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Effects of arbuscular mycorrhiza and phosphorus application on artemisinin concentration in *Artemisia annua* L.

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Abstract Annual wormwood (*Artemisia annua* L.) produces an array of complex terpenoids including artemisinin, a compound of current interest in the treatment of drug-resistant malaria. However, this promising antimalarial compound remains expensive and is hardly available on the global scale. Synthesis of artemisinin has not been proved to be feasible commercially. Therefore, increase in yield of naturally occurring artemisinin is an important area of investigation. The effects of inoculation by two arbuscular mycorrhizal (AM) fungi, *Glomus macrocarpum* and *Glomus fasciculatum*, either alone or supplemented with P-fertilizer, on artemisinin concentration in *A. annua* were studied. The concentration of artemisinin was determined by reverse-phase high-performance liquid chromatography with UV detection. The two fungi significantly increased concentration of artemisinin in the herb. Although there was significant increase in concentration of artemisinin in nonmycorrhizal P-fertilized plants as compared to control, the extent of the increase was less compared to mycorrhizal plants grown with or without P-fertilization. This suggests that the increase in artemisinin concentration may not be entirely attributed to enhanced P-nutrition and improved growth. A strong positive linear correlation was observed between glandular trichome density on leaves and artemisinin concentration. Mycorrhizal plants possessed higher foliar glandular trichome (site for artemisinin biosynthesis and sequestration) density compared to nonmycorrhizal plants. Glandular trichome density was not influenced by P-fertilizer application. The study suggests a potential role of AM fungi in improving the concentration of artemisinin in *A. annua*.

Keywords *Artemisia annua* · Artimisinin · Arbuscular mycorrhiza · Glandular trichome density · Phosphorus fertilization

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Introduction

Malaria is a major health problem in many developing countries, mostly in Africa and southeast Asia (Snow et al. 2005). With *Plasmodium falciparum* having become resistant to conventional antimalarial drugs, artemisinin-based combination therapy is considered a reliable optional treatment. The Roll Back Malaria program and Millennium Development Goal campaigns of WHO and United Nations Development Program, respectively, have made little headway, mainly due to paucity of artemisinin in the world market (Enserink 2005). Artemisinin, a sesquiterpene lactone containing a peroxide bridge, is isolated from the aerial parts of *Artemisia annua* L. (Avery et al. 1992). *Artemisia annua* (Asteraceae) is an aromatic plant that has been used for centuries in Chinese traditional medicine for the treatment of cerebral fever and malaria. It has been introduced to India, where it grows in the northern belt. The crop responds well to the chemical fertilizers (Ram et al. 1997).

Organic synthesis of artemisinin is possible, but the low yields make it economically nonviable as a means of drug production (Avery et al. 1992). The production of artemisinin by means of cell or tissue cultures has also not been successful (Nair et al. 1986). Ro et al. (2006) have put three plant genes into yeast (*Saccharomyces cerevisiae*) to produce artemisinic acid (precursor of artemisinin). Wu et al. (2006) have engineered high-level terpene produc-

tion in tobacco plants by diverting carbon flow from cytosolic or plastidic isopentenyl diphosphate through over expression in either compartment of an avian farnesyl diphosphate synthase and an appropriate terpene synthase. However, it may take several years for the process to get scaled up for industrial production. Therefore, improvement in naturally occurring artemisinin yield is an important area of investigation.

Arbuscular mycorrhizal (AM) fungi are known to play a pivotal role in the nutrition and growth of plants in many production-orientated agricultural systems, but little is known about their potential effect on secondary metabolites in medicinal and aromatic plants (Copetta et al. 2006; Kapoor et al. 2002a, b, 2004; Khaosaad et al. 2006). Members of the Asteraceae are usually both mycorrhizal and responsive to the symbiosis (Blanke et al. 2005; Bryla and Duniway 1997; Ultra et al. 2007; Warcup and Mc Gee 1983). To our knowledge, no study has been carried out on the effect of AM in the production of artemisinin in *A. annua*. We therefore conducted an experiment with the objectives to (1) compare the effects of two AM fungi *Glomus macrocarpum* and *Glomus fasciculatum* on plant growth and production of artemisinin in *A. annua*, (2) exclude a simple phosphorus-mediated effect through mycorrhization, and (3) determine if phosphate fertilization would alter AM effects on artemisinin production. As the subcuticular space of glandular trichomes on leaves is a site for sequestration of artemisinin produced by *A. annua*, we also studied the effect of mycorrhization and phosphate fertilization on trichome density of leaves.

