Scanning electron microscopy indicates Pseudomonad strains facilitate AMF mycorrhization in litchi (*Litchi chinensis* Sonn.) air-layers and improving survivability, growth and leaf nutrient status

Amit Visen, Pramod Narayan Singh, Binayak Chakraborty, Anand Singh, Tejpal Singh Bisht

Keywords:
Litchi
PGPR
AM fungi
Air-layer
Leaf nutrient

**ABSTRACT**

The efficacy of two plant growth promoting rhizobacteria (PGPR) viz. Pseudomonas jessenii strain R62 and Pseudomonas syxantha strain R81 was examined for mycorrhization of arbuscular mycorrhizal (AM) fungi (*Glomus intraradices*), survivability, growth and leaf nutrient status in litchi air-layer system. Therefore, the litchi air-layers were inoculated with PGPR i.e., Pseudomonad strains and AM fungi alone and with combination during the preparation of air-layers on the mother tree and planting of air-layers in root trainers just after detachment of the fresh air-layers from the mother tree. The scanning electron microscopy of the litchi roots indicated that Pseudomonad strains enhanced the process of mycorrhization of AM fungi and accounted near about 11.5 (tree inoculation) to 14.5 (root trainer inoculation) per cent increase in colonization over the sole inoculation of AM fungi in respective air-layers. No sign of mortality in any air-layered plants was noted in PGPR + AM fungi and sole AM fungi inoculated air-layers up to 18 months of growing. Significantly the highest shoot and root dry weight, and root length were recorded in the air-layers inoculated with both PGPR and AM fungi. This co-inoculation of PGPR with AM fungi was also responsible for the significant enrichment of the primary (N, P and K) and micro (Zn, Cu and Fe) nutrient concentration of the leaves in the litchi air-layers. However, the inoculation of air-layers with these microorganisms failed to produce any significant effects on leaf secondary (Ca, Mg and S) nutrient content. Further, the inoculation treatments had an adverse impact on leaf Mn content. The fresh air-layers inoculated after detachment from the mother tree were performed better for most of the studied parameters than the tree inoculated air-layers.

1. Introduction

Mycorrhization is an essential event during the entire life cycle in litchi (*Litchi chinensis* Sonn.) tree. This association is not only helping the tree in acquisition of essential elements from soil (Smith and Read, 2008) but providing a better tolerance level to environmental stresses under field conditions also (Begum et al., 2019). The causes of mortality of young litchi tree under field conditions are reported to be either the poor development of sublateral roots (Kumar et al., 2019) and/or slow rate of the mycorrhization process in these lateral roots (Visen et al., 2017). Since, mycorrhization in litchi is only taking place on short-lived sublateral roots. Therefore, to stimulate the process of mycorrhization, mixing of pit soil with the soils of old litchi orchards is a common practice by the litchi growers in India during establishment of a new orchard by planting of litchi saplings in a barren land (Singh et al., 2011). However, the success of this practice depends upon the presence of the arbuscular mycorrhizal fungi (AM fungi) inoculum in the introduced soils i.e., soils of old litchi orchard, proper mixing of both new and old orchards soils and several other operational and biological factors.

Worldwide, young litchi trees are multiplied by the technique of air-
layering, a method of induction of adventitious root system in a pen-to-
pencil thickness branch when it is still hanging in air to the mother tree (Visen et al., 2017). Therefore, there is a very less chance of mycorrhiza in the newly formed roots of the litchi air-layers on the mother trees. However, a preliminary symbiotic process could be initiated at this stage in the young litchi plants with the manipulation of propagating practices like mixing of AM fungi inoculum with the moisture retaining materials used to cover up the exposed bark portion of the litchi branch during the process of air-layering or mixing of AM fungi inoculum with the soils of root trainers used to unfold the super coiling of newly formed adventitious root system in the air-layers before planting it to the main field. The time course needed for this symbiotic association is also depending upon the status of the host. The process of mycorrhization may further be enhanced by the use of mycorrhiza helper bacteria (Frey-Klett et al., 2007).

Several bacterial communities have been identified which have the ability to colonize the hyphal surfaces of AM fungi. The interaction between bacteria-fungi has been reported to modulate the behaviour of either or both of the interacting partners (Deveau et al., 2010). These bacteria have either promoted or inhibited the process of mycorrhization. The bacteria which stimulate the spore germination, develop mycelial growth and subsequent AM fungi colonization in host roots are referred as mycorrhiza helper bacteria (MHB) (Frey-Klett et al., 2007). The mycorrhization of AM fungi species in the roots of litchi is reported to be enhanced by co-inoculation with Azotobacter chroococcum (Sharma et al., 2009; Kumar et al., 2018). Besides Azotobacter, several soil bacteria (for example: Pseudomonas, Rhizobium, Paenibacillus and Bacillus) are fallen in the category of MHB (Frey-Klett et al., 2007). This study aims to find out the impact of co-inoculation of florescent Pseudomonad strains with the most commonly found AM fungi species i.e., Glomus sp. on mycorrhization in the roots of litchi air-layers through scanning electron microscopy. 

