World J Microbiol Biotechnol (2016) 32:19
DOI 10.1007/s11274-015-1972-5

ORIGINAL PAPER

Dual symbiosis between *Piriformospora indica* and *Azotobacter chroococcum* enhances the artemisinin content in *Artemisia annua* L.

Monika Arora[1] · Parul Saxena[2] · Devendra Kumar Choudhary[1] · Malik Zainul Abdin[2] · Ajit Varma[1]

Received: 23 July 2015 / Accepted: 12 November 2015 / Published online: 8 January 2016

Abstract At present, *Artemisia annua* L. is the major source of artemisinin production. To control the outbreaks of malaria, artemisinin combination therapies (ACTs) are recommended, and hence an ample amount of artemisinin is required for ACTs manufacture to save millions of lives. The low yield of this antimalarial drug in *A. annua* L. plants (0.01–1.1 %) ensues its short supply and high cost, thus making it a topic of scrutiny worldwide. In this study, the effects of root endophyte, *Piriformospora indica* strain DSM 11827 and nitrogen fixing bacterium, *Azotobacter chroococcum* strain W-5, either singly and/or in combination for artemisinin production in *A. annua* L. plants have been studied under poly house conditions. The plant growth was monitored by measuring parameters like height of plant, total dry weight and leaf yield with an increase of 63.51, 52.61 and 79.70 % respectively, for treatment with dual biological consortium, as compared to that of control plants. This significant improvement in biomass was associated with higher total chlorophyll content (59.29 %) and enhanced nutrition (especially nitrogen and phosphorus, 55.75 and 86.21 % respectively). The concentration of artemisinin along with expression patterns of artemisinin biosynthesis genes were appreciably higher in dual treatment, which showed positive correlation. The study suggested the potential use of the consortium *P. indica* strain DSM 11827 and *A. chroococcum* strain W-5 in *A. annua* L. plants for increased overall productivity and sustainable agriculture.

Keywords *Artemisia annua* · Artemisinin · Artemisinin biosynthesis genes · *Azotobacter chroococcum* (W-5) · Dual symbiosis · *Piriformospora indica*

Introduction

Artemisinin (Qinghaosu) is isolated from the aerial parts of herb *Artemisia annua* L. belonging to the family Asteraceae. It is popular as a potent antimalarial compound, acting against both drug-resistant and cerebral malaria causing strains of *Plasmodium* sp. (Abdin et al. 2003; Abdin and Alam 2015). Artemisinin and its derivatives, delivered in the form of artemisinin-based combination therapies (ACTs), are currently the best treatment option for malaria (Rathore et al. 2005; Yakasai et al. 2015).

Globally, it is estimated that 3.3 billion people are at risk of being infected with malaria and developing disease, and 1.2 billion are at high risk (>1 in 1000 chance of getting malaria in a year). According to the latest estimates, 198 million cases of malaria occurred globally in 2013 (uncertainty range 124–283 million) and the disease led to 584,000 deaths (uncertainty range 367,000–755,000). The burden is heaviest in the World Health Organization (WHO) African Region, where an estimated 90 % of all malaria deaths occur, and in children aged less than 5 years, who account for 78 % of all deaths (WHO 2014).

At present, ACTs are recommended by WHO as first-line treatment for *Plasmodium falciparum* malaria, as they offer significant advantages over alternatives. Most malaria parasites are still sensitive to artemisinin, and since it rapidly clears the malaria parasite from the patient's blood,

✉ Ajit Varma
ajitvarma@amity.edu

[1] Amity Institute of Microbial Technology (AIMT), Block 'E-3', 4th Floor, Amity University Campus, Sector-125, Gautam Buddha Nagar, Noida, UP 201313, India

[2] Centre for Transgenic Plant Development, Faculty of Science, Hamdard University, New Delhi, India

even before treatment is completed, transmission by biting mosquitoes is also reduced. The slower-acting partner drug in the combination therapy is then on hand to kill any remaining parasites and provide post-treatment prophylaxis for at least 1 month (Price and Douglas 2009; WHO 2014). Globally, there is a wide gap between the demand of artemisinin (289 tonnes) and its supply (232–262 tonnes) for the use in manufacturing of ACTs due to low content of artemisinin in *A. annua* L. plants (Ahlawat et al. 2014). Current cost of this drug is US$250–$300 per kg (Kindermans et al. 2007).

Consequently, efforts have been made worldwide to enhance the concentration of artemisinin in *A. annua* L. using molecular, physiological and biochemical approaches (Kapoor et al. 2007; Davies et al. 2009; Aftab et al. 2010). In addition, biotechnological methods such as in vitro cultivation of hairy roots, plant cell cultures and fermentation with microbes have been employed, but have not been found effective in terms of enough artemisinin production to meet with the global demand (Wang et al. 2001; Ro et al. 2006; Durante et al. 2011). Hence, improvements in artemisinin yield in cultivated plants remain an active area of research (Chaudhary et al. 2008).

Piriformospora indica is a root endophytic fungus, belonging to the family Sebacinaceae, isolated from rhizosphere soil of xerophytic shrubs in Thar desert, India. It functions as bioregulator, biofertilizer, bioprotector against abiotic and biotic stresses, confers resistance to toxins and heavy metal ions, and stimulates overall productivity of plants (Verma et al.1998; Varma et al. 2012). *P. indica* is functionally similar to arbuscular mycorrhiza fungi (AMF) except that it can grow axenically. The fungus has been studied for its impact on growth and biomass in approximately 150 plant species including several economically and medicinally important plants (Varma et al. 2001; Das et al. 2012; Satheesan et al. 2012). It also has been shown to promote secondary metabolite accumulation along with higher biomass in in vitro cultures of different plant species. *P. indica* as well as AMF individually have been shown to enhance artemisinin content in *A. annua* L. plants previously (Kapoor et al. 2007; Chaudhary et al. 2008; Rapparini et al. 2008; Awasthi et al. 2011; Varma et al. 2012; Sharma and Agrawal 2013; Mandal et al. 2014).

Artemisinin, a sesquiterpene lactone, comes under the family of terpenoids. In the biosynthesis of terpenoids, isopentenyl diphosphate (IPP) and its isomer dimethyllallyl diphospahte (DMAPP) are the primary precursors which are synthesized from two different pathways, mevalonic acid (MVA) and methylerythritol phosphate (MEP) pathway (Fig. 1). Several studies reported that *P. indica* as well as AMF induced up-regulation of MEP pathway genes (Strack and Fester 2006; Schäfer et al. 2009; Mandal et al. 2014).

