6

Artemisia annua: Botany, Horticulture, Pharmacology

Jorge F. S. Ferreira, James E. Simon, and Jules Janick
Center for New Crops and Plant Products
Department of Horticulture
Purdue University
West Lafayette, Indiana, USA

I. Introduction
II. Botany
  A. Taxonomy
  B. Floral Biology
  C. Glandular Trichomes
  D. Secondary Metabolites
    1. Aromatic Volatiles
    2. Nonvolatile Sesquiterpenes
    3. Chemotaxonomy
III. Horticulture
  A. Field Cultivation
    1. Germplasm
    2. Crop Culture
    3. Harvest
    4. Postharvest Handling
  B. In Vitro Culture
  C. Genetic Improvement
IV. Pharmacology of Artemisinin and Derivatives
  A. The Challenge of Malaria
  B. Chemistry
  C. Isolation and Synthesis
  D. Detection and Quantification
    1. Sample Preparation
    2. Chemical Detection
    3. Immunoassays

Horticultural Reviews, Volume 19, Edited by Jules Janick
ISBN 0-471-16529-8 © 1997 John Wiley & Sons, Inc.

E. Biosynthesis
F. Mode of Action
1. Plant Growth Regulator
2. Antimalarial
G. Drug Development
1. Drug Availability
2. The Future of Artemisinin as an Antimalarial
V. Conclusions
Literature Cited

INTRODUCTION

Artemisia annua L. (Asteraceae, formerly Compositae), 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 a steppe vegetation in the northern parts of Chahar and Suiyuan provinces (40° N, 109° E) in China, at 1000 to 1500 m above sea level (Wang 1961). The plant now grows wild in many countries, such as Argentina, Bulgaria, France, Hungary, Romania (where it is cultivated for its essential oil), Italy, Spain, the United States, and the former Yugoslavia (Klayman 1989, 1993). Although it is unclear how or when *A. annua* was brought to the United States, it was naturalized around Nashville, Tennessee, in the nineteenth century (Gray 1884) and now occurs in waste areas in east and central North America (Bailey and Bailey 1976). *Artemisia annua* is used for the crafting of aromatic wreaths, as a flavoring for spirits such as vermouth, as a source of essential oils, and most recently as a source of artemisinin, the most important natural antimalarial after quinine.

The generic name *Artemisia* refers to Artemis (Greek name for Diana), goddess of maternity, because in antiquity, plants of this genus, probably *A. absinthium* (wormwood), were used to control the pangs of childbirth, to regulate women's menstrual disorders, and as an abortifacient (Riddle and Estes 1992). Many *Artemisia* species are cited by early herbalists including Theophrastus in the third century B.C., (Einarson and Link 1976), Pliny (Bostock and Riley 1855–1857), and Dioscorides (Gunther 1959) in the first century, and Gerard (1597). Wormwood, probably *A. judaica*, is mentioned in the Bible (Rev. 8:10, 11). In A.D. 340, Ge Hong prescribed aerial parts of *Artemisia* for treatment of fevers in the Chinese *Handbook of Prescriptions for Emergency Treatments*, and in 1527, Li Shi-Zhen, a Chinese herbalist/pharmacologist, mentioned the use of *huang hua hao* (or yellow flower, later identified as *A. annua*) for children's fever, and *qinghao* (*A. apiacea*) as a treatment for the disease now known as malaria (Klayman 1993). Today, most references to *qinghao* refer to *A. annua*.

In 1967, Chinese researchers evaluated the effectiveness of several traditional herbal remedies against malaria and *A. annua* was one of the plants selected. While extractions of *A. annua* with hot water or ethanol had no antimalarial effect (Klayman et al. 1984) and a hot infusion made of the dried plant did not cure malaria-infected mice (Klayman 1989), in 1971, a low-temperature extraction of *A. annua* with diethyl ether yielded a complex with antimalarial activity on both infected mice and monkeys. The main active principle, named artemisinin, was isolated and its structure defined in 1972 in China (Anon. 1979). Artemisinin, formerly referred to as arteannuin, also known as *qinghaosu* (in Chinese: *su* = extract from, *qing hao* = green herb) is a rare sesquiterpene lactone endoperoxide belonging to the cadinane series (Brown 1993a). Although priority for the isolation of artemisinin is usually attributed to Chinese workers in 1972, the late D. L. Klayman (1993) reported that D. Jeremic′ first isolated artemisinin but reported an incorrect ozonide structure for the compound (see Jeremic′ et al. 1973). By the end of 1972, artemisinin and derivatives were tested in 10 regions of China with ca. 6000 patients (Klayman 1993).

The isolation and characterization of artemisinin has increased the interest in *A. annua* worldwide and, along with taxol, is considered one of the most novel discoveries in recent medicinal plant research. Artemisinin is the base compound for the synthesis of more potent antimalarial drugs with reduced toxicity for humans. Artemisinin is effective against both *Plasmodium vivax* and *P. falciparum*, two of the four species that cause human malaria, with *P. falciparum* responsible for the often fatal cerebral malaria, an advanced stage of the disease. Although total de novo synthesis of artemisinin has been achieved (Schmidt and Hofheinz 1983; Xu et al. 1986; Ravindranathan et al. 1990; Avery et al. 1992), the low yield and complexity of synthesis required make it apparent that *A. annua* is currently the more economically viable source of artemisinin.

General reviews on *A. annua* as a source of artemisinin have been made by Klayman (1985, 1989, 1993), Woerdenbag et al. (1990), Trigg (1990), and Ferreira and Janick (1995b). Early studies on the chemistry and clinical aspects of artemisinin have been reviewed (Anon. 1982; Luo and Shen 1987; Zhou and Xu 1989; Zaman and Sharma 1991). In 1993, an international symposium on artemisinin was convened in London (Baker 1994) and published information on artemisinin-related antimalarials was updated by Woerdenbag et al. (1994b). In this paper, we review the botany and horticulture of *A.*

annua, and the pharmacology of artemisinin, including chemistry, isolation and synthesis, detection, biosynthesis, mode of action and toxicity, and development of artemisinin-derived drugs.

II. BOTANY

A. Taxonomy

The Asteraceae is the largest dicotyledenous family of flowering plants with members widely distributed. The former name, Compositae, is derived from the many blossoms (or florets) combined into a flower head or capitulum. The plants in this family are mostly annual and perennial herbs, with a few woody species but not usually true trees. The family is composed of about 800 genera and 20,000 species, many highly aromatic and others extremely bitter due to the accumulation of terpenoids (Bailey 1951). Although sesquiterpene lactones occur in other families, the largest number of these compounds isolated in the last 30 years are from the Asteraceae (Fisher 1990).

The Asteraceae is divided into 2 subfamilies: Cichorioideae (Lactucoideae), composed of 8 tribes, and Asteroideae, composed of 9 tribes. *Artemisia* belongs to the tribe Anthemideae of the Asteroideae. Based principally on floral morphology, Hall and Clements (1923) divided the genus into 4 sections: *Abrotanum* Bess., *Absinthium* D.C., *Dracunculus* Bess., and *Seriphidium* Bess. and placed *A. annua* in section *Abrotanum*. Rydberg (1927) and Beetle and Young (1965) added a fifth section, *Tridentatae*, segregating from the section *Seriphidium*, all North American taxa with affinities to *A. tridentata*. Poljakov (1961) combined the sections *Abrotanum* and *Absinthium* into a new merged section *Artemisia*, which places it in conflict with the *Flora Europaea*, which chose instead to combine the sections *Absinthium* and *Seriphidium* into a single section *Artemisia*. The section *Dracunculus* is clearly separated from other subgenera by their sterile central disk florets and other morphological characters, such as hemispheric involucre of flower heads (Greger 1982). Recent taxonomical treatment by Yeou-ruenn (1994) provides yet another classification scheme in which the genus *Artemisia* contains 5 sections, including *Absinthium*, *Abrotanum*, *Artemisia*, plus 2 newer ones *Viscidipubes* Y. R. Ling, and *Albibractea* Y. R. Ling. *Seriphidium*, now elevated to a genus, rather than a section of *Artemisia*; contains 3 sections (*Seriphidium*, *Minchunensa* Y. R. Ling, and *Juncea* (Poljak., Y. R. Ling & C. J. Humphries).

The absence of hairs on the receptacle was the only morphological characteristic separating the sections *Abrotanum* from *Absinthium*. Recent studies by Ferreira and Janick (1995a) established that the trichomes on the capitulum of *A. annua* are the 10-cell biseriate type. There are no distinct chemical characteristics segregating the species into these 2 subgeneric sections, since both produce similar sesquiterpenes belonging to the eudesmanolides and guaianolides class. This may be the reason for Poljakov's (1961) decision to combine the sections *Abrotanum* and *Absinthium*.

The genus *Artemisia* includes ca. 400 species (Heywood and Humphries 1977) worldwide and 65 species north of Mexico in the Western Hemisphere. Species of *Artemisia*, usually shrubs, often with divided leaves and inconspicuous flowers, are known for their bioactive secondary compounds and essential oils used for flavorings, fragrances, and medicinals. Several artemisias had been used by Greek, Roman, Persian, and Arabic physicians as antihelmintics and stomachics (Mehrotra et al. 1990) and were the source of santonin, once a valuable antihelmintic drug, now obsolete due to its toxicity to humans. In addition to *A. annua*, there are several other widely recognized and related herbal species in this genus, including *A. abrotanum* (southernwood), *A. absinthium* (wormwood), *A. dracunculus* (French tarragon), and *A. vulgaris* (mugwort). A brief description of these well-known herbs based on various sources (e.g., Grieve 1971; Stuart 1979, and Simon et al. 1984) follows.

Artemisia annua is named for its annual cycle; other species, with the exception of *A. klotzschiana* Bess., are either biennial or perennial (Hall and Clements 1923). The chromosome number is $2n = 18$ (Bennet et al. 1982). **Description:** *A. annua* is a large shrub often reaching more than 2 m in height. It is usually single-stemmed with alternate branches. The aromatic leaves are deeply dissected and range from 2.5 to 5 cm in length. **Distribution:** The plant is native to China but is currently naturalized in many countries, including the United States (see Introduction). **Constituents:** At least 40 volatiles have been isolated from the essential oil of *A. annua* (see Section II.D), with the main aromatic volatiles including artemisia ketone, 1,8-cineole, camphor, and β-caryophyllene (Charles et al. 1991). The plant also contains several nonvolatile sesquiterpenes of interest, including artemisinic acid, arteannuin B, and artemisinin (Fig. 6.1). Artemisinin has been isolated from leaves and flowers of field-grown (Klayman et al. 1984; Ferreira et al. 1995a) and in vitro-grown plants (He et al. 1983; Nair et al. 1986; Martinez and Staba 1988; Whipkey et al. 1992; Ferreira et al. 1995b; Ferreira and Janick 1996b).

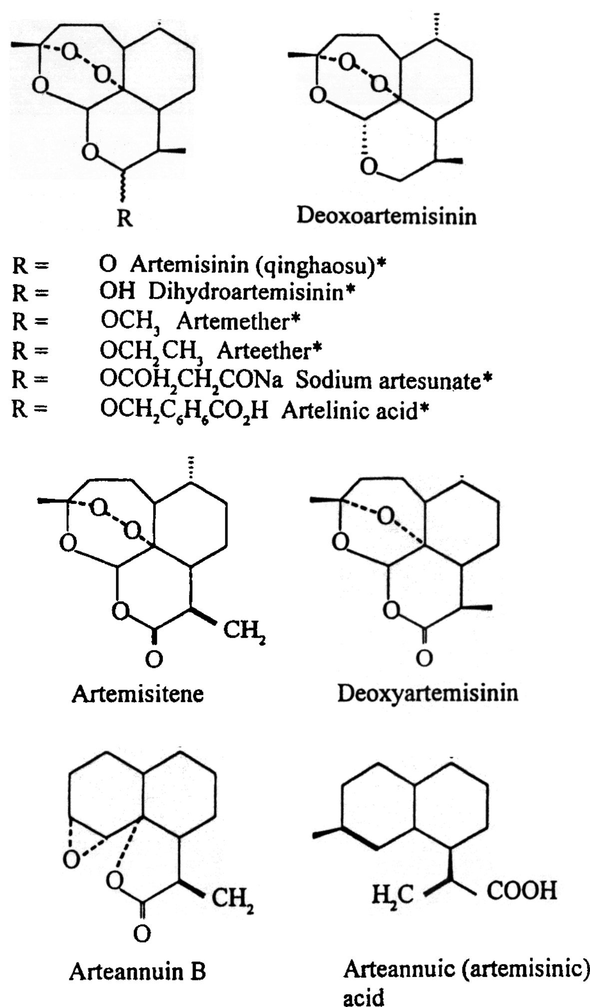

Fig. 6.1. Artemisinin and derivatives (antimalarial compounds indicated by asterisks).

Artemisinin has been detected in leaves, small green stems, buds, flowers, and seeds (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 (Ferreira et al. 1995a). The high-

est concentration of artemisinin is found in the inflorescence, which at anthesis may contain more than 10 times as much artemisinin as leaves (Ferreira et al. 1995a) (Fig. 6.2). The detection of artemisinin from seeds appears to be due to the presence of floral debris (Ferreira et al. 1995a). **Uses:** Used traditionally to treat fevers and hemorrhoids and currently as the source of the antimalarial artemisinin, which is the base compound for more stable, soluble, and potent antimalarial drugs (see Section IV.G); also used in aromatic wreaths.

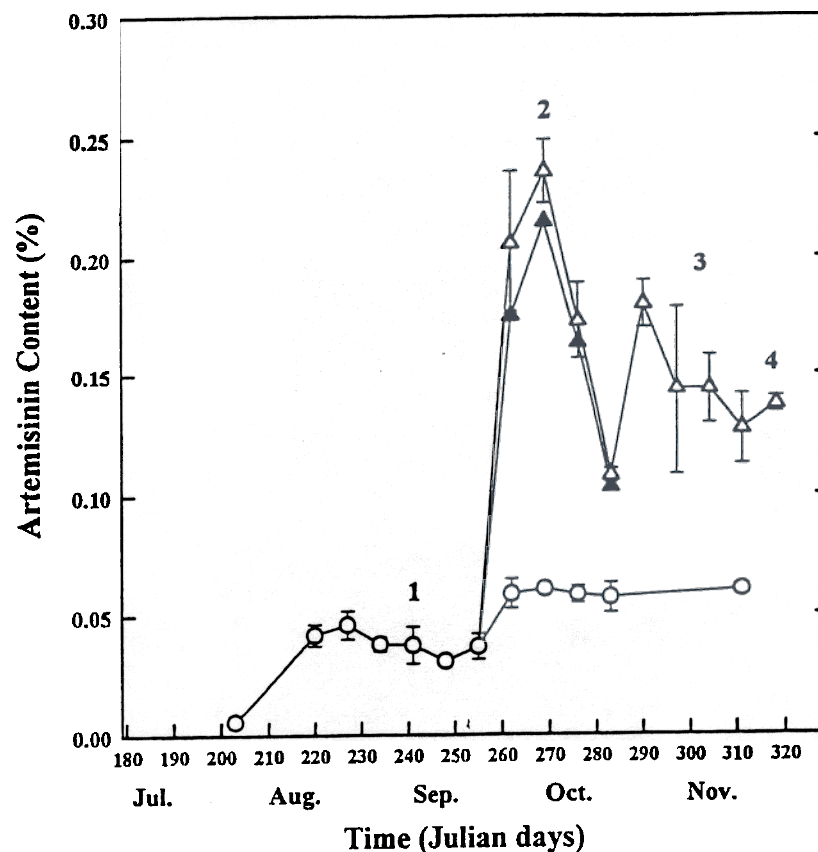

Fig. 6.2. Artemisinin content (% dry wt) of field-grown *A. annua* from leaves (○), inflorescences (△), and whole artemisia plants, in a weighed average basis (▲), sampled from 1 July to November 1992. Development stages, starting from flowering: 1, flower buds visible; 2, pollen shed; 3, immature seeds; and 4, mature seeds. (*Source*: Ferreira et al. [1995a].)

A. abrotanum L. (southernwood) is a popular ornamental and highly aromatic herb known as *garde robe* by the French due to its ability to repel insects such as moths. Field southernwood refers to a different species, *A. campestris* L., native to Europe and Asia. The name southernwood derives from the old English *therne-wudu* meaning a woody plant from the south (southern Europe origin). At one time, herbalists considered the plant to be an aphrodisiac, which might have been responsible for the change in common name from "old man" to "boy's love or lad's love." **Description:** The plant is a perennial erect shrub, 90 cm high, multibranched, with feathery gray-green leaves, 6 cm long, finely divided (thread-like), and somewhat downy, with a sweet, strong fragrance with a hint of lemon. Flowers are very small, inconspicuous, greenish-yellow, in loose panicles, and appear from late summer to early autumn. **Distribution:** The plant is native from Southern Europe but has been introduced in temperate zones as a garden plant, becoming naturalized in the eastern and central portions of North America and now widespread. **Constituents:** Essential oils, mainly absinthol; also an alkaloid called abrotine (Glasby 1991). **Traditional medicinal uses** (dried whole plant): Emmenagogue, febrifuge, antiseptic, antihelmintic, stimulant, and stomachic; used in aromatic baths and poultices for skin conditions. The leaves have been used in aromatic vinegars and can be rubbed on the skin as an insect repellent. Today, this plant is grown as an ornamental garden plant.

