *et al*., 2010) has proven tremendous impact on leguminous plants in natural and agricultural ecosystems. The micro-symbionts in both associations get benefits by acquiring photoassimilates from the macro-symbiont in return of nutrients supply (White *et al*., 2007; Graham, 2000; Gourion *et al*., 2011). This nonpathogenic association between prokaryote and eukaryote is a fascinating phenomenon for investigation of basic biological principles (Hirsch *et al*., 2001). There is growing evidence that diverse microbial populations in the rhizosphere play significant role in sustainability (Barea *et al*., 2002) and that the manipulation of AM and certain rhizobacteria is of great importance (Khan *et al*., 1995; Albrecht *et al*., 1999; Chalk *et al*., 2006). As legumes have the unique ability to establish both symbioses therefore they became general model system for investigating mutualistic plant microbe interactions. Furthermore, it is of imperative interest to understand the potential impact of these symbiotic associations which may have of agricultural importance. Functioning, degree of infection and structural development of these symbiotic agents is mainly affected by many factors including plant signals, root geometry, mineral elements level in the soil, soil type, presence of shade or light, temperature and pant growth rates (Finlay, 2008). Many authors have highlighted the multifunctional nature of mycorrhizal effects (Newsham *et al*., 1995; Finlay, 2004) including interactions with bacteria (Johanson *et al*., 2004), carbon cycling (Johnson *et al*., 2002), effects on plant communities (Van der Heijden *et al*., 1998) and mediation of plant responses to stress (Finlay, 2008). AM fungi are evident to usually enhance nodulation and nitrogen fixation in legumes, but the extent of these effects depends on AM species (Valdenegro *et al*., 2001). Moreover, the increase in total nitrogen in plant tissues has been linked with nitrogen fixation as a result of a higher phosphorus uptake through the AM hyphae rather than increased uptake of nitrogen from soil (Mortimer *et al*., 2008). The legumes and their association with bacteria (*Rhizobium* spp.) in the broad sense have always been extremely important agronomically. Although, there are many studies on the interactions between AM and bacteria, the underlying mechanisms behind these associations are not yet well understood. Moreover, the effects of mycorrhizal symbiosis are more difficult to reveal in the field and their functional properties still require further studies. Application of bacterial plant growth promoting bacteria is in practice now a days as biofertilizers in Pakistan and due to ubiquitous nature of AM fungi in soil it is worth noting the interactive effect of both microsymbionts on the plant growth and nutrient status. The study aimed to investigate interactions between different strains of *Bradyrhizobium* (nitrogen fixer), *Agrobacterium* (Indole acetic acid producer and phosphate solubilizer) and indigenous soil mycorrhiza to provide a clear understanding of this tripartite association and its impact on plant growth, nutrient content and AM root colonization. These multitrophic root associated microbial associations was studied through multi-disciplinary investigations of biochemical, ultrastructure and physiological methodologies.

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Materials and Methods

Legume host, microsymbiont and growth conditions: *Vigna radiata* (Mungbean) var. NM-92 requiring warm season of 90-100 days from seed sowing to maturity was selected for field trials in summer 2008 (June –September). Mean daily maximum day length was 14 h, mean daily maximum temperature was 40°C and mean daily minimum temperature was 28°C. *V. radiata* seeds obtained from mutation breeding division NIAB, Faisalabad, were inoculated by seed dressing with nodule forming and nitrogen fixing *Bradyrhizobium japonicum* strains MN-S & TAL-102 and growth hormone producing and phosphate solubilizing *Agrobacterium* sp. Ca-18. The *B. japonicum* MN-S and *Agrobacterium* sp. were obtained from BIRCEN culture collection of Plant Microbiology Division at NIBGE. Faisalabad. The *B. japonicum* Nif strain TAL-102 was obtained from TAL, Hawaii, USA. The inoculum of all bacterial inoculants was maintained on Yeast Extract Mannitol (YEM) agar plates (Somasegaran, 1985) containing Congo Red at incubation temperature of 28 ± 2ºC, pH 6.8 and autoclaved at 121ºC for 20 min. Bacterial inoculum was prepared by culturing selected colonies in broth culture medium of YEM. The cultures were grown with constant shaking at 100 rpm until maximum bacterial cell growth of approximately 10^8 cells mL^{-1}.

