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INFLUENCE OF SYMBIOTIC FUNGI ON *ARTEMISIA ANNUA* L.-A MEDICINAL PLANT

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ABSTRACT

Importance: Artemisinin is a potent antimalarial natural compound, obtained from the aerial portion of the plant - *Artemisia annua* L. The demand for artemisinin is exponentially increasing due to enhanced incidence of drug resistant malaria. However, the low yield of this anti-malarial drug is a major constraint in the commercialization. Several methods have been used since long but have not been found attainable on large scale. Hence outsized farming of *A. annua* is the only solution for the production of Artemisinin. **Observation:** A significant increase in artemisinin, an active compound in plant, used as anti-malarial drug under combination therapies recommended by WHO for treating multidrug resistance in malaria, has been achieved on inoculation with *Piriformospora indica* (*Serendipita indica*) and *Azotobacter chroococcum* in combination. The quantity of artemisinin was significantly higher (2.4 fold higher) on dual inoculation as compared to control. **Conclusion and Relevance:** The greenhouse and field trials have led enhanced plant growth and artemisinin content. At moment it is not clear the mechanism by which the artemisinin content is increased. The understanding of the mechanism will promise to help further enhancement artemisinin content. Past few years, efforts have been made to co-culture plant with symbiotic mycorrhizal fungi and azotroph. Results indicated a positive impact.

KEYWORDS: Artemisinin, *P. indica, A. chroococcum, A. Annua.*

INTRODUCTION

Piriformospora indica (Serendipita indica): an Endophytic Fungus

Is a mutualistic plant growth promoting fungus shows interaction with vascular Pteridophytes and Bryophytes.[1,2] The fungus was isolated from the sandy Thar Desert in north western Rajasthan, Jaisalmer, India.[3]

Most important advantage of *P. indica* over AM fungi is that a wide range of synthetic media can be used for its growth[4] (Patent 1and2). This fungus produces pear shaped thick walled chlamydospores having long shelf life of almost 12 months at room temperature. The fungus protects the plants against acidity, desiccation and heavy metals toxicity.[5] It acts as biofertilizer, bioprotector, plant regulator, fungicide and pesticide. The fungus has been commercialized under the trade mark "Rootonic".[7]

Morphology

The diameter of thick-walled hyphae of *P. indica* ranges from 0.7 to 3.5 µm, it is white and almost hyaline. The hyphae are highly interwoven and look like inter wined cords which adhere together. The length and width of pear shaped chlamydospores are 16-25 µm and 10-17 µm respectively and are observed as being single or in clusters (Fig. 1). 8-25 nuclei are present in each spore. Very thin hyaline wall is present in young spores and at maturity the spores have a wall thickness of 1.5 µm[4]. The green colour *P. indica* spores and filaments have been seen under confocal microscope when stained with WGA-Alexa 488 dye (Fig. 2a and 2b).

It belongs to phylum Hymenomycetes, class Basidiomycota and order Sebacinales. Sebacinale is split into 2 subgroups Sebacinaceae and Serendipitaceae (new family). Again, on the basis of molecular phylogenetic studies, it was concluded that *Piriformospora* belongs to Serendipita. But as *Piriformospora* belongs to genus anamorph i.e asexual stage so it was not possible to merge two different types of taxa International code of Botanical Nomenclature.[7] Finally, it was decided that the name for this new genus has to be *Serendipita indica.* Serendipitaceae contains only one genus i.e *Serendipita indica* with *Serendipita vermifera.*[8]

Cultivation of *P. indica*

Hill and Kaefer medium, Jaggery medium (Patent 1) and Mushroom span (Patent 3) medium are synthetic media used for its cultivation. For plate inoculation one disc (5mm diameter) was placed in the center of the Petri

plate containing 25ml solidified medium. After inoculation plates were incubated at 30˚C for 7days.

***Piriformospora indica* promotes plant growth - Mechanism**

Production of Auxin

P. indica produce auxin in liquid culture. Auxin affects the root growth that is responsible for promoting effect of *P. indica* on plant host. Although expression of auxin-regulated genes in *Arabidopsis* was not affected by the endophyte[9,10], such genes were induced in barley and in Chinese cabbage[11], and their induction was causative for the strong growth-promoting effect.

Suppress Ethylene Signaling

Ethylene inhibits plant growth, some rhizobacteria produce enzymes that degrade ethylene.[12] *P. Indica* inhibit ethylene signaling, which contribute plant growth promotion.[13] In *Arabidopsis*, mutations in ethylene signal transduction components resulted in increased root colonization and caused growth repression.[14] Hence, moderate interfering with ethylene signaling allow a certain degree of colonization by releasing the inhibiting effect of phytohormone.

