Tariq Aftab · Jorge F.S. Ferreira
M. Masroor A. Khan · M. Naeem *Editors*

Artemisia annua - Pharmacology and Biotechnology

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Contents

Chapter 10
Effect of Mineral Nutrition, Growth Regulators and Environmental Stresses on Biomass Production and Artemisinin Concentration of *Artemisia annua* L.

Tariq Aftab, M. Masroor A. Khan and J. F. S. Ferreira

Abstract Malaria is a mosquito-borne disease caused by different species of *Plasmodium*. It is the world's most severe parasitic infection and kills almost two million people a year, afflicting more than one-third of the global population. The burden of malaria has increased by the worldwide spread of multi-drug-resistant *Plasmodium falciparum*. *Artemisia annua* L. has been used for centuries in Chinese traditional medicine for the treatment for fever and malaria and is the only commercial source of artemisinin, a rare sesquiterpene lactone that is the only safe alternative therapy against multi-drug-resistant malaria. Because the chemical synthesis of artemisinin is very costly, the plant remains the only viable source of artemisinin for pharmacological use. Therefore, the enhanced production of artemisinin by the whole plant is highly desirable. Although artemisinin production a (*in planta*) is controlled mostly by genetic factors, the plant reacts to certain abiotic stresses by increasing artemisinin concentration. In the past 15 years, selection has increased artemisinin concentration in the plant from 0.3–0.5 % (g/100 g) to 1.0–1.8 %. However, artemisinin increase is still possible by applying selected stresses to the plant. In the present chapter, we are reviewing the various factors that affect biomass and artemisinin production of *A. annua*.

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10.1 Introduction

The most imperative concern of today is the success in achieving the highest possible level of human health and keeping diseases at bay in the global context. Of the several diseases of global concern, such as SARS, tuberculosis, influenza, HIV/AIDS, malaria is the one that causes the most losses in human lives. Malaria is an infectious disease caused by *Plasmodium falciparum*, a protozoan organism, which is carried by mosquitoes of the genus *Anopheles*. Worldwide, the most severe form of malaria is responsible for the incidence of 300–500 million clinical cases every year. Malaria is estimated to cause between 1.5 and 3 million deaths per year, mainly of African children under the age of 5 (Butler et al. 1997; Rinaldi 2004). In the past, the fight against malaria was based on two strategies:

1. Extermination of disease vector (mosquito) with DDT. This approach has undesirable side effects on the reproduction of birds and results in the persistent presence of pesticides in food chains. Also, although very effective when started, it also leads to changing behaviour of the vector to avoid surfaces treated with DDT.
2. Large-scale use of malaria drugs based on quinine (isolated from bark of *Cinchona*) and its cheap derivative chloroquine for the treatment for malaria patients. Unfortunately, *Plasmodium* spp. developed resistance to these drugs.

In 1960s, *P. falciparum* started showing signs of resistance against quinine-derived drugs. Such a resistance was reported from places as far apart as Brazil, Colombia, Malaysia, Cambodia and Vietnam, making it harder to control the disease. In addition, the mosquito species capable of transmitting the disease are found in many parts of the world (Fig. 10.1).

In 1969, the Chinese army found that the diethyl ether extract of *Artemisia annua* L., called "qinghao" in Chinese, had an excellent effect against malaria; in 1972, artemisinin (the bioactive sesquiterpene extracted from *A. annua* L.) was identified as the most active plant metabolite for malaria drugs (Klayman 1985). Artemisinin, responsible for antimalarial and anticancer activities, contains an endoperoxide bridge, which is rarely found in other secondary metabolites. It is a promising drug as it lacks cross-resistance with other antimalarial drugs and is known to have no adverse effects on humans, while providing fast clearance of blood parasites (24–48 h), compared to other available antimalarial drugs of malaria (Meshnick 2002). Complete chemical (*de novo*) synthesis of artemisinin has, so far, been achieved by several research groups (Ravindranathan et al. 1990; Avery et al. 1992). The procedure requires several steps and can start with different raw materials. However, because of low yield, complexity and high manufacturing cost, the isolation of artemisinin from *A. annua* is the most feasible and economic method for its commercial production. Since 2001, artemisinin-based combination therapies (ACTs) have been recommended by the World Health Organization (WHO 2010). This resulted in the enhanced demand for artemisinin that ultimately led to its supply shortages in the pharmaceutical markets (Cyranoski 2004).

