Synergy between Glomus fasciculatum *and a beneficial* Pseudomonas *in reducing root diseases and improving yield and forskolin content in* Coleus forskohlii *Briq. under organic field conditions*

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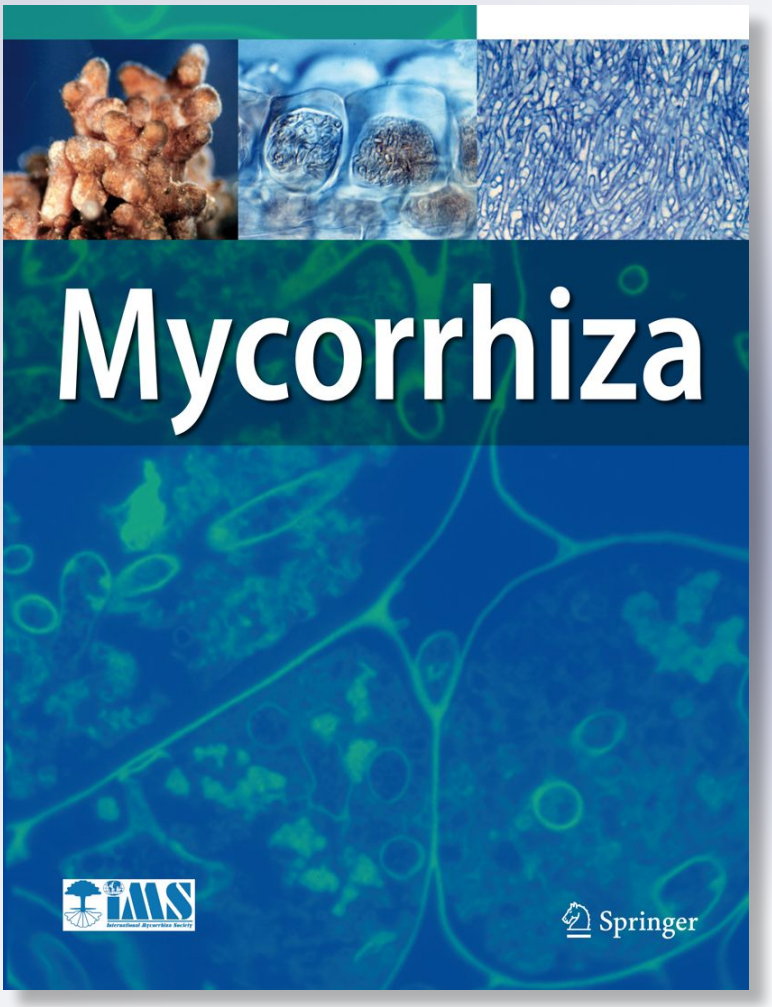

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ORIGINAL PAPER

Synergy between *Glomus fasciculatum* and a beneficial *Pseudomonas* in reducing root diseases and improving yield and forskolin content in *Coleus forskohlii* Briq. under organic field conditions

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Abstract Root rot and wilt, caused by a complex involving *Fusarium chlamydosporum* (Frag. and Cif.) and *Ralstonia solanacearum* (Smith), are serious diseases affecting the cultivation of *Coleus forskohlii*, a crop with economic potential as a source of the medicinal compound forskolin. The present 2-year field experiments were conducted with two bioinoculants (a native *Pseudomonas monteilii* strain and the exotic arbuscular mycorrhizal (AM) fungus *Glomus fasciculatum*) alone and in combination under organic field conditions in order to evaluate their potential in controlling root rot and wilt. Combined inoculation of *P. monteilii* with *G. fasciculatum* significantly increased plant height, plant spread, and number of branches; reduced disease incidence; and increased tuber dry mass of *C. forskohlii*, compared to vermicompost controls not receiving any bioinoculants. Increase in tuber yields was accompanied by an increase in plant N, P, and K uptake. Co-inoculation of *P. monteilii* with *G. fasciculatum* significantly improved the percent AM root colonization and spore numbers retrieved from soil. This suggests *P. monteilii* to be a mycorrhiza helper bacterium which could be useful in organic agriculture. The forskolin content of tubers was significantly increased by the inoculation treatments of *P. monteilii*, *G. fasciculatum,* and *P. monteilii* + *G. fasciculatum*.

Keywords *C. forskohlii* · PGPR · AM fungus · Vermicompost · Organic cultivation · Root rot/wilt

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Introduction

Coleus forskohlii Briq. (family Lamiaceae) grows perennially in tropical and subtropical regions of India, Pakistan, Sri Lanka, East Africa, and Brazil. Its tubers are the source of a labdane diterpene compound called forskolin (also called coleonol) which has a unique property to resensitize cell receptors by activating the enzyme adenylyl cyclase to increase the levels of cyclic adenosine monophosphate. Forskolin is also a potent vasodilatory, anti-hypertensive, and inotropic agent (Seamon 1984). The *Coleus* crop has economic potential as a source of forskolin for treating glaucoma, cardiac problems, and certain types of cancers (Shah et al. 1980; Kavitha et al. 2010). Its ethnomedicinal use for relief of cough, eczema, skin infections, tumors, and boils has been also reported (De Souza et al. 1986). Because of the continuous collection of tubers from wild sources, this plant has been included in the list of endangered species (Boby and Bagyaraj 2003). Recent interest in the cultivation of *C. forskohlii* to meet demands has resulted in an annual production of about 100 tons from 700 ha in India (Shivkumar et al. 2006).

