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

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ABSTRACT

The efficacy of two plant growth promoting rhizobacteria (PGPR) viz. Pseudomonas jessenni strain R62 and Pseudomonas synxantha 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-

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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 mycorrhization 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 (Ahemad 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 air-layers just before the final planting in order to supply healthy saplings for successful litchi orcharding.

2. Materials and methods

2.1. Preparation of litchi air-layers

A Litchi orchard (cv. Rose Scented) having 20 years old trees located at Horticulture Research Centre (G.B. Pant University of Agriculture and Technology, Pantnagar-India, 29° 02′ N and 79° 49′ E) was selected for the experiment. Healthy, pen-to-pencil thickness terminal litchi branch receiving good sunshine were identified for preparation of air-layers. A ring of bark measuring 2.50 cm in length was removed without disturbing the cambium layer about 40-45 cm below the apex 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 given 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 CO_2 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 Endresz et al. (2013). All the

Table 1
Detail of inoculation treatments.

Inoculants	Quantity applied
Control (without inoculation)	-
Only AM fungi (*Glomus intraradices*)	100 IP
Only PGPR (*Pseudomonas jessenni* strain R62 and *Pseudomonas synxantha* strain R81)	10^6 cfu g^{-1}
AM fungi (*Glomus intraradices*) and PGPR (*Pseudomonas jessenni* strain R62 and *Pseudomonas synxantha* strain R81)	100 IP + 10^6 cfu g^{-1}

Table 2
Physical properties and nutrient composition of soil used for root-trainers.

EC (dSm^{-1})	pH	Organic C (%)	N ($kg\ ha^{-1}$)	P	K	Ca	Mg	S	Cu ($mg\ ha^{-1}$)	Fe	Mn	Zn
0.13	6.4	1.12	141	10.7	47.5	14.5	38	6.5	1.24	13.2	3.16	3.3

