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The protective role of *Piriformospora indica* colonization in *Centella asiatica* (L.) *in vitro* under phosphate stress

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ABSTRACT

The core of present study was to determine potential role of *Piriformospora indica,* mutualistic Basidiomycete endosymbiont on co-cultivation with *Centella asiatica* (L.) Urb., a neutraceutical plant. The analysis of major secondary metabolite asiaticoside and differential expression of key genes involved in pathway under varying phosphate (P) concentrations maintained *in vitro* were carried out. Maximum asiaticoside content was documented in plants maintained in MS medium containing lowest level (62.5 μM) tried ($p < 0.001$). At phosphate concentration of 125 μ M (10% of normal P), significant stimulation of plant growth and asiaticoside production were observed in colonized plants. P levels at 125 μM however favored maximum fresh weight, root and shoot length, root and leaf number of plants. The transcript accumulation of β Amyrin Synthase, the key gene in asiaticoside pathway was recorded at the P level of 62.5 μM, which is significantly higher ($p < 0.001$) than those at other P concentrations tried which was evidenced by overproduction of auxin, Indole-3-Acetic Acid (IAA). A significantly higher level of IAA was recorded in *P. indica* colonized plants maintained in low (125 μM) P medium. The presence of *P. indica* under low phosphate concentrations has protective role in mitigating effects of stress, as evidenced by non-significant hydrogen peroxide production, acid/alkaline phosphatase activity, total phenolics and increased super oxide dismutase (SOD) activity in colonized plants. The study clarifies positive correlation of the asiaticoside production in *P. indica* colonized plants under low P conditions with significant up regulation of asiaticoside pathway gene transcripts with less stress.

1. Introduction

The axenically cultivable root endosymbiotic fungus *Piriformospora indica* possesses a broad host spectrum and positively affects different aspects of plant performance (Varma et al., 1999). This so far unique combination of attributes makes *P. indica* the extremely interesting tools for cultivation of various crops (Franken, 2012). The endophytic interaction of *P. indica* with plant roots is accompanied by an enormous acquisition of nitrogen and phosphorous from the environment (Yadav et al., 2010). In Arabidopsis, it regulates early flowering through regulation of photoperiod and Gibberellin pathways (Kim et al., 2017; Pan et al., 2017). Recently, enhanced antioxidant activity and expression of drought related genes were observed which confers drought tolerance on *Zea mays* L. (Xu et al., 2017). In the physiological characteristics and root morphology in wheat was significantly affected under stress conditions (Hosseini et al., 2017). Reports are also available on the role of *P. Indica* in plant water relations, gas exchange and growth of *chenopodium quinoa* at limited water availability (Hussin et al., 2017).

P is considered as the crucial element in plant biochemistry as it occurs in numerous macro molecules and has a major role in every energy transfer via the pyrophosphate (George et al. 2008). In tissue culture medium, the element is provided as potassium mono- and dihydrogen phosphates. It was recently reported that enhanced growth under P-depleted conditions in the presence of *P. indica* could be the manifestation of increased transport of P to the host plant by the fungal hyphae (Kumar et al., 2011). Uptake and transport of phosphorous, with diverse regulatory, structural, and energy transfer roles, are stimulated by the fungus in the colonized roots of maize (Yadav et al., 2010). A phosphate transporter was recently identified from the hyphae of *P. indica*, which is thought to enhance the nutritional efficiency of host plants (Pederson et al., 2013). Recently, the 25 Mb genome of *P. indica* was characterized and the transcriptional responses associated with the colonization process were predicted (Zuccaro et al., 2011).

Centella asiatica (L.) Urb. is one of the chief herbs for treating skin problems, to heal wounds, and for revitalizing the nerves and brain cells (Singh et al., 2010). The use of *Centella* in food and beverages has

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increased over the years basically due to its health benefits as an antioxidant, and anti-inflammatory agent, together with tremendous wound healing and memory enhancing capacity. In Ayurveda, the plant is effective in effective in treatment of stomach ulcers, mental fatigue, diarrhea, epilepsy, hepatitis, syphilis and asthma (Goldstein and Goldstein, 2012). Interest in the medicinal plant *C. asiatica* has increased over the years and studies on the increased production of centellosides and cloning of genes in the biosynthetic pathway have been attempted. Despite sincere efforts in different laboratories, the consistent overproduction of centellosides has not yet been achieved so far. Among the centellosides, asiaticoside are the major neutraceutical secondary metabolites in *C. asiatica*. Reports are available regarding the role of asiaticoside being neuroprotective, antidepressive, and anxiolytic in animal models (Puttarak et al., 2017). Attempts for micropropagation resulted in *in vitro* plants displaying lesser asiaticoside content than their *in vivo* counterparts and asiaticoside and madecassoside were undetectable in transformed roots and undifferentiated callus (Aziz et al., 2007).

The earlier efforts with *P. indica* were successful in significantly enhancing asiaticoside production by co-culture with *C. asiatica* (Jisha et al., 2012). The present report describes the salient observations of our efforts in the direction of assessing the influence of phosphate variation in *P. indica* colonization. The study was also extended to evaluate the effect of *P. indica* colonization on growth and asiaticoside, the major secondary metabolite production along with the analysis of oxidative stress in *C. asiatica.* Till date, there are no reports regarding the advantage of low phosphate medium for *C. asiatica-P. indica* co-culture system.

2. Materials and methods

2.1. Plant material and it's maintenance for phosphate treatments

C. asiatica plants were maintained at Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Thiruvananthapuram, Kerala, India, under uniform conditions of growth. Hydroponic MS media cultures were used for the present study. The P-sufficient control medium was the normal half strength Murashige and Skoog (MS) liquid medium (Murashige and Skoog, 1962), that contains 1250 µM KH_2PO_4 as the sole phosphate source. Medium with low P were made by adding 125 µM KH_2PO_4 (10% of normal) and 62.5 µM (5% of normal) phosphate to half strength MS medium instead of the normal dose. The overall level of potassium was maintained identical to MS (20 mM) by adding potassium sulphate in the required amounts. Higher than normal P levels were maintained by adding 2-fold (2500 µM) and 4-fold (5000 µM) of the normal KH_2PO_4 concentration to the medium. Control and treatment media contained sucrose (3%) and pH was adjusted to 5.7 before autoclaving. The autoclaving was done at 121°C for 15 min. The cultures were maintained in the plant tissue culture laboratory of Jawaharlal Nehru Tropical Botanic Garden and Research institute, Palode, Thiruvananthapuram. The control and *P. indica* colonized plant cultures were stored in sterile glass bottles at 23 ± 2°C with a 16/8-h photoperiod and a light intensity of 25 µmol m^{-2} s^{-1}, provided by white fluorescent tubes and 55–65% relative humidity.

2.2. Initiation and maintenance of Piriformospora indica culture

The cultures were maintained in Potato Dextrose Agar (PDA) medium at pH 7.0 and incubated in the dark at 28°C for a period of 10 days. Fungal hyphae (100 mg) were transferred to Potato Dextrose Broth (PDB) maintained under same growth conditions as above (Jisha et al., 2012).

2.3. P. indica –C. asiatica co-culture

Two weeks old *C. asiatica* plants maintained in MS liquid culture were transferred to medium containing MS and PDB (containing *P. indica*) in a 1: 1 ratio and incubated in 16 h: 8 h light/dark at 23 ± 2°C and 55–65% humidity and a light intensity of 25 µmol m-2 s −1 provided by white fluorescent tubes, for a period of 30–45 days in the Plant Tissue Culture Laboratory at Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Kerala. Plants maintained under identical conditions in MS - PDB (1: 1) liquid medium without *P. indica* co-cultivation provided as control (Jisha et al., 2012).

