
REVIEW**VARIOUS APPROACHES FOR ENHANCING THE ARTEMISININ CONTENT AND AROMATIC OIL OF ARTEMISIA ANNUA L.****Shivani*, Dutt B. and Sood M.***Department of Forest Products, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan Himachal Pradesh. India. Pin-173230**Email: shivaniibains7@gmail.com**Received-06.01.2024, Revised-17.01.2024, Accepted-30.01.2024*

Abstract: The *Artemisia annua* L. plant is well-known for yielding "artemisinin," a crucial ingredient in the treatment of malaria. Artemisinin, isolated from *Artemisia annua* L., is potentially being effective against multidrug-resistant strains of the malarial parasite, *Plasmodium*. The essential oils of the plant work well to keep mosquitoes away. The majority of efforts have been focused on increasing the content of artemisinin. Since wild *Artemisia* spp. produce limited amount of this metabolite, the artemisinin-based drugs remain exorbitantly costly despite tremendous efforts. An overview of the several initiatives to enhance these antimalarial chemicals is provided in this article.

Keywords: *Artemisia annua*, Artemisinin, Biotechnological tools, Fertilizers, Phytohormones

INTRODUCTION

The genus *Artemisia*, consists of small herbs and shrubs. It is one of the largest and widely distributed genera of the Asteraceae family. Amongst various species of the genus, *Artemisia annua* L. (sweet Annie, annual wormwood, qinghao, huang hua hao) is an annual aromatic plant which luxuriant in growth, erect with bright green foliage and inflorescence of loose panicles. This antibacterial herb destroys malarial parasites, lowers fevers and checks bleeding (Ozguven *et al.*, 2008). The antimalarial active compounds in *A. annua* include artemisinin, flavonoids, and aromatic oils (Namul *et al.*, 2018). It is a traditional medicinal herb, native to china and widely cultivated in Asia, America and Europe (Verma *et al.*, 2011).

Artemisinin, a sesquiterpene lactone containing a peroxide bridge, is isolated from aerial parts of the *Artemisia annua* plants and its derivatives such as artemether, arteether, artesunate and dihydroartemisinin are found effective against multidrug resistant *Plasmodium falciparum* strains (Jha *et al.*, 2011 and Verma *et al.*, 2011). The foliage and inflorescence of *A. annua* plants also yield an essential oil, which has potential to be used in perfumery, cosmetics and aromatherapy and has been reported to possess antifungal and antimicrobial activities (Wright, 2002). The content of artemisinin usually ranges from 0.01 to 2% dry weight (Cockram *et al.*, 2012). The aromatic oils range from 0.02 to 0.5% and 0.04 to 1.9% on the basis of fresh weight and dry weight, respectively (Damtew *et al.*, 2011).

Artemisinin was discovered by Youyou Tu in 1972, for which she was awarded the Nobel prize in 2015. Her extensive studies revealed that artemisinin is a colorless crystalline compound with the molecular formula $C_{15}H_{22}O_5$ (Dai *et al.*, 2017) and a melting point of 156–157 °C. Artemether is recommended by WHO for cerebral malaria (Wyk and Wink, 2004). Artemisinin and its derivatives are recommended by World Health Organisation (WHO, 2005) in artemisinin based combination therapy (ACT), which led to increase in demand of artemisinin. Due to increase in demand for artemisinin derivatives, leading to supply shortages that are not likely to be met soon (Brisibe *et al.*, 2008).

Organic manure and chemical fertilizers are known to play an important role in many production oriented agricultural systems. The effect of organic fertilizer application maintains and improves physical, chemical and biological soil fertility and plant productivity (Kuntyastuti *et al.*, 2020). The response of crops to organic manures is initially slow even though they contain all the necessary plant nutrients. This is because it takes time for them to transform the inaccessible nutrients into available forms following application. However, the application of organic manures has to be encouraged because of the lasting and positive impacts on soil characteristics. Combining the use of organic and inorganic fertilizers can improve yields and protect the environment (Moyin- Jesu EI, 2007).