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

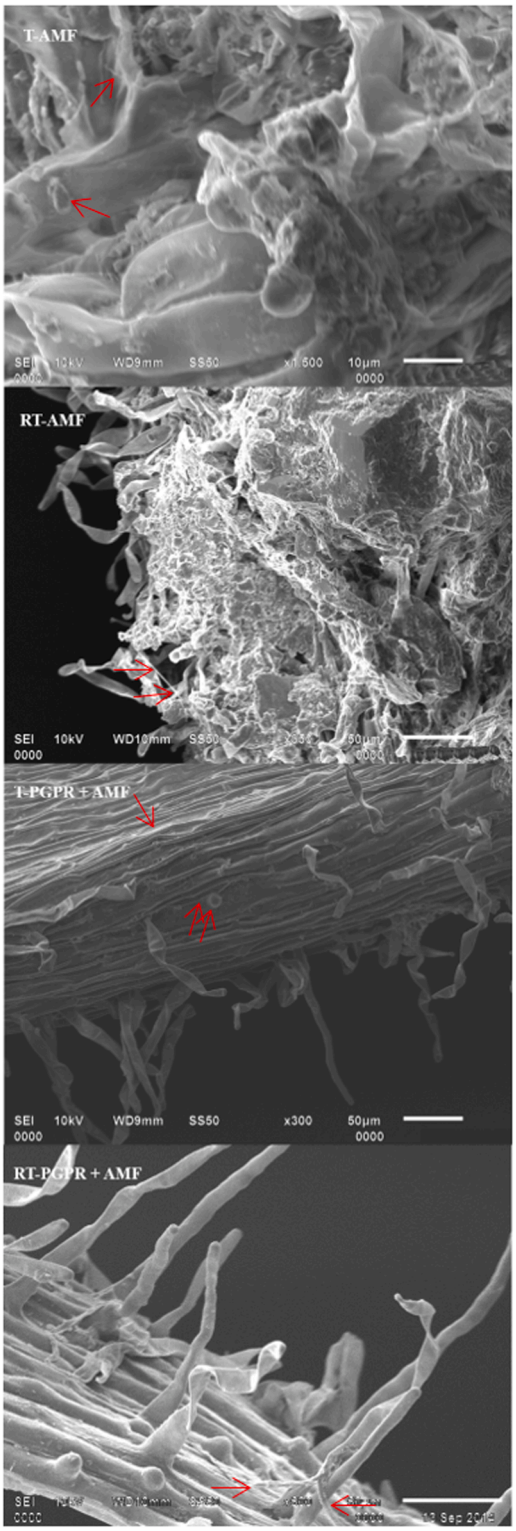

Fig. 1. Scanning electron microscopy of AM fungi (AMF) and AM fungi + *Pseudomonas jessenni* strain R62 and *Pseudomonas synxantha* strain R81 (AMF + PGPR) treated litchi roots [T= Tree inoculated and RT= Root Trainer].

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 uninoculated 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

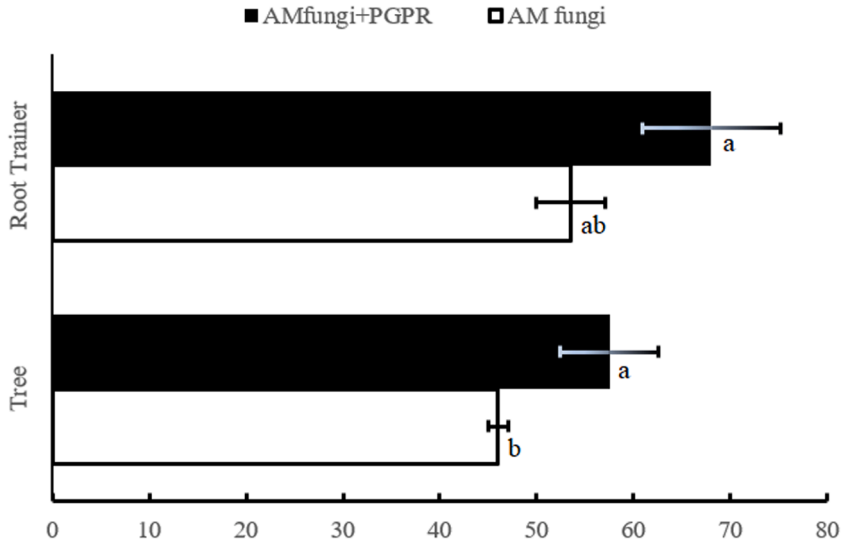

Fig. 2. AM fungi colonisation (%) in the litchi root. The vertical bar denotes standard error ($n = 8$) followed by the same letter are not significant at $p \leq 0.05$.