Harnessing the beneficial effects of this tripartite interactions between bacteria-fungi-plant are highly relevant in sustainable horticultural systems. Since, this is an eco-friendly and cost-effective way to improve the nutrient uptake, growth and overall health of the plants. Bacterial species, the so-called plant growth promoting rhizobacteria (PGPR) are able to solubilize the essential elements required for plants, synthesize plant growth promoting substances and provide abiotic and biotic stress tolerances to the plants (Ahamed and Kibret, 2014). On the other hand, AM fungi-plant interaction working together or individually helps to enhance plant health status by improving the absorption capacity of water and essential mineral elements for the plants. Since, AM fungi colonizes in the host-root cortex zone which ultimately increases the absorbing surface of roots (Begum et al., 2019). The fluorescent Pseudomonad strains have the ability to promote the plant growth and developmental processes by colonizing in the plant rhizosphere, and being widely used in the field of agriculture (Mäder et al., 2011). Here, in this experiment, we have used both these Pseudomonad strains and co-inoculated with AM fungi (Glomus intraradices) in two different stages of litchi propagation through air-layering technique and examine the survivability, root and shoot growth, and leaf nutrient status of the selected branch during the month of July-August. Then, the exposed bark portion was covered with a layer of moist sterilized sphagnum moss treated with AM fungi, PGPR and their combined inoculation (Table 1). A sum of 100 air-layers were prepared under each of the inoculant treatment. Additionally, 500 fresh air-layers were prepared without any inoculant treatments. The sphagnum moss cover was wrapped with 300-gauge transparent polythene sheet (20 × 25 cm) and tied firmly at both ends to ensure supply of moisture from the moist sphagnum moss cover to facilitate the development of roots on the wounded portion of branch.

2.2. Detachment of litchi air-layers from mother tree and subsequent operations

After 70 days of the air-layering operation, 40 successful air-layers from each inoculation treatments having profuse root system were detached from the mother tree for planting in root trainers filled with sterilized sandy loam soil (Table 2). The fresh successful air-layers (without inoculation) were detached from mother tree and inoculated with similar treatment combination as done on tree, just before planting in the root trainers. The air-layers inoculated at the time of air-layering operations on trees were also detached from mother tree and planted directly in root trainers. Thus, there were two sets of air-layers, one set consisted of tree inoculated air-layers and another set consisted of root trainer inoculated air-layers. One month after planting of air-layers in the root trainers, all the young litchi trees (both tree and root trainer inoculated) were transferred to perforated polybags having 2.5 kg soil capacity filled with same sterilized soil as used in the root trainers and grown for about 18 months. The polybag soil was fertilized with 2% urea for proper growth and development of litchi air-layers. The polybags were irrigated at 7 and 4 days intervals during winter and summer, respectively. Weeding and followed by loosing of upper layer of soil were carried out when required in the polybags.

2.3. Scanning electron microscopy and mycorrhizal colonisation

The samples for scanning electron microscopic studies were prepared following the method described by Hayat (2000). The fine, healthy roots of the litchi saplings were collected by careful removal of polythene followed by gentle loosening of the soil 18 months after growing in the polybags. Immediately after rinsing (2-3 times) with sterilized water, the roots were cut into several segments of 3-5 mm length and fixed in 2.5% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.2) for 10 h. Fixed root segments were subjected to drying with 30-100% ethanol series and kept in dry acetone. Further, the root segments were completely dried by using liquid CO₂ in a critical point drying apparatus. After drying, the samples were mounted on Al stubs with double coated carbon conductive tape and sputtered with gold. Observations and microphotographs were taken under SEM (Jeol JSM-6610 LV/A/LA) at voltage of 10 kV.

The root samples from 5 litchi saplings of tree inoculated air-layers and root trainer inoculated air-layers (uninoculated on tree) were collected 18 months after growing in root trainers. In both the cases, root samples of the air-layers were used to determine the mycorrhizal colonization percentage as described by Endress et al. (2003). All the

| Inoculants                                      | Quantity applied |
|------------------------------------------------|------------------|
| Control (without inoculation)                   |                  |
| Only AM fungi (Glomus intraradices)             | 100 IP           |
| Only PGPR (Pseudomonas jessenii strain R62 and Pseudomonas synxantha strain R81) | 10⁶ cfu g⁻¹       |
| AM fungi (Glomus intraradices) and PGPR (Pseudomonas jessenii strain R62 and Pseudomonas synxantha strain R81) | 100 IP + 10⁶ cfu g⁻¹ |

Table 1

Detail of inoculation treatments.
collected samples were kept at 5 °C dipped with 50.0 % ethanol until examination. From each specimen, 1-1.5 cm long 40 root segments were examined for estimation of colonisation percentage (i.e., percentage of AM fungi in each root segments).

