PLANT SCIENCE TODAY
Vol x(x): xx–xx
https://doi.org/10.14719/pst.10755

eISSN 2348-1900

RESEARCH ARTICLE

Application of root endophytes *Piriformospora indica* (*Serendipita indica*) and arbuscular mycorrhizal fungus *Glomus mosseae* enhances nutrient acquisition, growth and yield in cassava

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Received: 19 July 2025; Accepted: 14 October 2025; Available online: Version 1.0: 10 December 2025

Cite this article: Subhash AP, Ameena M, Suja G, Natarajan C, Rajan AS, Soumya VI, John J, Anith KN. Application of root endophytes *Piriformospora indica* (*Serendipita indica*) and arbuscular mycorrhizal fungus *Glomus mosseae* enhances nutrient acquisition, growth and yield in cassava. Plant Science Today (Early Access). https://doi.org/10.14719/pst.10755

Abstract

The study explores the application of the root endophytes *Piriformospora indica* and the arbuscular mycorrhizal fungus (AMF) *Glomus mosseae* as biofertilizers to enhance cassava growth and productivity. The successful colonization of cassava roots by *P. indica* and AMF was detected, confirming their effective association with the host plant. Root growth parameters were found to be significantly increased in the cassava plants colonized by *P. indica* when compared to uninoculated control plants. In a field study, the combination of 75 % of the recommended NPK dose along with *P. indica* was identified as the most effective treatment for enhancing growth and yield attributes. In contrast, the combination of 75 % of the NPK dose along with AMF showed the most significant improvement in total dry matter content and tuber quality parameters. The results showed that the nutrient dose can be reduced to a level of 75 % of the recommended dose, without compromising the growth and yield, if the chemical fertilizers are applied along with the bio-inoculants, either *P. indica* or AMF. The same treatments also resulted in higher total nitrogen uptake, while the combination of 100 % NPK and AMF resulted in better phosphorus and potassium uptake by the cassava plants. This is the first report demonstrating root colonization, growth promotion and improved nutrient acquisition in cassava following inoculation with the beneficial root endophyte *P. indica*.

Keywords: bio-inoculants; nutrient uptake; plant growth enhancement; root colonization; tapioca

Introduction

Climate change exacerbates abiotic stresses including nutritional deficiencies, leading to more frequent and severe events that challenge crop resilience in many agricultural crops. Root and tuber crops such as cassava, yams and sweet potatoes have shown notable resilience under tropical conditions. In the coming decades, promoting the cultivation of these crops can enhance and ensure food security, provide nutritional benefits and support local economies. Additionally, they can be vital in diversifying diets and improving livelihoods in rural communities. However, low productivity of these crops is an important issue that still needs to be addressed.

Cassava (*Manihot esculenta* Crantz), also known as Tapioca, a native to South America, is a woody perennial shrub belonging to the spurge family, Euphorbiaceae and mostly cultivated by small-scale farmers for its edible storage roots. Traditionally, it is one of the important food staples of the tropics. However, even under ideal growing conditions, its average yield (13 tonnes ha^{-1}) has been found to be below its productive potential, which can be up to 80 tonnes ha^{-1} (1). The low productivity is assigned to several unfavourable biotic and abiotic factors. This yield gap is attributed to several unfavourable biotic and abiotic factors. Intensive cassava cultivation often leads to rapid soil fertility depletion, requiring up to seven years of fallow to restore nitrogen and phosphorus levels (2).

The cassava crop is highly responsive to manures and fertilizers; however, the indiscriminate use of inputs especially chemical fertilizers can result in adverse environmental impacts. Excessive and constant usage of chemical fertilizers may degrade soil health and quality, affect rhizosphere micro-environment and cause nutrient imbalances (3). These adverse effects can be reduced by adopting new microbial-based technological practices, especially the use of biofertilizers, which are renewable and ecofriendly alternatives to chemical fertilizers that promote the growth of plants by enhancing the availability of plant nutrients (4). They also play a crucial role in the soil fertility conservation through nutrient recycling and energy transfer (5). Low input management strategies that reduce the fertilizer dosage and hence the cost of cultivation has become part of sustainable agricultural practices.

Piriformospora indica, a helper mycorrhiza like root colonizing endophytic Agaricomycetes fungus belonging to the order Sebacinales (Basidiomycota), was obtained from the rhizosphere of *Zizyphus nummularia* and *Prosopis juliflora*, woody shrubs in the Thar Desert in Northwest India. The fungus has recently gained significant attention due to its enormous abilities to promote plant growth efficiently, acquire nutrients and augment plant resilience against various environmental stresses (6-12). *P. indica* is known to colonize a wide spectrum of crop species, including various agronomical, horticultural, medicinal crops and ornamental plants (13-15). Numerous studies have reported its ability to stimulate growth, accelerate nutrient uptake and increase stress tolerance, making it a promising candidate for sustainable agricultural practices (16).

P. indica mediated root colonization leads to better nutrient-acquisition as well as improvement in plant growth and productivity as a result of enhanced root proliferation due to indole-3-acetic acid production, thereby providing better plant performance in all aspects (17). In many medicinal plant species, the application of *P. indica* leads to significant improvement in their growth and yield (13, 18). Improvement in growth, yield, piperine and oleoresin content of the black pepper berries has also been noted on inoculation with *P. indica* (14). Colonization further enhances nitrogen and phosphorus uptake in various crops (19, 20) and facilitates sulfur and phosphorus solubilization, similar to AMF (21). Our earlier work demonstrated its growth-promoting and biocontrol activity against taro leaf blight (22). However, its potential to improve productivity and nutrient acquisition in tropical tuber crops such as cassava remains unexplored.

AMF belonging to the Glomeromycota phylum, are predominant beneficial soil microorganisms with mutualistic interactions with the host plants in terms of providing the host plants with water and essential nutrients (23). Cassava has been reported as a highly mycorrhizal plant earlier (2, 24). Successful AMF colonization has been shown to enhance cassava growth and yield under field conditions (2) and to increase chlorophyll production under both well-watered and water-stressed conditions (24).

Many reports suggest that the AMF symbiosis with its host plant's roots helps to obtain essential macronutrients such as N, P, K, Ca and S as well as micronutrients like Cu and Zn. They can efficiently improve the host plant's growth by increasing the surface absorbing capability of roots which leads to enhanced production of photosynthates and accumulation of biomass (23, 25). In addition to this nutritional function, plant's tolerance to both biotic and abiotic stresses was also found to be enhanced (26, 27).

In this study, we report on the growth-promoting effects and enhanced nutrient acquisition in cassava following inoculation with the root endophytes *P. indica* and the AMF *Glomus mosseae*.

Materials and Methods

Cultivation of *P. indica* and AMF and inoculum preparation

P. indica was obtained from Dr. Ajit Varma (Jawaharlal Nehru University, New Delhi, India) and maintained on potato dextrose agar (PDA) at 28 °C. For mass multiplication, mycelial discs from freshly grown cultures were inoculated into sterile potato dextrose broth (PDB, pH 6.5) and incubated at 28 ± 2 °C with constant shaking (90 rpm) for 15 days. After incubation, the broth was filtered through muslin cloth, the mycelium was washed three times with sterile distilled water and the fresh weight was recorded. The inoculum was incorporated into the planting medium at 1 % (w/v).

