The use of medicinal plants has been a traditional health practice since ancient times. Even today people rely on plant-based medicines to cater for their primary healthcare needs. The bioactive ingredients of medicinal plants are also of great value in agriculture, food and beverage industry. However, the stable yield and quality of these bioactive ingredients in medicinal plants are often affected by several environmental conditions. Biotechnological techniques such as plant tissue culture, genetic manipulation and metabolic engineering have therefore been utilized for enhanced production and accumulation of these valuable compounds. In addition, the beneficial microorganisms have also been focused upon for rhizosphere engineering. A number of microorganisms like mycorrhiza, endophytes, and plant growth promoting rhizobacteria have been studied for their role in plant biomass enhancement and accumulation of plant bioactive compounds. The interactive effects of *Arbuscular mycorrhiza* (AM) with medicinal plants are well documented. However, the commercial production of AM fungi faces a challenge as the axenic cultivation of AM fungi has not been achieved. *P. indica*, a mycorrhiza like fungus having a wide host range, is known to stimulate plant growth and plant resistance to various biotic and abiotic stresses. It has also been shown to have beneficial effects like early flowering, enhanced seed production, and plant secondary metabolite content. Also, it can be cultivated on various complex and synthetic media. These features make *P. indica* a suitable candidate as microbial inoculums with diverse benefits. Rhizospheric bacteria have also been known to enhance plant growth by improving the phosphorus availability and by altering the hormonal balance of the plants. *A. chroococcum* is a plant growth promoting rhizobacteria, considered to have beneficial effects on associated plants. Recent studies have also established the symbiotic association of *P. indica* and *A. chroococcum* in a co-culture system, making them a promising bio-inoculant. Thus, use of this co-culture system can pave an alternative way for enhanced production and accumulation of commercially important bioactive compounds.
To date, *S. rebaudiana* is the only known source of sweet tasting steviol glycosides which are a good alternative to sugar and have also been demonstrated to act as an anti-diabetic, anti-hypertensive, anti-cariogenic and anti-diarrheal agent. The cultivation of *S. rebaudiana* faces a major problem due to its poor seed viability and low seed germination rate. Thus the vegetative propagation and micro propagation of this medicinally important plant has gained much importance to meet the increasing demand. Several studies have also been undertaken to improve the steviol glycoside content and sweetness quality of this plant. Therefore, the present study was designed to evaluate the synergistic interplay of the root endophytic fungus, *P. indica* and plant growth promoting rhizobacteria, *A. chroococcum*; singly and in combination on plant growth characteristics and stevioside yield in *S. rebaudiana*.

The establishment and *in vitro* multiplication of *S. rebaudiana* was undertaken by growing the sterilized nodal explants on MS media supplemented with different concentrations of cytokinins; BAP (0.5, 1.0, 1.5 and 2.0 mg l\(^{-1}\)) and Kn (0.5 and 1.0 mg l\(^{-1}\)). Study of shoot induction parameters (days to bud induction, percentage explants showing shoot proliferation and number of shoots per explant) showed that 0.5 mg l\(^{-1}\) Kn gave the best response for shoot initiation and multiplication. Successful root induction was achieved on MS basal medium and it was used for plant maintenance and further inoculation experiments.

Seven different spore concentrations of *P. indica* (6.25×10\(^7\), 1.25×10\(^7\), 2.5×10\(^6\), 5×10\(^5\), 1×10\(^5\), 2×10\(^4\) and 4×10\(^3\) spores ml\(^{-1}\)) were then applied to *in vitro* grown plants and studied for their effect on plant growth parameters and steviol glycoside (stevioside and rebaudioside-A) content. Plants were also treated with plant growth promoting rhizobacteria, *A. chroococcum* (10\(^2\) CFU ml\(^{-1}\)). To check the interaction effects under natural soil conditions, the *in vitro* grown *S. rebaudiana* plants were transferred to greenhouse after acclimatization and hardening, with a 77.5 % survival frequency. Greenhouse grown plants were also treated singly with *P. indica* (6.25×10\(^7\), 5×10\(^5\) and 4×10\(^3\) spores ml\(^{-1}\)) and *A. chroococcum* cell suspensions (10\(^2\) CFU ml\(^{-1}\)). The best
responsive concentrations of *P. indica* and *A. chroococcum* (5 × 10^5 spores ml^{-1} and 10^2 CFU ml^{-1}, respectively) were used for further studies involving combined inoculation in both *in vitro* and greenhouse grown plants.