2.4. Survivability, root and shoot growth analysis

The observations on survivability and growth of litchi saplings were taken after growing in polybags for 18 months. The successful air-layers per replication were counted to calculate the survival percentage under each treatment. The growth parameters were recorded based on average length of all the roots and dry weight of both shoot and roots and expressed in gram. After trimming the entire leafy portion and root parts from the shoot of the air-layer with a sharp Ni coated blade, the shoot and root parts were subjected to drying separately in hot air oven at 60 °C for about 72 h before recording the weight of each part with an electronic balance.

2.5. Leaf nutrient analysis

From each inoculation treatment, third composite leaf was harvested for leaf nutrients analysis after 18 months of growing (Singh and Chadha, 2009). The leaf samples were decontaminated by washing them in sequence with tap water to remove the dirt or soil, then in 0.2% detergent solution and in N/10 HCl solution to remove dust particles adhere on the leaf followed by washing in single and double distilled water. Excess water was removed by pressing the leaves between the folds of blotting paper and the leaf samples were dried in an oven at 60 °C for 72 h. After complete drying, the samples were powdered and stored in polycarbyl containers for analysis. The leaf samples were analysed for macronutrients (N, P, K, Ca, Mg and S) and micronutrients (Cu, Fe, Mn and Zn). Expect N, all other nutrients in leaf samples were digested in di-acid (9:4 ratio of HNO$_3$ and HClO$_4$) following standard analytical methods (Jackson, 1973). Nitrogen was estimated by Nessler’s reagent method, whereas P, K and S were analysed by vanado-molybdate, flame-photometer and turbidity methods, respectively (Chesnin and Yien, 1951). Ca, Mg and the micronutrients viz., Cu, Fe, Mn, and Zn were analysed by using atomic absorption spectrophotometer.

2.6. Statistical analysis

The experiment was laid out in factorial completely randomized design with eight replications (5 plants per replication) and least significant differences (LSDs) were calculated to compare significant effects at $p \leq 0.05$ (Snedecor and Cochran, 1967).

3. Results

3.1. Scanning electron microscopy and mycorrhizal colonisation

There was no mycorrhizal colonisation observed in the root samples taken from control and only Pseudomonad strains (PGPR) treated in either tree or root trainer inoculated air-layers. Hence, the photomicrographs by scanning electron microscopy of these treatments were omitted from Fig. 1. However, from rest of the photomicrographs, it could easily be deduced that when PGPR co-inoculated with the AM fungi (Glomus intraradices), the fungal hyphae were visible under ×300- ×500 zooming range and the colonisation was significantly better.
in the litchi air-layers of either from tree (11.5 %) or root trainers (14.5 %) over the sole inoculation of AM fungi when inoculated in respective air-layers (Fig. 2). The smaller number of fungal hyphae was observed in the roots of air-layers which were inoculated only with the AM fungi at the time of preparation on tree under zooming × 1500 and recorded the lowest fungal colonisation (46.0 %). The co-inoculation of PGPR with AM fungi had resulted mycorrhizal colonisation 57.5 % and 68.0 % in tree and root trainer inoculated air-layers, respectively.

3.2. Effect on survival percentage

No sign of mortality had been recorded in tree or pot inoculated litchi air-layers when AM fungi either individually or in combination was used as inoculation treatment (Fig. 3A). Inoculation with only PGPR also produced better survival percentage (93.75 %) as compared to control. Significant interaction effect was found between inoculation treatments and stage of inoculation for survival percentage of litchi air-layers. The mortality of air-layers was less when air-layers were inoculated in root trainers. It indicates that inoculation of air-layers with AM fungi and PGPR during planting would be more beneficial than inoculation at the time of air-layer preparation on tree for achieving higher survivability.

3.3. Effect on shoot and root

Inoculation treatments significantly affected the root length of air-layers (Fig. 3B). The longest length of root was recorded in air-layers inoculated with both AM fungi and PGPR. The root length of AM fungi inoculated air-layers was higher than the root length of only PGPR inoculated air-layers. The unoinoculated air-layers produced shortest length of root. Similar trend was observed for the shoot and root dry weight of litchi air-layers (Fig. 3C and D). The growth performances of root trainer inoculated air-layers were found to be higher than the air-layers inoculated during air-layering on mother tree.

3.4. Effect on leaf macronutrient content

Inoculation with AM fungi and PGPR significantly increased the leaf N, P and K content over the control (Table 3). However, the improvement of leaf NPK content with sole inoculation of Pseudomonas fluorescent strains or AM fungi had recorded statistically similar. The above three nutrients were found highest in the leaves of air-layers inoculated with both AM fungi and PGPR. Among NPK, the leaf phosphorous content was markedly increased. The leaf NPK level of litchi air-layers were almost similar among the air-layers inoculated with either AM fungi or PGPR individually. The time of inoculation had no effect on leaf NPK content. The secondary nutrients (Ca, Mg and S) were not significantly affected by both inoculation treatments and stages of inoculation (Table 4).