2.4. WGA staining for analysis of colonization and HPLC of C. asiatica extracts for phytochemical characterization

P. indica cultures were maintained in Potato Dextrose Broth (PDB) and *C. asiatica* hydroponic cultures [50 ml/cultures (25 ml of *P. indica* in PDB and 25 ml of *C. asiatica* in liquid MS)] were maintained under controlled *in vitro* conditions [light intensity (1000 lux), photoperiod (16 h light/8 h dark), uniform temperature in day and night (22 ± 2 °C) and relative humidity (50–60%)]. Rooted plantlets were transferred to half-strength MS basal liquid medium fortified with different P concentrations and 3% sucrose maintained at pH 5.8 in sterile culture tubes after a period of 2 weeks. The analyses were carried after a period of 45 days of *C. asiatica* and *P. indica* co-culture.

WGA staining for analysis of colonization was followed as reported in our previous study (Jisha et al., 2012) which include fixing the control and co-cultivated plant roots in trichloroacetic acid (TCA) fixation solution followed by boiling for 1 min with10% KOH and neutralized in 1X PBS. The root tissues were transferred to staining solution containing WGA-AF 488 (Invitrogen, Oregon, USA). During incubation, root segments were vacuum-infiltrated three times for 1 min at 50 mmHg and destained by incubating overnight in PBS after overnight incubation. The samples were viewed by confocal laser imaging on a multichannel TCS SP2 confocal system (Leica Microsystems, Bensheim, Germany). After 45 days of co-culture, root colonization was assessed as percentage colonization. The observations for the analysis of colonization by WGA-AF 488 staining and HPLC analysis were taken after a period of 45 days co-culture with *P. indica* in different P media under *in vitro* conditions.

HPLC was performed using High Performance Liquid Chromatographic system (Schimadzu, Kyoto, Japan) equipped with LC8A pump, SPD-M 10Avp Photo Array Detector in combination with Class LC 10A software. Chromatographic separation was performed using an Octyl silane C8 column (5 µ size, 250 × 4.6 mm in length) with water - acetonitrile (HPLC grade, Merck, Germany) as the mobile phase. Separation was carried out with a flow rate of 1.5 ml min^{-1}. The sample injection volume was 20 µl at 25°C (Jisha et al., 2012).

2.5. RNA isolation and Real Time PCR analyses of SQS and BAS

RNA isolation and Real Time PCR procedure were followed as already reported (Jisha et al., 2012). Total RNA was isolated from leaf tissues of control and *P. indica*-colonized plants after 15, 30, and 45 days of co-culture by Trizol method (Invitrogen, California, USA). Approximately 1 µg of DNase (Sigma, St. Louis, USA) treated RNA was used to prepare cDNA using MMLV-RT following the manufacturer's protocol (Promega). Gene-specific primers of *C. asiatica* SQS and BAS (Jisha et al., 2012, Table 1) were used for quantitative real-time PCR. The reaction was set up in a final volume of 20 µl containing 10 µl SYBR green PCR reagent (Applied Biosystems, California, USA), 1.5 µl of diluted cDNA (1:10 dilution), and 300 nM each of the designed primers; the conditions were: 50°C for 2 min initially followed by 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min in a real-time PCR machine (ABI 7500, Applied Biosystems). The plant-specific 5.8S rRNA gene served as control for constitutive gene expression in leaves. The observations were taken after a period of 45 days in molecular analyses. The procedures were followed as reported earlier (Jisha et al., 2012).

Table 1
List of primers used in Real Time PCR.

Primer names	Forward primer	Reverse primer
BAS RT	TCCCTCAGCAGGAAACAAC	GGTACTCTCCAAGTGCCCATA
SQSRT	CAAATTTTCCGTGGC	GGGTTTATTTCTCCAGAAGAC
CYSRT	ATGCCTGGTTTGGTTATCACT	AACCCCACCCACCATCTCTAT
5.8S rRNA RT	TCGATGGTTCACGGGATTC	TGAAGAACGGTAGCGAAATG
PITEF RT	TCGTCGCTGTCAACAAGATG	GAGGGCTCGAGCATGTTGT

2.6. Quantitative determination of auxin

Indole-3-Acetic Acid (IAA) was quantified by using the earlier reported protocol (Jisha et al., 2018) with slight modifications. Hundred milligram of fresh leaf tissues were collected from all 45-days old experimental and control samples and frozen using liquid nitrogen. The samples were ground in 5 ml of 80% methanol. Butylated hydroxyl toluene (BHT) (Sisco Research Laboratory, India) was added at a concentration of 10 μg ml^{-1}. The extraction was continued at −70°C for a period of 48 h. Sep-pak column (Waters Corporation, Milford, Massachusetts) was pre-wetted with 5 ml of 50% methanol followed by 5 ml of distilled water. The remaining traces of water were expelled by passing air. The clear methanol extract was passed through the column to remove lipids and other pigments. The column was washed with 3 ml of 50% methanol and the effluent collected. Pooled samples were evaporated, and the residue was dissolved in 1 ml of distilled water and partitioned thrice against an equal volume of dichloro methane (Merck, Germany) (1:1 v/v). The organic phase was evaporated, and the remaining residue was dissolved in 1 ml of TBS buffer (pH 7.5). Aliquots from this were used in immunoassay for auxin quantification using the Phytodetek Indole-3-Acetic Acid (IAA) enzyme immunoassay kit (Sigma, St. Louis, USA) following manufacturer's instructions.

2.7. Estimation of total phenols in methanol extracts of C. asiatica

Total phenols were determined following known procedure (McDonald et al., 2001) using Folin denis' reagent. A dilute extract of each plant extract (5 μl of 50 mg $ml^{-1}$of the extract with 495 μl of methanol) or gallic acid (Sisco Research Laboratory, India), was mixed with 5 ml of 1:10 diluted (with distilled water) Folin denis' reagent and 5 ml of 1M Na_2Co_3 (Sisco Research Laboratory, India). The mixtures were allowed to stand for 15 min and the totals phenols were determined by colorimetry at 765 nm. The standard curve was prepared using 25, 50, 100 and 200 mg l^{-1} solutions of gallic acid equivalent (mg g^{-1} of dry mass). All determinations were done in duplicates and the total phenolics content was expressed as mg g^{-1} gallic acid equivalent.

2.8. Acid phosphatase (EC 3.1.3.2, acid phosphomonoesterase) and alkaline phosphatase (EC 3.1.3.1, acid phosphomonoesterase) assay

Two hundred milligram of each of the sample was used for the assays. The samples were ground into fine powder using liquid nitrogen and 2 ml of distilled water was added to it. This diluted sample was centrifuged (Jouan Inc., Winchester, USA) at 5000 rpm after keeping it at 15 °C for 24 h at 200 rpm. The final supernatant was used for further assay using the acid phosphatase assay kit and alkaline phosphatase assay kit (Sigma, St. Louis, USA) according to manufacturer's protocol. One unit of acid/alkaline phosphatase will hydrolyze 1 μmole of 4-nitrophenyl phosphate per minute at pH 4.8 at 37 °C.

2.9. Hydrogen peroxide (H_2O_2) generation assay

Hundred mg of leaf tissues from each sample were ground into fine powder using liquid nitrogen and dissolved in 2 ml of double distilled water. The thoroughly mixed sample was centrifuged (Jouan Inc., Winchester, USA) at 1000 g for 5 min and the particulate pellet was removed. This crude preparation was filtered through sterile Millex syringe-driven filter unit (Millipore Corporation, Bedford, USA) and the filtrate used for the assay. 2–50 μl of sample was added to each microplate well and adjusted the volume to 50 μl using the assay buffer. The measurement of H_2O_2 for each sample was done by fluorometric method using the H_2O_2 assay kit (Abcam, United Kingdom).

