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ORIGINAL PAPER

Cumulative role of bioinoculants on growth, antioxidant potential and artemisinin content in *Artemisia annua* L. under organic field conditions

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Abstract Artemisia annua L. is mostly known for a bioactive metabolite, artemisinin, an effective sesquiterpene lactone used against malaria without any reputed cases of resistance. In this experiment, bioinoculants viz., *Streptomyces* sp. MTN14, *Bacillus megaterium* MTN2RP and *Trichoderma harzianum* Thu were applied as growth promoting substances to exploit full genetic potential of crops in terms of growth, yield, nutrient uptake and particularly artemisinin content. Further, multi-use of the bioinoculants singly and in combinations for the enhancement of antioxidant potential and therapeutic value was also undertaken which to our knowledge has never been investigated in context with microbial application. The results demonstrated that a significant ($P < 0.05$) increase in growth, nutrient uptake, total phenolic, flavonoid, free radical scavenging activity, ferric reducing antioxidant power, reducing power and total antioxidant capacity were observed in the *A. annua* treated with a combination of bioinoculants in comparison to control. Most importantly, an increase in artemisinin content and yield by 34 and 72 % respectively in the treatment having all the three microbes was observed. These results were further authenticated by the PCA analysis which showed positive correlation between plant macronutrients and antioxidant content with plant growth and artemisinin yield of *A. annua*. The present study thus highlights a possible new application of compatible bioinoculants for enhancing the growth along with antioxidant and therapeutic value of *A. annua.*

Keywords *Artemisia annua* · Artemisinin · Antioxidant · Bioinoculants · Nutrient uptake

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Introduction

The most urgent concern these days is the success in attaining the highest possible level of human health and keeping the diseases aside in the global context. In this context, the plant *Artemisia annua* L. is one such aromatic-antibacterial herb that effectively kills malarial parasites, lower down fever, and of which the secondary compound of significance is artemisinin, a sesquiterpene lactone containing an endoperoxide bridge. Artemisinin is an effective and safe alternative therapy against malaria (Liu et al. 2006) and its importance can be judged from its worldwide demand as an anti-malarial drug (Ferreira et al. 2013). Apart from artemisinin, *A. annua* is also a rich source of antioxidant flavonoids that are thought to play an imperative role in heightening the efficacy of artemisinin drugs against cancer and parasitic diseases (Liu et al. 2006). Recently, there is an increasing interest in nutrients and health promoting compounds especially polyphenols and antioxidants of natural origin, which have potential as protective and preventive activities against many chronic ailments (Aftab et al. 2013).

Due to the high global demand of artemisinin and its low yield (0.01–1 % dry weight), a significant effort has been made to enhance the production of artemisinin by chemical synthesis and genetic manipulation of genes involved in the synthesis of artemisinin (Avery et al. 1992; Martin et al. 2003; Ro et al. 2006). However, not much success could be achieved because of the expensive and

complex nature of the gene expression and its regulation in artemisinin biosynthesis. Thus, new approaches which are cheaper and more productive, is the need of hour for improving artemisinin production. In this context, over the last few years, there has been an expanding interest in application of bioinoculants with regard to their value addition in agriculture. It is now being realized that bioinoculants can also be beneficially utilized to impart value addition in agriculture by enhancing growth and yield of various crops (Pandey et al. 2011; Gupta et al. 2015b; Singh et al. 2016). An extension of this approach could be usage of combination of bioinoculants, which could enhance reliability and efficacy for plant growth promotion, induction of systemic responses, increased yield and disease protection (Singh et al. 2014; Gupta et al. 2016a).

Therefore, keeping in mind the importance of this enormously important anti-malarial drug plant and potentiality of the microbes, the present experiments were designed to determine the effects of bioinoculants viz., *Streptomyces* sp. MTN14 (KF699062), *B. megaterium* MTN2RP (KC978881) and *T. harzianum* Thu alone and in combinations on the crop productivity, nutrient uptake, modulation of phenolic and antioxidant components and artemisinin content in *A. annua* L.

Materials and methods

Preparation of bioinoculants

A single pure colony of *B. megaterium* MTN2RP and *Streptomyces* sp. MTN14 was individually inoculated in Luria Broth (LB) and Glucose Yeast Malt Broth (GYM), respectively and incubated at 30 ± 2 °C on a rotary shaker at 120 rpm for 3 days (for bacteria) and 7 days (for actinobacteria). The cells were centrifuged at 6000×*g* for 12 min and the pellet obtained was dissolved in saline (0.85 %) to obtain a cell density of 2×10^8 colony forming units (CFU) mL^{-1}. Similarly, the conidial suspension of fungal inoculants, *T. harzianum* Thu was sub cultured using Potato Dextrose Broth (Himedia, India). The culture was incubated on a rotary shaker at 120 rpm for 7 days at 28 ± 2 °C. After proper growth of the mycelial mat with conidia, it was homogenized and suspended in 100 mL of 0.1 M phosphate buffer (K_2HPO_4; KH_2PO_4) fixing cell density at 1.8×10^6 CFU mL^{-1}.

