

Review

## New cultivation approaches of *Artemisia annua* L. for a sustainable production of the antimalarial drug artemisinin

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*Artemisia annua* L. is an important medicinal plant from which artemisinin was extracted to treat malaria effectively. Artemisinin is isolated from the aerial part of the *A. annua* harvesting from the wild is causing loss of genetic diversity and habitat destruction. The use of controlled environments can overcome cultivation difficulties and could be a means to manipulate phenotypic variation in bioactive compounds and toxins. The aim of this review is to bring together most of the available scientific research papers about the cultivation and breeding conducted on the genus *A. annua*, which is currently scattered across various publications. Through this review the authors hope to attract the attention of all agronomist and breeders throughout the world to focus on the unexplored potential of *A. annua* species. Also, the future scope of this plant has been emphasized with a view of the importance of cultivation of *A. annua* for increasing of artemisinin content. New cultivation approach of *A. annua* offers the opportunity to optimize yield and achieve a uniform, high quality product.

**Key words:** *Artemisia annua*, breeding, artemisinin, cultivation, medicinal plant.

### INTRODUCTION

Since artemisinin was discovered as the active component of *A. annua* in early 1970s, hundreds of papers have focused on the anti-parasitic effects of artemisinin and its production (Bhattarai et al., 2007; Efferth et al., 2002; Singh and Lai, 2004; Utzinger and Keiser, 2004; Romero et al., 2005). The recent works of several groups show that artemisinin is currently the most effective malaria drug and is the World Health Organization's currently recommended medicine (Bhattarai et al., 2007)

in treating malaria. Artemisinin also shows promise as a potential therapeutic agent for other parasitic and viral diseases as well as for the treatment of certain cancers and the reduction of angiogenesis (Singh and Lai, 2004; Utzinger and Keiser, 2004; Romero et al., 2005). *A. annua* still remain one of the major sources of drug in the traditional and modern system of medicine throughout the world. Harvesting from the main source of raw material of *A. annua* is causing loss of genetic diversity and habitat

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and habitat destruction. Cultivation of this plant is a viable alternative and can overcome cultivation difficulties of it. At the present time, in the 21st century, malaria is still severely challenging people's health. Each year, more than one million people around the globe die of malaria and more than two billion people in over 100 countries are threatened by the disease (Bhattarai et al., 2007; WHO, 2008). In many developing countries, especially those in Africa, the mortality from malaria are still very high. The great majority of these are in children under the age of five years. Therefore, the world market for products including artemisinin derivatives is now growing rapidly, and the demand for artemisinin is increasing. At present, artemisinin compounds are derived from a raw substance extracted from the plant *A. annua* because artemisinin is very difficult to synthesize (Klayman, 1985; Singh et al., 1988). Enhanced production of artemisinin in the whole plant of *A. annua* is therefore highly desirable.

Many researchers have reported that the yields of extracted artemisinin is very poor (Singh et al., 1988; Charles et al., 1990) and much effort must be made to increase artemisinin content of *A. annua*. Because the plant material in wild stands is typically variable in its artemisinin content and plant biomass and this has an impact on drug extraction. Efforts are being made to increase its production in many ways such as plant tissue culture and biotechnological and agronomical practices (Jaziri et al., 1995). But artemisinin was not found to be accumulated in callus and cell suspension cultures, it is presumed that the biosynthesis of artemisinin to be restricted to the green part of the plant (Fulzele et al., 1991). At the present time, the biotechnological approach for the production of artemisinin remains disappointing, and the molecule must therefore still be extracted from *A. annua* plant grown outdoors.

## GENUS *ARTEMISIA* AND GEOGRAPHICAL DISTRIBUTION OF *A. ANNUA*

The genus *Artemisia* belongs to a useful group of aromatic and medicinal plants. It is one of the largest and most widely distributed genera of the family *Astraceae* comprised over 450 diverse species. These species are perennial, biennial and annual herbs or small shrubs (Watson et al., 2002; Iranshahi et al., 2007; Torrell et al., 2003). In the literature, artemisinin has been reported in *A. annua*, *A. apiacea* L., *A. lancea* L., *A. cina* L., *A. sieberi* L., *A. absinthium* L., *A. dubia* L. and *A. indica* L. (Tan et al., 1998; Iranshahi et al., 2007; Temraz and El-Tantawy, 2008). But *A. annua* is suitable for cultivation and has been described as containing 0.5 to 1.2% artemisinin in the dried plant material (Ferreira et al., 1997) also, *A. annua* is economically the only natural botanical source for artemisinin production.

