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Cultivation and genetics of *Artemisia annua* L. for increased production of the antimalarial artemisinin

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Abstract

Malaria has been treated for over 350 years with quinine and quinine-derived drugs. However, in several areas of the world, some strains of the malarial parasite *Plasmodium falciparum* have developed resistance against these drugs. Recently, the World Health Organization (WHO) recommended the use of artemisinin-combination treatments (ACT) as the first-line treatment for multidrug-resistant falciparum malaria. The WHO estimates that current supplies of artemisinin are sufficient for only 30 million ACT, and is foreseeing the need for 130–220 million ACT in 2005 (WHO, 2004). Current research on the production of synthetic artemisinin-like compounds by the Roll Back Malaria project, pharmaceutical companies and academia resulted in a promising synthetic artemisinin-like compound (OZ277) which is currently undergoing phase I clinical trials. In about 5 years this drug is expected to be approved and made available to the public, however, meeting current global demands for ACT depends on the immediate availability of affordable artemisinin-derived drugs. This will involve expansion of the area under cultivation of *Artemisia annua* and improved methods of cultivation and processing of raw material, associated with more efficient methods for extraction and purification of artemisinin from plant material. This review addresses the agricultural, environmental and genetic aspects that may be useful in the successful large-scale cultivation of *A. annua* and for producing the antimalarial artemisinin in areas where it is urgently needed today. It also includes geographic aspects (latitude and altitude), which will help make decisions about crop establishment in tropical countries, and includes a list of Good Agricultural and Collection Practices for *A. annua*.

Keywords: annual wormwood; *Artemisia annua*; artemisinin; cultivation; GAP (Good Agricultural Practice); genetics; *qinghao*; *qinghaosu*; malaria

Introduction

Bouts of chills (ague) and fever lasting several hours and at every 3 or 4 days, muscle ache, headache, diarrhoea and vomiting are symptoms commonly associated with malaria. If the disease is not treated, the spleen and the liver become enlarged, anaemia develops and jaundice appears. General debility, anaemia and clogging of the vessels of cerebral tissues are followed by coma, and eventually death (White, 1996). Cerebral malaria is most commonly seen in infants, pregnant women and non-immune travellers to endemic areas. The term malaria (*paludisme* in French, *paludismo* in Spanish and Portuguese) first entered the English medical literature in the first half of the 19th century and the word was derived from the Italian name for the disease (*mala* = bad, *aria* = air). Among infectious diseases, malaria is only

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second to AIDS, and costs Africa *ca* US$12 billion a year in lost gross national product (Roll Back Malaria, 2004). In recent years, the malaria epidemic has worsened, or at best stabilized, but not improved in any African country. Unfortunately, there are few success stories of malaria eradication, such as Taiwan and Jamaica, which had their growth accelerated after eradication: 1961 for Taiwan and 1958 for Jamaica (Gallup and Sachs, 2001). Each year, malaria afflicts over 300 million people worldwide, killing from 0.5 to 2.7 million, mostly children. Over 90% of these cases occur in the sub-Saharan Africa, but large areas of Asia, Central America and South America have high incidences of the disease (Nussenzweig and Long, 1994). Out of 37 countries and territories, members of the Pan American Health Organization (PAHO), 21 still have active malaria transmission (PAHO/WHO, 1998). Although there are four species of *Plasmodium* which can cause malaria, the most life-threatening species is *P. falciparum*.

In the 1960s, *P. falciparum* malaria started to show signs of resistance against quinine-derived drugs. This resistance was reported from places as far apart as Brazil, Colombia, Malaysia, Cambodia and Vietnam, making it harder to control the disease. In addition, over 1000 cases of malaria occur in the USA, and mosquito species capable of transmitting the disease are found in all 48 states of the continental USA. In 1969, the Chinese army found that a diethyl ether extract of *Artemisia annua* L., or *qinghao* in Chinese (Fig. 1), had an excellent effect against malaria, and in 1972 artemisinin was identified as the main active ingredient (Anonymous, 1982). Artemisinin (*qinghaosu*) (Fig. 1) is a sesquiterpene lactone of the cadinane series. In addition to a lactone group, artemisinin contains an endoperoxide bridge, which is rarely found in secondary metabolites, and is responsible for the antimalarial and anti-cancer activity. Complete chemical (*de novo*) synthesis of artemisinin was achieved by several research groups (Schmid

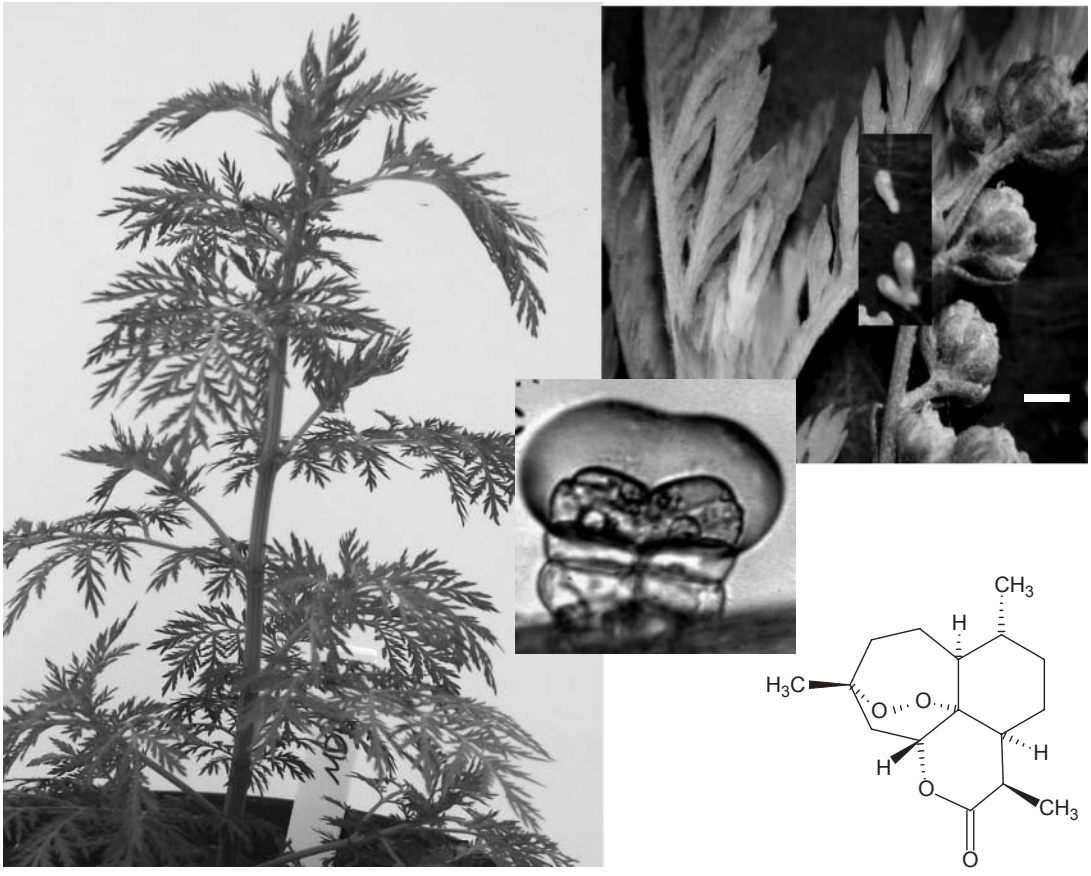

Fig. 1. Exomorphology of *Artemisia annua*. Left: 2-month old *Artemisia annua* plant (grown from Artemis seed, Mediplant, Switzerland). Right: upper insert—close-up showing details of leaves, capitula and hermaphroditic florets (bar = 2 mm) before anthesis; central insert—10-celled glandular trichome, found in leaves and flowers, accumulates essential oils and artemisinin in the upper subcuticular space (as pictured); lower insert—artemisinin (*qinghaosu*) molecule, a sesquiterpene lactone with a peroxide bridge, which is effective against malaria and cancer.

and Hofheinz, 1983; Xu *et al.*, 1986; Ravindranathan *et al.*, 1990; Avery *et al.*, 1992). The procedures require several steps, and can start from different raw materials. A comprehensive review on the chemistry, synthesis and semi-synthesis of artemisinin has been published by Ziffer *et al.* (1997). However, low yield, complexity and high cost indicate that the isolation of artemisinin from the plant is the most economically feasible method for its production at present.

Artemisinin, along with taxol, is considered one of the novel discoveries in recent medicinal plant research, and its isolation and characterization have increased the interest in *A. annua* worldwide. Increased production of artemisinin may also allow ready utilization of its recently established anti-cancer attributes (Efferth *et al.*, 2001). Artemisinin is the base compound for the synthesis of more potent and stable antimalarial drugs with reduced toxicity to humans. Artemisinin is effective against all *Plasmodium* species, including *P. vivax* and *P. falciparum*, two of the four species that cause human malaria. A multi-organizational approach launched in 1999, and organized by the Medicines for Malaria Venture involved academia, pharmaceutical companies and research institutes. This effort resulted in a synthetic triloxane peroxide named OZ277. The drug is effective, affordable and reported to last longer in the body than artemisinin (O'Neill, 2004; Vennerstrom *et al.*, 2004), but is still at least 5 years away from being commercially available. Due to the steady spread of chloroquine-resistant malaria, and the lack of affordable and efficient vaccines or alternative drugs, the search for effective, safe and affordable antimalarial drugs is one of the most pressing health priorities worldwide (Delhaes *et al.*, 2003). Previous reviews addressing cultivation aspects of *A. annua* for artemisinin production have been published by Laughlin (1994), Ferreira *et al.* (1997) and Laughlin *et al.* (2002). This review will focus on the agricultural, environmental and manufacturing aspects of artemisinin production, on recent advances in the genetics of *A. annua*, world demand and available drugs. Besides an updated literature, this review will discuss geographic aspects important for crop establishment around the world, and presents a tentative list of good agricultural and collection practices for *Artemisia annua*.

Artemisinin and *Artemisia annua*

The WHO estimates that *ca* 32 million doses of artemisinin-based antimalarial drugs are available and urges the production of 130–220 million by 2005 (WHO, 2004). The urgency in increasing the production of antimalarial drugs based on artemisinin calls for immediate action in increasing the area cultivated with *A. annua* in Asia, establishing its cultivation in Africa and in increasing efficiency in the production of artemisinin by maximizing its production per unit area and increasing the efficiency of processing *A. annua* leaves into artemisinin.

