Chapter 2

Review of literature

REVIEW OF LITERATURE

2.1 Malaria

In low income and developing nations, malaria is the fifth most prevalent infectious disease and the tenth overall cause of death. It is projected to remain at that level until at least 2030 (Mathers and Loncar, 2006). The global malaria situation is deteriorating faster today than at any time in the past century. The number of new cases of the disease has quadrupled in the past five years. Forty percent of the world population living in about 102 countries is at the risk of being infected and half of these live in sub-Saharan Africa. In addition to causing untold suffering and disability, malaria ranks as one of the world’s major killer, costing about 1.5 million people lives annually. The children are especially vulnerable, as they die from malaria than any other single disease. Primigravidae women are the next highest risk group for malaria in endemic areas. It is stated that malaria causes 0.96 million deaths of children per year in Africa alone (WHO, 2007). Most malarial infections occur in Africa. Countries in tropical Africa are estimated to account for 80% of all clinical cases and about 90% of all people, who carry the parasite (Kitua and Malebo, 2004). More recent global pattern of malaria distribution is presented in Fig. 1.

<table>
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<th>Malaria Statistics</th>
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<td>3.3 billion people at risk</td>
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<td>Over 216 million cases</td>
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<td>Over 655000 deaths</td>
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Fig. 1: Global pattern of malaria distribution (Source: World Malaria Report, 2011).
To the poor sub-Saharan countries of Africa, the WHO Roll Back Malaria has highlighted that:

- The cost of malaria control and treatments drains African economies. Endemic countries have to use hard currency on drugs, nets, and insecticides.
- Africa’s gross domestic product (GDP) today would be up to 32% greater, if malaria had been eliminated 35 years ago. According to estimates from Harvard study, malaria endemic countries are among the world’s most impoverished nations.
- A malaria-stricken family spends averages of over one quarter of its income on malaria treatment, as well as paying prevention costs and suffering loss of income.
- Workers suffering an attack of malaria can be incapacitated for at least 5 days.
- Malaria-afflicted families on an average can only harvest 40% of the crops harvested by healthy families.

To treat malaria, quinine and quinoline-based drugs such as chloroquine, mefloquine, and primaquine were widely used before the early sixties (Woerdenbag et al., 1990). Some *P. falciparum* strains, however, became resistant to these antimalarial drugs and multiple side effects also exhibited. Artemisinin and its derivatives have attracted more attention in 2001, When WHO recommended that Artemisinin-based Combination Therapies (ACTs) should be adopted to treat malaria (Mandelbaum-Schmid, 2005) due to little or no cross-resistance with other antimalarial drugs, rapid reduction of the parasite, and efficacious activity against *P. falciparum* strains (Meshnick et al., 1996).

The exponential increase in the number of countries adopting ACTs has led to a rapid increase in demand for artemisinin and its derivatives. The global consumption of ACTs has increased from a few thousands to tens of millions in 4 years (2001-2005). Thus, increased demand produced a global shortage of
artemisinin derivatives (artemether, artesunate/ lumefantrine) supply (WHO, 2001).

2.2 Artemisinin, structure and its clinical use

Artemisinin (also called Qinghaosu) is a sesquiterpene lactone with an unusual endoperoxide structure (Fig. 2). It was isolated from the herb *Artemisia annua* L. by Chinese scientists, when searching for novel antimalarial drugs in the 1970s (Li et al., 2006). Today, artemisinin derivatives provide the basis for the most effective treatments for malaria, particularly in the form of ACTs, which are advocated by the World Health Organization (WHO) in order to reduce the odds of developing resistance (White, 2008). It has an empirical formula of $C_{15}H_{22}O_5$ with a unique structure among antimalarial agents, lacking the nitrogen-containing heterocyclic ring system found in most antimalarial compounds. Artemisinin is currently the best therapeutic molecule against both drug-resistant and cerebral malaria-causing strains of *Plasmodium falciparum*. It is now widely used throughout Southeast Asia and Africa, where malaria has become resistant to nearly all anti-malarial drugs (Newton and White, 1999). Artemisinin and its more potent derivatives (Fig. 2), all contain an endoperoxide bridge that is required for therapeutic activity. Unfortunately, treatment courses, while effective, remain costly and thus are often unavailable to the people that need them most.