C. forskohlii is susceptible to many diseases, of which root rot and wilt are the most important, causing serious economic losses. The main pathogens causing disease have been identified as *Fusarium chlamydosporum* (Shyla 1998; Shivkumar et al. 2006; Singh et al. 2009) and *Fusarium oxysporum* (Khatun and Chatterjee 2011; Khatun et al. 2011). However, soil-borne diseases are complex because of the array of organisms associated with them, and *Ralstonia solanacearum* has also been reported to be causing vascular wilt of *Coleus* (Coelho Netto and Assis 2002). Organic and biological methods are being considered as a potential strategy for the management of diseases of medicinal plants in general and for the control of soil-borne

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pathogens in particular, because chemical methods result in accumulation of harmful chemical residues which may lead to serious ecological and health problems.

The ability of arbuscular mycorrhizal (AM) fungi not only to increase the absorbing surface area of the root for nutrients but also to induce suppression of root pathogens and reduce disease is well documented (Sampangi and Bagyaraj 1989; Mohan and Verma 1996; Azcón-Aguilar and Barea 1996; Prashanthi et al. 1997; Boby and Bagyaraj 2003; Harrier and Watson 2004; Whipps 2004). Another role of AM is to enhance plant quality by stimulating the synthesis of secondary metabolites/bioactive compounds (organosulfides, polyphenols, phytosterols, stilbenes, vitamins, lignans, and terpenoids including carotenoids) which can be important for plant tolerance to abiotic and biotic stresses, or beneficial to human health through their antioxidant activities (Gianinazzi et al. 2008, 2010). Pseudomonads can suppress a wide range of plant pathogens including *Fusarium* and *R. solanacearum* (Weller 1988; Nautiyal 1997; Johansson et al. 2003; Compant et al. 2005; Pal and Gardener 2006; Ramesh et al. 2009; Vanitha et al. 2009). This group of bacteria, also considered as plant growth-promoting rhizobacteria (PGPR), can exhibit multiple functional traits improving plant nutrient uptake, tolerance to stress, salinity, metal toxicity, and pesticides (Naik et al. 2008; Ramesh et al. 2009; Rani et al. 2009).

Synergistic interactions between PGPRs and AM fungi can have potentially beneficial functions in different plants, including growth promotion, disease suppression, and higher yields of secondary metabolites (Kloepper 1996; Awasthi et al. 2011; Bharadwaj et al. 2011; Liu et al. 2012; Hemashenpagam and Selvaraj 2011), and some pseudomonads have been shown to impact on AM formation and function (Barea et al. 1998, 2005). In *C. forskohlii*, the inoculation with AM fungi and/or PGPR has been reported to result in increased synthesis of secondary metabolites (Boby and Bagyaraj 2003; Sailo and Bagyaraj 2005; Singh et al. 2009, 2011, 2012).

A better understanding of the interactions between AM fungi and PGPRs will promote the development of strategies for sustainable management of soil fertility, crop protection, and production. Earlier, we have observed that an AM fungus (*Glomus fasciculatum*) and a PGPR (*P. fluorescens*) were effective in reducing root rot severity and improving tuber yields in *C. forskohlii* (Singh et al. 2009). Recently, we isolated a fluorescent pseudomonad, designated as CRC1, which showed a growth-promoting potential in *C. forskohlii* and a stronger antagonistic activity than the *P. fluorescens* strain used earlier (against *F. chlamydosporum* and *R. solanacearum*) under glass house conditions (unpublished data). Here, we identify the isolate CRC1 as *Pseudomonas monteilii* and describe a study of its synergism with *G. fasciculatum* in controlling root rot and wilt in *C. forskohlii* with the aim of improving forskolin yields specifically for organic/sustainable agriculture.

Materials and methods

Isolation of fungal and bacterial pathogens

F. chlamydosporum and *R. solanacearum* were isolated from infected root and shoot tissues of root rot and wilt-affected plants of *C. forskohlii* grown at Bangalore, India. The identity of the fungal pathogen *F. chlamydosporum* was confirmed by the Division of Mycology and Plant Pathology, Indian Agricultural Research Institute (Boby and Bagyaraj 2003), whereas the bacterial pathogen *R. solanacearum* was identified following the methods described by Kelman (1954) and Vanitha et al. (2009). The virulent *Ralstonia solanaceraum* (strain CIMAP-R7) and *F. chlamydosporum* (strain CIMAP-F6) isolates were purified and preserved at −80°C with 50 % glycerol (Sarvanakumar et al. 2009) in the culture collection of Central Institute of Medicinal and Aromatic Plants (CIMAP).

Isolation of plant growth promoting/pathogen antagonistic bacteria

Samples were collected from a disease suppressive soil of *C. forskohlii* fields located in Salem district of Tamil Nadu, India, where the incidence of root rot and wilt was negligible, in order to isolate microbes antagonist to *F. chlamydosporum* and *R. solanacearum*. From each field, 500-g soil was collected from the rhizosphere of *C. forskohlii* at five distant points (within an area of 40×30 m), pooled, and sealed in polyethylene bags prior to use. Soil samples were serially diluted to 10^{-4} and plated in triplicate on King's B agar medium liter^{-1} (distilled water 1 L, proteose peptone Difco 20 g, K_2HPO_4 1.5 g, glycerol 10 mL, agar 15 g, pH 7.0) to detect fluorescent pseudomonads (King et al. 1954). After 48 h, bacterial colonies producing a fluorescent yellow pigment under UV light (366 nm) were selected, and 12 strains were checked for antagonistic activity against *R. solanacearum* (strain CIMAP-R7) and *F. chlamydosporum* (strain CIMAP-F6) in vitro condition using agar diffusion and dual culture methods, respectively. One bacterium, CRC1, showing a maximum zone of inhibition (clear area of 23±2 mm) against both pathogens was selected for our study. A purified culture was preserved at −80°C with 50 % glycerol (Sarvanakumar et al. 2009) in the culture collection of CIMAP.