Effective root colonization by *P. indica* was established by PCR amplification of a 248 bp *P. indica* specific gene (*Pi*-Transcription elongation factor) from the genomic DNA of *P. indica* colonized roots. Visual observations after root staining were also carried out to confirm colonization by *P. indica*. The colonized roots showed detection of spores in extracellular spaces and within the root cells, which was not seen in the roots of control plants. However, different fungal spore concentrations did not show corresponding change on the root colonization percentage.

The plant growth parameters were recorded for *in vitro* and greenhouse grown plants, post-inoculation with *P. indica*, *A. chroococcum* and *P. indica + A. chroococcum*. Under *in vitro* conditions, varying effects on plant growth parameters were seen upon inoculation with different spore concentrations of *P. indica*. Higher spore concentrations (6.25×10^7 and 1.25×10^7 spores ml^{-1}) showed a reduction in plant growth parameters as compared to plants treated with lower spore concentrations of *P. indica* (2.5×10^6, 5×10^5, 1×10^5, 2×10^4 and 4×10^3 spores ml^{-1}). This could be due to nutrient competition and limited space under *in vitro* conditions as greenhouse grown plants showed no such effect. Under greenhouse conditions, the different *P. indica* inoculum concentrations (6.25×10^7, 5×10^5 and 4×10^3 spores ml^{-1}) did not show significant difference in plant growth parameters, amongst themselves. Inoculation experiments with *A. chroococcum* also showed an increase in plant growth parameters under both *in vitro* and greenhouse conditions, as compared to control. However, the change was not as significant as observed in *P. indica* treated plants. Combined inoculation studies with best responsive concentrations of *P. indica* (5 × 10^5 spores ml^{-1}) and *A. chroococcum* (10^2 CFU ml^{-1}) were also undertaken for both *in vitro* and greenhouse grown plants. A further enhancement in plant growth parameters was observed in plants treated with *P. indica + A. chroococcum* as compared to *P. indica* or *A. chroococcum* alone. The boost in plant growth parameters under dual inoculation could be
due to a conglomerated effect, as both *P. indica* and *A. chroococcum* are known to enhance plant biomass by improving the plant nutrient availability and uptake.

Further studies on the effect of the two microorganisms; *P. indica* and *A. chroococcum* (singly and/ or in combination) on steviol glycoside content of *S. rebaudiana* was undertaken. The methanolic extracts from leaves of *S. rebaudiana* were used to quantify the stevioside and rebaudioside-A contents by HPTLC. *In vitro* grown plants showed an increase in both stevioside and rebaudioside-A contents upon inoculation with *P. indica* (5×10^5 spores ml⁻¹). The results indicated that the optimal initiation of plant metabolites was not achieved at lower spore concentrations (4×10^3 spores ml⁻¹) of *P. indica*. Moreover, application of a still higher concentration of *P. indica* (6.25×10^7 spores ml⁻¹) led to significant reduction in plant secondary metabolite content. Plants grown under greenhouse conditions showed similar enhancement pattern in steviol glycosides upon inoculation with different spore concentrations of *P. indica*. Thus, the quantities for steviol glycosides showed correlation with the results obtained for plant growth parameters suggesting a direct association between nutrient availability and steviol glycoside content. Inoculation experiments with dual inoculation of *P. indica + A. chroococcum* showed further enhancement of steviol glycosides as compared with control, *P. indica* or *A. chroococcum* treatment. Also, the sweetening property of the plant was enhanced, with significant increase in rebaudioside-A to stevioside contents as compared to control. Thus, suggesting that *P. indica + A. chroococcum* are effective in providing appropriate nutrients that lead to further enhancement of stevioside content.

The number of trichomes are positively correlated with the steviol glycoside content in *S. rebaudiana*. Further validation of steviol glycoside contents, upon inoculation with *P. indica* and *A. chroococcum* (singly and combination), was done by SEM analysis of glandular trichome density in control and treated samples. For this the adaxial surface of the leaf from each treatment was observed and photographed with SEM and trichome density was calculated and expressed in terms of glandular trichomes per cm² of leaf sample. Similar to the results of steviol glycoside content, the highest trichome density was
observed in plants treated with *P. indica + A. chroococcum*, followed by single inoculation of *P. indica, A. chroococcum* and control.