A. absinthium L. (wormwood) is referred to in old herbals as a medicinal to counteract the effects of poisoning by hemlock and toadstools, to treat dyspeptic diseases, fever, and gastrointestinal worms (Wilbert 1991), and was used as an antifertility drug in antiquity (Riddle and Estes 1992). It also produces methyl jasmonate, a senescence-promoting substance (Ueda and Kato 1980). Wormwood is one of the bitterest herbs known ("as bitter as wormwood" is a very ancient proverb), and has been used as a principal ingredient in antiseptic fomentations. Roman wormwood (*A. pontica* L.) or small absinthe refers to another distinct herb. **Description:** Perennial, erect-growing underbrush 0.75–1.50 m high; multibranched, hairy stems bearing highly aromatic bipinnate and tripinnate gray-green leaves covered in down. Capitula are 3–4 mm in diameter, with gray-green bracts, and numerous yellow florets appearing from late summer to late autumn. **Distribution:** Central Europe, North America, and Asia. Widely introduced garden plant. Found native on waste ground, especially near the sea, in warm regions; adaptable to a wide range of soil types and soil pH. **Constituents:** Bitter principle is due to absinthin and anabsinthin; volatile oil (0.5–1.0% oil/fresh wt)

stimulates secretions and promotes appetite, contains α- and β-thujone, phellandrene, thyjyl alcohol, cadinene, and azulene; also a glucoside; resins and starch; antihelmintic action due to santonin, a toxic sesquiterpene. Also found in this species are lirioresinol A; 3,6- and 5,6-dihydrochamazulene; the diterpenoid absinthin; and the sesquiterpenoids anabsin, anabsinin, artabsin, artabsinolides A, B, C, and D, artemolin A and B (Glasby 1991). **Traditional medicinal uses:** Whole flowering plant and leaves used as antihelmintic, antipyretic, antiseptic, antispasmodic, carminative, stimulant, tonic, and stomachic. The tincture was formerly used to treat nervous diseases, and the crushed plants in liniments. Wormwood was also used as a pain reliever for women during labor and as a cardiac stimulant. Despite its toxicity in high doses, wormwood had been used by brewers in place of hops (*Humulus lupulus*), as a flavoring for vermouth, bitters, and liqueurs, and, until 1912, as the basis for absinthe, a liquor containing oil of wormwood, anise, and other aromatics, now banned because of its toxicity. **Side effects:** A central nervous system depressant; habitual use causes convulsions, stupor, nervousness, restlessness, and vomiting; overdose cause vertigo, cramps, intoxication, delirium and eventually death. The plant can cause contact dermatitis and is recognized as an insect (moth) repellent.

A. dracunculus L. cv. *sativa* (French tarragon) L. is used as a spice in French cuisine. Plants of the "true" French tarragon, or estragon, are difficult to obtain and maintain. Even under ideal circumstances, its delicate flavor may revert to the coarser flavor of Russian tarragon (Erichsen–Brown 1989). French and Russian tarragon both originated in Russia. Russian tarragon is hardier and the seeds are fertile. French tarragon rarely produces fertile seeds and is considered a sterile derivative of the wild Russian tarragon. Both tarragons are used on the commercial market as a condiment herb and as a culinary garden herb, but the French tarragon is considered to be the true tarragon and is the desired marketed type. Russian tarragon is continually introduced into commerce inadvertently as growers obtain seeds from seed companies marketing it as French tarragon. **Description:** Perennial, multibranched woody plant that can reach up to 1.5 m in height; the plant is slightly hairy, with narrow lanceolate leaves, 3 to 8 cm long and 2 to 10 mm broad. Leaves are green and smooth on both sides with 1 or 2 basal lobes in each side, but can also be finely pubescent. The flowers are greenish-white and 4 mm in size, numerous, brownish on drooping stems. The disk flowers are sterile, the ovaries abortive. **Distribution:** The plant is cultivated extensively in Southern Europe, Israel, and the United States. **Constituents:** Essential oils, sometimes referred to as estragole oil,

the fresh herb yields 0.5–2.5% oil, with dry tarragon yielding about 0.5–0.8% oil. The pale-yellow to amber oil contains aromatic volatiles with methyl chavicol (or estragole) as the principal component (>50% of the total oil). An oleoresin of tarragon is also commercially available. **Traditional medicinal uses:** Dried or fresh herb has no modern medicinal use, but it was once referred to as a "chief medicine" (Erichsen–Brown 1989). The root was formerly prescribed for toothaches and to stimulate the appetite; it was also considered a diuretic and emmenagogue. For culinary use, its delicate anise-flavored leaves are widely used as flavorings in vinegars, salads, beef, chicken, fish, stew, preserves and pickles, shellfish, herb butter, and eggs, and as an ingredient in mustards, perfumes, soaps, and liquors. Tarragon may also act as an antioxidant in foods.

A. vulgaris L. (mugwort or St. John's herb) is an ancient plant, deeply respected throughout Europe and Asia, and once known as the "mother of herbs" (*mater herbarum*). It was one of the 9 herbs employed to repel demons and venoms in pre-Christian times. Although used to flavor drinks, particularly beer, the common name derived from the old Saxon *muggia wort* , meaning midge root, after its ability to repel insects. Japanese mugwort comes from another species, *A. princeps* Pamp. **Description:** Erect, sparsely pubescent, perennial; stems grooved and reddish-purple, angular, reaching 1.75 m; leaves 2.5–5 cm long, pinnate or bipinnate with toothed leaflets, dark green on the adaxial surface and whitish and downy on the abaxial surface. Flowers brownish-yellow to red, numerous and small, arranged on panicles and appearing late summer to mid-autumn. According to Hall and Clements (1923), no other *Artemisia* is as variable in its morphology as *A. vulgaris.* **Distribution:** Asia and Europe. Naturalized in North America. Found in many soil types, along hedgerows, rivers, and streams. **Constituents:** Volatile oils, resin, tannin, and a bitter principle, absinthin, which stimulates digestion. **Traditional medicinal uses:** Dried flowering shoots, leaves, roots used as a diuretic, emmenagogue; stimulates the appetite and helps digestion. The Chinese use the heated leaves for rheumatism. Used also as tea, for stuffing fowl and meat or fish. Repels flies and moths. Leaves have been used to flavor tobacco. Formerly used in flavoring and clarification of beer. **Side effects:** Large dosage and prolonged use injures the nervous system.

B. Floral Biology

A. annua is a determinate short-day plant. Nonjuvenile plants are very responsive to photoperiodic stimulus and flower 2 weeks after

induction (Fig. 6.3). In greenhouse studies, nonjuvenile plants flowered 2 weeks after being moved to 8-, 10-, or 12-h photoperiods, while plants under 16-, 20-, and 24-h photoperiods remained vegetative. In field studies in the northern temperate zone, the first flower buds were visible on 4 September when daylength was 12:57 h at Lafayette, Indiana (40° 21' N). Greenhouse data suggested that induction occurred 2 weeks earlier when the daylength was 13:31 h (Ferreira et al. 1995a). However, temperature × photoperiod interactions have not been investigated.

The nodding flowers (capitula) are greenish-yellow and 2 to 3 mm in diameter, with calyces composed of numerous, imbricated bracts

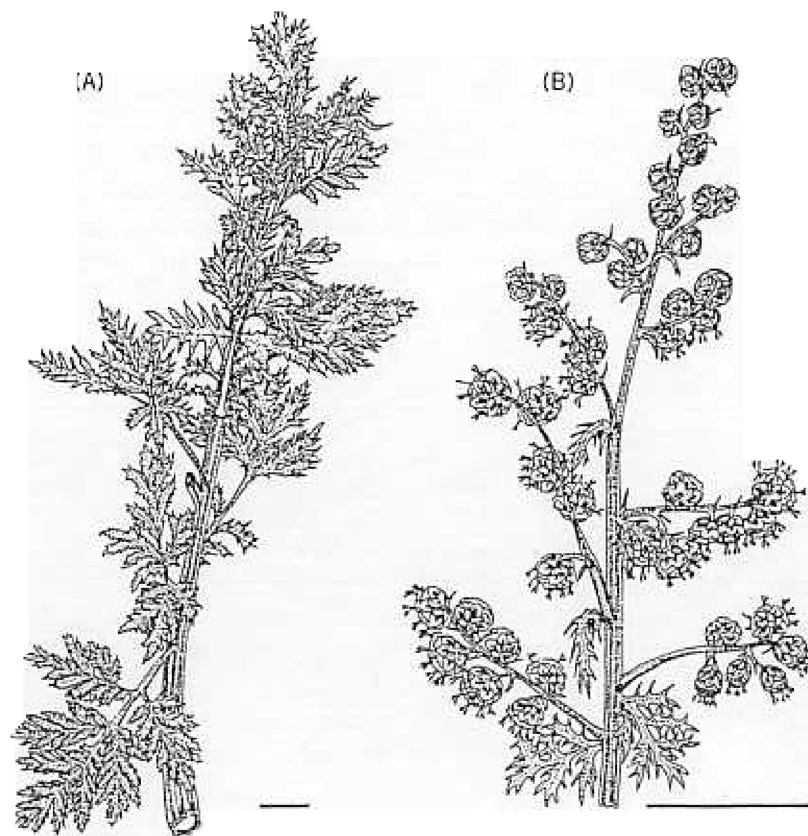

Fig. 6.3. Vegetative (A) and flowering (B) shoot of *Artemisia annua*. Bar size = 1 cm. (*Source*: Ferreira and Janick [1995a].)

(Fig. 6.4A–C). These capitula are displayed in loose panicles containing numerous bisexual flowers (florets) in the center and pistillate marginal florets, the latter extruding stigmas prior to the central flowers (Fig. 6.4B). Both flowers have a synpetalous tubular corolla with the top split into 5 lobes in the hermaphroditic florets and into 2–3 lobes in the pistillate florets. The receptacle is glabrous, not chaffy, and triangular in shape. The stigma is bifid (Fig. 6.4F) with modified acuminate epidermal cells that assist in the capture of the pollen and is referred to as a pollen presenter. The pollen presenter in the Asteraceae is considered to be of the active type, where the growth of the style pushes the pollen presenter past the anthers, causing the pollen to be collected and then extruded for presentation and dispersal by wind action (Ladd 1994). The 5 stamens have bilocular anthers, turned to the center of the floret (introrse), and with the connective attached to the bottom part of the corolla, inferior to the top of the style (Fig. 6.4F). Each stamen has a lanceolate appendix at the top, which alternates with the lobes of the corolla (Ferreira and Janick 1995a). Ovaries are inferior and unilocular and each generates one achene (Bailey 1951) ca.1 mm in length and faintly nerved. The pollen is tricolpate and relatively smooth, typical of anemophylous species (Fig. 6.4G,H). Pollen grains have an internal columellae–tecta complex configuration in the exine, which is common to all taxa of the tribe Anthemideae and seems to vary from 2–3 layers in *A. annua* (Skvarla and Larson 1965). The pollen is extremely allergenic as in other species of *Artemisia* (Mitchell 1975; Arora and Gangal 1991; Rantio–Lehtimaki et al. 1992). The allergenic protein has been shown to be present on the surface of the exine (Park et al. 1993).

The plant is naturally cross-pollinated by insects and wind action, which is unusual in the Asteraceae (McVaugh 1984). The floral morphology of *A. annua*, described on the basis of both light and scanning electron microscopy (Ferreira and Janick 1995a), indicate that the capitula is adapted to self-pollination. Experimental data, however, indicates that self-pollination is not only rare but difficult to achieve by bagging (Peter–Blanc 1992), which infers the presence of self-incompatibility in this species as in other members of the Asteraceae (North 1979).

C. Glandular Trichomes

Trichomes and glands, which contain volatile monoterpenes and sesquiterpenes, are common to the Asteraceae (Mehrotra et al. 1990).

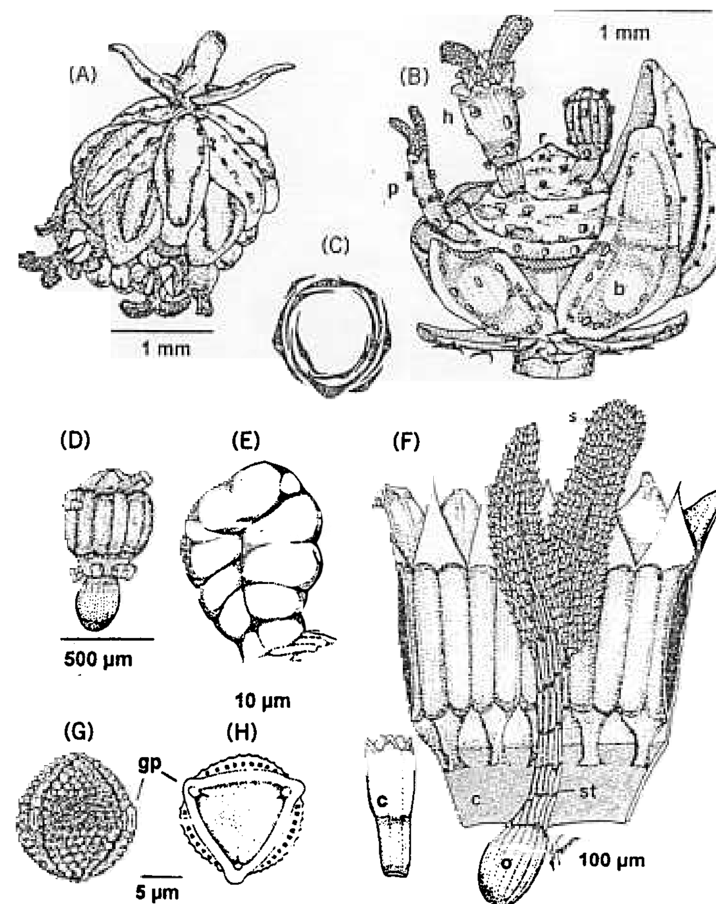

Fig. 6.4. Floral morphology of *A. annua.* (A) Nodding capitulum. (*B*) Expanded capitulum showing calyx with imbricated bracts (b), receptacle (r), marginal pistillate floret (p), and internal hermaphroditic (h) florets. Glandular trichomes are found abundantly on the receptacle, bracts, and florets of the capitulum. (*C*) Cross section of the involucre showing imbrication of bracts. (*D*) Unexpanded floret showing orientation of glandular trichomes. (*E*) Fully developed, turgid, glandular trichome, based on SEM. (*F*) Details of hermaphroditic floret with lobed anthers attached to basal portion of the corolla (c), pistil with bifid stigma (s), style (st), and ovary (o). Note that in a hermaphroditic floret, the stigma reaches this state of development only after pollen shed. (*G*) Tricolpate pollen grain with vestigial spines, characteristic of wind-pollinated species, and germination pores (gp) bulging from the furrows. (*H*) Pollen crosssection based on light microscopy shows details of bulging germination pores. (*Source:* Ferreira and Janick (1995a).)

These terpenoids are localized in the heart-shaped glandular trichome found in species of this family (Kelsey and Shafizadeh 1980; Rodriguez et al. 1976). Illustrations in floras indicate glandular trichomes on the florets of *A. paniculata* (Besser 1843), *A. mauiensis* (Degener 1939), *A. annua* (Makino 1960), and various other species (Hall and Clements 1923). Biseriate glandular trichomes have been reported on both leaf surfaces of *A. nova* (Kelsey and Shafizadeh 1980), in floral stalks of *A. tridentata* (Slone and Kelsey 1985), on both leaf surfaces and ovary surfaces of *A. umbelliformis* (Cappelletti et al. 1986), and in the adaxial leaf surface of *A. campestris* ssp. *maritima* (Ascensão and Pais 1987). Biseriate glands were reported to be isolated and collected from various *Artemisia* species by Slone and Kelsey (1985). Biseriate glandular trichomes were observed in the leaves of *A. annua* (Duke and Paul 1993) at the earliest stage of development, but later became obscured by filamentous T-trichomes. Both biseriate and filamentous trichomes are arranged in two rows, in troughs, along either side of the leaf midrib, but are arranged randomly on the abaxial surface of the leaf and stems.

Duke and Paul (1993) described the origin of biseriate glands in leaves, based on light, scanning, and transmission microscopy. The earliest stage of gland formation observed is the 1-cell stage, in which a single epidermal cell enlarges and protrudes above the leaf surface. After considerable expansion, this cell divides anticlinally, and then both of the resulting cells divide periclinally. In the 1- to 4-cell stage, vacuoles are relatively small and plastids are proplastids with only a few unstacked thylakoids. The 6-cell stage contains chloroplasts with few stacked thylakoids and no starch grains; lack of starch grains is the only distinguishing feature between chloroplasts of glands and mesophyll tissues. The final 10-cell stage is the result of further periclinal cell division of the 2 apical cell layers. After all 10 cells form, the cuticular surface of the gland begins to separate from the cell wall, near the tip of the gland. The onset of cuticular detachment was considered to be associated with the onset of secretory activity. Subcuticular space borders the 6 apical cells of the gland. Cytoplasm is denser at this stage than earlier and, at this point, the two basal cells contain chloroplasts and relatively large vacuoles. The apical cell pairs generally have no chloroplasts and the subapical 2- cell pairs (4 cells) contain large, amorphous chloroplasts without starch grains. Transmission and scanning electron photomicrographs of bisseriate glandular trichomes are shown in Fig. 6.5.