2.10. SOD (EC 1.15.1.1.) assay

Two hundred mg of each sample was ground into fine powder using liquid nitrogen. To which 2 ml of distilled water was added. This diluted sample was centrifuged at 5000 rpm after keeping at 15°C for 24 h at 200 rpm in a shaker (Lab-Therm). The supernatant was used for SOD determination (SOD assay kit, Sigma, St. Louis, USA) following the manufacturer's protocol. SOD activity was measured as inhibition rate %.

2.11. Determination of plant growth

Control plants as well as plants colonized with *P. super*Shoots and roots were separated and washed with distilled water. The length and the total number of roots as well as petioles and leaves were analyzed separately. For the determination of the dry weight, the materials were dried overnight in an oven at 40°C.

2.12. Experiment with P. indica in soil supplied with low P

Experiment under *in vitro* condition was repeated in the natural growing conditions. Uniform *in vivo* plants of *C. asiatica* maintained in sterilized soil under identical conditions (at 25 ± 2°C, 16 h light/8 h dark with fluorescent light intensity 1000 lux and relative humidity 70%) in a growth chamber (Conviron CMP6010; Controlled Environments Ltd., Canada) were transferred to sterile soil and maintained in green house conditions (under identical conditions of watering) for initiating pot experiments. Plants were observed for 45 days. Pot experiments were conducted in soil by keeping the plantlets in *P. indica* containing sterilized soil in which MS media with low and high P were supplied in the soil. Control non-colonized plants were maintained under identical conditions for all treatments.

2.13. Statistical analysis

Mean and the significant levels were carried out using the Graphpad Instat version 3.6 (Graphpad Software Inc., USA). $p > 0.05$ shows no significant levels in the values and $p < 0.001$ shows the maximum significance.

The experiments were performed *in vitro* and plants were arranged in a completely randomized design with three replicates for each growth parameter studied. For each experiment, 30 *P. indica* co-cultured plants were analyzed and the experiments were repeated twice. Thus, a total of 60 treated plants were studied in comparison to 10 control (untreated) plants. One plant each was maintained as a control for each growth parameter studied, i.e., five controls per experiment (5 × 2 = 10 control plants in total).

3. Results

3.1. Influence of varying growth medium phosphate levels on asiaticoside production and plant growth

Lower than normal P levels in the MS culture medium favorably influenced asiaticoside production in *C. asiatica* cultures, the maximum content being recorded in plants maintained in MS medium containing the lowest level (62.5 μM) tried (Fig. 1; $p < 0.001$). The significant favorable effect of *P. indica* colonization on growth of *C. asiatica* under normal P concentration was obvious in the present experiments, and

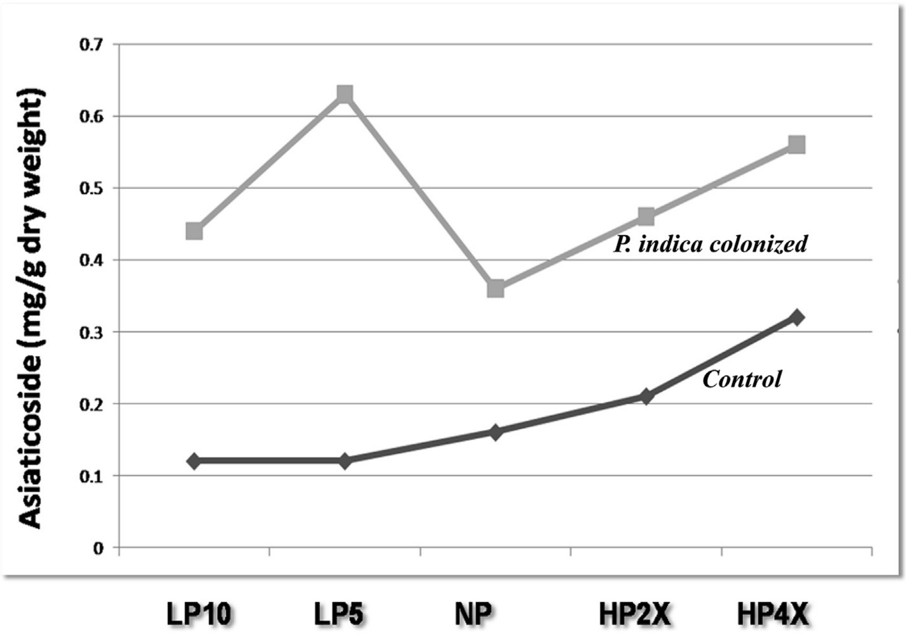

Fig. 1. Comparison of HPLC quantification of asiaticoside content. The amount of asiaticoside produced by control and *P. indica* colonized plants in low and high concentrations of P after a period of 45 days. Low phosphate 5 (LP5)- 62.5 µM (5% of normal MS); Low phosphate 10 (LP10) - 125 µM (10% of normal MS); Normal phosphate (NP) - 1250 µM (normal P as in MS medium); High phosphate 2X (HP2X)- 2.5 M (2-fold of normal MS); High phosphate 4X- 5M HP4X (4- fold of normal MS).

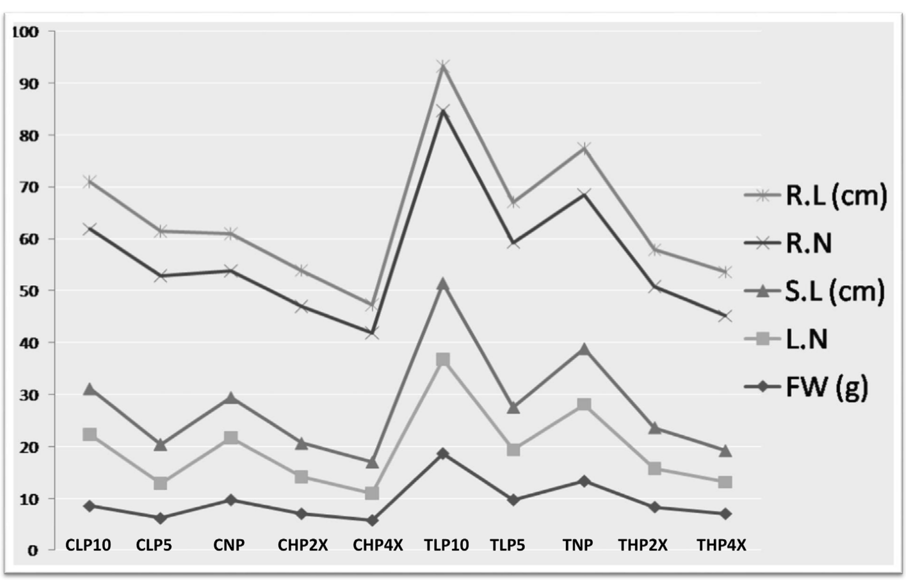

Fig. 2. Comparative analysis of total plant biomass and growth parameters. R.L.-root length (cm); R.N.-root number; S.L-Shoot length (cm); L.N-Leaf length and FW (g)-Fresh weight (g). The control non-colonized are CLP10-control low phosphate 10%; CLP5-control low phosphate 5%; CNP-control normal phosphate; CHP2X-control high phosphate 2-fold; CHP4X-control high phosphate 4-fold. The *P. indica* colonized (treated) *C. asiatica* include TLP10-treated low phosphate 10%; TLP5- treated low phosphate 5%; TNP- treated normal phosphate; THP2X- Treated high phosphate 2-fold; THP4X- Treated high phosphate 4-fold.

this effect was even more pronounced in cultures grown under low P (62.5 µM) medium (Fig. 2; TLP5 vs TNP). The fresh weights in all the treatments including the control non-colonized were reduced to 10%. Dry weights of all the treatments in P. indica colonized and control non-colonized were only the 10% of their fresh weights. [for example the TLP10 (10% of the normal half MS phosphate concentration showed around 20 g fresh weight, whereas its dry weight showed 0.2 g upon drying). Thus it can be concluded that around 90% of the plant biomass is composed of water. The results suggest that the asiaticoside promotory effect of *P. indica* is significantly enhanced under conditions of low P availability. P levels at 125 µM however favored maximum fresh weight (Fig. 2; CNP vs TLP10), root and shoot length, root and leaf number and of plants (Fig. 2; CNP vs TLP10). Plant dry weight was also recorded (Fig. 3), which also shows the beneficial role of *P. indica.* Lowering P concentration further had a negative impact on biomass growth (Fig. 2; CNP vs TLP5), and the effect was not significantly different ($p > 0.05$) from control. The results suggest that the growth promotory effect of *P. indica* is significantly enhanced under conditions of low P availability.