Azotobacter chroococcum [nitrogen fixing bacterium (NFB)] is a Gram negative, free living soil aerobic bacterium belonging to genus *Azotobacter*. It is one of the nitrogen fixing bacteria that fixes atmospheric nitrogen into nitrogen compounds (ammonium and nitrogen dioxide) which in turn are accessible to plants (Lakshminarayana et al. 1992). The ability to fix nitrogen has also stimulated interest in the use of *A. chroococcum* as bioinoculant to increase the crop yield and productivity. However, only recently, studies analyzing the synergistic effect of dual inoculation of *A. chroococcum* with *P. indica* or AMF on *Panicum miliaceum* (Shishehbor et al. 2013), *Oryza sativa* (Prajapati et al. 2008), *Stevia rebaudiana* (Vafadar et al. 2014), wheat (Behl et al. 2007) and apple (Sharma et al. 2012) have been conducted.

The synergistic effect of *A. chroococcum* and *P. indica* on *A. annua* L. plants has not been studied till date. In the present study, we have analysed and reported for the first time the results of effect of symbiotic consortium of endophytic fungi, *P. indica* strain DSM 11827 and an NFB, *A. chroococcum* strain W-5, on improving the biomass and artemisinin concentration in *A. annua* L. Transcript pattern of genes encoding key enzymes involved in MEP and MVA pathways of artemisinin synthesis were also evaluated using semi quantitative RT-PCR and are reported in this communication.

Materials and methods

Plant material and growth conditions

Artemisia annua L. seeds were obtained from IPCA Laboratories Limited, Ratlam, MP, India. Seeds were sown in earthen pots (12 inch high and 10 inch internal diameter) having mixture of soil, sand and compost in the ratio of 3:3:1. This mixture was sieved through 2.5 mm mesh and then autoclaved for 3 h at 121 °C with pressure 15 psi before filling the pots. The experiment was conducted in winter season from December to March, in a poly house at a temperature of 27 ± 2 °C with 13/11 h light and dark regime, and the relative humidity of 70 %. Soil used in the experiment was sandy loam with pH 7.8, EC 0.22 dsm^{-1}, organic carbon 0.17 %, available phosphorus (41.2 kg/ha), available potassium (113 kg/ha) and available nitrogen (230 kg/ha).

P. indica culture and inoculation with plant roots

Fungal strain *P. indica* (DSM11827) was obtained from Amity Institute of Microbial Technology, Amity University, Noida, India. The culture was maintained on Hill-Kaefer medium (Hill and Kaefer 2001) at pH 6.5 on an incubator shaker at 120 rpm and 28 ± 2 °C. For experiments, the

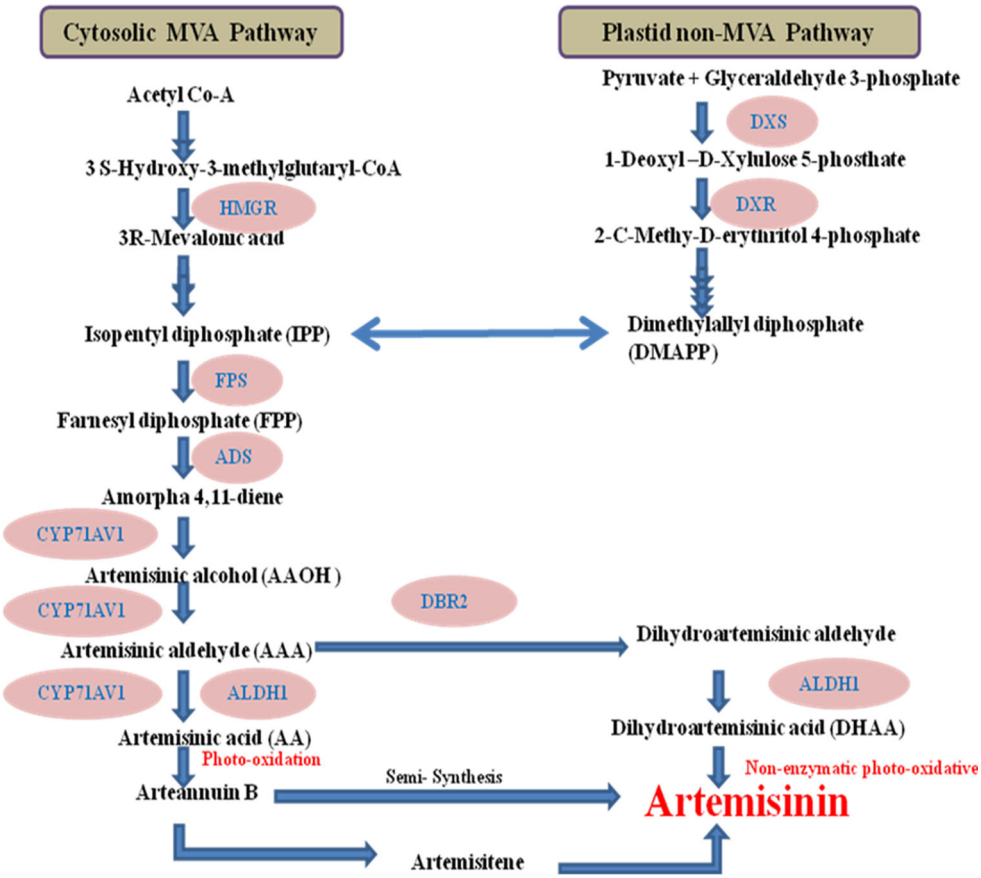

Fig. 1 Biosynthetic pathway of artemisinin. *HMGR* 3-hydroxy-3-methylglutaryl-CoA reductase, *DXS* 1-deoxy-D-xylulose-5-phosphate synthase, *DXR* 1-deoxy-D-xylulose-5-phosphate reductoisomerase, *FPS* farnesyl diphosphate synthase, *ADS* Amorpha-4,11-diene synthase, *CYP71AV1* cytochrome P450 monooxygenase, *DBR2* artemisinic aldehyde double bond reductase, *ALDH1* aldehyde dehydrogenase

culture was grown for 8 days till its late log phase. After 8 days, fungal mycelia were harvested from the liquid culture through filtration and washed with excess of double distilled water to remove adhered salts. This fungal biomass was utilized for co-cultivation with *A. annua* L. plants.

Selection of nitrogen fixing bacteria, *A. chroococcum*

Five different strains of *A. chroococcum* were collected from culture collection Division of Microbiology, IARI, New Delhi, India. These strains were maintained on Jensen's nitrogen free medium (Jensen 1942) on an incubator shaker at 200 rpm at 28 ± 2 °C for 48 h in shake flasks. Prior to conducting experiments, the compatibility between *A. chroococcum* strains towards fungus *P. indica* was done by the cross-streak assay (Sarma et al. 2011). Based on this screening, only *A. chroococcum* W-5 was selected because it did not show any inhibition zone towards *P. indica* as well as promoted fungal mycelial growth.