Ten-celled biseriate glandular trichomes are abundant in the bracts, receptacles, and florets of the capitulum in *A. annua*, as shown in

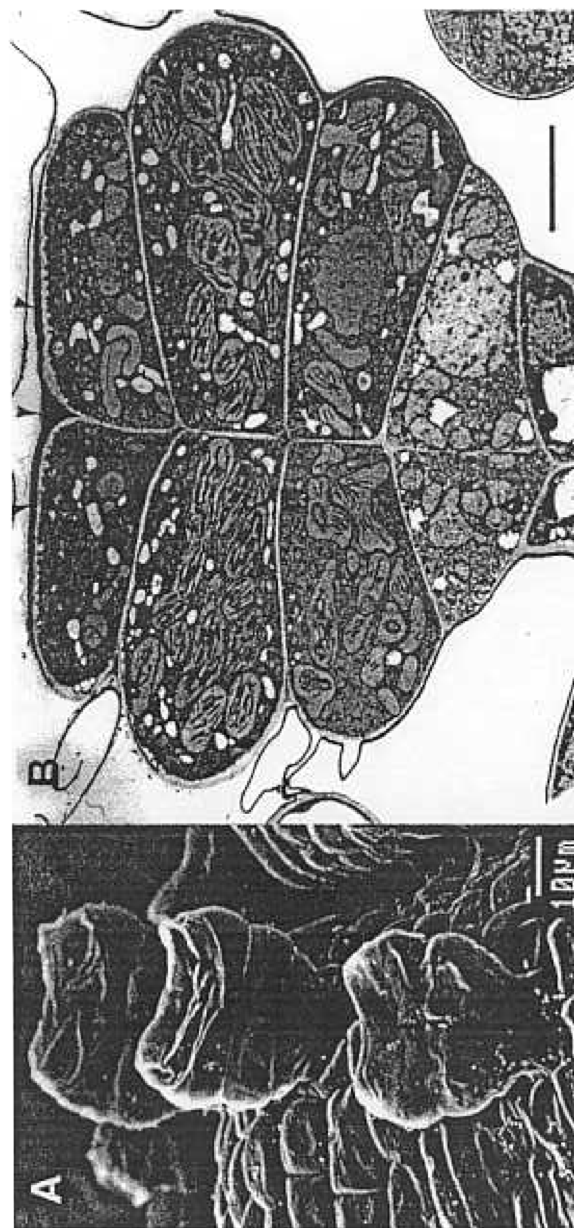

Fig. 6.5. Biseriate glandular trichomes of *A. annua*. (A) Scanning electron micrograph (SEM) of glandular trichome at the basal portion of the corolla with apical cells collapsed due to fixation process. (*Source:* Ferreira and Janick [1995a].) (B) Transmission electron micrograph (TEM) of a 10-cell stage gland. The arrows indicate the dense layer of osmiophilic material that comprises a portion of the cell walls of the two apical cells. The apical cell pair has no chloroplasts, but the subapical two cell pairs (4 cells) contain large, amorphous chloroplasts without starch grains. Bar size = 5 μm. (*Source:* Duke and Paul [1993].)

Fig. 6.4B,D,E (Ferreira and Janick 1995a). These glands are easily ruptured, releasing the volatile oils when florets are pressed, and occur in both pistillate and hermaphroditic florets from their earliest developmental stages. Five-celled T-shaped trichomes with an elongated top cell occur abundantly in stems, pedicels, and stipules but rarely in bracts of the capitulum.

The floral biseriate trichomes originate in the same manner as leaf glandular trichomes. During floral development, the subcuticular space at the apex of glandular trichomes enlarges to a sac-like structure that surrounds 4 to 6 apical cells. These apical cells discharge fluids into the sac-like structure, which expands and bursts during trichome disintegration. Intact glandular trichomes were seldom observed in florets after anthesis, but a few reached the final stage of flower development intact.

Duke et al. (1994) concluded that the subcuticular spaces of the biseriate glandular trichomes in leaves of *A. annua* are the sites of sequestration of artemisinin and artemisitene because both compounds were lacking in a glandless biotype, and virtually all artemisinin and artemisitene could be extracted by a 5-s leaf dip in chloroform, without visible damage to other leaf epidermal cells. Artemisinin was also extracted by a 1-min dip of inflorescences in petroleum ether or acetonitrile (Ferreira and Janick 1995a).

Artemisinin appears to be sequestered in biseriate foliar and floral glandular trichomes of *A. annua*. Although glandular trichomes are present in the early stages of flower formation, artemisinin content peaks when flowers approach full bloom (anthesis). This suggests that artemisinin must be produced or sequestered in these glands as they mature. Immunocytochemical localization of artemisinin will be necessary to confirm these glands as the sites of artemisinin synthesis.

D. Secondary Metabolites

1. Aromatic Volatiles. The highly aromatic volatile or essential oils of *A. annua*, composed primarily of terpenoids, with some phenylpropanoids and nonvolatile fatty acids, are abundant in the leaves and flowers, with only trace amounts found in the main or side stems and roots (Charles et al. 1991). Highest yields of essential oils have been reported at flowering (Simon et al. 1990). The volatiles of *A. annua* are extracted via hydrodistillation or steam distillation and have been chemically characterized by gas chromatography and GC/mass spectroscopy (Libbey and Sturtz 1989; Lawrence 1990;

Simon et al. 1990; Charles et al. 1991; Woerdenbag et al. 1993a; Ahmad and Misra 1994; Woerdenbag et al. 1994a). More than 60 constituents are reported in the distilled oil (Table 6.1), and significant variation in both oil content and oil composition within this species has been noted (Charles et al. 1991; Woerdenbag et al. 1993a, 1994a). The major volatile constitutents (>5% of the total essential oil, and highest reported values for each compound) include artemisia ketone (68.5%, Charles et al. 1991), 1,8-cineole (31.5%, Libbey and Sturtz 1989), camphor (27.5%, Charles et al. 1991); germacrene D (18.9%, Woerdenbag et al. 1994a), camphene hydrate (12%, Charles

Table 6.1. Essential oil constituents in *Artemisia annua*.

Allo-aromadendrene	γ-Cadinene
α-Copaene	γ-Elemene
α-Humulene	γ-Muurolene
α-Pinene	γ–Terpinene
α-Terpinene	Germacrene-D
α-Terpineol	Isoeugenol
Limonene	Isopinocamphone
α-Thujene	Linalool
α-Thujone	Longipinene
Artemisia alcohol	Myrcene
Artemisia ketone	Myrtenal
Benzyl isovalerate	Myrtenol
Benzyl 3-methyl butyrate	*n*-Nonacosane
Borneol	*n*-Pntacosane
β-Bisabolene	*p*-Cymene
β-Cadinene	*p*-Ethylcumene
β-Caryophyllene	Pinocarvone
β-Cubebene	Sabina ketone
β-Farnesene	Sabinene
β-Pinene	Sabinol
cadinene	Santolinatriene
Camphene	Terpinen-4-ol
Camphene hydrate	*trans*-2,7-Dimethyl-4,6-octadien-2-ol
Camphor	*trans*-Pinocarveol
Caryophyllene oxide	*trans*-Sabinene hydrate
Chrysantheone	4-Tetramethyl ether
cis-Chrysanthenol	3,7,7-Trimethylbicyclo[3.1.1]-2-heptene
cis-Chrysanthenol acetate	Unknown (MW 220)
cis-Sabinene hydrate	Yomogi alcohol
(*E*)-6-Methyl-3,5-heptadien-2-oney	1,8-Cineole
Fenchol	

Source: Anon (1982), Libbey and Sturtz (1989), Lawrence (1990), Charles et al. (1991), Woerdenbag 1993a, 1994a, and Ahmad and Misra (1994).

et al. 1991), α-pinene (16%, Charles et al. 1991), β-caryophyllene (8.6%, Woerdenbag et al. 1994a), myrcene (8.5%, Woerdenbag et al. 1994a); and artemisia alcohol (7.5%, Woerdenbag et al. 1993a). Borneol was erroneously reported as artemisia ketone in earlier literature reports (Ahmad and Misra 1994).

2. Nonvolatile Sesquiterpenes. Many of the sesquiterpenes of *A. annua* are nonvolatile, and, thus, not released upon water or steam distillation, and have little role in aroma or fragrance. These compounds can be denatured by the process of oil extraction. These nonvolatile sesquiterpenes can be recovered from the plant by solvent extraction, and some show high antimalarial activity.

At least 20 known sesquiterpenes are produced by *A. annua*, including arteannuin A (artemisinin), arteannuin B, artemisitene (Acton and Klayman 1985), artemisinic acid, artemisilactone, qinghaosu I, II, III, and IV (Glasby 1991), artemisinin G (Wei et al. 1992), annulide (Brown 1993a); deoxyisoartemisinin, deoxyisoartemisinin C, compound 5 (unnamed), 6,7-dehydroartemisinic acid, artemisinin C, qinghaosu VI, and deoxyartemisinin (Ranasinghe et al. 1993), plus 3-isobutyrl cadin-4-en-11-ol, cadin-4(7),11-dien-12-al, cadin-4(15),11-dien-9-one (Ahmad and Misra 1994). Almost all belong to the cadinane series (Brown 1993b), and many are bioactive (Duke et al. 1988), except for the last 3 cadinanes, which have not been evaluated. No other cadinanes have been reported outside this species. Further discussion on the chemistry of these compounds is found in Section IV.

3. Chemotaxonomy. The widespread occurrence of volatile and nonvolatile monoterpene and sesquiterpenes in the Asteraceae has led to the use of these compounds as possible taxonomic markers in the classification of this family as well as in the treatment of the genus *Artemisia*. In the earlier 1923 morphological classification of Hall and Clements, *A. annua*, *A. absinthium*, and *A. vulgaris* were all included in the section *Abrotanum* (Seaman 1982). Kelsey and Shafizadeh (1979) reviewed the systematics of *Artemisia* based on its chemical constituents and concluded that *A. annua* belongs within the section *Abrotanum*, because it contains novel nonvolatile sesquiterpenes such as arteannuin-B and artemisinin, but placed *A. absinthium* in the section *Absinthium*. Artemisinin and other cadinanes are unique to *A. annua*, with the possible exception of *A. apiacea* (Liersch et al. 1986). In contrast, many of the same monoterpenes are common to several species. For example, based on the volatile oils, *A. annua* could appear to be similar to *A. absinthium*

(section *Absinthium*), but the presence of artemisinin and related derivatives brings *A. annua* chemotaxomically closer to species belonging to the section *Abrotanum*. High concentrations of artemisia ketone and its derivatives in the volatile oils of *A. annua* suggest possible systematic significance since these compounds have not been found outside tribes of the Asteraceae, but some chemotypes of *A. annua* are devoid of this compound. Because of the widespread occurrence of both monoterpenes, and in particular certain sesquiterpenes in the Asteraceae, Seigler (1981) argued that the use of these chemical constituents are of limited value in establishing the phylogeny of higher plants.

Greger (1982) suggested that the distribution of different polyacetylenes, coumarin–sesquiterpene ethers, and sesamin-type lignans, rather than the aboveground monoterpenes, sesquiterpene lactones, or flavonoids, is a better chemical marker for differentiating species within the section *Artemisia*. The sections *Abrotanum* and *Absinthium* produce the most structurally diverse polyacetylenes and species of *Abrotanum* produce the most structurally diverse and biosynthetically complex sesquiterpene lactones within the Asteraceae (Kelsey and Shafizadeh 1979).

The differential accumulation patterns between spiroketalenol ethers, found in sections *Artemisia* and *Absinthium*, and that of pontica epoxides, as found in *Abrotanum* (in which *A. annua* resides), may also be useful markers in separating sections (Greger 1982). Yet, *A. annua*, which accumulates high epoxides, is also considered phylogenetically close to *A. biennis* Willd., *A. tournefortiana* Reich., and *A. klotzschiana*, all of which accumulate high concentrations of spiroketalenol ethers (Hall and Clements 1923; Poljakov 1961), making the differential accumulation of these compounds of lesser significance in classification. The sections *Drancunculus* and *Seriphidium*, in contrast, accumulate dehydrofalcarinone derivatives.

III. HORTICULTURE

A. Field Cultivation

Field cultivation of *A. annua* is presently the only commercially viable method to produce artemisinin because the chemical synthesis of this complex molecule, while possible, is uneconomical. Traditionally, *A. annua* has been collected from wild stands in China, which still provide much of the raw material that is harvested and processed for human clinical trials as a new antimalarial drug in

southeast Asia (WHO 1994). As interest and use of artemisinin has increased, a number of studies have been carried out to introduce *A. annua* as a cultivated crop, (Simon et al. 1990; Laughlin 1993, 1994). Because *A. annua* is a short-day annual, cultivation will be unadapted in the tropics, where plants will flower without achieving sufficient biomass. Trials on *A. annua* as a field crop have been carried out in Australia (Laughlin, 1993, 1994), Brazil (Magalhães 1994), India (Singh et al. 1986, 1988), Switzerland (Delabays et al. 1992, 1993), the United States (Simon and Cebert 1988; Simon et al. 1990; Morales et al. 1993, Ferreira et al. 1995a), and Vietnam (Woerdenbag et al. 1994a) with encouraging results.

1. Germplasm. Investigations on *A. annua* germplasm for artemisinin in many countries has indicated highly variable artemisinin content, sometimes as low as 0.01% (Trigg 1990). In the United States, strains have been detected with mean artemisinin concentrations ranging from 0.05 to 0.21% and individual plants producing up to 0.42% at the full flowering stage (Ferreira et al. 1995a). Swiss researchers reported clones of Chinese origin that produce 1.1% artemisinin (Delabays et al. 1993), but these clones have been unavailable to most researchers. Hybrids between these high artemisinin, low vigor, Chinese clones with vigorous, low artemisinin clones of Italian (0.04%), Yugoslavian (0.16%), and Spanish (0.22%) origin contained 0.7 to 0.8% artemisinin combined with high vigor (Delabays et al. 1993). The difficulty in developing high biomasss yielding lines or cultivars rich in artemisinin is due in part to the unavailability of germplasm sources, the absence of methods for hybridization, and the lack of an inexpensive, rapid screen for artemisinin and its derivatives.

Evaluation of germplasm of *A. annua* for essential oils has been conducted in the United States, and accessions rich in specific oil constituents such as artemisia ketone, camphor and 1,8-cineole have been identified (Charles et al. 1991). These accessions could be used to develop lines with custom designed oil profiles within the genetic limits of the germplasm. *Artemisia annua* of Chinese origin contains artemisia ketone as a major component (Charles et al. 1991, Ahmad and Misra 1994), while *A. annua* of Vietnamese origin lacked this compound (Woerdenbag et al. 1994a), suggesting large genetic variation in essential oil composition. Given the range of essential oil composition reported in germplasm collections, cultivars of *A. annua* with high oil or distinct volatile oil profiles could be developed for the flavor and fragrance industry.

2. Crop Culture. Although asexual propagation is easily achieved by cuttings from vegetative plants, clonal propagation from elite clones for field production would require that stock plants be maintained under long days to prevent flowering. Seed propagation, however, is the most practical and economic method for commercial propagation. Artemisinin seeds keep their vigor for at least 3 years if stored dry under cool conditions. In temperate zones the minuscule seeds that self sow, germinate early in the spring in the field of the following year. As such, *A. annua* has become naturalized as a weedy annual, adapted to both fertile farm land and waste areas. Specialized seed planters can uniformly sow the very small seed. The seeds can be coated or mixed with inert materials for improved sowing and seed distribution. Shallow planting coupled with frequent light irrigations has worked relatively well to achieve good stands. The most important problem to overcome in crop culture is uniform crop establishment and weed control.

Most researchers (Acton et al. 1985; Liersh et al. 1986; Singh et al. 1988; Laughlin 1993; Morales et al. 1993) transplant artemisia to the field at the 5–6 leaf stage. This usually requires 4 to 6 weeks of greenhouse growth. In areas with sufficiently long growing seasons, direct seed planting may produce similar dry matter and artemisinin yields (Laughlin 1993), and could be a practical alternative to transplanting with effective preplanting herbicide programs.

Although weed control is one of the major production costs, no herbicides are registered for use in the United States on *A. annua* and the prospects for new registrations appear bleak. Future production strategies must be developed with and without the application of herbicides. Early-season weed control is essential to allow vigorous crop development, since few weeds develop under the canopy. Several herbicides have been identified that could be used in production. Chloramben at 2.2 (a.i.) kg/ha before emergence or trifuralin at 0.6 kg/ha, incorporated before transplanting, followed by fluazifol at 0.2 + 0.2 kg/ha broadcast after emergence, and acifluorfen at 0.6 kg/ha after emergence gave good weed control without reduction of leaf yield or artemisinin content in Mississippi (Bryson and Croom 1991). Preplant application of 2.2 a.i. kg/ha napropamide resulted in good weed control without phytoxicity (Simon and Cebert 1988).

Studies on time of planting indicate that in northern temperate zones, late spring or early summer plantings are best to achieve high biomass yields. The key step to maximize artemisinin yields is to achieve high biomass before the onset of floral induction, which is

initiated when the photoperiod is ca. 13.5 h. Late-season planting of *A. annua* results in very short flowering plants with low yields. There is a possibility of fall sowing with the overwintering of young seedlings but little information is available on winter survival in geographical zones in which this technique might be successful. In India, establishment is best achieved by planting in the cool period of mid-December, while in Tasmania, October plantings doubled leaf dry matter as compared to November plantings (Laughlin 1994). In Indiana, plants were field transplanted every month from April through July and sampled biweekly for growth and oil accumulation. Maximum essential oil production occurred at peak flowering. May and June transplanting dates resulted in the highest essential oil yields (Simon et al. 1990).

