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A review of various efforts to increase artemisinin and other antimalarial compounds in *Artemisia Annua* L plant

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Abstract: The antimalarial active compounds in *Artemisia annua* include artemisinin, flavonoids, and aromatic oils. Artemisinin is the main antimalarial compound in *A. annua*, it used in the formulation of artemisinin-based combined therapies used to treat malaria. Artemisinin is largely obtained from *A. annua* plant but the content in it is very low and its production commercially is not cost effective worldwide. Flavonoids have a synergistic effect with artemisinin against malaria and are partly responsible for the prophylactic effect of *A. annua* herbal tea. Essential oils from *A. annua* are effective mosquito repellents. Most attempts have been made to try to raise artemisinin content. However, few or none has been tried to increase the flavonoids and aromatic oils. This article presents a review of various efforts that have been carried out to increase these antimalarial compounds.

Subjects: Environment & Agriculture; Bioscience; Food Science & Technology

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PUBLIC INTEREST STATEMENT

Dried leaf and herbal tea of *Artemisia annua*, also known as sweet wormwood, sweet annie, sweet sagewort—taken as a whole in its natural form or extract thereof, are one of the new hopes for treatment and prevention of malaria poor yet malaria burden countries in the world. The presence and quantity of active compounds against malaria in the plant material is very important for its effectiveness. A number of studies have reported that the content of artemisinin in most *A. annua* preparations is lower than attainable in pharmaceutical product. This is believed to limit the effectiveness of these preparations. Thus, effort should be put in to increasing the content of all active compounds present in the plant. This review article discusses some of the efforts that can be applied to increase various active compounds in the plant.

Keywords: artemisinin; biofertilizers; hormones; aromatic oils; flavonoids

1. Introduction

The antimalarial active compounds in *A. annua* include artemisinin, flavonoids, and aromatic oils. Artemisinin in form of artemisinin-based combination therapies (ACTs) is recommended for treatment of malaria caused by resistant *Plasmodium* species (WHO, 2006). The flavonoids have been reported to have synergistic effect with artemisinin against malaria (Bilia, De Malgalhaes, Bergonzi, & Vincieri, 2006; Ferreira, Luthria, Sasaki, & Heyerick, 2010; Liu, Yang, Roberts, Elford, & Phillipson, 1992; Weathers & Towler, 2012) and are believed to be responsible for the malaria prophylactic effect of *Artemisia annua* herbal teas (Jansen, 2006; Ogwang et al., 2012). Lastly, the *A. annua* aromatic oil is a mosquito repellant (Engeu, Omujal, Agwaya, Kyakulaga, & Celestino, 2015). Generally, the amount of the above antimalarial compounds varies from one area to another. The content of artemisinin usually ranges from 0.01 to 2% dry weight (Cockram et al., 2012; Keshavarzi, Mousavi-Nik, & Abdin, 2012; WHO, 2006). The aromatic oils range from 0.02 to 0.5% and 0.04 to 1.9% on basis of fresh weight and dry weight, respectively (Bagchi, Haider, Dwivedi, Singh, & Naqvi, 2003; Damtew et al., 2011; Malik, Ahmad, Mir, Ali, & Abdin, 2009). The flavonoids range from 0.68 to 5.72% on the basis of dry weight (Bilia et al., 2006; Engeu et al., 2015). Various investigations have been carried out in vitro in the laboratory (using roots and shoots) and in the field to increase the content of antimalarial compounds especially artemisinin in *A. annua* plant.

2. Efforts to increase artemisinin content

2.1. Use of fertilizers

Fertilizers are materials that are added to soils and plant tissues to supply more nutrients needed for plant growth. They can be subdivided into chemical fertilizers, biofertilizers, vermicompost and organic fertilizers.