Jasmonic acid is known to alter the epidermal differentiation and in turn increase the trichome density. The level of jasmonic acid content in leaves of *S. rebaudiana* was also determined to further validate the results as they are also known to mediate steviol glycosides enhancement by altering the genes of the MEP pathway. An HPTLC method was developed and validated for determination of jasmonic acid in leaf samples of *S. rebaudiana*. The results showed a correlation between steviol glycoside content, trichome density and jasmonic acid content of plants. Combined inoculation of *P. indica* and *A. chroococcum* showed enhanced jasmonic acid content as compared to single inoculation of *P. indica* and *A. chroococcum* and control.

The transcription profiling of eight key genes (DXR, GGDPS, KS, KO, KAH, UGT85C2, UGT74G1 and UGT76G1) of the steviol biosynthesis pathway was undertaken by relative quantification. RNA extraction was done followed by first strand synthesis using gene specific primers. Relative quantification was done by quantitative real-time PCR using β-Actin gene of *S. rebaudiana* as an internal control to normalize experimental gene expression. The results showed significant upregulation of all the genes, correlating the study to the enhanced concentration of steviol glycosides upon combined inoculation of *P. indica* and *A. chroococcum* as compared to single inoculations and control, under both in vitro and greenhouse conditions. The study showed induction of the MEP pathway gene, DXR and a cascading effect on the downstream genes; GGDPS, KS, KO and KAH. Enhanced transcript levels of UGT85C2, UGT74G1, and UGT76G1 exhibited the increase in stevioside and rebaudioside-A contents. The study provided evidence that the combined effects of *P. indica* and *A. chroococcum* causes induction of the steviol biosynthesis pathway and enhances production of steviol glycosides in *S. rebaudiana*.

The antioxidants, phenolics and flavonoids have a multifunctional role in plant microbe interaction and are also known to affect the taste of *S. rebaudiana*. The total phenolic and
flavonoid content was determined for in vitro and greenhouse grown plants by biochemical assays and expressed as mean phenolic and flavonoid content. The antioxidant potential was determined by DPPH free radical scavenging activity and the results were expressed as percentage inhibition and IC$_{50}$ value. Highest antioxidant activity, total phenolic acid and flavonoid content were seen in methanol extracts from P. indica + A. chroococcum treated plants followed by single treatments with P. indica and A. chroococcum.

S. rebaudiana plants with combined inoculations also showed increased levels of soluble sugar, mineral nutrient (phosphorus and nitrogen) and chlorophyll content which are known to modulate plant metabolic pathways and photosynthetic rate. Soluble sugar content was determined spectrophotometrically and expressed in terms of glucose equivalent. The combined treatment of P. indica and A. chroococcum showed the highest content of total soluble sugars compared to individual treatments of P. indica, A. chroococcum and control plants. The enhanced soluble sugar content can be correlated to the increased growth promotion in plants, as higher sugar concentrations in plants are known to promote carbohydrate storage and growth. Estimation of inorganic phosphate and nitrogen was carried out by spectrophotometry and Kjeldahl digestion respectively, in leaf samples from treated and untreated plants. Dual inoculation with P. indica and A. chroococcum showed maximum enhancement in phosphorus and nitrogen content, followed by single inoculations and control. Total chlorophyll content was also estimated in our studies as the leaf nitrogen content strongly influences the light use efficiency and photosynthetic rate. The chlorophyll a, chlorophyll b and total chlorophyll (mg g$^{-1}$ fresh weight) content was measured spectrophotometrically and calculated by Amon equations in treated and control samples. Changes in leaf nitrogen levels were found to be consistent with the chlorophyll content under single and dual inoculations. Leaf samples from plants after dual inoculation with P. indica and A. chroococcum showed higher total chlorophyll content as compared to single treatments and control.

The present study focused P. indica and A. chroococcum as plant growth promoting biological agents and successful inoculants for enhancement of steviol glycoside content
and sweetness ratio in *S. rebaudiana*. The study encompasses analysis of plant growth and yield attributing traits, biochemical and phytochemical analysis, and gene expression studies of key genes of steviol biosynthesis pathway suggesting the beneficial effects of the combined application of the endophyte, *P. indica* with plant growth promoting rhizobacteria, *A. chroococcum*. The study thus demonstrates *P. indica* and *A. chroococcum* as a suitable bio-inoculant for yield and steviol glycoside enhancement in medicinal plant *S. rebaudiana*, providing an important advancement for future successful commercial exploitation of this plant.