AM fungal spores were isolated by employing wet sieving and decanting method (Gerdemann & Nicolson, 1963) from the rhizospheric soil samples collected from the crops grown field areas of Faisalabad, Pakistan. AM spores were surface sterilized by immersing them in 2% (W/V) Chloramin T and 200 ppm Streptomycin for 15 minutes, followed by successive washing with sterilized distilled water till the removal of sterilant. The sterile spores were used to infect *Allium cepa* (onion) seedlings grown in earthen pots filled with sterilized substrate (soil and sand 1:1 V/V). The roots system of well infected seedlings along with the adhering soil were finally chopped and used as starter inoculum. The bulk inoculum was produced by infecting fresh seedling raised in sterilized soil inoculated with 5-10% of starter inoculums and mixing in the soil bed before seed sowing.

Experimental Design: A field experiment consisting of 10 bacterial and mycorrhizal treatments (Tables 1, 2) with 3 replications was conducted in a randomized complete block design (RCBD). The treatments were (1) *B. japonicum* MN-S, (2) *B. japonicum* TAL-102, (3) *Agrobacterium* sp Ca-18, (4) Mix bacterial inoculants, (5) *B. japonicum* MN-S + AM, (6) *B. japonicum* TAL-102 + AM, (7) *Agrobacterium* sp Ca-18 + AM, (8) Mix bacterial inoculants + AM, (9) AM alone and (10) Uninoculate control. Experimental unit was a plot of size, 12 m^2. Phosphate fertilizer Diammonium Phosphate (DAP) was added providing phosphorus @ 12kg $acre^{-1}$, as half the recommended dose and no additional nitrogen fertilizer was applied. A control without inoculation was also kept. The plants were irrigated with canal water, as per requirement.

Table 1. Effect of arbuscular mycorrhizae and bacteria (*Bradyrhizobium* & *Agrobacterium*) on nodule number, fresh weight and dry Weight of nodule plant^{-1} , fresh weight and dry of *Vigna radiata* plants.

Treatment	Nod. No. 45 DAS	Nod. F. wt (g) 45 DAS	Nod. D. wt (g) 45 DAS	P.F.wt (g) at maturity	P. D.wt (g) at maturity
T1 = *B. japonicum* MN-S	30 bc ± 8	0.149 bc ± 0.04	0.051 abcd ± 0.01	84.12 bc ± 9.84	37.67 bcd ± 2.26
T2 = *B. japonicum* TAL-102	27 bc ± 4	0.253 ab ± 0.10	0.071 ab ± 0.02	79.72 bc ± 7.69	39.61 bcd ± 14.41
T3 = *Agrobacterium* sp. Ca-18	21 cd ± 5	0.093 c ± 0.07	0.040 bcd ± 0.03	81.31 bc ± 9.55	46.12 abc ± 3.87
T4 = Mix bacterial inoculants	39 b ± 7	0.271 a ± 0.02	0.056 abc ± 0.01	109.70 a ± 8.48	49.83 ab ± 5.98
T5 = *B. japonicum* MN-S + AM	38 b ± 6	0.266 ab ± 0.04	0.060 abc ± 0.01	87.90 b ± 7.07	38.47 bcd ± 2.69
T6 = *B. japonicum* TAL-102 + AM	52 a ± 13	0.244 ab ± 0.01	0.068 ab ± 0.01	83.12 bc ± 5.93	40.15 bcd ± 1.16
T7 = *Agrobacterium* sp. Ca-18 + AM	27 bc ± 7	0.113 c ± 0.05	0.048 abcd + 0.02	86.05 bc ± 9.45	51.84 ab ± 3.94
T8 = Mix bacterial inoculants +AM	62 a ± 7	0.359 a ± 0.13	0.076 a ± 0.03	119.28 a ± 9.58	58.14 a ± 9.98
T9 = AM	28 bc ± 5	0.113 c ± 0.02	0.033 cd ± 0.01	72.40 c ± 3.66	32.46 cd ± 4.50
T10 = Uninoculated control	10 d ± 6	0.035 c ± 0.03	0.020 d ± 0.01	58.48 d ± 5.16	29.19 d ± 13.42
	LSD ≤ 0.05 = 20.205 **	LSD ≤ 0.05 = 0.109 ***	LSD ≤ 0.05 = 0.027**	LSD ≤ 0.05 = 13.468 ***	LSD ≤ 0.05 = 13.054 **

Nod = Nodule, No.= Number, F.wt = Fresh weight, D.wt = Dry weight, P = Plant, DAS = Days after sowing, Mix bacterial inoculants = *B. japonicum* MN-S, TAL-102 and *Agrobacterium* sp.Ca-18, LSD = Least significant difference.
** = More significant, *** = Highly significant

Table 2. Effect of arbuscular mycorrhizae and bacteria (*Bradyrhizobium* & *Agrobacterium*) on % N in grain, phosphorus contents and grain yield (kg ha^{-1}) at maturity (90 DAS).