Plant co-cultivation experiment

Experiments were done in a complete randomized design consisted of four treatments: (1) plants without any microbial treatment (C), (2) plants with *A. chroococcum* (Az), (3) plants with *P. indica* (Pi) and (4) plants with *P. indica* and *A. chroococcum* (Pi +Az). In total, the experiment consisted of 40 pots, ten pots per treatment containing one plant each. The seeds of *A. annua* L. were surface-sterilized with 3 % sodium hypochlorite solution for 15 min and then rinsed three times with sterile distilled water. After sterilization, the seeds were sown in sterile soil (autoclaved for 3 h at 120 °C and 15 psi) and moistened twice a week with autoclaved distilled water. After 4 weeks of sowing, seedlings with 2–3 leaves were transplanted in experimental pots containing autoclaved soil mixture. The groups were as follow: (a) Control: pots were filled with sterile soil and the seedlings were placed in the planting holes. The soil did not receive treatment with fungal or bacterial cultures. (b) Az: pots filled with sterile soil and comprised of biological mixture containing 2 mL of a 48 h old culture of *A. chroococcum* (having approximate 10^5–10^6 CFU/mL) and 5 g of soil deposited into the planting holes at the time of seedling transplantation. (c) Pi: pots filled with sterile soil and comprised of 2 % (w/w) *P. indica* (2 g of fungal mycelium per 100 g of soil), with the placement of a thin layer of fungal mycelia, 2 cm below soil surface, before planting seedlings. (d) Pi + Az: pots were filled with sterile soil comprised of 2 % *P. indica* that was placed 2 cm below the surface of soil along with a

mixture of 5 g of soil containing 2 mL of *A. chroococcum* bacterial suspension that was inserted in the planting holes before seedling transplantation.

During entire experimental period, roots samples were taken for detection of colonization with spores of external source of native AMF and no colonization was detected in non-mycorrhizal treatments. Pots were placed in poly house and were watered every alternate day with Hoagland's solution (Arnon and Hoagland 1940). Eight plant samples from each treatment consisted of 10 replicates were randomly harvested after 2 months of transplantation (60 days) for analysis of height, dry weights, chlorophyll, phosphorus, nitrogen and artemisinin content, also for studying the expression pattern of artemisinin biosynthetic pathway genes.

Analysis of plant growth parameters

The leaf, shoot and root were separated from each plant sample and dried in hot air oven at 40 °C for 72 h to determine dry weights. The total leaf yield, dry weight and height per plantlet in each treatment were recorded.

Determination of chlorophyll content

Chlorophyll estimation was performed according to the modified method of Hiscox and Israelstam (1979), with 1 g leaf tissue soaked in 10 mL 80 % acetone and incubated for 24 h in the dark. After incubation, absorbance of the green solution was read at 663 and 645 nm using a spectrophotometer. The total chlorophyll content was calculated according to formula developed by Arnon 1949: Chlorophyll a (mg/g) = $(12.72A_{663} - 2.59A_{645}) \times V/(m \times 1000)$, Chlorophyll b (mg/g) = $(22.88A_{663} - 4.67A_{645}) \times V/(m \times 1000)$. Where, V and m defines volume of solvent and mg of tissue respectively. Total chlorophyll content = Chl a + Chl b.

Estimation of phosphorus and nitrogen

Phosphorus concentration in leaves was measured following the method described by Gericke and Kurmies (1952). The nitrogen concentration was quantified in an elemental (CHNS) analyser (VARIO Elementar III) as per the instructions given in the user's manual.

Extraction and quantification of artemisinin

Samples for detection the artemisinin content in leaves of *A. annua* L. plants were taken from four treatments after 2 months of transplanting and dried in oven at 50 °C. One gram of dry leaf material from all the treatments was used for the estimation of artemisinin by using the method as described by Zhao and Zeng (1986). Derivatized artemisinin was analyzed and quantified by using High-performance liquid chromatography system (HPLC) attached to a diode array detector set at 260 nm. Reverse phase column (C18, 5 μm, 4.6 × 250 mm) was used for artemisinin detection. The mobile phase comprising of methanol and 100 mM K-phosphate buffer mixed in the ratio of 60:40, pH 6.5 was used at a constant flow rate of 1 mL/min. Artemisinin was quantified with the help of standard curve prepared by HPLC using different concentration of standard artemisinin (Sigma, Aldrich, India; Qian et al. 2005). Artemisinin content was expressed as percent (%) of dry weight.

Quantification of P. indica colonization and *A. chroococcum*

To determine the viable count of *A. chroococcum*, rhizospheric soil was taken from the plants inoculated with *A. chroococcum* alone or in combination with *P. indica*. One gram of rhizospheric soil was mixed with 10 mL of distilled water and standard serial dilution pour plate method was used. One millilitre of serial diluted soil solution was poured over the nitrogen free medium for determining the population count of *A. chroococcum*.

Piriformospora indica colonization was evaluated by randomly selected fine roots from 2 months old *A. annua* L. After rinsing the roots well in distilled water, the root segments were boiled in 10 % KOH at 60–70 °C for softening. Then the tissues were neutralized with 2 % HCl for 3–4 min. After cutting into 1 cm long pieces, the root segments were stained with 0.5 % Trypan blue in lactophenol blue solution and checked the *P. indica* colonization of plant roots in both *P. indica* alone and together with *A. chroococcum* treated plant roots (Phillips and Hayman 1970). Percentage colonization of *P. indica* was calculated using the formula as described by McGonigle et al. (1990).

RNA isolation and cDNA synthesis

Total RNA was isolated from 100 mg fresh leaf samples using RNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Quantity and quality of isolated total RNA samples were estimated using NanoDrop™ 8000 spectrophotometer (Thermo Scientific, USA) and formaldehyde-denatured agarose gel electrophoresis. DNase I (Fermentas, St Leo-Roth, Germany) was used to remove any traces of genomic DNA contamination. For cDNA synthesis, 1 μL of oligo (dT) primer, 50 ng of RNA sample, 4 μL of 5× long range RT buffer, 2 μL dNTP mix, 0.2 μL RNase inhibitor and 1 μL long range reverse transcriptase enzyme (MBI, Fermentas) were added in the reaction mixture and then water was mixed to make up the final volume to 20 μL. This mixture was then incubated at

42 °C for 90 min and finally the reaction was terminated by increasing the temperature to 85 °C for 5 min to inactivate the reverse transcriptase. The cDNA thus synthesized was subjected to RT-PCR analysis.

Semi quantitative RT-PCR Analysis

Expression profile of six genes, namely 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), amorpha-4,11-diene synthase (ADS), cytochrome P450 monooxygenase (CYP71AV1), artemisinic aldehyde double bond reductase (DBR2) and aldehyde dehydrogenase (ALDH1), was studied using semi-quantitative PCR method. Leaf samples from all the treatments were taken, snap frozen in liquid nitrogen, stored at −80 °C and further used for RNA isolation and cDNA synthesis as described above.