Artemisinin has been detected in leaves, small green stems, buds, flowers and seeds of *A. annua* (Acton *et al.*, 1985; Zhao and Zeng, 1985; Liersch *et al.*, 1986; Martinez and Staba, 1988; Singh *et al.*, 1988; Madhusudanan, 1989; Ferreira *et al.*, 1995a). Artemisinin has not been reported in roots of field-grown plants (Pras *et al.*, 1991; Klayman, 1993; Ferreira *et al.*, 1995a) or pollen, and the detection of artemisinin from seeds appears to be due to the presence of floral debris because seeds have no glandular trichomes (Ferreira *et al.*, 1995a). Artemisinin accumulates in glandular trichomes (Figs 1 and 2),

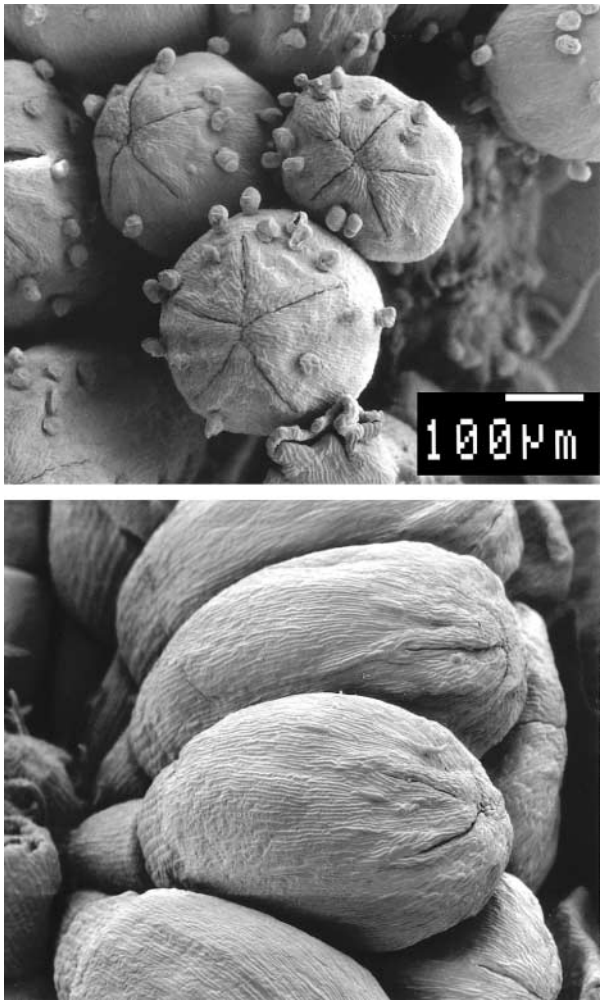

Fig. 2. Hermaphroditic florets in capitula of *Artemisia annua*. Top: wild-type *A. annua* with biseriate glandular trichomes described in detail by Duke *et al.* (1994) in leaves and Ferreira and Janick (1995a) in flowers. Bottom: glandless mutant, which contains no artemisinin or related compounds.

which are present only on leaves, stems and flowers of the plant (Duke *et al*., 1994; Ferreira *et al*., 1997).

Production of artemisinin under *in vitro* conditions has attracted the attention of several investigators due to the success in the production of a few natural compounds of medicinal value. Attempts to produce artemisinin and related compounds by tissue culture systems have been reviewed by Woerdenbag *et al*. (1994b), Ferreira *et al*. (1997) and Ferreira and Janick (2002), but the unstable and low yields are unattractive for commercial purposes.

Distribution and geographic range of *Artemisia annua*

Artemisia annua (family *Asteraceae*), also known as *qinghao* (Chinese), annual or sweet wormwood, or sweet Annie, is an annual herb native to Asia, most probably China (McVaugh, 1984). *Artemisia annua* occurs naturally as part of the steppe vegetation in the northern parts of Chahar and Suiyuan provinces (40°N, 109°E) in Northern China (now incorporated into Inner Mongolia), at 1000–1500 m above sea level (Wang, 1961). The plant now grows wild in many countries, such as Argentina, Bulgaria, France, Hungary, Romania (cultivated for its essential oil), Italy, Spain, USA and former Yugoslavia (Klayman, 1989, 1993). In addition, it has been introduced into experimental cultivation in India (Singh *et al*., 1986), Vietnam, Thailand, Myanmar, Madagascar, Malaysia, USA, Brazil, Australia (Tasmania) and in Europe into the Netherlands, Switzerland, France and as far north as Finland (Laughlin *et al*., 2002).

The geographic range of *A. annua* is paramount in determining areas for potential cultivation (Fig. 3). Although *A. annua* originated in relatively temperate latitudes it appears it can grow well at much lower tropical latitudes with lines which are either native to these areas (Woerdenbag *et al*., 1994a) or which have been adapted by breeding (Magalhães and Delabays, 1996). The current availability of late-flowering clones make it possible to cultivate *A. annua* in areas which were previously considered unsuitable due to their proximity to the equator, and short photoperiod. The high artemisinin concentrations (0.5–1.5%) in the leaves of some of these clones could allow high artemisinin yields in tropical latitudes, such as Vietnam, Madagascar and sub-Saharan Africa, even though the leaf biomass may not be as high as some strains of *A. annua* grown in temperate latitudes (see 'Crop planting time'). The beneficial influence that higher altitudes may have on the production of *A. annua* at tropical latitudes is a principle which could possibly be applied to parts of tropical Africa and elsewhere.

Currently, seizing the opportunity offered by the availability of late-flowering clones and the world demand for artemisinin, several international agencies, including the US Agency for International Development are carefully analysing the possibility of cultivating *A. annua* in tropical countries including Kenya and Tanzania. Artemisinin is the raw material needed to manufacture antimalarial drugs such as dihydroartemisinin, arteether, artemether and artesunate. Although it is generally stated that the main limitation factor in artemisinin production is its low levels in the plant, there are hybrids such as Artemis (a cross between Chinese and Vietnamese clones) that can produce from 1 to 2% artemisinin on a dry weight basis. Our view is that the current bottleneck for the feasible production of artemisinin in developing countries is the lack of affordable seeds from high-artemisinin parents. The seeds provided by Anamed.org have excellent germination (close to 100% in 3 days), but come at the high price of €40.00 per 1200 seeds, enough only to plant 0.1 ha. The seeds developed by the University of

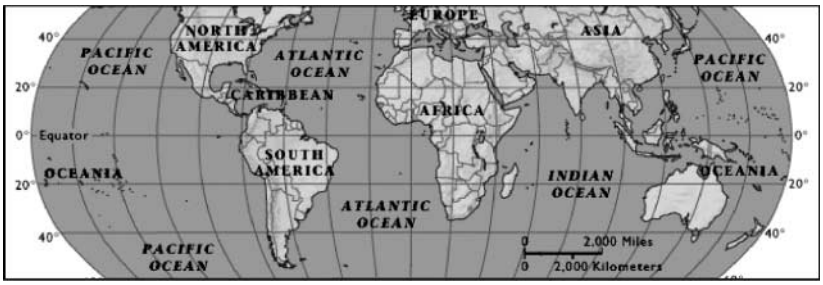

Fig. 3. Range of *Artemisia annua* cultivation in the world according to latitude, and altitude (when known). In North America: West Lafayette, IN (40°N, 184 m); Carbondale, IL (37°N). In Europe: Conthey, Switzerland (46°N, *ca* 1330 m). In Africa: Madagascar (18°52′S); Calabar, Nigeria (5°N, 60 m); Kenya (1°N); Tanzania (6°S). In Asia: Chongqing, China (29°N, 260 m); Penang, Malaysia (5°30′N); Lucknow (26°51′N) and Kashmir (32–36°N), India. In South America: Campinas, Brazil (23°S, 685 m); Teresina, Brazil (5°S, 75 m). In Australia: Devonport, Tasmania (43°S). The species occurs naturally in northern parts of Chahas and Suiyuan provinces, China (40°N). Sources: Singh *et al.* (1986), Delabays (1997), Ferreira *et al.* (1997), Laughlin *et al.* (2002); and personal communications from B. Moro (Brazil), P. Rasoanaivo (Madagascar), K. Mak (China) and A. Brisibe (Nigeria).

Campinas (Unicamp), Brazil were developed in collaboration with Mediplant and sell for US$40.00 per g (1 g contains *ca* 15,000 seeds), which is enough to plant 1 ha.

The cultivar Artemis, developed by Mediplant, has been selected for its biomass and high artemisinin content, and grows under photoperiods of less than 13 h light per day. The genotypes selected by Unicamp/Mediplant were used to generate hybrids named CPQBA, adapted for cultivation at latitudes of 20°S or N, if cultivated out of the inductive photoperiod for flowering. The recommended planting time for both North and South hemispheres would be after the equinox (Fig. 4). However, further selection is needed to isolate lines which will produce sufficient biomass and artemisinin at latitudes close to the equator, which have fewer hours of light a day compared to higher latitudes (Fig. 5). Even available improved genetic lines should be further improved to meet local and regional needs. *Artemisia* plants have been grown successfully in latitudes ranging from 42°S (Tasmania, Australia) to 40°N (Indiana, USA) (Fig. 3), according to Laughlin *et al.* (2002), Ferreira and Janick (1995b), Simon and Cebert (1988) and Singh *et al.* (1986). Recently, *A. annua* cultivars (CPQBA) bred in collaboration between Unicamp and Mediplant in Campinas, Brazil, have been grown in Teresina, Piaui (5°N) and Calabar, Nigeria (5°S). The plants from Teresina varied from 40 to 120 cm in height before flowering (M. Boro and L. A. da Silva, personal communication), while seeds grown in Calabar resulted in both early flowering and late flowering plants (A. Brisibe, personal communication). In such low latitudes, late flowering and tall plants with a high leaf-to-stem dry matter ratio should be selected for further planting and production of artemisinin. *Artemisia annua* plants (seeds from Kew Gardens, UK) were planted in Lucknow (26°51′N) and Kashmir valley (32–36°N) in India. The plants from Kashmir (temperate climate, altitude of 305 m or higher), although with a short season from mid-May to mid-June (plants at full bloom) produced 0.1% artemisinin, while the plants from the warmer climate and lower altitudes (123 m) of Lucknow produced a much lower, undisclosed, artemisinin concentration (Singh *et al.*, 1986). However, this Kew Garden *A. annua* strain was later established to be a poor grower, with early flowering and a growth span of about 75 days, when compared to a strain from Europe and another from the USA (Singh *et al.*, 1988).

Success in growing *A. annua* in tropical climates, such as Africa, currently relies on selection of clones for high leaf-to-stem dry matter ratio (possibly Artemis, Anamed-A3 or CPQBA) bred at latitudes varying from 0 to 20°N. With the current lines, cultivation is possible at a broader range of latitudes (N or S) up to 40°, although crop establishment becomes more challenging closer to the equator, where photoperiod is shorter (Fig. 5).

Cultivation of *Artemisia annua* and production of artemisinin

The ideal site for *A. annua* cultivation would depend on the scale of operation and the location of plants for commercial extraction. The concentration of artemisinin in *A. annua* is relatively low and the total dry herbage yield, from which the chemical is extracted, can be as high as 30 t/ha or higher (Laughlin *et al.*, 2002). For practical and commercial purposes, the

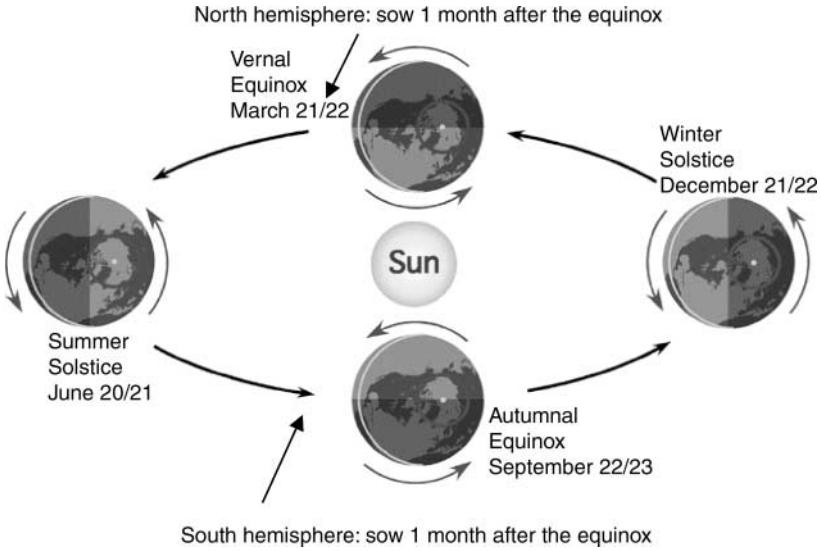

Fig. 4. Solstice and equinox with arrows showing recommended sowing times for *Artemisia annua* in the North and South hemispheres, according to the equinox, but observing the frost-free day where pertinent. Source:http://www.physicalgeography.net/fundamentals/6h.html.