Phyto-hormone Synthesis

Additional phyto-hormones synthesised by the root endophyte include cytokinins, gibberellins, abscisic acid and brassinosteroids.[10] In response to colonization, the abscisic acid pathway was proposed to enhance plant growth via calcium, phosphoinositide and particular protein kinases.[14] In summary, nearly the whole orchestra of phytohormones and phytohormone signalling networks seems to be involved in generating compatible interactions between the fungus and host, which lead to increased early root growth promotion and finally to greater biomass. β D-hydroxylase/myrosinase[15], a newly discovered phytohormone found responsible for seed promotion and plant growth in *Arabinodopsis* plant.

Azotobacter chroococcum

Azotobacter chroococcum belongs to Genus Azotobacter and family Azotobacteriaceae was first isolated from Holland soil in 1901.

Azotobacter is a free living nitrogen fixing bacterium which regulates plant nutrition and maintains soil fertility. *A. chroococcum* is an aerobic diazotroph which resides in rhizospheric area of maize (*Zea mays* L.), bajra (*Pennisetum glaucum*), sugarcane (*Saccharum officinarum* L.) and other vegetable crops[16]. It is easily cultured on synthetic Ashby's medium (Fig. 3).[17]

It enhances the uptake of NO_3^-, NH_4^+, $H_2PO_4^-$ and Fe that increases the nitrogen concentration[18]. It enhances the root development and growth of the plant by vitamins and amino acid secretion and through the formation of siderophores.[19]

Plant hormones are not only produced by higher plants, but many soil microorganisms are able to produce them. Phytohormone production i.e. auxins, gibberellins and cytokinins is one of the mechanisms by which rhizospheric bacteria can promote plant growth.[20] Effect of *Azotobacter chroococcum* on sugar beet was studied which showed an increase in the major element, nitrogen under greenhouse experiment.[21] *Azotobacter* spp. solubilizes and fixes inorganic phosphate makes it available to the plants.

Cultivation of *A. chroococcum*

Jensen's agar medium and Ashby's agar medium are synthetic media used for cultivation of *A. chroococcum*. The streak plate of culture was incubated at 28 ± 2˚C for 48 hrs.

Properties of PGPRs

Enhancement of Nitrogen Availability

Nitrogen fixing bacteria fix the atmospheric nitrogen to the more available form that can be easily accessible by plants. This fixation by PGPRs has a significant effect on the availability of nitrogen to plants by enhancing its growth.[22]

An AM fungus has ability to mobilize the inorganic form of phosphorus into simpler form, which helps in uptake of essential micronutrients and their indirect effect upon the nitrogen fixing system that plays an important role in plant growth enhancement. In plants that are colonized by AM fungi and PGPRs the rate of fixing nitrogen was found to be higher.[23,24]

Enhancement of Phosphorus Availability

Other feature of PGPRs is that they increase bioavailability of phosphorus. Bacteria involved in phosphate solubilising tend to release both organic and inorganic form of phosphate in soil, making it accessible to the AM fungi.[25] Organic form being unavailable to the plant, P solubilising bacteria and AM fungi convert it into inorganic state and its extraradical hyphae makes them available to the plants.[26] P solubilising bacteria does mineralize phosphate by secreting phosphatases. Hence, plants associated with both fungal and bacterial partner have shown to have greater biomass, together with P and N accumulation in comparison to plants grown in isolation.

Demands for Herbal Medicine

Plants being a rich source of bioactive compounds serve as a valuable lead for the novel drug design.[27] Gómez-Galera et al (2007), reported that till date at least 120 different chemical substances have been derived from more than 50,000 plant species that are used as important drugs for medicinal purpose.[28] Herbal molecules are safe and when in combined in the plant cell form suppress the effect of pathogenic microorganism.[29] Number of compounds has been identified from "ethnomedical" plant sources.[30] These immense properties of herbal molecules lead to increase in

demand of plant-based drugs creating a high load on selected medicinally important plants.