Materials and methods

Experimental design

The experiment was conducted in the Botanical Garden, Department of Botany, University of Delhi, in 18 microplots of 10 m^2 area each (microplots were separated from each other by a cemented wall about 50 cm thick and 150 cm in depth to avoid cross contamination). The 3×2 factorial experiment was designed with three mycorrhizal conditions viz. nonmycorrhizal, inoculated with *G. macrocarpum* Tul. and Tul. or inoculated with *G. fasciculatum* (Thaxter sensu Gerd.) Gerdemann and Trappe and combined with two concentrations of phosphorus (with and without P-fertilizer) in the soil. The treatment combinations were arranged in a completely randomized block design, with three microplots per treatment.

The plots were deep ploughed and the topsoil of the plots was fumigated by applying 0.1% commercial formaldehyde. The fumigated soil was covered with a black polythene sheet and left for 1 week. Thereafter, the polythene was removed to dissipate the fumigant. The soil had the following physico-chemical properties: loam texture, pH (H_2O) 7.6, EC (S/m at 32°C) 0.12; organic C 1.12%; total N 0.49%; available P, K, Na, Mg, Ca, Zn, and Cu 11.1, 55, 61.3, 45, 150, 55, and 22 mg/kg, respectively.

Seeds of *A. annua* L. EC 415012 were procured from the National Bureau of Plant Genetic Resources, Regional Station Bhowali (Uttaranchal). Seeds were surface sterilized with NaOCl and sown in raised nursery beds (sterile soil) in the month of October. Sowing was done in rows. Because the seeds are tiny, they were mixed with ash (autoclaved) to increase the volume to facilitate uniform distribution on the nursery bed. Seeds were spread on the surface of the bed and then covered with a thin layer of soil. The beds were kept moist until the seeds germinated. Light watering was provided once a day for the first 4 days. Later, the beds were flooded with water weekly.

Potassium dihydrogen phosphate (analytical reagent) was used as a source of phosphorus. The soil of a microplot was supplemented with 131 g of KH_2PO_4 equivalent to P 30 kg/ha. About 35-day-old seedlings were transplanted in rows (with 30 cm space between the plants in a row and 50 cm space between the rows to avoid intermingling of root systems) in the first week of December. Experiments consisted of six treatments, namely: (1) control (fumigated soil inoculated with rhizosphere soil of nonmycorrhizal sorghum), (2) inoculated with *G. macrocarpum* (GM − P), (3) inoculated with *G. fasciculatum* (GF − P), (4) soil supplemented with phosphorus fertilizer (NM + P), (5) inoculated with *G. macrocarpum* and supplemented with P-fertilizer (GM + P), and (6) inoculated with *G. fasciculatum* and supplemented with P-fertilizer (GF + P). Light irrigation was provided just after transplanting. The plants were allowed to grow and no fertilizer or pesticide was added to the soil during the course of the experiment. Hand weeding was done periodically to keep the field free from the weeds.

Mycorrhizal inoculation

Pot cultures of *G. macrocarpum* and *G. fasciculatum* were maintained on *Sorghum halepense* L. plants for 6 months. Five hundred grams of soil inoculum (50 spores/10 g soil), along with 200 mg of chopped AM sorghum roots (AM colonization level 80%), was placed in furrows made with the help of a drill in each microplot before planting the seedlings. Propagule infectivity was tested according to the method of Sharma et al. (1996). Nonmycorrhizal inoculum consisted of 500 g rhizosphere soil and 200 mg chopped nonmycorrhizal sorghum roots obtained by sowing surface sterilized seeds of sorghum in pot containing autoclaved soil.