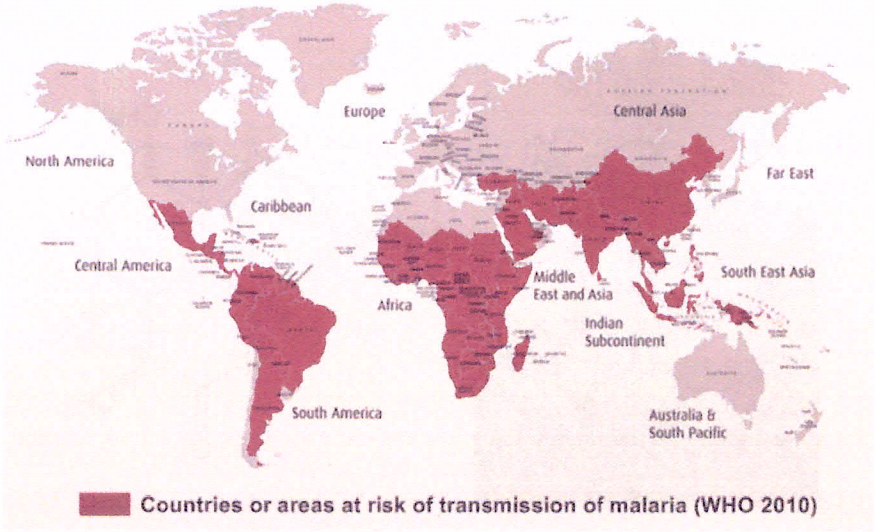

Fig. 10.1 Countries at risk of malaria

Although the situation has recently been reported to be under control (Roll Back Malaria 2004), it is still highly desirable to explore scientific methods to enhance the productivity of *A. annua* L. in conjunction with cutting short the manufacturing cost of artemisinin.

10.1.1 Artemisia annua

A. annua is a qualitative short-day plant (Ferreira et al. 1995a). The mature plant with a single stem can reach about 2 m in height. Aromatic leaves are about 2.5–5.0 cm long, deeply dissected and alternately branched around the stem (Fig. 10.2). At least 40 volatile compounds and several non-volatile compounds have been extracted from *A. annua* and identified. Artemisinin (Fig. 10.3) and other artemisinic compounds are the most important compounds isolated from this plant due to their pharmaceutical value (Ferreira and Janick 1996b). Artemisinin is stored in the glandular trichomes of *A. annua* and the glands of old leaves normally rupture open and release their stored materials, and thus, the artemisinin level in older leaves on whole plants is lower (Duke et al. 1994; Ferreira and Janick 1996b).

The major recommended use of artemisinin is for the production of artemisinin-based combination therapies (ACTs) in the treatment of malaria. ACTs have been shown to have rapid resolution to fever and parasitaemia and low toxicity and are well tolerated. The artemisinin compounds are effective against *P. falciparum* and *P. vivax*, including multi-drug-resistant strains. It has been recommended for use

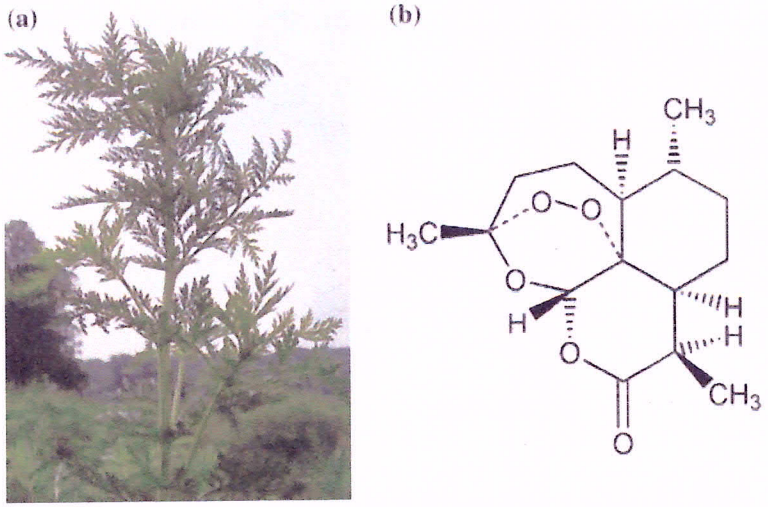

Fig. 10.2 **a** Vegetative top of *Artemisia annua* L. (*source* http://it.wikipedia.org/wiki/File:Artemisia_annua.jpg). **b** Chemical structure of artemisinin

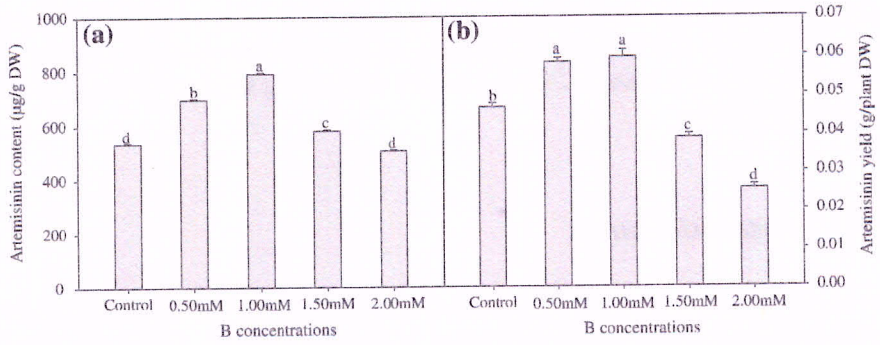

Fig. 10.3 Effect of different concentration of B on artemisinin content (**a**) and artemisinin yield (**b**) of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. *Error bars* (⊤) show SE

in herbal tea infusions for treatment of malaria. However, based on current knowledge, the recommendation of artemisinin teas is not acceptable by WHO as a replacement of ACTs.