The growth of herbal drug is increasing in the world market from US $62 billion, which is estimated to increase by US $5 trillion in 2050.[31]

Based on a molecule of plant origin more than 25% new drugs have been approved in the past 30 years. New pharmaceuticals were inspired by natural product and were originating from natural products.[32,33,34] About 50% of sold chemicals used in pharmacy are derived from plant secondary metabolites.[32]

Plants as source of antimalarial drugs

Plants have been used as a source of medicine since ancient times. Herbal medicines and natural products in healthcare preparations have shown their origins from plants.[35] Use of herbal medicines has a widespread use mainly in the developing countries with about 80% of the population relying on them.[36] In Ethiopia, a huge acceptability of local healers and pharmacopeias and lesser access to modern medicine facilities has lowered the cost of traditional medicines.[37,38] Siddha, Unani and Ayurveda are practised in the Indian medicinal system since ages and have had wide acceptance.[39]

The pharmacological use of *A. annua*, a medicinal plant as a source of antimalarial drug began in ancient Chinese medicine for treatment of fevers.[40] *A. annua* as a source of artemisinin has served as a source of antimalarial drug discovery. Emergence of resistance to quinine has emphasized on alternative drug delivery systems against malaria. Currently, the artemisinin-based combination therapies (ACTs) provide the basis for the most effective treatment against both chloroquine-resistant and sensitive strains of *Plasmodium* sp. causing cerebral malaria.[31] Artemisinin was discovered in 1971 by a Chinese medical scientist Youyou Tu, recently in 2015 received the Nobel Prize in Physiology and Medicine for her discovery and establishment of new therapies to treat malaria. Tu discovered that cold, not hot, water was to be used for extraction of artemisinin. With this extraction method, the team obtained good, reproducible, biological activity. The first (modern) human subject to test qinghao in 1971 was Youyou Tu herself. Artemisinin was to be used in fixed-dose combinations, for its own protection, with other existing medicines, such as lumefantrine and amodiaquine, and, later, mefloquine, piperaquine, and pyronaridine.

***Artemisia annua* L. Taxonomy and Morphology**

It is originated from China and grows mainly in the Eastern, Southern and Middle parts of Europe and the Northern, Eastern and Middle parts of Asia. In India, it is cultivated in medium to low-temperature conditions in Kashmir valley, Himachal Pradesh and in Uttar Pradesh to a limited scale. *Artemisia annua* belongs to the plant family of *Asteraceae* and is an annual short-day plant[42]. Its stem is erected brownish in color. The plant is hairless and naturally grows from 30 to 100 cm tall. The leaves are 3–5 cm in length and are divided into two or three small leaflets. The flowers are small, green-yellowish in color having a diameter of 2–2.5 mm and are arranged in loose panicles. The seeds are brown with a diameter of only 0.6–0.8 mm.

A. annua, has become one of the most popular medicinal plants in the world in the past couple of decades. Many species of Asteraceae produce terpenoids and phenolic compounds which are of medicinal importance.[43] *A. annua* an aromatic annual herb was brought into attention because of the discovery of artemisinin that proved to be a potential anti-malarial agent. Furthermore, the use of combined artemisinin therapy against malaria is widely recommended to confront drug- resistant *P. falciparum* by WHO in different countries including Ethiopia. Currently many countries have therefore approved the use of artemisinin or aqueous extracts of *A. annua* to treat malaria. The aromatic oils (essential oils) and artemisinin can be sequestered from glandular trichomes which have been reported to be present on leaf surfaces and floral stalks.[44]

Chemical constituents of *A. annua*

In the Asteraceae family, it is known that glandular trichomes located on the aerial tissues of the plants are responsible for the storage of the terpenoid compounds produced by the plant. This family produces two main types of terpenoids which accumulate in the trichome: Aromatic volatile monoterpenes and non-volatile sesquiterpenes. The composition of essential oil of *A. annua* was determined among different genotypes.[45,46] Although the relative amount of each constituent tends to vary among different genotypes, the same chemicals are present throughout.[45] Abundant monoterpenes found in the oil include camphor, borneol, pinocarveol, artemisia ketone, artemisia alcohol, and 1, 8-cineole. Abundant sesquiterpenes include germacrene D, deltacadinene and artemisinic acid/dihydroartemisinic acid (possible artemisinin precursors). Artemisinin is the most important chemical in the aspect of medicinal applications, however, it is present in *A. annua* at a very low level (0.01-0.8% dry weight).[45,46]

Artemisinin

Artemisinin is a potent drug against *Plasmodium falciparum* that causes malaria.[47] The structure of artemisinin is complex with 1, 2, 4-trioxane that consists of three fused rings, one of the three rings having seven atoms. A peroxide bridge is also present in one of the rings. Reduction of this ring gives rise to a structural analogue deoxyartemisinin which has important antimalarial activity. Biological targets of artemisinin in the host parasite are an intermediate PfATPase6, SERCA Ca^{2+}-ATPase which gets inhibited.[48] Artemisinin interacts with the heme complex forming free radicals which are toxic for the parasite.[49]