Experimental design and crop cultivation

The field trials were conducted in month of January to April 2014. In these experiments, the effect of bioinoculants along with the different combinations was studied. The bioinoculants were selected on the basis of their plant growth promoting potential (Gupta et al. 2015b). The seeds of a high yielding cultivar of *A. annua* L. 'CIM-Arogya' (Khanuja et al. 2008) were obtained from the National Gene Bank for CSIR-CIMAP, Lucknow (26.85° N, 80.92° E), India. The seeds were treated with pure culture of bioinoculants by mixing 150 seeds with 10 ml bioinoculants suspension and sown in sterilized soils filled pots in a greenhouse under natural light conditions. Following seven suspensions prepared were used in the experiment: (a) *Streptomyces* sp. MTN14 (STR), (b) *B. megaterium* (BM), (c) *T. harzianum* (TH), (d) STR + TH, (e) BM + TH, (f) STR + BM, and (g) STR + BM + TH. Untreated seeds sown in pots served as control. After 3–4 weeks, the treated seedlings of *A. annua* were gently removed according to height and number of leaves and transplanted into the fields at a spacing of 30 × 45 cm in beds laid out in randomized block design (RBD) with bioinoculants in plot (12 m^2) consisting of six rows of six plants. The experimental plot soil was sandy loam having alkaline reaction (pH 8.5, EC 0.40 dSm^{-1}). The available nitrogen (alkaline permanganate extractable, 165.00 kg ha^{-1}), potassium (neutral N ammonium acetate extractable, 105.00 kg ha^{-1}) and phosphorus (0.50 N $NaHCO_3$ extractable, 11.84 kg ha^{-1}) were determined according to Kiran and Patra (2003). On maturation of the crop (120 days) the data for different growth parameters were recorded. Nutrient uptake was determined by Jackson (1973) based on Nitrogen, Phosphorus and Potassium (NPK) concentration in dry matter samples.

Extraction and detection of artemisinin

The leaves samples from each treatment were shade dried and pulverized. The hexane extracts were prepared from 100 mg of each sample in three independent replicates. Artemisinin content was measured by high-performance thin layer chromatography (HPTLC) after extraction with solvent hexane (Gupta et al. 2002).

Sample preparation for phytochemical and antioxidant analysis

The leaves of plants from each treatment were randomly chosen for phytochemical analysis. The leaves samples were frozen in liquid nitrogen until used for antioxidant assays. About 2 g of homogenized leaves powder was suspended in 10 ml of a methanol (MeOH): water (1:1) mixture. The extracts were shaken for 1 h on a rotary homogenizer, and centrifuged at 8000×*g* for 20 min. The supernatant recovered was used for phytochemical analysis and measurement of antioxidant capacity. All enzymatic activities were analyzed using established spectrophotometric assays with some minor modifications.

Total phenol and flavonoid content (TPC and TFC)

Total phenolics were estimated by the Folin–Ciocalteu method (Carvalho et al. 2011) using gallic acid as the standard and absorbance was re-corded at 765 nm. Briefly, 100 µl of FC reagent and 7 % of sodium carbon-ate (0.5 ml) were mixed with 0.5 ml enzyme extract. The results were expressed as mg gallic acid equivalents (GAE) g^{-1} fresh weight (FW) of plant material. Total flavonoid content was determined using rutin as a standard (Weathers and Towler 2014). The 150 µl of 15 % sodium nitrite was added to 50 µl of extract. After 6 min, 2 ml of NaOH (4 %) was mixed with the reaction mixture and the final volume was made up to 5 ml with water. The absorbance was recorded at 510 nm after 15 min and the results were expressed as rutin equivalents per g FW (mg g^{-1}).

Measurement of antioxidant capacity

Free radical scavenging activity (FRSA) was measured using 1, 1-di-phenyl-2-picryl-hydrazil (DPPH) reagent according to (Carvalho et al. 2011). Briefly, 100 µl of the extracted sample was mixed with 400 µl Tris-HCl (0.1 M, pH 7.4) and 500 µl of freshly prepared methanolic DPPH solution (0.5 mM). The mixture was left at room temperature in dark conditions for 20 min and the absorbance was observed at 517 nm. The scavenging activity was calculated as following equation: Scavenging potential (%) = [(absorbance$_{control}$ − absorbance$_{sample}$) / (asbsorbance$_{control}$) × 100.

Total antioxidant capacity (TAC) was determined using ascorbic acid as a standard (Brisibe et al. 2009) and the results were expressed as mM ascorbic acid equivalents per g FW. Enzyme extract (100 µl) was added to 1 ml of reagent solution (sulphuric acid (3.3 ml), sodium phosphate (335 mg) and ammonium molybdate (78.416 mg) in 100 ml). The reaction mixture was boiled for 90 min at 95 °C for and absorbance was recorded at 695 nm.

The ferric reducing antioxidant power (FRAP) of the plant extracts was measured according to (Ferreira and Luthria 2010) with slight modifications and the results were expressed as mM ascorbic acid equivalents per g FW. Briefly, the reaction mixture consisted of acetate buffer (pH 3.6), 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl and 20 mM iron (III) chloride solution (10:1:1 v/v), respectively. The reaction mixture was incubated at 37 °C in a water bath. 50 µl of sample was added to 950 µl of the FRAP solution and the absorbance was observed at 593 nm after 30 min.