Artemisia annua has several anecdotal uses that include contraception, relief of joint pains, deworming, haemorrhoids, antiperiodic, antiseptic, digestive and febrifuge; an infusion of the leaves is used internally to treat fevers, colds, diarrhoea, etc. These uses, however, could be explained by current research showing artemisinin and flavonoid effects as anti-inflammatory, immune modulation and anticancer effects in vitro and in dogs (Dr. Tomi Sazaki, pers. comm.). So far,

apart from the antimalarial effect, the only use in humans supported by controlled clinical trials is the effect on schistosomiasis (Utzinger et al. 2002). An essential oil in the leaves is used as flavouring in spirits such as vermouth (Duke et al. 1994).

10.2 Various Factors Affecting Biomass and Artemisinin Yield

Although biomass and artemisinin production in *A. annua* are mostly regulated by genetic factors, it is still unknown if artemisinin follows a Mendelian inheritance or if the genetic control of artemisinin is multi-allelic. The plants referred to as "hybrids" are not true hybrids because the parents are not proved to be homozygous for artemisinin production. It is known, however, that artemisinin is mostly controlled by genetic factors, as indicated by broad-sense (Ferreira et al. 1995b) and narrow-sense (Delabays et al. 2001) heritability studies. However, there are several environmental conditions (e.g. abiotic stresses) that affect plant biomass and artemisinin yield. Currently, we do not know the direct effects of drought, mineral deficiency, growth hormones or salinity (among others) on artemisinin biosynthesis and why abiotic stresses have been more successful in increasing artemisinin than biotic stresses. It is currently accepted that the artemisinin precursor dihydroartemisinic acid (DHAA) may act as a scavenger of reactive oxygen species (ROS) in the plant, then getting transformed into artemisinin. However, artemisinin is only one of 16 molecules that are formed from DHAA (Brown 2010), indicating that scavenging for reactive oxygen species is not the main function of DHAA. However, abiotic stresses have reported to increase artemisinin in the plant, and we provide detailed description of individual stresses that affect this valuable plant, as follows.

10.3 Effect of Mineral Nutrients

Singh (2000) conducted a field experiment to study the effect of levels of nitrogen, phosphorus and potassium on herb, oil and artemisinin yield of *A. annua*. Herbage, essential oil and artemisinin yields increased significantly with application of 50 kg N ha^{-1} compared to control (0 kg N ha^{-1}), but were statistically similar at 100 kg N ha^{-1}. Application of 50 and 100 kg N ha^{-1} increased herb, oil and artemisinin yields by 26.2 and 40.1 % compared with control.

Kapoor et al. (2007) studied 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*. Although there was significant increase in concentration of artemisinin in non-mycorrhizal P-fertilized plants as compared to control, the increase was less compared to mycorrhizal plants grown with or without P-fertilization. They suggested that the

increase in artemisinin concentration may not be entirely attributed to enhanced P-nutrition and improved growth. The plants supplied with AM fungi and P-fertilizer produced up to tenfold more shoot biomass than control plant. Also, AM fungi and P-fertilizer combined resulted in significant increases in concentrations of chlorophyll-*a*, chlorophyll-*b* and carotenoids compared to their respective controls. Artemisinin content was also maximum in the plants fertilized with P, and inoculated with AM fungi.