Approaches to enhance the artemisinin and aromatic oil yield Biofertilizers

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A biofertilizer is a substance which contains living microorganisms which when applied to seeds, plants or soil, colonizes the rhizosphere or the interior of the plants and promotes plant growth by increasing the supply of nutrients to the host plant (Malusa and Vassilev, 2014). They improve soil fertility by fixing the atmospheric nitrogen and solubilizing insoluble phosphates and produce plant growth-promoting substances in the soil (Mazid and Khan, 2015). Biofertilizers consists of Nitrogen fixing bacteria (*Azotobacter*, *Azospirillum*, *Rhizobium*, Blue-green algae (*Cyanobacteria*) and *Azolla*), Phosphate solubilizing bacteria (*Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Acetobacter*, *Flavobacterium*, and *Erwinia*) and *Mycorrhizae* (Mahanty *et al.*, 2016). Kapoor *et al.* (2007) reported that the inoculation by two arbuscular mycorrhizal (AM) fungi, *Glomus macrocarpum* and *Glomus fasciculatum*, either singly or in combination with P-fertilizer (*Bacillus subtilis*) significantly increased concentration of artemisinin in *Artemisia annua* plant (Awasthi *et al.*, 2011; Rapparini *et al.*, 2008). In Huang *et al.* (2011) experiment, they observed the AM fungi *Glomus mosseae* and *G. versiforme* also increase the artemisinin content in *A. annua* stem, branch, and leaf by 32.8%, 15.2%, and 19.6%, and 26.5%, 10.1%, and 14.9%, and the volatile oil content in leaf was increased by 45.0% and 25.0%, respectively, compared with the control. The glandular density of *A. annua* was significantly increased by AM fungi infection (Kapoor *et al.*, 2007).

In natural conditions, the mycorrhizae are surrounded by diverse bacterial communities throughout their life span (Perotto & Bonfante, 1997; Frey-Klett & Garbaye, 2005). These bacterial populations, the so-called “mycorrhiza helper bacteria”, that support the mycorrhiza-plant symbiosis on a physiological, metabolic, and functional level (Garbaye, 1994) and belong to many groups and genera such as *Pseudomonas*, *Bacillus* and *Streptomyces* (Frey-Klett and Garbaye, 2005). Appropriate combinations of mycorrhizal fungi with soil bacteria communities, as a multitrophic mycorrhizal complex, are indeed recommended for the improvement of plant performance. Arbuscular Mycorrhizae increased artemisinin levels by increasing glandular trichome density and transcriptional activation of artemisinin biosynthesis genes (Mandal *et al.*, 2014).

The potential use of the consortium *Piriformospora indica* and *Azotobacter chroococcum* in *A. annua* L resulted an increase of Plant height, total dry weight and leaf yield by 63.51, 52.61 and 79.70 % respectively (Arora *et al.*, 2016). *Piriformospora indica* is an endophytic mycorrhiza like fungus, isolated from desert soil of Rajasthan (Verma *et al.*, 1998). It plays a vital role, not only in improving the overall growth of *A. annua* in terms of shoot, root and total biomass but also in enhancing the

artemisinin content in the cultures colonized with *P. indica* (Sharma and Agrawal, 2013). *Artemisia annua* yields 0.30±0.03% of essential oil when treated with *Azospirillum* (2ml/plant) and amounted 0.50±0.02% essential oil on fresh weight basis when treated with *Glomus* (Mycorrhizal soil 50g/plant)(Malik *et al.*, 2009). Further they suggested that *A. annua* treated with *Glomus* lacks glandular trichomes- the primary sites of synthesis of monoterpenes.

Organic fertilizers

Organic fertilizers comprise a variety of plant-derived materials that range from fresh or dried plant material to animal manures and litters to agricultural by-products. It has been reported in earlier studies that the addition of organic matter improves soil properties such as aggregation, water holding capacity, hydraulic conductivity, bulk density, fertility and resistance to water and soil erosion (Ewulo, 2005). Moreover, organic manure may be helpful in conserving soil moisture and lowering the soil temperature. This helps in absorbing more water and nutrients by the plants from soil, leading to the production of higher plant biomass (Saxena and Singh, 1998).

Some author such as Sajwan *et al.* (2020) revealed that organic manure affect the essential oil composition of the flower of *Artemisia annua* cv. CIM Arogya. The combined treatment of vermicompost fertilizer (150 g/plant) with mycorrhizal (10 g/plant) showed the highest average fresh (90.11 g and 91.66 g) and dry (19.66 g and 21.58 g) weight of *Artemisia annua* plant (Gaibi *et al.*, 2019). The results presented by Malik *et al.* (2012), suggested that different organic amendments such as SSB (sewage sludge biosolid), CPM (composted sugarcane pressmud) and FYM (farmyard manure) increased the glandular trichome density in *A. annua* by increase in essential oil content.