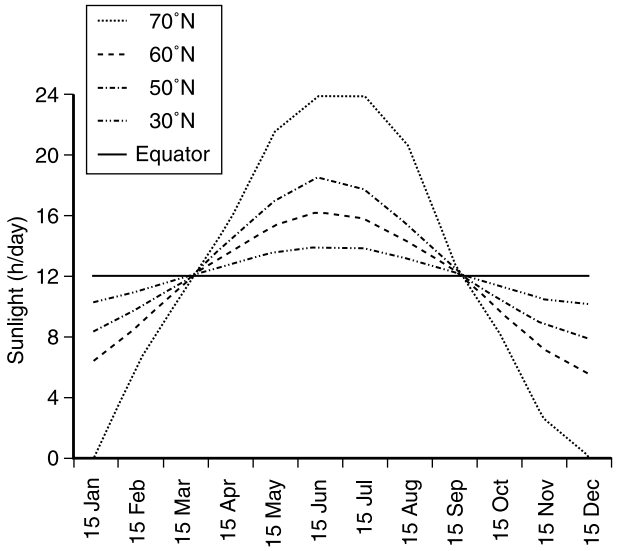

Fig. 5. Hours of sunlight per day according to the time of the year, in ascending order, ranging from 12 h (equator) to 24 h (at 70°N).

extraction and processing plant should be as close as possible to the area of production, or *vice versa*, depending on the circumstances. If mechanical harvesting is desired, the selection of relatively flat terrain would be appropriate. *Artemisia annua* has been grown in a wide diversity of soils and latitudes, showing its potential for adaptation. Apart from the intolerance of some *A. annua* selections to acid soils (pH 5.0–5.5), many soil types could be utilized.

Propagation of *A. annua* is normally done by seeds. Seeds remain viable up to 3 years if stored in dry, cool conditions (Ferreira *et al.*, 1997). Several researchers transplant *A. annua* to the field at about the 10-leaf stage, which requires 4–6 weeks of greenhouse growth (see 'Transplanting'). Vegetative propagation of *Artemisia* is normally achieved from cuttings. The shoots can be taken from juvenile or adult plants and have a rooting rate of 95–100%. Cuttings will root in about 2 weeks in a mist chamber (Ferreira, 1994). Although this method will produce homogenous plants regarding artemisinin content, it is not considered feasible for large areas destined for commercial production. On the other hand, seed-generated plants will vary widely in artemisinin content, which can range from 0.01% (Trigg, 1989) to about 1.5% (Debrunner *et al.*, 1996). Even seeds from the same parental origin, e.g. clones from half brothers from Campinas, Brazil, will produce plants that range in artemisinin content from 0.2 to 0.9% on a dry weight basis (J. F. S. Ferreira, unpublished data). Currently, there are lines that can produce up to 2% artemisinin (N. Delabays, unpublished data), but such high percentages are unusual in the currently available seed stocks. The economics of commercial development of artemisinin-derived drugs and their use in areas of greatest need hinge on plant raw material with high artemisinin content. Because of this, investigations have been carried out to select seed progenies having high artemisinin content and other desirable agronomic characteristics. These include good seed and plant vigour, high leaf-to-stem ratio with high dry matter leaf yield, disease resistance and desirable time of flowering appropriate to the region of production (Laughlin *et al.*, 2002), or lack of sensitivity to short photoperiods in locations close to the equator.

Germplasm comparison and plant selection have been carried out (i) on the basis of previous introduction and establishment of plants in the investigating countries and (ii) on the introduction of promising artemisinin-rich lines from countries where *A. annua* is native (e.g. China and Vietnam). However, commercial competition in the possession of high-artemisinin lines has limited the widespread availability of these lines at the present time. Likewise, the general access to hybrids which have incorporated the high artemisinin (1.1%) but low vigour of Chinese clones with the low artemisinin (0.04–0.22%) but high vigour of a range of European clones (Delabays *et al.*, 1993) are also generally unavailable for purposes other than research. Similarly, the more recent hybrids between Chinese and Vietnamese selections with even higher artemisinin (1.0–1.5%) are only available to a limited extent (Debrunner *et al.*, 1996). These shortages result mainly from the complex process of hybrid seed production which includes *in vitro* conservation and multiplication of the two parental clones, synchronization of the flowering, crossing, ripening and seed collection (Delabays, 1997). The cost and time involved in carrying out accurate assays for artemisinin have also limited progress in the screening and selection of *A. annua* lines. Rapid methods of artemisinin assay developed by Ferreira and Janick (1995b, 1996a) and the technique of appraising plantlets growing *in vitro* for artemisinin content (Pras *et al.*, 1991) are all strategies by which the process of screening *A. annua* germplasm could be accelerated. Germplasm assessment and studies of a range of other agronomic factors which have a bearing on the successful cultivation of *A. annua* have been carried out in Tasmania, Australia (Laughlin, 1993, 1994, 1995), Brazil (Magalhães, 1994, 1996), India (Singh *et al.*, 1986, 1988), Japan (Kawamoto *et al.*, 1999), Madagascar (Magalhães *et al.*, 1996), the Netherlands (Woerdenbag *et al.*, 1990), Switzerland (Delabays *et al.*, 1992, 1993; Debrunner *et al.*, 1996) and the USA (Simon *et al.*, 1990; Morales *et al.*, 1993; Ferreira *et al.*, 1995a). See Fig. 3 for the latitudinal range where *A. annua* has been cultivated around the globe.

Crop establishment

Natural stands versus cultivated crops

Although *A. annua* has been traditionally harvested in China from wild stands, the harvesting of raw material from wild stands for medicinal drug production is not recommended (Fritz, 1978; Franz, 1983). The plant material in wild stands is typically variable in its artemisinin content and plant biomass and this has an impact on the economics of drug extraction. In Madagascar, this variability in artemisinin content and plant morphology (0.3–2.0 m in height) has been reported by P. Rasoanaivo (personal communication). In addition, the overharvesting of wild stands may ultimately limit the ability for the plant to cross-pollinate and reseed naturally, eventually restricting the gene pool and genetic variability, which is vital to the development of improved seed lines. Another negative factor against utilization 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.

Although certain medicinal plants harvested from the wild achieve higher prices than cultivated crops (e.g. ginseng), the production of artemisinin from wild-grown versus cultivated *A. annua* crops is still being investigated. In China, where companies such as Holley produce Good Agricultural Practices (GAP) *Artemisia*, seeds from known *A. annua* parents are distributed to collaborating farmers who plant *ca* 6500 ha in three Chinese provinces. This cultivated area allows Holley to estimate the production of artemisinin for the upcoming harvest year to a minimum of 45 tons (K. Mak, Chongqing Holley China, personal communication).

Crop planting time

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 growth and biomass production were 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). In the temperate maritime climate of Tasmania, Australia, a field experiment compared transplanting in the spring months of October and November with early summer in December. Although all transplants flowered at the same time, the leaf dry biomass from October transplants was twice and four times as much as those obtained from November and December transplants, respectively. While artemisinin levels were similar for crops established from October to December, the concentration of artemisinic acid decreased by 25% and 50%, respectively, for crops established in November and December, compared to the crop established in October. However, when in field experiments winter transplanting (July and August) and spring transplanting (September and October) were compared, no differences in either leaf dry matter or in the yield of artemisinin or artemisinic acid were found (Laughlin, 1993). This experiment showed that there was no advantage in transplanting earlier than October in Tasmania, also avoiding problems associated with weed control.

In Indiana, USA, a greenhouse experiment at a constant temperature of 27°C with cuttings of a Chinese accession showed that *A. annua* from that accession was a short-day plant that flowered 2 weeks after exposure to photoperiods of 8, 10 and 12 h, but not of 16, 20 or 24 h. Subsequent field experiment showed that the critical photoperiod for that accession was 13 h 31 min. The flowering stimulus appears to be perceived at the apical meristem, and that flowering could be somewhat delayed by pinching the apical meristems and providing nitrogen fertilization (J. F. S. Ferreira, unpublished data). This technique, if applied early enough, will cause plants to branch out and potentially increase leaf biomass (Ferreira *et al.*, 1995a) if the season is 4–5 months long. Studies in Indiana, USA, showed that topping increased lateral branching but not the final yield. Under short growing seasons, topping was found to significantly lower biomass yields (J. Simon, personal communication). Although interactions between temperature and photoperiod were not possible under these greenhouse conditions, floral induction and flowering seem to show a very marked shift from this model in warmer tropical and subtropical climates. In Lucknow (26°52′N), India, seedlings of the same European *A. annua* selection which was used in the Tasmanian field experiment described previously (Laughlin, 1993) were transplanted in the relatively cool winter period of mid-December (Singh *et al.*, 1988). Under these conditions, bud formation occurred at a day length of 11 h 16 min with full flowering (anthesis) and maximum dry matter yield of leaves, flowers and artemisinin about 6 weeks later on 26 March at a day length of 12 h 15 min. In contrast to the Tasmanian field experiment, in which artemisinin peaked at the late vegetative stage, artemisinin concentration reached its peak during full flowering.

Another field experiment near Hanoi (21°02′N), Vietnam, was carried out with a Vietnamese strain of *A. annua* to establish the pattern of dry matter yield and artemisinin content over a growing season (Woerdenbag *et al.*, 1994a). Plants were sown into field plots at a density of 25 plants/m^2 (200 mm × 200 mm) in January. Maximum

dry matter yield of leaves (5.3 t/ha), maximum artemisinin concentration (0.86%) and maximum artemisinin yield (45.4 kg/ha) occurred at the vegetative stage on 15 June at a day length of 13 h 24 min. The plants remained in vegetative phase until 15 October (budding stage) with a dry leaf matter yield of 3.8 t/ha and artemisinin concentration of 0.42% at a day length of 11 h 41 min. Thus, for this Vietnamese strain, under Hanoi's environmental conditions, maximum leaf yield and artemisinin concentration were achieved 4 months before the onset of flowering. Similar results were obtained for a Brazilian hybrid (CPQBA 3M × POP) bred at the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA), Campinas, Brazil, and grown in southern Illinois (J. F. S. Ferreira, unpublished data). The above Indian and Vietnamese experiments illustrate the point that the response of *A. annua* cultivated at low latitudes may be quite different from the responses observed at higher latitudes (for instance at 40°N or S).

Vietnamese plants propagated by tissue culture were grown under greenhouse conditions in mid-December 1994. These plants were transferred to the field to a number of locations near Devonport in mid-February 1995 and remained in the vegetative stage throughout the autumn. They survived the winter and continued to grow throughout the following spring, with some plants achieving a height of 2 m. Flowering did not start until late February 1996, and a few plants that flowered did not set viable seeds (Laughlin *et al.*, 2002), indicating their single plant source since self-pollinated *A. annua* plants, or stands originated from a single plant by vegetative propagation, do not produce viable seeds (Delabays *et al.*, 1992).