Özgüven et al. (2008) evaluated yield, yield components and artemisinin content of *A. annua* grown under four nitrogen applications (0, 40, 80 and 120 kg ha^{-1}) for two successive years. In their study, nitrogen doses had no significant effect on plant height, number of branches, fresh herbage yield, dry herbage yield, fresh leaf yield, dry leaf yield or essential oil content. However, artemisinin concentration of the dried leaves were significantly increased by nitrogen applications and ranged from 6.3 to 27.5 mg 100 g^{-1} among the treatments. Peyvandi et al. (2009) investigated the effects of different nitrogen and phosphorus on the plant growth parameters, yield and essential oil composition of *A. annua*. They observed that differences between the average height, number of branches and dry weight were significantly increased by the treatments. The maximum number of branches and plant height increased in $N_{80}P_{40}$ treatment. Increasing P-fertilizer more than 40 kg ha^{-1} decreased the growth parameters significantly. They did not observe the changes in artemisinin content due to the treatments. Davies et al. (2009) examined the effect of various concentrations of nitrogen and potassium on artemisinin concentration and yield responses of *A. annua*. The nutrients were supplied in irrigation water to plants in pots, and after a growth period, biomass production and leaf artemisinin concentration were measured. Nitrogen nutrition enhanced plant nitrogen concentration and biomass production successively up to 106 mg N L^{-1} for biomass and 206 mg N L^{-1} for leaf nitrogen; further increases in nitrogen had no influence on biomass. Artemisinin concentration in dried leaf material was maximum at a nitrogen application of 106 mg L^{-1}, but declined at higher concentrations. Increasing potassium application from 51 to 153 mg L^{-1} increased total plant biomass, but not at higher applications. Potassium application enhanced leaf potassium concentration, but there was no effect on leaf artemisinin concentration or leaf artemisinin yield. They suggested that maximization of artemisinin yield (amount per plant) requires optimization of plant biomass via control of nitrogen nutrition.

Aftab et al. (2011a) evaluated varying levels of soil-applied nitrogen with foliar GA_3. Application of GA_3 proved effective in increasing growth, photosynthesis and enzyme activities of *A. annua*. However, N levels combined with GA_3 led to further improvement in shoot lengths and dry weights, and photosynthetic rate. Furthermore, N combined (80 mg kg^{-1} soil) with GA_3 augmented the content and yield in the treated plants over the control (soil had 47.46 mg N kg^{-1}). Jha et al. (2011) assessed the effect of organic manure and chemical fertilizers on the accumulation of artemisinin and biomass in various plant parts through the developmental stages of *A. annua*. Phosphorus and potassium fertilizers (40 kg ha^{-1} each) were applied at the time of transplantation, while nitrogen and sulphur fertilizers at the rate of

80 and 30 kg ha^{-1} were applied in two equal splits, one at the time of transplantation and second at bolting stage, respectively. They found that artemisinin content and artemisinin yield of dried leaves increased significantly (27.3 and 53.6 %, respectively) at pre-flowering stage in the plants treated with NPKS and NPK (18.2 and 33.5 %, respectively) when compared with control. Maximum dry leaf yield ranged from 2,596 to 3,141 kg ha^{-1} at pre-flowering stage with various treatments.

The work of Davies et al. (2011) aimed to determine the response of *A. annua* through dry matter and artemisinin concentration in response to different levels of phosphorus (P) and boron (B). Mineral nutrients were supplied in irrigation water to potted plants, and after a period of growth, dry matter production and leaf artemisinin concentration were determined. Increases in P application enhanced plant growth and total dry matter production up to 30 mg P L^{-1}. Although P applications had no influence on leaf artemisinin concentration, optimal yields of artemisinin per plant were achieved at P rates from 30 to 60 mg L^{-1}, reflecting the increase in biomass caused by P applications. Increasing B application rate had no significant effect on dry matter production. Leaf artemisinin concentration significantly increased by 20 % (0.65–0.78 %) with B increases from 0.1 to 0.6 mg B L^{-1}. This increase in B also increased artemisinin from 0.36 to 0.46 g $plant^{-1}$ (28 %). Increasing B concentration to 0.9 mg L^{-1} had no further effect on artemisinin concentration or yield.

Thus, to our knowledge, little to no increase in artemisinin has been achieved by providing the plant with ideal (or surplus) rates of macro- and micronutrients. Ideally, a primary experiment should be done to establish the ideal mineral requirement for vegetative growth of *A. annua* in a certain soil. Then, the effect of providing additional nutrients could be evaluated against the ideal mineral fertilization previously established. That would establish if the increase in yield was indeed a result of additional fertilization instead of a response caused by feeding a starved plant with proper minerals. Next, an economical evaluation could establish the cost-effectiveness of the investment in extra fertilization versus the additional benefit of the achieved artemisinin yield increase. To date, we could find no such economic studies.

10.4 Effect of Plant Growth Regulators

Shukla et al. (1992) determined artemisinin and herbage yield of *A. annua* plants after application of triacontanol (Tria) and 2-chloroethyl trimethyl ammonium chloride (chlormequat). Tria produced a statistically significant increase on artemisinin concentration as well as on plant height, leaf and herbage yield. Chlormequat also increased artemisinin level, decreased the plant height at higher concentrations and increased the leaf and herbage yield at lower concentrations. They suggested that the effect of Tria on artemisinin yield seems to be mediated through GA- and ABA-like activities on plant growth. Smith et al. (1997) explored the effect of gibberellic acid (GA_3) on the growth and artemisinin production of