2.1.1. Chemical fertilizers

Chemical fertilizers are derived from inorganic materials which may be exclusively or partially synthesized and when added to the soil, they add nutrients to the soil and promote plant growth. Most chemical fertilizers are aimed at increasing nitrogen, phosphorus and potassium in the soil. Singh (2000) studied the effect of different levels of nitrogen, phosphorus and potassium (at 0, 50 and 100 kg/ha) and he did not see any effect with phosphorus and potassium but nitrogen (at 50 kg/ha) increased herbage. Many other authors (Davies et al., 2009; Lulie, Nigussie, & Chala, 2017; Omer, Abou Hussein, Hendawy, Ezz El-Din, & Gendy, 2014) have also reported an increase in artemisinin by increasing concentration in nitrogen (50–80 kg/ha). Increase in potassium has been reported to have either no effect on leaf artemisinin concentration (Davies et al., 2009; Singh, 2000) or decrease artemisinin percentage for example, Omer et al. (2014) reported the percentage of artemisinin as 0.471 and 0.448 at O and 25 kg K/ha, respectively. With reference to phosphorus, results of Aftab et al. (2014) and Lulie et al. (2017) were positive (artemisinin significantly increased) at 40 kg P/ha and 10 kg P/ha, respectively, but not like those reported by Singh (2000). Furthermore, Jha et al. (2011) reported that artemisinin content increased significantly at pre-flowering stage in plants treated with combined chemical fertilizers, that is, NPKS (nitrogen, phosphorus, potassium and sulphur) and NPK (nitrogen, phosphorus, potassium) than those not treated. Artemisinin in the leaf of the treated was 0.70% (NPKS) and 0.65% (NPK) while in the untreated was 0.55%. However, chemical fertilizers are non-biodegradable (ecofriendly), toxic and not cost-effective, thus their use is discouraged and biofertilizers are emerging as the efficient alternative (Hisamuddin, Akhtar, & Sharf, 2015; Kumar, 2014; Panwar, 2015).

2.1.2. Use of biofertilizers

Biofertilizers are also called microbial inoculants or bio inoculants (Sivasakthivelan & Saranraj, 2013). According to Kumar (2014), the term biofertilizer refers to formulations based on beneficial microbes and/or biological product that either fixes atmospheric nitrogen or enhances the solubility of soil nutrients and has potential to increase the yield of crops. Fungi that are used in biofertilizers have the ability to establish a symbiotic association with the plant roots forming what is referred to as mycorrhiza. Mycorrhiza help in nutrient recycling, increase nutrient (especially phosphorus) uptake and safeguard the plant from different forms of stress (Azcón-Aguilar & Barea, 1997; Rapparini, Llusià, & Peñuelas, 2007). This relationship is essential where one puts in little during cultivation but his gains are high. Mycorrhiza are divided into two depending on the plant tissues they colonise, that is, endomycorrhizal such as arbuscular mycorrhizal fungi (colonise intracellulary) and ectomycorrhizal fungi (colonise extracellulary). Most of the major plant families have the ability to form mycorrhiza and AMF is the commonest mycorrhizal type involved in agricultural systems (Azcón-Aguilar & Barea, 1997). In *A. annua* farming also AMF is the most applied however for most of them, their mechanism of action is not known. Mandal et al. (2014) reported that AMF (specifically *Rhizophagus intraradices*) enhanced production of secondary metabolites in shoots and in case of artemisinin, AMF increased glandular trichome density, jasmonic acid and transcriptional activation of artemisinin biosynthesis genes.

The common AMF that have been shown to increase artemisinin production include mainly those that belong to the genus *Glomus* such as *Glomus mosseae* (Awasthi et al., 2011; Rapparini et al., 2007), *Glomus macrocarpum* (Chaudhary, Kapoor, & Bhatnagar, 2008; Kapoor, Chaudhary, & Bhatnagar, 2007), *Glomus fasciculatum* (Awasthi et al., 2011; Chaudhary et al., 2008; Kapoor et al., 2007), *Glomus intraradices* (Awasthi et al., 2011; Rapparini et al., 2007), *Glomus viscosum* (Rapparini et al., 2007) *Glomus aggregatum* (Awasthi et al., 2011). In addition, also a mycorrhiza-like fungus *Piriformospora indica* (*P. indica*) increased artemisinin when co cultivated with *A. annua* shoots (Sharma & Agrawal, 2013) and with *A. annua* plant, (Arora, Saxena, Choudhary, Abdin, & Varma, 2016). The artemisinin in the leaves was about 0.33% in the control experiment and 0.45% in the plant inoculated with *P. indica*.

The common bacteria that have been used as biofertilizers to increase artemisinin content include *Azotobacter* such as *Azotobacter chroococcum* (Arora et al., 2016; Keshavarzi et al., 2012), *Azospirillum* (Keshavarzi et al., 2012), *Bacillus* (Keshavarzi et al., 2012) and *Pseudomonas* such as *Pseudomonas fluorescens* (Keshavarzi et al., 2012; Rapparini et al., 2007), *Bacillus subtilis* (Awasthi et al., 2011; Rapparini et al., 2007), *Streptomyces* spp., *Radiobacter* spp. (Rapparini et al., 2007), *Stenotrophomonas* spp. (Awasthi et al., 2011). These bacteria (particularly *Bacillus* and *Pseudomonas*) have the capability of dissolving insoluble phosphates (Keshavarzi et al., 2012). Synergism between fungal and bacterial species has been shown to highly increase artemisinin content than when single species are used (Arora et al., 2016; Awasthi et al., 2011; Rapparini et al., 2007). In Arora et al. (2016) experiments, they observed that artemisinin concentration in *A. annua* plants treated with a combination of *P. indica* and *A. chroococcum* was about 0.58% when compared with when each species was used alone, that is, 0.42% (*A. chroococcum*) and 0.45% (*P. indica*). Awasthi et al. (2011) reported that the artemisinin content observed when *G. mosseae* (Gm) and *B. subtilis* (Bs) were combined was higher than when each species was used alone, that is, Gm + B s (0.77%), Gm (0.061) and Bs (0.68).