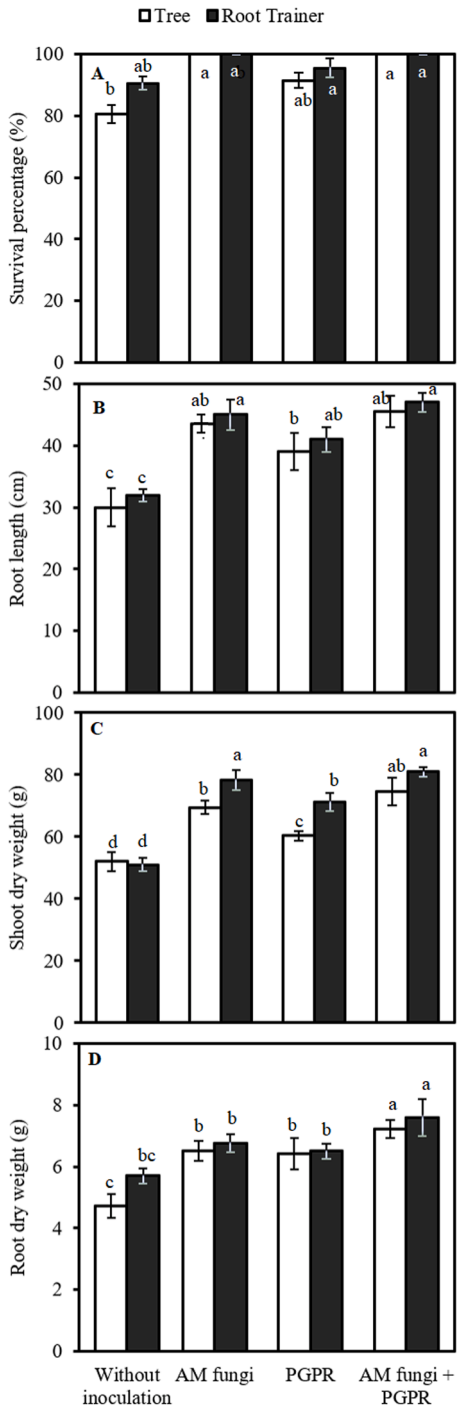

Fig. 3. Effect of AM fungi (*Glomus intraradices*) and PGPR (*Pseudomonas jessenni* strain R62 and *Pseudomonas synxantha* strain R81) on survival percentage (A), root length (B), shoot dry weight (C) and root dry weight (D) of the litchi air-layers under two different stages of propagation. The vertical bar denotes standard error ($n = 8$) followed by the same letter are not significant at $p \leq 0.05$.

(Plett and Martin, 2012). In addition, the plant defence related JA (jasmonic acid) signalling is also down regulated by the mycorrhizal formation (Kurth et al., 2015) and modulation of such functions occurs to be a higher extent in the presence of *Pseudomonas fluorescence* as reported in aspen seedlings (Shinde et al., 2019). The *Pseudomonas* strains might act as a mediator in all these interactions and influencing both the plant root and fungus for colonisation. The secondary metabolites such as auxofuran produced by the bacteria is known to stimulate the mycorrhizal formation (Kinkel et al., 2011). However, such metabolites produced by the *Pseudomonas* species is yet to be discovered. The *Pseudomonas* is able to solubilize inorganic phosphates in soil which also favours the plant-fungus physical contact by stimulating the lateral root formation. Labbe et al. (2014) found that *Pseudomonas* regulates strain-specific gene expression which helps in metabolic restructuring of the fungus in favour of mycorrhization. Scientific evidences have also confirmed that thiamine produced by *Pseudomonas fluorescens* BBc6R8 promotes the fungal growth *in vitro* (Deveau et al., 2010). Increased root colonisation of AM fungi with the use of *Pseudomonas fluorescence* have already been documented in aspen seedling (Shinde et al., 2019). The results of this experiment also suggested that Pseudomonas jessenni 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 *Psuedomonas* 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

Table 3
Effect of AM fungi (*Glomus intraradices*) and PGPR (*Pseudomonas jessenni* strain R62 and *Pseudomonas synxantha* strain R81) on primary nutrient content in the leaves of litchi air-layers under two different stages of propagation.

Inoculant	Nitrogen (%)		Phosphorous (%)		Potassium (%)	
	Tree	Root Trainer	Tree	Root Trainer	Tree	Root Trainer
Without inoculation	1.32 ± 0.04^{f}	1.34 ± 0.01^{f}	$1.36 \pm 0.01 \times 10^{-1e}$	$1.44 \pm 0.02 \times 10^{-1e}$	$6.30 \pm 0.12 \times 10^{-1ab}$	$6.50 \pm 0.01 \times 10^{-1a}$
AM fungi	1.50 ± 0.02^{de}	1.66 ± 0.16^{b}	$1.54 \pm 0.02 \times 10^{-1ab}$	$1.76 \pm 0.01 \times 10^{-1a}$	$6.55 \pm 0.02 \times 10^{-1b}$	$6.84 \pm 0.02 \times 10^{-1a}$
PGPR	1.55 ± 0.24^{d}	1.60 ± 0.04^{c}	$1.45 \pm 0.02 \times 10^{-1b}$	$1.55 \pm 0.11 \times 10^{-1ab}$	$6.50 \pm 0.03 \times 10^{-1b}$	$6.70 \pm 0.02 \times 10^{-1a}$
AM fungi + PGPR	1.52 ± 0.02^{d}	1.70 ± 0.02^{a}	$1.55 \pm 0.03 \times 10^{-1ab}$	$2.05 \pm 0.02 \times 10^{-1a}$	$6.65 \pm 0.01 \times 10^{-1ab}$	$7.15 \pm 0.10 \times 10^{-1a}$