hairy roots of *A. annua*. They used six different concentrations of GA_3 to determine the optimum concentration. GA_3 levels of 0.01–0.001 mg L^{-1} (28.9–2.89 μM) provided the most significant increase in biomass and 0.01 mg L^{-1} (28.9 μM) produced the highest amount of artemisinin. They also studied growth kinetics and found that the use of GA_3 at 0.01 mg L^{-1} (28.9 μM) increased the growth rate of hairy roots of *A. annua* by 24.9 %. Wang et al. (2002) focused their research on artemisinin accumulation in hairy root cultures of *A. annua* by (22S, 23S)-homobrassinolide (SSHB). They observed that when 1 μg L^{-1} of SSHB was added to the hairy root cultures, the production of artemisinin reached 14 mg L^{-1}, an increment of 57 % over the control. Furthermore, artemisinin accumulation in hairy roots was found to be dose dependent as well in the treatment with SSHB. They also found that the SSHB treatments at 0.1–10 μg L^{-1} increased the root biomass up to 12–15 g L^{-1} from that of the control. However, if SSHB concentrations were higher than 100 μg L^{-1}, a decline in growth was detected with some root browning, an indication of the cell necrosis.

Studying root cultures, Weathers et al. (2005) focused on the effect of a broad range of phytohormones on growth and secondary metabolism of *A. annua*. They measured growth, development and production of the antimalarial drug, artemisinin, in *A. annua* hairy roots in response to the five main hormones: auxins, cytokinins, ethylene, gibberellins (GA) and abscisic acid (ABA). Single roots grown in six-well plates in medium B5 with 0.01 mg L^{-1} GA_3 produced the highest values overall in terms of the number of lateral roots, length of the primary root, lateral root tip density, total lateral root length and total root length. When the total root lengths were compared, the best conditions for stimulating root elongation was with 0.01 mg L^{-1} GA. Bulk yields of biomass were inversely proportional to the concentration of each hormone tested in their study. All root cultures provided with ABA yielded the highest amount of biomass. Both 6-benzylaminopurine and 2-isopentenyladenine inhibited root growth, however, only 2-isopentenyladenine stimulated artemisinin production, more than twice to that of the B5 controls, and more than any other hormone studied.

Regarding shoot cultures, Ferreira and Janick (1996a) used the growth regulators benzyladenine (BA), kinetin, chlormequat (CCC) and daminozide to induce shoot development. Shoot proliferation was increased by BA at 0.5 and 5.0 μM, but decreased root production at all concentrations. A highly significant correlation was observed between shoot artemisinin concentration and the number of roots ($r = 0.775^{**}$), while shoot number and artemisinin were unrelated ($r = -0.198$). These authors reported that the highest levels of shoot artemisinin (0.29 %) in shoot cultures were obtained with hormone-free medium (control), when root production was maximized. In the same study, removal of roots from shoot cultures grown in hormone-free medium reduced shoot artemisinin in 53 % and arteannuin B by 60 %, confirming the pivotal role of roots in artemisinin biosynthesis. The use of BA in shoot cultures, even at 0.5 μM, can cause vitrification and is not needed because shoot cultures grown in hormone-free medium produced artemisinin shoot concentrations at similar levels found in greenhouse and field-grown clones (Ferreira et al. 1995b).

The research of Pu et al. (2009) provided evidence that salicylic acid (SA) can activate artemisinin biosynthesis in *A. annua*. They observed that exogenous application of SA to *A. annua* leaves was followed by a burst of reactive oxygen species (ROS) and the conversion of dihydroartemisinic acid into artemisinin. Within 24 h from application, SA led to a gradual increase in the expression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*) gene and a temporary peak in the expression of the amorpha-4, 11-diene synthase (*ADS*) gene. However, the expression of the farnesyl diphosphate synthase (*FDS*) gene and that of the cytochrome P450 monooxygenase (*CYP71AV1*) gene changed little. At 96 h after SA (1.0 mM) treatment, the concentration of artemisinin, artemisinic acid and dihydroartemisinic acid were 54, 127 and 72 % higher than that of the control, respectively. On the basis of their results, they suggested that SA induces artemisinin biosynthesis in at least two ways: by increasing the conversion of dihydroartemisinic acid into artemisinin caused by a burst in ROS and by upregulating the expression of genes involved in artemisinin biosynthesis.