Chemical fertilizers

Chemical fertilizers are synthetic substances that are applied to crops to provide essential nutrients for growth and productivity. They are widely used in modern agriculture to increase crop yields and meet the demands of a growing population. Overuse of chemical can cause a decline in soil fertility, reduce crop yields over time, and increase the risk of soil erosion and runoff (Phad, 2023).

Jha *et al.* (2011) revealed an increase in artemisinin content and artemisinin yield, when plant treated with NPKS (N₄₀₊₄₀, P₄₀, K₄₀, S₁₅₊₁₅ kg/ha; nitrogen, phosphorous, potassium and sulphur) at pre-flowering stage. Further in Ozguven *et al.* (2008) experiments, nitrogen doses (80 and 120) kg/ha enhances the artemisinin content (6.32 to 27.50 mg/100g) of dried leaves and also the essential oil content of *Artemisia annua* (Ayanoglu *et al.*, 2002; Omer *et al.*, 2014). Lulie *et al.* (2017) observed that, the maximum essential oil yield was obtained by the

interaction of nitrogen and phosphorous content at the rate of 46 kg/ha and 10 kg/ha. Further Malik *et al.* (2009), studied the amount of volatile oil increases to $0.32 \pm 0.03\%$ of fresh weight when treated with basal N,P,K and S application (in the form of urea, P_2O_5 and K_2O and gypsum at the rate of 120, 50, 100 and 50 kg/ha, respectively). N content (80mg/kg soil) augmented the artemisinin content (21.8%) and yield (55.8%). Davies *et al.* (2009) revealed that, nitrogen nutrition (106 mg /L) enhanced plant biomass production and artemisinin concentration. Increasing potassium application from 51 to 153 mg/L increased total plant biomass, but was no effect on leaf artemisinin concentration. Kapoor *et al.* (2007) studied that the application of phosphorous @ (at the rate of) 30 kg/ha significantly increase the artemisinin content. Singh (2000) reported significant increase in artemisinin yield by application of nitrogen @ 50 and 100 kg/ha. The highest value of artemisinin percentage (1.4 %) was observed in (60*60) cm planting distance with 50% chemical fertilization (8g urea, 24g calcium superphosphate, and 12g potassium sulphate/plant) + 50% of the organic compost (7.5 m³/ Fed) (Mohamed *et al.*, 2023).

Phytohormones

Phytohormones (PHs) play major role in regulation of various physiological and biochemical processes that govern plant growth and yield under optimal and stress conditions (Ayman *et al.*, 2022). There are some interesting studies suggesting that plant hormones can play important roles in altering root growth and secondary metabolism. At present, nine types of PHs have been identified (Su *et al.*, 2017), including auxins, the first phytohormone discovered (Darwin and Darwin, 1880), salicylates (SA), ethylene (ET), cytokinins (CKs), gibberellins (GAs), brassinosteroids (BRs), jasmonates (JA), abscisic acid (ABA), and strigolactones (SL), the last PHs to be discovered (Gomez-Roldan *et al.*, 2008) Smith *et al.* (1997) studied the effect of gibberellic acid (GA_3) on the growth and artemisinin production of hairy roots of *Artemisia annua*. They used six different concentration of GA_3 to determine the optimum concentrations of GA_3 levels of 0.01-0.001 mg/L (28.9-2.89 μ M) provided the significant increase in biomass and 0.01 mg/L (28.9 μ M) slightly increases the amount of artemisinin. Salicylic acid (SA) acts as a potential plant growth regulator and plays a major role in regulating a number of plant physiological and biochemical processes. Foliar application of salicylic acid at 1.00 mM increased the content and yield of artemisinin by 25.8 and 50.0%, respectively (Aftab *et al.*, 2010).