Molecular characterization of the bacterial isolate CRC1

Bacterial genomic DNA was extracted from overnight grown cells using standard procedures (Chachaty and

Saulnier 2000), electrophoretically separated on 0.8 % agarose gel in TAE buffer, and visualized under UV (Uvitec, Bangalore Genei, India) to check for integrity. The extracted DNA was quantified spectrophotometrically (Nanodrop ND1000). The universal primers (forward 5′-AGAGTTTGATCCTGGCT CAG-3′ and reverse 5′-ACGGCTACCTTGTTACGACTT-3′) described earlier (Wood et al. 1998; Awasthi et al. 2011) were used to amplify the 16S rRNA gene from the bacterial strain. Approximately 25 ng of bacterial genomic DNA and 10 pmol of forward and reverse primer, 0.6 U of Taq polymerase, and 2.5 μL of 10× buffer (Bangalore Genei, India) were used for amplification in a Mastercyler gradient (Eppendorf) programmed as 94°C for 5 min; 34 cycles of 94°C for 1 min, 57.4°C for 1 min, 72°C for 2 min; 72°C for 10 min; 4°C for an infinite period. PCR products were checked in 1.26 % agarose gels in TAE buffer stained with ethidium bromide (0.5 mg mL^{-1}) and visualized under UV. The PCR product was purified using PCR Cleanup Kit (Genaxy) according to the manufacturer's instructions and directly sequenced using the forward universal primer and Big Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) on a 3130xl Genetic Analyzer (Applied Biosystems, USA) using the manufacturer's protocol. Sequence analysis was carried out using the nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The 16S rRNA gene sequence of isolate CRC1 was aligned with those of 25 other fluorescent pseudomonads using ClustalW alignment tool (accessed through the MEGA, version 5; Tamura et al. 2011). A phylogenetic tree was constructed using the bootstrapped neighbor-joining tree method from MEGA5 which gave maximum similarity of CRC1 with *P. monteilii* (see "Results"). The 16S rRNA gene sequence of strain CRC1 was submitted to NCBI GenBank database (accession number HQ995498).

Preparation of bio-inoculums

The AM fungus *G. fasciculatum* (Thaxter) Gerdemann and Trappe emend Walker and Koske (isolate GF1), obtained from microbial culture collection of CSIR-CIMAP, was propagated on maize roots (*Zea mays* L.) for 10 weeks in a 1:1 (*v*/*v*) mix of sterilized sand and soil (5 kg) of low phosphorus content (7.5 kg ha^{-1}) and subsequently left to shade dry for 2 weeks. AM roots (colonized 70 %) were extracted from the cultures, cut into 1-cm segments, and thoroughly re-mixed into the sand–soil substrate from the pot culture, and the composite inoculum was stored at 5°C until use. The inoculum potential of *G. fasciculatum* used in this study was calculated to be 5.3± 1.3 infective propagules g^{-1} of sand–soil substrate by the method of Liu and Luo (1994).

The cultures of the bacterial isolate CRC1 were prepared in nutrient broth for 24 h on an incubator shaker (210 rpm). The bacterial suspension was centrifuged at 8,000 rpm for 10 min, the supernatants were discarded, and the pellets containing bacterial cells were suspended in 500 mL of 100 mM phosphate buffer to give a colony forming unit (CFU) of 9.1×10^{8} mL^{-1}.

Nursery preparation and growth conditions

Cuttings of *C. forskohlii* were raised in 15×6 cm polyethylene bags containing 200 g of either a soil/vermicompost (9:1 *v*/*v*) potting mixture or soil alone. Healthy stem cuttings were dipped in the CRC1 bacterial culture suspension for half an hour before planting, and *G. fasciculatum* inoculum (10 g $cutting^{-1}$) was placed adjacent to the cut-end of stem cuttings at planting. The bacterial culture suspension (5 mL bag^{-1}) was also poured into their respective treatments after planting. Planted cuttings were placed in agrinet shade of 60 % for 50 days for rooting (Singh et al. 2009). The plants were kept for 5–7 days in open conditions to harden before transplanting to the field.

Transplanting to the field

The experiment was conducted in a certified (ECOCERT) organic farm of the CIMAP, in Bangalore (India), located at 12 °58′ N 77 °35′ E and 930 m above mean sea level, where *C. forskohlii* was grown continuously for the last 3 years, and disease incidence was more than 80 %. The soil of the experimental field was a red sandy loam with pH 6.4, 125 kg ha^{-1} available N, 11.5 kg ha^{-1} available P, 123.7 kg ha^{-1} available K, and 0.44 % organic carbon. Treatments were disposed in four replicate plots (1.8×3.6 m) each arranged in a randomized block design in the naturally infected soil. Each plot was separated with 50-cm flat ridges (guard row) to mitigate the effect of adjoining plots. Initial soil samples (200 g) were collected using a soil auger (0–15 cm) from five points of each replicated treatment, pooled, and mixed; a subsample was used to record the initial microbial population (beneficial and pathogenic) prior to transplanting of the *C. forskohlii* cuttings. The soil was uniformly infested by *F. chlamydosporum* (CFU $6.2\pm0.2\times10^{4}$ g^{-1} soil) and *R. solanacearum* (CFU $3.6\pm0.2\times10^{6}$ g^{-1} soil) throughout the field. The 55-day-old rooted nursery cuttings of *C. forskohlii* were planted on both edges of ridges at a spacing of 60×45 cm (row spacing was 60 cm, 45 cm between plants within each row, and each ridge separated by 20-cm channel used for irrigation). There were four rows per plot. The *C. forskohlii* crop was maintained in the same plots with same treatments for 2 years. *C. forskohlii*-rooted cuttings were re-transplanted twice during the 2-year period. The total N requirement (40 kg ha^{-1}) of plants was met by the application of vermicompost as an organic nutrient supplement, whereas plots receiving vermicompost only (no bioinoculants) served as control. The vermicompost was produced from a distillation waste mixture of

lemongrass, patchouli, and geranium produced in a vermicomposting unit using *Eudrilius euginiae*, an epigeic species of earthworms, at our certified organic farm. The vermicompost contained 1.10 %N, 0.75 % P, and 0.81 %K.