3.5. Effect on leaf micronutrient content

Significant variations in leaf micronutrient content of litchi air-layers were recorded with different inoculation treatments (Fig. 4). The concentration of studied leaf micronutrients (Zn, Cu and Fe) except Mn were significantly the highest in the air-layers inoculated with AM fungi and PGPR. The leaf Fe concentration was better in PGPR inoculated air-layer than AM fungi. However, inoculation treatments had a negative impact on leaf Mn concentration. However, the leaf Mn content of AM fungi + PGPR inoculated air-layers was statistically similar with the untreated control air-layers. The lowest leaf Mn content was in the PGPR inoculated air-layers. Overall, all the studied micronutrients were significantly higher in the leaves when air-layers were inoculated during the time of placing in root trainers.

4. Discussion

4.1. Scanning electron microscopy and mycorrhizal colonisation

No evidence of mycorrhization in the roots of control and only PGPR inoculated both tree and root-trainer air-layers might be due to the use of sterilized sphagnum moss and soils, respectively. The presence of Pseudomonad strains (PGPRs) had a positive impact on mycorrhization of Glomus intraradices in the roots of litchi air-layers. Such an increase might be described from the bacteria-induced changes in metabolism of fungus or host and their interactive effects (Plett and Martin, 2012). The initiation of mycorrhization involves a complex of molecular signals between both plants and the AM fungi and the perception of these signals by both the symbiotic partners. The plant releases strigolactones and peptide molecules which are responsible for mycorrhizal hyphal branching and subsequent attraction of fungal hyphae to the plant roots, respectively (Parniske, 2005; Horii et al., 2009). The lipochito-oligosaccharides produced by the AM fungi are able to alter the root host morphology in favour of colonisation (Maillet et al., 2011). Further, the antigenic components such as chitin present in the cell wall of host plant for providing immunity to the host against fungal pathogen, is reported to be regulated by the colonising fungal propagules.

Fig. 2. AM fungi colonisation (%) in the litchi root. The vertical bar denotes standard error (n = 8) followed by the same letter are not significant at p ≤ 0.05.
auxofuran produced by the bacteria is known to stimulate the growth of roots (Ahemad and Kibret, 2014). Additionally, many florescent Pseudomonad species are also reported to produce cytokinin (Pallai et al., 2012). This plant hormone has a regulatory role in plant growth and development. Further, inhibition on synthesis of ethylene due to increase ACC-deaminase activity in Pseudomonas inoculated air-layers might also have a stimulatory effect on the root growth of litchi air-layers (Nadeem et al., 2010). The P is known to responsible for secondary root formation (Kurth et al., 2015) and modulation of such functions occurs to be a higher extent in the presence of Pseudomonas fluorescens as reported in aspen seedlings (Shinde et al., 2019). The results of this experiment also suggested that Pseudomonas jesseni strain R62 and Pseudomonas synxantha strain R81 could also play a role as helper bacteria for better mycorrhization of AM fungi in the litchi roots.

4.2. Survivability of litchi air-layers

The primary reason of mortality of litchi air-layers is reported to be the inability of plants to develop strong fibrous root system (Kumar et al., 2019). Phosphorous nutrition at establishment stages of plants helps in development of stronger root system. The mortality percentage was lower in Pseudomonas strains inoculated air-layers than the uninoculated control air-layers. Since, these air-layers might be able to form strong root system through improve P nutrition by the Pseudomonas strains (Mäder et al., 2011). It is well established fact that AM fungi improves P acquisition from soil to plants (Bagyaraj et al., 2015). Therefore, early root development in AM fungi inoculated air-layers might have helped in better water uptake, proper nutrition in plants and resulting higher survivability than untreated (control) air-layers. Further, an elevated level of P nutrition might have helped the development of more stronger root system in the air-layers treated with both AM fungi and Pseudomonas strains and resulted cent percent alive plants. Sharma et al. (2009) also reported that co-inoculation of AM fungal species with A. chroococcum strains also helped for better adaptation of litchi air-layers.

4.3. Growth of litchi air-layers

The higher shoot and root dry weight were directly linked to vigorous growth habit of inoculated air-layers. Many Pseudomonas species have the ability to synthesize indole acetic acid (IAA), plant hormone known to stimulate the growth of roots (Ahemad and Kibret, 2014). Additionally, many florescent Pseudomonad species are also reported to produce cytokinin (Pallai et al., 2012). This plant hormone has a regulatory role in plant growth and development. Further, inhibition on synthesis of ethylene due to increase ACC-deaminase activity in Pseudomonas inoculated air-layers might also have a stimulatory effect on the root growth of litchi air-layers (Nadeem et al., 2010). The increased in length and dry weight of root in PGPR inoculated air-layers were also responsible for the higher above ground biomass production and resulting increased the shoot dry weight over untreated control air-layers. The growth promotion of roots in AM fungi inoculated air-layers might be attributed to higher acquisition of phosphorous (Bagyaraj et al., 2015). The P is known to responsible for secondary (lateral) root network development in plants (Visen et al., 2017). All the positive impacts of both Pseudomonas and AM fungi on growth of plants might have resulted the highest root as well as shoot growth in PGPR + AM fungi inoculated air-layers. Increased in length of litchi air-layered root by 81.39% over uninoculated control plants with co-inoculation of AM fungi (G. fasciculatum) and Azotobacter spp. had already been documented (Sharma et al., 2009).