At an elevation of *ca* 1500 m above sea level in Anatananarivo, Madagascar (18°52′S), *A. annua* hybrid plants resulting from a cross between Chinese and Vietnamese plants were transplanted into field plots on 12 March at a spacing of 50 × 70 cm. Mature plants were harvested on 4 August, and produced dry leaf biomass of 4.7 t/ha and an artemisinin yield of 41.3 kg/ha (Magalhães *et al.*, 1996). Other hybrids (CPQBA 3M × POP and CPQBA 5 × 2/39) planted in Campinas, Brazil, produced 3 tons of dried leaves and 25 kg of artemisinin per hectare (P. M. De Magalhães, unpublished data). Although specifics of this breeding programme in Brazil have not been published, a similar breeding programme developed by Mediplant has been discussed in more detail elsewhere (Delabays *et al.*, 1993, 2001). In Penang, Malaysia (5°30′N), seeds of *A. annua* obtained from Hanoi were used in field studies to evaluate plant performance at low latitudes and altitudes. Three-week-old plantlets were transferred into field plots and flowering occurred 14 weeks after transplanting with maximum artemisinin (0.39%) 1 week before flowering (Chan *et al.*, 1995). Although leaf dry matter yields were not obtained in this experiment, plants grew to 1 m tall. Similar results were reported when the CPQBA *A. annua* developed in Campinas, Brazil (23°S) was planted in Teresina, Brazil (5°S) at a low altitude, producing plants from 0.3 to 1.2 m in height (B. Moro and L. A. da Silva, personal communication). It appears that if *A. annua* is to be grown under such low latitudes and near the equator, further selection under local conditions must be done, based mainly on plant height, high biomass yield and high artemisinin accumulation. It may also be possible, in these tropical climates with very rapid growth, to consider planting two crops per year as has been suggested for Vietnam (Woerdenbag *et al.*, 1994a) and Brazil (Magalhães, 1996).

Transplanting

Supplies of *A. annua* seeds from high-artemisinin parents have been generally limited, partly due to increased interest by the pharmaceutical community, and further due to limited numbers of sources which make improved seedstocks available. Thus, most experimental field programmes have utilized transplanting as the preferred method of establishment (Acton *et al.*, 1985; Liersch *et al.*, 1986; Simon and Cebert, 1988; Singh *et al.*, 1988; Delabays *et al.*, 1993; Laughlin, 1993; Ferreira *et al.*, 1995a). In a few cases transplants have been generated from cuttings obtained through tissue culture or greenhouse propagation (Ferreira *et al.*, 1995a), but in most other studies the investigators have used a cellular tray system such as Speedlings® (pyramid-shaped cells) and a wide range of other cell trays used commonly in vegetable transplant production. The Speedlings® system involves germinating *A. annua* seeds in shallow (5–6 cm deep) seed trays using a sterilized potting mix such as 2:2:1 (sand: peat: vermiculite) with the pH adjusted to 6.0–7.0 and a low to moderate dose of a complete (macro- and micro-nutrient) fertilizer added after sterilization. The seeds are sprinkled uniformly on to the surface of this mixture, covered lightly with vermiculite (1–2 mm) and germinated in a greenhouse. The surface must be kept moist until germination and root establishment. When the seedlings are about 2 cm high (four or five true leaves)—or 3 weeks later—they are carefully uprooted and transplanted into the cellular trays, using the same mixture as above, or any available suitable container. The plants are then grown in a greenhouse for approximately 4 weeks or until they have about 10 true leaves and are about 10–14 cm in height. At this stage, the plants are acclimated outside or in a shade-house for 3–5 days and then transplanted to the field. There is no published information on the

optimum size for transplanting *A. annua* plantlets, but plants that have a developed and strong root system, in which the growth media and roots remain intact during transplanting, and the seedlings are about 15 cm in height (10-leaf stage), have led to successful field establishment. If mechanically transplanted, plants should be sufficiently robust to cope with such operation. Until the young plants start to actively grow, the soil should be kept moist. Approximately 800 ml of water per plant, three times a week, proved enough for field establishment of *A. annua* in Illinois and Indiana, USA. If irrigation is to be used, more delicate nozzle and water droplet size is advised to minimize direct water impact on the young transplant and to increase transplanting success. To lower production costs, several groups use outside nursery beds to start the seeds rather than greenhouses. Once the plants emerge and reach transplantable size they are collected and moved, bare-rooted, to the final field destination (J. Simon, personal communication). While the use of transplants has been successful, the expensive and time-consuming nature of the operation makes it desirable to develop more efficient and direct seeding methods, suitable for commercial cultivation.

Sowing

If supplies of seed were freely available, direct sowing would be the most economical method of plant establishment, provided the length of the growing season and other environmental factors were suitable and weeds could be controlled (see 'Herbicides' and 'Crop density'). Currently, prices of *A. annua* seeds from known origin are expensive for commercial-scale planting. Anamed.org sells limited amounts of seeds for €40.00 per 1200 seeds (covered in kaolin), Mediplant sells Artemis seeds from US$55.00 to 70.00 per g (depending on percentage germination) and CPQBA sells their seeds for US$40.00 per g (1 g = 12,000–15,000 seeds). These seeds have to be ordered at least 8 months ahead due to short supplies. Seeds from China and Vietnam are currently unavailable. A germination rate higher than 90% was obtained with seeds from Anamed.org in 3–5 days (J. F. S. Ferreira, unpublished data) but this rate decreases as the seed ages. Six-year-old Mediplant seeds have been reported to have a germination rate of *ca* 60% (TechnoServe, 2004). In Tasmania, Australia, sowing plants gave leaf dry matter and artemisinin yields very similar to the yields from transplanting, in two out of three field experiments (Laughlin, 1993). In these experiments sowing and transplanting took place in mid-October (spring). Both treatments matured at the same time and were harvested at the early bud stage in late February (summer), four and a half months later. There is little information on the possibility of autumn sowing and the over-wintering survival of *A. annua* seedlings in geographical zones where this technique may be appropriate (Ferreira *et al.*, 1997). However the performance of a self-sown Yugoslavian strain of *A. annua*, which germinated in the field in autumn (April) in Tasmania (Forthside, 41°12′S), may bring some light to this issue. These seeds germinated in late April and the young seedlings survived the winter very well. They grew strongly in the spring, but did not flower until late February of the following year (Laughlin *et al.*, 2002). Throughout that year, temperatures at Forthside never reached below 3.5°C or above 21.2°C (Table 1 in Laughlin *et al.*, 2002). Although these temperatures might appear inappropriate for plant growth, *A. annua* has recently been determined to be a C3 plant, thus having the capability of thriving mostly in temperate climates because it synthesizes carbohydrates through C3 photosynthetic mechanism (Marchese *et al.*, 2005). In Germany *A. annua* has been sown successfully in field experiments with seeding taking place in the third week of June and full bloom in late August (Liersch *et al.*, 1986). Also in Vietnam, *A. annua* was sown in January with floral induction in October (Woerdenbag *et al.*, 1994a) (see 'Crop planting time').

Sowing of *A. annua* seed can be carried out by various methods ranging from the basic hand application, or simple hand-pushed single-row seeders, to sophisticated combined multi-row seed and fertilizer drills. In all of these operations the soil needs to be ploughed to a fine tilth and consolidated by rolling where appropriate. Because *A. annua* seed is small (10,000–15,000 seeds/g) it needs to be mixed either with some inert material or with an appropriate neutral fertilizer. In Tasmanian experiments, the technique of mixing a 50:50 blend of fine ground limestone and superphosphate with *A. annua* seed resulted in successful establishment (Laughlin, 1993). This technique is useful in many situations ranging from basic hand sowing to machine drilling and has the advantage of not requiring a sophisticated drill with a specialized small seed attachment. Although the 50:50 fertilizer–limestone blend is neutral, the seed should only be mixed through it immediately before drilling. Ideally, the drilled area should be irrigated soon after sowing if soil moisture is less than optimal. In mixing seed and fertilizer, one should consider the current (i) seed germination rate and (ii) field survival in order to use a seed sowing rate that avoids either very sparse or very thick stands, which may require thinning. The technique of seed pelleting should be assessed for its possible adaptation to *A. annua* cultivation. Although *A. annua* seeds are very small (10,000–15,000 seeds/g), tobacco seeds (14,000 seeds/g) have been successfully pelleted, and

the equipment for pelleting is available (Cantliffe, 1997; Lee, 2004). Pelleting consists of the application of clay particles plus a binder to small seeds. The seeds are enlarged and have a spherical shape, which facilitates their precise dispersal into trays (Caruso *et al.*, 2001).

Depth of sowing is also critical for crops with small seeds such as *A. annua*. In Tasmanian studies, 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 emerging seedlings do not suffer moisture stress or fertilizer 'burn' (see 'Water requirements'). An alternative technique to mixing seed and fertilizer has been the sowing of a fine mixture of seed and other inert floral parts of *A. annua* (Galambosi, 1982). Ultimately, the success of any technique of direct sowing depends on a detailed knowledge of local soil and its physical characteristics, on environmental factors such as rainfall and temperature (Laughlin *et al.*, 2002), and on seed viability, which usually decreases after 3 years if seeds are not stored under dry and/or cold conditions.

Crop density

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 (Holliday, 1960; 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 0.5–1.0 m may be appropriate. Similarly, wide intra-row spacing may also be appropriate. However, if effective herbicides were applied, yield per unit area could be increased by using higher plant densities. In earlier studies, low densities of 1 plant/m^2 (Maynard, 1985; WHO, 1988) and 2.5 plants/m^2 (Delabays *et al.*, 1993) gave yields of 1–4 t/ha of dried leaf. Simon *et al.* (1990), in Indiana, USA, compared 3, 7 and 11 plants/m^2 and obtained the highest biomass at the highest density. In Tasmania, Australia, a field experiment with a Yugoslavian strain compared 1, 5, 10, 15 and 20 plants/m^2 at a November transplanting and found that leaf dry matter yield increased up to a density of 20 plants/m^2. However, 10 plants/m^2 allowed about 90% of the maximum yield of 6.8 t/ha (Laughlin, 1993). A high density of 25 plants/m^2 was also used in a field experiment in Vietnam which gave a maximum leaf dry matter yield of 5.3 t/ha (Woerdenbag *et al.*, 1994a). In the above Australian study (Laughlin, 1993), plant population density had no effect on the concentration of either artemisinin or artemisinic acid in the Yugoslavian strain of *A. annua* used. In the north Indian plains it has been recommended that *A. annua* should be cultivated at a high plant density of about 22 plants/m^2 (Ram *et al.*, 1997). The effect of variation in rectangularity (the ratio of inter- to intra-plant spacing) at constant plant population density (Chung, 1990) has not been studied with *A. annua* and may also be worth investigating. Considering the current lack of affordable seeds of high-artemisinin hybrids, it seems practical not to surpass 10–12 plants/m^2, which can produce 90% of maximum yields and make rational use of the cultivated area and applied resources.

Water requirements

Whether transplanted as seedlings or directly seeded into the field *A. annua* requires adequate soil moisture. If not established at the beginning of the rainy season, frequent light irrigations are necessary to ensure good crop establishment. The irrigation frequency will depend on soil type, climate and season. Another reason to avoid soil moisture drop at crop establishment is the possibility of fertilizer 'burn'. This problem is caused by a detrimental osmotic effect from high concentrations of soluble mobile elements such as nitrogen and sometimes potassium (Simon *et al.*, 1990; Laughlin and Chung, 1992). After plants are established, and at the end of the vegetative cycle, 1 week of drought could be desirable to hasten the field drying process, but the effect of such a drought on artemisinin accumulation needs to be investigated. Water stress data, measured through soil water potential during the 2 weeks before harvest of *A. annua*, indicated ($r^2 = 0.24$) that leaf artemisinin concentration might decrease (Charles *et al.*, 1993). Marchese (1999) subjected 84-day-old *A. annua* (CPQBA, Brazil) plants to water stress under both growth chamber and greenhouse conditions. Irrigation in growth chamber studies was suspended for 14, 38, 62 and 86 h. Only plants stressed for 38 h showed a significant increase in artemisinin compared to the control (0.54% versus 0.42%, and 0.82 versus 0.15%). In the greenhouse work, 147-day-old greenhouse plants had irrigation suspended for 18.5, 42.5, 66.5, 90.5 and 114.5 h. Additionally, plants from the 114.5 h treatment were re-hydrated and further deprived of water for 72 and 144 h. None of the treatments showed significant changes in artemisinin content. Although these plants were generated from seeds, their average artemisinin content ($n = 8$) ranged from 0.81 to 1.16% on a dry weight basis. These results suggest that seeds from the CPQBA cultivar used had a high heritability for artemisinin content, but water management and artemisinin accumulation, for *A. annua* in general, will be better conducted with clonally propagated plants to prevent error caused by artemisinin variability observed

in plants generated from seeds. Thus, a short drought before harvesting (or allowing the plants to sun dry after harvest) may not decrease artemisinin concentration.