Treatment	% N in grain	P in grain (mg g^{-1})	Yield (kg ha^{-1})
T1 = *B. japonicum* MN-S	3.466 bc ± 0.17	3.366 ef ± 0.23	1165.843bc ± 86.01
T2 = *B. japonicum* TAL-102	3.513 bc ± 0.09	3.590 def ± 0.63	1094.300c ± 11.80
T3 = *Agrobacterium* sp. Ca-18	3.366 cd ± 0.02	4.201 bcde ± 0.67	1244.593ab ± 60.49
T4 = Mix bacterial inoculants	3.596 b ± 0.87	5.060 ab ±0.22	1253.135ab ± 61.28
T5 = *B. japonicum* MN-S + AM	3.843 a ± 0.12	4.521 abcd ± 0.466	1262.302a ± 62.54
T6 = *B. japonicum* TAL-102 + AM	3.813 a ± 0.03	3.667 def ± 0.187	1256.677 ab ± 38.18
T7 = *Agrobacterium* sp. Ca-18 + AM	3.590 b ± 0.14	4.681 abc ± 0.946	1289.177 a ± 58.79
T8 = Mix bacterial inoculants + AM	3.906 a ± 0.04	5.356 a ± 0.281	1322.302a ± 15.37
T9 = AM	3.408bcd ± 0.03	3.949 cdef ±0.705	1246.052ab ± 80.31
T10 = Uninoculated control	3.246 d ± 0.17	3.153 f ± 0.095	937.7158d ± 55.24
	LSD≤0.05=0.184** *	LSD ≤ 0.05= 0.884 ***	LSD ≤ 0.05 = 83.598 ***

Mix bacterial inoculants = *B. japonicum* MN-S, TAL-102 and *Agrobacterium* sp. Ca-18, DAS = Days after sowing, LSD = Least significant difference, *** = Highly significant, P = Phosphorus, N = Nitrogen

Soil analysis: Before planting, soil samples up to a 20cm depth were collected from experimental area of NIBGE, Faisalabad. The soil was found sandy loam with pH 7.5, total nitrogen (Nelson & Sommers, 1973) 0.05% and available phosphorus (Olsen *et al*., 1954) 10mg kg^{-1}.

Parameter studied: Various parameters studied were nodule number $plant^{-1}$, fresh and dry weight of nodules $plant^{-1}$, fresh and dry weight $plant^{-1}$, nitrogen and phosphorus contents in grain and total grain yield ha^{-1}. Data regarding nodulation was collected at 45 days after sowing (DAS). Fresh and dry weight $plant^{-1}$, grain yield ha^{-1}, nitrogen and phosphorus contents in grain were measured at maturity (90 DAS).

Light and transmission electron microscopic study: *Allium cepa* roots were chosen as a reference crop while *V. radiata* roots were selected as test crop to evaluate mycorrhizal colonization in roots through light microscopy. The root system was checked microscopically (Nikon Optiphot II fitted with a Leica DC 500 equipped with digital CCD camera) by staining with Trypan blue (Phillips & Hayman, 1970) for uniform colonization at different time intervals (after 7 days).

For transmission electron microscopic studies root and nodule samples of *V. radiata* were washed with distilled water twice to remove soil particles and blot dried. Specimens (1 x 4 mm) fixed in 5% gluteraldehyde (v/v in PIPES buffer) vacuum infiltrated and left to fix for 18h. Fixed specimens were washed for 2 x 30 min with PIPES buffer. Osmium tetra oxide 0.2% (in 0.2% PIPES buffer, pH 6.8) was put in for 16-18h, washed with sterilized distilled water for 2 x 30min, treated with 5% aqueous uranyl acetate for 16-18h and dehydrated through a graded ethanol series, infiltrated with 1:1 acetone / spur resin for 24-48 h, then in pure resin for 24h. Samples were then transferred to flat embedded moulds and polymerized for 72h at 65-70^{o}C. Ultrathin sections of 120-200nm cut on ultra-microtome RMC-7000 and lifted on copper grids. Double stained with uranyl acetate for 30 min and with lead citrate for 10 min, washed with deionized water, dried and observed under TEM JEOL 1010 and recorded on micrographs.