The reducing power (RP) was determined according to Apati et al. (2003). Briefly, extract (50 µl) was added to 1 ml of a 0.2 M phosphate buffer (pH 6.6) and 1 % (w/v) solution of potassium ferricyanide (2.5 ml). The reaction mixture was warmed at 50 °C for 20 min in a water bath after which the reaction was stopped by the addition of 2.5 ml of a 10 % (w/v) trichloroacetic acid. The mixture was centrifuged at 1500×g for 10 min. The 2.5 ml of the supernatant was combined with equal volume of distilled water and 0.5 ml of a 0.1 % (w/v) solution of ferric chloride for initiating the reaction and the absorbance of the final colored solution was measured at 700 nm. RP was expressed as mM ascorbic acid equivalent ml^{-1} (ASE). Observations for all biochemical assays were recorded in triplicate independent measurements and mean values were calculated.

Statistical analysis

For statistical analysis of data, Analysis of variance (ANOVA) techniques were performed by SPSS package (SPSS V16.0, SPSS Inc., Chicago, IL) and means were separated using Tukey's multiple comparison test at ($P < 0.05$). Linear correlation coefficient was evaluated among biomass (fresh and dry), leaf yield, artemisinin content, artemisinin yield and all antioxidant parameters. Principal component analysis (PCA) was applied to produce components suitable to be used as response variables in the present analysis (Jolliffe 2002).

Results

Effect of potential bioinoculants on biomass yield, NPK and artemisinin content

The inoculation of selected bioinoculants, STR, BM and TH individually improved the plant growth as indicated by plant weight (fresh and dry) and leaf yield of the plants (Table 1). However, when inoculated in combination, co-inoculation with STR + BM + TH was highly effective in improving the plant weight (fresh and dry) and leaf yield of plants with an observed increase of 42.96, 47.50, and 59.16 %, respectively over the uninoculated control. Although, alone bioinoculants also enhanced the leaf yields, but significant and more promising results were observed when inoculated in combinations probably because of the synergistic effect of microbes. The concentration of N was found to increase by 66.66 % in presence of STR + BM + TH followed by dual bioinoculants (54.17–57.36 %) as compared to control (Table 2). Likewise, the plant P content was increased significantly by 76.17 % in presence of STR + BM + TH in comparison to control plants. On an individual basis, bioinoculants also improved the content of P significantly. Further enhancement in content of K was also noted in STR + BM + TH treated plants (46.60 %) as compared to the control plants.

Table 1 Analysis of *A. annua* plant growth parameters under various bioinoculant treatments

Treatments	Fresh wt (t ha^{-1})	Dry wt (t ha^{-1})	Leaf yield (t ha^{-1})
Control	13.36 ± 0.13^{e}	6.02 ± 0.16^{e}	0.95 ± 0.02^{f}
STR	16.29 ± 0.15^{cd}	7.09 ± 0.39^{d}	1.14 ± 0.03^{e}
BM	15.57 ± 0.49^{d}	6.44 ± 0.16^{e}	1.03 ± 0.02^{f}
TH	16.85 ± 0.37^{bc}	7.30 ± 0.13^{cd}	1.21 ± 0.01^{de}
STR + TH	17.74 ± 0.49^{b}	8.22 ± 0.03^{b}	1.36 ± 0.04^{b}
BM + TH	17.47 ± 0.57^{b}	7.79 ± 0.19^{bc}	1.25 ± 0.02^{cd}
STR + BM	17.63 ± 0.22^{b}	8.06 ± 0.17^{b}	1.33 ± 0.04^{bc}
STR + BM + TH	19.10 ± 0.50^{a}	8.88 ± 0.15^{a}	1.52 ± 0.05^{a}

Results are means of three replicates ±SD and means within a column followed by the same letter are not significantly different ($P \leq 0.05$). Control: untreated; *Streptomyces* sp. MTN14 (STR); *B. megaterium* (BM) and *T. harzianum* Thu (TH) single as well as in combinations

Table 2 Analysis of *A. annua* macronutrients concentration in leaves under various bioinoculant treatments

Treatments	Nitrogen (%)	Phosphorus (%)	Potassium (%)
Control	1.17 ± 0.26^{b}	0.30 ± 0.02^{c}	2.06 ± 0.18^{d}
STR	1.51 ± 0.23^{ab}	0.32 ± 0.02^{c}	2.44 ± 0.12^{cd}
BM	1.31 ± 0.46^{ab}	0.31 ± 0.01^{c}	2.24 ± 0.10^{cd}
TH	1.66 ± 0.17^{ab}	0.32 ± 0.01^{c}	2.58 ± 0.08^{bc}
STR + TH	1.80 ± 0.13^{ab}	0.34 ± 0.01^{c}	2.61 ± 0.09^{bc}
BM + TH	1.84 ± 0.16^{a}	0.35 ± 0.03^{c}	2.39 ± 0.17^{cd}
STR + BM	1.81 ± 0.13^{ab}	0.46 ± 0.01^{b}	2.96 ± 0.20^{ab}
STR + BM + TH	1.95 ± 0.10^{a}	0.54 ± 0.03^{a}	3.02 ± 0.09^{a}

Results are means of three replicates ± SD and means within a column followed by the same letter are not significantly different ($P \leq 0.05$). Control: untreated; *Streptomyces* sp. MTN14 (STR); *B. megaterium* (BM) and *T. harzianum* Thu (TH) single as well as in combinations

On an individual basis, all the bioinoculants were also found useful in increasing the artemisinin content. However, in all the dual bioinoculant combinations and triple microbes treatment (STR + BM + TH), a significant increase of 20.23–28.57 and 34.52 % in artemisinin content, respectively was observed when compared with the untreated-control (Table 3). The total yield of artemisinin (Kg ha^{-1}) was found to increase by 72 % in STR + BM + TH treated *A. annua* plants. A positive and direct association was found among fresh and dry plant wt ($r = 0.964^*$), leaf yield with dry plant weight and artemisinin yield, ($r = 0.995^*$, $r = 0.975^*$), respectively (Table 4).