*A. annua* originated from China and is widely distributed

in the Northern Hemisphere but poorly represented in the Southern Hemisphere. It grows mainly in the middle, eastern and southern parts of Europe, in the northern, middle and eastern parts of Asia and in North Africa (Simon et al., 1984; Ferreira and Janick, 1996). It is also distributed in the temperate, cool temperate, subtropical zones and Mediterranean region of the world. The plant is not grown the tropics because flowering will be induced when the plants are very small (Ferreira and Janick, 1996).

## Botany *A. annua*

*A. annua* is shrub and usually single-stemmed reaching about 2 m in height with alternate branches. The plant is extremely vigorous and essentially disease and pest free. *A. annua* is a short-day plant and very responsive to short photoperiodic stimuli and flowers about two weeks after induction. They require about 1000 h of sunlight per year. Annual sunlight time is a critical factor for the growth of *A. annua* (Simon et al., 1984). The fruit of *A. annua* is an achene with a single seed inside. The seeds are approximately 1 mm in length, oblong and the 1000 seed weight is approximately 0.03 g. The seeds do not have a dormant phase and can be used in the same year or in the year following collection (Ferreira and Janick, 1996). During the vegetative growth of *A. annua* trichome numbers increased per unit area on the adaxial leaf surface until leaf expansion ceased, at which point trichome numbers began to decline, apparently as a result of their collapse (Lommen et al., 2006). Leaves had 89% of the total artemisinin in the plant with the uppermost foliar portion of the plant containing almost double that of the lower leaves (Charles et al., 1990).

## Cultivation of *A. annua*

As we mentioned earlier, artemisinin cannot be synthesized, so it is still extracted from *A. annua* aerial parts. Therefore, the science of commercial cultivation of *A. annua*, to maximize artemisinin yields, should be well developed (Laughlin et al., 2002). The overharvesting of wild stands may restrict the ability for the plant to cross-pollinate and reseed naturally, eventually limiting the gene pool and genetic variability, which is critical to the development of improved seed lines. Another negative factor against application of wild stands is that transport distances often become uneconomic with a crop such as *A. annua*, which has relatively low artemisinin content and requires large biomass production.

In order to maximize the yield of artemisinin, the critical factor is day length, because the plant usually grows in the long summer days at high latitudes and flowers when the day length shortens. In the Tropics, where days are shorter than in Northern summers, flowering occurs earlier.

reducing the biomass achieved. However, yields can be maximized at higher altitudes and with late-flowering varieties (Laughlin et al., 2002; Ferreira et al., 1995). In wild-type plants, the greatest concentration of artemisinin is found in the inflorescence, although it occurs in all other aerial parts of the plant, except the seed (Ferreira et al., 1997). Seed varieties have been adapted by breeding for lower latitudes, and cultivation has been successfully achieved in many tropical countries, for example, in Congo (Mueller et al., 2000), India (Mukherjee, 1991) and Brazil (Milliken, 1997). The range of artemisinin content of *A. annua* harvested from different production areas is wide. The highest content of artemisinin that can be reached is up to 0.5 to 1.2% expressed as dry weight of leaves of *A. annua*. Although, the content of artemisinin is affected by numerous factors such as geographical conditions, harvesting time, temperature and fertilizer application, harvesting at the appropriate time is critically important to ensure optimum content of artemisinin in *A. annua*. Therefore, the best time for harvesting of *A. annua* should be determined by a study of the weather conditions, artemisinin accumulation and local harvesting experience. The yield of *A. annua* leaves and the content of artemisinin are reduced if harvesting is too early or is delayed.

For cultivation of this medicinal plant, it is important to plan the crop establishment for the beginning of the rainy season, which will enable fast growth at the crop's early stages and the production of higher biomass before flowering. In Switzerland, good biomass production was obtained from planting in late spring (Delabays et al., 1993) and early summer in Germany (Liersch et al., 1986) and the USA (Charles et al., 1990). If supplies of seed were freely available, direct sowing would be the most economical method of plant establishment, provided the environmental factors were suitable. For sowing of *A. annua*, the soil needs to be ploughed to a fine tilth and consolidated by rolling where appropriate. Because *A. annua* seed is small and it needs to be mixed either with some inert material. Depth of sowing is also critical for *A. annua*. A depth of drilling of 5 mm below the surface of finely prepared soil resulted in good emergence and establishment (Laughlin et al., 2002). Also, it is important to irrigate soon after sowing so that young seedlings do not suffer water stress. The irrigation frequency will depend on soil type, climate and season.