AMF G. *mosseae* was obtained from the culture collection of the Department of Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. Mass multiplication of fungus was done by maintaining the purified isolate of *G. mosseae* by growing it in the Guinea grass roots in vermiculite perlite-based medium, which was prepared by mixing vermiculite, perlite, farm yard manure (FYM) and soil in the ratio 60:10:5:5. The medium was filled in cement tanks (100 x 100 x 45 cm dimension) and sterilized using formaldehyde (12). For this, small pits were made in the mixture at 10 cm intervals and 30 mL of formaldehyde (2.5 L/tank) was added in each pit. The tank was then covered with a tarpaulin sheet for 10 days and later kept open for 7 days for the complete evaporation of formaldehyde. The isolated single spores of *G. mosseae* were deposited into the planting pits (15 x 15 cm spacing) at a depth of 5-10 cm and Guinea grass slips (5 slips/pit) were planted over the spores in each pit, so that when the roots were formed, it came into intimate contact with the fungal inoculum and thus multiplied.

Colonization of *P. indica* in cassava roots

Multiplication of *P. indica* was done in PDB medium and mycelium was incorporated at 1 % (w/v) in the planting medium as described above. Preparation of the planting medium was done by mixing coir pith and farmyard manure in a 3:1 ratio and sterilization was done by autoclaving for 1 hr each at 121 °C for three successive days. Minisetts of Sree Jaya, Sree Suvarna and Aswathy (local) varieties of cassava stem (2 nodes) were planted in protrays (50 cells capacity, having a cell size of 3x 3 x 3 cm) filled with the planting medium in the glass house. The plants were irrigated twice daily and after two weeks, the roots were excavated and examined for the presence of chlamydospores microscopically. Light microscopy using trypan blue staining as well as confocal imaging using WGA-AF 488 (Wheat germ agglutinin- Alexa flour 488) staining were done (12, 22). Further confirmation was done by molecular detection with the help of the PiTEF gene primer using a PCR reaction.

Trypan blue staining

Staining of roots was done as per the procedure described in a previous study (14). The excavated roots were first washed thoroughly in running tap water to remove the adhering planting medium and were cut into small fragments of one cm length. These root pieces were then softened by boiling in freshly prepared 10 % (w/v) potassium hydroxide (KOH) for 10 min. Followed by washing with distilled water thrice, these bits were then acidified for 5 min with 1 N HCl and were transferred to the lactophenol-trypan blue staining solution for 10 min. Finally, destaining was carried out in lactophenol solution for 10 min. Pieces of roots were placed on a slide, a coverslip placed over them and observed under a compound bright field light microscope (Olympus-U-CMAD3, Japan). The presence of chlamydospores represents a positive indication of root colonization by *P. indica*. The *P. indica* root colonization percentage was calculated using the formula (12).

$$\text{Percentage root colonization} = \frac{\text{No. of root bits with chlamydospores}}{\text{Total no. of root bits observed}} \times 100$$

Confocal imaging

P. indica treated plant roots as well as roots of control plants without inoculation were collected, washed thoroughly in running tap water and cut into small fragments of one cm length. These root bits were then fixed using 0.15 % (w/v) trichloroacetic acid (TCA) in 4:1 (v/v) ethanol/chloroform. Followed by washing with 1X phosphate buffer saline (PBS, pH 7.4) for 5 min, these root bits were boiled with 10 % KOH for 1 min, followed by neutralization in 1X PBS. Further, these bits were transferred to 100 µg mL^{-1} WGA-AF 488 stain (Invitrogen, Oregon, USA) dissolved in 1X PBS (pH 7.4) staining solution. These root bits were then destained in 1X PBS by incubating overnight. The conjugated WGA-AF 488 stain was excited at 488 nm wavelength and detected at 500—600 nm using confocal laser imaging on a multichannel TCS SP2 confocal system (Leica Microsystems, Germany), available at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram (22).

Molecular detection of P. indica in cassava roots

Fresh roots were collected after one month of planting from both *P. indica* inoculated Sree Suvarna and Sree Jaya plants as well as their control counterparts without *P. indica* inoculation. Using the CTAB method, total genomic DNA was isolated from the roots. CTAB buffer with 0.2 % β-mercaptoethanol and 2 % PVP was warmed at 60 °C for 5 min in a water bath. Using a mortar and pestle, 100 mg of root sample were ground to a fine powder with the help of liquid nitrogen and 1 mL of pre-warmed CTAB buffer was also added and mixed. The suspension was transferred to a 2 mL sterile microcentrifuge tubes and was incubated at 60 °C for 30 min in a water bath, followed by centrifugation (13000 rpm, 10 min RT). After transferring the supernatant to new tubes, an equal volume of chloroform: isoamyl alcohol mixture (24:1 ratio) was added and mixed by inversion. Further, these tubes were centrifuged (13000 rpm, 10 min) in a 4 °C pre-cooled centrifuge and the aqueous phase was transferred to new 1.5 mL centrifuge tubes and 0.8 x the volume of ice-cold isopropanol was added. The tubes were then incubated at -20 °C for 40 min. Further these tubes were centrifuged (13000 rpm, 15 min, 4 °C) and 500 µL 70 % ethanol was added to the pellet after discarding the supernatant and was mixed by tapping. Ethanol wash was done by centrifugation (13000 rpm, 10 min, 4 °C), followed by discarding the supernatant and the pellet was air-dried. Further, the pellet was dispersed by adding 30 µL nuclease-free water and was stored at -20 °C. Agarose gel electrophoresis (BioRad, USA) was performed with 0.8 % gel to visualise the isolated DNA. The concentration and purity of the isolated DNA were also determined using a NanoDrop 2000C spectrophotometer (Thermo Scientific, USA). Using species specific primer, DNA of *P. indica* was amplified as described previously (28). The reaction mix was optimized. The used primer sequences are listed below.

PiTEF forward primer: 5'TCGTCGCTGTCAACAAGATG3'

PiTEF reverse primer: 5'GAGGGCTCGAGCATGTTGT3'

Polymerase chain reaction (PCR) was done in a BioRad C1000 Touch thermal cycler using the following PCR programme: initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 sec (denaturation), 55 °C for 1 min (annealing) and 72 °C for 1 min 30 sec (extension); a final extension at 72 °C for 8 min. A reaction of total volume 20 µL was set up using 10 µL Taq DNA Polymerase master mix, 2 µL forward primer, 2 µL reverse primer, 1 µL template DNA and remaining with nuclease free water, mixed by gentle vortexing and the products were loaded in a 1.5 % agarose gel containing 3 µL ethidium bromide for visualisation, with the GeneRuler 1 kbPlus DNA ladder (ThermoScientific, USA) and using the Gel Doc System (BioRad, USA), gel image was documented.

Root initiation by *Piriformospora indica* inoculation in cassava

Minisetts (2 nodes) of cassava varieties Sree Jaya and Aswathy were raised in plastic cups of 8 cm diameter and 10 cm height, filled halfway with unsterile planting medium (coir pith and cow dung in 3:1 ratio) to analyse root and shoot parameters such as root length, number of basal roots, nodal roots, total number of roots, plant height and leaf count per minisett. *P. indica* application was done as described earlier. Control plants were also kept without *P. indica* inoculation. Twelve replications were maintained for each treatment in a Completely Randomized Design (CRD). The plants were kept in a glass house, irrigated twice daily and after one month they were uprooted to assess various shoot and root growth parameters.