Effect of bioinoculants on total phenolic (TPC) and flavonoid contents (TFC)

In the present study, highest increment in TPC (44.85 %) was observed in the combination of STR + BM + TH followed by STR + BM (33.37 %) with respect to untreated control (Fig. 1a). Similarly, the flavonoid content was highest in plants treated with the microbial combination STR + BM + TH which was 61.95 % followed by dual combination of bioinoculants (29.74–52.98 %) and alone treatments (8.37–24.74 %) as compared to untreated control plants, which can be positively correlated to the increased FRSA, FRAP and RP (Fig. 1b). The flavonoid content was found to be directly and positively associated with the total phenolic content as observed by regression analysis ($r = 0.983^*$) (Table 4).

Effect of bioinoculants on free radical scavenging assay (FRSA) and total antioxidant capacity (TAC)

The bioinoculants showed higher activity of FRSA and TAC in *A. annua* leaves compared to their activities in untreated control plants (Fig. 2a, b). The FRSA activity was assayed in all bioinoculants treatments which ranged from 63.47 to 79.73 % (Fig. 2a). The maximum activity was observed in the treatment STR + BM + TH which was 81.99 % higher in comparison to untreated control plants, suggesting that these plants possessed maximum accumulation of natural antioxidants. Similarly, a significant increase in TAC was also observed in bioinoculants treated *A.annua* plants, being maximum in the treatments

Table 3 Effect of various bioinoculant treatments on artemisinin content of *A. annua*

Treatments	Artemisinin content (%)	Artemisinin yield (kg ha^{-1})
Control	0.84 ± 0.02^{c}	8.01 ± 0.20^{d}
STR	0.95 ± 0.03^{bc}	10.27 ± 0.56^{bc}
BM	0.87 ± 0.03^{c}	9.20 ± 0.19^{cd}
TH	1.02 ± 0.02^{ab}	10.67 ± 0.28^{bc}
STR + TH	1.08 ± 0.03^{a}	11.83 ± 0.66^{b}
BM + TH	1.01 ± 0.02^{ab}	10.21 ± 0.21^{bc}
STR + BM	1.07 ± 0.06^{ab}	11.49 ± 0.44^{b}
STR + BM + TH	1.13 ± 0.08^{a}	13.85 ± 1.41^{a}

Results are means of three replicates ±SD and means within a column followed by the same letter are not significantly different ($P \leq 0.05$). Control: untreated; *Streptomyces* sp. MTN14 (STR); *B. megaterium* (BM) and *T. harzianum* Thu (TH) single as well as in combinations

Table 4 Correlation study among plant biomass (fresh and dry), leaf yield, artemisinin content, Artemisinin yield and all the antioxidant parameters in *A. annua*

	Fresh wt	Dry wt	Leaf yield	Artemisinin content	Artemisinin yield	TPC	TFC	FRSA	TAC	FRAP	RP
Fresh wt	1	0.964	0.958	0.947	0.935	0.843	0.901	0.963	0.890	0.896	0.861
Dry wt		1	0.995	0.980	0.955	0.920	0.955	0.896	0.958	0.952	0.934
Leaf yield			1	0.984	0.975	0.931	0.963	0.892	0.953	0.941	0.938
Artemisinin content				1	0.946	0.891	0.949	0.895	0.916	0.895	0.908
Artemisinin yield					1	0.903	0.925	0.899	0.930	0.876	0.902
TPC						1	0.983	0.774	0.942	0.948	0.987
TFC							1	0.847	0.938	0.947	0.988
FRSA								1	0.840	0.785	0.807
TAC									1	0.927	0.937
FRAP										1	0.952
RP											1

Correlation is significant at the $P < 0.05$

TAC total antioxidant content, *FRSA* free radical scavenging activity, *FRAP* ferric reducing antioxidant power, *TPC* total phenolic content, *TFC* total flavonoid content, *RP* reducing power

having a synergistic combination of microbes (Fig. 2b). Correlation results further validated positive interactions of FRSA with phenolics and flavonoid content respectively (r = 774* and r = 0.847*) and TAC (r = 942* and r = 0.938*) (Table 4).

Effect of bioinoculants on ferric reducing antioxidant power (FRAP) and reducing Power (RP)

The bioinoculants treated *A. annua* plants resulted in a significant ($P < 0.05$) increase in FRAP and RP in all treatments compared to the control plants (Fig. 3a, b). The maximum FRAP activity of 91.97 % was recorded for STR + BM + TH followed by 62.11 % in STR + BM as compared to the control set. Interestingly, the FRAP activity in alone bioinoculants treatments of STR, BM and TH was also high. In general, FRAP increased between 9.79 and 91.97 % in bioinoculants treated *A. annua* leaves as compared to untreated control (Fig. 3a). The present findings revealed a significant increase in RP activity of 87.19 % in combination of bioinoculants (STR + BM + TH) treated plants followed by dual (38.33–75.55 %) and single bioinoculant (7.78–20.97 %) treatments, as compared to the untreated control (Fig. 3b). The increased RP may be positively correlated with the enhanced scavengers and reductants viz., phenols, flavonoids, FRAP which were highly induced in the bioinoculants treated plants as compared to their alone and dual counterparts. The RP was also directly and positively associated with the FRAP (r = 0.952*) and also strongly correlated with phenolic (r = 0.987*) and flavonoid (r = 0.988*) content (Table 4).