Jing et al. (2009) evaluated the effect of different concentrations of abscisic acid (ABA) on artemisinin concentration in *A. annua*, under tissue culture conditions. Artemisinin content in plants treated with 10 μM ABA was 65 % higher than that in control plants (1.1 % on a dry weight basis) and ranged from 1.5 % to 1.84 %. They also studied gene expression analysis and showed that in both ABA-treated plants and cell suspension cultures, the important genes in the artemisinin biosynthetic pathway, such as *HMGR*, *FPS*, *CYP71AV1* and *CPR* (cytochrome P450 reductase), were significantly induced. While only a slight increase in ADS expression was observed in ABA-treated plants, no expression of ADS was detected in cell suspension cultures. They suggested that there is probably a crosstalk between the ABA signalling pathway and artemisinin biosynthetic pathway and that *CYP71AV1*, which was induced most significantly, may play a key regulatory role in the artemisinin biosynthetic pathway. Aftab et al. (2010a) investigated the effects of foliar sprays of triacontanol (Tria) alone and in combination with gibberellic acid (GA_3) on growth attributes, photosynthesis, enzymatic activities, essential oil and artemisinin content and yield of *A. annua*. The results indicated that combination of Tria and GA_3 significantly increased activities of nitrate reductase and carbonic anhydrase by 25.9 and 21.5 %, and net photosynthetic rate, stomatal conductance and internal CO_2 by 25.4, 14.1 and 15.4 %, respectively, when compared to unsprayed plants. Combination of Tria and GA_3 also significantly enhanced artemisinin content and yield.

In a study by Banyai et al. (2011) involving cloned plants grown in mixed soil potting medium in a growth chamber, the production of artemisinin and leaf biomass in *A. annua* (varieties 007 and 253-2) was significantly increased by exogenous GA_3 applied to the soil. They also worked out the effects of GA_3 application on expression of key enzymes involved in artemisinin biosynthesis. They postulated that the increased artemisinin content (close to 1 % DW) from exogenous GA_3 treatment was associated with increased expression of key enzymes in the artemisinin biosynthesis pathway. Interestingly, exogenous GA_3 continuously enhanced artemisinin content from the vegetative stage to flower

initiation in both plant lines involved and gave significantly higher leaf biomass than in control plants. Consequently, the artemisinin yield in GA_3-treated plants was much higher than in control plants. In their work, although the maximum artemisinin content was found at the full blooming stage, the highest artemisinin yield in GA_3-treated plants was obtained during the flower initiation stage. This was 26.3 and 27.8 % higher, respectively, than in non-treated plants 007 and 253-2.

Wang et al. (2010) reported the effects of exogenous foliar methyl jasmonate (MJ) on artemisinin biosynthesis and secondary metabolites in *A. annua* under greenhouse conditions. They found a 49 % increase in artemisinin concentration on day 8 after treatment with MJ, associated with an 80 % increase in artemisinic acid and 28 % in dihydroartemisinic acid, the latter currently accepted as being the main precursor of artemisinin. In addition, they also worked out some other secondary metabolites using metabolite profiling after exogenous methyl jasmonate treatment. Their content also changed significantly after MJ treatment, including a 50 % increase in methyl artemisinic acid, a 67 % increase in squalene and a 60 % increase in an unidentified sesquiterpenoid. They argued that these compounds may be promising targets for further studies on artemisinin biosynthesis.

Aftab et al. (2010b) showed that salicylic acid (SA) acts as a potential plant growth promoter and plays an important role in regulating a number of plant physiological and biochemical processes under field conditions. Four levels of SA (0, 0.25, 0.50 and 1.00 mM SA) were applied in the form of diluted aqueous sprays on the aboveground plant parts in that study. Plant height, dry weight, chlorophyll and carotenoid contents were improved significantly as the level of SA increased. Furthermore, significant enhancement in net photosynthetic rate (31.7 %), the activity of nitrate reductase (17.2 %) and carbonic anhydrase (10.9 %) was noticed as the level of SA application was increased from 0 to 1.00 mM SA. Most importantly, the content and yield of artemisinin were increased by 25.8 and 50.0 %, respectively, after treatment with SA.

10.5 Effect of Environmental Stresses

Prasad et al. (1998) observed the effect of soil salinity (mixture of cations and anions) on the growth, yield, mineral composition and artemisinin concentration of *A. annua* cultivar from Kew, England. They noticed that plant height decreased as salinity stress increased, and the leaf-to-stem ratio was generally increased in salinized plants as compared with control plants not subjected to salinity stress. The vegetative yield of shoots increased significantly with increasing salinity stress up to 6.0 dS/m, but further increases in salinity decreased shoot yield. Artemisinin content in vegetative tissue was around 0.01 % (g/100 g dry weight) and was not influenced by salinity levels from 0.9 to 10.4 dS/m, but decreased to 0.006 % at 14 dS/m. The concentration of nitrogen was significantly higher, and the concentrations of phosphorus and calcium were lower in plants subjected to salinity stress. Potassium concentration and the potassium-to-sodium ratio in

shoots decreased, while the sodium and magnesium concentrations increased with salinity stress to 6.0 dS/m. The potassium-to-sodium ratio was significantly and negatively correlated with the dry weight of shoot.