3.2. Effect of P concentration on P. indica colonization

In consonance with the earlier observation (Jisha et al., 2012) the significant favorable effect of *P. indica* colonization on growth of *C. asiatica* under normal P concentration was obvious in the present experiments, and this effect was more prominent in cultures grown under low P (62.5 µM) medium (Fig. 4; TLP5 vs TNP). Maximum fungal colonization as indicated by maximum expression of PITEF transcripts was observed in colonized plants grown under low phosphate condition (Fig. 4; TLP10). Further reduction in P concentration (62.5 µM; TLP5) less favored *P. indica* colonization. Confocal images (Fig. 5) reveal the extent of colonization in response to varying P concentration in the medium. Confocal images also show TLP10 with maximum *P. indica* colonization. Higher than normal levels of P concentrations [2-fold (2500 µM) and 4-fold (5000 µM) of the normal KH_2PO_4] were found inhibitory to *P. indica* colonization (Fig. 5 E & F).

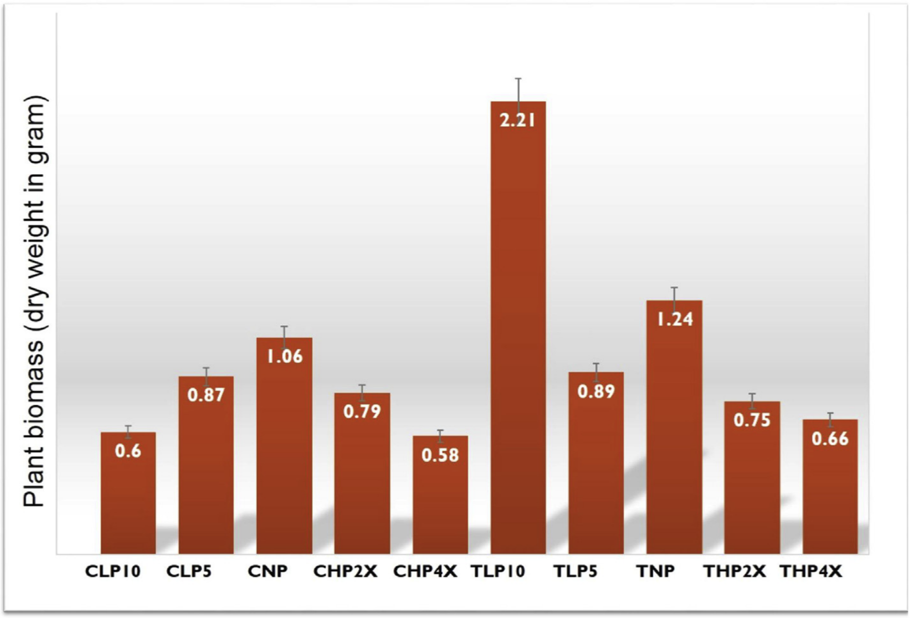

Fig. 3. Real-Time RT-PCR analysis of PITEF 1 transcripts in response to P concentration. Analysis was performed in the roots of non-colonized *C. asiatica* (C); *P. indica* colonized *C. asiatica* in 125 µM P [10% of the normal P concentration (TLP10)]; *P. indica* colonized *C. asiatica* in 62.5 µM P [5% of the normal P concentration (TLP5)]; *P. indica* colonized *C. asiatica* in 1250 µM P [normal P concentration in MS (TNP)]; *P. indica* colonized *C. asiatica* in 2.5 M P [2-fold P concentration (THP2X)] and *P. indica* colonized *C. asiatica* in 5M P [4-fold P concentration (THP4X)]. Samples were isolated after 45 days in culture. Relative quantification represents the fold expression levels of PITEF 1. Number of replications (n) = 3. *** depicts significance at $p < 0.001$.

3.3. Effect of P on transcript accumulation of SQS and BAS

In Real Time experiments, it was observed that colonization by *P. indica* invariably favored accumulation of BAS transcripts under all P levels tried (Fig. 6A). Maximum BAS transcript accumulation was recorded at the P level of 62.5 µM, which is significantly higher ($p < 0.001$) than those at other P concentrations tried (Fig. 6). Transcript up regulation of SQS gene was most favored by low phosphate levels in colonized and non-colonized (control) plants (Fig. 6B). The P level of 125 µM displayed the maximum transcript expression of SQS followed by P level of 62.5 µM. Increasing the P levels in the medium did not induce the accumulation of both BAS and SQS in control and colonized plants. It was recorded that *P. indica* also enhances SQS and ABS transcript expression in all concentrations of P tried, eventhough its effect in higher P levels are not found significant. Melting curves for all the genes analyzed are also included (ESM1).

3.4. Influence of P levels on auxin (IAA) production

A significantly higher level of IAA ($p < 0.001$) was recorded in *P. indica* colonized plants maintained in low (125 µM) P medium (Fig. 7; LP10 Treatment). Further decrease in P concentration resulted in a concomitant decrease in IAA (Fig. 7; LP5 Treatment) in colonized plants. In non-colonized plants, auxin (IAA) concentration was maximum at normal P concentration (1250 µM) (Fig. 7; NP Control). The increased and decreased concentrations of P in MS media did not support the biosynthesis of IAA. Both the higher concentrations of P (HP2X and HP4X) could not exhibit enhanced IAA in control and *P. indica* colonized *C. asiatica*. The standard curve for the quantification of IAA is also provided (ESM2).

3.5. Phenolic content

Any of the treatment produced a significant enhancement in the total phenolic content.Total phenolic content of non-colonized and

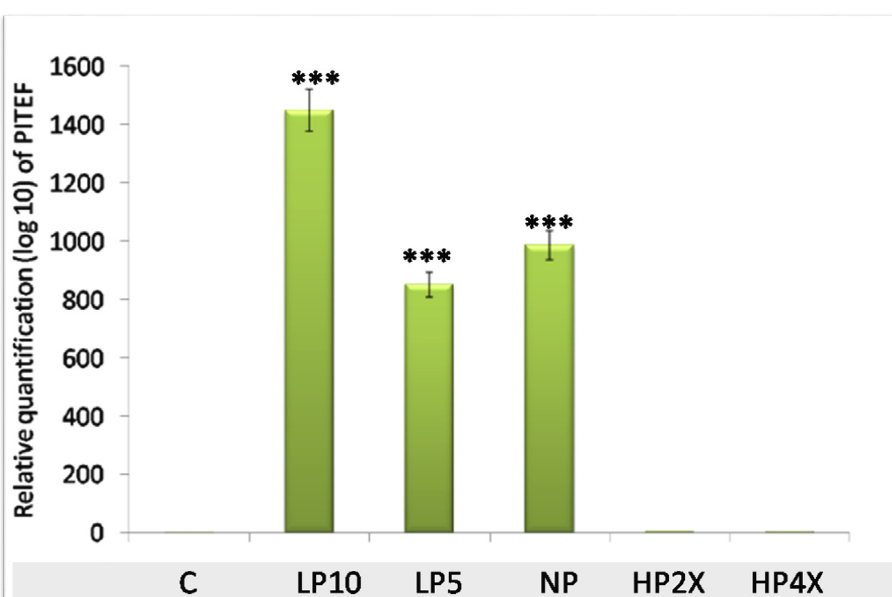

Fig. 4. Confocal images after WGA-AF 488 staining of roots of *C. asiatica* after 45 days of co-culturing with *P. indica*. A- Control non-colonized *C. asiatica*, B - *P. indica* colonization in low phosphate (10% of normal concentration), C- *P. indica* colonization in low phosphate (5% of normal concentration), D- *P. indica* colonization in normal phosphate concentration, E− *P. indica* colonization in 2-fold high phosphate concentration and F - *P. indica* colonization in 4-fold high phosphate concentration.