A. annua responds well to balanced fertilizers, and appears to be responsive to nitrogen (relative to growth), but there are few data available on the accumulation of artemisinin relative to fertility. Srivastava and Sharma (1990) reported that micronutrients such as boron may increase artemisinin concentration, but it is unknown whether the soils used were deficient or whether artemisia is responsive to boron. Field experiments in Tasmania (Laughlin 1993) indicated that lime increased dry matter yields in soils of pH 5.0. In greenhouse trials, leaf yields were optimal and remained relatively constant from pH 5.5 to 7.4, and pH had little effect on artemisinin content. Water stress during the 2 weeks before harvest led to reduced leaf artemisinin content (Charles et al. 1993). Shukla et al. (1992) reported that exogenous application of plant growth regulators may increase artemisinin content as well as plant height, but further studies are needed to determine whether growth regulators can be used effectively.

Initial artemisinin yield studies in Switzerland were carried out at plant densities of 2.5 plants/m^2 (Delabays et al. 1993). In Tasmania, dry matter yields increased from 2.0 to 6.8 t/ha as density increased from 1 to 20 plants/m^2; plant density did not affect artemisinin or artemisinic acid content (Laughlin 1994). In Indiana, biomass yield increased from 2.7 to 11.1 plants/m^2 at 0, 67, and 134 kg N/ha, but optimum biomass was achieved at 67 kg N/ha (Simon et al. 1990). Hybrids obtained from Switzerland and tested in Campinas, Brazil, between September and November produced 2.15 t of dried leaves/ha, 6 kg/ha of artemisinin, and 14.6 kg/ha of artemisinic acid when spaced 0.3 × 0.5 m (Magalhães 1994).

Increasing density also increased essential oil production per area as a direct result of the increase in total biomass (Simon et al. 1990).

For commercial oil production, plants can be field cut, allowed to partially air dry, and then be steam distilled with excellent results. The mechanized systems used in commercial mint production and distillation work well with *A. annua*.

For the herbal trade as a dried aromatic wreath, production recommendations differ, since individual phenotypic characteristics are critical for the drying and shaping of individual plants. Here, greater spacings between plants results in higher number of side or lateral shoots, and an increased dry weight gain per plant, desirable traits for the ornamental industry. Studies examining the influence of topping on subsequent plant growth resulted in a significant reduction in final plant height, biomass yield, and lateral shoot length or spread (Simon and Cebert 1988). Plants have been identified that exhibit different growth habits and flowering times, indicating the potential to select for ornamental traits in a given population of *A. annua*.

3. Harvest. Studies by Ferreira et al. (1995a) in which artemisinin was evaluated separately in leaves and inflorescences clearly indicate that artemisinin content is highest in inflorescences with maximum concentration close to or at anthesis. This is confirmed in a number of greenhouse and field trials, where peak artemisinin was achieved during full flowering (Singh et al. 1988; Pras et al. 1991; Morales et al. 1993; Ferreira et al. 1995a), although others report artemisinin content highest with harvest just before flowering (Acton et al. 1985; Liersch et al. 1986; El-Sohly 1990; Woerdenbag et al. 1991, 1994a; Laughlin 1993).

Because artemisinin content is relatively low, harvesting is best carried out when artemisinin content per unit area is at a maximum, in order to reduce extraction and processing costs. Thus, the optimum time of harvest must take into consideration maximum artemisinin content as well as biomass yield. Recently, there has been some interest in producing artemisinin from artemisinic acid (arteannuic or qinghao acid), which is 8- to 10-fold more abundant than artemisinin (Roth and Acton 1987; Jung et al. 1990a; Laughlin 1993; Vonwiller et al. 1993). If this technique should prove feasible, optimum harvest will have to take both compounds into consideration.

The concept of harvesting artemisia for both essential oils and artemisinin or artemisinin derivatives warrants exploration. If artemisinin is the targeted product this may not be possible, since steam distillation could destroy the peroxide bridge. If artemisinic acid, which does not contain a peroxide bridge but can be converted

to artemisinin (Sangwan et al. 1993), is the targeted chemical, then dual procurement of essential oils and artemisinic acid may be feasible.

The harvest of *A. annua* for the floral and craft trade is much different than that for the extraction of secondary products. Plants need to be harvested 15–25 cm above the ground and should be taller than 1.5 m. For the craft trade, plants are harvested at full bloom, prior to seed maturation, as the color changes from dark green to a more golden color. For the floral trade, plants are harvested later as the seeds develop. Once the plants are harvested, they are usually dried in a shaded area or under forced heated air, not to exceed 35°C. Quality of this product is based mainly on aroma and visual appearance (color, size, shape of plant, and stage of development).

4. Postharvest Handling. Because of the tremendous bulk of biomass produced by *A. annua*, plant material has to be at least partially dried before processing. Ferreira et al. (1992) compared artemisinin content by HPLC-EC from freeze-dried, oven-dried (40°C), and indoor air dried *A. annua* shoots. Highest artemisinin yields were obtained with indoor air drying (0.13%) as compared to oven drying (0.10%) and freeze drying (0.02%). Time of air drying (2, 4, 6, or 8 days) did not affect artemisinin content.

Microwave (2 min, 100% power) drastically reduced or eliminated artemisinin from 5.0 g fresh wt leaf samples, and artemisinin was reduced by ca. 50% when the samples were microwave dried for 5 min at 50% power (Ferreira et al. 1992). However, microwaving of standard artemisinin solutions for 5 min at 50% power caused no artemisinin loss as determined by HPLC-EC (J. F. S. Ferreira, unpublished).

B. In Vitro Culture

Many investigators have reported successful in vitro propagation of *A. annua* via shoot cultures (He et al. 1983; Nair et al. 1986; Martinez and Staba 1988; Fulzele et al. 1991; Elhag et al. 1992; Whipkey et al. 1992; Woerdenbag et al. 1993c; Brown 1993b; Ferreira and Janick 1996b). Shoots are easily cultured using standard protocols and cytokinin supplementation. Benzyladenine (BA) and coconut water are effective in inducing shoot formation (Whipkey et al. 1992). In vitro-grown plants readily acclimate to soil. However, many plants derived from tissue culture appear to have cytokinin abnormalities, being highly branched and bushy without apical dominance. Such plants do not flower unless their growth habit reverts to normal (Ferreira 1994).

Callus can be obtained with media supplemented with combinations of auxin and cytokinins (Martinez and Staba 1988; Kim et al. 1992; Brown 1993; Ferreira and Janick 1996b), but nonfriable callus is usually obtained (Nair et al. 1986). The highest yields of friable callus were obtained with 4.4 μM BA and 4.5 μM 2,4-D, but only 10% of the clones generated callus (Ferreira et al. 1996). Cell cultures can be obtained with difficulty from callus cultures in liquid callus induction medium. Artemisinin appears only in trace amounts in undifferentiated callus and cell cultures (Nair et al. 1986; Kudakasseril et al. 1987; Liu et al. 1992, Woerdenbag et al. 1992, Ferreira et al. 1996) or not at all (He et al. 1983; Tawfiq et al. 1989; Fulzele et al. 1991; Brown 1993b; Ferreira and Janick 1996b), suggesting that a certain degree of differentiation is required for artemisinin production (Martinez and Staba 1988, Fulzele et al. 1991, Brown 1993b). The medium from cell cultures had no detectable levels of artemisinin (Fulzele et al. 1991; Ferreira and Janick 1996b), but Nair et al. (1986) reported low levels of artemisinin from culture medium from callus.

There are inconsistencies in the literature regarding the presence of artemisinin in different organs of in vitro-grown plants. Artemisinin is produced by differentiated shoot cultures, i.e., shoots plus roots (Martinez and Staba 1988; Fulzele et al. 1991; Whipkey et al. 1992), but occurs at trace levels, if at all, in shoots without roots (Martinez and Staba 1988; Jha et al. 1988; Fulzele et al. 1991; Woerdenbag et al. 1993b; Paniego and Giulietti 1994, Ferreira and Janick 1996b). Most workers (Martinez and Staba 1988; Tawfig et al. 1989; Fulzele et al. 1991; Kim et al. 1992; Ferreira et al. 1996) did not detect artemisinin in roots in vitro, although Nair et al. (1986) and Jha et al. (1988) reported trace amounts. Recently, hairy root cultures of *A. annua* have been produced by transformation with *Agrobracterium rhizogenes* (Weathers et al. 1994; Jaziri et al. 1995). Weathers et al. (1994) reported high artemisinin levels (0.4%), but those hairy root cultures have been unstable (P.J. Weathers, personal communication) and Jaziri (1995) could not confirm the presence of artemisinin in hairy root cultures. Because artemisinin is highly phytotoxic (Duke et al. 1987) and appears to be sequestered only in glandular trichomes (Duke et al. 1994; Ferreira and Janick 1995a), the reports of high artemisinin in root cultures appears suspect, but, if true, could have a profound effect on our understanding of artemisinin biosynthesis.

A number of studies have evaluated the effect of growth substances on the production of artemisinin from shoot cultures. Artemisinin increased 7 times in shoot cultures treated with 100 μg/mL of

miconazole for 6 weeks (Kudakasseril et al. 1987). Woerdenbag et al. (1993b) reported 0.16% artemisinin in shoot cultures maintained in Murashige and Skoog medium supplemented with 0.2 mg/L BA, 0.05 mg/L NAA, and 1% sucrose. Artemisinin increased with 10 mg/L GA_3 (54%), 0.5 g/L casein hydrolysate (69%), and 10 or 20 mg/L naftine (40%), while other growth regulators, such as miconazole and terbinafine; elicitors, such as cellulase, chitosan, glutathion, and nigeran; the precursor mevalonic acid; and gene regulators, such as 5-azacytidine and colchicine had a negative effect or no effect on shoot artemisinin production.

Whipkey et al. (1992), based on unreplicated data, reported that 6-benzylaminopurine (BA) at 0.4 μM (1.0 mg/L) and kinetin at 46.5 μM (10 mg/L) increased the yield of artemisinin in shoot cultures about 30%, but this increase was due to an increase in dry matter production, which overcame a decrease in artemisinin content (in mg/g dry wt). Daminozide at 0.6, 6.2, and 62.4 μM and chlormequat (CCC) at 0.6, 6.3, and 63.4 μM were evaluated because growth retardants which influence gibberellins have been shown to increase terpene formation (El-Keltawi and Croteau1986, 1987). Daminozide and CCC greatly reduced dry weight of shoot cultures at all concentrations, but increased artemisinin content (mg/g dry wt) at all but the lowest concentration of CCC. This experiment was repeated using replicated treatments and an improved HPLC-EC procedure for detection and quantification of artemisinin (Ferreira and Janick 1996a). None of the growth regulators significantly increased artemisinin accumulation, although artemisinin content with CCC at 6.3 μM was higher than the control (0.019 vs. 0.014%). Artemisinin was undetected in the presence of BA at 4.4 μM and kinetin at 4.6 and 46.5 μM.

In this experiment, there was a significant correlation over all treatments ($r = .775$, $P = 1\%$) between root number and artemisinin content. In a separate study on the effect of BA (0.0, 0.5, 5.0, and 50 μM), shoot production was maximal at 5.0 μM BA, but rooting and artemisinin decreased as BA increased with rooting and artemisinin highest in BA-free medium. BA also increased shoot vitrification. Martinez and Staba (1988) also reported an increase in artemisinin when plants developed a root system. Roots do not contain artemisinin but evidently enhance its production in cultured shoots. Removal of roots from shoots cultured in hormone-free, liquid medium reduced shoot artemisinin by 53% (Ferreira and Janick 1996b). Shoot cultures in hormone-free medium consistently produced roots and contained artemisinin levels as high as 0.28% in some clones (Ferreira et al. 1995b), the highest reported leaf artemisinin derived

from differentiated shoot cultures. Thus far, observations from in vitro culture of *A. annua* indicate that this process is unlikely to be feasible for the commerical production of artemisinin.

Tissue culture was evaluated by Ferreira et al. (1995b) as a system to store clones and as a system for selection of high artemisinin. Artemisinin content was ca. 40% higher for greenhouse than for tissue culture-grown plants, but the correlation between artemisinin content for the same clones grown in vitro and in the greenhouse was $r = .50$, ($P = 5\%$), indicating that only 25% of the variability was accounted for by regression. When tissue-cultured plants were reanalyzed after 2 years of culture, artemisinin content was 20% lower and the correlation coefficient between the two analyses was $r = .61$, ($P = 1\%$). These relatively low correlation coefficients indicate that evaluation of artemisinin content from tissue-cultured plants is an unreliable procedure to estimate artemisinin content. The results may be an artifact of tissue culture, possibly due to somaclonal variation or adaptive changes.

C. Genetic Improvement

Broad-sense heritability for artemisinin production, the ratio of genetic to total variation (genetic + environmental) was estimated from asexually propagated clones derived from a random mating population in greenhouse and field trials (Ferreira et al. 1995b). Heritability estimates varied from 0.910 (greenhouse, individual basis) to 0.985 (combined field and greenhouse, family basis). These high broad-sense heritability estimates indicate that artemisinin content in *A. annua* has a high genetic component, i.e., heritability plays a key role in the trait. Delabays et al. (1993) fertilized a high-yielding (over 1%), low-vigor, Chinese clone of *A. annua*, which was induced to flower under controlled greenhouse conditions with pollen from Italian, Yugoslavian, and Spanish origins. These hybrids produced on average 0.64, 0.73, and 0.95% artemisinin, respectively, from a yield of dry leaves of about 2000 kg/ha. The fact that artemisinin content between high and low lines is intermediate suggests that artemisinin content is controlled by additive genetic factors.

Artemisinin content of nonflowering plants grown under long days in the greenhouse was found to be highly correlated ($r = .93$ to $.95$, $P = 1\%$) with the same clones grown under the long days in the field (Ferreira et al. 1995b). Thus, greenhouse evaluation of *A. annua* under long days has the potential to be an efficient system to select for high-artemisinin-yielding clones. Superior clones could be induced

to flower under short days and could then be intercrossed. Repetition of this cycle on progeny would be expected to lead to genetic gain for artemisinin production.

IV. PHARMACOLOGY OF ARTEMISININ AND DERIVATIVES

A. The Challenge of Malaria

Malaria, a disease known in antiquity, was referred to in Egyptian writings of the sixteenth century B.C., with symptoms of shivering, fever, and spleen enlargement. Followers of Hippocrates in the fifth century B.C. described the recurrence of fevers at regular intervals and observed a connection of the disease to marshes (Klayman 1989). In the seventeenth century, Italians believed that breathing bad air (*mal aria*) arising from swamps was responsible for the disease, and in the first half of the nineteenth century the term malaria entered the English medical literature. The names of the disease in French (*paludisme*) and Spanish (*paludismo*) are derived from the Latin *palus*, meaning marsh. In 1899, the mystery of malaria transmission was solved independently, by Ronald Ross, an English physician working in India, and Giovanni Battista Grassi, an Italian physician, who proved that the disease was spread by the bite of the female *Anopheles* mosquito (De Kruif 1939). The existence of malaria in pre-Columbian America is controversial; some suggest that the disease first appeared with the importation of slaves from Africa (Klayman 1989).

Human malaria is caused by four major *Plasmodium* species: *falciparum*, *vivax*, *malariae*, and *ovale*. *Plasmodium falciparum*, the prevailing parasite species in most of the world, causes the most severe form of malaria (cerebral), which often is fatal to children (Wirth et al. 1986) and nonimmune adults. Malaria, one of the most devastating diseases in the tropical world, is on the increase (Miller 1992). Over 300 million clinical cases occur worldwide, resulting in up to 2.7 million deaths annually. Most of these cases occur in Africa, but large areas of Asia, Central, and South America have high incidences of the disease (Nussenzweig and Long 1994). Malaria has been essentially eradicated from the United States and Cuba, but about 1000 cases are reported annually in the United States, mainly travelers, migrant workers, and military personnel, and, rarely, in people living around international airports. Malaria poses a major barrier to the economy of developing tropical countries, and its control is an important goal for improved world health (Wirth et al. 1986).

Immunity to malaria builds up only after several years of recurring infections but is only partially effective. An effective vaccine, if released at affordable price, may offer the best long-term control option for malaria. However, until this occurs, the best approach appears to be the use of quinine-derived drugs, in areas where resistant strains of *Plasmodium* are not present, or alternative drug therapies, such as semi-synthetic derivatives of artemisinin, with proved efficacy against multidrug-resistant strains of *P. falciparum* and little or no side effects.

B. Chemistry

Artemisinin is a sesquiterpene lactone belonging to the cadinane series. In addition to a lactone group, artemisinin contains an endoperoxide bridge, which is rare in secondary metabolites and, unlike other antimalarials, lacks a nitrogen-containing ring. The integrity of the stereo structure of the ring system has also been reported as vital for antimalarial activity (Anon. 1982; Luo and Shen 1987), and artemisinin-related compounds without the peroxide or with only one oxygen in the bridge (deoxyartemisinin and derivatives) are devoid of antimalarial properties. Artemisinin is an odorless and colorless compound and forms crystals with a melting point (mp) of 156–157°C. Its molecular weight, determined by high-resolution mass spectroscopy, is *m/e* 282.1742 M+ (Luo and Shen 1987). It has an empirical formula of $C_{15}H_{22}O_5$ and shows no absorption in most of the UV range. In the IR region there is a peak at 1745 cm^{-1} that corresponds to a strong gamma lactone function, and there are other peaks at 831, 881, and 1115 cm^{-1}, which correspond to the peroxide function (Anon. 1982). The ^1H-NMR and ^{13}C-NMR spectra led to the assignments of three methyl groups (one tertiary and two secondary), and an acetal function (Luo and Shen 1987). Artemisinin is surprisingly stable in neutral solvent heated up to 150°C (mp), or up to 50°C above its melting point (200°C) for 2.5 min in pure form (Lin et al. 1985). The molecule is also light stable (Anon. 1982).