4.4. Leaf macronutrient content

Inoculation treatments significantly enriched the leaf NPK content in
Pseudomonas was reported earlier (Mader et al., 2011). The Pseudomonas is described to be a strong P solubilizers (Visen et al., 2017). The process involves to bring the soluble P from insoluble phosphates are acidification through releasing succinic, oxalic, gluconic, citric and α-ketogluconic acids (Chen et al., 2006), chelatiation with Fe nutrition in the air-layers. Increase in the phyto-availability of Zn and Cu in litchi air-layers as evident by the second-best inoculation treatment. Whereas, the role of AM fungi on Mn nutrition in agricultural crops has been well recognized (Pichardo et al., 2011). The high concentration of leaf Mn in uninoculated air-layers is the survivability, shoot length and dry weight, and leaf micronutrient content. The improvement in leaf Mn content with the inoculation of AM fungi and PGPR might have helped in acquisition of Fe by the litchi air-layers. Further, AM fungi provided more surface area in host root for acquisition of nutrient elements by spreading the fungal hyphae network both into the root and rhizosphere (Smith and Read, 2008). The AM fungi acidifies the rhizosphere by releasing organic acids like citric and oxalic acids and helps in bioavailability of diffusion-limited metal elements (Pichard et al., 2012). Further, AM fungi provided more surface area in host root for acquisition of nutrient elements by spreading the fungal hyphae network both into the root and rhizosphere (Smith and Read, 2008). Siderophores released by both, Pseudomonas and AM fungi might have helped in acquisition of Fe by the litchi air-layers. Since, microbial siderophores have the ability to reduce Fe$^{3+}$ to Fe$^{2+}$ and thereby increases the bioavailable form of Fe in the rhizosphere (Rashid et al., 2011). The negative impact AM fungi on Mn nutrition in agricultural crops has been well recognized (Pichard et al., 2012). The highest concentration of leaf Mn in uninoculated air-layers is also supporting this fact. However, air-layers inoculated with both AM fungi and Pseudomonas resulted second highest leaf Mn content, which might be due to the influential effects of Glomus intraradices on the population of Pseudomonad species. Since, the mycorrhizal fungal hyphae contains high level of trehalose, a disaccharide which is known to be acted as a chemoattractant and promoted the growth of the helper bacteria (Devoue et al., 2010). Improvement in leaf Mn content with the use of Pseudomonas is also reported in melon (Martinez et al., 2019).

### 4.5. Leaf micronutrient content

The improvement in leaf micronutrient content was recorded for Zn, Cu and Fe with co-inoculation of Pseudomonas strains and AM fungi. The role of AM fungi was found to be more instrumental for uptake of Zn and Cu in litchi air-layers as evident by the second-best inoculation treatment. Whereas, the role of Pseudomonas strains was more influential for Fe nutrition in the air-layers. Increase in the phyto-availability of Zn and Cu with the association of AM fungi have been reported earlier (Smith and Read, 2008). The AM fungi acidifies the rhizosphere by releasing organic acids like citric and oxalic acids and helps in bioavailability of diffusion-limited metal elements (Pichard et al., 2012). Further, AM fungi provided more surface area in host root for acquisition of nutrient elements by spreading the fungal hyphae network both into the root and rhizosphere (Smith and Read, 2008). Siderophores released by both, Pseudomonas and AM fungi might have helped in acquisition of Fe by the litchi air-layers. Since, microbial siderophores have the ability to reduce Fe$^{3+}$ to Fe$^{2+}$ and thereby increases the bioavailable form of Fe in the rhizosphere (Rashid et al., 2011). The negative impact AM fungi on Mn nutrition in agricultural crops has been well recognized (Pichard et al., 2012). The highest concentration of leaf Mn in uninoculated air-layers is also supporting this fact. However, air-layers inoculated with both AM fungi and Pseudomonas resulted second highest leaf Mn content, which might be due to the influential effects of Glomus intraradices on the population of Pseudomonad species. Since, the mycorrhizal fungal hyphae contains high level of trehalose, a disaccharide which is known to be acted as a chemoattractant and promoted the growth of the helper bacteria (Devoue et al., 2010). Improvement in leaf Mn content with the use of Pseudomonas is also reported in melon (Martinez et al., 2019).

### 4.6. Performance of inoculation at different stages of litchi propagation

The survivability, shoot length and dry weight, and leaf micronutrient concentrations were significantly better when air-layers were inoculated during planting in root trainers. The poor performances by the tree inoculated air-layers might be due to the fact that, removal of bark (girdling) from the branch of the litchi tree during the process of air-layering breaks the carbohydrate supply channel. Poor supply of

---

**Table 3**

Effect of AM fungi (Glomus intraradices) and PGPR (Pseudomonas jessenii strain R62 and Pseudomonas syxaxantha strain R81) on primary nutrient content in the leaves of litchi air-layers under two different stages of propagation.