Colonization of AMF in cassava roots

Cassava minisetts (2 nodes) of Sree Jaya, Sree Suvarna and Aswathy varieties were planted in protrays (50 cells capacity, having a cell size of 3 x 3 x 3 cm). Preparation of sterile planting medium was done as described earlier (12). This planting medium amended with the mycorrhizal inoculum at 1 % (w/v) was used for filling the portrays and was kept in the glass house. The root bits of Guinea grass containing the *G. mosseae* culture were used as the inoculum. The plants were irrigated twice daily and after two weeks, the roots were excavated and examined for the presence of vesicles or arbuscules microscopically. Staining of roots for AMF colonization was done as per the standard procedure (29). Minisett roots were initially washed in running tap water to remove the adhering soil particles and cut into 1-2 cm root fragments. These root fragments were softened by boiling in 10 % (w/v) KOH. Further washed with distilled water thrice to remove KOH, followed by acidification with dilute HCl. They were then stained in 0.05 % (v/v) lactophenol-trypan blue for 10 min. Destaining was done using lactophenol to remove the excess stain and observed under a compound bright field microscope. The presence of vesicles and arbuscules was considered evidence of colonization. The root colonization percentage was calculated using the formula (12).

$$\text{Percentage root colonization} = \frac{\text{No. of root bits with vesicles or arbuscules}}{\text{Total no. of root bits observed}} \times 100$$

Nutrient acquisition, plant growth promotion and yield enhancement in cassava

A field experiment was conducted at the ICAR- Central Tuber Crops Research Institute, 8°29' N, 76°57' E, 52 m altitude), Thiruvananthapuram, Kerala, India with the cassava variety Sree Suvarna, as per the Kerala Agricultural University Package of Practices recommendations for cassava cultivation (30). The experimental field was with lateritic soil belonging to the order Ultisol having a slightly acidic pH of 5.5-6.0. The land was cleared, ploughed and ridges were taken mechanically using a tractor drawn rotary tiller. Healthy, disease-free stems of Sree Suvarna variety were cut into 15-20 cm length setts (six nodes) and were used for planting in the ridges, leaving 2-3 nodes above-ground. The experimental design adopted was Randomized Block Design (RBD) with a plot size of 3.6 m x 3.6 m with a plant spacing of 90 cm x 90 cm with 3 replications (4 plants/replication).

Inoculation of *P. indica* and AMF independently with 50 %, 75 % and 100 % of the recommended NPK dose, control with full recommended dose of fertilizer (100 % NPK fertilizers without *P. indica* or AM fungus) and absolute control (without NPK fertilizers, *P. indica* or AMF) were the treatments of the experiment. *P. indica* multiplication was done in PDB and the fungal mycelium was harvested and incorporated at 1 % (w/v) in the planting pits (20 g/pit), prior to the planting of cassava setts. AMF application was done using the root bits of Guinea grass containing the *G. mosseae* culture at 5 g/pit at the time of planting.

Farmyard manure was applied at 12.5 tonnes ha^{-1} during the land preparation, so as to provide about 1 kg of organic manure per plant. The general NPK recommendation for cassava; 100:50:100 kg N, P_2O_5, K_2O ha^{-1} with ½ N + full P + ½ K as basal dose and ½ N + ½ K at 45 days after planting was followed. The different NPK fertilizers, Urea (46 % N), Rajphos (20 % P_2O_5) and Muriate of potash (MOP- 60 % K_2O) at 217:250:167 kg ha^{-1} were used according to the treatment schedule as fertilizer sources in appropriate quantities. One week after planting, the first dose of fertilizers was applied. Plants were irrigated initially for a period of two months as and when required. Removal of dried or unsprouted setts as well as gap filling was done within 15 days after planting (DAP). Excess shoots were removed, and half doses of N and K were given after weeding and earthing up at 45 DAP. Hand weeding was done at 1 month after planting (MAP), 3 MAP and 5 MAP. Harvesting was done manually at 9 MAP.

Biometric and physiological parameters viz. plant height, number of leaves/plants, chlorophyll content, leaf retention, girth of plant, leaf weight/plant and stem weight/plant and quality parameters of tubers such as tuber starch, sugar and crude protein contents were recorded. Leaf retention was calculated based on a scale of 1 to 5, where scale 1 indicates the number of retained leaves within a range of 0-25, scale 2 (25-50), scale 3 (50-75), scale 4 (75-100) and scale 5 (100-125). The yield attributes viz. number of tubers/plant, mean tuber length, mean tuber girth, mean tuber weight, tuber yield/plant, tuber dry matter content and harvest index were noted. Apart from leaf weight/plant and stem weight/plant, all other biometric and physiological parameters were recorded at 3 months intervals (3 MAP, 6 MAP and 9 MAP) whereas quality and yield parameters were noted at the harvest time (9 MAP). Analyses for soil N, P and K status at the initial stage and after harvest of the crop, as well as N, P and K uptake by the plants of the different treatments were recorded. Before initiation of the experiment, composite soil samples were collected separately from different sites of the experimental field and the available NPK status was determined by alkaline potassium permanganate method (31) for available N, Bray colorimetry for available P_2O_5 and extraction using the neutral ammonium acetate method for available K_2O (32). Analyses of soil samples for NPK content after harvest of the crop were also done separately for representative treatment plots. To determine the total N, total P and total K contents in various plant parts such as stems, leaves and tubers, the modified microkjeldahl method, colorimetry (vanadomolybdo phosphoric yellow colour in sulphuric acid) and flame photometry (32) respectively were employed. The contents were calculated and expressed in percentage. The plant NPK uptake was calculated by finding the products of the total nutrient contents in the various plant parts (stems, leaves and tubers) with their respective dry weights and expressed as kg per plant.

Statistical analysis

Statistical analysis was done using the statistical package GRAPES 1.0.0 developed by the Kerala Agricultural University (https://www.kaugrapes.com). The results of root initiation studies were compared using *Student's t* test at 5 % level of significance ($\alpha = 0.05$). The field experiment data were analysed using one-way analysis of variance (ANOVA) and the means were compared using Least Significant Difference (LSD) test ($P \leq 0.05$).

Results

P. indica and AMF colonization in cassava roots

The colonization by *P. indica* was found to be in the range of 60 % in Sree Suvarna variety in sterile planting medium and 70 % in the unsterile medium, whereas in Sree Jaya and Aswathy varieties 60 % colonization was observed in sterile medium and 55 % in the unsterile medium (Fig. 1). No chlamydospores were found in the root cortex of control plants. Confocal laser imaging also provided evidence for the fungal colonization in the root cortex region (Fig. 2). PCR reaction with PiTEF1 primer produced a 250 bp amplicon from the genomic DNA of the roots colonized by *P. indica*, which again confirmed the successful colonization of the fungus in both Sree Suvarna and Sree Jaya cassava varieties. No bands were visualized with amplified product from the uninoculated control plants. Successful colonization of AMF was found in all varieties of cassava plants when treated with the inoculum (Fig. 3). The root colonization percentage was found to be 100 %. No mycorrhizal structures such as vesicles/arbuscules were found in the roots of uninoculated control plants.