Transplanting of rooted *C. forskohlii* cuttings was done by placing intact ball of earth (soil mixture) into planting holes having a depth of 10–12 cm and diameter of 8–10 cm. Each bed contained 32 plants, but only 12 net plants (non-peripheral plants to avoid border effects) were considered for the various observations in five treatments: plots receiving cuttings treated with (a) CRC1−*P. monteilii* (PM), (b) *G. fasciculatum* (GF), (c) *P. monteilii* (PM) + *G. fasciculatum* (GF), (d) plots receiving cuttings not treated with bioinoculants but supplemented with vermicompost [vermicompost (VC) control], and (e) plots receiving untreated cuttings grown in soil only (without vermicompost).

Plant growth and wilt observations in field conditions

Five randomly tagged plants out of 12 net plants from each plot were considered for growth parameters (plant height, plant spread, and number of branches). Plant growth parameters such as plant height (measured from soil surface to the growing tip of the plant), number of branches, and plant spread were recorded at the time of harvesting. Percent wilt incidence (PWI) (yellowing and drooping of leaves, browning of vascular stem tissues) was assessed in the field with net plants of each replicate plot before harvest as numbers of wilted plants / total number of plants ×100.

Harvesting

Harvesting was done 140 days after transplanting each year. Plants were manually uprooted with proper care of not damaging the tubers which constitute more than 90 % of total root biomass, although some finer roots are always attached. Root rot disease severity was measured at harvest (see below). Fresh and dry root and shoot weights were recorded from each replicate net plot. Samples (shoot and root) were taken randomly and mixed, and then a subsample of 200 g of fresh shoot was dried in a hot air oven at 80°C for 24 h, whereas fresh root samples (200 g) were chopped into 1–2 cm of small pieces and shade-dried for 5–7 days to determine dry matter yields, moisture content, and nutrient concentration. Powdered dry roots (pass through 0.2-mm sieve) were used to measure nutrient and forskolin content. At the time of harvesting, rhizospheric soil samples (200 g) were also collected in the same manner as in initial soil sampling to determine beneficial and pathogenic microbial populations from each plot. Nutrient concentration (NPK) in dried root tubers, with some attached fine roots, and in shoots was determined as described by Jackson (1973). Total nutrient uptake by plants was determined from the nutrient concentrations and total dry matter yield.

Percent disease index

Root rot disease severity was measured on a 0–4 scale from 0 = healthy roots (no symptoms) to 4 = >75 % root tubers affected by rot (blackening, oozing, and putrefaction of roots and plant death). The percentage disease index (PDI) was calculated based on root rot severity scores for net plants in all three replicates of each treatment as: PDI = (sum of numerical grading recorded / number of roots observed × highest numerical rating) ×100.

Forskolin estimation

Forskolin in the *C. forskohlii* tubers with attached fine roots was estimated by HPLC. One gram of powdered dried tubers was transferred into a conical flask (25 mL) to which 10 mL of acetonitrile was added. The samples were sonicated four times for 15 min each and filtered after each sonication. The four extracts of each sample were combined, concentrated, and diluted to the final volume of 10 mL with acetonitrile. Samples were filtered (0.45 μm nylon filter membrane), and 25 μL was injected into a Shimadzu LC10ATVP HPLC equipped with a Phenomenex Luna 5 μm, C18(2) 100 Å, 250 mm×4.6 ID. Operation parameters were oven temperature 30°C, mobile phase acetonitrile/water with a flow rate of 1 mL min^{-1}. Standards preparation, calibration, and gradient elution were done as per the method reported by Schaneberg and Khan (2003). Forskolin yield was calculated based on percent forskolin content and dry tuber mass.

Microbial population estimations

To assess AM fungal colonization, fine roots from host plants were cut into 5-mm-long pieces, cleared with 10 % KOH, stained with 0.05 % trypan blue, and percentage root calculated as described by McGonigle et al. (1990) based on the presence of aseptate hyphae/vesicles/arbuscules. The wet sieving and decanting method was used to isolate AM fungal spores and estimate abundance (Gerdemann and Nicolson 1963). *Pseudomonas* populations in the root zone soil were determined by the serial dilution technique with 0.85 % saline solution using King's B medium. The density of pathogen populations (*Fusarium* and *Ralstonia*) was estimated using PCNB peptone (Nash and Snyder 1962) and 2,3,5-triphenyl tetrazolium chloride semi-selective (Kelman 1954) media, respectively.

Statistical analysis

For all parameters, data from five treatments with three replicates in the two trials during the 2-year period were

analyzed statistically by the analysis of variance method (ANOVA) for randomized complete block design. The experimental data from the two trials had a similar variance value; hence, the data were combined for further analysis. Significant differences among treatments were based on the *F* test in ANOVA, and treatment means were compared using least significant difference at $P \leq 0.05$ (Snedecor and Cochran 1989). The data on percent root colonization by AM fungus were statistically analyzed using arcsine square-transformed values. The standard error of means in vertical bar charts was computed using Sigma Plot 11.