Statistical analysis: The data regarding different plant characters under study were subjected to one way analysis of variance to determine significance of mean among the treatments (Steel & Torrie, 1986). The means were separated by Duncan's multiple range test and comparison of treatment means accomplished by least significant difference (LSD) test at 0.05 level of significance using Costat software (Cohort, Berkeley, calif.).

Results and Discussion

Growth studies: The application of beneficial microorganisms can enhance the growth and increase the yield of crops. In this context, we analyzed the growth, yield and nutrient status of *V. radiata* L. in a field experiment. One of the main objectives of our study was to explore the beneficial effects of potential bacterial inoculants (nitrogen fixers and phospate solubilizer) and indigenous mycorrhizal fungus that can be used as biological fertilizers. Considering this property, *Agrobacterium* sp. Ca-18 was applied singly and as multi strain inocula with *B. japonicum* MN-S and TAL-102 as biofertilizer to explore its potential for increased growth and grain yield. In the present study, the combine inoculation of nitrogen fixers and phosphate solubilizing bacteria increased the measured parameters substantially over the control or single inoculation treatment, Moreover, consortium of these potential bacterial inoculants with AM too showed remarkable increase in all growth parameters over single bacterial and mycorrhizal treatment. In general, nodules number $plant^{-1}$, fresh and dry weight of nodule $plant^{-1}$, fresh and dry weight of plant, grain yield, nitrogen and phosphorus contents in grain were found significantly higher in mycorrhizal than those of non-mycorrhizal plants (Tables 1, 2). These results are supported by Khan *et al*., (2008) who studied the effect of arbuscular mycorrhizae on the growth and nutrients uptake of *Medicago sativa.* El-Azouni *et al*., (2008) also reported the same associative effects of AM with *Bradyrhizobium* as biofertilizers on growth and nutrient uptake of *Arachis hypogaea*.

Nodulation: Nodulation was observed in all the treatments including uninoculated control plants. The presence of nodules in uninoculated plant roots, indicate the presence of indigenous rhizobial population in the soil. However, the number and size of the nodules in uninoculated control plants was relatively less than most of the other treatments. Data regarding nodulation was collected at 45 days after sowing, showed higher number of nodules in treatment T8 (Mix bacterial inoculants + AM). T1 (*B. japonicum* MN-S) and T2 (*B. japonicum* TAL-102) were comparable with 30 and 27 number of nodule $plant^{-1}$. Among the bacterial inoculations, T4 (Mix bacterial inoculants) was at top with 39 number of nodule $plant^{-1}$. Bacterial inoculation along with mycorrhizal inoculation in T6 (*B. japonicum* TAL-102 + AM) and T8 (Mix bacterial inoculants + AM) were significantly higher than those of non mycorrhizal plants. Average fresh and dry weight of nodule was observed 0.359 and 0.359g $plant^{-1}$, respectively in T8 (Mix bacterial inoculants + AM) those are higher than all other treatments. Fresh and dry weight of nodules $plant^{-1}$ was significantly different between nitrogen fixers (*B. japonicum* MN-S and TAL-102), but non- significant difference among them was observed when they were applied in combination with mycorrhizae. Moreover, mycorrhizal plant showed greater values of nodule fresh and dry weigh $plant^{-1}$ compared to non mycorrhizal plants. The number of nodules is positively correlated with fresh and dry weight of roots directly and that of plant indirectly. Increased nodulation is connected with plant growth promotion (Dey *et al*., 2004). A positive correlation exist between nodule number, fresh and dry weight of plant indicating positive influence of nodulation on plant growth, development and biomass through biological nitrogen fixation (Badri *et al*., 2010). Meghvansi & Mahna (2009) also found dual inoculation of AM + *B. japonicum* superior, over single inoculation of AM or *B. japonicum* and approved the suitability of AM and *B. japonicum* to be used in combination for enhanced yield and soil fertility. We observed in our experiment that nodule number, fresh and dry weight of nodule $plant^{-1}$ at 45 DAS was higher in mycorrhizal plant than non mycorrhizal plants. Moreover, lowest values of nodule number, fresh and dry weight of nodule $plant^{-1}$ were recorded for *Agrobacterium* sp. Ca-18 and uninoculated control, respectively.