![Fig. 2: Chemical structure of artemisinin (A) and its derivatives (B).](image)
Artemisinin has a relatively short half-life thus requiring significant quantities in prescribed doses, and, thus, there is a large and increasing need for this important compound (Arsenault et al., 2008). Since artemisinin is relatively safe drug with other anti-microbial activity and no obvious adverse reactions or serious side effects, even for pregnant women (WHO, 2003), it is of particular interest in the possible treatment of a variety of diseases. However, the drug is in short supply, and because of its complex structure it is still extracted from plants. Although others are working on a synthetic trioxolane (Vennerstrom et al., 2004) and bacterial derived artemisinin precursors (Martin et al., 2003), that may replace artemisinin as an inexpensive therapeutic molecule. A. annua L. plants are currently the only source of the drug and even modest annual targets of 20–22 T yr\(^{-1}\) of artemisinin are not being achieved (WHO, 2005).

### 2.2.1 Artemisinin: uses other than malaria

Besides malaria, artemisinin and its derivatives have been shown to be effective against a number of viruses including human cytomegaloviruses, herpes simplex, and hepatitis B and C (Romero et al., 2005; Efferth, 2009). The drug is also effective against other prokaryotic and eukaryotic pathogens such as *Pnuemocystis carinii*, *Toxoplasma gondii*, a number of human cancer cell lines (Efferth, 2007), and a variety of other parasitic tropical diseases including schistosomiasis (Utzinger et al., 2001), leishmaniasis (Sen et al., 2007), Chagas disease, and African sleeping sickness (Mishina et al., 2007).

Efficacy of the drug against cancer apparently stems from the anti-angiogenesis activities of artemisinin along with its possible role in protein alkylation in high iron environments (Chen et al., 2003). Cancer cells maintain a higher iron content than do non-cancerous cells, with the exception of erythrocytes, making this a viable working hypothesis for the specific activity of artemisinin. In addition, dihydroartemisinin has been shown to induce cell cycle arrest and apoptosis in certain cancer cell lines (Efferth, 2007). Limited clinical trials are in progress using artesunate in conjunction with standard chemotherapies as a treatment for uveal melanoma with some positive results.
(Berger et al., 2005). Artemisinin and its derivatives, therefore, may have wide applicability in the treatment of various cancers. The drug also appears to be useful in treating some animal diseases (Ferreira and Gonzalez, 2008).

2.3 *Artemisia annua* L.

*Artemisia annua* L. is also known as sweet wormwood in the United States, and Qing Hao in China. As a Chinese annual herb, the pharmaceutical value of *A. annua* L. has been recognized since 168 B.C. and it has been used to treat fevers, haemorrhoids, and malaria in China for centuries (Abdin et al., 2003). *A. annua* L. is a qualitative short day plant (Ferreira and Janick, 1995). The mature plant with a single stem can reach about 2 m in height. Aromatic leaves are about 2.5-5.0 cm long, deeply dissected and alternately branched around the stem. At least 40 volatile compounds and a lot of non-volatile compounds have been extracted and identified from *A. annua* L. (Ferreira and Janick, 1996). Artemisinin and other artemisinic compounds are the most important compounds isolated from this plant due to their pharmaceutical value (Ferreira and Janick, 1996).