Results

16S rRNA gene sequence-based identification of the bacterial isolate CRC1

The BLAST analysis of the 16S rRNA gene sequence from the CRC1 isolate revealed that the bacterium belongs to the genus *Pseudomonas*, and its phylogenetic position in relation to other related *Pseudomonas* is given in Fig. 1. Phylogenetic analysis showed that isolate CRC1 has a maximum similarity with *P. monteilii* strain WAPP53 (accession no. FJ905913); therefore, isolate CRC1 was designated as *P. monteilii*. This identification was confirmed by CSIR-IMTECH, Chandigarh, India, and the bacterium is deposited with MTCC as strain no. 9796.

Effect of bioinoculants on growth parameters of *C. forskohlii* under field conditions

At harvest, plants treated with *P. monteilii*, *G. fasciculatum*, or *P. monteilii* + *G. fasciculatum* showed a significant increase in plant height of 18, 20, and 38 %, respectively, compared to the vermicompost control plants. Plant spread and number of branches showed a similar trend, with maximum plant spread (38 %) and number of branches (65 %) with co-inoculation of *P. monteilii* + *G. fasciculatum* (Fig. 2).

Effect of bioinoculants on tuber and shoot yield and on forskolin concentration in *C. forskohlii* under field conditions

Plants inoculated with *G. fasciculatum*, *P. monteilii*, or *P. monteilii* + *G. fasciculatum* had significantly higher dry tuber yields (32, 38, and 103 %, respectively) and higher dry shoot mass (27, 34, and 54 %, respectively) than the vermicompost control plants (Fig. 3). Forskolin content was significantly influenced by inoculation with *G. fasciculatum*, *P. monteilii*, or *P. monteilii* + *G.*

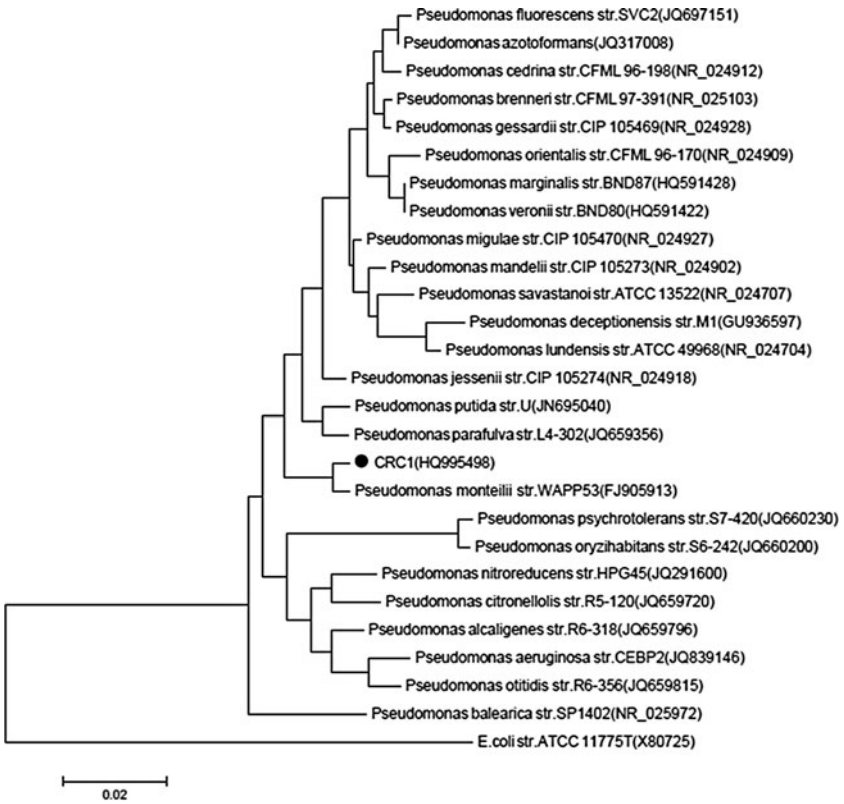

Fig. 1 Phylogenetic tree constructed from the 16S rRNA gene of strain CRC1 and related organisms using neighbor-joining algorithm from an alignment of 801 nucleotides. Accession numbers of corresponding sequences are given in *parentheses* and *scale bar* represents 1 base substitution per 20 nucleotide positions. The bootstrap probabilities calculated from 1,000 replications. *E. coli* str. ATCC 11775T was taken as an out-group

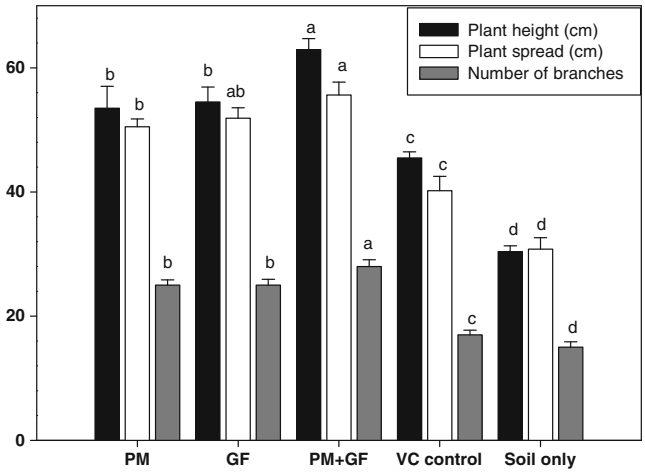

Fig. 2 Effect of *P. monteilii* (*PM*) (strain CRC1) and *G. fasciculatum* (*GF*) alone and co-inoculated (*PM* + *GF*) on growth characteristics of *C. forskohlii*. *PM P. monteilii*, *GF G. fasciculatum*, *PM* + *GF* co-inoculation of *P. monteilii* and *G. fasciculatum*, *VC* vermicompost control. *Error bars* shown as standard error of mean (SE). *Different letters above the error bars* indicate a significant difference at $P \leq 0.05$