Plasmodium the causative organism of malaria is carried by female Anopheles mosquito.[50] Malaria is a prevalent disease in the tropics affecting huge populations in Africa and India.[51] The treatment of malaria which was effectively administered was quinine, chloroquine, mefloquine, and primaquine until cases of resistance of this protozoon were registered against the drug.[52] The main cause of resistance was the genetic mutations caused in the malarial parasites.[53] The discovery of this important plant metabolite and its action mechanism envisioned combinational formulations to treat malaria.[54] Artemisinin-based Combination Therapies "ACT" have since been developed in combination with chloroquine and sulfdoxime/pyrimethamine[55] (Fig. 4). These therapies have shown to be highly effective against drug resistant malaria.[56]

Distribution of Artemisinin in *A. annua*

Artemisinin, an antimalarial drug present plant *A. annua* plant accumulate in leaves, main stem, lateral branches, roots, inflorescence and seeds.[57,58,59] It has been reported that the content of artemisinin more present in leaves and flower neither artemisinin nor its precursors were present in roots and pollens.[60,61] Due to the presence of floral remnants the occurrence of artemisinin was seen in the achene (seed). The concentration of artemisinin was changed over the time during plant moves from vegetative phase into flowering phase and also change in plant profile from top to bottom of the plant. According to some reports major concentration of artemisinin is present in flower but contradictory reports showed that higher artemisinin content in leaves compared to other plant parts.[62,63,64,65,66]

Biosynthesis of artemisinin occurred in special cell structures of *A. annua*. Biseriate glandular trichomes were present along both sides of the leaf midrib as well as on abaxial surfaces of the leaf and on the stem[67] (Fig. 5)

Although some authors reported artemisinin being highest during pre-flowering stage, others reported artemisinin reaching its peak during flowering.[59,62] At flowering stage, it was reported that the artemisinin content in *A. annua* plants grown under both green house and field condition reaches its peak. Charles et al., was reported that 89% of the total plant artemisinin was in leaves just prior to flowering and only about 10% in the lateral branches.[68] Distribution pattern of artemisinin was reported in different organs of plants containing 0.15% in the upper leaves, 0.04% in side shoots and 0.04% of artemisinin in seeds after flowering and trace amounts in the main stem and none in the roots.[59,68]

Apart from malaria artemisinin and its derivatives have shown to be effective against schistosomiasis disease caused by the protozoan *Schistosoma japnicum*, *S. mansoni* and *S. haematobium*. About 1.5 million disabilities have been estimated from this disease each year.[69] It has also shown effectiveness against other protozoan diseases like leishmaniasis.[54] Uses of artemisinin have also been seen in elimination of necrotic material from the body which are formed during ulcerative colitis, and Crohn's disease.[70] Artemisinin have also shown to be effective against hepatitis B and C viruses.[71,72]

Biosynthetic Pathway of Artemisinin

Artemisinin belongs to the terpenoid family. The primary precursor of terpenoids is isopentenyl diphosphate (IPP) and its isomer dimethyllallyl diphospahte (DMAPP). Two different biosynthetic pathways lead to the production of IPP, the mevalonic acid dependent (MVA) present in the cytosol or independent (MEP) pathway present in the plastid.[73] Mauji et al. showed that competitive inhibition of DXR, the rate limiting enzyme of MEP pathway has resulted into the reduced synthesis of 14.2% artemisinin as compared to 80.4% reduction in artemisinin synthesis when HMGR of the MVA pathway was inhibited by mevinolin, thus indicating that mevalonate pathway is the major contributor of carbon required for artemisinin biosynthesis.[74]

Condensation of the C5 substrate, DMAPP and IPP leads to the biosynthesis of C15 product, farnesyl diphosphate (FPP) via farnesyl diphosphate synthase (FPS). FPP can be converted through enzymatic catalysis to produce various isopreniods such as artemisinin and sterols. The first committed step in artemisinin biosynthesis is catalyzed by a sesquiterpene cyclase ADS to form cyclic sesquiterpenoid, amorpha-4, 11-diene in *A. annua* L. (Fig.6). A cytochrome P450, CYP71AV1 clone was characterized, catalyzing the oxidation of amorpha-4, 11-diene, artemisinic alcohol and artemisinic aldehyde.[75] Characterization of a double bond reductase, DBR2 with specificity for artemisinic aldehyde to form dihydroartemisinic aldehyde showed the presence of a reduction pathway in artemisinin biosynthesis.[76] Further oxidation of dihydroartemisinic aldehyde to dihydroartemisinic acid *via* an aldehyde dehydrogenase, Aldh1 was proposed.[77] Through substrate specificity and expression pattern analysis it was shown that Aldh1 had some activity on dihydroartemisinic aldehyde. RED1, a broad substrate oxidoreductase that converts dihydroartemisinic aldehyde to dihydroartemisinic alcohol acting as a competing metabolic pathway.