Plant biomass: In general fresh and dry weight plant^{-1} were significantly higher in mycorrhizal plants compared to non mycorrhizal plants and the effect was more pronounced in treatment T8 (Mix bacterial inoculants + AM). Treatment T4 (Mix bacterial inoculants) with 109.70 and 49.83 g fresh and dry weight plant^{-1}, respectively proved more efficient for its ability to improve plant biomass among bacterial inoculations. Moreover, *Agrobacterium* sp. being phosphate solubilizer showed higher dry weight plant^{-1} in single as well as dual inoculation with mycorrhizae. Nitrogen fixers *B. japonicum* MN-S and TAL-102 were not very much different from each others both in single and with mycorrhizal inoculation. The observed increase in biomass of mycorrhizal plant are in agreement with previous findings which indicate the effect of mycorrhizae on plant growth (Feddermann *et al*., 2008). The result was also in close conformity with the findings of Jalaluddin (2005) showing increased root nodulation, fresh and dry weights and seed weight with co-inoculation of mycorrhizae and *Bradyrhizobium*.

Nitrogen and phosphorus in grain: Statistically significant difference for nitrogen and phosphorus contents in grain was observed among different treatments with higher values in the dual inoculation of bacteria and mycorrhizae. Negligible difference among *B. Japonicum* MN-S and TAL-102 was observed for % nitrogen in grains of *V. radiata.* Moreover, *Agrobacterium* sp. Ca-18 in combination with mycorrhizae also significantly improved the nutrient status of grain (3.843% N, 4.681mg g^{-1} P). Treatment T8 (Mix bacterial inoculants + AM) with 3.90% N and 5.356 mg g^{-1} P in grain was at top followed by 3.843% N in T5 (*B. Japonicum* MN-S + AM) and 4.681 mg g^{-1} P in T7 (*Agrobacterium* sp. Ca-18 + AM), respectively.

Phosphate solubilizing bacteria (PSB) inhabiting soil or rhizosphere, play fundamental roles in biogeochemical phosphorus cycling and ultimately in growth and development of plants in agro-ecosystems. Nitrogen fixation is another important phenomenon that changes nitrogen to ammonia by microorganisms (Kim & Rees, 1994)and represents an economically beneficial and environmentally sound alternative to chemical fertilizers (Ladha *et al*., 1997). The combined inoculation of Nitrogen fixers, phosphate solubilizing bacteria and mycorrhizal fungi could be more effective than single organism for providing a more balanced nutrition (Zaidi *et al*., 2010).

Sailo & Bagyaraj (2003) conducted a glass house investigation to study the influence of the arbuscular mycorrhizal fungus and plant growth promoting rhizobacteria alone and in combination, on the growth and nutrition of medicinal plants. Individual inoculation of these organisms significantly enhanced plant biomass but biomass was significantly greater in the plants co-inoculated with AM and PGPRs. Phosphorus contents were also greater in the same treatment. Tavasolee *et al*., (2011) found significant interactions of AM and bacterial inoculations in chick pea. Nitrogen and phosphorus contents were again found significantly high in the treatment where bacterial inoculants were applied in combination with mycorrhizae.

In another study response of *Pisum sativum* cv. Avola to arbuscular mycorrhizal fungi (AM) species and *Rhizobium* inoculation was evaluated regarding growth, photosynthesis, nodulation and nitrogen fixation activity (Geneva *et al*., 2006)**.** The dual inoculation significantly increased the plant biomass, photosynthetic rate, nodulation and nitrogen fixation activity in comparison with single inoculation of *Rhizobium.* On the other hand, coinoculation significantly increased the total phosphorus content in plant tissue, acid phosphatase activity and percentage of root colonization**.**

Grain yield: Maximum grain yield was again observed in the plants inoculated with consortium of bacterial inoculants plus mycorrhizae in T8 (Mix bacterial inoculants + AM) with 1322.302 kg ha^{-1} grain yield. Treatment T5 (*B. Japonicum* MN-S + AM) was found superior with 1262.302kg ha^{-1} grain yield over T6 (*B. Japonicum* TAL-102 + AM) with 1256.677kg ha^{-1} grain yield. Moreover *Agrobacterium* alone and in combination with mycorrhizae showed 33% and 37% increase over control, respectively. In the present study, the combined inoculation of nitrogen fixing and phosphate solubilizing bacteria suggested that the nitrogen fixer, phosphate solubilizing bacteria and AM were compatible microbes, exhibiting a synergistic interaction amongst each other and contribute substantially by improving the nutrition of *V. radiata* plants.