Qureshi et al. (2005) reported the effects of NaCl (0–160 mM) and lead acetate (0–500 μM) on 90 (S1 treatment)- and 120-day-old (S2 treatment) *A. annua* plants. Treated plants were evaluated for lipid peroxidation rate, photosynthetic rate (Pn), chlorophyll content, artemisinin concentration and artemisinin yield, and for total biomass accumulation, through leaf samples, at 100, 130 and 160 days after sowing (DAS) in S1, and at 130 and 160 DAS in S2 treatments. Treatments enhanced lipid peroxidation at all stages of plant growth and increased the concentration and yield of artemisinin at 100 and 130 DAS in S1 and S2, respectively, while other parameters declined at all growth stages. The magnitude of changes was greater in lead-treated than in salt-treated plants. Both treatments induced oxidative stress, which might have damaged the photosynthetic apparatus resulting in a loss of chlorophyll content and a decline in photosynthetic rate, biomass accumulation and artemisinin production. They postulated that increase in artemisinin content, observed during the early phase of plant growth, might be due to a sudden conversion of artemisinin precursors into artemisinin by activated oxygen species (ROS) under oxidative stress. However, no work so far has quantified ROS in *A. annua* or directly correlated artemisinin increase with increased ROS in response to abiotic stress.

Ferreira (2007) reported that a cloned, greenhouse-grown *A. annua* (Artemis) subjected to an acidic soil and macronutrient deficit was evaluated for artemisinin production. Lack of lime (L) and macronutrients (N, P and K) reduced leaf biomass accumulation. When L was provided, the highest average leaf biomass was achieved with the "complete" (+N, +P, +K and +L) treatment, and the least biomass was achieved with the untreated (−N, −P, −K and −L) treatment. The macronutrient least required for biomass accumulation per plant was K (49.0 g), followed by P (36.5 g) and N (14.3 g). The artemisinin concentration (g/100 g) was significantly higher (75.5 %) in −K plants when compared to plants under the complete treatment. Although the artemisinin total yield was 21 % higher in −K plants, it was not significantly different from plants under the complete treatment, due to the lower biomass accumulation caused by deficiency of K. There were no marked treatment effects for concentration or yield of both dihydroartemisinic acid and artemisinic acid, although higher levels were achieved in plants under the complete or −K treatments. There was a positive and significant correlation between artemisinin and both artemisinic acid and dihydroartemisinic acid, in g/100 g and g/plant. This is the first report where potassium deficiency significantly increases the concentration (%) of artemisinin. These results were confirmed with a different set of cloned Artemis plants under the same GH conditions, same macronutrient deficiencies and same soil, but with the addition, or lack, of tannic acid to soils. Potassium deficiency (regardless the addition or lack of tannic acid) significantly increased both artemisinin concentration and yield (Ritchey and Ferreira unpublished).

A. annua plants were cultivated by Marchese et al. (2010) in growth chambers and submitted to five water deficit treatments. Water deficits of 38 and 62 h increased leaf artemisinin content, but only 38 h led to a significant increase in both leaf and plant artemisinin, with no detriment to plant biomass production. The other treatments had no effect on, or decreased artemisinin accumulation. *A. annua* plants tolerated well water deficit treatments, including the most severe water deficit applied and recovered their turgor pressure after rehydration. They concluded that moderate water deficit prior to harvesting the crop may not only reduce time and costs in drying the crop, but can also induce artemisinin accumulation, both of which increase crop profit margins.

Aftab et al. (2010c) studied the effect of increasing levels of boron (B) on oxidative stress, antioxidant defence response and changes in artemisinin content in *A. annua*. Toxicity caused by B reduced growth parameters such as stem height, fresh weight and dry weight. Treatments induced oxidative stress resulting in lower net photosynthetic rate, stomatal conductance, internal CO_2 and total chlorophyll content. The increased activities of antioxidant enzymes like CAT (catalase), POX (peroxidase) and SOD (superoxide dismutase) were also noted in response to increasing levels of B stress. However, H_2O_2 and artemisinin content were found to be high up to 1.00 mM concentration of boron compared to control, and on applying higher doses, further reduced contents were obtained. Their results suggest that a mild stress of B can be utilized to enhance artemisinin production.