Though study of kinetic parameters has shown that Aldh1 more efficiently converts dihydroartemisinic aldehyde to dihydroartemisinic acid than Red1 that converts dihydroartemisinic aldehyde into dihydroartemisinic alcohol.[78] Finally, the formation of artemisinin occurs in a hypothesized non-enzymatic photo-oxidative step.

The level of artemisinin in *A. annua* is found to be very low (0.01-0.8 %), therefore, to increase the production of artemisinin and to meet its demand in the medical market a number of strategies can be adopted.[45]

CASE STUDY

Effect of *P. indica, A. chroococcum* in combination on growth of *A. annua* plant

When plantlets of *A. annua* was co-cultivated *in vitro* with *P. indica (*disc of 4mm size in diameter), *A. chroococcum* (cells 500 µl (10^9 CFU/ml) were found to produce higher number of leaves, increased shoot length, biomass per plantlet and artemisinin content after 6 weeks of culture (Table 1).70 % enhancement was observed in artemisinin content in leaves of *in vitro*, Poly house and field plants treated with microbial consortium of *P. indica* and *A. chroococcum* followed by 37 and 30% enhancement in leaves of *P. indica* and *A. chroococcum* alone treated plants.

RT -RCR Analysis

RT-PCR analyses revealed that when *A. annua* plants colonized with *P. indica* alone or in combination with *A. chroococcum* showed higher expression levels of both *hmgr* and *ads* genes, when compared with Control (Fig. 7 a & b).

When *A. annua* plants colonized with consortium of *P. indica* and *A. chroococcum* under *in vitro* conditions, poly house and field condition, it was observed that consortium not only improved the overall growth of the plant but also enhancing the artemisinin content. 70 % enhancement in artemisinin content was recorded in *A. annua* plants colonized with dual treatment (Table 1). In roots of colonized plants, the fungus synthesizes the precursors of artemisinin such as artemisinic acid and dihydroartemisinin which are subsequently translocated to the leaves for bioconversion into artemisinin possibly by following the process of photo-oxidation.[79] This could be the reason behind the enhancement of artemisinin in the *A. annua* plants treated with *P. indica*. The RT-PCR analyses showed that the expression of HMGR transcript and ADS was higher in leaves of plants inoculated with *P. indica* and *A. chroococcum* alone or in combination (Fig. 11 a and b).

Fitter and Garbaye, reported that rhizobacteria increased the capacity of AMF to colonize the roots of plants[24]. In rhizosphere of mycorrhizal plants increase and microbial population has showed by free living bacteria such as *Azotobacter* and *Azospirillum* species.[79] Similar results were found when combined application of AMF and PGPR increased growth and development in inoculated plants.[79]

In central Africa successful cultivation of hybrid form of *A. annua* containing 0.63 - 0.2% of artemisinin was reported.[80] Sharma et al (2013) also reported the significant increase in artemisinin content in shoots of plants when co-cultivated with *P. indica.*[81]

Legend for Figures

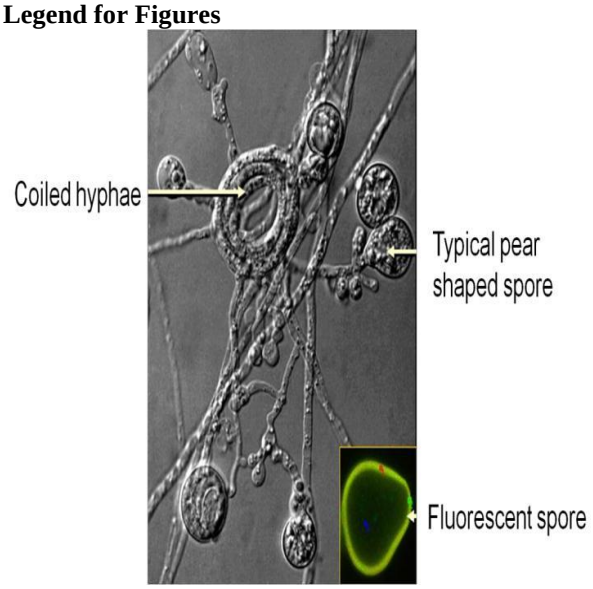

Fig. 1 An EM View of *Piriformospora indica* (Source: Prasad et al., 2008).