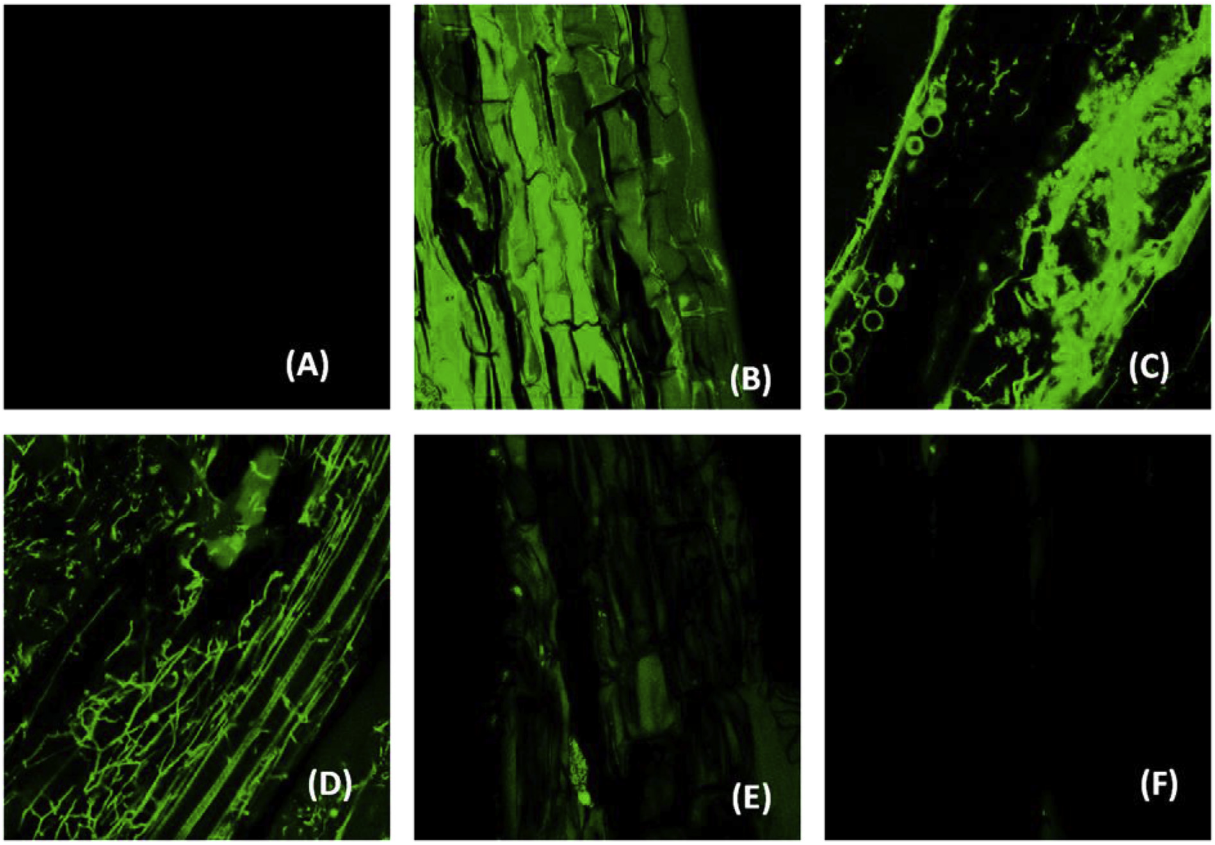

Fig. 5. Real-time RT-PCR analysis of BAS (A) SQS (B) expressed as fold expression in leaves of *C. asiatica*. Analysis was performed in leaves of control and *P. indica* colonized *C. asiatica* (Treatment) in 62.5 μM P[low phosphate 5% (LP5)]; 125 μM P [low phosphate 10% (LP10)]; 1250 μM P [Normal phosphate as in MS medium (NP)]; 2.5 M P [2-fold of normal MS, high phosphate 2X (HP2X)] and 5 M P [4-fold of normal MS, high phosphate 4X (HP4X)]. Samples were isolated after 45 days in culture. Relative quantification represents the fold expression levels of BAS. Number of replications (n) = 3. *** depicts significance at $p < 0.001$.

colonized plants was comparable under normal phosphate conditions ($p > 0.05$; Fig. 8), which was however more favorably influenced by P variations in the growth medium in colonized plants. A maximum of 12.5 and 14 $mg\,l^{-1}$ methanol extract was recorded in colonized plants incubated in the lowest (62.5 μM) and highest (5000 μM) P concentrations respectively (Fig. 8; LP5 and HP4X Treatment). The standard curve for phenol quantification was provided (ESM3).

3.6. Acid phosphatase and alkaline phosphatase activity

Acid phosphatase and alkaline phosphate levels in noncolonized and colonized plants were almost identical ($p > 0.05$) under normal phosphate conditions (Fig. 9; NP). In noncolonized plants, lowering the phosphate concentration had a profound effect on acid phosphatase activity, and maximum enzyme activity was recorded in the presence of 1250 μM P [NP; Fig. 9A]. In colonized plants, decreasing P concentrations in the medium did not have a significant influence on acid phosphatase activity [Fig. 9A; LP5 and LP10 Treatments], where alkaline phosphatase showed a slight increase in activity in all the treatments tested [Fig. 9B].

3.7. H_2O_2 generation and SOD activity in response to P stress

H_2O_2 generation under conditions of normal P is apparently similar in control and colonized plants. Lower and higher P concentration in the medium significantly increased H_2O_2 production in control plants (Fig. 10A), whereas *P. indica* colonization induced lesser production of H_2O_2 under lower P concentrations, which was like colonized control plants maintained at normal P conditions (Fig. 10A). Higher than normal P levels in the growth medium induced H_2O_2 generation in control and colonized plants. overall, the presence of *P. indica* apparently reduces H_2O_2 generation in *C. asiatica.* The standard graph for the detection of H_2O_2 is provided in ESM4. Under normal P condition, *P. indica* colonization significantly favored SOD activity compared to control ($p < 0.001$; Fig. 10B). Higher and normal P concentrations in the growth medium increased SOD activity in the control (non-colonized) and colonized plants, and maximum SOD was recorded in *P. indica* colonized plants maintained in the presence of lowest phosphate concentration.

3.8. Effect of low P along with P. indica under natural conditions

P. indica co – cultivation with low and normal concentrations of P analyzed in field conditions showed enhancement in plant growth in comparison with the control *C. asiatica* (Fig. 11). The growth parameters were recorded, which was identical to the results observed in the *in vitro* experiments. The plants in the high concentrations of P did not survive for the specific periods tested, which might be due to the high concentration of P within the soil.