2.1.3. Use of organic fertilizers and vermicompost
Organic fertilizers are fertilizers that are obtained from animal and plant waste. Few authors such as Jha et al. (2011) have reported the use of organic manure to increase artemisinin content. Furthermore, also the reports on the use of Vermicompost (formed from compositing action of earth worms) to increase artemisinin content are scarce yet when Keshavarzi et al. (2012) compared vermicompost with chemical fertilizers and various biofertilizers. Vermicompost (V) combined with chemical fertilizers was the most effective in increasing artemisinin content, that is, V + 80 kg N/ha + 80 P kg/ha) (0.334 μg/ml), V (0.27 μg/ml) and 80 kg N/ha) + 80 kg P/ha) (0.289 μg/ml).

2.2. Use of plant growth regulators
Plant growth regulators are substances that control plant growth. Examples of these hormones include cytokinins, gibberellic acid (GA), ethylene, auxins, abscisic acid, jasmonates etc. Reports on

the increase of artemisinin using hormones have employed a few of those above-mentioned examples. Long ago, in 1986, Liersch et al. reported that chlormequat increased artemisinin content by 30% when compared to the untreated plants and that the plant growth regulator had slight effects on the morphology of glandular trichomes. GA has been used by various authors and has been reported to be effective in increasing artemisinin production (Banyai, Mii, & Kanyaratt, 2011; Paniego & Giulietti, 1996; Woerdenbag et al., 1993). According to Banyai et al. (2011), GA causes a rise in the expression of the key enzymes involved in artemisinin yield. Furthermore, Aftab et al. (2011) applied methyl jasmonate (a methyl ester of jasmonic acid, MJ) and the artemisinin content was increased by 58.8% in methyl jasmonate-treated plants under boron toxicity of 1.00 mM, thus this hormone alleviated the toxicity. His observations with MJ were similar to those reported by Guo, Yang, Yang, and Zeng (2010) and Wang et al. (2010). Furthermore, Guo et al. (2010) reported that *A. annua* plants incubated with salicylic acid (50μM) gave rise to a higher yield of artemisinin (two-fold higher than the control). In another study, Aftab et al. (2014) observed that an irradiated growth promoting polysaccharide derived from brown algae (irradiated sodium alginate/ISA) enhanced artemisinin yield by 61.7% over the control at flowering stage when it was mixed with phosphorus (ISA 80 + P40).

2.3. Variation of growth conditions

The growth conditions (such as water, light and macro and micro nutrient) necessary for proper growth of *A. annua* plant have been varied to try to enhance artemisinin production. In his study, Ferreira (2007) varied macronutrients (nitrogen, phosphorus, and potassium) and observed that generally their absence reduced leaf (the main source of artemisinin) biomass accumulation but mild potassium deficiency significantly increased the concentration (g/100 g) of artemisinin. His results correlate with those of, Ayanoglu, Mert, and Kirici (2002) as the artemisinin content was not affected by the different low doses of the nitrogen (0, 6, 12 and 18 kg/da). However, when higher doses of nitrogen like 70–80 kg/ha (Magalhaes, Raharinaivo, & Delabays, 1996) and 120 kg/ha (Özgüven et al., 2008) are used, artemisinin content increases.

On the other hand, when Srivastava and Sharma (1990) varied micronutrients, they reported that deficiency in manganese, copper, iron, copper, and boron decreased artemisinin content by 25–30%. Among micronutrients, boron also has been studied by other authors. Srivastava and Sharma (1990) reported that boron deficiency inhibited flowering (one of the best times to extract artemisinin) and as a result artemisinin concentration decreased by approximately 50%. However, in 2010, Aftab et al. observed that artemisinin content increased when up to 1.0 mm of boron concentration was used (mild stress) but higher doses showed similar results as Srivastava and Sharma (1990). Furthermore, Han et al. (2010) and Li, Zhao, Guo, and Huang (2012) showed that Cadmium enhanced the accumulation of artemisinin and its precursors in *A. annua*. The mechanism for cadmium is that it up-regulates the relative expression levels of key enzyme genes involved in artemisinin biosynthesis (Zhou et al., 2016). Thus from these studies, more artemisinin is obtained if micronutrients are present in the right quantity.