| Inoculant       | Nitrogen (%) | Root Trainer | Phosphorous (%) | Root Trainer | Potassium (%) |
|-----------------|--------------|--------------|-----------------|--------------|---------------|
| Without inoculation | 1.32 ± 0.04a | 1.34 ± 0.01f | 1.36 ± 0.01 × 10⁻⁴ | 1.44 ± 0.02 × 10⁻⁴ | 6.40 ± 0.12 × 10⁻¹a |
| AM fungi        | 1.50 ± 0.02ab | 1.66 ± 0.16d | 1.54 ± 0.02 × 10⁻¹a | 1.76 ± 0.01 × 10⁻¹a | 6.55 ± 0.02 × 10⁻¹b |
| PGPR           | 1.55 ± 0.24d  | 1.60 ± 0.04e  | 1.45 ± 0.02 × 10⁻¹b | 1.55 ± 0.11 × 10⁻¹ab | 6.50 ± 0.03 × 10⁻¹b |
| AM fungi + PGPR | 1.52 ± 0.02bd | 1.70 ± 0.02d  | 1.55 ± 0.03 × 10⁻¹ab | 2.05 ± 0.02 × 10⁻¹a  | 6.65 ± 0.01 × 10⁻¹ab |

For each measurement corresponding mean ± standard error (n=8) followed by the same letter are not significantly different at p ≤ 0.05.

**Table 4**

Effect of AM fungi (Glomus intraradices) and PGPR (Pseudomonas jessenii strain R62 and Pseudomonas syxaxantha strain R81) on secondary nutrient content in the leaves of litchi air-layers under two different stages of propagation.

| Inoculant       | Ca (%) | Root Trainer | Mg (%) | Root Trainer | S (%) | Root Trainer |
|-----------------|--------|--------------|--------|--------------|-------|--------------|
| Without inoculation | 1.16 ± 0.02a | 1.20 ± 0.01a | 2.52 ± 0.03 × 10⁻¹a | 2.55 ± 0.12 × 10⁻¹a | 5.85 ± 0.01 × 10⁻²a | 5.75 ± 0.10 × 10⁻²a |
| AM fungi        | 1.17 ± 0.04a | 1.21 ± 0.01a | 2.53 ± 0.02 × 10⁻¹a | 2.71 ± 0.04 × 10⁻¹a | 5.79 ± 0.05 × 10⁻²a | 5.70 ± 0.02 × 10⁻²a |
| PGPR           | 1.16 ± 0.01a | 1.23 ± 0.03a | 2.55 ± 0.11 × 10⁻¹a | 2.75 ± 0.03 × 10⁻¹a | 5.65 ± 0.03 × 10⁻²a | 5.95 ± 0.06 × 10⁻²a |
| AM fungi + PGPR | 1.18 ± 0.03a | 1.23 ± 0.02a | 2.57 ± 0.02 × 10⁻¹a | 2.73 ± 0.05 × 10⁻¹a | 6.00 ± 0.02 × 10⁻²a | 6.20 ± 0.03 × 10⁻²a |

For each measurement corresponding mean ± standard error (n=8) followed by the same letter are not significantly different at p ≤ 0.05.
different stages of propagation. The vertical bar denotes standard error (n is reported to be more beneficial than inoculation at the time of microbial inoculation after detachment of litchi air-layers from mother tree downregulated the process of mycorrhization (Shu et al., 2016). Micronutrient content [Zn (A), Cu (B), Fe (C) and Mn (D)] in the leaves of the litchi air-layers under two strain R62 and Fig. 4.

5. Conclusion

The mortality of young litchi plants under field condition is the major concern to the litchi growers. This problem could be minimized by producing healthy air-layers with profuse root mass. The results of our experiment suggested an alternative way of development of healthy litchi plantlets with a satisfactory root system by harnessing the beneficial effects of plant-fungi-bacteria interactions.

CRediT authorship contribution statement

Amit Visen: Conceptualization, Data curation, Formal analysis.

Pramodh Narayan Singh: Conceptualization, Methodology, Supervision, Project administration.

Binayak Chakraborty: Conceptualization, Formal analysis, Software, Writing – original draft.

Anand Singh: Software, Validation, Data curation.

Tejpal Singh Bish: Writing – review & editing.

Declaration of Competing Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

Ahemad, M., Kibret, M., 2014. Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. J. King Saud Univ. Sci. 26, 1–20. https://doi.org/10.1016/j.jksus.2013.05.001.

Bagyaráj, D.J., Sharma, M.P., Maiti, D., 2015. Phosphorus nutrition of crops through arbuscular mycorrhizal fungi. Curr. Sci. 108, 1288–1293.

Begum, N., Qin, C., Ahanger, M.A., Rana, S., Khan, M.I., Ashraf, M., Ahmed, N., Zhang, L., 2019. Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. Front. Plant Sci. 10, 1068. https://doi.org/10.3389/fpls.2019.01068.

Chen, Y.P., Sekha, P.D., Aran, A.B., Shen, F.T., Lai, W.A., Young, C.C, 2006. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl. Soil Ecol. 34, 33–41. https://doi.org/10.1016/j.apsoil.2005.12.002.