Plant population density and its components of inter- and intra-row spacing are important in determining yield and the practicability of both weed control and harvesting (Willey and Heath, 1969; Ratkowsky, 1983). If inter-row cultivation is intended for the control of weeds before the rows close, then inter-row spacing of 0.5 to 1.0 m may be appropriate. Similarly, wide intra-row spacing may also be appropriate. In earlier studies, low densities of 1 plant/m<sup>2</sup> (WHO, 1988) and 2.5 plants/m<sup>2</sup> (Delabays et al., 1993) gave yields of 1 to 4 t/ha of dried leaf.

Very little published work exists on the vegetative growth

responses of *A. annua* to the nitrogen, phosphorus and potassium or of their effects on the concentration of artemisinin and related compounds. Significant increase of total plant and leaf dry matter (1 to 3 t/ha) was obtained in Mississippi, USA, where a complete fertilizer mixture containing 100 kg N, 100 kg P and 100 kg K/ha was broadcast and worked uniformly through the soil (WHO, 1988). In China, a range of growing media and micronutrients were tested for their effect on the synthesis of artemisinin. There were no effects on artemisinin from any of these treatments (Chen and Zhang, 1987).

*A. annua* has been grown in a wide diversity of soils and latitudes, showing its potential for adaptation. If the *A. annua* cultivar and geographic region allow for a long vegetative cycle, more than one harvest can be performed to increase the final yield of leaves and artemisinin. In a study by Kumar et al. (2004) with the *A. annua* cultivar Jeevanraksha, carried out in a subtropical climate in India for 3 years the crops were harvested once, twice, three times and four times, respectively, during a 1-year growth cycle. *A. annua* can be grown under a wide range of soil pH (5.0 to 8.0), depending on the plant origin, but there are only a few studies on the effect of soil pH on the vegetative growth and artemisinin concentration in *A. annua*. It has been shown that some strains of *A. annua* are sensitive to soil pH below 5.0 to 5.5 (Laughlin, 1994). Weeds are a constant problem for crop production throughout the world and any system of *A. annua* cultivation must give careful thought to weed control. Weed control in the early stages of growth is critical for *A. annua*. Once plants become established, and with good early season weed control, the canopy shade will provide good weed control. There have been no serious pests or diseases yet reported to be a problem associated with *A. annua* (Simon and Cebert, 1988).

Many researchers used to think that sun and oven drying reduced the artemisinin content and that it was best to air-dry leaves in the shade (Laughlin et al., 2002; Ferreira and Luthria, 2010). However, comparisons between sun-drying, shade-drying and oven-drying at 60°C have shown that natural sun-drying is the best method (Ferreira and Luthria, 2010). Simonnet et al. (2001) found that sun-drying plants in the field increased the artemisinin content, but that if drying continued for more than a week, leaves were lost, decreasing the overall yield. The optimum would therefore seem to be drying in the field for 1 week, followed by air-drying in the shade. Harvesting time has to be established according to the cultivar of *A. annua* used because peak artemisinin can be achieved before or at full flowering, but it is generally accepted that the leaves should have no more than 12 to 13% relative humidity to realize optimum recovery of artemisinin. A drought a week before harvesting the plants could shorten the time needed to bring the cut plants to 12 to 13% relative humidity, but the effect of such a drought on artemisinin accumulation needs

to be investigated. The whole aerial part should be harvested, but leaves are the main source of artemisinin. These can be separated from the stems by threshing the whole plant over a plastic tarp. Leaves can then be sieved using a 5-mm mesh and then a 3-mm mesh (TechnoServe, 2004). Fine grinding of leaves is not necessary for the extraction of artemisinin because the compound is located in glandular trichomes found in both leaves and flowers (Ferreira and Janick, 1995).