Nutrient requirements for plant growth

Macronutrients

Very little published work exists on the vegetative growth responses of *A. annua* to the specific macronutrients 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 (1–3 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). Similarly in Tasmania, Australia, dry leaf yields of 6–12 t/ha were obtained in experiments with a mixed fertilizer containing 60 kg N, 60 kg P and 50 kg K/ha pre-drilled in bands 150 mm apart and about 50 mm below seed and 75 mm below transplants (J. C. Laughlin, unpublished data). The technique of banding fertilizer is to be generally recommended in soils where phosphorous fixation is a problem. The manoeuvre of pre-drilling fertilizer in 150 mm rows prior to sowing or transplanting plantlets allows very simple and inexpensive sowing equipment to be used and obviates the need for sophisticated and expensive sowing practices which place fertilizer and seed in one operation. In this technique the fertilizer bands can never be more than 75 mm (half the row width) laterally displaced from the plant row when seed is sown at random in a second operation parallel to the fertilizer and closer to the surface (Laughlin, 1978).

Although there is no published evidence on field responses of *A. annua* to phosphorus or potassium, there is some work on the response of *A. annua* to nitrogen under field conditions, and phosphorus and potassium in tissue culture. Regarding the response of *A. annua* to phosphorus and potassium in tissue culture, Liu *et al.* (2003) reported that KH_2PO_4 increased plant biomass and artemisinin content (0.05–0.20%) up to 200 mg/l. Beyond 200 mg/l, there was a slight increase in plant biomass, but artemisinin content decreased to original levels. Trials in Indiana, USA, compared three rates of nitrogen (0, 67 and 134 kg N/ha) and three plant densities (27,778, 55,555 and 111,111 plants/ha). The study indicated that both optimum essential oil (85 kg/ha) and fresh whole plant biomass (30 t/ha) were achieved at 67 kg N/ha at medium plant density. Although the highest plant density (at 67 kg N/ha) provided 35 t/ha of plant biomass, the plants had a lower leaf-to-stem ratio (Simon *et al.*, 1990). Hydroponic studies in Brazil concluded that the omission of nitrogen or phosphorus drastically reduced plant growth and dry matter production (Figueira, 1996). Later field trials with nitrogen fertilizer compared 0, 32, 64 and 97 kg N/ha applied as urea (Magalhães *et al.*, 1996). In this trial, the leaf dry matter yield of 2420 kg/ha and artemisinin yield of 26 kg/ha obtained at 0 kg N/ha increased to 4690 kg/ha dry leaves and 41 kg/ha artemisinin at 97 kg N/ha, although the concentration of artemisinin per plant was reduced by 22% at this high N rate. The rate of 64 kg N/ha resulted in a leaf biomass of 3880 kg/ha and artemisinin yield of 40.4 kg/ha (a 51% increase from the 0 kg N/ha), thus being the most cost-effective N rate for artemisinin production. In the same study, no significant differences were found for leaf biomass and artemisinin production when ammonium sulphate and ammonium nitrate were compared as the sources of nitrogen. Because nitrogen is a very mobile element, it can be easily leached out of the root zone, especially in areas of high or concentrated rainfall periods. This leaching effect may well be very significant in tropical and sub-tropical regions and in these situations the method and timing of nitrogen fertilization may be very important. Banding of nitrogen near the seed or plant row may give less leaching than broadcasting and uniform mixing. Split applications of nitrogen or slow-release nitrogen may be other means to minimize leaching, especially in long growing seasons. For example, in the growth pattern of the Vietnamese *A. annua*, the plants remained in the vegetative phase from seed drilling in January until September (Woerdenbag *et al.*, 1994a). At the optimum harvest time in mid-June the mean total rainfall, between January and June, had been 533 mm, but more than twice this pluviosity is possible in very wet years (Takahashi and Arakawa, 1981). Under such rainy weather, splitting the total nitrogen to be applied into two, three or more doses is recommended. Ideally some form of leaf or tissue analysis to determine the critical concentration of N at which a vegetative growth or artemisinin (or artemisinic acid) concentration response would be obtained could be the ultimate aim because soil analyses for N (unlike P and K) are often unreliable (Laughlin *et al.*, 2002).

It has been shown that some strains of *A. annua* are sensitive to soil pH below 5.0–5.5 (Laughlin, 1994) (see 'Soil pH'). A comparison of different forms of nitrogen such as urea and the neutral calcium ammonium nitrate with the more acidic ammonium sulphate and ammonium nitrate may be useful. Ammonium sulphate has been compared with ammonium nitrate in a field experiment on sandy soil in Switzerland (Magalhães *et al.*, 1996). When 90 kg N/ha was applied, both forms of nitrogen increased leaf dry matter yield and artemisinin yield by about 50%. However, a hydroponic

nutrient culture experiment suggested that a higher proportion of nitrate than ammonium may induce better leaf biomass (Magalhães *et al.*, 1996).

Micronutrients

In China, a range of growing media and nutrient treatments 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). However, sand culture experiments with a strain of *A. annua* (Washington, DC) showed that low levels of the micronutrients Fe, Mn, Cu, Zn and B, when compared to upper levels, resulted in significant decreases in plant height (26–63% shorter), fresh weight (19–45% lighter) and dry matter accumulation (18–49% lighter). Artemisinin level on control plants was on average 0.02% (w/w), but decreased significantly in plants submitted to low levels of Fe (twice as low), Cu (20 times lower), Zn (10 times lower) and B (10 times lower), according to Srivastava and Sharma (1990).

Soil pH

Artemisia annua can be grown under a wide range of soil pH (5.0–8.0), 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*. In Tasmania, Australia, the effect of zero and 10 t/ha of fine ground limestone (calcium carbonate) was evaluated for the growth of Chinese and Yugoslavian strains of *A. annua* field grown in a red krasnozem soil of pH 5.0 in the top 500 mm (Laughlin, 1993, 1994). The 10 t/ha limestone treatment increased soil pH from 5.0 to 5.5. The leaf dry matter yield of the Yugoslavian strain increased from 1.0 to 6.5 t/ha while the Chinese strain increased from 4.5 to 8.0 t/ha. The concentrations of neither artemisinin nor artemisinic acid were affected by the change in soil pH. These results suggested that there were large differences in strain (genotype) susceptibility to soil pH. The responses of the Chinese and Yugoslavian strains of *A. annua* to a wide range of soil pH were later studied under greenhouse conditions using plants potted in the same krasnozem soil from the field experiment. Fine ground calcium hydroxide at the equivalent of 0, 1, 2.5, 5, 10, 20 and 40 t/ha was uniformly mixed through the soil to give mean soil pH values of 5.0, 5.2, 5.3, 5.4, 6.0, 7.4 and 8.2, respectively. Both strains of *A. annua* grew well at a pH range of 5.4–7.4. However, the Chinese strain was more tolerant of both high (8.2) and low (5.0) pH conditions than the Yugoslavian strain (Laughlin, 1993, 1994). In Indian pot culture experiments, with *A. annua* grown on soils of widely varying pHs, essential oil yields at pH 4.9 and 9.9 were, respectively, about 75% and 25% of those grown on soils with pH 7.9–8.9 (Prasad *et al.*, 1998).

The work of Srivastava and Sharma (1990) correlated boron and copper with artemisinin concentration, and may also have implications for soil amendment practices by lime application. On some light soils the application of lime can lower the availability of boron. Although there might be advantages in evaluating correlations between copper, boron and lime application, an alternative strategy to the costly correction of soil pH may rest in the selection of strains of *A. annua* which are not only adapted to the local environment but also tolerant to extremes of soil pH below 5.5 and above 7.5 (Laughlin *et al.*, 2002). Although no gain in artemisinin levels has been shown with pH amendment, a lot can be gained by increasing biomass production and, thus, total artemisinin yield.

Herbicides

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. In small areas of cultivation in developing countries, manual weeding may be appropriate if row spacing is wide enough to allow it. Similarly, if inter-row cultivation by hand-pushed or tractor-drawn implements is to be used, row spacing must be to allow easy access while the crop is small and before the rows close. This system may also be the only practical one available even in developed economies where strict regulations governing the registration of herbicides for new crops demand lengthy lead times and investigations (Ferreira *et al.*, 1997).

If *A. annua* is to be established from seed, weed control in the early stages of growth is even more critical than with transplants. The young seedlings of *A. annua* are very small and can easily be outgrown by weeds in their early growth stages. In such cases, chemical weed control is certainly the most convenient and efficient method. Application of 2.2 kg active ingredient (a.i.)/ha napropamide before transplanting gave good weed control without phytotoxicity in the USA (Simon and Cebert, 1988). More detailed field studies have been carried out in Mississippi, USA where a range of herbicides was tested (Bryson and Croom, 1991). Chloramben was very effective when applied at 2.2 kg a.i./ha before emergence. Also, a good weed control was achieved with trifluralin at 0.6 kg a.i./ha incorporated before transplanting followed by fluazifol at 0.2 + 0.2 kg a.i./ha broadcast

after emergence and acifluorfen at 0.6 kg a.i./ha after emergence. All of these treatments gave good weed control without any significant reduction in leaf yield or concentration of artemisinin (Bryson and Croom, 1991). Once plants become established, and with good early season weed control, the canopy shade will provide good weed control.

Insects and pathogens

There have been no serious pests or diseases yet reported to be a problem associated with *A. annua*, according to Simon and Cebert (1988). In Tasmania, Australia, from a wide range of experiments, the only disease observed in some trials was a very low incidence (<1% of plants) of *Sclerotinia* stem infection on the lower third of the plant (Laughlin *et al.*, 2002). The symptoms took the form of conspicuous white fungal patches on the surface of the main stem. The possibility of *Sclerotinia* stem infection and appropriate control measures should be considered when *A. annua* is grown in plantations with high densities. Under these conditions the build up of localized humidity could induce the infection (Laughlin and Munro, 1983). In Saudi Arabia, *Orobanche cernua* was identified as a root parasite of *A. annua* with the potential to cause losses in yield (Elhag *et al.*, 1997).

Growth hormones

Applications of 50 mg/l gibberellic acid (GA_3) to field-grown plants increased artemisinin content from 0.77 to 1.10 mg/g; kinetin (10 and 20 mg/l) increased leaf yield and oil content, but decreased artemisinin content; and triacontanol had no effect on artemisinin content (Farooqi *et al.*, 1996). The levels of artemisinin increased from 0.77 to 1.3% when 80 mg/l GA_3 was applied to field crops, but artemisinin levels were not correlated to the levels of GA_3 applied to the crop (Siyapata-Ntakirutimana *et al.*, 1996). The beneficial effect of GA_3 on artemisinin accumulation has recently been supported by the findings of Zhang *et al.* (2005), who treated Chinese *A. annua* plants with half-strength Hoagland's solution containing GA_3. The authors reported that 14 μM GA_3 increased artemisinin content from 0.14 (control) to 0.64% (w/w) when applied to 74-day-old plants. The authors also observed that artemisinic acid decreased as artemisinin increased during plant development, reaching the peak at the full flowering stage. From a recent review on production of artemisinin under *in vitro* conditions (Ferreira and Janick, 2002), it was established that no other growth regulator, besides GA_3, produced any significant increase in artemisinin. Salinity stress did not influence artemisinin production (Prasad *et al.*, 1997). However, caution and more experimentation are needed before suggesting GA_3 as a cost-effective treatment to increase artemisinin content under field conditions.