Root initiation by *P. indica* inoculation in cassava

Inoculation of *P. indica* in the variety Sree Jaya showed significant increase in all roots and shoot parameters, such as root length, number of basal roots, number of nodal roots, total number of roots, plant height as well as the number of leaves per minisett when compared to the control plants (Table 1 and 2; Fig. 4). In the case of the variety Aswathy (local), except for the number of nodal roots and plant height per minisett, all other parameters were found to be significantly

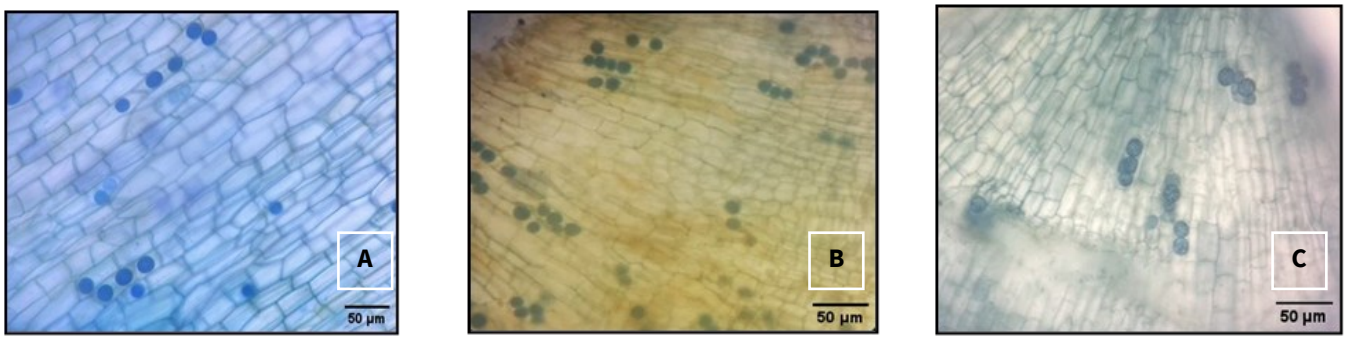

Fig. 1. Chlamydospores of *P. indica* in the root cortex cells of different varieties of cassava.

A: Aswathy; B: Sree Jaya; C: Sree Suvarna.

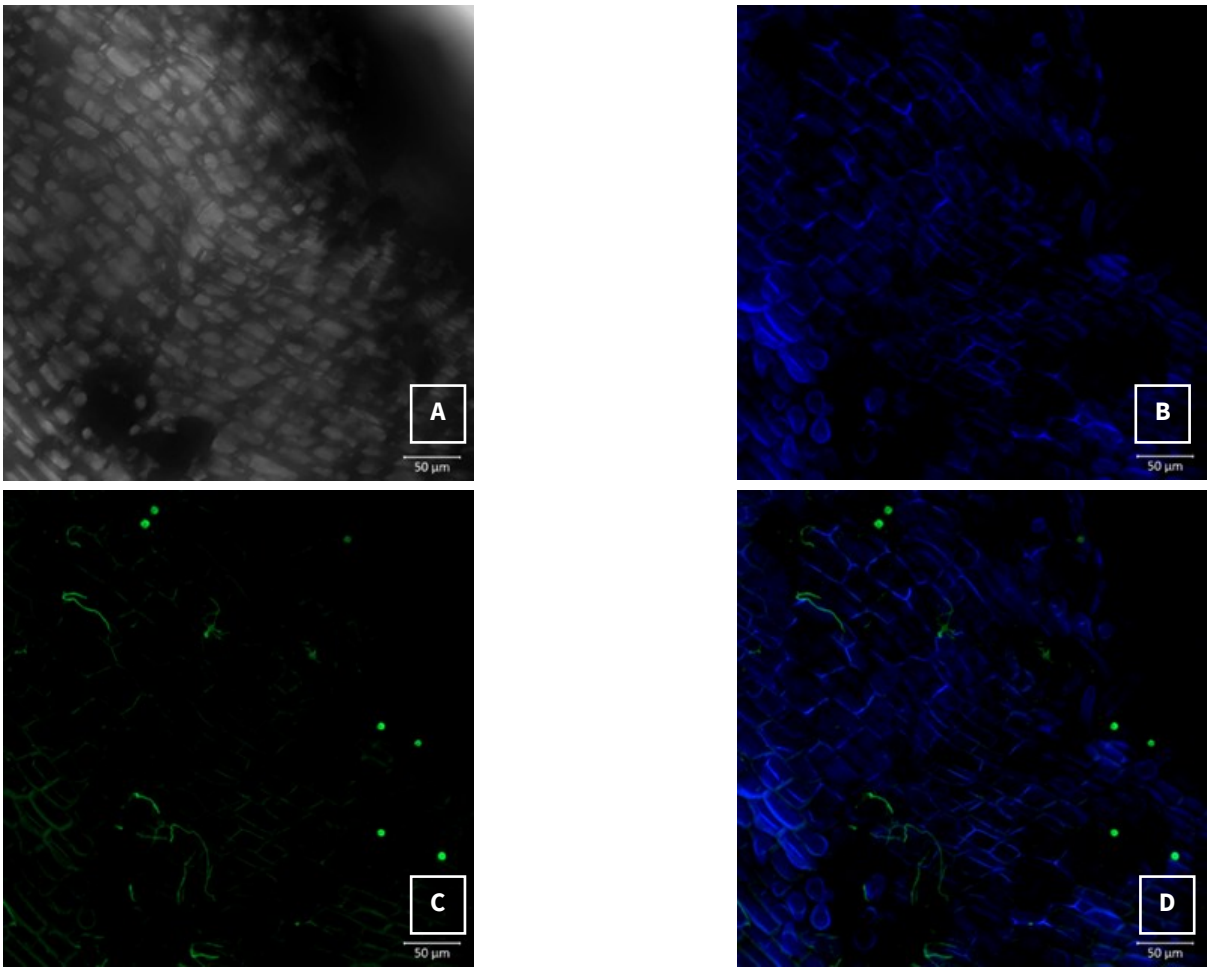

Fig. 2. Confocal image of *P. indica* spores stained with WGA-AF 488, indicating its colonization in the roots of cassava plants. A, B: Bright field image. C: WGA-AF 488 stained spores and intercellular hyphae excited at 488 nm. D: Overlay of C with B.

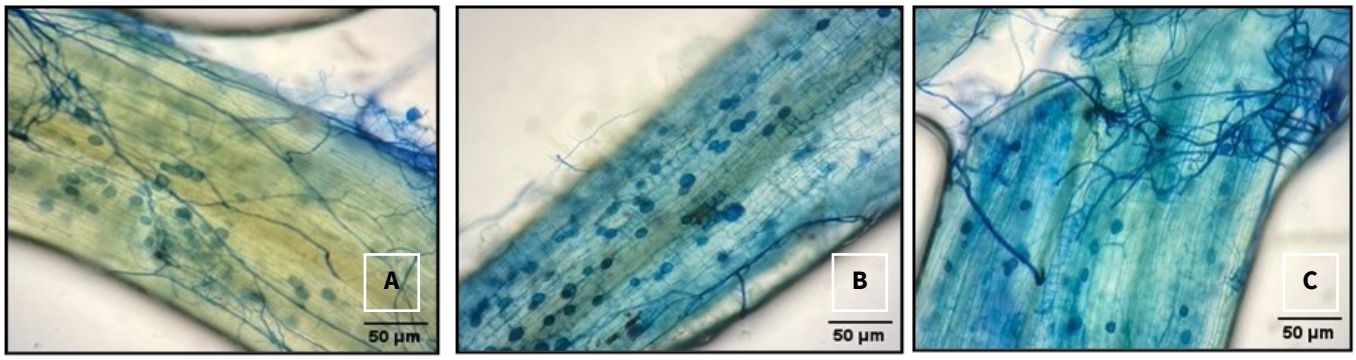

Fig. 3. Vesicles and hyphae of AMF in the roots of different varieties of cassava.

A: Aswathy; B: Sree Jaya; C: Sree Suvarna.

increased in the colonized plants.