The peroxide bridge is considered the most chemically reactive moiety of artemisinin. Catalytic hydrogenation of the molecule produces deoxyartemisinin, an epoxide devoid of antimalarial activity (Anon. 1982). When reduced with sodium borohydride ($NaBH_4$), the lactone function of the molecule is converted into a lactol (hemiacetal) known as dihydroartemisinin in which the peroxide bridge is intact. When dihydroartemisinin is crystalline, it is in the β form, and when in solution, it is a mixture of α and β epimers.

Dihydroartemisinin can be converted into either ethers or esters (Luo and Shen 1987). Reduction of artemisinin with $NaBH_4$, using boron trifluoride as a catalyst and dry tetrahydrofuran as the solvent, resulted in 71% deoxoartemisinin. This compound is ca. 8 times more active against chloroquine-resistant malaria in vitro than artemisinin (Jung et al. 1990b).

C. Isolation and Synthesis

The first published laboratory procedure for isolation of artemisinin has been described by Klayman et al. (1984). Air-dried leaves were extracted with petroleum ether (bp 30 to 60°C), which was subsequently removed in vacuo. The residue was redissolved in chloroform to which acetonitrile was added to precipitate inert plant components such as waxes. The concentrated extract was then chromatographed on a column of silica gel. Fractions with a high artemisinin content crystallized readily; recrystallization was achieved with cyclohexane or 50% ethanol.

Complete chemical (de novo) synthesis of artemisinin was achieved by Schmidt and Hofheinz (1983), Xu et al. (1986), Ravindranathan et al. (1990), and Avery et al. (1992). Each procedure for the chemical synthesis of artemisinin requires a final photooxidative step. Low yield, complexity, and high cost of the de novo synthesis suggests that isolation from the plant is the optimum system. However, there have been great advances in the synthetic chemistry of artemisinin and related compounds, which have enabled the radiolabeling of compounds to be used in studies on pharmacokinetic, metabolism, mode of action, and toxicity. Synthesis of greatly modified structures has provided a better understanding of structure–activity relationships and led to the synthesis of analogs with considerably increased antimalarial activity.

Although artemisinin is the starting material for the synthesis of other more soluble and stable compounds, the most abundant sesquiterpene in *A. annua* is artemisinic acid (arteannuic acid, qinghao acid), which occurs at specific content 8- to 10-fold higher than artemisinin (Roth and Acton 1987; Jung et al. 1990a), followed by arteannuin B (Klayman 1993). The usual extraction method for artemisinin neglects artemisinic acid, but recently Vonwiller et al. (1993) devised an efficient method to extract both artemisinic acid and artemisinin from the same material. Artemisinic acid can then be converted to artemisinin (Xu et al. 1983), which greatly increases the yield of artemisinin.

D. Detection and Quantification

1. Sample Preparation. Shoots of *A. annua* should be air dried before sample preparation and analysis. A low-temperature (ca. 40°C), air-flow oven can be used for drying, if a large biomass is involved, and if the humidity is too high for air drying. Fresh samples should not be used for extraction and analysis of artemisinin because artemisinin content detected in fresh samples may be lower than dried samples. This appears to be an artifact of the extraction because, although artemisinin is poorly soluble in water (Lin et al. 1989), part may be lost in the warm (45°C) aqueous phase under the petroleum ether fraction. Extraction of fresh samples might be acceptable for rapid screening of clones by enzyme immunoassays if the samples are dissolved in DMSO, methanol, or other water-miscible solvent. High-performance chromatography with electrochemical detection (HPLC-EC) was able to detect artemisinin in dried samples of 10 mg but samples smaller than 500 mg are less reliable (Ferreira et al. 1992).

2. Chemical Detection. Artemisinin has been detected and quantified by many different methods in the last 20 years. These include thin-layer chromatography (TLC) (Tu et al. 1982; Klayman et al. 1984; Luo and Shen 1987; Roth and Acton 1989; Tawfiq et al. 1989; Pras et al. 1991), high-performance liquid chromatography with UV detection (HPLC-UV) (Zhao and Zeng 1985; Acton and Klayman 1985; Liersh et al. 1986; Singh et al. 1988; Pras et al. 1991), high-performance liquid chromatography with electrochemical detection (HPLC-EC) (Acton et al. 1985; Zhou et al. 1988; Charles et al. 1990; Melendez et al. 1991; Ferreira et al. 1994), gas chromatography (GC) (Fulzele et al. 1991; Sipahimalani et al. 1991; Woerdenbag et al. 1993a), GC combined with mass spectrometry (GC/MS) (Banthorpe and Brown 1989; Woerdenbag et al. 1991; Woerdenbag et al. 1993b) or MS/MS (Ranasinghe et al. 1993), radioimmunoassay (RIA) (Song et al. 1985; Zhao et al. 1986), and enzyme-immunoassay (ELISA) (Jaziri et al. 1993; Ferreira and Janick 1995b, 1996a).

TLC is neither sensitive nor precise enough to quantify artemisinin in crude plant extracts without interference from other compounds, although some authors (Tu et al. 1982; Klayman et al. 1984; Luo and Shen 1987; Roth and Acton 1989; Tawfiq et al. 1989; Pras et al. 1991) have quantified artemisinin by this method. To be analyzed by HPLC-UV, artemisinin needs to be derivatized due to its lack of chromophores. This process may also derivatize other compounds present in the crude extract and mask the results. Artemisinin can be ana-

lyzed and quantified by GC alone or GC/MS through its degradation products since these methods usually use oven temperatures beyond artemisinin stability (ca. 150°C). Arteannuin B, when present in the plant extracts, generates a peak that appears with the same retention time as one of the artemisinin degradation peaks (Sipahimalani et al. 1991; Ferreira et al. 1994). HPLC-EC is a sensitive way of detecting and quantifying artemisinin in crude plant extracts without molecular breakdown, or interference from other related compounds such as artemisitene, and does not require previous derivatization or sample purification. Compounds without a peroxide bridge (such as arteannuin B and artemisinic acid) or with only one oxygen in the bridge (deoxyartemisinin) are undetected by this method.

Enhanced selective and sensitive detection and quantification of peroxides, using high-performance liquid chromatography combined with electrochemical detection, at a negative potential, was initially demonstrated with benzoyl peroxide by Funk et al. (1980). Acton et al. (1985) first described the use of this method for detection (3 ng readily detected) and quantification of artemisinin from crude plant extracts. An improved HPLC-EC method, originally based on Acton's method, has been described by Ferreira et al. (1994). Absence of the degassing step was considered to be the key step in improving the efficiency of the HPLC-EC method since it hastened the analysis, eliminated baseline drifting, and simplified the analysis in that the system no longer required forcing the solution into the 10-μL loop under gas pressure. Absence of sample degassing did not cause any oxygen peak to show up in the chromatogram. In addition, the heating of the mobile phase for recycling was reduced from 2 h (Acton et al. 1985) to 15 min, allowing a second cycle of analysis/day using the same mobile phase. The detector setting at −1.0 V, with the mobile phase being constituted of 45% acetonitrile:55% 0.1 N amonium acetate, and a flow rate of 1.5 mL/min, brought the artemisinin retention time down to ca. 7 min. This improved HPLC-EC method effectively separated and quantified dihydroartemisinin (mobile phase changed to 35% acetonitrile:65% 0.1 N amonium acetate), artemisitene, artemisinin, and dihydroartemisinin carboxymethylester.

This HPLC-EC method was compared to a GC method by Ferreira et al. (1994). The GC analysis degraded artemisinin to three other compounds, which generated peaks named A (identified as artemisinin G by EI mass spectrometry), B, and Z. Although peak A was the largest peak, it coincided with the peak generated by arteannuin B and thus could not be trusted for artemisinin quantification, which was possible only through peak B. Although Woerdenbag et al. (1991, 1993a) reported the separation of artemisinin from arteannuin B through GC/MS or CG alone, without degradation of artemisinin, HPLC-EC was found to be ca. 10 times more sensitive than GC and provided a better separation of dihydroartemisinin, artemisitene, and artemisinin (Ferreira et al. 1994).

3. Immunoassays. Radioimmunoassays (RIAs) and enzyme-linked immunosorbant assays (ELISAs) are more sensitive than the chemical methods discussed above, although they are more laborious, since generation of polyclonal or monoclonal antibodies is required. A radiommunoassay for artemisinin has been described by Song et al. (1985) and Zhao et al. (1986) but great importance has been given to enzyme immunoassays because they do not involve the problems of legal use and disposal, instability, high prices, or health hazards associated with radioactive labels. Because artemisinin is a very small molecule and thus nonimmunogenic, it needs to be linked to a carrier protein which will function as the primary immunogen. To be attached to the protein, artemisinin must first be derivatized to a compound with a reactive group that will act as a chemical handle. The first step in the derivatization of artemisinin is its reduction to dihydroartemisinin by sodium borohydride, which does not destroy the peroxide group. Dihydroartemisinin can then be derivatized to dihydroartemisinin carboxymethylester, which is then hydrolyzed to dihydroartemisinin carboxymethylether (a carboxylic acid). This compound, which has a reactive carboxyl group, is then linked to free amino groups of lysine residues in the carrier protein (Song et al. 1985; Zhao et al. 1986; Ferreira and Janick 1996a). Dihydroartemisinin can also react with succinic anhydride to generate 10-succinyldihydroartemisinin, which has the desired reactive carboxyl group (Jaziri et al. 1993) and can be linked to a protein using the mixed anhydride reaction (Song et al. 1985; Zhao et al. 1986) or by a reaction using a water-soluble carbodiimide (Staros et al. 1986). Zhao et al. (1986) used tritiated dihydroartemisinin to determine that an average molar ratio of 15:1 artemisinin to protein carrier had been achieved, and successfully generated polyclonal antibodies against artemisinin.

One of the polyclonal antibodies obtained by Ferreira and Janick (1996a) produced a near linear standard curve that enabled the quantification of artemisinin from crude extracts of *A. annua*. The ELISA correlated highly with HPLC-EC in discriminating samples of tissue-cultured clones but overestimated artemisinin by 3- to 10-fold.

This overestimation was due to cross-reactivity of polyclonal antibodies with artemisinin-related compounds, a problem also encountered by Jaziri et al. (1993). Cross-reactivity with compounds produced by root extracts from *A. annua*, diluted 10 to 20×, suggested that structural similarity, as well as the peroxide bridge, is important for antibody recognition. Cross-reactivity with root extracts was circumvented by diluting samples 100 to 500×. Development of ELISAs using monoclonal antibodies might eliminate the cross-reactivity problem but may bring other problems such as false negative reactivity of antibodies caused by destruction or obstruction of the epitope involved in antibody recognition.

E. Biosynthesis

Although the complete biosynthetic pathway for artemisinin and some of its precursors has not been established, some biotransformation steps have been elucidated, in vitro and in vivo. Farnesyl pyrophosphate (FPP) is considered to be the precursor for sesquiterpenes (Akhila et al. 1987). These authors proposed a complete biosynthetic pathway for artemisinin, starting from isopentenyl pyrophosphate (IPP). A four-step pathway for artemisinic acid proposed by Akhila et al (1990) is based on feeding the plant with [$^{14}C/^{3}H$] mevalonic acid and starts from the cyclization of *cis*-FPP. Akhila's studies did not acknowledge artemisinic acid as a precursor for artemisinin but others (El-Feraly et al. 1986; Jung et al. 1990a; Roth and Acton 1987, 1989; Kim and Kim 1992; Sangwan et al. 1993) consider artemisinic acid to be a possible biogenetic precursor for both arteannuin B and artemisinin, sequentially or independently.

Arteannuin B occurs naturally in *A. annua* and has been considered as another precursor for artemisinin (Nair et al. 1985; Roth and Acton 1989). Arteannuin B was converted into artemisinin in cell-free extracts of *A. annua* leaves (Nair et al. 1985). El-Feraly et al. (1986) demonstrated that artemisinic (arteannuic) acid could be converted to arteannuin B, in vitro, by singlet oxygen ($^{1}O_2$) through dye-sensitized photooxygenation. Artemisinic acid was converted into artemisinin (Roth and Acton 1989) and other artemisinin derivatives and correlated compounds such as artemisitene and arteannuin B. However, Nair et al. (1986) suggested that artemisinin and arteannuin B are produced independently from artemisinic acid.

Kim and Kim (1992) reported the transformation of dihydroartemisinic acid into artemisinin by *A. annua* tumor cell-free extracts but not by leaves or callus cell-free extracts. Tumor cell-free

extracts, however, failed to transform artemisinic acid into dihydroartemisinic acid, both of which are found in crude plant extracts.

Sangwan et al. (1993) reported in vivo and in vitro transformation of artemisinic acid to arteannuin B and artemisinin. Using an in vitro system, artemisinic acid was transformed into arteannuin B (1.58%) and artemisinin (3.59%). Horseradish peroxidase in the presence of H_2O_2 further enhanced the yields to 4.68 and 7.19%, respectively. Peroxidases are known to catalyze a variety of reactions in the metabolism of natural products, such as oxidation of substrates with H_2O_2 and introduction of oxygen into a substrate (Barz and Koster 1981). Although in vitro and in vivo studies suggest that artemisininic acid may serve as a biogenetic precursor for the synthesis of arteannuin B and artemisinin, the intermediate products or enzymes after FPP and before artemisinic acid, arteannuin B, and artemisinin, have not been isolated in vivo.

F. Mode of Action

1. Plant Growth Regulator. Artemisinin and other sesquiterpene lactones from the genus *Artemisia* have been shown to regulate plant growth (Duke et al. 1988). Arbusculin-A and other sesquiterpene lactones isolated from *A. tridentata* var. *vaseyana* and other species of *Artemisia* inhibited lateral root growth but stimulated respiration in *Cucumis sativus* (McCahon et al. 1973).

Artemisinin inhibited germination of lettuce (*Lactuca sativa*) and *A. annua* and root and shoot growth of lettuce, redroot pigweed (*Amaranthus retroflexus*), pitted morning glory (*Ipomoea lacunosa*), *A. annua*, and common purslane (*Portulaca oleracea*) at 33 μM, although this concentration did not affect velvetleaf (*Abutilon theophrasti*) or grain sorghum (*Sorghum bicolor*) (Duke et al. 1987). Artemisinin was not as effective as 2,4-D, but was more effective than glyphosate, when tested as an herbicide in the mung bean (*Vigna radiata*) bioassay at 5, 10, and 20 μM (Chen and Polatnick 1991). However, its herbicidal mode of action has not been elucidated.

2. Antimalarial. Based on studies in monkeys, artemisinin has proven effective in impairing the life cycle, and thus transmission, of *Plasmodium cynomolgi* B (simian malaria) within 18–24 h even in small (5 mg/kg) single intramuscular doses (Dutta et al. 1989). Pharmacological studies and clinical observations in every type of malaria infection indicate that artemisinin has direct parasiticidal

action on *Plasmodium* in the erythrocytic stage but is ineffective in the exoerythrocytic (liver) stage. In cases of uncomplicated falciparum malaria, artemisinin-derived drugs, such as artemether and artesunate (Fig. 6.1), require a 5-day treatment to avoid recrudescence. Under these conditions, artesunate or artemether, in a total dose of 600 mg given over 5 days, produced cure rates of over 90%. However, the cure rates depend on the severity of the disease—the more severe, the lower the cure rate (Looareesuwan 1994). The same dose over the same period, produced a cure rate of only 76% in severe malaria, when intramuscular artemether was administered, and addition of chloroquine or sulfadoxine-pyrimethamine resulted in no improvement in these severe cases. However, the addition of mefloquine (25 mg/kg at the end of the course of artesunate) or doxycycline produced higher cure rates when combined with artesunate at 600 mg total dose over a 5-day period (Bunnag et al. 1992). For the treatment of severe and complicated malaria, various formulations of artemisinin, artemether, and artesunate have been used. In patients who are comatose or vomiting, the administration of intramuscular artemether, at a dose of 3.2 mg/kg on the first day followed by 1.6 mg/kg per day until oral therapy can be given, has been effective (WHO 1994). All the artemisinin-related drugs (e.g., artesenuate, and artemether) when injected intramuscularly or intravenously, act faster than most antimalarial drugs and are well tolerated, without evident toxicity (White 1994). Studies carried out by the Chinese Qinghaosu Antimalaria Coordinating Research Group, reported by Dutta et al. (1989), indicated that artemisinin is relatively nontoxic, with a LD_{50} in mouse of 5.1 g/kg orally and 2.8 g/kg intramuscularly.

Studies on the initial mechanism of action have established that artemisinin causes structural changes in the erythrocite stage of the parasite, that affect the membranes surrounding the food vacuole, the nucleus, the mitochondria, endoplasmic reticulum, and nucleoplasm. Such changes lead to the formation of autophagic vacuoles and the loss of cytoplasm, which kill the parasites (Anon. 1979; Maeno et al. 1993). It is well known that the peroxide function in artemisinin and related compounds is vital for antimalarial activity, and results of biological activity studies in vitro indicate that the carbonyl function is not necessary for antimalarial activity (Jung et al. 1990b).