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

Table 4
Effect of AM fungi (*Glomus intraradices*) and PGPR (*Pseudomonas jessenni* strain R62 and *Pseudomonas synxantha* strain R81) on secondary nutrient content in the leaves of litchi air-layers under two different stages of propagation.

Inoculant	Ca (%)		Mg (%)		S (%)	
	Tree	Root Trainer	Tree	Root Trainer	Tree	Root Trainer
Without inoculation	1.16 ± 0.02^{a}	1.20 ± 0.01^{a}	$2.52 \pm 0.03 \times 10^{-1a}$	$2.55 \pm 0.12 \times 10^{-1e}$	$5.85 \pm 0.01 \times 10^{-2a}$	$5.75 \pm 0.10 \times 10^{-2a}$
AM fungi	1.17 ± 0.04^{a}	1.21 ± 0.01^{a}	$2.53 \pm 0.02 \times 10^{-1a}$	$2.71 \pm 0.04 \times 10^{-1a}$	$5.70 \pm 0.05 \times 10^{-2a}$	$5.70 \pm 0.02 \times 10^{-2a}$
PGPR	1.16 ± 0.01^{a}	1.23 ± 0.03^{a}	$2.55 \pm 0.11 \times 10^{-1a}$	$2.75 \pm 0.03 \times 10^{-1a}$	$5.65 \pm 0.03 \times 10^{-2a}$	$5.95 \pm 0.06 \times 10^{-2a}$
AM fungi + PGPR	1.18 ± 0.03^{a}	1.23 ± 0.02^{a}	$2.57 \pm 0.02 \times 10^{-1a}$	$2.73 \pm 0.05 \times 10^{-1a}$	$6.00 \pm 0.02 \times 10^{-2a}$	$6.20 \pm 0.03 \times 10^{-2a}$

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

litchi air-layers. The improvement of leaf N content in PGPR inoculated air-layers might be attributed to ammonia production by the *Pseudomonas* strains. An increase in raw protein content in wheat grains indicating improvement in N-status of plant with the inoculation of same *Pseudomonas* strains has been reported earlier (Mäder 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 phosphates by carboxyl and hydroxyl group of these weak acids (Miller et al., 2010) and exchange reactions with P at adsorption site of soil by anions of these low molecular weight acids (Jones and Oburger, 2011). This PGPR also releases a variety of organic compounds to the rhizosphere such as oxalic acid and lactic acid which are thought to be responsible for dissolving the rock K or chelated silicon ions to bring the K into rhizosphere and resulted enhanced K uptake in plants (Kour et al., 2020). AM fungi are reported to be capable of absorbing free amino acids and various organic N-containing compounds from soil and resulting higher N content in host plants (Hodge and Storer, 2015). Further, AM fungi provides plant derived C and P for the non-symbiotic N_2 fixers and thus indirectly improves N_2-fixation in rhizosphere (Jones and Oburger, 2011). The P acquisition ability of AM fungi has been well established (Bagyaraj et al., 2015). AM fungi absorbs P through a well-developed hyphal network beyond P depletion zone from the root surface which permits for the scavenging of spatially available P (Bagyaraj et al., 2015). Potassium is considered as an 'indirect-trophic' element under AM fungi symbiosis. Since, a strong positive correlation has been demonstrated between K^+ and phosphorus (P) nutrition during this type of symbiosis (Garcia and Zimmermann, 2014). However, organic acids such as oxalate, malate, citrate secreted by AM fungi might also take part in the K solubilization process. Further, this process is also enhanced by release of H^+ and CO_2 by the AM fungi (Rashid et al., 2011). The translocation, mineralization and mobilization of NPK in plants were further enhanced when air-layers were co-inoculated with PGPR and AM fungi. The basic mechanisms involved in bioavailability of NPK under bacteria-fungi interactions are N fixation, P and K mobilization through production of organic acids (Rashid et al., 2011). Since, *Pseudomonas* strains positively influenced the mycorrhization of the AM fungi in litchi roots and therefore, increased the effectiveness of nutrient availability for the host plant. The same *Pseudomonas* strains are reported to be enhanced the NPK content of wheat plants when co-inoculated with AM fungi (Mäder et al., 2011). No significant improvement in leaf secondary nutrient (Ca, Mg and S) content in the leaves of litchi air-layers was gained with the inoculation of either PGPR or AM fungi or both. However, previous report indicated a lower concentration of Ca and Mg in the litchi leaves with the use of AM fungi (Janos et al., 2001).