Harvesting, drying and commercialization

Various methods of harvesting and drying *A. annua* have been tested in practical and experimental situations. Traditionally, manual harvest followed by various periods of sun and shade drying have been used in China and Vietnam. 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 12–13% relative humidity to realize optimum recovery of artemisinin. Depending upon the weather condition it may be appropriate to leave the harvested plants in the field for 5–10 days to achieve drying to the desired relative humidity under field conditions (TechnoServe, 2004). A drought a week before harvesting the plants could shorten the time needed to bring the cut plants to 12–13% relative humidity, but the results of water stress on artemisinin content are scarce and only one work is available (Marchese, 1999), which tested water stress in *A. annua* up to 6 days (see 'Water requirements').

Regarding temperature effects, Wallaart *et al.* (2000) recorded that the levels of artemisinin, in a Vietnamese *A. annua* cultivar, increased nearly 58% after a night-frost period, even though plants were showing a gradual decrease in artemisinin over time. This is possibly a stress-induced phenomenon and needs to be confirmed under controlled environment conditions. Wallaart *et al.* (2000) stated that the increased artemisinin after a frost is consistent with the hypothesis that stress will trigger the conversion of dihydroartemisinic acid to artemisinin, the final step in the biochemical pathway. These authors also suggest that the presence of high levels of dihydroartemisinic acid may be an adaptation to stress conditions (e.g. night frost), during which relatively high levels of O_2 are formed, and that dihydroartemisinic acid might protect the plant by reacting with these reactive oxygen species and yielding artemisinin as a stable end-product. Marchese (1999) submitted *A. annua* plants from Brazil (CPQBA, Campinas) to temperatures ranging from 18–28°C. These plants accumulated more artemisinin, although not significantly, than plants submitted to 11–20°C (0.41% versus 0.36%). These results were based on the average artemisinin analysis of plants generated from seeds. Ferreira *et al.* (1995a) also indicated that a decrease in artemisinin content (0.04–0.01%) observed

in a Chinese clone of *A. annua*, under greenhouse conditions, was due to higher temperatures observed from April to July, compared to cooler greenhouse temperatures observed from January to March. Singh *et al.* (1986) also found artemisinin to be higher in *A. annua* plants (seeds from Kew Gardens, UK) grown in the temperate climates of Kashmir than in the subtropical climate (0.1% versus 0.06%) of Lucknow, India. Although water stress and temperature regimen cannot be controlled under field conditions, these results indicate that *A. annua* plants can tolerate some changes in water availability and temperature without a drastic decrease in artemisinin content. However, it is important to note that results in artemisinin content may vary depending on the origin of the *A. annua* cultivar and on the regional environmental conditions.

The whole aerial part should be harvested, but leaves (or flowers) are the main source of artemisinin. These can be separated from the stems by threshing the whole plant (like rice threshing) 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 protruding glandular trichomes found in both leaves and flowers (Duke *et al.*, 1994; Ferreira and Janick, 1995a). Ferreira and Janick (1996b) analysed seven clones of a Chinese *A. annua* at two harvesting times (September and October) in Indiana, USA, and established that there was no significant difference in artemisinin content analysed from the bottom, middle or top part of the plants. Mechanized mowing and binding were first tried in the USA (Maynard, 1985) and harvesting after sun-drying whole plants for 1 week was tried in Australia (Laughlin *et al.*, 2002). The effect of various methods and times of drying have been reviewed by Laughlin *et al.* (2002) but there is little published information on the effect of drying on the new high-artemisinin strains of *A. annua*. A recent study by Simonnet *et al.* (2001) made a valuable contribution to this problem. A clone of a Vietnamese strain of *A. annua* was used in a field experiment in the Valais region of Switzerland. Whole plants were harvested at the late vegetative stage and sun-dried in the field for 31 and 29 days, respectively, in succeeding years. Leaf artemisinin concentration increased by 30% and 26%, respectively, in these two experiments, in which artemisinin levels immediately after harvest were in the range of 1.0–1.5%. In a similar field study in Tasmania, Australia, whole plants of a Chinese strain of *A. annua* were dried under full sun for 21 days. Leaf artemisinin concentration in this low-level strain (*ca* 0.1%) was increased by *ca* 100% (Laughlin, 2002). It may be important to assess to what extent these results from continental and temperate environments would apply to sub-Saharan Africa and other tropical regions where potential leaf loss and fungal diseases may be more likely due to the hot and humid weather.

Regarding the paying mechanism used for *A. annua* growers, crops are priced according to their artemisinin content (K. Mak, Chongqing Holley China, personal communication) or payment can be divided in three installments based on expected artemisinin content for the crop. For instance, a base payment of US$250/ton is suggested for a crop with 0.5% artemisinin. A second installment of US$100/ton is suggested after extraction is completed and a third installment (as a bonus, if applicable) of US$40/ton per every additional full 0.1% above the expected 0.5% artemisinin content (TechnoServe, 2004). It is important to consider that leaf artemisinin content may decrease if dried leaves are stored outside a dry and cool environment and that processed leaves (crude artemisinin) might be desirable for transport and commercialization over dry leaves. Therefore, processing plants, ideally, should be available to growers, at close proximity to the production site.

Good Agricultural and Collection Practices (GACP)

All medicinal plants are now recommended to be produced and/or collected using a process known as Good Agricultural and Collection Practices (GACP). These practices can increase the level of quality and traceability of the target botanical, and ensure that the correct botanical species is used. GACP will assure that the collection and/or cultivation practices result in a consistent quality of the raw botanical product. While several countries have produced versions of their own general GAP or GACP guidelines, and while none have yet been produced specifically for *A. annua*, the 'WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants' (see http://www.who.int/medicines/library/trm/medicinalplants/agricultural.shtml) provide a rather comprehensive list of practices that should be followed. As *A. annua* is still both collected from the wild in several countries as well as cultivated, it is important that the collection be performed in a manner that does not lead to the degradation of the environment and/or loss of the plant's biodiversity. Following such a GACP will assure ecologically sound cultivation protocols, in which the main objectives include: (i) quality assurance of medicinal plant materials used as source for herbal medicines or raw material for pharmaceuticals; (ii) guidance to the formulation of GACP guidelines and monographs at regional and national levels; (iii) encouragement and support of the sustainable cultivation and collection of medicinal plants of good quality in ways that respect and support the conservation of medicinal plants and the environment.

It is important to note that only the *A. annua* species has been reported to contain the antimalarials. Thus, the assurance that only this species enters into processing is important for quality control and to avoid any sales of *A. annua* teas and/or herbal products which would contain, intentionally or unintentionally, undesirable species.

GACP guidelines, as applied to the cultivation of *A. annua*, would focus on several issues related to the cultivation and harvest/post-harvest aspects of the crop, which include, but are not limited to:

- Site selection: soil that has not been previously contaminated with hazardous waste that might be absorbed by the plant and may end up in the final product. This also includes water sources free of contaminants that could harm the crop or consumers (e.g. high arsenic levels in ground water). Water tests are advisable.
- Use of seeds that are of known origin and reliable source, which will assure that the crop is indeed *A. annua*, and not a related species of different (or no) medicinal value. This will also give the producer an idea of the average artemisinin level that can be achieved with plants originated from such seeds.
- Conservation agricultural techniques that should be followed to foster organic matter, soil moisture and adoption of no-tillage systems.
- Environmental conditions that would foster plant development such as duration of sunlight during the months the crop is being established and undergoing vegetative growth (more than 13 h/day desirable). Information on the rainfall potential for the area. The altitude of the location might bring advantages in latitudes close to the equator.
- Adequate physical (drainage, organic matter content) and chemical (pH, nutrient availability) characteristics of the soil to host the crop.
- Adopting planting densities that will increase yield without fostering the development of fungal diseases, or be so sparse as to allow weeds to grow rampantly. In this aspect, the efficient use of herbicides might be investigated, if the economics of the crop allows such control, with special concern that national laws are followed with regard to the application and use of both herbicides and pesticides.
- Harvest should occur at optimal seasons to ensure the highest level of artemisinin in the final product, and lowest content of water (without harming artemisinin biosynthesis) possible to minimize costs and labour during post-harvest processing. Optimal drying conditions of the plant material (leaves) should be determined under field conditions to maintain (or increase) levels of the desired compound. In regions where long growing seasons are possible, it seems that several harvests conducted on the same plants allow higher leaf yield than one harvest at the end of the long season (Kumar *et al.*, 2004). Drying under full sun versus drying under shade needs further investigation. Regarding storage, moisture content of *ca* 13% seems appropriate for short-term storage of plant material without decreasing artemisinin content, although long-term storage is not recommended at this humidity level due to the possibility of fungal contamination.
- Personnel involved should be trained for the functions they are expected to conduct in the growing/harvesting pipeline. For instance, harvest crews should avoid mixing *A. annua* with potential toxic plants that might be present in the same cultivated area.

Given the increased global interest in the production of *A. annua*, we predict that such international cultivation manual(s) and specific GACP on this crop will be developed.

Genetics of *Artemisia annua* and artemisinin production

Broad sense heritability compared the artemisinin content of 24 clones of *A. annua* grown simultaneously under tissue culture, greenhouse and field conditions, and indicated that artemisinin production is controlled mainly by genetic factors (Ferreira *et al.*, 1995b). Recently, narrow-sense heritability studies (Delabays *et al.*, 2001, 2002) confirmed the results previously reported by Ferreira and collaborators and provided further evidence that artemisinin is mostly controlled by genetic factors. *Artemisia* plants can be kept in a vegetative growth phase under long photoperiods or induced to flower under short days in a greenhouse. Thus, genetic gain can be achieved, and maintained, from intercrossing high-artemisinin clones selected in the field and induced to flower in a greenhouse. Flowering of different lines often is mismatched under field conditions. A Chinese selection could be induced to flower after 2 weeks of short photoperiods (Ferreira *et al.*, 1995a). These results were confirmed for the same Chinese selection by Marchese *et al.* (2002) but a Vietnamese selection tested in the same study required an average of 33 days of short photoperiod to flower under growth chamber conditions. *Artemisia annua* plants are naturally wind-pollinated and favour outcrossing over selfing (Delabays, 1997). Crossing of a late-flowering clone of Chinese origin, rich in artemisinin (1.1%, w/w), with European plants by Mediplant in Switzerland led to progenies containing between 0.64% and 0.95% artemisinin, with dry leaf