Growth measurement and nutrient concentration

Plants were grown under natural field conditions for 12 weeks. For each parameter studied, six plants were randomly harvested from three microplots of each treatment. Roots were washed and dried on blotting paper. Fresh weight of shoot per plant was determined. Shoot samples were oven-dried at 72°C for 48 h for determination of dry weight (g/plant). Later, the same dried shoot samples were analyzed for their mineral concentration. Dried samples were ground, digested in concentrated acid (HNO3/HClO4, 2:3 v/v) at 140–160°C. After cooling, the extracts were diluted with 1 N HCl and made up to 25 ml (Allen 1989). Reagent blanks were prepared by carrying out the whole extraction procedure but in absence of the sample. The phosphorus in the digested sample was estimated by the molybdate method (Allen 1989) at 700 nm using a UV spectrophotometer. The concentrations of Mg, Cu, Zn, Mn, and Fe were determined using atomic absorption spectrophotometry (ShimadzuAA-130, AAS, lamp current 7 mA, slit width of 1.9 cm and burner height of 7 cm) at wavelengths 285.2, 324.8, 213.9, 279.5, and 248.3 nm, respectively. Working standards were procured from Sigma Aldrich, St. Louis, MO, USA.

Determination of root colonization

To visualize AM fungal colonization, fresh root samples were cleared with 10% KOH and stained with Trypan blue (0.1%) in lactoglycerol (Phillips and Hayman 1970). The percent root colonized by AM fungi in plants was assessed and scored (100× magnification) following Bierman and Linderman's (1981) method. Selected colonized root segments were examined for colonization pattern (400× magnification) using a compound microscope.

Photosynthetic pigment concentration

Chlorophyll and carotenoid concentrations were determined in fresh leaves using the method of Hiscox and Israelstam (1979). Photosynthetic pigments were extracted in dimethyl sulfoxide, and absorbance of the decant was taken at 480, 510, 645, and 663 nm. Chlorophyll-a, chlorophyll-b and carotenoid concentrations (mg/g, fresh weight) were calculated using the equations given by Arnon (1949).

Glandular trichome density

The youngest terminal leaf of about 5 cm in length on each plant was removed for sampling. The leaf selection process yielded terminal leaves of similar sizes. Two leaf discs (0.6 cm diameter) were punched midway between the leaf tip and leaf base, one on either side of the midrib, taking care to preserve trichomes on the leaf surface. The number of glandular (capitate) trichomes was determined by stereomicroscopy (SMZ1000 Nikon, Tokyo, Japan). Other trichomes were not examined in this study.

Artemisinin extraction and estimation

Standard artemisinin was purchased from Sigma Aldrich, Bangalore, India (purity 98%). Standard solutions of artemisinin were prepared by following Qian et al. (2005). Water, methanol, ethanol, and acetonitrile were chromatographic grade (Sigma Aldrich, Bangalore, India). All other chemicals were analytical-grade reagents. Artemisinin was extracted from oven (36°C)-dried leaves by dipping 10-g dried leaves in 50 ml 100% chloroform for 8 s, filtering with Whatman no.1 filter paper, and evaporating the chloroform to dryness at 40°C in a rotary evaporator. The residue was cooled to room temperature, dissolved in ethanol, and vacuum filtered. Ten milliliters of the filtrate was mixed with 0.2% (by weight) NaOH, then acidified with 0.08 M acetic acid. The sample was checked for absorbance maxima at 260 nm and submitted directly to chromatographic analysis.

High-performance liquid chromatography system: A 250×4.6-mm, reverse-phase C18 silica column was used. The mobile phase was 45–10–45 (by volume) methanol–acetonitrile–0.9 mM Na_2HPO_4–3.6 mM Na_2HPO_4 buffer (pH 7.76). Each sample was filtered through a 0.45-μm filter and injected 10 μl each time. The detection wavelength was 260 nm. All chromatographic analyses were performed isocratically at 30°C.

Statistical analysis

One-way analysis of variance was carried out for each parameter studied. Tukey's post hoc multiple mean comparison test was used to test for significant differences between treatments (at 5% level). All statistical analyses were performed with Statistical Package for Social Sciences (version 10).

Results

Plant growth and nutrient concentration

Both the *Glomus* species successfully colonized the roots of *A annua* (Table 1) forming *Arum*-type mycorrhiza. No AM colonization was observed in those plant roots that were not inoculated with AM fungi. Percentage of root colonization varied between the fungi and decreased in plants grown in P-fertilized soil (Table 1).