Gene specific primers for all the six genes were designed using Primer 3 software and synthesized by Sigma-Aldrich, USA and Roche, USA (Table 1). The plant specific β-actin gene was used as endogenous control and amplified in parallel with the target genes. The RT-PCR cycling conditions for amplification of each cDNA sample included an initial denaturation step of 95 °C for 10 min for melting of template followed by 40 cycles with denaturation at 95 °C for 15 s, annealing at 55 °C for 1 min, elongation on 72 °C for 1 min. Additional final extension at 72 °C for 5 min was carried out to extend any premature synthesis of cDNA. After completion of all the steps, the expression levels of the six genes were observed on 1 % agarose gel in 1× TAE buffer with respect to the endogenous control β-actin gene. A 100 bp DNA ladder molecular weight marker (Fermentas) was run on every gel to confirm expected molecular weight of the amplification product.

Statistical analysis

Data for morphological parameters such as average height, total dry weight, leaf yield, nitrogen, phosphorus, chlorophyll and artemisinin content (%) were examined using One-way ANOVA. A Tukey's post hoc multiple mean comparison test was used to establish significant differences between treatments ($P < 0.05$).

Results

Effect of treatments on plant height and biomass

Piriformospora. indica and *A. chroococcum* inoculation, either singly or in combination, significantly enhanced the plant biomass. The increase was evident in the entire measured plant growth parameters, viz., plant height, total biomass and total leaf yield per plant as compared with control plants (Table 2). The plants treated with dual inoculation showed a significant increase of 63.51, 52.61 and 79.70 %, respectively in these parameters over the control.

Root colonization by *P. indica* and population of *A. chroococcum* in rhizospheric soil of *A. annua* L. plants

On individual basis, *A. annua* L. plants showed 50.23 % colonization by *P. indica* and 18.33×10^5 CFU/g soil in *A. chroococcum* co-cultivated plants, whereas dual treated plants not only exhibited better root colonization of 78.99 % by *P. indica,* but also high population of *A. chroococcum* (21.12×10^5 CFU/g soil) in the rhizospheric soil.

Chlorophyll content in leaves

Chla, chlb and total chlorophyll content was quantified in leaves of *A. annua* L. and found significantly increased in plants treated with *P. indica*, *A. chroococcum* alone or in combination as compared to the control plants. Chla showed values of 4.5 and 4.7 mg/g, respectively, for plant treated

Table 1 List of primers sequences used for RT-PCR analysis

Name of gene with accession number	Forward primer (5′–3′)	Reverse primer (3′–5′)
1-Deoxyxylulose 5-phosphate reductoisomerase (DXR) (AF182287.2)	CTTGATCTGTTCCGCAAG	CACTGGTTCTATTGGGACTC
3-Hydroxy-3-methylglutaryl-CoA (HMGR) (U14625.1)	GGTCAGGATCCGGCCCAAAACATT	CCAGCCAACACCGAACCAGCAACT
Amorpha-4,11-diene synthase (ADS) (HQ315833.1)	AAAAGAGGAGGTGCAGAGAC	CATACCAACCACTGAAGAGC
Cytochrome P450 monooxygenase (CYP71AV1) (AB706288.1)	GCTCAGCTACAAGGTTATCG	GAGATACTGAGGCAAACTGG
Double bond reductase 2 (DBR2) (KC505370.1)	GAGCCACAGCTTGTAACC	GGAAGTGAGGAGGAGGAAGTAGC
Aldehyde dehydrogenase (ALDH1) (FJ809784.1)	CTGTAAGGGGAGTATGTTCG	TCGGAGTAGTTGGTCACATC
Actin (EU531837.1)	AGCTCCTGCTCATAGTCAAG	CCTATCTACGAAGGGTATGC

Table 2 Effect of *P. indica*/*A. chroococcum* alone and dual treatment on biomass of *A. annua* L. plants

Plant growth parameters	C	Az	Pi	Pi + Az
Plant height (cm)	60.4 ± 3.36[a]	74.74 ± 4.42[b]	79.37 ± 2.76[b]	98.76 ± 2.68[c]
Total dry weight per plant (g)	57.71 ± 3.23[a]	64.84 ± 3.56[b]	76.14 ± 2.47[b]	88.07 ± 4.53[c]
Leaf yield per plant (g)	7.93 ± 1.26[a]	10.04 ± 1.05[b]	12.13 ± 1.03[b]	14.25 ± 1.14[c]

Plants were grown with *P. indica* designated as Pi, with *A. chroococcum* as Az, with both *P. indica* + *A. chroococcum* as Pi +Az and control plant without *P. indica* or *A. chroococcum* designated as (C). Values are presented as means (n = 8) ± SD. Different letters indicate significant differences between each treatment ($P \leq 0.05$) by Tukey's post hoc test

with *A. chroococcum* and *P. indica,* separately, and 5.6 mg/g fresh weight for plant dual treated with *P. indica* and *A. chroococcum* together. Similarly, the content of chlb exhibited values of 0.7 and 0.8 mg/g, respectively, for plant treated with *A. chroococcum*, and *P. indica*, separately, and 1.0 mg/g fresh weight for plant dual treated with *P. indica* and *A. chroococcum* together. The plants dual treated *P. indica* and *A. chroococcum* together also enhanced total chlorophyll content by 57.91 % than control plants (Fig. 2). However, the chlorophyll content of *A. annua* L. plants treated with *P. indica* and *A. chroococcum*, separately was not significantly different.

Phosphorus and nitrogen content

Treatment with *P. indica* significantly increased P content by 65.95 % and with *A. chroococcum* resulted in 31.90 % higher P content in *A. annua* L. plants compared to the control plants respectively. The colonization of *A. annua* L. with *P. indica* + *A. chroococcum* resulted in 86 % increase in P content (Fig. 3a). Likewise, plants treated with *P. indica* significantly

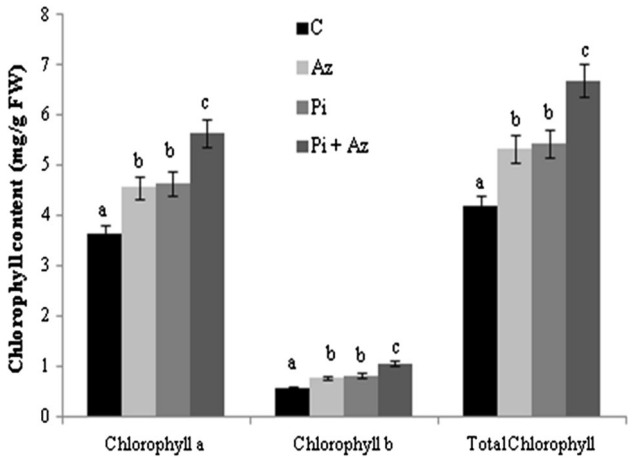

Fig. 2 Chlorophyll content (mg/g fresh weight) in leaves of *A. annua* L. plants, grown for 2 months after transplanting, under poly house conditions. Columns with *different letter* is indicating significant differences between each treatment at 5 % probability level according to Tukey's post hoc test, and the *error bars* represent the standard error. *A. annua* plants were grown with control (C), inoculated with either *A. chroococcum* (Az) or *P. indica* (Pi) alone or in combination (Pi + Az)

increased N content by 13.27 % and with *A. chroococcum* resulted in 29.20 % higher N content in *A. annua* L. plants compared to the control plants respectively. The colonization of *A. annua* L. with *P. indica* + *A. chroococcum* resulted in 55.75 % increase in N content (Fig. 3b).