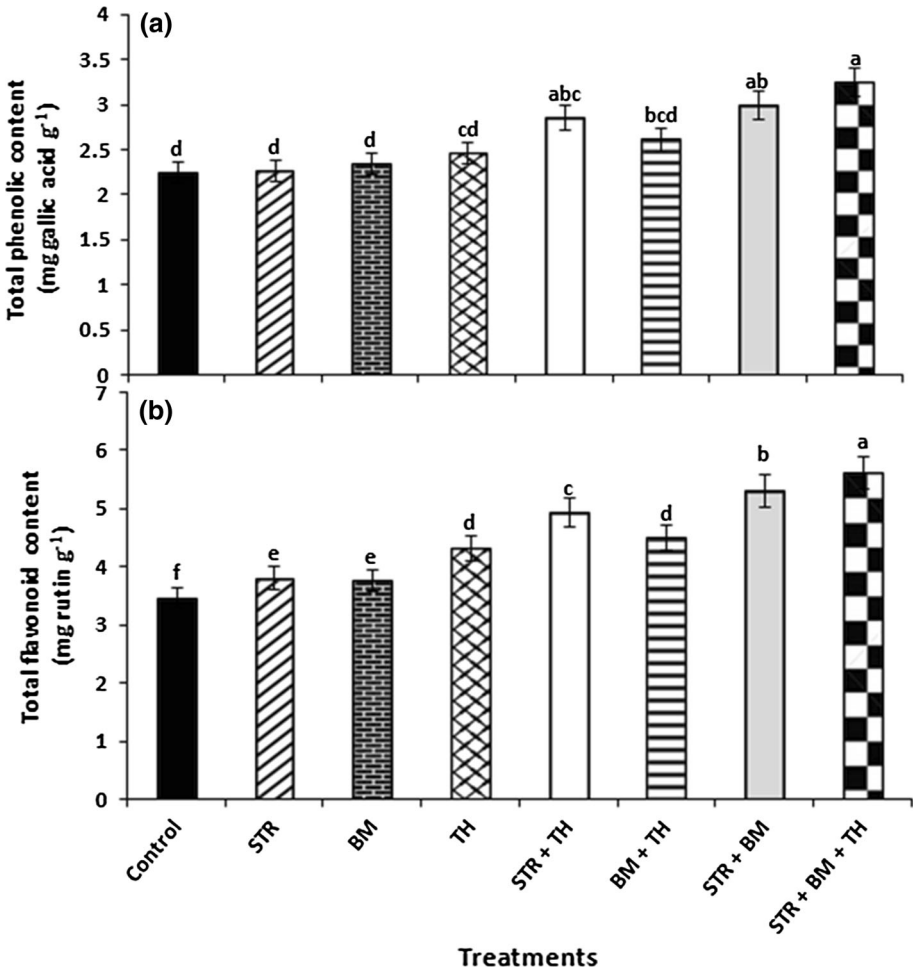

Fig. 1 Effect of bioinoculants on **a** Total phenolic and **b** flavonoid content of *A. annua*. *Bars* on top of columns represent standard error of the means (n = 3). *Different letters* indicate significant differences among treatment as determined by Tukey's multiple comparison test at $P < 0.05$. Control: untreated; *Streptomyces* sp. MTN14 (STR); *B. megaterium* (BM) and *T. harzianum* Thu (TH) single as well as in combinations

Principal component analysis

PCA contributes 95 % of the total variance. PC1 contributed 91 %, and PC2 contributed 4 % of the total variance. Results obtained from the present study were further authenticated by PCA as clustering of treatments revealed formation of four subgroups in which first subcluster had only control (T1), second: STR (T2), BM (T3) and TH (T4), third: STR + TH (T5) and BM + TH (T6) and fourth: STR + BM (T7) and STR + BM + TH (T8). The treatments STR + BM and STR + BM + TH with synergistic combinations formed a separate group where vegetative growth, plant major nutrient and antioxidant production of plant were significantly enhanced (Fig. 4).

Discussion

High degree of renewed interest has been placed on the exploitation of bioinoculants for the enhancement of pharmacological properties of *A. annua*, used in the treatment of haemorrhoids, fevers associated with malaria. Apart from this, some of the biologically active compounds isolated from the plant have also been proven to have very effective anti-microbial, anti-inflammatory and anti-tumour properties. The present study demonstrated that the selected bioinoculants, STR, BM and TH individually and in combination improved the plant growth as indicated by plant weight and leaf yield of the plants. Though alone bioinoculants enhanced the leaf yields, but more promising results were observed when inoculated in combinations probably because of the added beneficial effect of microbes when they are applied in synergism.