In this study we used non-sterile soil due to the fact that in many researches, plant growth promotion with PGPRs was observed only under controlled conditions (Cattelan *et al*., 1999) where these bacteria do not have to compete with a range of indigenous soil microbes. However, different environmental factors may also affect performances of PGPRs and their interactions with the host plant. Therefore, to examine the potential and way that PGPRs follow to exert their effects on plants under field conditions, we conducted the experiment in field conditions.

Light microscopic studies: Before the application of *Allium cepa* roots as source of inoculum for *V. radiata* field experiments, the roots were checked for mycorrhizal infection by light microscopy. *A. cepa* roots stained with trypan blue, showed high intensity of arbuscular mycorrhizal infection under light microscope (Figs. 1A, 1B). Different morphological stages of arbuscular mycorrhizae like vesicles, spores and arbuscules with attached hyphae were observed with high frequency in the *A. cepa* root cells. Arbuscular mycorrhizal infection was also localized in the root and nodules of the *V. radiata* by simple staining with trypan blue (Figs. 2A, 2B). An interesting finding of the presence of AM spores in the nodules of *V. radiata* root have been achieved during the course of study but we did not check the nitrogen fixing ability of the under study nodules. The same results were found by Scheublin & van der Heijden (2006) but the nodules colonized with AM were not nitrogen fixing. The presence of arbuscular mycorrhizal spores in the cortical tissue, as well as in the nodules, indicates the dispersed arbuscular mycorrhizal infection in various tissue types of the *V. radiata* root. This study confirms the capability of AM for colonization of root nodules in legume plants as observed in previous studies ((Baird & Caruso, 1994; Vidal- Dominguez *et al*., 1994).

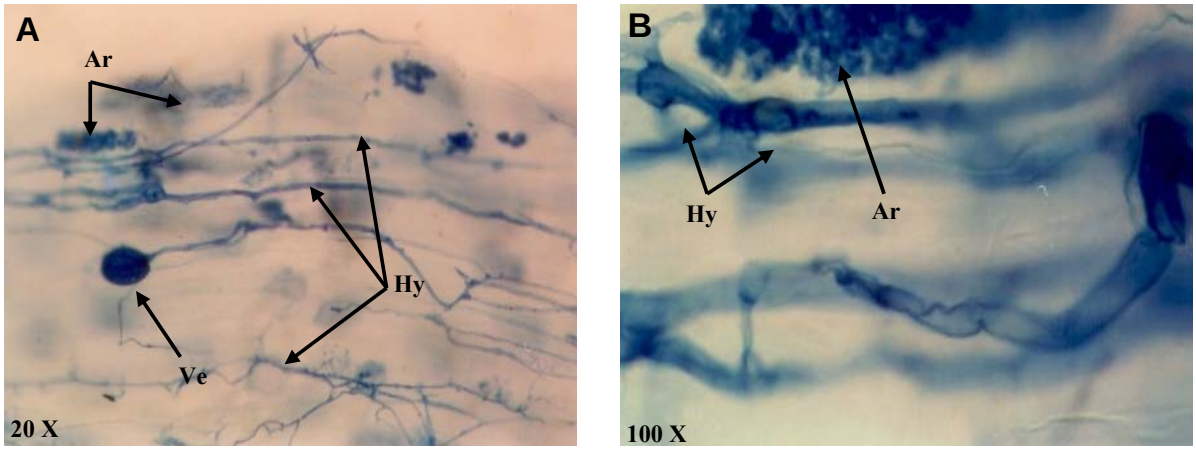

Fig. 1 Light micrograph of arbuscular mycorrhizal infection in the trypan blue (0.05 % in lactoglycerole) stained *Alium cepa* roots showing different morphological forms of mycorrhizae i.e. vesicle (Ve) with attached hyphae, arbuscules (Ar) and network of hyphae (Hy).