Nutrient acquisition, plant growth promotion and yield enhancement in cassava

Inoculation with the endophytes showed significant improvement in plant growth parameters when compared to that of the control plants (Table 3-8).

Tuber yield

The combination of 75 % NPK with *P. indica* produced the highest tuber yield (3.17 kg/plant), mean tuber weight (237.25 g) and tuber number (13.41/plant), corresponding to increases of 81.14 %, 37.31 %

Table 1. Root and shoot parameters of minisetts of variety Sree Jaya

Treatment	Root length/ minisett (cm)	No. of basal roots/ minisett	No. of nodal roots/ minisett	Total no. of roots/ minisett	Plant height (cm)	No. of leaves
P. indica	13.86 ± 3.13*	22.5 ± 3.94*	4.17 ± 1.11*	26.67 ± 4.16*	18.06 ± 1.62*	7.50 ± 1.44*
Control	7.95 ± 1.48	15.0 ± 4.47	2.17 ± 0.71	17.00 ± 4.59	13.30 ± 1.81	5.50 ± 1.00
*t*_value	5.91	4.35	5.22	5.40	6.78	3.94

The values presented are means (±SD) of twelve replicates. *Significant at $p<0.05$ according to *Student's t* test.

Table 2. Root and shoot parameters of minisetts of variety Aswathy

Treatment	Root length/ minisett (cm)	No. of basal roots/ minisett	No. of nodal roots/ minisett	Total no. of roots/ minisett	Plant height (cm)	No. of leaves
P. indica	14.95 ± 1.92*	31.16 ± 12.75*	1.83 ± 0.71	33.00 ± 12.89*	14.68 ± 2.79	7.66 ± 1.43*
Control	10.85 ± 3.40	10.00 ± 4.00	1.50 ± 1.00	11.50 ± 3.60	12.31 ± 4.13	5.83 ± 2.58
*t*_value	3.62	5.48	0.93	5.56	1.64	2.14

The values presented are means (±SD) of twelve replicates. *Significant at $p<0.05$ according to *Student's t* test.

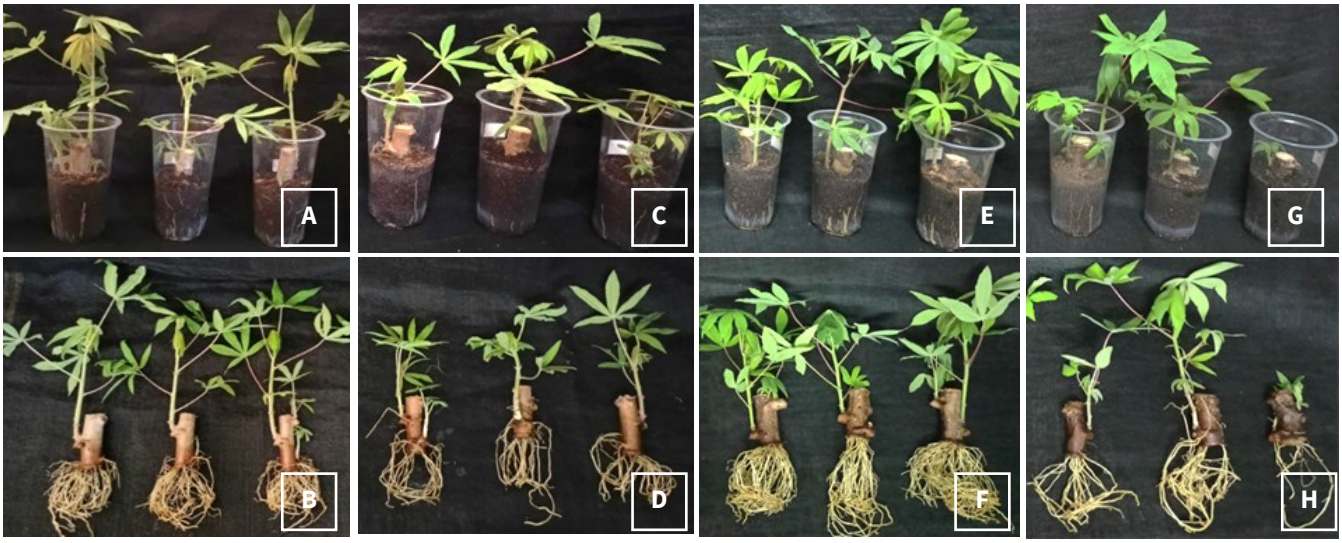

Fig. 4. Screening for growth of minisetts on inoculation with fungal bioagents.

A, B: Variety Sree Jaya inoculated with *P. indica*. C, D: Sree Jaya uninoculated.

E, F: Variety Aswathi inoculated with *P. indica*. G, H: Aswathi uninoculated.

Table 3. Biometric observations of cassava plants at 3 months interval on inoculation with bioagents and application of different doses of NPK fertilizers

Treatments	Plant height (cm)			Plant girth (cm)		
	3 MAP	6 MAP	9 MAP	3 MAP	6 MAP	9 MAP
50 % NPK + *P. indica*	77.67 ± 2.51^{c}	154.33 ± 4.04^{c}	183.67 ± 2.08^{c}	5.83 ± 0.25^{c}	7.73 ± 0.20^{d}	8.73 ± 0.25^{c}
75 % NPK + *P. indica*	92.67 ± 2.51^{a}	178.33 ± 2.51^{a}	205.33 ± 5.03^{a}	6.40 ± 0.17^{ab}	9.16 ± 0.30^{a}	10.83 ± 0.28^{ab}
100 % NPK + *P. indica*	85.00 ± 2.00^{b}	167.67 ± 2.51^{b}	193.00 ± 3.60^{b}	6.63 ± 0.15^{a}	8.13 ± 0.15^{c}	9.76 ± 0.25^{a}
50 % NPK + AM fungus	71.00 ± 1.00^{d}	151.67 ± 3.51^{c}	180.67 ± 1.52^{c}	5.90 ± 0.20^{c}	7.06 ± 0.15^{e}	8.50 ± 0.30^{c}
75 % NPK + AM fungus	85.67 ± 3.05^{b}	164.67 ± 4.16^{b}	191.00 ± 3.60^{b}	6.26 ± 0.15^{b}	8.56 ± 0.11^{b}	11.00 ± 0.50^{b}
100 % NPK + AM fungus	78.00 ± 3.00^{c}	161.33 ± 3.21^{b}	183.00 ± 3.05^{c}	6.13 ± 0.15^{bc}	7.20 ± 0.10^{e}	9.36 ± 0.41^{bc}
Control 100 % NPK	65.33 ± 0.20^{e}	144.33 ± 4.04^{d}	167.33 ± 2.08^{d}	5.30 ± 0.20^{d}	6.33 ± 0.20^{f}	7.43 ± 0.45^{d}
Absolute control	56.67 ± 1.52^{f}	109.00 ± 3.60^{e}	114.67 ± 4.50^{e}	4.63 ± 0.15^{e}	5.67 ± 0.25^{g}	6.30 ± 0.20^{e}
SE (m)	1.33	2.12	2.08	0.10	0.11	0.20
CV (%)	3.02	2.38	2.04	3.14	2.74	3.95

Values presented are means (±SD) of three replicates. Values in a column with same superscripts do not differ significantly LSD ($p<0.05$). MAP: Months after planting

Table 4. Biometric observations on inoculation with bioagents and application of different doses of NPK fertilizers