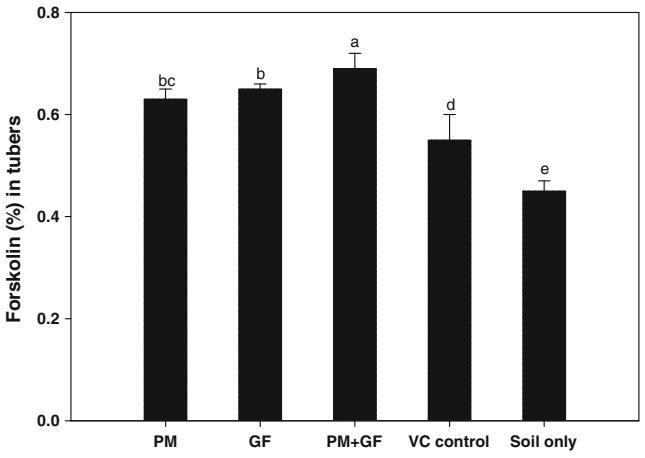

Fig. 4 Effect of *P. monteilii* (*PM*) (strain CRC1) and *G. fasciculatum* (*GF*) alone and co-inoculated (*PM* + *GF*) on forskolin content (percent) in root tubers of *C. forskohlii*. *PM P. monteilii*, *GF G. fasciculatum*, *PM* + *GF* co-inoculation of *P. monteilii* and *G. fasciculatum*, *VC* vermicompost control. *Error bars* shown as standard error of mean (SE). *Different letters above the error bars* indicate a significant difference at $P \leq 0.05$

fasciculatum, showing an increase of 15, 18, and 25 %, respectively (Fig. 4).

Effect of bioinoculants on the incidence and severity of root rot or wilt under field conditions in *C. forskohlii*

All the bioinoculants alone or in combination significantly reduced (52–68 %) wilt incidence (PWI) in *C. forskohlii* compared to vermicompost control plants, with a maximum reduction in plants treated with *P. monteilii* + *G. fasciculatum* (68 %) (Fig. 5). The treatments *P. monteilii*, *G. fasciculatum*, and *P. monteilii* + *G. fasciculatum* also significantly reduced (56–63 %) the severity of root rot as indicated by the PDI, as compared to vermicompost control plants (Fig. 5). The highest reduction in disease index was observed in plants co-inoculated with *P. monteilii* + *G. fasciculatum* (63 %) (Fig. 5). In addition, significant reductions were observed in PWI and PDI in the treatment with vermicompost (no bioinoculants) as compared to soil alone (no bioinoculants or vermicompost; Fig. 5), indicating a role of the vermicompost in reducing the incidence and severity of disease.

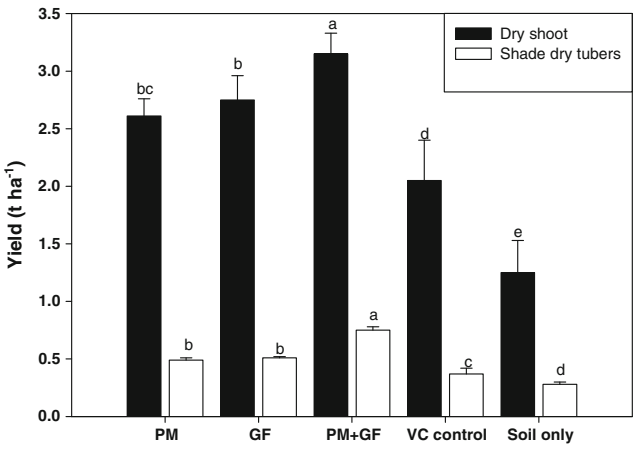

Fig. 3 Effect of *P. monteilii* (*PM*) (strain CRC1) and *G. fasciculatum* (*GF*) alone and co-inoculated (*PM* + *GF*) on yield of *C. forskohlii*. *PM P. monteilii*, *GF G. fasciculatum*, *PM* + *GF* co-inoculation of *P. monteilii* and *G. fasciculatum*, *VC* vermicompost control. *Error bars* shown as standard error of mean (SE). *Different letters above the error bars* indicate a significant difference at $P \leq 0.05$

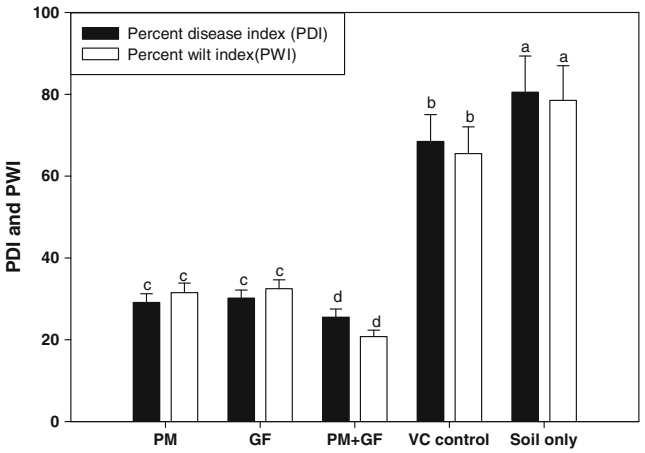

Fig. 5 Effect of *P. monteilii* (*PM*) (strain CRC1) and *G. fasciculatum* (*GF*) alone and co-inoculated (*PM* + *GF*) on percent disease index (*PDI*) and percent wilt incidence (*PWI*) of *C. forskohlii*. *PM P. monteilii*, *GF G. fasciculatum*, *PM* + *GF* co-inoculation of *P. monteilii* and *G. fasciculatum*, *VC* vermicompost control. *Error bars* shown as standard error of mean (SE). *Different letters above the error bars* indicate a significant difference at $P \leq 0.05$