Table 1 Effects of AM fungi and phosphate fertilizer on various growth parameters and per cent AM colonization in roots of *A. annua*

	Treatments					
	Con	GM − P	GF − P	P-fert	GM + P	GF + P
Fresh weight of shoot (g/plant)	11.9 a	47.0 b	69.1 c	49.5 b	81.6 d	119.4 e
Dry weight of shoot (g/plant)	1.9 a	5.1 c	5.5 cd	4.0 b	6.2 d	11.6 e
Per cent AM colonization in roots	00.00	65	62	00.00	43	51

Values are mean of six replicates. Within a row, values followed by the same letters are not significantly different (P=0.05) by Tukey's post hoc test using Statistical Package for Social Sciences software
Con control, *P-fert* soil supplemented with phosphorus fertilizer

AM fungal inoculation and/or phosphorus fertilization had a significant effect on all measured plant growth variables (Table 1). However, the level to which plant growth was enhanced varied between the fungal inoculants. *Glomus fasciculatum*-colonized plants performed consistently better than *G. macrocarpum*-inoculated and nonmycorrhizal P-fertilized plants. Mycorrhizal inoculation of plants in the P-enriched soil further increased the shoot biomass. The treatment GF + P produced up to tenfold more shoot biomass than noninoculated control plants.

Mycorrhizal plants consistently accumulated more quantities of phosphorus in their shoots than the nonmycorrhizal plants. However, the differences were not significant in plants grown in P-fertilized soil (Table 2). AM fungi and P-fertilizer application together resulted in higher concentrations of Zn and Fe in shoots. Conversely, a distinct decrease was observed in Mn concentrations after inoculation with AM fungi and, to some extent, also after addition of P to soil. The concentration of Cu was not influenced by AM or addition of P-fertilizer

Glandular trichome density in leaves

AM fungal inoculation of *A. annua* induced increased densities of glandular trichomes on leaves (Fig. 1), while significant i.e. P-fertilizer had no significant effect on trichome densities. Highest densities of trichomes were present on leaves produced by GF + P and GF − P plants. Trichome density was 1.5-fold higher on leaves of *G. fasciculatum*-inoculated plants than that on control plants. A highly positive linear correlation between glandular trichome density in leaf and shoot artemisinin concentration (r^2=0.985) was observed (Fig. 2).

Concentration of photosynthetic pigments

Mycorrhizal inoculation or P-fertilizer application alone did not significantly influence the concentrations of chlorophyll-a (1.5 mg/g fresh weight), chlorophyll-b (0.4 mg/g fresh weight), or carotenoids (0.8 mg/g fresh weight) significantly. However, AM fungi and P-fertilizer together resulted in significant increases in concentrations of chlorophyll-a (1.9 mg/g fresh weight), chlorophyll-b (0.6 mg/g fresh weight), and carotenoids (0.9 mg/g fresh weight) compared to their respective controls.

Concentration of artemisinin in leaves

Concentration of artemisinin in the foliage was significantly higher in all treatments compared to controls. The artemisinin concentration was highest in GF + P and GF − P plants, which did not differ significantly. The next best

Table 2 Effects of AM fungi and phosphate fertilizer on mineral nutrient concentration in shoots and roots of *A. annua*

Shoot	Treatments					
	Cont	GM − P	GF − P	P-fert	GM + P	GF + P
Phosphorus (%)	0.72 a	1.17 b	1.15 b	1.20 b	1.33 b	1.32 b
Zinc (ppm)	13.6 a	16.5 b	18.7 c	17.0 bc	16.6 bc	17.0 bc
Iron (ppm)	90.9 a	92.7 bc	93.5 c	91.5 ab	94.2 d	93.6 c
Manganese (ppm)	12.4 d	9.5 c	9.1 bc	11.3 d	7.4 a	8.0 ab

Values are mean of six replicates. Within a row, values followed by the same letters are not significantly different (P=0.05) by Tukey's post hoc test using Statistical Package for Social Sciences software
Con control; *P-fert* soil supplemented with phosphorus fertilizer