Artemisinin content

Artemisinin concentration in the leaves of *A. annua* L. plants determined by HPLC was higher in all the treatments as compared to the control. Artemisinin concentration (%) in *A. annua* L. plants treated with combination of *P. indica* and *A. chroococcum* was approximately 70 % higher when compared with the control. *P. indica* colonization or *A. chroococcum* inoculation independently enhanced artemisinin content to approximately similar levels (Fig. 4).

RT-PCR analysis

The expression pattern of the genes encoding HMGR, which catalyzing the final step in MVA pathway, and DXR, an important enzyme which plays a central role in MEP pathway, was studied by using semi-quantitative RT-PCR. Not major difference was found in the HMGR transcript profile in any treatments as compared with control. However, maximum transcript profile of DXR was found in dual treated plants (Fig. 5). Expression profile of genes ADS, CYP, DBR2, and ALDH1, which are responsible for artemisinin biosynthesis pathway, was also studied by reverse transcriptase-PCR. The relative transcript profiles of the above genes showed appreciable variation in different treatments and found significantly higher in leaves of *A. annua* L. plants treated with dual treatment (Fig. 5). This maximum transcript level of genes was well correlated with the observed increase in artemisinin contents in the plants treated with beneficial microbes.

Discussion

In the present study, significant enhancements in the plant growth, phosphorus and nitrogen content, concentrations of photosynthetic pigments, artemisinin content and

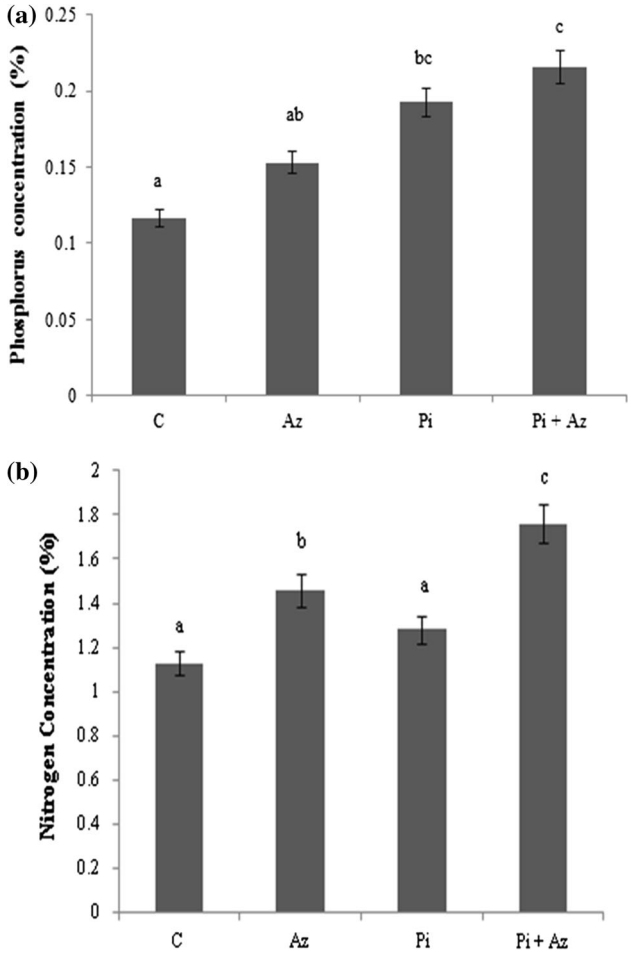

Fig. 3 Phosphorus (**a**) and nitrogen (**b**) concentration (%) in leaves of *A. annua* L. plants, grown for 2 months after transplanting, under poly house conditions. Columns with *different letter* is indicating significant differences between each treatment at 5 % probability level according to Tukey's post hoc test, and the *error bars* represent the standard error. *A. annua* plants were grown with control (C), inoculated with either *A. chroococcum* (Az) or *P. indica* (Pi) alone or in combination (Pi + Az)

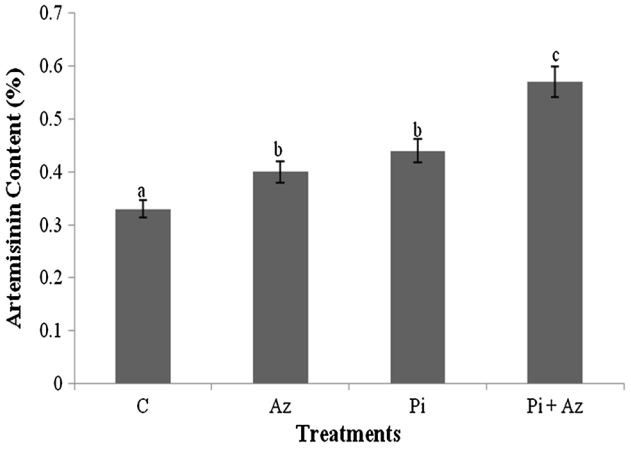

Fig. 4 Artemisinin content (%) in leaves of *A. annua* L. plants, grown for 2 months after transplanting, under poly house conditions. Columns with *different letter* is indicating significant differences between each treatment at 5 % probability level according to Tukey's post hoc test, and the *error bars* represent the standard error. *A. annua* L. plants were grown with control (C), inoculated with either *A. chroococcum* (Az) or *P. indica* (Pi) alone/or in combination (Pi +Az)