In the whole plant, artemisinin has been reported to accumulate in the shoots and seeds with highest levels in leaves and flowers. Neither artemisinin nor its precursors, however, were detected in roots (Abdin et al., 2003). Artemisinin content in flowers was 4–5 times higher than in leaves (Ferreira et al., 1995). Artemisinin is sequestered in the glandular trichomes (Duke et al., 1994), and glandless trichomes appear to produce no artemisinin (Ferreira and Janick, 1995). Artemisinin yields vary with plant development revealing a possible positive correlation between plant age and artemisinin content. Reports on the distribution of artemisinin throughout the plant, however, have been inconsistent. Artemisinin has been reported to be higher at the top of the plant in some varieties (Charles et al., 1990; Laughlin, 1995) and equally distributed in others (Laughlin, 1995). Some contend that artemisinin content is highest just prior to flowering, while others noted peak production during full bloom. Artemisinin concentration in *A. annua* L. is low, in the range of 0.01–0.8%
(Abdin et al., 2003); however, concentrations in some strains may be as high as 1.5%. Although chemical synthesis is possible, it is complicated and economically not viable due to low yields. Thus, the plant remains the only commercial source of the drug, and its relatively low yield in *A. annua* L. is a serious limitation to its commercialization (Abdin et al., 2003; Bertea et al., 2005). The enhanced production of artemisinin either in tissue cultures or in whole plants of *A. annua* L. is thus highly desirable and should be achievable with a better understanding of the biosynthetic pathway and its regulation by both exogenous and endogenous factors. Furthermore, once the pathway genes and their regulatory controls have been elucidated, metabolic engineering can be employed to enhance artemisinin biosynthesis and its accumulation in *A. annua* L.

*A. annua* L. is required to first produce the bulk artemisinin starting material and eventually the finished product. The global and Indian status of demand and supply of artemisinin are given in Fig. 3.

![Fig. 3: The demand and supply of artemisinin at global level (Source: A2S2 market intelligence, 2012).](image)

2012 MSF-2012 Minimum supply forecast.
2012 MaxSF-2013 Maximum supply forecast.
2013 MDF-2013 Minimum demand forecast.
2013 MaxDF-2013 Maximum demand forecast.
2.3.1 Biosynthesis of artemisinin

Artemisinin is an endoperoxide sesquiterpene lactone. It belongs to isoprenoid group of compounds that are synthesized by isoprenoid pathway, the most important and widely encountered biosynthetic pathways in plants (Fig. 4). These compounds are derived from two common precursors, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP). It has been established that higher plants have two independent biosynthetic pathways leading to the formation of IPP; the cytosolic mevalonate pathway and the plastid-localized mevalonate independent pathway (Liu et al., 2005; Towler and Weathers, 2007). Recently, the two gene clones, encoding for deoxy-D-xylulose-5-phosphate synthase (DXPS) and deoxy-D-xylulose-5-phosphate reductoisomerase (DXPR) have been isolated from transformed hairy roots of A. annua L. These are the key enzymes of alternate pathway for IPP synthesis. The cloning of the genes encoding these enzymes, thus demonstrated the plastid-localized terpenoid biosynthetic pathway (Souret et al., 2002; Krushkal et al., 2003). It is now well established, however, that ~80% of the carbon for artemisinin biosynthesis is made available by mevalonate pathway, while ~14% of carbon requirement is met by non-mevalonate pathway (Maujiram et al., 2010).

2.4 Approaches for the production of artemisinin

At least three different approaches have been used to increase production of artemisinin: heterologous, nontransgenic and transgenic systems. The heterologous systems include insertion of key artemisinin biosynthetic genes into organisms other than A. annua L. Nontransgenic approaches include selective breeding, alteration of nutrients and environmental conditions, use of in vitro cultures, and exploitation of plant’s natural defense system through elicitation. Transgenic A. annua L. plants have also been produced using a variety of different genes.
Fig. 4: Possible routes of artemisinin biosynthesis (Towler and Weathers, 2007; Zhang et al., 2008).
Abbreviations; ADS, amorpha-4,11-diene synthase; ALDH1, aldehyde dehydrogenase 1; CYP71AV1, cytochrome P450 monooxygenase; DBR2, artemisinic aldehyde reductase; DXP, 1-deoxy-d-xylulose 5-phosphate; DMAPP, dimethyl allyl pyrophosphate; DXS, 1-deoxy-d-xylulose 5-phosphate synthase; DXR, 1-deoxy-d-xylulose 5-phosphate reductoisomerase; G3P, d-glyceraldehyde-3-phosphate; HMGR, 3-hydroxy-3-methylglutaroyl coenzyme A reductase; IPP, isopentenyl diphosphate; ISPH, Artemisia annua IPP/DMAPP synthase gene; MEP, 2-Cmethyl-d-erythritol-4-phosphate.