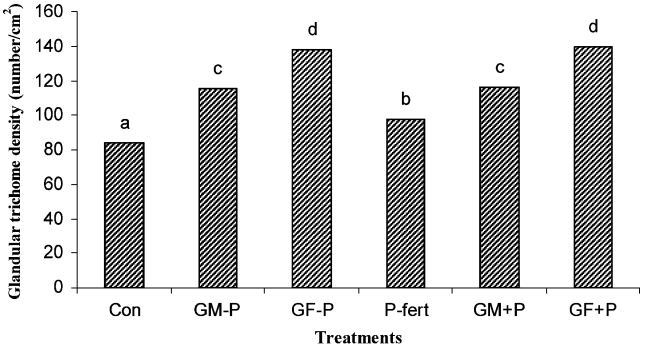

Fig. 1 Effect of AM fungi and P-fertilizer on glandular trichome density (number/cm^2 leaf area) in leaves of *A. annua*. *Histograms with different letters* are significantly different at 5% probability level according to Tukey post hoc test. *Con*: control; *P-fert*: soil supplemented with phosphorus fertilizer

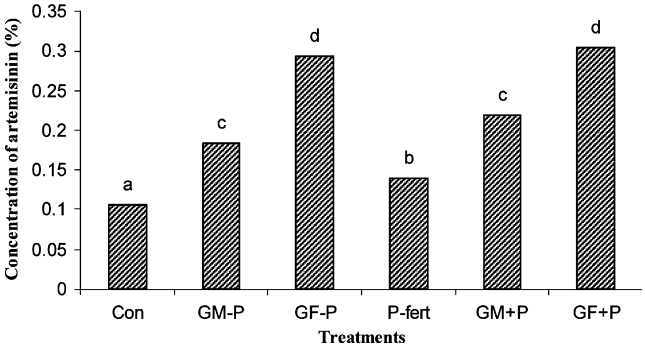

Fig. 3 Effect of AM fungi and P-fertilizer on the concentration of artemisinin (%, dry weight) in foliage of *A. annua*. *Histograms with different letters* are significantly different at 5% probability level according to Tukey Post hoc test. *Con*: control; *P-fert*: soil supplemented with phosphorus fertilizer

treatments were GM + P and GM − P, followed by nonmycorrhizal plants grown in P-enriched soil (Fig. 3). Artemisinin concentration showed positive correlation with shoot P (r^2=0.64) (Fig. 4).

Discussion

A significant increase in growth and artemisinin concentration in *A. annua* was recorded in plants from all treatments relative to the controls. Increased growth and development in AM plants compared to nonmycorrhizal ones has been reported for many plant species, including members of Asteraceae (Bryla and Duniway 1997; Ultra et al. 2007). Different effects on plant development were observed depending on the fungal species and P-status of the soil. *Glomus fasciculatum*-inoculated plants registered higher yield at both levels of phosphorus in soil. The greatest growth effect was observed with *G. fasciculatum* in the P-enriched soil. The results of the present work are consistent with reports of variations in plant growth and active principles in other medicinal plants (Copetta et al. 2006; Sailo and Bagyaraj 2005). Certain combinations of host and fungus are more or less compatible than others (Ravnskov and Jakobsen 1995), and species or strains of AM fungi can vary in their capacity to take up P from soil and transfer it to the host plant (Burleigh et al. 2002; Smith et al. 2000). It is acknowledged that there is high functional diversity among AM fungal species that results in variations in plant responses (Munkrold et al. 2004).

Mycorrhization or P-fertilization alone did not influence the concentration of photosynthetic pigments. These results are not in agreement with results obtained in some earlier works (Giri et al. 2003; Kapoor and Bhatnagar 2007). Taiz and Zeiger (1998) correlated enhanced concentrations of chlorophyll to Cu uptake. Cu is involved in the electron transport system and is a component of the chlorophyll protein plastocyanin. Thus, nonsignificant differences in concentrations of chlorophyll-a and chlorophyll-b observed in the present study may be attributed to similar Cu

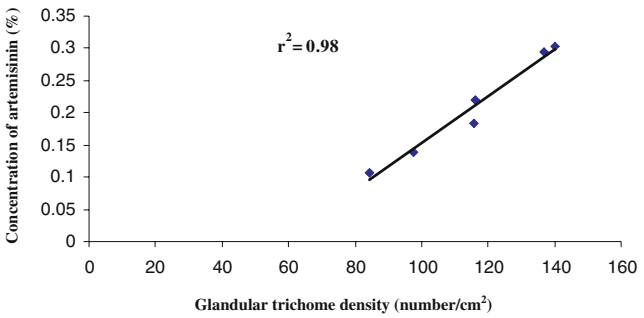

Fig. 2 Relationship between glandular trichome density (number/cm^2 leaf area) on leaves and concentration of artemisinin (%) in leaves of *A. annua*