Further, all the bioinoculants were able to improve nutrient uptake in the plants individually as well as in combinations. On an individual basis, all the bioinoculants were also found useful in increasing the artemisinin content. The results are in conformity with the study of other researchers who also showed promising effect of microbes on enhancement of crop yield and their bioactive compounds (Gupta and Pandey 2015; Gupta et al. 2015a, 2016b). All growth parameters and artemisinin yield were consistently higher in the bioinoculants treated plants with respect to control. These results are in

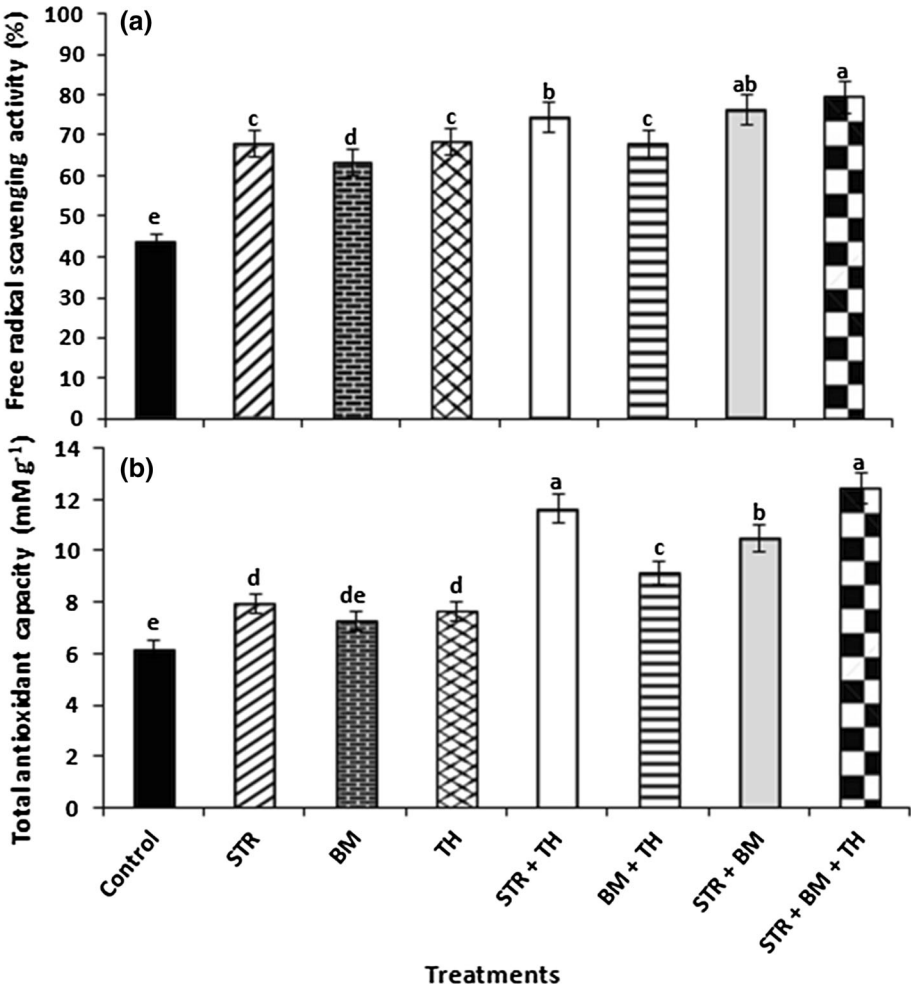

Fig. 2 Effect of bioinoculants on **a** Ferric reducing antioxidant power and **b** reducing power of *A. annua*. *Bars* on top of columns represent standard error of the means (n = 3). *Different letters* indicate significant differences among treatment as determined by Tukey's multiple comparison test at $P < 0.05$. Control: untreated; *Streptomyces* sp. MTN14 (STR); *B. megaterium* (BM) and *T. harzianum* Thu (TH) single as well as in combinations

agreement with the findings of other researchers who had previously worked on various cultivated plants (Singh et al. 2013; Gupta et al. 2015b). Looking into the results, it might be speculated that the bioinoculants improved crop performance by providing higher supplies of metabolites, plant hormones and nutrients to plants. The potentiality of synergistic mixture of microbes can be deduced from the fact that yield was maximally enhanced in treatments having mixture of microbes over their individual counterparts.

All plants possess an array of antioxidants majorly phenols and flavonoids that scavenge the free radicals and defend cells from unfavorable conditions. These antioxidants not only defend plants from damage but also enhance their therapeutic value. The bioinoculants elicit antioxidant machinery and therefore, can aid the growth and therapeutic value of pharmaceutically important *A. annua* even under tough environmental conditions. In the present study, the total phenolic and flavonoid contents in different microbial treatments were found to be significantly higher compared with the control. Phenols are well known biomolecules known to play an important role in plant defense which are also intimately connected with free radical scavenging activity. These polyphenols interact with the receptors or enzymes involved in signal transduction and thus aid in modifying the redox status of the plant cell (Halliwell et al. 2005). An increased phenolic content is in accordance with previous documentation where an increase in total phenol was observed in *Trigonella foenum-graecum* upon treatment with *B. lentimorbus* (Nautiyal et al. 2008). These flavonoids are well known to enhance antioxidant properties in foods by inhibiting the activity of alternative oxidase (Shahidi et al. 1992; Shimoji and Yamasaki 2005). As nutrient supplement, these flavonoids lessen the oxidant-induced lipid peroxidation and potassium permeability generated because of impeded membrane function in the isolated erythrocytes (Maridonneau-Parini et al. 1986). These results suggest that the bioinoculants play a vital role in boosting the phenolic and flavonoid contents in *A. annua* and can be effectively used for strengthening the plants reactive oxygen species scavenging system. Moreover, combination of bioinoculants