2.4.1 Heterologous production of artemisinin

Heterologous systems have been identified as potentially more productive platforms for pathway engineering. The process of artemisinin production through synthetic biology, the metabolism of the microbes (Sacchromyces cerevisae) engineered to produce artemisinic acid, a precursor of artemisinin (Fig. 5; Ro et al., 2006) is an important step in artemisinin production and its yield enhancement. The artemisinic acid is chemically converted into artemisinin. Once artemisinin is produced, it must be further chemically converted into a derivative such as artesunate or artemether, which are integrated into ACTs for the treatment of malaria. Fortunately, ACTs are not always available for every malaria victim in the endemic regions, because artemisinin is produced in Artemisia annua L. (sweet wormwood) in trace
amounts (Duke and Paul, 1993; Duke et al., 1994), which makes it a substantial bottleneck for meeting the demand of over 100 million courses of ACTs each year (Mutabingwa, 2005).

The shift of artemisinin production from plants to microbes will allow the industrial process of artemisinin synthesis, ultimately enabling ACTs accessible at an affordable price to all patients in poor nations. For this purpose, practical approaches have been developed to transplant artemisinin biosynthetic genes from A. annua L. into genetically tractable microbial hosts; for example, *Escherichia coli* and *Saccharomyces cerevisiae* (Khosla and Keasling, 2003). Such metabolic engineering platform is readily established for terpene production as most catalytic steps for terpene. As a eukaryotic microbe, *S. cerevisiae* offers promise for heterologous expression of plant genes including those encoding the membrane-bound proteins that have difficulty functionalizing in bacterial systems (DeJong et al., 2006). One of the early successful examples is expression of the gene coding for taxane 10b-hydroxylase from *Taxus* sp. in *S. cerevisiae* (Schoendorf et al., 2001). While choosing yeast for high-level production of artemisinin, its known high rate of isoprene metabolism must be taken into account. An ideally engineered yeast strain should have increased FPP production and decreased sterol accumulation. The Brodelius group has compared the effects of two different formats of A. annua L. *ads* gene expression in yeast on the production of amorpha-4,11-diene. First, the *ads* gene has been introduced into yeast cells on an episomal plasmid using a galactose-inducible promoter. Second, the *ads* gene has been inserted into the yeast genome by homologous recombination. The plasmid and genome-transformed yeasts produced 600 and 110 µg l⁻¹ of the artemisinin precursor, amorpha-4,11-diene, respectively during 16 days batch cultivation (Lindahl et al., 2006). It is apparent that the production of sesquiterpene in yeast is positively correlated with the gene dosage, but the insufficient substrate (FPP) pool is most likely a limiting factor. Engineered yeast strains capable of producing artemisinic acid have been achieved through multi-gene transfer as reported by Ro et al. (2006). The *ads* and *hmgr*
genes have been transferred into yeast cells for generating amorpha-4,11-diene and increasing the FPP pool, respectively.