The biochemical action of artemisinin depends on two sequential steps (Meshnick 1994): (1) activation, which comprises an iron-mediated cleavage of the endoperoxide bridge generating an unstable organic free radical and/or other electrophilic species; and (2) alkylation, which involves the formation of covalent adducts between the drug and proteins synthesized by the *Plasmodium*. Since iron readily catalyzes the cleavage of artemisinin endoperoxide bridge, the same reaction is thought to occur in vivo, catalyzed by the heme group of hemoglobin. *Plasmodium* multiplication occurs inside the erythrocytes and is dependent on heme groups of hemoglobin. If these heme groups form complexes with the peroxide group of artemisinin, they would be unavailable for the *Plasmodium*, thus impairing its cycle.

Activation. This iron-mediated decomposition mechanism of artemisinin has been elucidated by Posner and Oh (1992); data indicate that iron activates artemisinin into a free radical. However, since there are various iron pools in the infected red blood cell, it is not known for sure which ones are responsible for artemisinin activation (Meshnick 1994). The observation that chloroquine, which binds heme (Chou et al. 1980), antagonizes the antimalarial activity of artemisinin against *P. falciparum* (Stahel et al. 1988) suggests that the free heme pool might be important. This hypothesis is reinforced by the fact that iron chelators, which bind free iron, have been observed to antagonize the effect of artemisinin (Kamchonwongpaisan et al. 1992; Meshnick et al. 1993).

Alkylation. After artemisinin-related drugs are converted to a reactive free radical, they can covalently bind to proteins. Radiolabeled artemisinin was shown to bind covalently mostly with free amino groups in human serum albumin (Yang et al. 1993). Radiolabeled drug is also taken up by isolated red cell membranes, where it forms covalent bonds with various membrane proteins. In contrast, when artemisinin is incubated with intact erythrocytes, there is no uptake or protein alkylation (Asawamahasakda et al. cited by Meshnick [1994]). However, the alkylation of heme appears to have little biological significance since, when infected red blood cells are exposed to high levels of artemisinin, there is no diminution in haemozoin content (Chang et al.; Asawamahasakda et al. cited by Meshnick [1994]).

A more recent series of water-soluble artemisinin derivatives has been developed in which the linkage of the water-solubilizing function to dihydroartemisinin was mediated by an ether, rather than an ester, functional group. The most active compound of this series is sodium artelinate, which, although less potent than sodium artesunate, compares favorably with this drug both in vitro against

P. falciparum and in vivo against *P. berghei* and is more stable than artesunate, remaining available in solution longer. Sodium artesunate, in alkaline solution, hydrolyzes into the insoluble dihydroartemisinin in a few hours, while sodium artelinate is detected with minor decomposition after 3 months (Lin et al. 1987). Arteether, artemether, sodium artesunate, artelininic acid (Fig. 6.1), and sodium artelinate are more stable and effective against malaria than artemisinin and dihydroartemisinin (dihydroqinghaosu), but the latter two compounds were less cytotoxic when tested in vitro (Woerdenbag et al. 1993c). However, in whole animal systems, dihydroartemisinin is by far the most potent and the most toxic compound, and although artemisinin is the least effective, it is also the easiest compound to manufacture. Artemisinin has been of great use in Vietnam, where the technology to derivatize it has not yet been available (D. Davidson, personal communication).

G. Drug Development

Artemisinin is the main active principle of *A. annua* effective against malaria. The peroxide moiety of the molecule appears to be responsible for the antimalarial activity, although other compounds produced by the plant, without the peroxide bridge, might have helped activate the antimalarial activity of plant extracts originally used by the Chinese. The first clinical studies conducted in China in 1972 showed excellent activity against malaria caused by both *P. falciparum* and *P. vivax*. Artemisinin was shown to clear parasitemia faster than chloroquine besides being effective against chloroquine-resistant *Plasmodium* and against potentially fatal cases of cerebral malaria.

Artemisinin formulations in China were suspended in oil or water for intramuscular injection, or were prepared as tablets and suppositories. However, due to its poor solubility in water or oil, researchers from the Walter Reed Army Institute have tried to develop semisynthetic derivatives of artemisinin with better solubility, such as arteether, artemether (both soluble in oil), and sodium artesunate and sodium artelinate (both water soluble). However, both artemether and artesunate are susceptible to breakdown by humidity, light, or acidic conditions. At room temperature, an aqueous solution of sodium artesunate at pH 7–8 hydrolyzes within hours to dihydroartemisinin. Sodium artesunate, a water-soluble salt derivative, is available commercially in China and Vietnam. It is 5-fold more effective than artemisinin, is well tolerated by test animals, and is

far less harmful to the heart than chloroquine. The prescribed dosage is 60 mg (or 1.2 mg/kg) for adults, administered intravenously or intramuscularly and repeated 4, 24, and 48 h after the initial dose. Sodium artelinate was developed by Lin et al. (1987) and is more potent and stable than artemisinin, remaining available, undegraded, longer in the bloodstream, and providing a better control against *Plasmodium berghei* and *P. falciparum*. Sodium artesunate and sodium artelinate have special application for treatment of the potentially fatal cerebral malaria (Klayman 1993). Though still under clinical investigation, sodium artesenuate is available and routinely used as an injectable treatment for malaria. Artemether in oil solution, ready for intramuscular injection, is available commercially in the People's Republic of China and is now registered in countries in Africa, Asia, and Latin America under the name Paluther, but it is still awaiting approval in others (Rhône–Poulenc Rorer Inc., personal communication; Roche and Helenport 1994).

Other artemisinin-related drugs including arteether and artelinic acid are potential candidates for commercial drug development. Artelinic acid is a second generation, water soluble derivative with improved stability in solution. It has been effective in rodent malaria when administered orally, parenterally, or transdermally. An intramuscular formulation of arteether, an ethyl ether of artemisinin, is now undergoing phase-I clinical trials and the synthetic analog yinghaosu is in phase-II trials. Other related compounds such as the trioxane and dioxane artemisinin derivatives are at earlier stages of development (WHO 1994).

1. Drug Availability. At the present, a number of formulations of artemisinin and its derivatives are being marketed. The most widely available preparations are (WHO 1994):

Oral

Artemisinin tablets	250 mg
Artemisinin capsules	250 mg
Artemether capsules	40 mg
Artesunic acid (artesunate) tablets	50 mg
Dihydroartemisinin tablets	60 mg

Intramuscular

Artemether oily solution	80 mg/L mL ampoule
Artesunate (anhydrous powder)	60 mg/L mL ampoule (reconstituted in 0.6 mL of 5% (w/v) sodium bicarbonate and diluted in 5.4 mL of dextrose solution or dextrose in saline just before use, because of the instability of the acid)

Intravenous
Artesunic acid identical to the formulation for intramuscular administration
Suppositories
Artemisinin 100 mg

In China, artemisinin is no longer used clinically, but dihydroartemisinin in tablet form has been approved for marketing in 1993 (WHO 1994). Vietnam produces artemisinin tablets, capsules, and suppositories as well as artesunate tablets and capsules, which are authorized for domestic use, as are artesunate intravenous preparations imported from China (WHO 1994). Raw artemisinin is purchased from Vietnam for derivation and drug formulation by a Belgian pharmaceutical enterprise. In Myanmar, Thailand, and Brazil, artesunate tablets and intravenous formulations, as well as injectable artemether preparations, are imported. In a few African countries, the parenteral formulation of artemether has been registered (e.g, Paluther), but by June 1995 it was not yet authorized for use by the malaria programs by most Health Ministries. Although about 1000 cases of malaria are registered per year in the United States, no artemisinin-derived drug is approved by the Food and Drug Administration.

Most of the information on the use of these derivatives relates to adults, but there are sufficient data to conclude that children also tolerate the drugs well and that their therapeutic response resembles that of adults having similar levels of immunity (Hien et al. 1991; White et al. 1992; Taylor et al. 1993). In an ongoing trial in Vietnam, 90 children have been treated in a three-way comparison of intramuscular artesunate, intravenous quinine, and artemisinin suppositories. Interim analysis indicates that the two artemisinin compounds are equivalent and more effective than intravenous quinine as an antimalarial (WHO 1994).

2. The Future of Artemisinin as an Antimalarial. Effective control of malaria is complicated by the fact that the mosquito vector develops resistance to available insecticides and *Plasmodium falciparum* develops resistance to the currently used antimalarial drugs. The best solution for malaria would be an effective, low-cost vaccine. This, however, has not yet been achieved and cannot be expected in the foreseeable future because of the different stages of the life cycle of *Plasmodium* in humans and the mutation frequency of this protozoa. Recently, the Pan American Health Organization reported a plasmid DNA vaccine, raised by producing antibodies and lymphocytes,

with a 68% effectiveness in mice. Another vaccine (SPf66) was developed in Colombia under the supervision of M. Patarroyo and collaborators (Valero et al. 1993) and has been shown to induce a substantial antibody response with three doses. It is well tolerated and safe for children but its safety and immunogenicity against malaria remain to be determined in a phase III trial in Kilombero, Africa, where malaria is highly endemic (Teuscher et al. 1994).

At present, short-term solutions to malaria involve: (1) providing early diagnosis and prompt treatment; (2) planning and implementing selective and sustainable preventive measures, including vector control; (3) encouraging early detection and prevention of epidemics; (4) strenthening of local capacities in basic and applied research to permit and promote regular assessment of a country's malaria situation, in particular the ecological, social, and economic determinants of the disease. The development of drug-resistant strains of *Plasmodium* has greatly complicated drug therapy. Artemisinin-derived drugs, used in association with other antimalarials, and strategies to control transmission of *Plasmodium* may be the only effective means of malaria control. Artemisinin-derived drugs are now available commercially in a few countries but they are restricted because of high cost; e.g., oral artesunate currently costs about $5–6 per treatment, with about 5 doses, as compared to $1.85 for mefloquine and 7¢ for drugs such as chloroquine and amodiaquine. Injectable artesunate and artemether cost even more, compared to quinine injections at less than $2.00 per treatment. The fear that the *Plasmodium* will develop resistance to artemisinin has led to the suggestion (WHO/MAL 1994) that these drugs be restricted to those areas where multidrug-resistant *Plasmodium* is endemic in order to avoid evolution of new artemisinin-resistant strains.

V. CONCLUSIONS

Artemisia annua is an example of an ancient medicinal plant that has steadfastly remained a traditional Chinese herb, and whose virtues are only now being recognized. Although prized in the United States for the dried flowers and use in the craft trade, the plant has recently received enormous attention as the only practical source of artemisinin that is effective against drug-resistant strains of *Plasmodium falciparum*, the pathogen responsible for the most severe form of malaria. As malaria has increased, so too has medicinal and agricultural research on *A. annua* and artemisinin. The discovery of the

plant's antimalarial activity and the isolation and identification of the main bioactive compound, artemisinin, is among the most exciting and successful breakthroughs in medicinal plant drug development in the past two decades.

China remains the world's major supplier of artemisinin and, at present, most is extracted from natural stands, although some lines are now being cultivated as a crop in Szechwan Province. Unfortunately, crop information and crop statistics have been unavailable, because the Chinese consider this proprietary information. The potential market for artemisinin and related derivatives has prompted the establishment of many research programs to develop *A. annua* as a commercial crop around the world including Australia, Brazil, India, The Netherlands, Switzerland, the United States, and Vietnam, which, in 1995, exported artemisinin at the price of $300–400/kg. These crop studies are in their early stages, and their success is closely linked with the potential of industrial commercialization. Agricultural field production of *A. annua* rather than in vitro culture appears to be the most practical method to produce artemisinin.

The low yield of artemisinin may be overcome by the breeding of high-yielding, adapted clones. Early success toward this goal has been reported in Switzerland, but little is known concerning the genetics or breeding behavior of this species, and research in this area is needed. In addition to artemisinin, other closely related sesquiterpenes, such as artemisinic acid, may also be of clinical interest in the development of antimalarials. Harvesting the plant for both artemisinin and artemisinic acid, which occurs in levels 8 to 10 times higher than artemisinin, is feasible.

Artemisinin is associated with the glandular trichomes in leaves and flowers, with the highest concentrations being associated with the inflorescence. Because *A. annua* is a short-day plant with a critical photoperiod of about 13.5 h, and unless day neutral genotypes are discovered, the likely areas of production will be temperate areas in which the daylength is long enough to achieve sufficient biomass development before flowering. Although it is clear that artemisinin is sequestered in glandular trichomes, it is not certain if actual synthesis takes place in these glands. Immunolocalization studies, now made possible through the development of antibodies against artemisinin, could resolve this question. In vitro studies suggest that roots may play a role in production of artemisinin and this warrants further study.

Agronomic research carried out to date indicates that *A. annua* can be easily grown in temperate climates. High biomass yields have

resulted from relatively high plant densities (5 to 11 plants/m^2) and the plant is responsive to nitrogen fertilization. The optimum time of harvest must take into consideration the maximum yields of artemisinin per unit area, balanced against extraction costs. At the present time, harvest close to anthesis, which coincides with the maximum content per plants, appears to be optimum. The development of efficient production systems will require effective strategies in weed control, mechanical sowing and harvesting, and postharvest handling. The low content of artemisinin, even in the best lines, suggest that processing facilities should be close to production areas. Despite the positive attributes of *A. annua*, the pollen, as in many other species of the Asteraceae, causes allergic rhinitis (hay fever), and is of concern.

As *A. annua* generates greater research based on artemisinin production, we predict that wider medical and agricultural uses will be identified. For example, the bioactivity of the volatile and nonvolatile oils of this plant also are of interest. Artemisinin and 1,8-cineole have been identified as possible allelopathic agents exhibiting activity against the seed germination of other weed species. Although *A. annua* is presently marketed as a limited-volume essential oil, this could change with a significant increase in field production for artemisinin. Large quantities of such essential oil could stimulate new uses in beverages and fragrances, and generate new markets. The development of a processing system whereby both the artemisinic acid and the essential oil could be extracted from the same harvested plant material would increase the economic viability of production.

Progress toward approval and commercialization of artemisinin and its antimalarial derivatives as pharmaceuticals has been slow. Although artemisinin has been substituted for dihydroartemisinin in China, more potent and stable artemisinin-derived antimalarials are still undergoing clinical trials. Artemether is now approved and available in Africa as Paluther (Rhône–Poulenc–Rorer) and is awaiting approval in other countries. Although over 1000 cases of malaria per year are reported in the United States, none of the artemisinin derivatives have been approved for human use by the Food and Drug Administration, despite their low toxicity. In some countries, these derivatives reach the population, at inflated prices, through the black market. This constitutes a serious hazard to the control of malaria, since the indiscriminate use of artemisinin-related drugs raises the risk of creating zones of artemisinin-resistant *Plasmodium* in areas where quinine-derived drugs are still effective. The emergence and

spread of drug resistance by *Plasmodium* is a serious threat to effective treatment, particularly in Southeast Asia and Latin America. Complete or partial resistance to chloroquine has now spread to all endemic areas, including Africa, although the drug is still useful in some areas.

Malaria control was once based on the control of the mosquito with DDT, but this approach is no longer practical, safe, or cost-effective. The global malaria control strategy adopted in 1992 emphasized the need for early diagnosis, appropriate treatment with antimalarial drugs, and selective use of preventive measures, including elimination of potential sites for mosquito breeding and use of mosquito nets. The improvement and approval of artemisinin-derived drugs and the possible release of a malaria vaccine are the current hope for the control of this scourge of humankind.

LITERATURE CITED

Acton, N., and D. L. Klayman. 1985. Artemisitene, a new sesquiterpene lactone endoperoxide from *Artemisia annua*. Planta Med. 51:441–442.

Acton, N., D. L. Klayman, and I. J. Rollman. 1985. Reductive electrochemical HPLC assay for artemisinin (qinghaosu). Planta Med. 51:445–446.

Ahmad, A., and L. M. Misra. 1994. Terpenoids from *Artemisia annua* and constituents of its essential oil. Phytochemistry 37:183–186.

Akhila, A., R. S. Thakur, and S. P. Popli. 1987. Biosynthesis of artemisinin in *Artemisia annua*. Phytochemistry 26:1927–1930.

Akhila, A., K. Rani, and R. S. Thakur. 1990. Biosynthesis of artemisinic acid in *Artemisia annua*. Phytochemistry 29:2129–2132.

Anon. 1979. Qinghaosu Antimalarial Coordinating Research Group. Antimalarial studies on qinghaosu. Chin. Med. J. 92:811-816.

Anon. 1982. China Cooperative Group on Qinghaosu and Its Derivatives as Antimalarials. Chemical studies on qinghaosu (Artemisinin). J. Trad. Chin. Med. 2:3–8.

Arora, N., and S. V. Gangal. 1991. Liposomes are vehicle for allergen presentation in the immunotherapy of allergic diseases. Allergy 46:386–392.

Ascensão, L., and M. S. S. Pais. 1987. Glandular trichomes of *Artemisia campestris* (ssp *Maritima*): ontogeny and histochemistry of the secretory product. Bot. Gaz. 148:221–227.

Avery, M. A., W. K. M. Chong, and C. Jennings-White. 1992. Stereoselective total synthesis of (+)-artemisinin, the antimalarial constituent of *Artemisia annua* L. J. Am. Chem. Soc. 114:974–979.

Bailey, L. H. 1951. Manual of cultivated plants. Macmillan, New York.

Bailey, L. H., and E. Z. Bailey. 1976. Hortus third. Macmillan, New York.