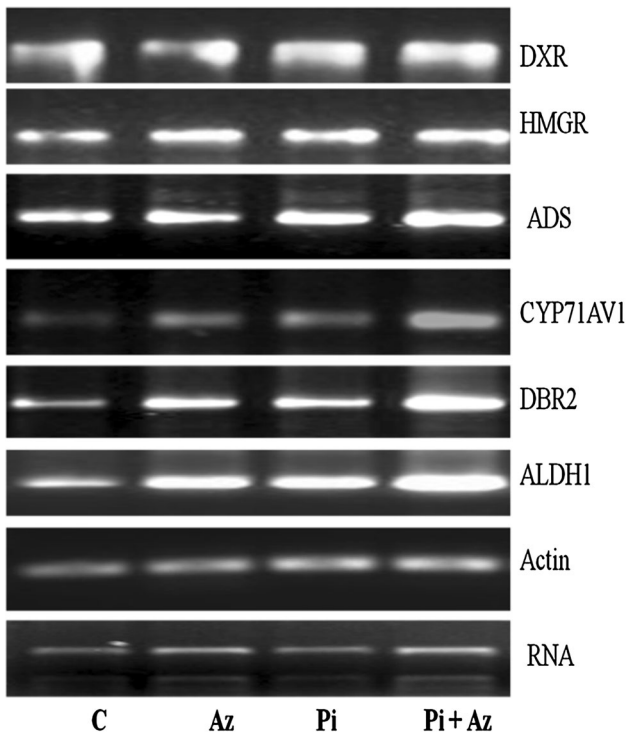

Fig. 5 Reverse Transcriptase polymerase chain reaction amplification of *DXR*, *HMGR*, *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* using cDNA from leaves of *A. annua* L. plantlets where control (C), inoculated with either *A. chroococcum* (Az) or *P. indica* (Pi) alone/or in combination (Pi +Az). Total RNA from each treatment was used for quantification. Actin was kept as internal control

expression patterns of genes were observed, when *A. annua* L. plants were subjected to dual treatment with *P. indica* and *A. chroococcum* as compared to the control plants. A similar result in plant biomass increase was observed in case of rice plants treated with dual inoculation of *A. chroococcum* and *P. indica* along with vermicompost (Prajapati et al. 2008). In addition, the improved plant growth in present study is in partial agreement with an earlier finding, where AMF (*Glomus mossea*) and NFB (*Bacillus subtilis*) improved the height, plant weight and total leaf yield by 57.78, 62.64 and 66.6 % respectively, compared to the control in *A. annua* L. plants (Awasthi et al. 2011). Likewise, the *Stevia rebaudiana* plants treated with combination of *A. chroococcum* and *Glomus intraradices* showed remarkably improved fresh weight (53.7 %), dry weight (57 %) and height (37 %) (Vafadar et al. 2014). These investigations also revealed the effective *P. indica* colonization in the roots and increased *A. chroococcum* density in the rhizospheric soil of *A. annua*

L. plant receiving dual inoculation. This may be due to compatibility and synergistic effect of *P. indica* with *A. chroococcum,* which increased population of NFB by altering root exudation in rhizosphere. Garbaye (1994) reported that rhizobacteria increased the capacity of AMF to colonize the roots of plants by secreting certain enzymes that soften the cell wall and middle lamella between cells of plant root cortex. These observations are partially consistent with the findings of Awasthi et al. (2011), where root colonisation by AMF was improved by 50.32 % in the presence of NFB. Although, the NFB population was increased by 18.5 % in the presence of AMF. Likewise, when *Zea mays* L. cultivar PR37Y15 was treated with combination of *Pseudomonas fluorescens* F113 with either *Azospirillum lipoferum* CRT1 or *G. intraradices* JJ291, the cell number of *P. fluorescens* F113 was increased in the maize rhizosphere (Walker et al. 2012). The improvement in phosphorous and nitrogen contents of individually treated plants or with combination of *P. indica* and *A. chroococcum* is consistent with earlier reports (Awasthi et al. 2011; Vafadar et al. 2014). *P. indica* treated plants displayed a 66 % increase in phosphorous content. *A. chroococcum* alone however, improved phosphorous content by approximately 32 % in comparison with the control.

Increased plant growth parameters in treated plants could be attributed to the production of higher quantities of growth promoting substances and complementary effect of enhanced phosphate availability by both symbiotic fungus and nitrogen fixing bacteria. Growth promoting activity of *P. indica* is largely influenced by the enhanced nutritional uptake by the plant. It has been revealed that a high affinity phosphate transporter in *P. indica* is involved in phosphate transfer to the host plant (Yadav et al. 2010). Kumar et al. (2011) showed that the expression of *P. indica* phosphate transporter at low levels of soil phosphate was found higher than those in enriched levels leading to active transportation of P to the plant. Similarly, the nitrogen concentration in shoot biomass of plant treated with *A. chroococcum* was found 29.2 % higher and with *P. indica* 13.27 % higher than control. *A. chroococcum,* a NFB, largely increases nitrogen uptake by the plants (Narula et al. 2007). An increase in N and P uptake was reported, while studying the effect of two NFB strains *A. chroococcum* and *Pseudomonas agglomerans* on wheat (Narula et al. 2007). Also in *S. rebaudiana, A. chroococcum* was found to stimulate N uptake by 27 % when compared with the control (Vafadar et al. 2014). *P. indica* has also been shown to promote growth of *Arabidopsis* and tobacco seedlings by up regulating nitrate reductase gene expression (Sherameti et al. 2005). Co-inoculation with both *P. indica* and *A. chroococcum* recorded maximal N and P contents in *A. annua* L. plants over the control. The synergistic action of AMF and NFBs has been reported to enhance N and P contents as high as 48.76 and 82.39 % by Vafadar et al. (2014). Artursson et al. (2006) had previously reported that in plants inoculated with the nitrogen fixing bacteria and AMF, the two micro symbionts interact effectively to promote the plant growth, nutritional uptake and inhibition of pathogenesis by other microbes. *P. indica* is known to enhance phosphorous uptake in plants, which in turn might enable more energy available for nitrogen fixation by *A. chroococcum*. Chlorophyll content of plants treated with combination was more pronounced than individual treatments and control as well. More chlorophyll content in the plants is attributed to the fact that increase in plant nutrition by an increase in P and N uptake will optimize the rate of photosynthesis by increasing the amount of plant chlorophyll, which will lead to an increase in biomass production by C fixation from CO_2. N is one of the elements to form chlorophyll (Awasthi et al. 2011; Vafadar et al. 2014).

The symbiotic effectiveness was much evident when artemisinin content was recorded 70 % higher in *A. annua* L. plants subjected to dual inoculation, which is again consistent with Awasthi et al. (2011) finding in which artemisinin content increased from 0.61 % in control to 0.77 % in leaves of *A. annua* plants treated with dual inoculation of *G. mosseae* and *B. subtilis*. It is well documented that both arbuscular mycorrhizal fungi and endophytic fungi play an important role in enhancing artemisinin content in leaves of *A. annua* plants (Kapoor et al. 2007; Sharma and Agrawal 2013; Mandal et al. 2014). *A. annua* plants treated with *P. indica* showed maximum growth and increased secondary metabolite concentration (Sharma and Agrawal 2013). The enhanced concentration of artemisinin by dual treatment may be due to improved growth and nutrient status of the plants. Recent reports suggesting enhancement of secondary metabolites both mycorrhiza fungus and rhizobacteria include saponin from *Chlorophytum* sp. (Gosal et al. 2010), artemisinin from *A. annua* L. (Awasthi et al. 2011) and steviosides from *S. rebaudiana* (Vafadar et al. 2014).