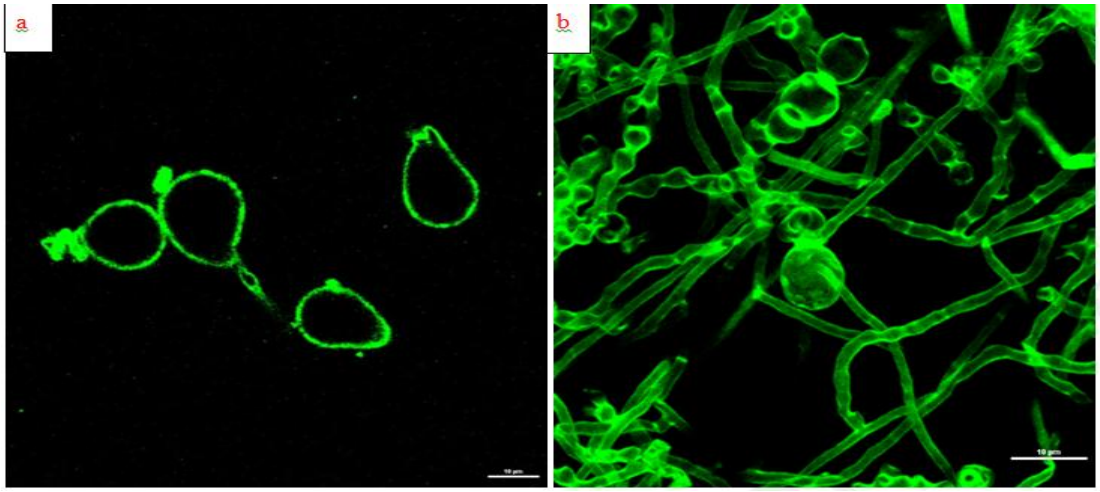

Fig. 2 a *P. indica* spores and b *P. indica* filaments, stained with WGA-Alexa 488, photograph by confocal microscope (Nikon, Confocal A1).

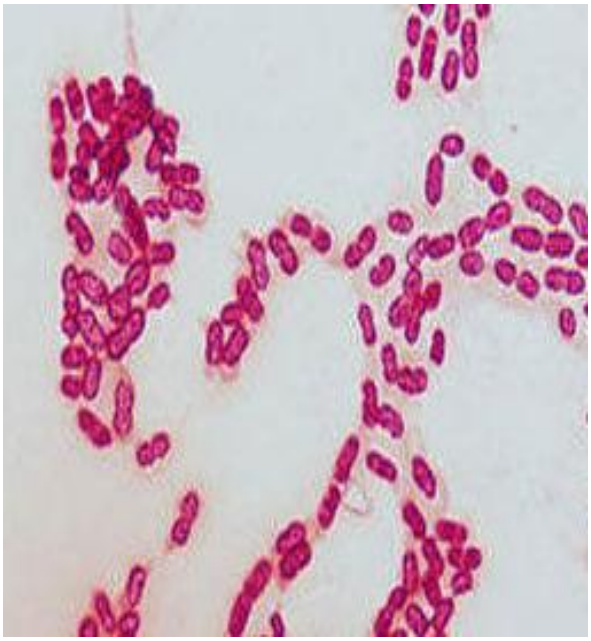

Fig. 3 *Azotobacter chroococcum* under microscope (100X).

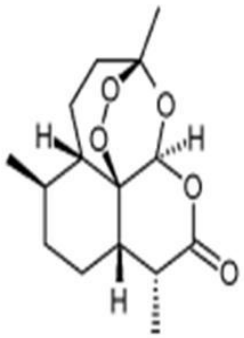

Fig. 4 Structure of Artemisinin (Source: Covello et al., 2007).