yields between 14 and 21 t/ha (Delabays *et al.*, 1993). Numerous other hybridizations between Chinese and Vietnamese clones have been carried out to generate hybrids such as Artemis, containing up to 1.4% artemisinin, and allowing the achievement of a potential artemisinin production of 38 kg/ha (Delabays *et al.*, 2001). Some of these hybrid populations have been further improved to produce progenies with 2% artemisinin (N. Delabays, unpublished data). All these hybrids are obtained through the crossings between two high-artemisinin parental clones (although neither was back-crossed to the homozygous stage and cannot be called 'true hybrids'—J. A. Marchese, personal communication). These so-called hybrids were maintained and scaled up by vegetative propagation under long photoperiod (14 or more hours of light per day). According to Hirt (2001), a stock of progenitor plants needs to be maintained for production of hybrid seeds. According to this author, if second-generation seeds are taken from the hybrid plants only a few will germinate, and these will result in weaker plants with approximately 30% less artemisinin. Although artemisinin will always be present in plant tissues, artemisinin final yield is not solely dependent on the artemisinin genetic potential of the plant, but also on the total biomass production, accounted for mainly by leaves and flowers because roots are devoid of artemisinin and branches are low in artemisinin (Ferreira *et al.*, 1995a). The Institute of Materia Medica in Vietnam has been breeding plants for high artemisinin levels and high plant biomass (Dong and Thuan, 2003). Although a population which is uniform in artemisinin content cannot be achieved by sexual propagation, the original parents should be used whenever fresh seeds are needed. Outcrossing F1 plants to produce seeds (or F2 plants) will lead to loss of hybrid vigour in subsequent populations (F3, etc.). Selecting for plants that reach the peak in artemisinin before, or at the onset of, flowering (e.g. Artemis, Anamed, CPQBA) also allows for harvesting to be performed 3–4 months after planting and allows for at least two crops a year in tropical climates. Plants with a high leaf-to-stem ratio would also be desirable. However, the synchronization of flowering is one of the foreseeable problems to be overcome. Plants from different origins might have different requirements for photoinductive cycle, i.e. the number of short days the plant has to be exposed to before flowering. For instance, a Chinese *A. annua* has been reported as a short-day plant, which flowered 2 weeks after being exposed to the inductive photoperiod of 13.3 h of light under greenhouse and field conditions in Indiana, USA (Ferreira *et al.*, 1995b). The same Chinese line was compared to a Vietnamese line of *A. annua* for the inductive photoperiod in southern Brazil (Marchese *et al.*, 2002). These authors confirmed the requirement of 14 days (or cycles) of short days (13–15 h) for the Chinese line to flower under growth chamber and field conditions (26°11′S and 760 m altitude). However, the Vietnamese line required an average of 33 short days before flowering. Also, 100% of the Chinese plants flowered under average temperatures of 37°C maximum and 19°C minimum, compared to *ca* 33% of the Vietnamese plants. In order for 100% of the Vietnamese plants to flower, the average temperatures had to be 29°C maximum and 13°C minimum and the photoperiod had to be of either 7 or 9 h of light per day. The percentage of flowering Vietnamese plants decreased to 83.3% with an 11-hour photoperiod, while 100% of the Chinese plants flowered with photoperiods of 7, 9, 11 and 13 h of light per day.

Tetraploid Artemisia annua and artemisinin

Tetraploid *A. annua* ($2n = 36$) was obtained with the mitotic inhibitor colchicine by Wallaart *et al.* (1999) with an efficiency of approximately 20%. The content of artemisinin (0.46% dry wt) in the tetraploid *A. annua* during one vegetation period was *ca* 39% higher than in the diploid parental clone of *A. annua* (0.33% artemisinin). However, the average production of essential oils was *ca* 32% lower than in the diploid parental clone, indicating a possible inverse correlation between artemisinin and essential oil production. The authors indicated that the higher production of artemisinin might be achieved at the expense of the essential oil level and concluded that the *A. annua* tetraploid did not achieve the higher levels of secondary metabolites achieved by tetraploid *Atropa beladona* (68% higher), *Datura stramonium* (105% higher) or *Cinchona succirubra* (110% higher). The biomass accumulation of the tetraploid *A. annua* was also lower than the one obtained by the diploid parental clone, which decreased the total yield of artemisinin of the tetraploid by 25%. Because the glandular trichomes of *A. annua* have been indicated as the site for artemisinin accumulation (Duke and Paul, 1993; Ferreira and Janick, 1995a) it would be interesting to investigate the density and size of these glandular trichomes in the tetraploid plants. De Jesus-Gonzalez and Weathers (2003) also obtained stable tetraploid *A. annua* root cultures with colchicine and an efficiency of 10%, but growth in tetraploid root cultures was slower than in diploid cultures. Root diameter of tetraploid roots was larger than in diploids. Although artemisinin production of tetraploid hairy-root clones was from three to six times higher than the production achieved by diploid clones, the levels of artemisinin in those cultures (μg/g dry wt) did not have commercial potential. An additional setback was that the production of artemisinin in the diploid clones isolated by the authors in the early 1990s has

been declining ever since. This decrease and instability in artemisinin production was previously reported by Ferreira *et al.* (1995b) in whole plant (differentiated) cultures, kept in tissue culture for 2 years. The decrease in artemisinin and loss of apical dominance were attributed to epigenetic changes induced by tissue culture abnormal growth conditions, compared to greenhouse or field crops. This indicates that field-selected clones are better maintained under greenhouse than under tissue culture conditions.

Cloning and characterization of cDNAs involved in artemisinin biosynthesis

The biosynthetic pathway of artemisinin has been broadly defined (Wang *et al.*, 1988; Akhila *et al.*, 1990), but the proper order and structure of the biochemical intermediates is still being verified. In the past 10 years, considerable effort has been made to clone and characterize genes involved in the regulation of biosynthetic enzymes with the hope of increasing artemisinin in *A. annua*. Farnesyl pyrophosphate (FPP), as the primary metabolic precursor to artemisinin, was the initial 'target' molecule for this strategy. Matsushita *et al.* (1996) cloned farnesyl pyrophosphate synthase (FPPS) from *A. annua* and demonstrated that the recombinant enzyme has authentic FPPS activity in *Escherichia coli*.

The first committed step of artemisinin production is the initial cyclization of FPP to the corresponding sesquiterpene amorpha-4,11-diene. Several sesquiterpene synthase cDNAs have been cloned and characterized from *A. annua* including 8-epicedrol synthase (Hua and Matsuda, 1999), *epi*-cedrol synthase (Mercke *et al.*, 1999), (3*R*)-linalool synthase (Jia *et al.*, 1999) and β-caryophyllene synthase (Cai *et al.*, 2002). Each of the cDNAs were expressed in *E. coli* and demonstrated to have authentic sesquiterpene synthase activity. The enzymes 8-*epi*-cedrol synthase and *epi*-cedrol synthase generate a mechanistically complex class of compounds known as cedranes. However, cedranes are not thought to be involved in artemisinin biosynthesis. Similarly, (3*R*)-linalool synthase and β-caryophyllene synthase do not generate sesquiterpene precursors of artemisinin. Also, cedranes are postulated to be involved in resistance to plant pathogens. For instance, (3*R*)-linalool synthase steady-state transcript accumulation increases in response to wounding in *A. annua* seedlings (Jia *et al.*, 1999), and β-caryophyllene synthase steady-state transcript accumulation increases in response to a fungal elicitor (*Verticillium dahliae*) in *A. annua* seedlings (Cai *et al.*, 2002). However, manipulation of genes that control enzymes of the essential oils (e.g. caryophyllene and linalool) pathway by fungal elicitors cannot be extrapolated to the branch of the pathway leading to the biosynthesis of sesquiterpenes such as arteannuin B, artemisinic acid and artemisinin. Presently, there is no evidence that artemisinin production can be increased by fungal attack. The conversion of FPP to amorpha-4,11-diene by amorpha-4,11-diene synthase (ADS) is postulated to be the first committed step in artemisinin biosynthesis. Mercke *et al.* (2000) cloned and characterized the enzyme ADS from *A. annua* tissues. This enzyme was cloned by screening an *A. annua* cDNA library using the full-length *epi*-aristolochene synthase cDNA from tobacco as probe. Characterization of the ADS enzyme demonstrates a low K_m for FPP (0.7 μm), and that the major product is amorpha-4,11-diene (91%) although other related compounds such as α-bisabolol and β-sesquiphellandrine were produced at less than 1%. Martin *et al.* (2003) circumvented the poor performance of plant terpene cyclases in *E. coli* by expressing a codon-optimized synthetic cyclase gene, which improved the production of FPP over 100-fold. They also used an engineered mevalonate pathway from yeast, which performed from 30 to 90 times better than the normal *E. coli* pathway. Combination of both approaches increased the production of amorpha diene by *ca* 10^5-fold. So far, the authors have created a high-flux isoprenoid pathway in a bacterial system capable of generating significant amounts of amorpha-4,11-diene. However, because both artemisinic acid and arteannuin B are bacteriostatic against *E. coli* (Dhingra *et al.*, 2000), it will be an interesting challenge to move further than amorpha diene in this bacterial system.

Selecting for other secondary metabolites

Artemisinic acid (*qinghao* acid), a precursor of artemisinin, has been reported in *A. annua* at concentrations up to 10-fold that of artemisinin (Laughlin, 1993) and can be converted to artemisinin with an efficiency up to 40% (Roth and Acton, 1989; Haynes and Vonwiller, 1991). However, due to the existence of different chemotypes of *A. annua* from different origins, producing artemisinin from artemisinic acid is not always possible. Some lines have only traces of artemisinic acid. *A. annua* also contains an essential oil in its herbage which is used in perfumery and as an anti-microbial (Lawrence, 1990), and can add commercial value to the crop if demand increases. Although the demand for *Artemisia* essential oils is small in the Western hemisphere, in the Eastern hemisphere several toiletry items contain *A. annua* essential oil. Strains of *A. annua* with essential oil profiles of commercial interest were identified in studies in the USA (Charles *et al.*, 1991), although to date these strains have not been explored commercially.

The screening of *A. annua* germplasm for both artemisinic acid and essential oil content may be a useful strategy to increase the cost–benefit ratio of the extraction process by aiming for both oil and artemisinic acid, but not artemisinin, in one operation (Laughlin, 1994), when using super-critical CO_2 extraction. Essential oils are extracted by steam distillation at temperatures which destroy most of the artemisinin in the tissue, as reported by Magalhães (1996) and Laughlin (2002). It is important to note that if artemisinin is extracted in water below the boiling point, up to 75% of the artemisinin will be present in the aqueous extract (J. F. S. Ferreira, unpublished data). A similar extraction procedure produced over 50% artemisinin, while boiling water produced 30% artemisinin or less (Magalhães, 1996). It seems that clones of *A. annua* high in artemisinin and dihydroartemisinic acid are low in artemisinic acid, and that chemotypes with low levels of artemisinin and dihydroartemisinic acid are high in artemisinic acid, the direct precursor of artemisinin (Wallaart *et al.*, 2000).

Currently, the resurgence of the use of *Artemisia* tea as an alternative treatment for malaria with an efficacy similar to, and bioavailability higher than, pure artemisinin, points to a possible synergistic effect of other components present in the decoction or infusion obtained from dried plant material. This indicates that selection of germplasm should head towards lines or clones not only high in artemisinin, but also with fairly high contents of artemisinin-related compounds such as artemisitene, also with a peroxide group, and compounds which might increase the biological activity of artemisinin.

Increasing artemisinin in *Artemisia annua*

Manipulation of the artemisinin biosynthetic pathway to increase artemisinin production would greatly decrease costs of artemisinin-derived drugs and contribute to the understanding of terpene biosynthesis. FPP, a central molecule in plant metabolism, is the direct precursor of sesquiterpenes (C_{15}) and triterpenes (C_{30}), and is related to the biosynthesis of monoterpenes (C_{10}) and tetraterpenes (C_{40}). As demonstrated by Martin *et al.* (2003), ADS is capable of converting available FPP to amorpha-4,11-diene, indicating that FPP is not limiting to artemisinin biosynthesis. Thus, *in vivo*, Ferreira *et al.* (2004) postulated that competition for FPP by a variety of sesquiterpene synthases will be the critical control point. Some possible approaches to increase production of artemisinin in the plant, proposed by Ferreira *et al.* (2004), were (i) over-express ADS in *A. annua* to shunt FPP into the artemisinin pathway; (ii) knock-out other known sesquiterpene synthase genes (such as 8-epicedrol synthase, *epi*-cedrol synthase, (3*R*)-linalool synthase and β-caryophyllene synthase) to see if a reduced demand for FPP by other sesquiterpene (C_{15}) biosynthetic pathways would increase artemisinin; (iii) combine approaches (i) and (ii); (iv) use macro- or micro-arrays to determine the suite of genes expressed in wild-type *A. annua* under physiological and developmental states when artemisinin accumulation occurs. When compared to similar data from wounded or pathogen-challenged *A. annua*, it should be possible to identify 'master genes' capable of increasing carbon flux specifically to artemisinin biosynthesis. However, although expression analysis suggests that wounding and/or pathogen attack might increase carbon flux through the sesquiterpene (C_{15}) biosynthetic pathway (Jia *et al.*, 1999; Cai *et al.*, 2002), wounding or disease might not increase artemisinin biosynthesis.