Treatments	No. of retained leaves	No. of fallen leaves	Total no. of leaves	Leaf retention	Leaf weight/plant (kg)	Stem weight/plant (kg)
50 % NPK + *P. indica*	98.00 ± 3.00^{bc}	135.00 ± 3.60^{a}	233.00 ± 1.00^{d}	4.33 ± 0.57^{bc}	0.20 ± 0.011^{d}	0.75 ± 0.012^{d}
75 % NPK + *P. indica*	113.66 ± 2.30^{a}	133.34± 4.16^{a}	247.00 ± 2.00^{b}	5.00 ± 0.00^{a}	0.27 ± 0.012^{b}	0.89 ± 0.004^{a}
100 % NPK + *P. indica*	87.66 ± 11.71^{d}	151.00 ± 8.18^{b}	238.67 ± 3.78^{cd}	4.33 ± 0.57^{bc}	0.23 ± 0.007^{c}	0.78 ± 0.011^{c}
50 % NPK + AM fungus	102.00 ± 6.55^{d}	139.67 ± 2.08^{a}	238.34 ± 3.05^{cd}	4.67 ± 0.57^{ab}	0.25 ± 0.005^{c}	0.73 ± 0.020^{e}
75 % NPK + AM fungus	117.00 ± 1.73^{a}	138.00 ± 2.64^{a}	255.00 ± 4.00^{a}	5.00 ± 0.00^{a}	0.30 ± 0.012^{a}	0.82 ± 0.020^{b}
100 % NPK + AM fungus	92.33 ± 3.05^{cd}	150.67 ± 3.05^{b}	243.00 ± 5.29^{bc}	4.00 ± 0.00^{c}	0.23 ± 0.006^{c}	0.78 ± 0.010^{c}
Control 100 % NPK	56.66 ± 1.52^{e}	165.67 ± 2.08^{c}	222.33 ± 3.05^{e}	3.00 ± 0.00^{d}	0.18 ± 0.008^{d}	0.65 ± 0.013^{f}
Absolute control	42.66 ± 2.51^{f}	172.00 ± 2.64^{c}	214.67 ± 4.16^{f}	2.00 ± 0.00^{e}	0.11 ± 0.011^{e}	0.42 ± 0.027^{g}
SE (m)	2.89	2.39	2.06	0.19	0.005	0.008
CV (%)	5.65	2.80	1.51	8.53	4.20	1.98

The values presented are means (± SD) of three replicates. Values in a column with same superscripts do not differ significantly. LSD ($p < 0.05$).

Table 5. Chlorophyll content of cassava plants at 3 months interval on inoculation with bioagents and application of different doses of NPK fertilizers

Treatments	Chl a (mg/g of fresh tissue)			Chl b (mg/g of fresh tissue)			Total Chl (mg/g of fresh tissue)		
	3 MAP	6 MAP	9 MAP	3 MAP	6 MAP	9 MAP	3 MAP	6 MAP	9 MAP
50 % NPK + *P. indica*	0.12 ± 0.002^{c}	1.70 ± 0.01^{cd}	1.67 ± 0.02^{b}	0.12 ± 0.002^{c}	1.10 ± 0.006^{ab}	1.05 ± 0.03^{ab}	0.24 ± 0.004^{c}	2.81 ± 0.01^{b}	2.72 ± 0.05^{ab}
75 % NPK +	0.14 ± 0.006^{a}	1.84 ± 0.02^{a}	1.73 ± 0.02^{a}	0.16 ± 0.004^{a}	1.18 ± 0.062^{a}	1.14 ± 0.05^{a}	0.31 ± 0.010^{a}	3.02 ± 0.06^{a}	2.87 ± 0.07^{a}
100 % NPK + *P. indica*	0.12 ± 0.002^{bc}	1.73 ± 0.01^{bc}	1.69 ± 0.01^{b}	0.14 ± 0.002^{b}	1.11 ± 0.021^{ab}	1.08 ± 0.04^{ab}	0.27 ± 0.001^{b}	2.84 ± 0.03^{b}	2.77 ± 0.04^{ab}
50 % NPK + AM fungus	0.12 ± 0.002^{bc}	1.69 ± 0.01^{d}	1.62 ± 0.01^{c}	0.14 ± 0.002^{b}	1.06 ± 0.052^{bc}	1.01 ± 0.06^{ab}	0.27 ± 0.001^{b}	2.75 ± 0.05^{b}	2.63 ± 0.06^{b}
75 % NPK + AM fungus	0.13 ± 0.003^{b}	1.75 ± 0.03^{b}	1.62 ± 0.02^{c}	0.15 ± 0.004^{b}	1.08 ± 0.049^{ab}	1.03 ± 0.08^{ab}	0.28 ± 0.006^{ab}	2.83 ± 0.05^{b}	2.65 ± 0.10^{b}
100 % NPK + AM fungus	0.12 ± 0.003^{c}	1.69 ± 0.01^{d}	1.62 ± 0.03^{c}	0.11 ± 0.002^{d}	1.02 ± 0.108^{bc}	1.00 ± 0.09^{b}	0.23 ± 0.001^{c}	2.71 ± 0.11^{b}	2.62 ± 0.11^{b}
Control 100 % NPK	0.10 ± 0.006^{d}	1.43 ± 0.01^{e}	1.37 ± 0.02^{d}	0.11 ± 0.005^{d}	0.93 ± 0.125^{c}	0.86 ± 0.14^{c}	0.23 ± 0.044^{c}	2.37 ± 0.13^{c}	2.23 ± 0.12^{c}
Absolute control	0.07 ± 0.005^{e}	1.36 ± 0.01^{f}	1.23 ± 0.03^{e}	0.08 ± 0.004^{e}	0.70 ± 0.019^{d}	0.65 ± 0.06^{d}	0.16 ± 0.008^{d}	2.06 ± 0.02^{d}	1.88 ± 0.09^{d}
SE (m)	0.002	0.01	0.01	0.002	0.041	0.046	0.01	0.04	0.05
CV (%)	3.43	1.09	1.54	2.17	6.85	8.05	6.58	2.89	3.53

The values presented are means (±SD) of three replicates. Values in a column with same superscripts do not differ significantly LSD ($p < 0.05$)
MAP: Months after planting.

Table 6. Yield attributes of cassava plants on inoculation with bioagents and application of different doses of NPK fertilizers