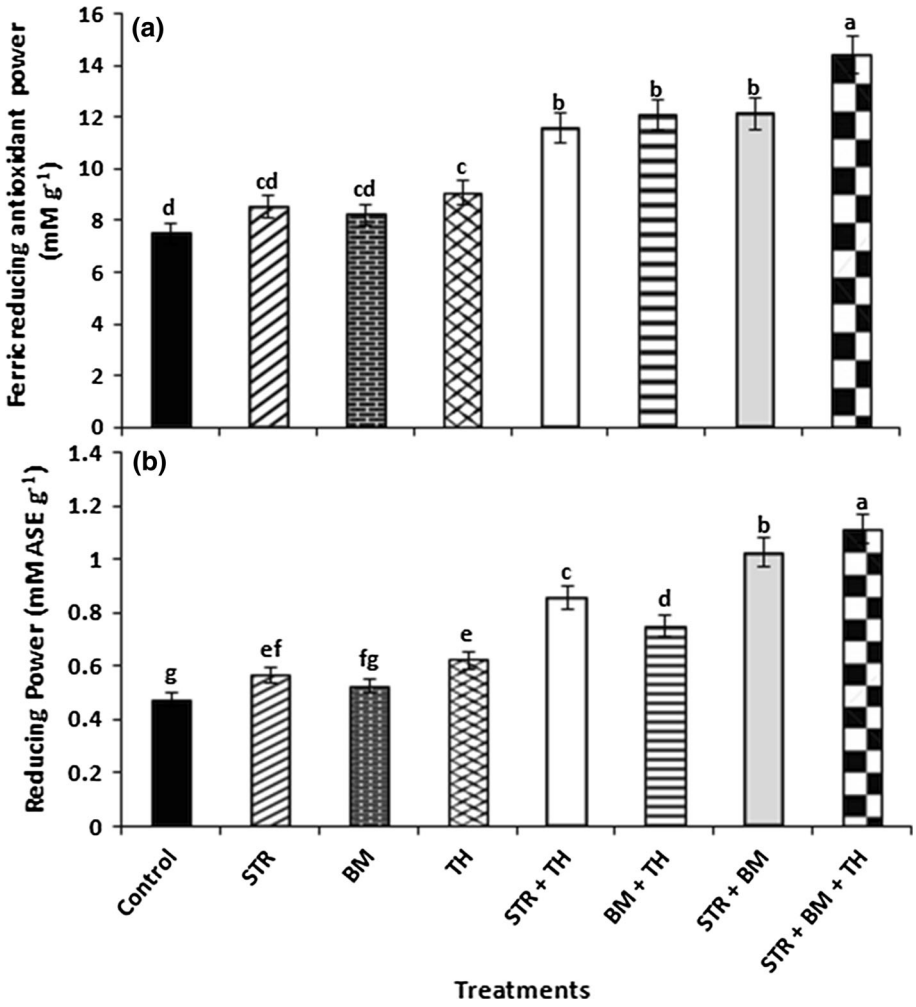

Fig. 3 Effect of bioinoculants on **a** Free radical scavenging activity and **b** total antioxidant activity of *A. annua. Bars* on top of columns represent standard error of the means (n = 3). *Different letters* indicate significant differences among treatment as determined by Tukey's multiple comparison test at $P < 0.05$. Control: untreated; *Streptomyces* sp. MTN14 (STR); *B. megaterium* (BM) and *T. harzianum* Thu (TH) single as well as in combinations

enhanced accumulation of phenols and flavonoids in comparison to others treatment signifying advantages of exploiting microbes for enhancement of antioxidant status of plants (Singh et al. 2014). Recently, our group also reported that mixture of bioinoculants viz., *T. harzianum ThU, Glomus intraradices* and *B. subtilis CIM* ameliorated phenolic and flavonoid content of an aromatic and medical plant *Pelargonium graveolens* L'Hér (Gupta et al. 2016a).

It is an accepted fact that antioxidants, which include several biomolecules including phenols and reductants reduce the risk of cancers and other chronic disease by scavenging the free radicals (Pourmorad et al. 2006). The combination of bioinoculants treated plants showed higher levels of FRSA and TAC possibly because of the presence of higher amounts of phenols and flavonoids, imparting *A. annua* with enhanced free radical scavenging properties. In similar studies, enhanced free scavenging activity was also observed in plants treated with microbes (Nautiyal et al. 2008; Singh et al. 2014). The variation in these activities may be due to variation in the content of antioxidant induced by these microbes when treated alone or in combination mode. Previously, similar correlations have been established with increase in FRAP and enhanced accumulation of phenols and flavonoids (Gupta et al. 2015a). The bioinoculants treated plants resulted in higher increment of FRAP possibly owing to accumulation of higher amounts of phenols and flavonoids thus, strengthening the plants with better equipped and enhanced scavenging properties.