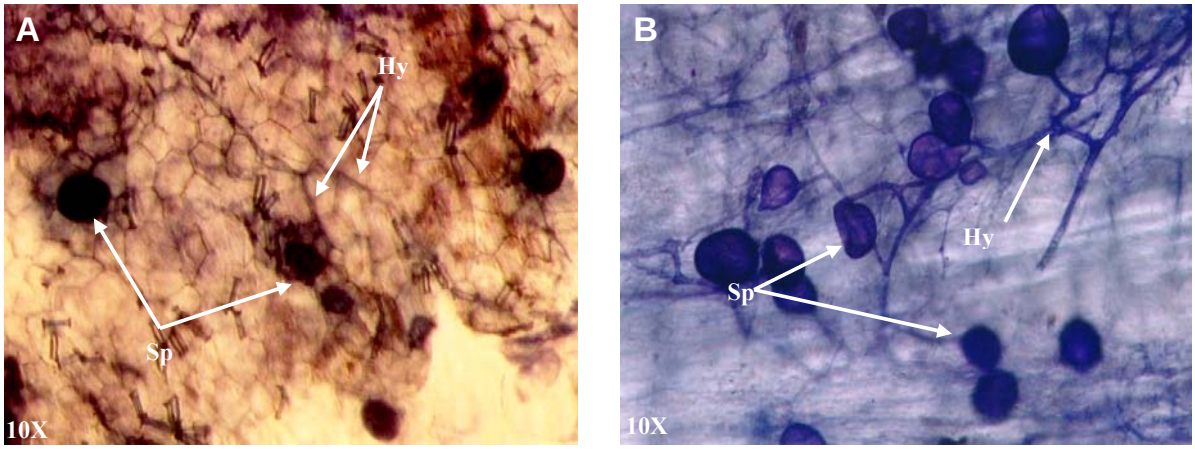

Fig. 2. Light micrograph of trypane blue (in 0.05 % lactoglycerole) stained *Vigna radiata* (A) nodule cells showing spores (Sp) with attached hyphae of arbuscular mycorrhizal fungi (B) high intensity of dispersed spores in the inner cortical tissues of *Vigna radiata* root along with hyphal network.

Ultra structural studies: Transmission electron microscopic observation of *Vigna radiata* root showed mycorrhizal spores localization in the rhizosphere and mycorrhizae helping bacteria (MHB) inside the root cells as well as in the rhizosphere. The presence of arbuscular mycorrhizae (AM) was found in the uninfected cells (Fig. 3A) as well as in the intercellular spaces of nodule (Fig. 3B). It is notable that no mycorrhizal infection was found in the cells infected with bacteria, in this study. Spores, which are the reproductive structures of AMF, were abundantly found in roots and comparatively less number of spores was found in nodules. Mycorrhizal infection was either visualized in the intercellular spaces or in unoccupied empty cells interspersed with bacterial occupied cells. (Figs. 3A, 3B). Stimulation of nitrogen fixation by AM fungi, which has been reported by previous studies (Asimi *et al*., 1980; Azcón *et al*., 1991), probably be an indirect effect that do not directly involved in nitrogen fixation but results the increased nutrient supply to the whole plant.

However, large number of food granules (FG) could also be localized within the uninfected zone in close proximity of the infected cells (Fig. 3A). Arbuscular mycorrhizal infection was also localized in rhizosphere of *V. radiata* showing high number of characteristically thick wall mycorrhizal spores (Fig. 4A) along with relatively small mycorrhizae helping bacteria (MHB) (Fig. 4B). Characteristically thick walled property of spore is confirmed by the findings of Maia & Kimbroughz (1994). A great number of fungi produce spores outside the roots but some peculiar fungi produce the spores inside the root and according to a report if AM fungi grow for 16 wk in association with *A. cepa* L., it results the quantity of spores that is about 5.5% of the root mass (Jarstfer, 1993).

Conclusively, increase in nodule number due to co-inoculation with non-nodulating *Agrobacterium* sp. Ca-18 with nodulating *B. japonicum* MN-S and TAL-102 shows beneficial effect of *Agrobacterium* sp. Ca-18 as a phosphate solubilizer and *B. japonicum* MN-S and TAL-102 as nitrogen fixer. The present study suggests that the combined application of mix bacterial inoculants and AM fungus was more effective than other inoculation treatments and are suggested to be important bio-resource for efficient bio-inoculant development for *V. radiata* productivity.