Treatments	Mean tuber length (cm)	Mean tuber girth (cm)	Mean tuber weight (g)	No. of tubers/ plant	Tuber yield/ plant (kg)	Total Dry Matter (TDM) content (%)	Harvest Index (HI)
50 % NPK + *P. indica*	25.34 ± 0.99^{b}	11.25 ± 0.86^{bc}	184.96 ± 26.65^{bc}	13.33 ± 2.24^{c}	2.43 ± 0.36^{b}	42.90 ± 0.64^{a}	0.34 ± 0.003^{cd}
75 % NPK + *P. indica*	27.09 ± 0.92^{a}	12.75 ± 0.23^{a}	237.25 ± 41.73^{a}	13.41 ± 0.38^{a}	3.17 ± 0.46^{a}	42.25 ± 1.63^{a}	0.40 ± 0.003^{a}
100 % NPK + *P. indica*	23.60 ± 0.36^{c}	12.05 ± 0.35^{ab}	220.11 ± 31.51^{ab}	12.41 ± 1.42^{b}	2.70 ± 0.11^{b}	40.83 ± 2.58^{ab}	0.38 ± 0.006^{b}
50 % NPK + AM fungus	24.55 ± 0.79^{bc}	10.75 ± 0.26^{c}	180.13 ± 26.50^{bcd}	11.11 ± 1.09^{c}	2.26 ± 0.15^{b}	41.80 ± 1.60^{ab}	0.35 ± 0.004^{c}
75 % NPK + AM fungus	23.39 ± 0.94^{c}	11.85 ± 1.19^{ab}	184.89 ± 34.74^{bc}	13.30 ± 1.38^{b}	2.43 ± 0.27^{b}	43.39 ± 1.39^{a}	0.38 ± 0.003^{b}
100 % NPK + AM fungus	24.14 ± 1.16^{bc}	10.54 ± 1.15^{c}	208.95 ± 25.51^{abc}	11.16 ± 1.60^{c}	2.30 ± 0.06^{b}	40.63 ± 2.07^{ab}	0.38 ± 0.007^{b}
Control 100 % NPK	21.99 ± 0.39^{d}	10.56 ± 0.33^{c}	172.79 ± 11.80^{cd}	10.16 ± 0.84^{d}	1.75 ± 0.18^{c}	38.98 ± 0.74^{b}	0.34 ± 0.008^{d}
Absolute control	19.58 ± 0.73^{e}	9.13 ± 0.58^{d}	139.30 ± 5.50^{d}	8.50 ± 1.09^{e}	1.18 ± 0.16^{d}	35.72 ± 0.70^{c}	0.31 ± 0.007^{e}
SE (m)	0.44	0.36	15.02	0.74	0.14	0.94	0.003
CV (%)	3.22	5.61	13.62	11.07	11.26	3.99	1.55

The values presented are means (±SD) of three replicates. Values in a column with same superscripts do not differ significantly LSD ($p < 0.05$).

Table 7. Soil and plant nutrient analysis on inoculation with bioagents and application of different doses of NPK fertilizers

Treatments	Soil N (kg/ha) *	Soil P (kg/ha)	Soil K (kg/ha) *	Plant N uptake (kg/plant)	Plant P uptake (kg/plant)	Plant K uptake (kg/plant)
50 % NPK + *P. indica*	229.97 ± 38.32	29.57 ± 2.93^{b}	145.60 ± 33.60	184.47 ± 4.96^{d}	9.12 ± 2.00^{bcd}	51.03 ± 5.92^{b}
75 % NPK + *P. indica*	246.69 ± 47.49	75.96 ± 35.18^{a}	134.40 ± 33.60	291.87 ± 22.27^{a}	10.00 ± 0.11^{bc}	74.61 ± 21.56^{a}
100 % NPK + *P. indica*	332.41 ± 6.27	35.75 ± 12.65^{b}	100.80 ± 33.60	246.10 ± 13.41^{b}	11.53 ± 0.48^{abc}	82.74 ± 8.87^{a}
50 % NPK + AM fungus	284.33 ± 97.43	42.35 ± 29.53^{b}	134.40 ± 19.39	267.44 ± 9.56^{ab}	12.01 ± 0.28^{abc}	67.63 ± 17.86^{ab}
75 % NPK + AM fungus	242.51 ± 52.22	27.37 ± 8.32^{b}	104.53 ± 17.10	291.38 ± 25.44^{a}	12.94 ± 2.69^{ab}	75.79 ± 4.39^{a}
100 % NPK + AM fungus	334.50 ± 50.69	23.89 ± 2.01^{b}	182.93 ± 25.86	248.55 ± 5.17^{b}	14.17 ± 4.95^{a}	85.79 ± 10.90^{a}
Control 100 % NPK	351.23 ± 54.67	29.63 ± 14.33^{b}	138.13 ± 63.68	191.07 ± 3.39^{c}	8.50 ± 1.01^{cd}	71.72 ± 5.22^{a}
Absolute control	367.95 ± 52.22	31.47 ± 6.90^{b}	112.00 ± 11.20	126.62 ± 11.06^{d}	5.81 ± 0.73^{d}	50.73 ± 9.13^{b}
SE (m)	33.36	9.98	19.10	8.51	1.27	6.75
CV (%)	19.34	46.74	25.14	6.38	21.05	16.70

The values presented are means (± SD) of three replicates. Values in a column with same superscripts do not differ significantly LSD ($p<0.05$)
*Treatment means are not significantly different.

Table 8. Quality parameters of cassava tubers on inoculation with bioagents and application of different doses of NPK fertilizers

Treatments	Tuber starch content (%)	Tuber sugar content (%)	Crude protein (%) *
50 % NPK + *P. indica*	55.18 ± 1.53^{cd}	6.12 ± 0.11^{c}	4.37 ± 0.29
75 % NPK + *P. indica*	63.17 ± 0.92^{b}	5.56 ± 0.01^{d}	5.25 ± 0.29
100 % NPK + *P. indica*	57.20 ± 0.42^{c}	6.82 ± 0.29^{b}	4.57 ± 0.60
50 % NPK + AM fungus	54.65 ± 0.77^{d}	7.10 ± 0.53^{b}	4.08 ± 0.58
75 % NPK + AM fungus	65.53 ± 0.55^{a}	7.94 ± 0.31^{a}	4.76 ± 1.10
100 % NPK + AM fungus	53.04 ± 0.95^{de}	5.48 ± 0.34^{d}	3.69 ± 0.44
Control 100 % NPK	51.11 ± 0.89^{e}	6.21 ± 0.07^{c}	5.05 ± 0.44
Absolute control	45.35 ± 2.23^{f}	6.71 ± 0.44^{b}	4.57 ± 0.16
SE (m)	0.72	0.15	0.32
CV (%)	2.24	4.25	12.34

The values presented are means (± SD) of three replicates. Values in a column with same superscripts do not differ significantly LSD ($p < 0.05$).
*Treatment means are not significantly different.

and 31.99 % respectively compared to uninoculated plants under 100 % NPK. With AMF, 75 % NPK increased tuber yield/plant by 38.86 % compared to 100 % NPK. All the parameters showed statistically significant differences among the treatment means.

Vegetative growth and tuber quality

Vegetative characters like the number of retained leaves, total number of leaves, leaf weight/plant and quality parameters such as tuber starch and sugar content were more with the treatment of a combination of 75 % NPK and AMF. Control plants with 100 % NPK and without bio inoculation as well as absolute control plants recorded the maximum number of fallen leaves when compared to other treatments.

Nutrient uptake

Soil analyses of the experimental field recorded an average N status of 188.35 kg/ha, P content of 13.88 kg/ha and K content of 142.40 kg/ha at the initial stage. There was a significant increase in the total NPK uptake by cassava plants when chemical fertilizers were applied in combination with bio inoculation, compared to the control plants. It was found that plants that had a combination of 75 % NPK and *P. indica* as well as combination of 75 % NPK and AMF had higher total N uptake and plants with a combination of 100 % NPK and AMF accumulated higher amounts of total P and K.