There are few studies on the effects of exogenous ethylene on hairy root cultures, possibly because ethylene is known to promote senescence and application of a gas is generally challenging. Fulzele *et al.* (1995) concluded that exogenous addition of ethylene to the culture medium also stimulated

terpenoid synthesis. Sa *et al.* (2001) reported that transgenic *A. annua* plants produced more artemisinin when endogenous cytokinins were increased via an ipt gene transfer. Weathers *et al.* (2005) studied the growth, development and production of the artemisinin in *Artemisia annua* hairy roots in response to the five main hormones: auxins, cytokinins, ethylene, gibberellins (GA), and abscisic acid (ABA). Bulk yields of biomass were inversely proportional to the concentration of each hormone tested. They concluded that, ABA yielded the highest amount of biomass. Both 6-benzylaminopurine and 2-isopentenyladenine inhibited root growth, however, only 2-isopentenyladenine stimulated artemisinin production. GA_3 had a significant positive effect on growth, physiological and biochemical parameters as well as on artemisinin content. The net photosynthetic rate, stomatal conductance and internal CO_2 were 26.2, 17.7 and 13.1% higher than control plants when 10^{-6} M GA_3 was applied. Overproduction of artemisinin content (23.9% more compared to control) was observed in plants when grown with 10^{-6} M GA_3 (Aftab *et al.*, 2011).

The phytohormone ABA is a phytohormone with the sesquiterpene structure, that plays important roles in several biological processes, such as senescence, seed germination and root elongation, as well as responses to cold, drought and salt stress (Zang *et al.*, 2013; Bi *et al.*, 2017; Sun *et al.*, 2018).

Kaminska, (2021) reported another group of growth regulators with hormone like properties namely; Jasmonates (JAs), which are widely distributed in plant tissues with high activity and growth rate, such as the stem tips, root tips, young leaves, flowers and unripe fruits. Jasmonic acid (JA) and its methyl ester (methyl jasmonates, MeJA) are linolenic acid (LA)-derived cyclopentanone-based compounds that belong to oxylipins (Creelman and Mullet, 1995). JAs do not work independently but work as a part of a complex signaling network with other phytohormones. MeJA is a signaling molecule belonging to the jasmonate family of plant hormones. It performs a variety of physiological roles, such as serving as a cellular transporter to activate specific defense-related genes. It elicits the production of compounds related to the plant's defense response. They often serve to elicit and stimulate the synthesis of secondary metabolites and are employed to trigger response during injury (Pauwels *et al.*, 2008). Many studies have investigated that MeJA can induce terpenoid synthesis. Wang *et al.* (2010) studied the effects of heterologous MeJA on the biosynthesis of artemisinin and several other terpenoid compounds. They concluded that 300 μ mol/L of MeJA could raise artemisinin content by 38% in 8 days. In Liu *et al.* (2023) experiment, resulted an *AaMYB108-like* gene which is a positive regulator induced by light and jasmonic acid for glandular secretory trichomes initiation in *A. annua*. These glandular secretory

trichomes (GSTs) secrete and store artemisinin on the leaves of *A. annua*. MYB (myeloblastosis) transcription factor, the *AaMYB108-like*, which is co-induced by light and jasmonic acid, and positively regulates GST initiation in *A. annua*. Overexpression of the *AaMYB108-like* gene in *A. annua* increased GST density and enhanced the artemisinin content, whereas anti-sense of the *AaMYB108-like* gene resulted in the reduction in GST density and artemisinin content.

Biotechnological approaches

Artemisinin was derived from the plant terpenoid biosynthesis pathway by using IPP (isopentenyl diphosphate) and its isomer, DMAPP (dimethylallyl diphosphate), as raw materials, which were produced by the MVA (mevalonate) pathway in the cytoplasm and the MEP (methylerythritol phosphate) pathway in the plastid. Derivatives of the MVA pathway were precursors to sesquiterpenes, triterpenes and polyterpenoids, while the MEP pathway was generally involved in the biosynthesis of monoterpenes, diterpenes and tetraterpenes (Mercke *et al.*, 2000). A precursor of artemisinin, farnesyl pyrophosphate (FPP, C₁₅), is synthesized from two molecules of C-5 isoprenoid units and one molecule of isoprenoid unit, which entered the downstream metabolic pathway of artemisinin biosynthesis (Towler and Weathers, 2007; Schramek *et al.*, 2010). FPP is cyclized to amorpha-4,11diene by amorpha-4, 11-diene synthase (ADS) Billa *et al.*, 2008; Ma *et al.*, 2008; Woerdenbag *et al.*, 1993) the precursor of artemisinin. The next step is the oxidation of amorpha-4, 11-diene to artemisinic alcohol by amorpha-4, 11-diene₁₂ monooxygenase (CYP71AV1). This enzyme also catalyzes the oxidation of artemisinic alcohol to artemisinic aldehyde and artemisinic acid (Teoh *et al.*, 2006; Ro *et al.*, 2006). Starting from artemisinic aldehyde, an additional branch emerged. Artemisinic aldehyde was catalyzed by DBR2 (artemisinic aldehyde Δ 11(13) reductase) to generate DHAAA (dihydroartemisinic aldehyde), and DHAAA was converted to DHAA (dihydroartemisinic acid) by ALDH1. Artemisinic acid and DHAA were thought to be the final compounds formed by the enzyme-catalyzed reactions in this pathway (Ikram and Simonsen, 2017; Al-Khayri *et al.* 2022). The next step of artemisinin biosynthesis is the reduction of artemisinic aldehyde into dihydroartemisinic aldehyde by artemisinic aldehyde D11(13) reductase (DBR2). Then, ALDH1 oxidizes dihydroartemisinic aldehyde into dihydroartemisinic acid, which is converted non-enzymatically into artemisinin (Zhao *et al.*, 2022).