Plant nutrient uptake and rhizospheric bioinoculant populations

The data on nutrient uptake are presented in Table 1. N uptake improved significantly in *C. forskohlii* plants with inoculation of *P. monteilii* (13 %), *G. fasciculatum* (16 %), or *P. monteilii* + *G. fasciculatum* (26 %). Uptake of P and K was also significantly higher in the treatments with *P. monteilii* (16 and 20 %, respectively), *G. fasciculatum* (25 and 37 %, respectively), and *P. monteilii* + *G. fasciculatum* (60 and 42 %, respectively).

The initial populations of fluorescent pseudomonads and AM fungi were 3.1×10^4 CFU g^{-1} soil and 55 spores 50 g^{-1} soil, respectively, in the experimental field. In the case of *G. fasciculatum*-treated plants of *C. forskohlii*, root colonization (67 %) and number of AM fungal spores (240 spores 50 g^{-1} soil) were higher in the rhizosphere, as compared to control plants (in vermicompost or soil), and higher populations of pseudomonads (1.21×10^5 CFU g^{-1} soil) were also observed in the root zone (Table 2). Significantly higher numbers of AM fungal spores (255 spores 50 g^{-1} soil), percent root colonization (71 %), and pseudomonad populations (1.65×10^5 CFU g^{-1} soil) were also observed in treatment receiving both *P. monteilii* + *G. fasciculatum* (Table 2).

Efficacy of bioinoculants in reducing plant pathogen populations

The population (CFU per gram of soil) of *Fusarium* and *Ralstonia* prior to transplanting into the field was 6.3×10^4 and 3.7×10^6, respectively. The maximum reduction in the *Fusarium* population (CFU per gram of soil) was observed with plants co-inoculated with *P. monteilii* + *G. fasciculatum* (65 %) followed by *P. monteilii* (54 %), then *G. fasciculatum* (51 %), as compared to control plants (in vermicompost or soil). The *Ralstonia* population followed the same trend as for *Fusarium* (Table 2).

Discussion

Tuberous roots are the main economic part of *C. forskohlii*. A considerable loss in root yields occurs because of root rot and wilt, especially under organic field conditions. Efforts were made to minimize these losses through the application of the microbial inoculants *P. monteilii* and *G. fasciculatum* in order to manage root rot and wilt and increase *C. forskohlii* yields under certified organic field conditions. Inoculation with *G. fasciculatum* and *P. monteilii* alone or co-inoculation of *G. fasciculatum* with *P. monteilii* resulted in significantly higher tuber yields and improved tuber contents of forskolin compared to vermicompost or soil control grown plants. A *P. monteilii* strain (97AN) has previously been shown to improve plant growth and yield of soybean crop (Rani et al. 2009), and Edwards et al. (1998) reported that *P. fluorescens* performed better in terms of improving growth of tomato (*Lycopersicon esculentum*) and leek (*Allium porrum*) when the bacterium was co-inoculated with *Glomus mosseae*.

C. forskohlii derives its medicinal value from the presence of forskolin in its tubers. The present observations on the effect of bioinoculants in increasing forskolin contents under diseased field conditions are in agreement with those from pot experiments by Singh et al. (2009) where inoculation of *P. fluorescens* or *G. fasciculatum* alone significantly improved the content of forskolin in the presence of low pathogen (*F. chlamydosporum*) levels. Positive effects of AM on medicinal compound content have been reported in other plants (Gianinazzi et al. 2010). For example, Kapoor et al. (2007) observed a significant increase in the density of glandular trichomes (site for biosynthesis and sequestration of antimalarial artemisinin) in the medicinal plant *Artemisia annua* following inoculation with the AM fungi *Glomus macrocarpum* and *G. fasciculatum*, contributing to an enhanced artemisinin content in the plant. However, Awasthi et al. (2011) reported that while individual inoculation with an AM fungus (*G. mosseae*) or *Bacillus subtilis* was not

Table 1 Effect of *P. monteilii* (PM) (strain CRC1), *G. fasciculatum* (GF), and co-inoculated (PM + GF) on nutrient uptake of *C. forskohlii*

Treatments	Shoot uptake (kg ha^{-1})			Root (tubers + fibrous) uptake (kg ha^{-1})			Total uptake (kg ha^{-1})		
	N	P	K	N	P	K	N	P	K
PM	28.75 c	18.75 c	25.55 b	1.75 b	2.45 b	2.75 b	30.50 b	21.20 c	28.30 c
GF	29.55 b	20.45 b	29.45 a	1.80 b	2.45 b	2.80 b	31.35 b	22.90 b	32.25 b
PM + GF	31.45 a	25.45 a	30.45 a	2.55 a	3.75 a	3.12 a	34.00 a	29.20 a	33.57 a
VC	25.55 d	16.45 d	21.45 c	1.45 c	1.85 c	2.14 c	27.00 c	18.30 d	23.59 d
Soil only	20.12 e	12.50 e	18.45 d	1.25 c	1.25 d	1.85 d	21.37 d	13.75 e	20.30 e

Values in each column followed by different letters are significantly different at $P\leq0.05$

PM P. monteilii, *GF G. fasciculatum*, *PM + GF* co-inoculation of *P. monteilii* and *G. fasciculatum*, *VC* vermicompost control