Discussion

P. indica, a beneficial fungal root endophyte with a broad host range has now been reported to colonize cassava plants successfully. Root colonization was reported to begin by intracellular chlamydospore germination by *P. indica*, later, extracellular hyphal mats were formed, leading to simultaneous penetration of the rhizodermal and root cortical cells (33). Later on, the extracellular hyphae densely cover the roots and harbor through inter and intracellular networks, but it never invades the vascular tissues. The living root cells are colonized by this fungus through its direct penetration at the cellular level (34). Different cassava varieties, such as, Sree Jaya, Sree Suvarna and Aswathy were checked for colonization by *P. indica* and all of them were successfully colonized by the endophytic fungus. Chlamydospores which are pear shaped structures, were observed within the root cortical tissues on microscopy after trypan blue staining at 14 days after inoculation. This was considered as a preliminary indication of root colonization. Further, WGA-AF 488 staining and confocal imaging showed that this beneficial fungus mostly colonizes on the outer epidermal layers of root cortical cells. This approach is widely used in fungal-plant interaction studies because WGA binds specifically to chitin residues in the fungal cell wall, resulting in bright, selective fluorescence of fungal structures, with minimal labelling of plant tissues. This helps in providing a strong contrast of fungal hyphae (green channel) inside roots, facilitating discrimination of fungal vs plant structures (33). Additionally, PCR analysis with PiTEF1 primers confirmed the presence of *P. indica* in the colonized roots of cassava plants. Successful colonization of *P. indica* in taro roots, another tropical tuber crop, was also reported by our group (22). To the best of our knowledge, this is the first report of successful *P. indica* colonization in cassava roots.

P. indica application on minisetts of cassava was found to have a significant positive impact on root and shoot growth. The fungus under investigation promotes root proliferation and may act as a substitute for phytohormone (35). The root growth stimulation as well as alteration in the root architecture by microbes involves auxins or intervenes with the auxin metabolism which includes processes such as elevation of auxin biosynthesis, release of auxin from stores or conjugates, stimulation of its transport, or activation of genes involved in the induction of auxin in various tissues that are required for cell growth or proliferation or with signalling in the roots (36). Similarly, activation of the F-box protein TIR1 receptor, involved in the auxin-induced signalling pathway leads to ubiquitination-based degradation of transcriptional repressors and complex transcriptional reprogramming (37). Similarly, root branching was reduced by the inhibition of auxin transport which further hinders the effect of beneficial microbes (38). In addition to this, several studies indicate that the microbially synthesized auxin or auxin related compounds trigger the plant auxin signalling pathway (39). The increase in the active auxin level mediated by *P. indica* helped to enlarge the surface of roots as well as to promote root hair development (40). Our findings align with earlier reports in rice, maize, tobacco, tomato, bacopa, artemisia, parsley, bush pepper and poplar plants, where *P. indica* doubled root and shoot biomass compared to controls (6, 9-11, 14).

Cassava is highly mycorrhizal and AMF colonization enhances productivity by improving photosynthesis, nutrient uptake and water relations (2, 41). The cassava plant growth and yield are known to be raised by the colonization of AMF through increased net photosynthesis, improved water and nutrients uptake (42). Earlier reports also showed a significant increase in the dry yield components of cassava plants upon inoculation with AMF. The growth parameters (plant height, foliar surface area, total dry and total fresh matter) of cassava plants were significantly improved after four months in the green house on inoculation with AMF (2). The hyphal network formed by AMF with the roots of its host plants significantly increases the access of roots to a large soil surface area, leading to plant growth improvement (43). In our study, the

combination of AMF with 75 % NPK similarly improved vegetative growth and tuber yield, consistent with earlier greenhouse studies.

In the field experiment, the bio-inoculants were applied at the time of planting and the chemical fertilizers were applied one week after planting, so as to reduce the toxic effects of chemicals on them. Adequate quantities of organic manure were also applied along with the bioinoculants to ensure better survival, growth and activity of the introduced microbial inoculum in the soils, which was slightly acidic in nature. Even at a reduced level of fertilizer application, the plants with fungal inoculation had improved growth and yield. It was proposed that the *P. indica* mediated growth promotion is accompanied by a co-regulated stimulation of enzymes involved in nitrate and starch metabolisms (44). An increase in the growth and yield parameters of black pepper plants on *P. indica* inoculation was reported earlier, and a higher level of chlorophyll content was one of the attributes that contributed to the same (14). The chlorophyll a, chlorophyll b and total chlorophyll content of cassava leaves showed significant differences between the treatment means of *P. indica* as well as AMF inoculated and uninoculated control plants when analysed during 3 MAP, 6 MAP and 9 MAP. Enhanced chlorophyll content in *P. indica* and AMF treatments likely contributed to the observed improvements in photosynthesis and yield.

Application of the fungal inoculants could enhance the nutrient uptake by plants even when the fertilizer application was reduced to 75 % of the recommended dose. This can be attributed to the ability of these beneficial fungi in effectively helping plants to take up nutrients from the soil by increasing the surface absorbing capacity of the roots, regulating root elongation, root hair formation and improving the root density of the host plant. *P. indica* is reported to activate nitrate reductase (NR), the key enzyme involved in the nitrate acquisition in plants. Co-cultivation of *P. indica* with the seedlings of *Nicotiana tabaccum* and *Arabidopsis* leads to the increased transfer of nitrogen from the agar plates into the aerial part of the seedlings (19).

The amounts of acid phosphatases produced by *P. indica* can solubilize the insoluble condensed or complex forms of phosphate reserve in the soil to soluble forms and thereby helps in plant absorption and the acid phosphatases were found to mobilize and assist *Lycopersicon esculentum* in phosphate acquisition and growth promotion (44). The fungal endophyte also expresses a phosphate transporter, *P. indica*-mediated phosphate transporter (PiPT), that can promote phosphate uptake by plant roots, providing P nutrition to the host plants (20). Potassium uptake is supported by fungal K^+ transporters (*Si*HAK1, *Si*TRK1, *Si*TRK2, *Si*TOK1), with *Si*HAK1 strongly induced under K^+ deficiency (45).

AMF contribute substantially to N nutrition, often supplying 20 %-75 % of host N, primarily as ammonium and amino acids. This aligns with our observation of increased N uptake in AMF-inoculated cassava, even under reduced fertilizer application (46). Reports have shown that AMF mostly prefers ammonium rather than nitrate from the soil and that amino acids represent the major compounds that helps to transfer N to the host plant (47). Effective trapping of nitrogen in chlorophyll molecules results in higher chlorophyll contents in AMF plants due to increased nitrogen contents in them (48). Interaction between phosphorus fertilizer and AMF was reported earlier. Reports have shown that both P fertilizers and AMF inoculant application increased the harvestable root dry yield of cassava plants (49). Growth and P uptake by cassava plants in phosphorus deficient soil in response to AMF inoculation was found to be increased when the soil was disinfected (42). Similarly, in sweet potato, the application of potassium as well as mycorrhizal inoculation has been proven to exert greater effects on the root morphology, regulation as well as nutrient acquisition (50). Our data showed that AMF improved P and K uptake, consistent with reports in cassava and sweet potato where AMF enhanced nutrient acquisition and root morphology under nutrient-limited soils.

Conclusion

The present study describes an eco-friendly method that utilizes the abilities of two root colonizing endophytic beneficial fungi *P. indica* and the AMF *G. mosseae,* to promote growth as well as yield in cassava. Both fungi enhanced nutrient uptake in cassava, even when fertilizer input was reduced to 75 % of the recommended dose. *P. indica* and AMF root endophytism can be an important strategy to reduce the quantum of chemical fertilizers, without affecting the growth and yield of cassava plants. They also help in maintaining soil fertility and environmental sustainability. Our findings highlight the potential of *P. indica* and AMF to be developed as biofertilizers for sustainable cassava production.

Acknowledgements

Authors are grateful to the Kerala Agricultural University for providing facilities for carrying out the research.

Authors' contributions

APS carried out the experiments, collected and analysed the data and drafted the original manuscript. MA helped in designing the experiments, reviewed and edited the manuscript. CN, SAR and VIS provided resources for conducting the experiments. JJ and GS also provided resources and helped in designing the experiments. KNA conceptualized and supervised the research, drafted, reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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