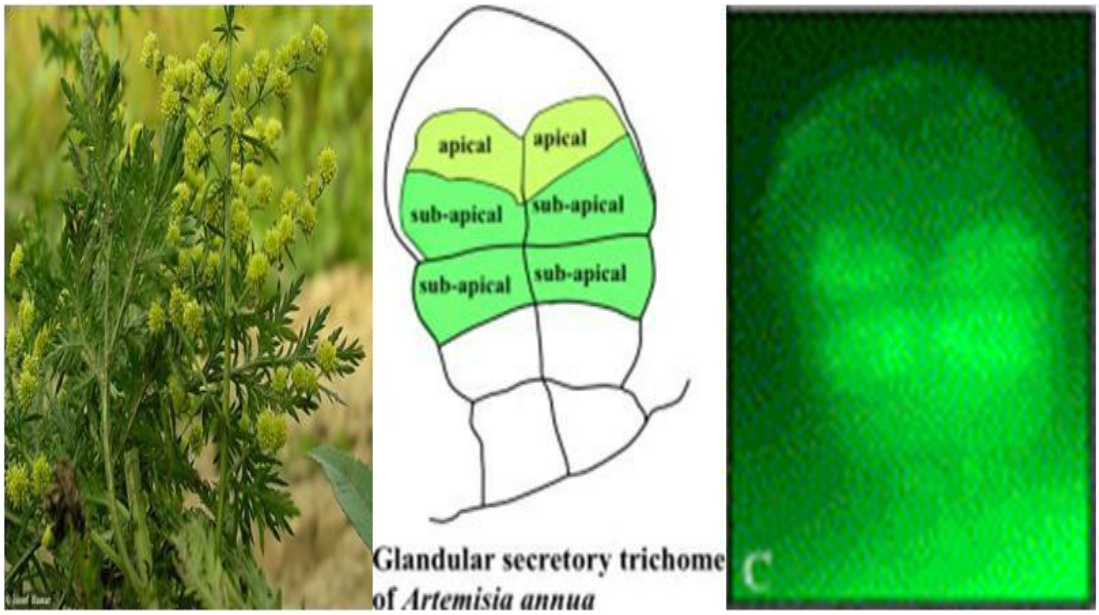

Fig. 5 Glandular that secret artemisinin in *A. annua* (Source: Arora 2013).

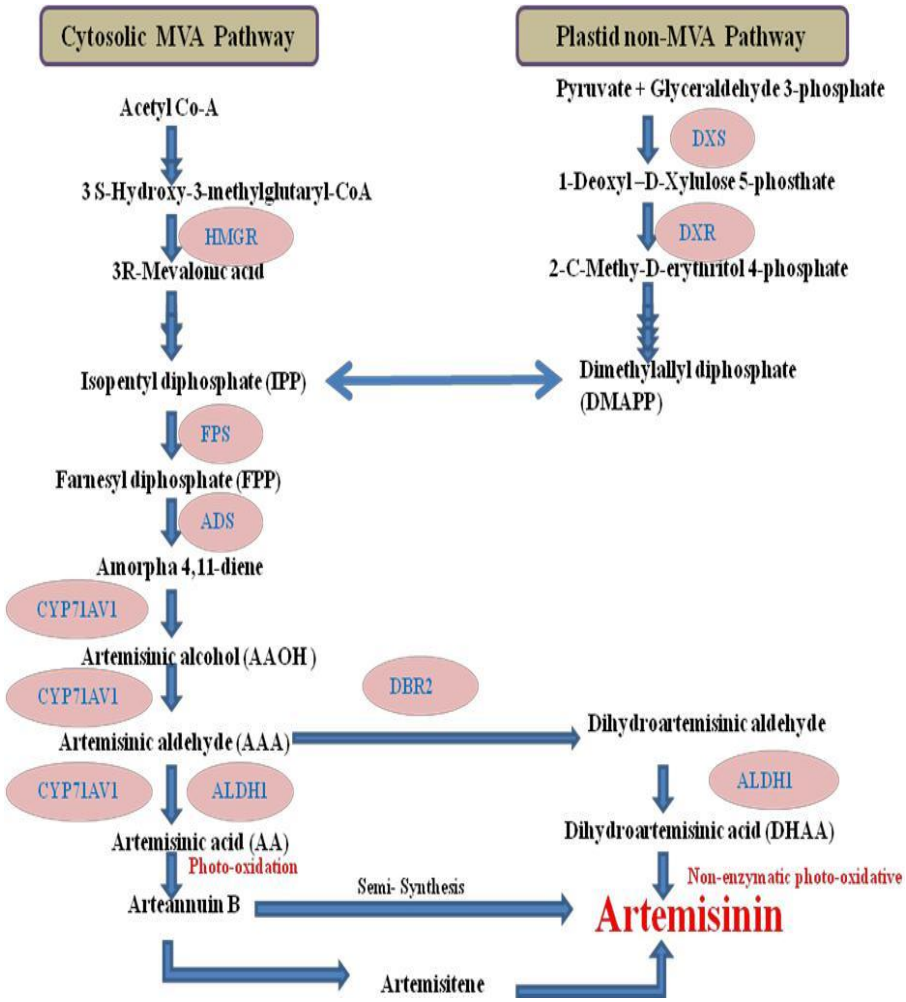

Fig. 6 Artemisinin biosynthesis by the cytosolic MVA pathway and the plastidal MEP pathway[75]