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 (Pichardo 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 (Pichardo 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 (Deveau et al., 2010). Improvement in leaf Mn content with the use of *Pseudomonas* is also reported in melon (Martínez 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

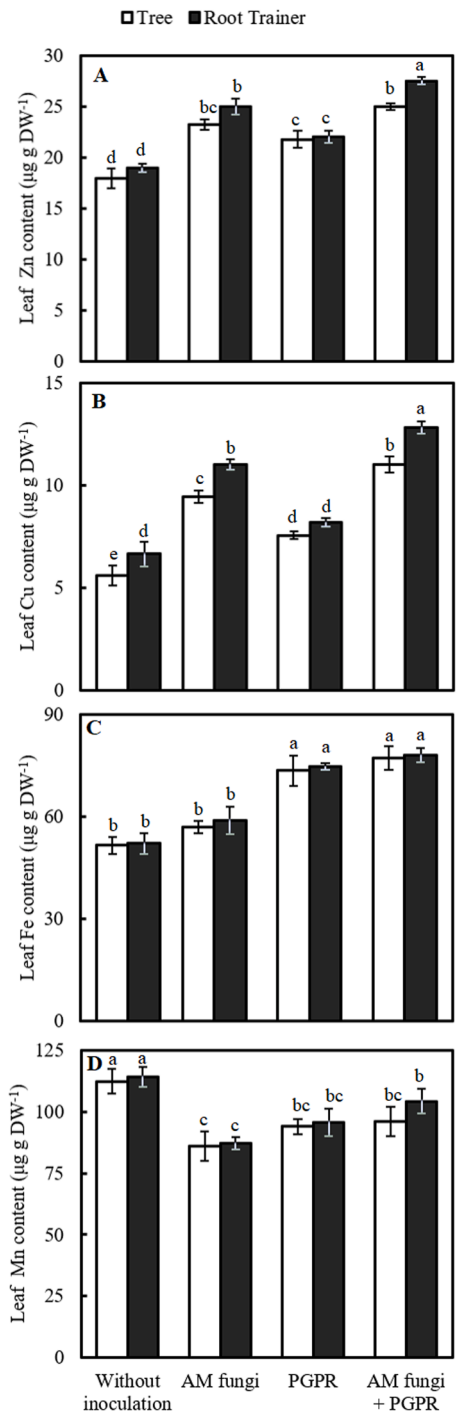

Fig. 4. Effect of AM fungi (*Glomus intraradices*) and PGPR (*Pseudomonas jessenni* strain R62 and *Pseudomonas synxantha* strain R81) on micronutrient content [Zn (A), Cu (B), Fe (C) and Mn (D)] in the leaves of the litchi air-layers under two different stages of propagation. The vertical bar denotes standard error (n = 8) followed by the same letter are not significant at $p \leq 0.05$.

carbohydrate molecules in the girdled portion of the branch might downregulated the process of mycorrhization (Shu et al., 2016). Microbial inoculation after detachment of litchi air-layers from mother tree is reported to be more beneficial than inoculation at the time of air-layering process (Visen et al., 2017).

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 Bisht:** Writing – review & editing.

Declaration of Competing Interest

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

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