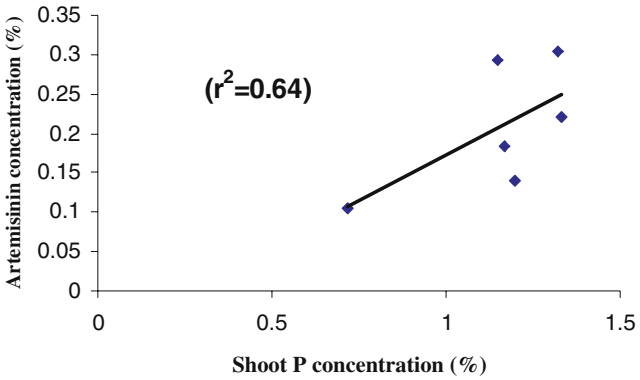

Fig. 4 Relationship between shoot P concentration (%) and concentration of artemisinin in leaves (%) of *A. annua*

concentrations in leaves of AM-inoculated and nonmycorrhizal plants.

The concentration of artemisinin in leaves of *A. annua* ranged between 0.1% (control) and 0.3% (GF + P), which is consistent with earlier published values (Charles et al. 1990). Artemisinin has been reported to accumulate in leaves (89% of the total artemisinin in the plant; Ferreira and Janick 1995). Therefore, substances that improve leaf development and shoot growth are presumed to increase artemisinin yield. Moreover, biosynthesis of terpenoids is dependent on primary metabolism, e.g., photosynthesis and oxidative pathways for carbon and energy supply (Singh et al. 1990). According to Fitter (1988) and Giri et al. (2003), net photosynthesis of mycorrhizal plants can increase as a result of improved plant nutritional status. Factors that increase dry matter production may influence the interrelationship between primary and secondary metabolism, leading to increased biosynthesis of secondary products (Shukla et al. 1992). It appears that significant improvement in plant biomass results in greater availability of substrate for artemisinin biosynthesis (van Gelgre et al. 1997). The enhanced concentration of artemisinin by mycorrhization and/or P-fertilization may be due to improved growth and nutrient status of the plants. Consequently, the increase in artemisinin concentration depends on the species of AM fungus used, with *G. fasciculatum* being a more efficient symbiont for *A. annua* than *G. macrocarpum*.

Although shoot P concentrations in mycorrhizal and nonmycorrhizal plants were not significantly different in P enriched soil, mycorrhizal plants had significantly higher contents of artemisinin. This indicates that, in spite of a positive linear correlation between shoot P concentration and artemisinin concentration (Fig. 4), the increase in artemisinin concentration in mycorrhizal plants may not be entirely attributed to enhanced P nutrition. The majority of evidence indicates that the terpenoids are produced by cells of the gland rather than by other leaf cells (Wagner 1991). This is further supported in the present study by the highly positive correlation between glandular trichome density on leaves and shoot artemisinin concentration in *A. annua* (r^2=0.985). Duke et al. (1994) provided evidence that these glandular trichomes are the sole sites of artemisinin accumulation, based on their extraction from leaves by a 5-s dip in chloroform, without observable damage to other leaf epidermal cells, and their absence from extracts of a glandless biotype. Leaves of mycorrhizal plants had higher glandular trichome density compared to nonmycorrhizal plants. Moreover, P-fertilization had no effect on glandular trichome density. Higher numbers of glandular trichomes in mycorrhizal plants in comparison to nonmycorrhizal plants have been reported earlier in other plant species (Copetta et al. 2006). This greater number of glands may be related to alterations in the hormonal balance of plants due to increased levels of auxins, cytokinins, and gibberellins in AM plants (Dixon et al. 1988).

In conclusion, the present study demonstrates the effectiveness of AM fungi in improving the concentration of artemisinin in *A. annua*. Inoculation with suitable AM fungal species along with P-fertilization produces significant increases in shoot production, resulting in significant increases in artemisinin content of individual plants. Thus, introduction of mycorrhizal technology in conjunction with P-fertilizers will be helpful in developing low-cost cultivation of *A. annua* and production of artemisinin.

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