Although the levels of artemisinin achieved by the plant are mostly linked to genetic factors, the work of Simonnet *et al.* (2001) indicate that it is possible to increase artemisinin by up to 30% after harvested plants are left to dry under full sun for 20–30 days. Similar patterns of increase in artemisinin concentration from sun-drying whole plants of *A. annua* for 21 days were recorded by Laughlin (2002). This increase might be due to photo-oxidative stress undergone by the plants during the drying process. It is possible that artemisinin precursors (artemisinic acid and arteannuin B) are transformed into artemisinin during that time.

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 this regard, the work of Kumar *et al.* (2004) with the *A. annua* cultivar Jeevanraksha, carried out in a subtropical climate in India for 3 years, resulted in yields of *ca* 28, 27, 40 and 74 kg/ha if the crops were harvested once, twice, three times and four times, respectively, during a 1-year growth cycle.

Available drugs and world demand

China and Vietnam are the main producers of artemisinin and its derivatives either for oral or parenteral use. Malaria control programme officials have distributed, between 1991 and 1998, 31.6 million tablets of artemisinin, 10.5 million of artesunate and 793,500 vials of injectable artesunate in Vietnam. Although recent data from China are not available, sales of artesunate tablets rose from 185,000 to 2,545,000 between 1991 and 1995. In Thailand, consumption of artesunate rose from 2880 tablets in 1993 to 653,199 tablets in 1997. Artemisinin, artemether, arteether, artesunate and dihydroartemisinin can all be purchased as drug substances from producers

in China while artemisinin, artemether and artesunate can be purchased from Vietnam (WHO/MAL, 1998).

According to the WHO, artemisinin and its derivatives are widely registered as antimalarial drugs in countries where malaria is endemic. Currently, Artekin (dihydroartemisinin-piperaquine), Duo-cotexin (dihydroartemisinin-piperaquine phosphate) and Coartem® (artemether + lumefantrine) are the artemisinin-combination drugs available for the treatment of malaria caused by *P. falciparum* strains resistant to chloroquine. The first two drugs are produced by Chongqing Holley Holdings Co., and have been approved for use as a combination treatment for malaria in Africa and Asia. Coartem® is produced by Novartis from raw material produced by Holley. Zambia was the first African country to adopt Coartem® as a first-line treatment for malaria with Zambia's Central Board of Health receiving 2.1 million treatments in 2003 and, supposedly 3.4 million treatments in 2004 on a not-for-profit basis (Anonymous, 2004). Intramuscular artemether can be made available in France and Denmark upon request. Countries such as Bangladesh and the Philippines have no problem with malaria caused by multidrug-resistant strains of *P. falciparum*, and artemisinin-derived drugs are unavailable. However, countries such as Myanmar and Vietnam require the use of artemisinin drugs due to the existence of multidrug-resistant *Plasmodium* strains. The world demand for artemisinin-derived drugs is currently dictated by the development and spread of multidrug-resistant *P. falciparum*. Unfortunately, the high demand for artemisinin not only holds the price up, but also leads to counterfeit drugs such as the fake artesunate reported by Newton *et al.* (2001). Artesunate is manufactured by Guilin Pharma and is widely used in South-East Asia and other areas to fight malaria, which is still a major killer in developing countries. The real artesunate is identified by a circular hologram incorporated into the foil of the blister packs. The fakes are very similar in appearance and packaging to the genuine medication, but they lack the active ingredient artesunate, which makes them deadly if used to treat complicated malaria. Both real and fake artesunate are depicted in Newton *et al.* (2001). To date, these fakes have been found in Laos and Cambodia. This problem is so serious that PAHO has recommended a field method to test artesunate as follows:

- Scrape 1/100 of tablet into tubes.
- Add 0.5 ml of 1 N NaOH mix.
- Wait for 5–20 min, room temperature.
- Add 1 ml of 1.1 M acetic acid.
- Add 0.5 ml of Fast TR red salt (5 mg/ml in distilled water).

If a yellow colour appears in 5 min, there is real artesunate in the sample. One should keep in mind that this method is only qualitative, not allowing the user to know how much artesunate is present in the sample. Although we could not find a reference for this test, a similar test was published by Green *et al.* (2001). Their method can be used quantitatively if a spectrophotometer is available.

Laboratory and commercial extraction of artemisinin

Artemisinin is an odourless, non-volatile compound, which is purified as white crystals with a melting point of 156–157°C. Its molecular weight is *m/e* 282.1742 M+ (Luo and Shen, 1987), with an empirical formula of $C_{15}H_{22}O_5$.

Artemisinin can be easily extracted with petroleum ether (bp = 45°C), hexane (bp = 60°C), or other miscible solvents such as chloroform, acetonitrile and ether which have boiling points lower than the critical temperature for artemisinin stability. Artemisinin was established to be stable up to 150°C in neutral solvents (Lin *et al.*, 1985). However, during tea preparation artemisinin decreases when leaves are boiled for as little as 5 min, but seems to remain stable if leaves are extracted with water before the boiling point (Magalhães, 1996). The first published laboratory procedure for isolation of artemisinin was described by the late Dr Daniel Klayman (Klayman *et al.*, 1984). Extraction of artemisinin and artemisinic acid is also achieved by an improved method of supercritical CO_2, with optimal pressure of 15 MPa, temperature of 50°C, methanol (or ethanol) concentration of 3%, flow rate of 2 ml/min and extraction time of 20 min (Kohler *et al.*, 1997), although the economics of this extraction method have not been discussed for its commercial-scale use. A feasibility report performed by TechnoServe (2004) considered the costs of large-scale extraction of artemisinin with (i) mixed solvents, including petroleum ether (or hexane) and ethyl ether; (ii) ethanol; and (iii) CO_2, and concluded that mixed solvents and CO_2 are economically attractive, but the initial investment would be large for a 2600-ton capacity plant, and none of the options consider the price of a quality control laboratory, equipped with a high-performance liquid chromatograph, to monitor leaf artemisinin content of different batches. Prices and economic study numbers are undisclosed, but can be obtained by contacting Mr Thomas Dixon (Thomas.Dixon@tnstanzania.org).

The first large-scale extraction procedure of artemisinin was published by ElSohly (1990). Unground, dried leaves (400 kg) were extracted to produce 485 g (0.12% yield) of artemisinin, 2.12 kg (0.53% yield) of artemisinic acid and

170 g (0.04% yield) of arteannuin B. Commercial procedures in general involve the initial extraction with petroleum ether or hexane (low-boiling solvents). As petroleum ether and hexane are non-polar solvents, all the waxes are extracted with artemisinin. This crude paste is later refined through column chromatography to separate artemisinin from undesirable debris and from other desirable compounds such as artemisinic acid and arteannuin B (if present). A major artemisinin producer in China (Holley) recycles the petroleum ether so that it can be reused in subsequent extractions. Holley achieves an average yield of pure artemisinin per ton of dried leaves of *ca* 85% (K. MaK, personal communication).

In the attempt to protect ecosystems from pollution by solvents derived from petroleum, clean methods and good manufacturing practices need to be considered. Although initial costs for installation and routine acquisition of liquid CO_2 might be seen as disadvantageous, the advantages, when compared to a gasoline extraction method, can be found in the yield (0.62% versus 0.3%), extraction time (20 h versus 120 h), total cost (19% cheaper), safety and low pollution of the environment (WHO, 2002). In addition, the possibility of using small-scale super-critical fluid extraction units can alter the economic model and the benefits of introducing such environmentally friendly, recyclable solvents such as CO_2.

Conclusions

Artemisia annua is the main source of artemisinin, the most potent and efficacious antimalarial after quinine. Recently, artemisinin has also been proved to be a selective anti-cancer drug (Moore *et al.*, 1995; Efferth *et al.*, 2001). Currently, the limited availability of artemisinin and the lack of real competition among producers of raw material seem to be the major barriers to scaling-up production and are partially responsible for its high price (World Bank, 2003). Also, the lack of affordable certified seeds hampers the extension of *A. annua* cultivation around the world. Breeding high-yielding, late-flowering cultivars of *A. annua* adapted to the tropics, where malaria is endemic, is a desirable approach that needs to be pursued. Reports about the antimalarial efficacy of tea prepared with leaves of artemisinin-rich plants (Mueller *et al.*, 2000, 2004) offer hope to people who are isolated from immediate health care. Anamed.org presents detailed protocols for tea preparation from leaves, but we cannot overstress the need of physician assistance to assure that the treatment will be carried out to completion. Also, the tea alternative should be used as an immediate source of artemisinin to alleviate symptoms and fight *Plasmodium* reproduction until a full-course treatment for malaria, preferably with artemisinin-combination therapy, can be provided. However, this approach should not be used in areas where chloroquine-resistant *Plasmodium* is in existence. Scientists are currently trying to understand the intricate and self-regulated biosynthetic pathway of artemisinin, its potential increase by the manipulation of a bacterial system and by the overexpression of terpene cyclase genes, although commercially feasible results are still to be seen.

Currently, the hope to curb malaria rests on hampering the spread of the disease by mosquito vectors, on the availability of an effective and affordable vaccine, on the widespread use of insecticide-treated nets, on new antimalarial drugs effective against multidrug-resistant *Plasmodium*, and on meeting the world demand for artemisinin-combination treatments. Of course this last factor depends on a steady production of artemisinin, at affordable prices, to meet global demand.

Recently, the entire genome of *Plasmodium falciparum* has been deciphered, revealing that the resistance to chloroquine rests on one single gene. Interestingly, the same mutation renders *Plasmodium* more susceptible to quinine and artemisinin (Gardner *et al.*, 2002). Genetics has played a major role in the control of malaria through the breeding of more productive *A. annua* cultivars and by helping to find a chink in the armour of the malarial parasite. It is now up to researchers to translate this knowledge into actions that can alleviate the suffering of people afflicted with malaria. Genetic improvement of *A. annua* to generate plants with high artemisinin and high biomass in the tropics, mainly close to latitude zero, is an essential goal that must be pursued immediately.

Although field production of *A. annua* is presently the most commercially feasible approach to produce artemisinin and related compounds, farmers must have access to good-quality seed generated from high-artemisinin parents. Although these seeds do not constitute 'true hybrids' because the parents are not homozygous, artemisinin content found currently in seeds available for research is approximately twice as high as it was 10 years ago (1.0% compared to less than 0.5%). Also, the agricultural aspects of artemisinin production such as soil fertility and pH, plant density, water availability, latitude and altitude, hormones, harvesting and drying protocols must be fine-tuned for each geographic area where artemisinin is to be produced as a raw material. In addition, factors that affect temporal (when artemisinin reaches its maximum) or spatial (tissue localization) accumulation must not be ignored when evaluating the commercial potential of *Artemisia annua* as a new crop for tropical or temperate regions.

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