Chemin, L., Yen, C.H, 1951. Turbidimetric determination of available sulphates. Soil Sci. Soc. Am. J. 15, 149–151. https://doi.org/10.2136/sssaj1951.0361599500150001C0032x.

Deveau, A., Brudé, C., Palin, B., Campmartin, D., Rubini, P., Garbaye, J., Sarniguet, A., Frey-Klett, P., 2010. Role of fungal trehalose and bacterial thiamine in the improved survival and growth of the ectomycorrhizal fungus Laccaria bicolorS23BN and the helper bacterium Pseudomonas fluorescensB6c848. Environ. Microbiol. Rep. 2, 566–568. https://doi.org/10.1111/j.1758-2229.2010.00145.x.

Endreze, G., Somodi, I., Kalapos, T., 2013. Arbuscular mycorrhizal colonisation of roots of grass species differing in invasiveness. Commun. Ecol. 14, 67–76. https://doi.org/10.1556/comec.14.2013.1.8.

Frey-Klett, P., Garbaye, J., Tarkka, M., 2007. The mycorrhiza helper bacteria revisited. New Phytol. 176, 22–36. https://doi.org/10.1111/j.1469-8137.2007.02191.x.

Garcia, K., Zimmermann, S.D, 2014. The role of mycorrhizal associations in plant water stressed bahia grass is an attractant for individual plants through to ecosystems. Plant Soil 386, 1–19. https://doi.org/10.1007/s11104-014-2162-1.

Horii, S., Matsumura, A., Kuramoto, M., Ishii, T., 2009. Tryptophan dimer produced by Pseudomonas synxantha. World. J. Microbiol. Biotechnol. 25, 1207–1215. https://doi.org/10.1007/s11299-010-0361-5.

Jackson, M.L., 1973. Soil Chemical Analysis. Prentice Hall of India Pvt. Ltd., New Delhi, p. 498.

Janos, D.P., Schroeder, M.S., Schaffer, B., Crane, J.H, 2001. Inoculation with arbuscular mycorrhizal fungi. Curr. Sci. 108, 1288–1293.

Jones, D.L., Oburger, E., et al., 2011. Solubilization of phosphorus by soil rhizobacteria: current perspective. J. King Saud Univ. Sci. 25, 1207–1215. https://doi.org/10.1016/s1124-6099(10)00039-9.

Kour, D., Rana, K.L., Kaur, T., Yadav, N., Halder, S.K., Yadav, A.N., Sachan, S.G., Saxena, A.K, et al., 2020. Potassium solubilizing and mobilizing microbes: Biodiversity, mechanisms of solubilization and biotechnological implication for alleviations of abiotic stress. In: Rastegari, et al. (Eds.), New and Future Developments in Microbial Biotechnology and Bioengineering: Trends of Microbial Biotechnology for Sustainable Agriculture and Biomedicine Systems: Diversity and Functional Perspectives. Elsevier, pp. 177–202. https://doi.org/10.1016/B978-0-12-820526-6.00012-9.

Fig. 4. Effect of AM fungi (Glomus intraradices) and PGPR (Pseudomonas jessenii strain R62 and Pseudomonas sypyancha strain R81) on micronutrient content [Zn (A), Cu (B), Fe (C) and Mn (D)] in the leaves of the litchi air-layers under two different stages of propagation. The vertical bar denotes standard error (n = 8) followed by the same letter are not significant at p ≤ 0.05.
Kumar, V., Anal, A.K.D., Nath, V., 2018. Growth response of litchi to arbuscular mycorrhizal co-inoculation with Trichoderma viride, Azotobacter chroococcum and Bacillus megaterium. Indian Phytopathol. 71, 65–74. https://doi.org/10.1007/s42360-018-0010-6.

Kumar, V., Kumar, A., Anal, A.K.D, 2019. Mortality of air-layered plants of litchi (Litchi chinensis) and histopathology of affected roots in Bihar, India. Indian J. Agric. Sci. 89, 168–170.

Kurth, F., Feldhahn, L., Bonn, M., Herrmann, S., Buscot, F., Tarkka, M.T. 2015. Large scale transcriptome analysis reveals interplay between development of forest trees and a beneficial mycorrhiza helper bacterium. BMC Genom. 16, 658. https://doi.org/10.1186/s12864-015-1856-y.

Labbe, J.L., Weston, D.J., Dunkirk, N., Pelletier, D.A., Tuskan, G.A, 2014. Newly discovered endophytes in Gnetum mobilis and Pseudotsuga menziesii are capable of producing ACC-deaminase may mitigate salt stress in wheat. Soil Sci. Soc. Am. J. 74, 533–542. https://doi.org/10.2136/sssaj2008.00420.

Maillet, F., Poinsot, V., Andradas, P., Kaiser, F., Adholeya, A., Singh, R., Uppal, H.S., Sharma, A.K., Srivastava, P.C., Kumar, S., Sharma, A.K., 2016. Scale transcriptome analysis reveals interplay between development of forest trees and a beneficial mycorrhiza helper bacterium. BMC Genom. 16, 658. https://doi.org/10.1186/s12864-015-1856-y.