In the agronomy point of view, novel production methods are required to accommodate the ever-growing need for this important drug. The future work should focus on releasing high artemisinin content varieties and improved cultivation approaches. Cultivation of this plant requires at least 6 months, and extraction, processing and manufacturing of the final product require at minimum of 2 to 5 months depending on the product formulation. Research is also needed on the development of new cultivation practices methods to robust yield of new breeding lines. Such methods will require collaboration between Agronomists, breeders, farmers, and social scientists in developing techniques for initiating and sustaining farmer participation in yield improvement.

### **BREEDING FOR INCREASING ARTEMISININ CONTENT OF *A. annua***

A new breeding strategy of comprehensive integration of biotechnology and DNA marker applications with conventional backcross breeding techniques for *Artemisia* improvement should be developed. In designing breeding programs, germplasm collections are important reservoirs of genetic diversity and this diversity should exploit for artemisinin improvement. Artemisinin enhancing alleles may exist in wild *Artemisia* germplasms. Furthermore, this technique enables us to select potential parent lines of *A. annua*. Breeders should propose strategies for introgressing artemisinin alleles into elite cultivars. Three approaches are considered in this review. These are: 1. Conventional breeding programs; 2. Mutation breeding and 3. Molecular breeding approaches.

#### **Conventional breeding programs**

The scientific studies have shown that artemisinin content can vary widely among different genotypes of *A. annua* from different origins (Wallaart et al., 2001), so breeding for this trait may be feasible. The genetic basis of this variation has been investigated by many researchers. Additive genetic components were predominant, resulting in a high narrow-sense heritability estimate. This trait exhibits high heritability therefore it appears possible to breed *A. annua* with a higher level of artemisinin. For breeding purposes, crosses can be only made between

individuals of *A. annua* that those have high artemisinin content. Plant breeders must therefore select varieties capable of producing well in favorable and unfavorable conditions. Selection criteria to be used in order to obtain high yields under various environments should be determined by breeders. Authors believe that there are 4 major breeding targets that may result in improved artemisinin yield: increasing leaf yield potential, increasing number of branches, increasing artemisinin content and improving number of trichome in plants.

Since the plant *A. annua* is highly cross pollinated like the members of family Asteraceae the chemical character like 'artemisinin content' segregate like any other phenotypic characters as multigenic characters always segregate in the progeny population. Every individual of its population can be expected to be homozygous at many loci, but also heterozygous at many loci. As a consequence of the segregation and recombination alleles of many loci are reshuffled and regrouped into vast numbers of multilocus allelic configurations each generation. Due to this all the progeny plants of the high artemisinin containing plant may not yield same amount of the artemisinin. Some will be high and some very low. The authors believe that the pyramiding of independent artemisinin-promoting segments can lead to novel commercial varieties of *A. annua*. A hybrid variety of *A. annua* is made by cross-pollinating two specific parent varieties. This first generation of offspring is referred to as the F1 hybrid.

#### **Mutation breeding**

Induced mutations have great potentials and serve as a complimentary approach in genetic improvement of crops for greater yield and quality traits (Ahloowalia and Maluszynski, 2001). The generation of genetic variability by induced mutagenesis provides a base for strengthening plant improvement programs (Rekha and Langer, 2007). For any mutation-breeding program, selection of effective and efficient mutagen is very essential to recover high frequency of desirable mutations (Solanki and Sharma, 1994). The mutagenesis approach is attractive alternate method for enhancement of artemisinin *in vivo* as well as *in vitro*. The results of many studies showed that mutation breeding has the capacity to release mutants with high artemisinin content (Rekha and Langer, 2007). Also, there is evidence that mutagens (radiations) stimulate the metabolic activity of plants such as respiration glycolysis and oxidative phosphorylation (Mergen and Johnson, 1964) and cytochrome oxidase and catalase activity which may ultimately influence and enhance synthesis of plant products. The main advantage of mutation breeding is the possibility of improving one or two characters without changing the rest of genotypes. Induced mutation breeding, which has been recognized as a valuable supplement

to conventional breeding in crop improvement, has been least applied in *A. annua*. The authors believed that mutation breeding can induce enormous variability in the *A. annua* through physical and chemical mutagenesis, because genetic variation is the starting point of any breeding program. Produced new mutant plants have great potential to be incorporated in further breeding programmes for upgrading new productive cultivars.