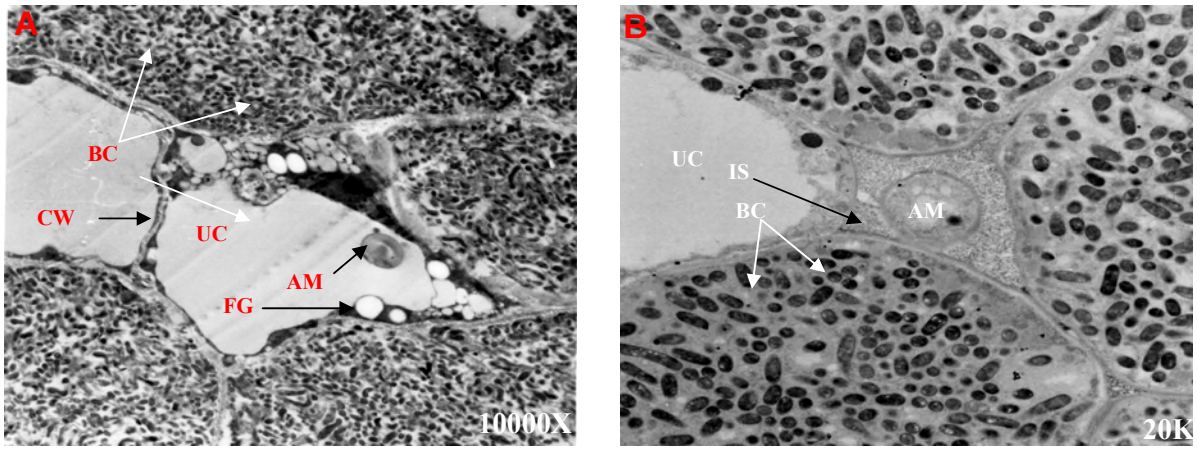

Fig. 3. Transmission Electron Micrograph (TEM) of *Vigna radiata* root nodule (A) from field experiment infected with bacteria and arbuscular mycorrhizal fungi showing over view of nodule cells at low magnification with mycorrhizal infection in empty surrounded by bacterial occupied and unoccupied cells (B) from growth pouches infected with bacteria and arbuscular mycorrhizal fungi showing mycorrhizal infection in the intercellular space surrounded by bacterial occupied and unoccupied cells. No mycorrhizal infection was ever found in the bacterial occupied cell in our study. Mycorrhizal infection was frequently found in the unoccupied nodule cells or in intercellular spaces interspersed with the bacterial occupied cells.

AM=Arbuscular mycorrhizal infection, UC = Uninfected cells, BC = Bacterial cells, IS = Intercellular spaces, CW = Cell wall, FG = Food granules

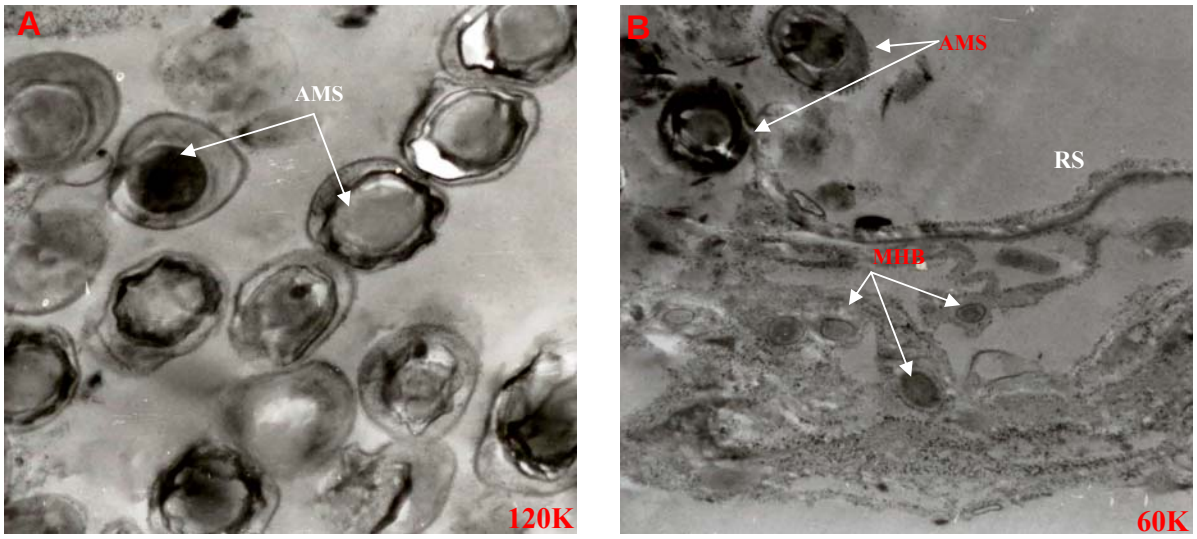

Fig. 4. Transmission Electron Micrograph (TEM) of characteristically thick walled arbuscular mycorrhizal spores (AMS) (A) in the rhizosphere (RS) of *Vigna radiata* (B) along with mycorrhizal helping bacteria (MHB).

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