Baker, J. (ed). 1994. Artemisinin: proceedings of a meeting convened by the Wellcome Trust, 25–27, April 1993. Trans. R. Soc. Trop. Med. Hyg. 88, Suppl. 1, London, UK.

Banthorpe, D. V., and G. D. Brown. 1989. Two unexpected coumarin derivatives from tissue cultures of Compositae species. Phytochemistry 28:3003–3007.

Barz, W., and J. Köster. 1981. Turnover and degradation of secondary (natural) products. p. 35–84. In: E. E. Conn (ed.), The biochemistry of plants. Academic, New York.

Beetle, A. A., and A. Young. 1965. A third subspecies in the *Artemisia tridentata* complex. Rhodora 67:405–406.

Bennett, M. D., J. B. Smith, and J. S. Heslop-Harrison. 1982. Nuclear DNA amounts in angiosperms. Proc. R. Soc. London Ser. B 216:179–199.

Besser, W. S. J. G. 1843. Monographiae Artemisiarum, Section 1 (Dracunculi). Mémoires, L'Académie Impériale des Sciences de Saint Petersbourg.

Bostock, J., and H. T. Riley (Transl.). 1855–1857. The natural history of Pliny (6 vols.). Henry G. Bohn, London (see Vol. 5, Book XXV, Chapter 36, p. 106–107).

Brown, G. D. 1993a. Annulide, a sesquiterpene lactone from *Artemisia annua*. Phytochemistry 32:391–393.

Brown, G. D. 1993b. Production of anti-malarial and anti-migraine drugs in tissue culture of *Artemisia annua* and *Tanacetum parthenium*. Acta Hort. 330:269–276.

Bryson, C. T., and E. M. Croom Jr. 1991. Herbicide inputs for a new agronomic crop, annual wormwood (*Artemisia annua*). Weed Technol. 5:117–124.

Bunnag, D., J. Karbwang, and T. Harinasuta. 1992. Artemether in the treatment of multiple drug resistant falciparum malaria. Southeast Asian J. Trop. Med. Pub. Health. 23:762–768. (Cited by Looareesuwan [1994]).

Cappelletti, E. M., R. Caniato, and G. Appendino. 1986. Localization of cytotoxic hydroper-oxyeudesmanolides in *Artemisia umbelliformis*. Biochem. System. Ecol. 14:183–190.

Charles, D. J., J. E. Simon, K. V. Wood, and P. Heinstein. 1990. Germplasm variation in artemisinin content of *Artemisia annua* using an alternative method of artemisinin analysis from crude plant extracts. J. Nat. Prod. 53:157–160.

Charles, D. J., E. Cebert, and J. E. Simon. 1991. Characterization of the essential oil of *Artemisia annua* L. J. Ess. Oil Res. 3:33–39.

Charles, D. J., J. E. Simon, C. C. Shock, E. B. G. Feibert, and R. M. Smith. 1993. Effect of water stress and post-harvest handling on artemisinin content in the leaves of *Artemisia annua* L. p. 640–642. In: J. Janick and J. E. Simon (eds.), New crops. Wiley, New York.

Chen, P. K., and M. Polatnick. 1991. Comparative study on artemisinin, 2,4-D, and glyphosate. J. Agr. Food Chem. 39:991–994.

Chou, A., R. Chevli, and C. D. Fitch. 1980. Ferriprotoporphyrin IX fulfills the criteria for identification as the chloroquine receptor of malaria parasites. Biochemistry 19:1543–1549.

Degener, O. 1939. Flora Hawaiiensis, Vol. 6, New York.

De Kruif, P. 1939. Ross vs. Grassi, Malaria. p. 290–323. In: H. G. Grover (ed.), Microbe hunters. Harcourt, Brace, New York.

Delabays, N., C. Blank, and G. Collet. 1992. The culture and the selection of *Artemisia annua* L. for the production of artemisinin. Fed. Agr. Res. Sta. Changins. Rev. Suisse Vitic. Arboric. Hort. 24:245–251.

Delabays, N., A. Benakis, and G. Collet. 1993. Selection and breeding for high artemisinin (qinghaosu) yielding strains of *Artemisia annua*. Acta Hort. 330:203–207.

Duke, M. V., R. N. Paul, H. N. Elsohly, G. Sturtz, and S. O. Duke. 1994. Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua* L. Int. J. Plant Sci. 155:365–372.

Duke, S. O., and R. N. Paul. 1993. Development and fine structure of glandular trichomes of *Artemisia annua* L. Int. J. Plant Sci. 154:107–118.

Duke, S. O., K. C. Vaughn, E. M. Croom, Jr., and H. N. Elsohly. 1987. Artemisinin, a constituent of annual wormwood (*Artemisia annua*), is a selective phytotoxin. Weed Sci. 35:499–505.

Duke, S. O., R. N. Paul, Jr., and S. M. Lee. 1988. Terpenoids from the genus *Artemisia* as potential pesticides. p. 318–334. In. H. G. Cutler (ed.), Biological active natural products: potential use in agriculture. ACS Symp. Series 380. Am. Chem. Soc., Washington, DC.

Dutta, G. P., R. Bajpai, and R. A. Vishwakarma. 1989. Antimalarial efficacy of arteether against multiple drug resistant strain of *Plasmodium yoelii nigeriensis*. Pharmacol. Res. 21:415–419.

Einarson, B., and G. K. K. Link. (Transl.). 1976. Theophrastus: De causis plantarum (3 vol.). Harvard Univ. Press, Cambridge, MA.

El-Feraly, F. S., I. A. Al-Meshal, M. A. Al-Yahya, and M. S. Hifnawy. 1986. On the possible role of qinghao acid in the biosynthesis of artemisinin. Phytochemistry 25:277–2778.

Elhag, H. M., M. M. El-Domiaty, F. S. El-Feraly, J. S. Mossa, and M. M. El-Olemi. 1992. Selection and micropropagation of high artemisinin producing clones of *Artemisia annua* L. Phytother. Res. 6:20–24.

El-Keltawi, N. E., and R. Croteau. 1986. Influence of phosphon D and cycocel on growth and essential oil content of sage and pepermint. Phytochemistry 25:1603–1606.

El-Keltawi, N. E., and R. Croteau. 1987. Influence of foliar applied cytokinins on growth and essential oil content of several members of the Lamiaceae. Phytochemistry 26:891–895.

El-Sohly, H. N. 1990. A large scale extraction technique of artemisinin from *Artemisia annua*. J. Nat. Prod. 53:1560–1564.

Erichsen–Brown, C. 1989. Medicinal and other uses of North American Plants: an historical survey with special reference to eastern indian tribes. Dover, New York.

Ferreira, J. F. S., 1994. Production and detection of artemisinin in *Artemisia annua* L. Ph.D. Thesis, Purdue Univ., West Lafayette, IN.

Ferreira, J. F. S., and J. Janick. 1995a. Floral morphology of *Artemisia annua* with special reference to trichomes. Int. J. Plant Sci. 156:807–815.

Ferreira, J. F. S. and J. Janick. 1995b. Production and detection of artemisinin from *Artemisia annua*. Acta Hort. 390:41–49.

Ferreira, J. F. S., and J. Janick. 1996a. Immunoquantitative analysis of artemisinin from *Artemisia annua* using polyclonal antibodies: Phytochemistry 41:97–104.

Ferreira, J. F. S. and J. Janick. 1996b. Roots as an enhancing factor for the production of artemisinin in shoot cultures of *Artemisia annua*. Plant, Cell, Tissue Organ Cult. 44:211–217.

Ferreira, J. F. S., D. Charles, J. E. Simon, and J. Janick. 1992. Effect of drying methods on the recovery and yield of artemisinin from *Artemisia annua* L. HortScience 27:650 (Abstr. 565).

Ferreira, J .F. S., D. J. Charles, K. Wood, J. E. Simon, and J. Janick. 1994. A comparison of gas chromatography and high performance liquid chromatography for artemisinin analyses. Phytochem. Anal. 5:116–120.

Ferreira, J. F. S., J. E. Simon, and J. Janick. 1995a. Developmental studies of *Artemisia annua*: Flowering and artemisinin production under greenhouse and field conditions. Planta Med. 61:167–170.

Ferreira, J. F. S., J. E. Simon, and J. Janick. 1995b. Relationship of artemisinin content of tissue-cultured, greenhouse-grown, and field-grown plants of *Artemisia annua*. Planta Med. 61:351–355.

Fisher, N. H. 1990. Sesquiterpene lactones: Biogenesis and biomimetic transformations. p. 161–201. In: G. H. N. Towers and H. A. Stafford (eds.), Biochemistry of the mevalonic acid pathway. Plenum, New York.

Fulzele, D. P., A. T. Sipahimalani, and M. R. Heble. 1991. Tissue cultures of *Artemisia annua*: organogenesis and artemisinin production. Phytother. Res. 5:149–153.

Funk, M. O., M. B. Keller, and B. Levinson. 1980. Determination of peroxides by high performance liquid chromatography with amperometric detection. Anal. Chem. 52:771–773.

Gerard(e), J. 1597. The herball or generall history of plants. John Norton, London. Chapters 432–438.

Gray, A. 1884. Synoptical flora of North America, Vol. 1, Part II. Smithsonian Institution, Washington, DC. Univ. Press, John Wilson and Son, Cambridge, UK.

Greger, H. 1982. New chemical markers within *Artemisia* (Compositae-Anthemidae). p. 153–163. In: N. Margaris, A. Koedam, and D. Vokou (eds.), Martinus Nijhoff, Hague.

Grieve, M. 1971. A modern herbal, Vol. II. C. F. Leyel (ed.). Dover, New York.

Glasby, J. S. 1991. Dictionary of plants containing secondary metabolites. Taylor & Francis, New York.

Gunther, R. T. (ed.). 1959. p. 259–263; 357–360. In: The Greek herbal of Dioscorides (illustrated by Byzantine A.D. 512, Translated by John Goodyear A.D. 1655, edited and first printed A.D. 1933). Hafner, New York.

Hall, H. M., and F. E. Clements. 1923. The phylogenetic method in taxonomy. The North American species of *Artemisia, Chrysothamnus* and *Atriplex*. Carnegie Inst. Wash., Washington, DC.

He, X-C., M-Y. Zeng, G-F. Li, and Z. Liang. 1983. Callus induction and regeneration of plantlets from *Artemisia annua* and changes of qinghaosu contents. Acta Bot. Sin. 25:87–90.

Heywood, V. H., and C. J. Humphries. 1977. Anthemideae-systematic review. p. 851–898. In: V. H. Heywood, J. B. Harborne, and B. L. Turner (eds.), The biology and chemistry of the Compositae, Vol. II, Academic, New York.

Hien, T. T., D. T. H. Tam, N. T. K. Cuc, and K. Arnold. 1991. Comparative effectiveness of artemisinin suppositories and oral quinine in children with acute falciparum malaria. Trans. R. Soc. Trop. Med. Hyg. 85:210–211.

Jaziri, M., B. Diallo, M. Vanhaelen, J. Homes, K. Yoshimatsu, and K. Shimomura. 1993. Immunodetection of artemisinin in *Artemisia annua* cultivated in hydroponic conditions. Phytochemistry 33:821–826.

Jaziri, M., K. Shimomura, K. Yoshimatsu, M.-L. Fauconnier, M. Marlier, and J. Homes. 1995. Establishment of normal and transformed root cultures of *Artemisia annua* L. for artemisinin production. J. Plant Physiol. 145:175–177.

Jeremic', D., A. Jockic', A. Behbud, and M. Stefanovic'. 1973. A new type of sesquiterpene lactone isolated from *Artemisia annua* L. Tetrahedron Lett. 32:3039–3042.

Jha, J., T. B. Jha, and S. B. Mahato. 1988. Tissue culture of *Artemisia annua* L.: a potential source of an antimalarial drug. Curr. Sci. 57:344–346.

Jung, M., H. N. ElSohly, and J. D. McChesney. 1990a. Artemisinic acid: a versatile chiral synthon and bioprecursor to natural products. Planta Med. 56:624.

Jung, M., X. Li, D. A. Bustos, H. N. Elsohly, J. D. McChesney, and W. K. Milhous. 1990b. Synthesis and antimalarial activity of (+)-deoxoartemisinin. J. Med. Chem. 33:1516–1518.

Kamchonwongpaisan, S., N. Vanitcharoen, and Y. Yuthavong. 1992. The mechanism of action of artemisinin (qinghaosu). p. 363–372. In: A.S.H. Ong and L. Packer (eds.), Lipid-soluble antioxidants: biochemistry and clinical applications. Basel:Birkhauser.

Kelsey, R. G., and F. Shafizadeh. 1979. Sesquiterpene lactones and systematics of the genus *Artemisia*. Phytochemistry 18:1591–1611.

Kelsey, R. G., and F. Shafizadeh. 1980. Glandular trichomes and sesquiterpene lactones of *Artemisia nova* (Asteraceae). Biochem. System. Ecol. 8:371–377.

Kim, N-C., and S-U. Kim. 1992. Biosynthesis of artemisinin from 11,12-dihydroarteannuic acid. J. Korean Agr. Chem. Soc. 35:106–109.

Kim, N-C., J-G. Kim, H-J. Lim, T-R. Hahn, and S-U. Kim. 1992. Production of secondary metabolites by tissue culture of *Artemisia annua* L. J. Korean Agr. Chem. Soc. 35:99–105.

Klayman, D. L. 1985. Qinghaosu (artemisinin): an antimalarial drug from China. Science. 228: 1049–1055.

Klayman, D. L. 1989. Weeding out malaria. Nat. Hist. Oct.:18-26.

Klayman, D. L. 1993 *Artemisia annua*: From weed to respectable antimalarial plant. p. 242–255. In: A. D. Kinghorn and M. F. Balandrin (eds.), Human medicinal agents from plants. Am. Chem. Soc. Symp. Ser. Washington, DC.

Klayman, D. L., A. J. Lin, N. Acton, J. P. Scovill, J. M. Hoch, W. K. Milhous, and A. D. Theorides. 1984. Isolation of artemisinin (qinghaosu) from *Artemisia annua* growing in the United States. J. Nat. Prod. 47:715–717.

Kudakasseril, G. J., L. Lam, and E. J. Staba. 1987. Effect of sterol inhibitors on the incorporation of ^{14}C-isopentenyl pyrophosphate into artemisinin by a cell-free system from *Artemisia annua* tissue cultures and plants. Planta Med. 28:280–284.

Ladd, P. G. 1994. Pollen presenters in the flowering plants: Form and function. Bot. J. Linn. Soc. 115:165–195.

Laughlin, J. C. 1993. Effect of agronomic practices on plant yield and antimalarial constituents of *Artemisia annua* L. Acta Hort. 331:53–61.

Laughlin, J. C. 1994. Agricultural production of artemisinin: A review. Trans. R. Soc. Trop. Med. Hyg. 88 (Suppl. 1):21–22.

Lawrence, B. M. 1990. Progress in essential oils. Perfumer Flavorist 15:63–64.

Libbey, L. M., and G. Sturtz. 1989. Unusual essential oils grown in Oregon, II: *Artemisia annua* L. J. Ess. Oil Res. 1:201–202.

Liersch, R., H. Soicke, C. Stehr, and H-U. Tüllner. 1986. Formation of artemisinin in *Artemisia annua* during one vegetation period. Planta Med. 52:387–390.

Lin, A. J., D. L. Klayman, and J. M. Hoch. 1985. Thermal rearrangement and decomposition products of artemisinin (qinghaosu)[1]. J. Org. Chem. 50:4504–4508.

Lin, A. J., D. L. Klayman, and W. K. Milhous. 1987. Antimalarial activity of new water-soluble dihydroartemisinin derivatives. J. Med. Chem. 30:2147–2150.

Lin, A. J., M. Lee, and D. L. Klayman. 1989. Antimalarial activity of new water-soluble dihydroartemisinin derivatives. 2.[1,2] Stereospecificity of the ether side chain. J. Med. Chem. June 1989:1249–1252.

Liu, K.C-S.C., S. L. Yang, M. F. Roberts, B. C. Elford, and J. D. Phillipson. 1992. Antimalarial activity of *Artemisia annua* flavonoids from whole plants and cell cultures. Plant Cell Rep. 11:637–640.

Looareesuwan, S. 1994. Overview of clinical studies on artemisinin derivatives in Thailand. Trans. R. Soc. Trop. Med. Hyg. 88 (suppl. 1):9–11.

Luo, X. D., and C. C. Shen. 1987. The chemistry, pharmacology, and clinical applications of qinghaosu (artemisinin) and its derivatives. Med. Res. Rev. 7:29–52.

Madhusudanan, K. P. 1989. Mass spectral studies on artemisinin, dihydroartemisinin, and arteether. Indian J. Chem. 28B:751–754.

Maeno, Y., T. Toyoshima, H. Fujioka, Y. Ito, S. R. Meshnick, A. Benakis, W. K. Milhous, and M. Aikawa. 1993. Morphological effects of artemisinin in *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 49:485–491.

Magalhães, P. M. 1994. A experimentação agrícola com plantas medicinais e aromáticas. Atualidades Científicas 3:31–56 (CPQBA/UNICAMP, Campinas, Brazil).

Makino, T. 1960. Makino's new illustrated flora of Japan. Hokuryukan Co., Tokyo, Japan.

Martinez, B. C., and J. Staba. 1988. The production of artemisinin in *Artemisia annua* L. tissue cultures. Adv. Cell Cult. 6:69–87.