### Molecular breeding approaches

During the past 30 years, the continued development and application of plant biotechnology, molecular markers, and genomics has established new tools for the creation, analysis, and manipulation of genetic variation and the development of improved cultivars and new varieties (Sharma et al., 2002; Collard and Mackill, 2008). Presently, development of large numbers of molecular markers, high density genetic maps, and appropriately mapping populations are routined for many crop species. This technique, known as marker-assisted selection (MAS), is theoretically more reliable than selection based on phenotype. A vast majority of literature has considered the utility of molecular marker-assisted selection and its fit with different breeding methods (Dekkers and Hospital, 2002; Collard and Mackill, 2008). Molecular markers that are either within genes or tightly linked to QTL influencing traits under selection can be employed as a supplement to phenotypic observations in a selection index (Lande and Thompson, 1990). Use of molecular markers can significantly increase breeding efficiency and enhance genetic gain for traits where the phenotype is difficult to evaluate because of its expense or its dependence on specific environmental conditions.

Molecular marker technology has so far failed to be extensively used as a breeding tool by *Artemisia* breeders, because of the fact that no QTL responsible for a large enough effect on leaf yield has been discovered. In *A. annua*, there are now attempts to employ markers linked to leaf artemisinin content, trichom density and flowering delay. Only a very small fraction of the available *A. annua* germplasm has been assayed for alleles that might improve artemisinin content. It is likely that *A. annua* from many areas regarded as a wild plant and such plants have rarely been used as parents in QTL mapping studies. A more extensive survey of artemisinin content of *A. annua* germplasm will hopefully lead to the identification of lines carrying major genes conferring high artemisinin content. One of the objects of the molecular breeders is to develop an efficient marker system to be used in breeding *A. annua* for high artemisinin content. This marker system can distinguish plant tending to synthesize high amount of artemisinin when the biosynthetic system of the plant is functional. Other objective of the invention should generate a breeding and selection method using the marker assisted breeding to

increase the content of artemisinin in the plants. Individual loci with large effects on artemisinin content should be identified. The characterization of such genes and their anatomical, physiological, and molecular genetic effects, will be key factors in the application of molecular marker technology to the development of high artemisinin varieties.

### PERSPECTIVES IN THE STUDY OF *A. annua*

In this review we discussed the possibility of increasing the amount of artemisinin in *A. annua* by cultivation and breeding approaches. To date progress in cultivation and breeding of *A. annua* focusing on artemisinin content has made little progress. This may be explained simply by the lack of investment. Research is needed on the development of new cultivation practices methods to robust yield of *A. annua*. Such methods will require collaboration between agronomist, breeders, farmers, and social scientists in developing techniques for initiating and sustaining farmer participation in cooperative testing networks. The research on *A. annua* is expected to require a substantial amount of resources. The improvement on *A. annua* will be faster and easier accomplished by an international and multidisciplinary collaboration among agronomists, breeders, traditional geneticist, molecular biologists, etc. If it is pursued by different groups, including academia, governmental and non-governmental organizations, interested in making a real contribution in the field of cancer, malaria, and other chronic and parasitic diseases, the result on *A. annua* will be better.

There is a need to develop high artemisinin content varieties that will produce acceptable yields under favorable and unfavorable environments. Many traits are known to contribute to improving leaf yield and artemisinin content, but the anatomical, physiological, and molecular pathways controlling them are not well understood. A better understanding of the genetic basis of artemisinin content will probably be achieved by using more diverse mapping populations and by precisely identifying the genes affecting variation in artemisinin content through fine-mapping, microarray analyses and proteomics. Germplasm collections of *Artemisia* are important reservoirs of genetic diversity, but very little of this diversity has been exploited for its improvement. Artemisinin enhancing alleles may exist in wild germplasm. A methodology for introgression of artemisinin enhancing loci from wild species to the cultivated germplasm should be developed. Scientists should propose strategies for simultaneously mapping and introgressing these alleles into elite cultivars. Development of new varieties of *Artemisia* with high artemisinin content will both reduce the costs of cultivation and the costs of extraction associated with more artemisinin production. It will also produce better returns to farmers who grow the crop. The development of artemisinin-rich genotypes of *A. annua*

has encouraged some to promote cultivation and local use of the plant to treat malaria.

It is hoped that the publication of this paper will be helpful for the prevention and treatment of malaria around the globe. The anti-malarial drug, artemisinin, production can be maximized by breeding of new varieties and new well developed cultivation systems. These species seem to hold great potential for in depth investigation for various biological activities. The future studies focused on the breeding of new varieties could be beneficial.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

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