The demand (101–119 metric tonnes) for artemisinin is exponentially increasing every year because of increased incidence of drug-resistant malaria throughout the world, especially in Africa and Asia. However, the presence of low concentrations (0.01–1.1 %) of the compound in *A. annua* L. plants poses a major constraint in the commercialization of ACTs recommended by WHO for the treatment of multidrug-resistant and cerebral malaria. Further, the improvement in the yield of artemisinin through conventional breeding, in vitro culture, cell suspension culture and total organic synthesis still poses a challenge. However, possibilities are there to enhance the artemisinin biosynthesis either by overexpression of the genes encoding enzymes associated with the rate-limiting steps of the

mevalonate and artemisinin biosynthetic pathways or by the suppression of genes encoding enzymes of other competing pathways. Based on the current understanding of the pathway and cloning of the related genes, efforts have been made in the past few years to increase the artemisinin content in *A. annua* L., *Cichorium intybus* L. and microbes through metabolic engineering (Abdin and Alam 2015). Artemisinin biosynthesis derives 80 % of the carbon from mevalonate pathway and remaining 20 % from the non-mevalonate pathway (Ram et al. 2014). However, in the present study transcript profile of HMGR gene remained unaltered in all the treatment, which is similar with the observations by Mandal et al. (2014). Schäfer et al. (2009) reported that almost all genes of the MEP pathway are induced by *P. indica* that means the mycorrhiza treated plants derive carbon from MEP pathway rather than MVA pathway. Higher transcript profile of DXR1 gene was seen in plants treated with *P. indica* alone or in combination with *A. chroococcum* and supports this hypothesis.

In addition, the present study showed that plants treated with *P. indica* and *A. chroococcum* enhanced transcript profiles of artemisinin biosynthesis genes, viz., ADS, CYP71AV1, DBR2, and ALDH1, which consequently increased the biosynthesis of artemisinin. The higher transcript level of CYP71AV1, ADS genes and artemisinin content have been reported to be notably higher in *A. annua* plants treated with mycorrhiza (Mandal et al. 2014) and endophytic actinomycetes (Li et al. 2012). Maximum up-regulation of ADS, CYP71AV1, DBR2, ALDH1 genes have been reported by Ahlawat et al. (2014) in *A. annua* L. hairy root culture treated with *P. indica* in combination with methyl jasmonate. Also, the favoured artemisinin biosynthesis accompanied with changes in transcript profiles of genes that are responsible for artemisinin biosynthesis is in line with observations for *Centella asiatica* wherein induction of root colonization by *P. indica* leads to enhanced asiaticoside production in *C. asiatica* (Satheesan et al. 2012).

The obtained results demonstrate that consortium of *P. indica* and *A. chroococcum* improves the plant biomass and concentration of artemisinin in *A. annua* L. plants. The combinatorial application of *P. indica* with *A. chroococcum* induces reprogramming of many cellular activities like phyto-hormone biosynthesis, nutrient acquisition and secondary metabolite synthesis in *A. annua* L. leading to higher biomass, enhanced artemisinin content and yield. The use of this microbial consortium as bio-fertilizer in place of chemical fertilizers hence, presents a viable option for increased artemisinin availability.

Acknowledgments This research is funded by Department of Biotechnology grant. M.A. is gratefully acknowledges the support from Department of Biotechnology project as Junior Research Fellowship. Authors are grateful to Prof. M.Z. Abdin from Jamia Hamdard for providing laboratory facility and expertise to carry out this work.

References

Abdin MZ, Alam P (2015) Genetic engineering of artemisinin biosynthesis: prospects to improve its production. Acta Physiol Plant 37:33

Abdin MZ, Israr M, Rehman RU, Jain SK (2003) Artemisinin, a novel antimalarial drug: biochemical and molecular approaches for enhanced production. Planta Med 69:289–299

Aftab T, Khan MMA, Idrees M et al (2010) Boron induced oxidative stress, antioxidant defence response and changes in artemisinin content in *Artemisia annua* L. J Agro Crop Sci 196:423–430

Ahlawat S, Saxena P, Alam P, Wajid S, Abdin MZ (2014) Modulation of artemisinin biosynthesis by elicitors, inhibitor, and precursor in hairy root cultures of *Artemisia annua* L. J Plant Interact 9:811–824

Arnon DI (1949) Copper enzyme in isolated chloroplast polyphenoloxidase in *Beta vulgaris* L. Plant Physiol 24:1–15

Arnon DI, Hoagland DR (1940) Crop production in artificial solutions and soil with special reference to factors influencing yields and absorption of organic nutrients. Soil Sci 50:463–484

Artursson V, Finlay RD, Jansson JK (2006) Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ Microbiol 8:1–10

Awasthi A, Bharti N, Nair P et al (2011) Synergistic effect of *Glomus mosseae* and nitrogen fixing *Bacilluc subtilis* strain Daz26 on artemisinin content in *Artemisia annua* L. Appl Soil Ecol 49:125–130

Behl RK, Ruppel S, Kothe E, Narula N (2007) Wheat x Azotobacter x VA mycorrhiza interactions towards plant nutrition and growth—a review. J Appl Bot Food Qual 81:95–109

Chaudhary V, Kapoor R, Bhatnagar AK (2008) Effectiveness of two arbuscular mycorrhizal fungi on concentrations of essential oil and artemisinin in three accessions of *Artemisia annua* L. Appl Soil Ecol 40:174–181

Das A, Kamal S, Shakil NA et al (2012) The root endophyte fungus *Piriformospora indica* leads to early flowering, higher biomass and altered secondary metabolites of the medicinal plant, *Coleus forskohlii*. Plant Signal Behav 7:103–112

Davies MJ, Atkinson CJ, Burns C et al (2009) Enhancement of artemisinin concentration and yield in response to optimization of nitrogen and potassium supply to *Artemisia annua*. Ann Bot 104:315–323

Durante M, Caretto S, Quarta A et al (2011) Beta-Cyclodextrins enhance artemisinin production in *Artemisia annua* suspension cell cultures. Appl Microbiol Biotechnol 90:1905–1913

Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210

Gericke S, Kurmies B (1952) The colorimetric determination of phosphoric acid ammonium vanadate molybdate and its application in plant analysis. J Plant Nutr Soil Sci 159:11–21

Gosal SK, Karlupia A, Gosal SS, Chhibba IM (2010) Biotization with *Piriformospora indica* and *Pseudomonas fluorescens* improves survival rate, nutrient acquisition, field performance and saponin content of micropropagated *Chlorophytum* sp. Ind J Biotechnol 9:289–297

Hill TW, Kaefer E (2001) Improved protocols for aspergillus medium: trace elements and minimum medium salt stock solutions. Fungal Genet News Lett 48:20–21