4. Discussion

Owing to its immense medicinal value, *C. asiatica* has been subjected to various approaches for enhancing centelloside production including optimization and elicitation of cell suspension cultures (Bouhouche et al., 1998), and identification and cloning of relevant genes in the asiaticoside biosynthetic pathway (Kim et al., 2005). Despite encouraging results obtained, the scope of widespread application of biotechnological approach for sustained overproduction of asiaticosides is far from enough. The instability of cell cultures and requirement

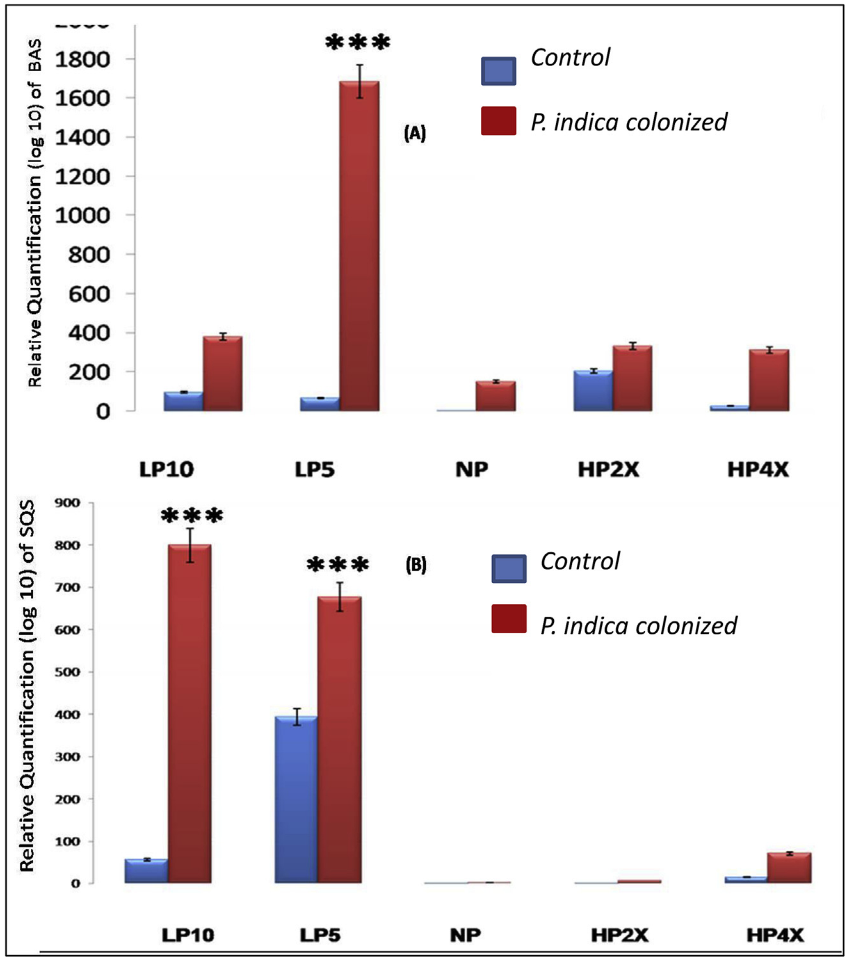

Fig. 6. Quantification of Auxin, IAA in the leaves of *P. indica* colonized *C. asiatica*. Estimation of the endogenous levels of hormone auxin-IAA in the leaves of control, *P. indica* co-cultivated *C. asiatica in vitro* cultures in 62.5 μM P[low phosphate 5% (LP5)]; 125 μM P [low phosphate 10% (LP10)]; 1250 μM P [Normal phosphate as in MS medium (NP)]; 2.5 M P [2-fold of normal MS, high phosphate 2X (HP2X)] and 5 M P [4-fold of normal MS, high phosphate 4X (HP4X)]. Samples were isolated after 45 days in culture. *** depicts significance at $p < 0.001$.

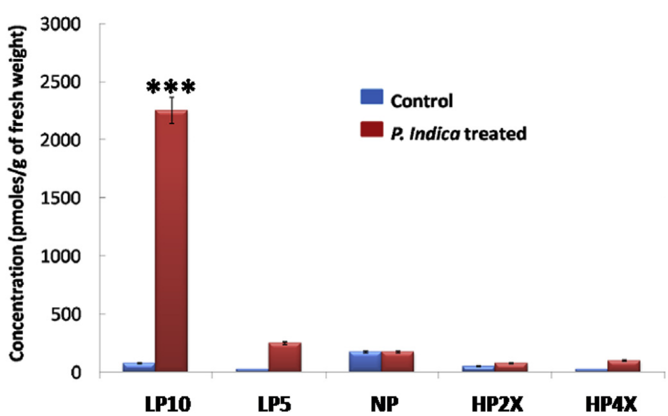

Fig. 7. Analysis of total phenolics. Gallic acid was used as the systematic equivalent and the estimation of total phenols from the methanolic extracts of control and *P. indica* colonized (Treatment) plants in 62.5 μM P[low phosphate 5% (LP5)]; 125 μM P [low phosphate 10% (LP10)]; 1250 μM P [Normal phosphate as in MS medium (NP)]; 2.5 M P [2-fold of normal MS, high phosphate 2X (HP2X)] and 5 M P [4-fold of normal MS, high phosphate 4X (HP4X)]. Values represent the average from 3 replications. The values were not significant ($p > 0.05$).

of specialized or differentiated structures for optimum production of asiaticosides are often factors that limit the *in vitro* production (James and Dubery, 2009). Earlier study reported the considerable favorable impact of co-culture with the growth –promoting fungus - *P. indica* on asiaticoside production *in vitro* (Jisha et al., 2012) and the impact of *P. indica* cell wall extract on asiaticoside production (Jisha et al., 2018). The present work reports the influence of *P. indica* co-culture and varying P concentrations on asiaticoside biosynthesis, and its influence on biochemical activity of enzymes and metabolites of the host plant, which could help in obtaining a wider physiological perception of the process of colonization.

The main physiological basis for a plant-fungal symbiotic association is bidirectional nutrient transfer, wherein plants supply the fungi with sugar and the fungi enhance the ability of plants to scavenge for scarce and immobile nutrients, particularly P (Smith and Smith. 1990). The analyses were conducted in whole plants exposed to different P levels Smith for 45 days, to ensure an effective colonization by *P. indica*, which is maximum after 30–45 days of coculture. Maintaining test plants for these many days will most likely induce a systemic P signaling response to P variations in the medium, though the primary sensing site is the root. This inference is driven by a recent observation that under P stress, a primary systemic root-derived signal is delivered via xylem, where they are perceived by systemic sensors, and produced as secondary signals in the shoots and again transported to the roots (Chiou and Lin, 2011).

In the present experiments, a low concentration of P (125 μM) in the

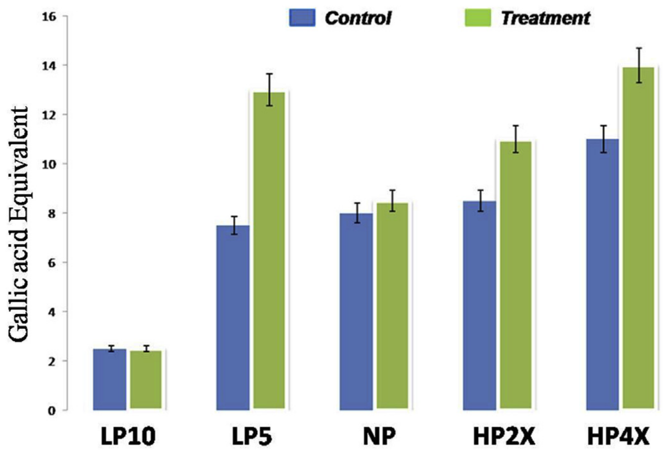

Fig. 8. Acid/alkaline phosphatase assay. Estimation of acid phosphatase activity (A) and alkaline phosphatase activity from the aqueous extracts of control and *P. indica* colonized (Treatment) plants in 62.5 μM P [low phosphate 5% (LP5)]; 125 μM P [low phosphate 10% (LP10)]; 1250 μM P [Normal phosphate as in MS medium (NP)]; 2.5 M P [2-fold of normal MS, high phosphate 2X (HP2X)] and 5 M P [4-fold of normal MS, high phosphate 4X (HP4X)]. Values represent the average from 3 replications. The values were not significant ($p > 0.05$).

growth medium induced maximum growth of plants which also correlated with a remarkable accumulation of the auxin IAA, agreeing with previous observations that *P. indica* colonization results in auxin overproduction (Lahrmann et al. 2012). Growth and yield benefits are largely attributed to the production of the phytohormones IAA. However, the part of auxin and auxin signaling pathways in controlling the interactions between different plants and microbes is enigmatic (Sukumar et al., 2013). Increased lateral root formation and primary root emergence were observed in *C. asiatica* plants in response to *P. indica* colonization, which also coincided with increased auxin content. *P. indica* colonized Chinese cabbage showed two-fold higher concentrations of auxins when compared to the noncolonized plants and found that the auxin synthesized is not of fungal origin (Lee et al., 2011). It is established that *P. indica* increases auxin signaling in order to change the root morphology for enhanced nutrient sequestration and may itself produce auxin (Sirrenberg et al., 2007).