The RP of the extracts was measured in terms of green color formed by the reduction of Fe^{3+} ferricyanide complex to Fe^{2+} in the presence of reductants (antioxidants) using potassium ferricyanide method. The results obtained are well supported by the study of Yang et al. (2000) and Moktan et al. (2008) who reported that application of *Acetobacter* sp., *Lactobacillus* sp., *Saccharomyces* sp. and *Streptomyces* sp. significantly enhanced the reducing power activity of fermented soybean broth.

The PCA showed that antioxidant parameters such as TPC, TFC, FRAP, RP, TAC and macro plant nutrients (P and K) influenced the plant vegetative parameters viz., plant weight (fresh and dry), leaf yield, artemisinin yield

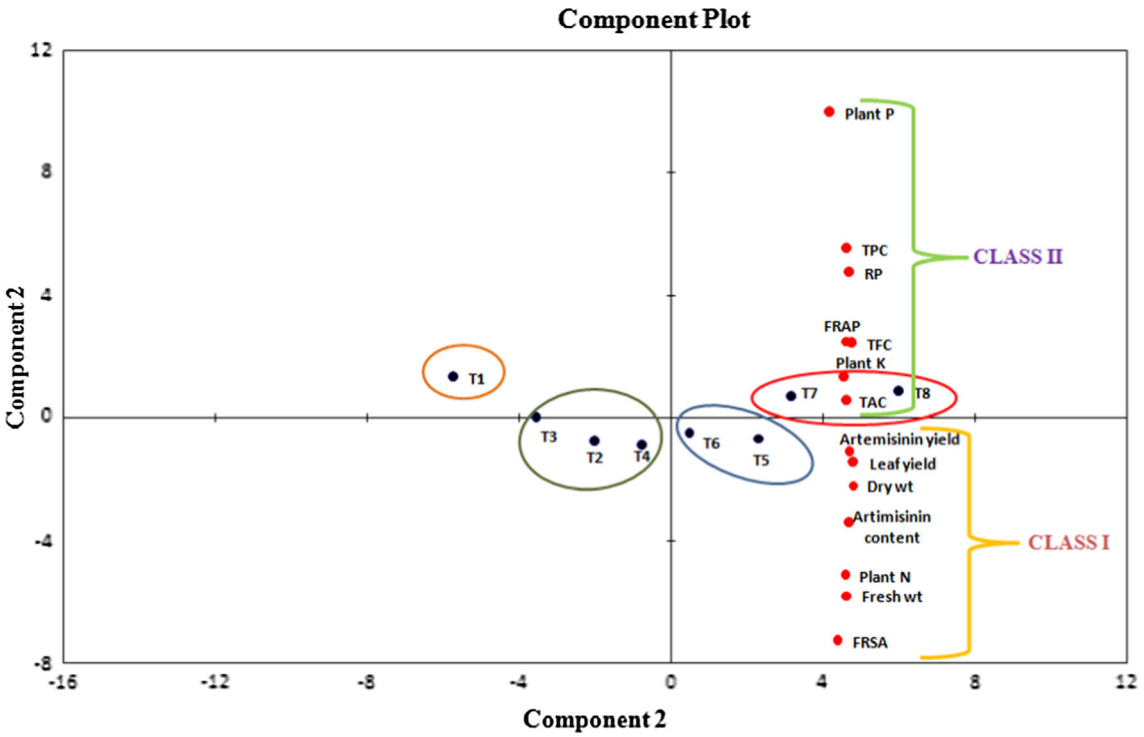

Fig. 4 Principal component analysis (PCA) for plant biomass (fresh and dry), leaf yield, artemisinin content, artemisinin yield, nutrient content (NPK) in plant, and all the antioxidant parameters of *A. annua* with respect to various treatments (T1–T8). Treatments T1: Control; T2: *Streptomyces* sp. MTN14 (STR); T3: *B. megaterium* (BM); T4: *T. harzianum* Thu (TH), T5: STR + TH, T6: BM + TH, T7: STR + BM and T8: STR + BM + TH. TAC: Total antioxidant content; FRSA: Free radical scavenging activity; FRAP: Ferric reducing antioxidant power; TPC: Total phenolic content: TFC: Total flavonoid content and RP: Reducing power

and its free radical scavenging properties that provided the plant with better immune system to cope up with the various stress. Thus, the PCA analysis concluded that plant macronutrients and antioxidant content positively affected the plant growth and artemisinin yield of *A. annua*. In general, the combination of bioinoculants especially STR + BM and STR + BM + TH supplied the plants all the required elements and antioxidants in a better way for the enhanced growth and artemisinin yield to the plant.

The enhancement of therapeutic properties of plants is becoming very significant as it plays a key role in human disease prevention. The results derived from the present study of *A. annua* are clear indications of the role of beneficial microbes in enhancing the antioxidant and therapeutic potential of the plant thereby effectively expanding the therapeutic uses. Further, the study highlights the probable role of bioinoculants as an efficient tool for the enhancement of growth, nutrient uptake, antioxidant as well as artemisinin content. To our knowledge, the use of bioinoculants alone and in combinations for improving therapeutic and antioxidant value of the "wonder plant" has been shown for the first time, indicating their multifaceted benefits. Therefore, it could be said that in near future bioinoculants will play important role in protecting plant from various stresses along with increasing the antioxidant status of the plants.

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Compliance with ethical standards

Conflict of interest All authors declare that there exists no conflict of interest among them.

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