The availability of water influences the accumulation of secondary metabolites. In a study conducted by Marchese, Ferreira, Rehder, and Rodrigues (2010), they observed that artemisinin content increased with water deficits of 38 and 62 h but decreased with water deficit of 86 h and proposed that moderate water deficit prior to harvesting the crop induces artemisinin accumulation. Their results correlated with what was reported by Charles, Simon, Shock, Feibert, and Smith (1993) when they studied the effect of water stress on artemisinin accumulation.

Light is vital for plant functioning, it provides energy for moving electrons during photosynthesis and it is a signal received by photoreceptors to regulate photomorphogenesis (growth, differentiation, and metabolism) (Wang, Zhang, Zhao, & Yuan, 2001). Light effects are influenced by its quality, duration and wavelength. Wang et al. (2001) compared the effect of various light wavelength (ranging from 385 nm to 790 nm) on artemisinin production in *A. annua* hairy roots and in descending order, that is, red (660 nm) > white > blue > yellow > green light. Thus, red light is the best option as it influences the enzymes that facilitate artemisinin production (Y. Wang et al., 2001).

2.4. Selection of high yielding strains/clones

Research has shown that various strains yield different artemisinin content. Artemisinin is obtained mainly from the leaves hence if the leaf yield is good also the artemisinin yield will be good. Poor leaf yield in a given strain is mainly influenced by three factors, that is, short plant structure, early flowering and short growth span (Singh, Vishwakarma, & Husain, 1988). A study carried out on three different strains obtained from Washington, Europe, and Kew Gardens (London) concluded that different strains had various stages at which artemisinin yield was maximum (European strain – 50% flowering while Washington strain- full flowering) (Singh et al., 1988). In relation to harvesting, in his patent (US 6,393,763 B1), Kumar et al. (2002) suggested multiple harvesting schedules. Furthermore, the content in a given strain may also be affected by the planting time (Ram, Gupta, Naqvi, & Kumar, 1997) and transplanting time (Kumar et al., 2002). Thus, to obtain excellent amounts of artemisinin, one should know the stage at which to harvest the plant and the time to plant/transplant. But there are also high-yielding cultivars or transgenic plants obtained from selection and crossing, in wild populations, of genotypes with high artemisinin concentration (Delabays, Simonnet, & Gaudin, 2001).

Furthermore, *in vitro* studies have shown that polyploidy affects the production of secondary metabolites, for example, hairy roots of *A. annua* that were tetraploid produced six times more artemisinin than those that were diploid (De Jesus-Gonzalez & Weathers, 2003). The same observation was also later reported in tetraploid *A. annua* plants by Lin et al. (2011). In their study, during the vegetative period, the average level of artemisinin in tetraploid *A. annua* plants increased from 39% to 56% than the level in the diploids. Lin et al. (2011) attributed the high artemisinin in the tetraploids to up-regulated expression of some key enzyme genes related to the biosynthesis pathway of artemisinin.

3. Efforts to increase aromatic oil content

Fertilizers have been used to increase the oil content of *A. annua*. Malik et al. (2009) used biofertilizers and they reported that untreated (control) plants and those treated with *Glomus* and *Azospirillum* yielded $0.28 \pm 0.04\%$, $0.30 \pm 0.03\%$ and $0.50 \pm 0.02\%$ essential oil on fresh weight basis respectively. On the other hand, various authors have used chemical fertilizers to increase the *A. annua* oil. Malik et al. (2009) reported that the essential oil from plants treated with basal N, P, K and S application and the untreated amounted to $0.32 \pm 0.03\%$ and $0.28 \pm 0.04\%$ of fresh weight, respectively. Singh (2000) also observed an increase in oil content, that is, 84.6, 76.26 and 60.42 kg/ha with 100, 50 and 0 kg/ha of nitrogen. Furthermore, also Ayanoglu et al. (2002), Özgüven et al. (2008) and Omer et al. (2014) reported an increase in oil content when nitrogen was applied at 12 kg/da, 80 kg N/ha and 75 kg N/fed, respectively. In relation to potassium, Singh (2000) applied 50 kg K/ha and oil content was not affected but with 25 kg K/fed, Omer et al. (2014) reported 3.49 ml of oil per plant compared to 2.35 ml per plant observed with the control. Lastly, in relation to phosphorus, Lulie et al. (2017) and Singh (2000) reported no significant increase in oil content with an increase in phosphorus concentration.

4. Conclusion

This review reveals that a lot of effort such as the use of plant growth regulators, chemical fertilizers, biofertilizers, organic fertilizers, use of high-yielding strains, etc. have been reported to increase artemisinin content. Thus, individuals interested in obtaining the highest artemisinin and aromatic oil content in *Artemisia annua* plants could apply any of those efforts. Furthermore, researchers can apply the above to increase flavonoids as these are vital in malaria prophylaxis activity of the herbal tea.

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