McCahon, C. B., R. G. Kelsey, R. P. Sheridan, and F. Shafizadeh. 1973. Bull. Torrey Bot. Club 100:23. (Cited by Rodriguez et al. [1976]).

McVaugh, R. 1984. In: W. R. Anderson (ed.). Flora Novo-Galiciana: a descriptive account of the vascular plants of Western Mexico, Vol. 12 (Compositae). Univ. of Michigan Press, Ann Arbor.

Mehrotra, S., B. N. Mehrotra, B. S. Aswal, and H. P. Sharma. 1990. Leaf surface studies of some medicinal artemisias. Int. J. Crude Drug Res. 28:103–119.

Melendez, V., O. Peggins, T. G. Brewer, and A. D. Theorides. 1991. Determination of the antimalarial arteether and its deethylated metabolite dihydroartemisinin in plasma by high-performance liquid chromatography with reductive electrochemical detection. J. Pharm. Sci. 80:132–138.

Meshnick, S. R. 1994. The mode of action of antimalarial endoperoxides. Trans. R. Soc. Trop. Med. Hyg. 88 (Suppl. 1):31–32.

Meshnick, S. R., Y. Z. Yang, V. Lima, F. Kuypers, S. Kamshonwongpaisan, and Y. Yuthavong. 1993. Iron-dependent free radical generation and the antimalarial artemisinin (qinghaosu). Antimicrobial Agents Chemother. 37:1108–1114.

Miller, L. H. 1992. The challenge of malaria. Science 257:36–37.

Mitchell, J. C. 1975. Contact allergy from plants. p. 119–139. In: V. C. Runeckles (ed.), Recent adv. phytochem. Vol. 9. Plenum, New York.

Morales, M. R., D. J. Charles, and J. E. Simon. 1993. Seasonal accumulation of artemisinin in *Artemisia annua* L. Acta Hort. 344:416–420.

Nair, M. S. R., N. Acton, D. L. Klayman, K. Kendrick, and S. Mante. 1985. Tissue culture and cell-free extract studies on qinghaosu. Abstracts. Int. Congr. Natural Products, Chapel Hill, NC, 7–12, July 1985 (Abstr. 75).

Nair, M. S. R., N. Acton, D. L. Klayman, K. Kendrick, D. V. Basile, and S. Mante. 1986. Production of artemisinin in tissue cultures of *Artemisia annua*. J. Nat. Prod. 49:504–507.

North, C. 1979. Plant breeding and genetics in horticulture. Macmillan, London.

Nussenzweig, R. S., and C. A. Long. 1994. Malaria vaccines: multiple targets. Science 265:1381–1383.

Nyquist, N. E. 1991. Estimation of heritability and prediction of selection response in plant populations. Crit. Rev. Plant Sci. 10:235–322.
Paniego, N. B., and A. M. Giulietti. 1994. *Artemisia annua* L.: dedifferentiated and differentiated cultures. Plant Cell Tiss. Organ Cult. 36:163–168.
Park, H. S., J. W. Kim, and C. S. Hong. 1993. Immunoelectron-microscopic localization of IgE binding site of mugwort pollen. J. Korean Med. Sci. 8:30–33.
Peter-Blanc, C. 1992. Developpement et biologie de la reproduction de l'*Artemisia annua* L. Travail de diplome. Univ. de Lausanne, Switzerland.
Poljakov, P. P. 1961. Systematic studies in the genus *Artemisia* L. Trudy Inst. Bot. Akad. Navk. Kasakh., SSR. Alma Ata 11:134. (Cited by Seaman [1982].)
Posner, G., and C. H. Oh. 1992. A regiospecifically oxygen-18 labelled 1,2,4-trioxane: a simple chemical model system to probe the mechanisms for the antimalarial activity of artemisinin (qinghaosu). J. Am. Chem. Soc. 114:8328–8329.
Pras, N., J. F. Visser, S. Batterman, H. J. Woerdenbag, T. M. Malingré, and C. B. Lugt. 1991. Laboratory selection of *Artemisia annua* L. for high artemisinin yielding types. Phytochem. Anal. 2:80–83.
Ranasinghe, A., J. D. Sweatlock, and R. G. Cooks. 1993. A rapid screening method for artemisinin and its congeners using ms/ms: search for new analogs in *Artemisia annua* L. J. Nat. Prod. 56:552–563.
Rantio-Lehtimaki, A., M. L. Helander, and K. Kaihu. 1992. Does cutting of mugwort stands affect airborne pollen concentrations? Allergy. 47:388–390.
Ravindranathan, T., M. A. Kumar, R. B. Menon, and S. V. Hiremath. 1990. Stereoselective synthesis of artemisinin+. Tetrahedron Lett. 31:755–758.
Riddle, J. M., and J. W. Estes. 1992. Oral contraceptives in ancient and medieval times. Am. Scient. 80:226–233.
Roche, G., and J. P. Helenport. 1994. The view of the pharmaceutical industry. p. 57–58. In: Artemisinin: Trans. R. Soc. Trop. Med. Hyg., Proc. Vol. 88, Suppl. 1.
Rodriguez, E., G. H. N. Towers, and J. C. Mitchell. 1976. Biological activities of sesquiterpene lactones. Phytochemistry 15:1573–1580.
Roth, R. J., and N. Acton. 1987. Isolation of arteannuic acid from *Artemisia annua*. Planta Med. 53:501–502.
Roth, R. J. and N. Acton. 1989. A simple conversion of artemisinic acid into artemisinin. J. Nat. Prod. 52:1183–1185.
Rydberg, P. A. 1927. Carduaceae (Carduales), Liabeae, Neurolaeneae, Senecioneae (Pars.) N. Am. Fl. 34:289. (Cited by Seaman [1982].)
Sangwan, R. S., K. Agarwal, R. Luthra, R. S. Thakur, and N. Singh-Sangwan. 1993. Biotransformation of arteannuic acid into arteannuin B and artemisinin in *Artemisia annua*. Phytochemistry 34:1301–1302.
Schmid, G., and W. Hofheinz. 1983. Total synthesis of qinghaosu. J. Am. Chem. Soc. 105:624–625.
Seaman, F. C. 1982. Sesquiterpene lactones as taxonomic characters in the Asteraceae. Bot. Rev. 48:121–592.
Seigler, D. S. 1981. Terpenes and plant phylogeny. p. 117–148. In: D. A. Young and D. S. Siegler (eds.), Phytochemistry and angiosperm phylogeny. Praeger, New York.
Shukla, A., A. H. Farooqi-Abad, Y. N. Shukla, and S. Sharma. 1992. Effect of triacontanol and chlormequat on growth, plant hormones and artemisinin yield in *Artemisia annua* L. Plant Grow. Regul. 11:165–171.

Simon, J. E., and E. Cebert. 1988. *Artemisia annua:* A production guide. p. 78–83. In: J. E. Simon and L. Z. Clavio (eds.), Third National Herb Growing and Marketing Conference. Purdue Univ. Agr. Exp. Sta. Bull. 552.
Simon, J. E., A. F. Chadwick, and L. E. Craker. 1984. Herbs: an indexed bibliography: the scientific literature on selected herbs, and aromatic and medicinal plants. Archon Books, Hamden, CT.
Simon, J. E., D. Charles, E. Cebert, L. Grant, J. Janick, and A. Whipkey. 1990. *Artemisia annua* L.: a promising aromatic and medicinal. p. 522–526. In: J. Janick and J. E. Simon (eds.). Advances in new crops. Timber Press, Portland, OR.
Singh, A., V. K. Kaul, V. P. Mahajan, A. Singh, L. N. Misra, R. S. Thakur, and A. Husain. 1986. Introduction of *Artemisia annua* in India and isolation of artemisinin, a promising antimalarial drug. Indian J. Pharmaceutical Sciences. 48:137–138.
Singh, A., R. A. Vishwakarma, and A. Husain. 1988. Evaluation of *Artemisia annua* strains for higher artemisinin production. Planta Med. 54:475–476.
Sipahimalani, A. T., D. P. Fulzele, and M. R. Heble. 1991. Rapid method for the detection and determination of artemisin by gas chromatography. J. Chromatogr. 538:452–455.
Skvarla, J. J., and D. A. Larson. 1965. An electron microscopy study of pollen morphology in the Compositae with special reference to the Ambrosiinae. Grana Palinol. 6:210–269.
Slone, J. H., and R. G. Kelsey. 1985. Isolation and purification of glandular secretory cells from *Artemisia tridentata* (ssp. vaseyana) by percoll density gradient centrifugation. Am. J. Bot. 72:1445-1451.
Song, Z. Y., K. C. Zhao, X. T. Liang, C. X. Liu, and M. G. Yi. 1985. Radioimmunoassay of qinghaosu and artesunate. Acta Pharm. Sinica 20:610–614.
Srivastava, N. K, and S. Sharma. 1990. Influence of micronutrient imbalance on growth and artemisinin content in *Artemisia annua*. Indian J. Pharm. Sci. 52:225–227.
Stahel, E., P. Druilhe, and M. Gentilini. 1988. Antagonism of chloroquine with other antimalarials. Trans. R. Soc. Trop. Med. Hyg. 82:221.
Staros, J. V., R. W. Write, and D. M. Swingle. 1986. Enhancement by *N*-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. Anal. Biochem. 156:220–222.
Stuart, M. 1979. The encyclopedia of herbs and herbalism. Crescent Books, New York.
Tawfiq, N. K., L. A. Anderson, M. F. Roberts, J. D. Phillipson, D. H Bray, and D. C. Warhurst. 1989. Antiplasmodial activity of *Artemisia annua* plant cell cultures. Plant Cell Rep. 8:425–428.
Taylor, T. E., B. A. Wills, P. Kazembe, M. Chisale, J. J. Wirima, E. Y. E. C. Ratsma, and M. E. Molyneux. 1993. Rapid coma resolution with artemether in Malawian children with cerebral malaria. Lancet 341:661–662.
Teuscher, T., J. R. M. Armstrong Schellenberg, I. Bastos de Azevedo, N. Hurt, T. Smith, R. Hayes, H. Masanja, Y. Silva, M. C. Lopez, A. Kitua, W. Kilama, M. Tanner, and P. L. Alonso. 1994. SPf66, a chemically synthesized subunit malaria vaccine, is safe and immunogenic in Tanzanians exposed to intense malaria transmission. Vaccine 12:328–336.
Trigg, P. I. 1990. Qinghaosu (artemisinin) as an antimalarial drug. Econ. Med. Plant Res. 3:20–55.

Tu, Y-Y., M-Y. Ni, Y. R. Zhong, L-N. Li, S-L. Cui, M-Q. Zhang, Y. Z. Wang, Z. Ji, and X-T. Liang. 1982. Studies on the constituents of *Artemisia annua* part II. Planta Med. 44:143–145.
Ueda, J., and J. Kato. 1980. Isolation and identification of a senescence-promoting substance from wormwood (*Artemisia absinthium* L.). Plant Physiol. 66:246–249.
Valero, M. V., L. R. Amador, C. Galindo, J. Figueiroa, M. S. Bello, L. A. Murillo, A. L. Mora, G. Patarroyo, C. L. Rocha, M. Rojas, J. J. Aponte, L. E. Sarmiento, D. M. Lozada, C. G. Coronell, N. M. Ortega, J. E. Rosas, P. L. Alonso, and M. E. Patarroyo. 1993. Vaccination with SP_f66, a chemically synthesised vaccine, against *Plasmodium falciparum* malaria in Columbia. The Lancet. 341:705–710.
Vonwiller, S. C., R. K. Haynes, G. King, and H. J. Wang. 1993. An improved method for the isolation of qinghao (artemisinic) acid from *Artemisia annua*. Planta Med. Lett. 59:562–563.
Wang, C-W. 1961. The forests of China, with a survey of grassland and desert vegetations. p. 171–187. In Harvard Univ. Maria Moors Cabot Foundation No. 5. Harvard Univ., Cambridge, MA.
Weathers, P. J., R. D. Cheetham, E. Follansbee, and K. Tesh. 1994. Artemisinin production by transformed roots of *Artemisia annua*. Biotech. Lett. 16:1281–1286.
Wei, Z-X., J-P. Pan, and Y. Li. 1992. Artemisinin G: a sesquiterpene from *Artemisia annua*. Planta Med. 58:300.
Whipkey, A., J. E. Simon, D. J. Charles, and J. Janick. 1992. In vitro production of artemisinin from *Artemisia annua* L. J. Herbs Spices Med. Plants. 1:15–25.
White, N. 1994. Foreword (Artemisinin meeting). Trans. Royal Soc. Trop. Med. Hyg. 88(suppl. 1):1.
White, N., J., D. Waller, J. Crawley, F. Nosten, D. Chapman, D. Brewster, and B.,M. Greenwood. 1992. Comparison of artemether and chloroquine for severe malaria in Gambian children. Lancet 339:317–321.
WHO (World Health Organization). 1994. The role of artemisinin and its derivatives in the current treatment of malaria. World Health Organization.
Wilbert, S. 1991. Thesis. Universitat Bonn. Cited by Rucher, G., D. Manns, and S. Wilbert. Phytochemistry. 31:340–342.
Wirth, D. F., W. O. Rogers, R. Barker, Jr., H. Dourado, L. Susebang, and B. Albuquerque. 1986. Leishmaniasis and malaria: new tools for epidemiologic analysis. Science 234:975–979.
Woerdenbag, H. J., C. B. Lugt, and N. Pras. 1990. *Artemisia annua* L.: a source of novel antimalarial drugs. Pharm. Weekblad Sci. 12: 169–181.
Woerdenbag, H. J., N. Pras, R. Bos, J. F. Visser, H. Hendriks, and T. M. Malingré. 1991. Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua* L. by combined gas chromatography/mass spectrometry. Phytochem. Anal. 2:215–219.
Woerdenbag, H. J., N. Pras, W. Van Uden, A. DeBoer, S. Batterman, J. F. Visser, and T. M. Malingré. 1992. High peroxidase activity in cell cultures of *Artemisia annua* with minute artemisinin contents. Nat. Prod. Lett. 1:121–128.
Woerdenbag, H. J., R. Bos, M. C. Salomons, H. Hendriks, N. Pras, and T. M. Malingré. 1993a. Volatile constituents of *Artemisia annua* L. (Asteraceae). Flav. Frag. J. 8:131–137.
Woerdenbag, H. J., J. F. J. Lüers, W. van Uden, N. Pras, T. M. Malingré, and A. W. Alfermann. 1993b. Production of the new antimalarial drug artemisinin in shoot cultures of *Artemisia annua* L. Plant Cell Tiss. Organ Cult. 32:247–257.

Woerdenbag, H. J., T. A. Moskal, N. Pras, T. M. Malingré, F. S. El-Feraly, H. M. Kampinga, and A. W. T. Konings. 1993c. Cytotoxicity of artemisinin-related endoperoxides to ehrlich ascites tumor cells. J. Nat. Prod. 56:849–856.
Woerdenbag, H J., N. Pras, N. G. Chan, B. T. Bang, R. Bos, W. van Uden, P. Van Y., N. V. Boi, S. Batterman, and C.B. Lugt. 1994a. Artemisinin, related sesquiterpenes, and essential oil in *Artemisia annua* during a vegetation period in Vietnam. Planta Med. 60:272–275.
Woerdenbag, H. J., N. Pras, W. Van Uden, T. E. Wallaart, A. C. Beekman, and C. B. Lugt. 1994b. Progress in the research of artemisinin-related antimalarials: an update. Pharm. World Sci. 16:169–180.
Xu, X. X., J. Zhu, D. Z. Huang, and W. S. Zhou. 1983. The stereocontrolled synthesis of qinghaosu and deoxyqinghaosu from artennuic acid. Huaxue Xuebao 42:940. (Cited by Luo and Shen [1987].)
Xu, X. X., J. Zhu, D. Z. Huang, W. S. Zhou. 1986. Total synthesis of arteannuin and deoxyarteannuin. Tetrahedron 42:819–828.
Yang, Y. Z., W. Asawamahasakda, and S. R. Meshnick. 1993. Alkylation of human albumin by the antimalarial artemisinin. Biochem. Pharmacology 46:336–339.
Yeou-ruenn, L. 1994. The genera *Artemisia* L. and *Seriphidium* (Bess.) Poljak. in the world. Comp. Newslett. 25:39–45.
Zaman, S. S., and R. P. Sharma. 1991. Some aspects of the chemistry and biological activity of artemisinin and related antimalarials. Heterocycles 32:1593–1638.
Zhao, K. C., C. X. Liu, X. T. Liang, Y. Mingguang, and Z. Y. Song. 1986. Development of radioimmunoassay for determination of artesunate, a derivative of the antimalarial qinghaosu. Proc. Chinese Acad. Med. Sci., Pekin Union Medical College 1:213–217.
Zhao, S. S., and M. Y. Zeng. 1985. Spektrometrische hochdruck-flussigkeits-chromatographische (HPLC) untersuchungen zur analytic von qinghaosu. Planta Med. 51:233–237.
Zhou, W. S., and X. X. Xu. 1989. The structure, reactions and syntheses of arteannuin (qinghaosu) and related compounds. In: Atta-Ur-Rahman (ed.). Studies in natural products chemistry. Stereoselective Synth. 3B:495–527.
Zhou, Z., Y. Huang, G. Xie, X. Sun, Y. Wang, L. Fu, H. Jian, X. Guo, and G. Li. 1988. HPLC with polarographic detection of artemisinin and its derivatives and aplication of the method to the pharmacokinetic study of artemether. J. Liq. Chromatogr. 1:1117–1137.