Mader, P., Kainer, F., Adholeya, A., Singh, R., Uppal, H.S., Sharma, A.K., Srivastava, R., Sahai, V., Aragno, M., Wiemken, A., Johri, B.N., Fried, P.M, 2011. Inoculation of root microorganisms for sustainable wheat-rice and wheat-black gram rotations in India. Soil Biol. Biochem. 43, 609–619. https://doi.org/10.1016/j.soilbio.2010.11.031.

Maillot, F., Poinot, V., André, O., Puech-Pages, V., Haouy, A., Gueunier, M., Croma, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E.A., Driguez, H., Bader, P., Kaiser, F., Adholeya, A., Singh, R., Uppal, H.S., Sharma, A.K., Srivastava, P.C., Kumar, S., Sharma, A.K., 2016. Scale transcriptome analysis reveals interplay between development of forest trees and a beneficial mycorrhiza helper bacterium. BMC Genom. 16, 658. https://doi.org/10.1186/s12864-015-1856-y.

Miller, S.H., Browne, P., Priegent-Combaret, C., Combes-Meynet, E., Morrissey, J.P., 2012. Phytohormone production and solubilization in Pseudomonas species. Environ. Microbiol. Rep. 4, 403–411. https://doi.org/10.1111/j.1758-2229.2009.00105.x.

Nadeem, S.M., Zahair, Z.A., Naveed, M., Asghar, H.N., Asghar, M., 2010. Rhizobacteria capable of producing ACC-deaminase may mitigate salt stress in wheat. Soil Sci. Soc. Am. J. 74, 533–542. https://doi.org/10.2136/sssaj2008.0240.

Pallai, R., Hynes, R.K., Verma, B., Nelson, L.M, 2012. Phytohormone production and colonization of canola (Brassica napus L.) roots by Pseudomonas fluorescens 6-8 under gnotobiotic conditions. Can. J. Microbiol. 58, 170–178. https://doi.org/10.1139/11-120.

Pamidhe, M., 2005. Plant–fungal associations: cue for the branching connection. Nature 435, 750–751. https://doi.org/10.1038/435750a.

Pichardo, S.T., Su, Y., Han, F.X, 2012. The potential effects of Arbuscular Mycorrhizae (AM) on the uptake of heavy metals by plants from contaminated soils. J. Bioremed. Biodeg. 3, e124. https://doi.org/10.4172/2155-6199.1000e124.

Plett, J.M., Martin, F., 2012. Mutualistic effectors: architects of symbiosis. In: Martin, F, Kamoun, S (Eds.), Effectors in Plant-Microbe Interactions. Wiley, Hoboken, pp. 295–326. https://doi.org/10.1002/9781119949138.ch12.

Rashid, M.I., Majawara, L.H., Shahzade, T., Almeebdia, T., Islamia, M.I., Ovesaa, M., 2011. Bacteria and fungi can contribute to nutrients bioavailability and aggregate formation in degraded soils. Microbiol. Res. 183, 26–41. https://doi.org/10.1016/j.micres.2015.11.007.

Sharma, S.D., Kumar, P., Raj, H., Bhardwaj, S.K, 2009. Isolation of arbuscular mycorrhizal fungi and Azotobacter chroococcum from local litchi orchards and evaluation of their activity in the air-layers system. Sci. Hortic. 123, 117–123. https://doi.org/10.1016/j.scienta.2009.07.019.

Shinde, S., Zerbs, S., Collart, F.R., Cumming, J.R., Noiroit, P., Larsen, P.E, 2019. Pseudomonas fluorescens increases mycorrhization and modulates expression of antifungal defence response genes in roots of aspen seedlings. BMC Plant Biol. 19, 4. https://doi.org/10.1186/s12870-018-1610-0.

Shu, B., Li, W., Liu, L., Wei, Y., Shi, S., 2016. Transcriptomes of arbuscular mycorrhizal fungi and litchi host interaction after tree girdling. Front. Microbiol. 7, 408. https://doi.org/10.3389/fmicb.2016.00408.

Singh, B., Chadha, K.I., 2009. Standardization of leaf sampling technique in litchi. Indian J. Hortic. 66, 445–448.

Singh, G., Nath, V., Pandey, S.D., Ray, P.K, 2011. Good Management Practices in litchi. Ministry of Agriculture, Gol, National Research Centre for litchi, Bihar, India. 30.

Smith, S.E., Read, D.J, 2008. Mycorrhizal Symbiosis. Academic Press, London.

Snedecor, G., Cochran, W.G, 1967. Statistical Methods. Oxford and IBH Publ. Co, New Delhi.

Visen, A., Bohra, M., Singh, P.N., Srivastava, P.C., Kumar, S., Sharma, A.K., Chakraborty, B., 2017. Two pseudomonad strains facilitate AMF mycorrhization of litchi (Litchi chinensis Sonn.) and improving phosphorus uptake. Rhizosphere 3, 196–202. https://doi.org/10.1016/j.rhisp.2017.04.006.