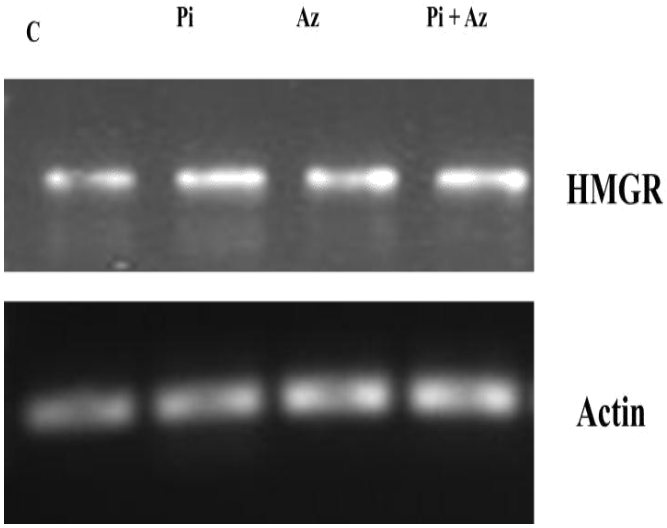

Fig. 7 a Reverse Transcriptase Polymerase chain reaction amplification of various transcripts using cDNA from leaves of *A. annua* plantlets co-cultivated with *P. indica* and *A. chroococcum* alone, and dual culture with both HMGR and Actin- specific primers (Arora; 2013).

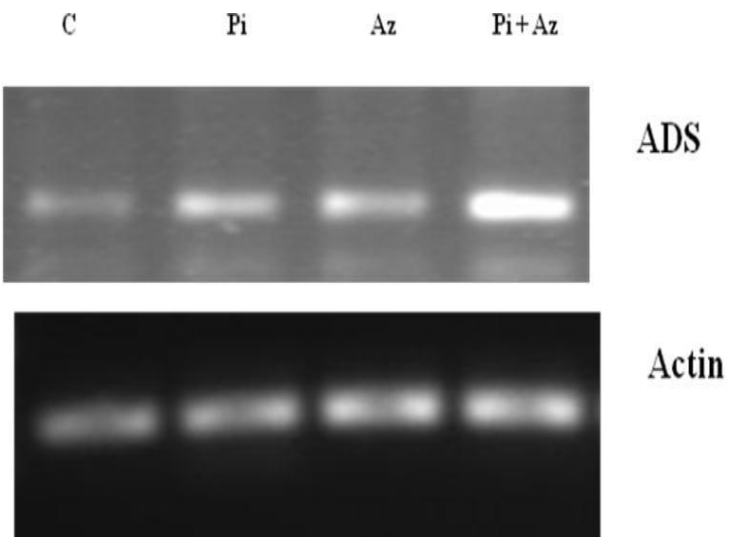

Fig. 7 b: Reverse Transcriptase Polymerase chain reaction amplification of various transcripts using cDNA from leaves of *A. annua* plantlets co-cultivated with *P. indica* and *A. chroococcum* alone, and dual culture. Agarose gels showing RT-PCR results for both ADS and Actin- specific primers (Arora; 2013).

Table 1: Evaluation of Artemisinin Content (%) in leaves of *A. annua* L. plants co-cultivated with *P. indica* /*A. chroococcum* alone and dual treatment under *in vitro*, Poly house and Field condition (Arora; 2013).

Treatments	Artemisinin Content (%)					
	In vitro	% enhancement	Poly house	% enhancement	Field	% enhancement
Control	0.08 ± 0.005	0.0	0.33 ± 0.006	0.0	0.45 ± 0.005	0.0
P. indica	0.11± 0.06	37.5	0.44 ± 0.03	33.33	0.62 ± 0.03	37.7
A. chroococcum	0.106 ± 0.005	32.49	0.399 ± 0.009	20.9	0.588± 0.02	30.6
***P. indica* +**	**0.136** ± **0.07**	**70**	**0.57** ± 0.005	**72.72**	**0.77** ± 0.003	**71.11**
A. chroococcum						

Data given is the mean value of the three replications

CONCLUSION

The overall conclusion drawn from the different environmental studies showed that consortium of *P. indica* and *A. chroococcum* not only improving the overall growth of the plant but also enhanced the artemisinin content. 70 % enhancement in artemisinin content was recorded in *A. annua* plants when colonized with dual treatment.

Future Prospective

In future, in-depth study of genes involved in biosynthetic pathway and their expression levels can be done by advanced quantitative techniques. Proteomic studies can be also carried out to check the effect of consortium on plants.

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Patent

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