Further augmented levels of FPP and amorpha-4,11-diene have been achieved through the reduction of sterol biosynthesis by repressing sesquiterpene synthase gene expression with a methionine-repressible promoter. Then, cyp71av1 and cpr genes have been introduced into yeast cells to enable a three-step oxidation from amorpha-4,11-diene producing artemisinic acid. When evaluated step-by step, the expression of ads gene alone only results in a relatively lower quantity of amorpha-4,11-diene (4.4 mg amorpha-4,11-diene l$^{-1}$), while co-expression of a truncated hmgr12 gene improves the yield of amorpha-4,11-diene by approximately five-fold. Introduction of a methionine repressible promoter down-regulates the sterol biosynthetic gene, erg9, and leads to an additional two fold increase of amorpha-4,11-diene. Down-regulation of the erg9 gene and over-expression of upc2-1, a dominant mutated gene encoding the UPC2P transcription factor capable of regulating sterol biosynthesis has increased amorpha-4,11-diene production to 105 mg l$^{-1}$. Integration of an additional copy of the hmgr gene into the chromosome further increases amorpha-4,11-diene production to 149 mg l$^{-1}$. fpps (erg20) gene decreases cell density and, in turn, increases amorpha-4,11-diene yield by about additional 10% (w/w). All these multi-gene manipulations lead to a total enhancement in amorpha-4,11-diene biosynthesis up to 153 mg amorpha-4,11-diene l$^{-1}$, an elevation of nearly 500-fold of what has been reported previously (Jackson et al., 2003). These molecular strategies together with over-expression of other downstream genes of artemisinin biosynthesis in yeast may ultimately lead to the production of artemisinin using this recombinant yeast system.
2.4.2 Nontransgenic efforts

Much work is being done to increase the artemisinin supply, including breeding high yielding *A. annua* L. strains (Graham et al., 2010). Although total chemical synthesis of artemisinin has been achieved, it is not cost effective (Haynes, 2006).

The commercial sources of artemisinin are from field grown leaves and flowering tops of *A. annua* L. plants, which are subjected to seasonal and somatic variation and infestation of bacteria, fungi, and insects that can affect the functional medicinal content of this plant (Klayman, 1985; Luo and Shen, 1987). The total organic syntheses are very complicated with low yields, and are economically unattractive (Xu et al., 1986; Avery et al., 1992). Harvesting
of *A. annua* L. takes place before flowering, when the artemisinin yield is highest, and is completed within a month. Extraction takes place during four to six months after the harvest, which occurs around six months after planting. Thus a period of 12 months from the time of planting of *A. annua* L. is required to first produce the bulk artemisinin starting material and eventually the finished product.

In view of these problems, an alternative to field-grown crops or to chemical synthesis for artemisinin production has been large scale *in vitro* plant tissue culture (Dhingra et al., 2000). The biosynthesis of artemisinin was studied in the calli, suspension cells, and shoots of *A. annua* L. during their cultivation *in vitro* (He et al., 1983; Nair et al., 1986; Tawfiq et al., 1989; Teo et al., 1995; Paniego and Giulietti, 1996; Liu et al., 1997).

### 2.4.2.1 Artemisinin production from root culture, shoot culture and whole plants

Paniego and Giuletti (1994) reported trace amounts of artemisinin in the multiple shoot cultures of *A. annua* L. Woerdenbag et al. (1993) reported a high percentage of artemisinin content in *A. annua* L. shoots cultured on 1/2 MS medium supplemented with 0.05 mg l\(^{-1}\) naphthaleneacetic acid, 0.2 mg l\(^{-1}\) benzyladenine (BA), and 2% sucrose. The flowering of *A. annua* L. was observed *in vitro* by supplementing with gibberellic acid (GA\(_3\)), where artemisinin content reached 0.1% in *A. annua* L. plantlets, and the highest artemisinin content in the plantlets was observed in full bloom (Gulati et al., 1996). Most groups did not find artemisinin in root part of *A. annua* L. plants. However, artemisinin content in the shoot part of cultured plants was higher than that in the cultured shoots without roots (Martinez and Staba, 1988; Ferreira and Janick, 1996).