Hiscox JD, Israelstam GF (1979) A method for the extraction of chlorophyll from leaf tissue with out maceration. Can J Bot 57:1332–1334

Jensen HL (1942) Pro Line Soc. NSW 57:205–212
Kapoor R, Chaudhary V, Bhatnagar AK (2007) Effects of arbuscular mycorrhiza and phosphorus application on artemisinin concentration in *Artemisia annua* L. Mycorrhiza 17:581–587
Kindermans J-M, Pilloy J, Olliaro P, Gomes M (2007) Ensuring sustained ACT production and reliable artemisinin supply. Malar J 6:125
Kumar M, Yadav V, Kumar H et al (2011) *Piriformospora indica* enhances plant growth by transferring phosphate. Plant Signal Behav 6:723–725
Lakshminarayana K, Narula N, Hooda IS, Faroda AS (1992) Nitrogen economy in wheat (*Triticum aestivum)* through use of *Azotobacter chroococcum*. Ind J Agric Sci 62:75–76
Li J, Zhao GZ, Varma A et al (2012) An endophytic *Pseudonocardia* species induces the production of artemisinin in *Artemisia annua*. PLoS ONE 7:e51410. doi:10.1371/journal.pone.0051410
Mandal S, Upadhyay S, Wajid S et al (2014) Arbuscular mycorrhiza increase artemisinin accumulation in *Artemisia annua* by higher expression of key biosynthesis genes via enhanced jasmonic acid levels. Mycorrhiza. doi:10.1007/s00572-014-0614-3
McGonigle TP, Miller MH, Evans DG et al (1990) A new method which gives an objective measure of colonisation of roots by vesicular arbuscular mycorrhizal fungi. New Phytol 115:495–501
Narula N, Remus R, Deubel A et al (2007) Comparison of the effectiveness of wheat roots colonization by *Azotobacter chroococcum* and *Pantoea agglomerans* using serological techniques. Plant Soil Environ 53:167–176
Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and VAM fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161
Prajapati K, Yami KD, Singh A (2008) Plant growth promotional effect of *Azotobacter chroococcum*, *Piriformospora indica* and Vermicompost on Rice Plant. Nepal J Sci Tech 9:85–90
Price RN, Douglas NM (2009) Artemisinin combination therapy for malaria: beyond good efficacy. Clin Infect Dis 49:1638–1640
Qian GP, Yang YW, Ren QL (2005) Determination of artemisinin in *Artemisia annua* L. by reversed phase HPLC. J Liq Chromatogr Relat Technol 22:705–712
Ram M, Jain DC, Mishra H et al (2014) Recent advances to enhance yield of artemisinin: a novel antimalarial compound, in *Artemisia annua* L. plants. In: Aftab T et al (eds) *Artemisia annua*—pharmacology and biotechnology. Springer, Berlin, pp 173–204
Rapparini F, Llusia J, Penuelas J (2008) Effect of arbuscular mycorrhizal (AM) colonization on terpene emission and content of *Artemisia annua* L. Plant Biol 10:108–122
Rathore D, McCutchan TF, Sullivan M et al (2005) Antimalarial drugs: current status and new developments. Expert Opin Investig Drugs 14:871–883
Ro DK, Paradise EM, Ouellet M et al (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440:940–943
Sarma MV, Kumar V, Saharan K et al (2011) Application of inorganic carrier-based formulations of fluorescent pseudomonads and *Piriformospora indica* on tomato plants and evaluation of their efficacy. J Appl Microbiol 111:456–466
Satheesan J, Narayanan AK, Sakunthala M (2012) Induction of root colonization by *Piriformospora indica* leads to enhanced asiaticoside production in *Centella asiatica*. Mycorrhiza 22:195–202
Schäfer P, Pfiffi S, Voll LM et al (2009) Manipulation of plant innate immunity and gibberellin as factor of compatibility in the mutualistic association of barley roots with *Piriformospora indica*. Plant J 59:461–474
Sharma G, Agrawal V (2013) Marked enhancement in the artemisinin content and biomass productivity in *Artemisia annua* L. shoots co-cultivated with *Piriformospora indica*. World J Microbiol Biotechnol 29:1133–1138
Sharma SD, Sharma NC, Sharma CL et al (2012) Glomus-Azotobacter symbiosis in apple under reduced inorganic nutrient fertilization for sustainable and economic orcharding enterprise. Sci Hortic 146:175–181
Sherameti I, Shahollari B, Venus Y et al (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. J Biol Chem 280:26241–26247
Shishehbor M, Madani H, Ardakani MR (2013) Effect of vermicompost and biofertilizers on yield and yield components of common millet (*Panicum miliaceum*). Ann Biol Res 4:174–180
Strack D, Fester T (2006) Isoprenoid metabolism and plastid reorganization in arbuscular mycorrhizal roots. New Phytol 172:22–34
Vafadar F, Amooaghaie R, Otroshy M (2014) Effects of plant growth promoting rhizobacteria and arbuscular mycorrhizal fungi on plant growth, stevioside, NPK, and chlorophyll content of *Stevia rebaudiana*. J Plant Interact 9:128–136
Varma A, Singh A, Sahay S et al (2001) *Piriformospora indica*: an axenically culturable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) Mycota IX. Springer, Berlin, pp 123–150
Varma A, Sherameti I, Tripathi S et al (2012) The symbiotic fungus *Piriformospora indica*: review. In: Hock B (ed) Fungal associations, 2nd edn, the mycota, vol IX. Springer, Berlin, pp 231–254
Verma S, Varma A, Rexer KH et al (1998) *Piriformospora indica*, gen. et sp. Nov., a new root-colonizing fungus. Mycologia 90:896–903
Walker V, Couillerot O, Felten AV et al (2012) Variation of secondary metabolite levels in maize seedling roots induced by inoculation with *Azospirillum*, *Pseudomonas* and *Glomus* consortium under field conditions. Plant Soil 356:151–163
Wang JW, Zhang Z, Tan RX (2001) Stimulation of artemisinin production in *Artemisia annua* hairy roots by the elicitor from the endophytic *Colletotrichum* sp. Biotechnol Lett 23:857–860
WHO (2014) World malaria report 2014. World Health Organization, Geneva
Yadav V, Kumar M, Deep DK et al (2010) A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in phosphate transport to the host plant. J Biol Chem 285:26532–26544
Yakasai AM, Hamza M, Dalhat MM (2015) Adherence to artemisinin-based combination therapy for the treatment of uncomplicated malaria: a systematic review and meta-analysis. J Trop Med. doi:10.1155/2015/189232
Zhao SS, Zeng MY (1986) Determination of qinghaosuin in *Artemisia annua* L. by high performance liquid chromatography. Chin J Pharma Anal 6:3–5