The role of salicylic acid (SA) in inducing salinity tolerance was studied in *A. annua* L., by Aftab et al. (2011b). When applied to leaves at 1.0 mM, SA provided considerable protection against salt stress imposed by 50, 100 or 200 mM NaCl to soil. Salt stress negatively affected plant growth as assessed by length and dry weight of shoots and roots. Salinity also reduced the values of photosynthetic attributes and total chlorophyll content and inhibited the activities of nitrate reductase and carbonic anhydrase. Furthermore, salt stress significantly increased electrolyte leakage and proline content. Salt stress also induced oxidative stress as indicated by the elevated levels of lipid peroxidation compared to the control. A foliar spray of SA at 1.0 mM promoted the growth of plants, independent of salinity level. The activity of antioxidant enzymes, namely CAT, POX and SOD, was upregulated by salt stress and was further enhanced by SA treatment. Artemisinin concentration increased at 50 and 100 mM NaCl but decreased at 200 mM NaCl. The application of SA further enhanced artemisinin concentration when applied with 50 and 100 mM NaCl by 18.3 and 52.4 %, respectively. Their results indicate that moderate saline conditions can be exploited to obtain higher artemisinin content in *A. annua* plants, whereas the application of SA can be used to maintain plant growth and induce its antioxidant defence system under salt stress (Fig. 10.4).

Aftab et al. (2011b) conducted a study to determine whether the exogenous application of methyl jasmonate (MJ) on *A. annua* could counteract the ill effects of excessive B present in the soil. According to the results obtained, B toxicity induced oxidative stress and reduced stem height, as well as fresh and dry masses of the plant significantly. The excessive amounts of soil B also lowered the net

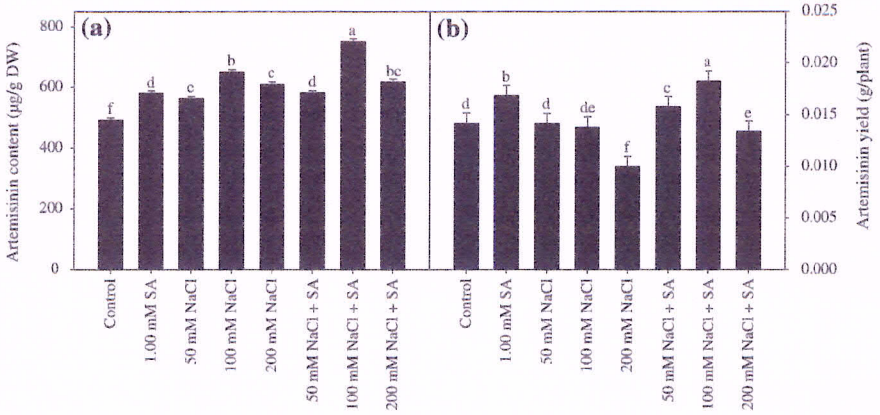

Fig. 10.4 Effect of SA and different concentrations of salinity on artemisinin content (**a**) and artemisinin yield (**b**) of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. *Error bars* (⊤) show SE

photosynthetic rate, stomatal conductance, internal CO_2 concentration and total chlorophyll content in the leaves. In contrast, the foliar application of MJ enhanced the growth and photosynthetic efficiency in both the stressed and non-stressed plants. The excessive B levels also increased the activities of antioxidant enzymes, such as CAT, POX and SOD. Endogenous H_2O_2 and O_2^- levels were also high in stressed plants. However, MJ application on stressed plants reduced the amount of lipid peroxidation and stimulated the synthesis of antioxidant enzymes, enhancing the concentration and yield of artemisinin as well. They concluded that MJ might be utilized in mitigating B toxicity and improving both concentration and yield of artemisinin in *A. annua*.

Aftab et al. (2012) reported the effects of B and aluminium (Al) contamination in soil, carried out with or without application of exogenous sodium nitroprusside or SNP (a nitric oxide donor), on various plant processes in *A. annua*, including changes in artemisinin content. The addition of B or Al to soil significantly reduced the yield and growth of plants and lowered the values of net photosynthetic rate, stomatal conductance, internal CO_2 concentration and total chlorophyll content. The follow-up treatment of NO donor favoured growth and improved the photosynthetic efficiency in stressed and non-stressed plants. Artemisinin content was enhanced by 24.6 and 43.8 % at 1.0 mol of soil-applied B or Al. When SNP was applied at 2.0 mol concentration with either 1.0 mol of B and/or Al, it further stimulated artemisinin biosynthesis compared to the control. Application of B + Al + SNP proved to be the best treatment combination to increase artemisinin concentration in *A. annua* leaves.

Although we cannot safely conclude that *A. annua* responds to stress by using dihydroartemisinic acid as a scavenger of ROS, the response of the plant to SA, MJ, GA_3, B, K deficiency, water stress, etc., all seem to cause slight to significant

increases in artemisinin biosynthesis. These responses, associated with the reports that rootless shoot cultures have traces of artemisinin and that well-fed crops increase biomass but no artemisinin indicate that, although roots do not seem to be a site of artemisinin accumulation (as glandular trichomes are), artemisinin could be part of *A. annua* chemical system of response abiotic stresses sensed by the roots. Although we found one study that reports artemisinin increase after frost, there are no other studies confirming such effect of low temperatures, what would obfuscate the idea that roots are an important organ that senses stress and triggers increased artemisinin biosynthesis.

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