Bioengineering approaches for enhancing artemisinin production

Overexpression of artemisinin biosynthetic genes results an improvement in artemisinin content of *A. annua* and blocking key genes in competitive pathways of artemisinin biosynthesis, or regulating

the expression of transcription factors involved in artemisinin biosynthesis.

Jain *et al.* (1996) reported that, the micro-cloned progenies of the *Artemisia annua* cv. Asha were tested for their growth, morphology and artemisinin yield and homology in respect to these characters with the respective parent selections and in vitro regeneration response to a micropropagation procedure using young inflorescence segments as explants. They concluded that the clones of selected plants yield 3.2- and 2.5-fold more artemisinin per plant as compared to the parent cultivar. Recently the hybrid of *A. annua* is generated from the combination of high-artemisinin-producing and vigorous cultivars to increase the production yield of artemisinin to more than 2 % dry weight (Al-Khayri *et al.*, 2022; Ikram and Simonsen, 2017; Zhao *et al.*, 2022). Jasmonic acid (JA) application induces artemisinin biosynthesis by increasing expression of AaMYC2, a transcription factor that binds to the DBR2 and CYP71AV1 promoters. Overexpression of AaMYC2 in *A. annua* significantly activated the transcript levels of CYP71AV1 and DBR2, which resulted in an increased artemisinin content and AaMYC2 suppression decreased artemisinin biosynthesis (Shen *et al.*, 2016). Yuan *et al.*, (2022) reported a novel ABA (Abscisic acid) and JA (Jasmonic acid) induced bHLH TF, AabHLH113, which positively enhance the artemisinin biosynthesis by directly binding to the promoters of artemisinin biosynthetic genes, DBR2 and ALDH1. The artemisinin and dihydroartemisinic acid content increased by 1.71- to 2.06-fold and 1.47- to 2.23-fold, respectively, in AabHLH113 overexpressed *A. annua*, whereas they decreased by 14-36% and 26-53%, respectively. Overexpression of AaGSWI in *A. annua* significantly improves artemisinin and dihydroartemisinic acid contents and AaGSWI can be directly regulated by AaMYC2 and AabZIPI1, which are positive regulators of jasmonate (JA)- and abscisic acid (ABA)-mediated artemisinin biosynthetic pathway (Chen *et al.*, 2016). Some other authors also suggested that, the overexpression of key terpenoid genes encoding for the enzymes IDI, FPS, HMGR, the plastid targeted DXR and HDR have increased production of arteannuin B and artemisinin significantly (some by 2 to 3 fold) in *A. annua* (Ma *et al.*, 2017). Ghassemi *et al.* (2021) showed that, the different concentration of chitosan nanoparticles could trigger the production of artemisinin metabolite.

CONCLUSION

This review emphasizes the various approaches, such as the use of bofertilizers, organic manures, plant growth regulators, chemical fertilizers, biotechnological tools, etc. to increase artemisinin content and aromatic oil in *Artemisia annua* plant. This article provides the individual with different

efforts to enhance the secondary metabolites and aromatic oil with latest literatures on a single platform.

AUTHOR CONTRIBUTIONS

All authors equally contributed to the preparation of the review, revised the text at different stages of the writing process and read and approved the current manuscript.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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