Table 2 Mean population of microbes in the root zone soil of *Coleus forskohlii* at the time of harvesting

Treatments	Root zone microbial population				
	Fluorescent pseudomonads ($\times 10^4$ g^{-1} soil)	Mycorrhizal spores 50 g^{-1} soil	Percent root colonization	*Fusarium* ($\times 10^4$ g^{-1} soil)	*Ralstonia* ($\times 10^6$ g^{-1} soil)
PM	13.2 b	231 c	58 c	2.90 b	1.61 c
GF	12.1 c	240 b	65 b	2.91 b	1.59 c
PM + GF	16.5 a	255 a	71 a	2.21 c	1.23 d
VC	4.01 d	61 d	33 d	6.30 a	3.51 b
Soil only	3.15 e	55 e	28 d	6.34 a	3.70 a

Values in each column followed by different letters are significantly different at $P \leq 0.05$

PM P. monteilii, *GF G. fasciculatum*, *PM + GF* co-inoculation of *P. monteilii* and *G. fasciculatum*, *VC* vermicompost control

effective in increasing artemisinin content, co-inoculation of the two microbes (*G. mosseae* + *B. subtilis*) significantly increased the content of artemisinin.

The higher tuber yields in *C. forskohlii* plants inoculated with *G. fasciculatum* and/or *P. monteilii* under field conditions may result from the effectiveness of the bioinoculants in reducing phytopathogen attack and improving the availability of nutrients to the plants (Singh et al. 2011; Boby and Bagyaraj 2003). *G. fasciculatum* and pseudomonads have been documented as inhibitors of pathogens like *Fusarium* and *Ralstonia* (Shivkumar et al. 2006; Ramesh et al. 2009; Singh et al. 2009). According to Cook and Sequeira (1991), *R. solanacearum* is a poor competitor outside the plant system, giving an ample opportunity for biological control. Although root rot and wilt disease was reduced (>50 %) by single or co-inoculations, co-inoculation of *G. fasciculatum* and *P. monteilii* resulted in maximum reductions in the populations of *Ralstonia* and *Fusarium*. This better performance when present together may be due to the bioinoculants working by different modes in association with *C. forskohlii*. Plots receiving vermicompost performed better in terms of disease control when compared with plots not receiving any bioinoculants and vermicompost (soil only). Vermicomposts produced from distillation waste of aromatic plants have been shown to reduce the severity of some phytopathogens (Pandey and Kalra 2010; Singh et al. 2012). The presence of organic manure like vermicompost could also help in improving the population of beneficial microbes (Kalra et al. 2010).

Single or combined inoculation of *G. fasciculatum* and *P. monteilii* significantly influenced the nutrient uptake (NPK) by *C. forskohlii*. Plants inoculated with *P. monteilii* + *G. fasciculatum* showed the highest NPK uptake. Similar findings were reported in plants like *Eucalyptus hybrid* and *Ficus benjamina* inoculated with beneficial fungal and bacterial consortia (Sastry et al. 2000; Srinath et al. 2003). *P. monteilii* has been referred to as a P solubilizer (Naik et al. 2008; Rani et al. 2009) which could have contributed to its effect in terms of improved phosphorus uptake by *C. forskohlii*. *P. monteilii* could be an exceptionally useful microbe in organic or sustainable farming where phosphorus is a limiting factor by playing a vital role together with AM fungi in solubilizing inorganic phosphate that could be transported by hyphae of the mycorrhizal fungi to the plants. Also, its antagonism towards *F. chlamydosporum* and *R. solanacearum* and its compatibility with *G. fasciculatum* to form an effective microbial consortium could be useful in organic farms for nutrient and disease management.

The highest mycorrhizal root colonization, number of AM fungal spores, and pseudomonad population were observed when *G. fasciculatum* and *P. monteilii* were co-inoculated onto *C. forskohlii* plants. Under field conditions, higher AM colonization might influence the species composition of the soil microbial community by increasing the populations of beneficial microbes (Krishnaraj and Sreenivasa 1992). Ames et al. (1984) and Bansal and Mukerjii (1994) demonstrated a positive correlation between AM root colonization and rhizospheric bacterial populations, and Edwards et al. (1998) reported that the presence of *G. mosseae* increased the population of *P. fluorescens* in the rhizosphere of tomato (*L. esculentum*) and leek (*A. porrum*), although the bacterium had no effect on the AM fungus. In the present study, the increased population of AM fungi or fluorescent pseudomonad following co-inoculation of *P. monteilii* and *G. fasciculatum*, as compared to single inoculations, probably contributed to the better performance of *C. forskohlii* in the presence of both microbes. Consequently, *P. monteilii* strain CRC1 could be designated as a new mycorrhiza helper bacterium (MHB), with a similar role as those involved in the ectomycorrhizal symbiosis (Garbaye 1994; Kataoka and Futai 2009). Duponnois and Plenchette (2003) reported that another *P. monteilii* strain (HR13) promoted mycorrhization of *Acacia mangium* and *Acacia auriculiformis*. Srinath et al. (2003) have suggested that MHB favors AM development by producing hydrolytic enzymes which dilate the cortical cells and provide wider

intercellular space, enabling AM fungi to penetrate and ramify more easily in the root system.

In the present study, synergy between *G. fasciculatum* and *P. monteilii* strain CRC1 was observed under organic field conditions in reducing root rot and wilt and in increasing tuber and forskolin yield of *C. forskohlii*. In general, consortia of beneficial microorganisms with different modes of action may improve biological management of nutrients and plant diseases leading to yield enhancement and sustainability. Such a management approach will be particularly useful under organic farm conditions or especially in medicinal plants where the use of chemicals is restricted because of health and residue considerations.

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