Attempts have been made to improve the artemisinin production by omittance or addition of medium components like plant growth regulators, casein hydrolysate, and by inclusion of precursors of artemisinin biosynthetic pathway, elicitors and by addition of sterol synthesis inhibitors. Casein
hydrolysate, a source of aminoacids and oligopeptides, in low concentration enhances the artemisinin production, but its prolonged exposure negatively affects the biomass production and growth of shoots. Kumar (1997) reported that incorporation of boron, casein hydrolysate and gibberillic acid enhances artemisinin production by 25%, 36% and 65%, respectively. No effect on growth was however, observed. Fulzele et al. (1995) reported the stimulation of terpenoid synthesis in plantlet cultures from \textit{A. annua} L. by addition of GA$_3$. However, other scientists found only a slight effect on biomass production of plants treated with 40 and 80 mg l$^{-1}$ GA$_3$ (Martinez and Staba, 1988; Charles et al., 1990; Fulzele et al., 1995; Smith et al., 1997; Weathers et al., 2005). A combination of benzylaminopurine (1mg l$^{-1}$) and kinetin (10 mg l$^{-1}$) increased the yields of artemisinin \textit{in vitro} by 3.6 and 2.6 fold, respectively, due to increase in dry matter production which can overcome a concurrent decrease in the artemisinin content (Whipkey et al., 1992).

Enhancing the artemisinin production by precursor feeding was also investigated. Addition of artemisinin precursors to the medium used for tissue cultures of \textit{A. annua} L. resulted in a four fold increase of artemisinin in the tissue and an 11 fold increase of artemisinin in the spent medium (Weathers et al., 1994). The feeding of mevalonic acid alone, however, did not induce an enhancement of artemisinin production (Woerdenbag et al., 1993). But the addition of some compounds such as naphtiphine (an inhibitor of the enzyme squalene epoxidase) to the medium improved the artemisinin production. Other additions, such as 5-azacytidine (a gene regulator), colchicine (a gene regulator), miconazole (an inhibitor of sterol demethylase), and terbinaphine (an inhibitor of the enzyme squalene epoxidase), were too toxic for the cultures to induce an enhancement of the artemisinin production (Woerdenbag et al., 1993). Kudakasseril et al. (1987), however, reported a concentration-dependent increase in the levels of artemisinin and growth of shoot cultures with miconazole. Other sterol inhibitors, such as chlorocholine chloride, 2-isopropyl-4-(trimethylammoniumchloride)-5-methylphenylpiperidinecarboxylate, and 4-chloro-2-(2-diethylaminoethoxyphenyl)-2-(4-methylphenyl)
benzeneethanol increased both the incorporation of 14C-IPP into artemisinin by cell-free extracts and the production of artemisinin in shoot cultures of *A. annua* L.

### 2.4.2.2 Artemisinin production from cell suspension culture

Several-fold enhancement in yield of secondary metabolites has been reported for many cell cultures (Panda et al., 1992; Sakamoto et al., 1993; Sato et al., 1996). Paniego and Giuletti (1994) however, reported no artemisinin in cell suspension cultures of *A. annua* L. In later studies the yields of artemisinin obtained in cell suspension cultures of *A. annua* L. were not commercially attractive (Liu et al., 2006; Covello, 2008). Nevertheless, this approach is fundamental to identify chemical and molecular factors that could have a role in artemisinin biosynthesis (Baldi and Dixit, 2008; Wang et al., 2009).

*A. annua* L. cell cultures were established by Durante et al. (2011) and effect of cyclodextrins were observed. Native β-cyclodextrins as well as the chemically modified heptakis(2,6-di-O-methyl)-β-cyclodextrin (DIMEB) and 2-hydroxypropyl-β-cyclodextrins were added to the culture medium of *A. annua* L. suspension cultures, and their effects on artemisinin production were analysed. 50 mM DIMEB as well as a combination of 50 mM DIMEB and 100 μM methyl jasmonate was highly effective in increasing the artemisinin levels in the culture medium. The observed artemisinin level (27 μmol g⁻¹ DW) was about 300 fold higher than that observed in untreated suspensions. Effect of methyl jasmonate (MeJA) and miconazole was observed by Caretto et al. (2011), where 22 μM MeJA induced a three fold increase of artemisinin production in around 30 min; while 200 μM miconazole induced a 2.5 fold increase of artemisinin production after 24 h. These treatments, however, had severe effects on cell viability. The influence of these treatments on expression of biosynthetic genes was also investigated. MeJA induced up-regulation of *cyp71av1*, while miconazole induced up-regulation of *cpr* and *dbr2*. The major constraint with cell cultures is the gradual decrease (deteriorations) in
biosynthetic capacity of cell suspension cultures resulting in low yields of secondary metabolites during subcultures.