Asiaticoside biosynthesis was best favored in the presence of lowest P concentration in the medium (62.5 μM), which was least favorable to growth as judged by the decrease in biomass. The negative correlation between growth and asiaticoside content at extreme P variations could be linked to the physiological stress response of the host plants under P stress, which is further strengthened by the indication of high phenolic content at this P level. When plants are stressed, secondary metabolite production may increase at the expense of growth, since the carbon fixed is predominantly allocated to secondary metabolite production (Seigler, 1998). The marked effect of nutrient stress on phenolic levels is well documented (Chalker-Scott and Fuchigami, 1989) and has been explained in terms of increased accumulation of phenyl propanoids and lignifications resulting from nutrient stress (Dixon and Paiva, 1995). The current data also indicate that *P. indica* colonization under low phosphate conditions can have a tremendous effect on asiaticoside accumulation. The presence of *P. indica* invariably favors host plant growth, including root formation, and this effect is further accentuated by decrease in concentration of P upto an optimum level, beyond which the plant manifests a reduction in growth, and stress response. It seems reasonable to assume that the presence of *P. indica* in the roots, protects the plant from the negative effects of P stress. On the contrary, the endophytic colonization in rice has been extensively studied (Gill et al., 2016) and *P. indica* colonization expressively influences mechanisms involved in osmotic stress in WC-297 (drought tolerant), Caawa (moderately drought tolerant) and IR-64 (drought susceptible) rice varieties (Saddique et al., 2018).

The mRNA transcripts of BAS and SQS, the two key genes in the triterpenoid pathway consistently showed upregulation corresponding to an increased accumulation of asiaticoside, corresponding to *P. indica* colonization and decrease in P concentration in the medium. But an interesting observation in the present study was the significant upregulation of BAS transcripts, concomitant with and asiaticoside production. This is contradictory to all earlier reports that higher expression of BAS transcripts and resulting higher terpenoid synthesis (Bonfill et al., 2011). Role of phytosterols in alleviating harmful effects of nutrient stress has been documented earlier (Basayuni et al., 2007), and possibly explains the present observation of concomitant overproduction of β-amyrin and phytosterols in response to P stress in *C. asiatica*.

Phosphatase is important for many physiological processes, including regulation of phosphorous efficiency (Duff et al., 1994). External and internal acid and alkaline phosphatase activities are increased under low phosphorous availability in plants (Tadano et al., 1993). Secretion of acid phosphatase under P stress liberates P from organic sources *in vivo*. During P starvation, increased activity of phosphatases is found throughout the plant tissues and in the rhizosphere (Hubel et al. 1996). The increased activity of this enzyme in noncolonized plants of C. asiatica, under low P availability supports this view. The role of P. indica in uptake of P and its mobilization has been reported earlier (Varma and Podila, 2005). The increased P efficiency of P. indica colonized plants may explain the lack of acid and alkaline

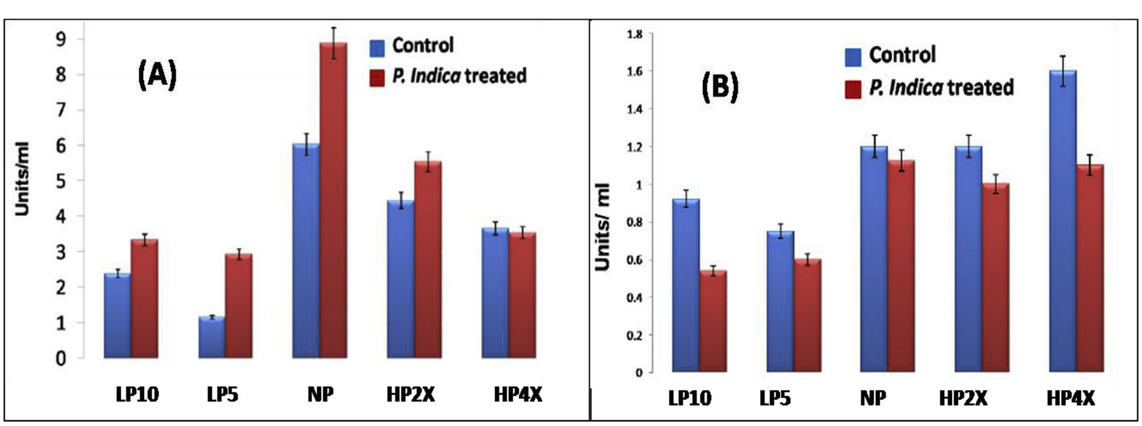

Fig. 9. Quantition of H_2O_2 (A) and Effect of *P. indica* colonization on SOD activity (B). H_2O_2 (pmoles μl^{-1}) generation assay in control and *P. indica* colonized (Treatment) plants in in 62.5 μM P[low phosphate 5% (LP5)]; 125 μM P [low phosphate 10% (LP10)]; 1250 μM P [Normal phosphate as in MS medium (NP)]; 2.5 M P [2-fold of normal MS, high phosphate 2X (HP2X)] and 5 M P [4-fold of normal MS, high phosphate 4X (HP4X)].*** depicts significance at $p < 0.001$.

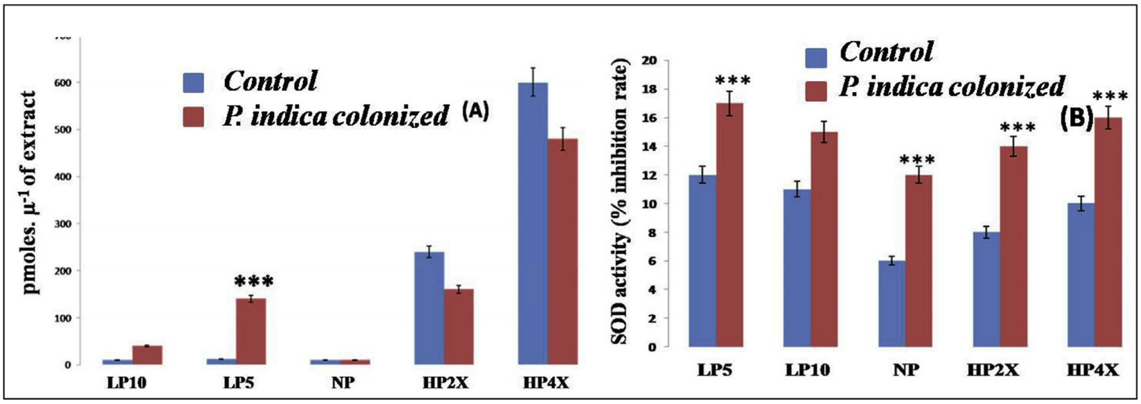

Fig. 10. *C. asiatica* plants grown under varying concentrations of P in field conditions. A – control *C. asiatica* in(1250 μM); B - control *C. asiatica* in 62.5 μM P; C - control *C. asiatica* in 125 μM P; D - *P. indica* colonized *C. asiatica* in basal MS medium (1250 μM); E − *P. indica* colonized in 62. 5 μM and F - *P. indica* colonized *C. asiatica* in 125 μM P.

phosphatase induction under conditions of low phosphate. Enhanced acid and alkaline phosphatase were reported in rhizosphere soils as a marker of P nutrition in nodulated Cyclopia and Aspalathus species in the Cape Fynbos of South Africa. Identical to VAM colonization (Koide, 1991), it can be speculated that P. indica colonization increases the rate of P accumulation which is utilized subsequently during conditions of P limitation, thereby rendering colonized plants to perform better under conditions of P deprivation.

A general observation was the significantly reduced H_2O_2 generation at P level variations. H_2O_2 is a hall mark of Reactive Oxygen

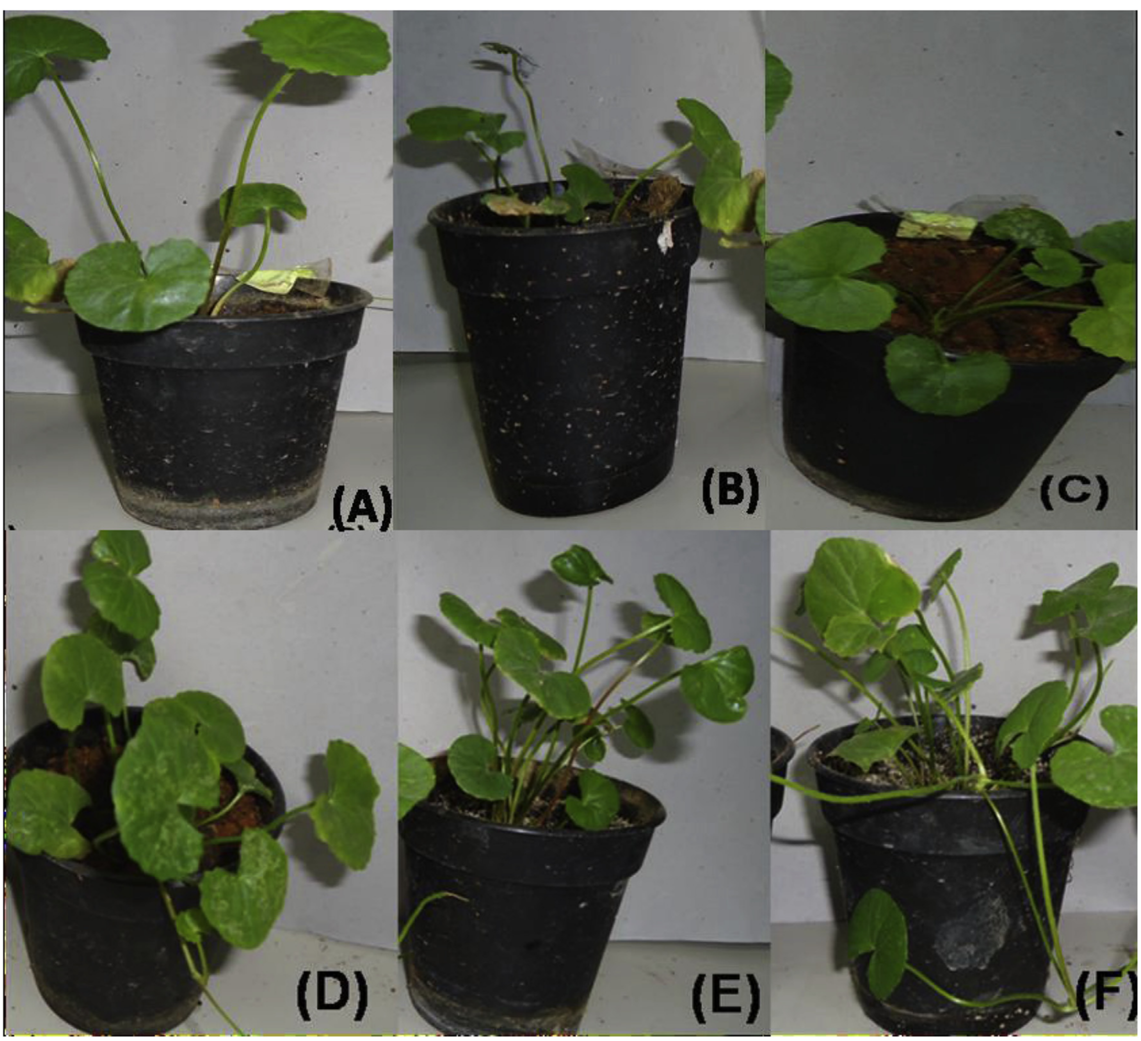

Fig. 11. *Centella asiatica* plants grown under selected best grown concentrations of P. A – control *C. asiatica* in basal MS medium (1250 μM); B - control *C. asiatica* in 62.5 μM P; C - control *C. asiatica* in 125 μM P; D - *P. indica* colonized *C. asiatica* in basal MS medium (1250 μM); E - *P. indica* colonized in 62. 5 μM and F - *P. indica* colonized *C. asiatica* in 125 μM P.

species (ROS)-mediated stress manifestation and therefore suggests a protective role for *P. indica* in lessening H_2O_2 generation under conditions of nutrient stress associated with very low and high levels of P in the growth medium. Increase in SOD in response to *P. indica* colonization and phosphate limiting growth conditions could possibly be explained in terms of the detoxification of H_2O_2 produced (Kumar et al., 2009). Exposure of plants to unfavorable environmental conditions can increase the production of ROS. To protect themselves against toxic oxygen intermediates like H_2O_2, plants activate its cellular antioxidant machinery. SOD is the most effective intracellular enzymatic antioxidant and it has been proposed to provide the first line of defense against the toxic effects of elevated levels of ROS (Gill and Tuteja, 2010). It is worth mentioning in this context that phenolic compounds are considered as the major contributors to the antioxidative activities of *C. asiatica* (Zainol et al., 2003), and treatment with *C. asiatica* significantly increased the level of antioxidant enzymes including SOD in animal models (Jayashree et al., 2003). It is interesting that presence of *P. indica* in low phosphate conditions potentially increases the antioxidant status of the plant in terms of phenolic and SOD accumulation, which sequentially confers increased stress tolerance in *C. asiatica.*

The potential of *P. indica* in host plant growth promotion and stress tolerance was also observed in pot experiments with low P. It is evident from the experiment that *P. indica* is appropriate for the biomass growth in phosphate deficient soils rather than in the P containing soils. Arbuscular mycorrhizal fungi, AMF, the root-interacting predominant microbiota also play an indispensable role in improving plant growth vigour and survival (Davies, 1995). However, the non-availability of the axenic culture in arbuscular mycorrhizal fungus is a great bottleneck for the fundamental studies and their biotechnological applications (Singh et al., 2000).

5. Conclusions

The present study determined the potential role of *P. indica* on cocultivation with *C. asiatica* (L.) Urb. under boosted and reduced P levels under *in vitro* conditions. The plant biomass was strongly promoted by *P. indica* under alleviated P concentrations. Major secondary metabolite asiaticoside and expression of the key genes involved in this secondary metabolite pathway were evaluated under varying concentrations of phosphate. In addition, the presence of *P. indica* under low phosphate concentrations has a protective role in mitigating the effects of stress, which was evidenced by non-significant hydrogen peroxide production, acid and alkaline phosphatase activity, total phenolics and increased super oxide dismutase activities in *P. indica* colonized plants under low phosphate concentrations. The results obtained in *C. asiatica* under phosphate stress strongly supports the recent prediction that *P. indica* has great potential in agriculture and especially for healthier establishment of tissue culture raised plants. The present studies provide clues on the potential role of *P. indica* in low phosphate conditions and the role of *P. indica* colonization in enhancing asiaticoside production in *C. asiatica*. Thus *P. indica* can be supplied in phosphate deficient soils in order to enhance the plant biomass growth as well as its secondary metabolites.

Conflicts of interest

The author declares no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101088.

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