2.4.3 Transgenic approach

2.4.3.1 Artemisinin production from bioengineered *A. annua* L. plants

Genetic engineering of the *A. annua* L. plants is one of the possibilities for improving artemisinin production. In the last decade, and especially, in the last 4 years, excellent progress has been made in elucidating the biosynthetic pathway of artemisinin (Bertea et al., 2005; Covello et al., 2007; Zhang et al., 2010). There has been a certain amount of confusion, however, in the literature regarding artemisinin biosynthesis (Li et al., 2006). Most efforts in the study of artemisinin biosynthesis have concentrated on elucidating the enzymes and genes in the artemisinin biosynthetic pathway beginning with the cyclization of FPP by sesquiterpene cyclase.

Based on the information available on artemisinin biosynthesis and the genes encoding enzymes catalyzing various steps of not only artemisinin biosynthesis, but also those competing with it, the approaches developed and used to enhance artemisinin production in transgenic *A. annua* L. plants are discussed below:

2.4.3.2 Over-expression of endo and exogenous genes

It is envisaged to produce high artemisinin yielding transgenic strains of *A. annua* L. plants by either the optimization of enzymes catalyzing rate limiting steps of artemisinin biosynthesis or suppressing the enzymes catalyzing first step of competing pathways or both. To achieve these objectives, the researchers have successfully transferred and over-expressed a number of endo and exogenous genes into *A. annua* L. to ensure high production of artemisinin. In another approach they have down-regulated the expression of genes encoding the enzymes of competing pathways for artemisinin precursors. In recent years, remarkable progress has been made in the understanding of molecular biology of artemisinin biosynthesis and its
regulation (Bouwmeester et al., 1999; Weathers et al., 2006). The genes of the key enzymes involved in the biosynthesis of artemisinin, such as, HMG-CoA reductase (HMGR), farnesyl pyrophosphate synthase (FPS), amorpha-4,11-diene synthase (ADS), and isopentyl transferase (IPT) have been cloned from A. annua L. (Mercke et al., 2000; Wallaart et al., 2000; Abdin et al., 2003; Liu et al., 2003; Alam et al., 2010; Alam and Abdin, 2011). On the other hand the farnesyl diphosphate synthase gene (fps) from Gossypium arboretum and hmgr from Catharanthus roseus have been over-expressed as exogenous gens in to Artemisia annua L. (Aquil et al., 2009; Nafis et al., 2011). Vergauwe et al. (1996) developed an A. tumefaciens mediated transformation system for A. annua L. plants with high transformation rates (75% regenerants harboring foreign gene). Artemisinin content in the leaves of regenerated plants was 0.17%, a little bit higher than that present in the leaves of normally cultured plants (0.11% DW). They further investigated the factors influencing transformation efficiency viz., the age of explants, A. tumefaciens strain and plant genotype (Vergauwe et al., 1998). Later, Han et al. (2005) established a high efficiency genetic transformation and regeneration system for A. annua L. via A. tumefaciens.

In the isoprenoid biosynthesis pathway, farnesyl pyrophosphate synthase (FPS) catalyzes the two sequential 1-4 condensations of IPP with DMAPP to produce GPP and with GPP to give FPP, which is then utilized by isoprenoid pathway and artemisinin biosynthetic pathway to produce isoprenoids and artemisinin, respectively (Cane, 1990). The cytokinin biosynthetic gene (ipt) codes for the isopentenyl transferase, which catalyzes the condensation of isopentenyl pyrophosphate and adenosine monophosphate (AMP) to yield isopentenyl-AMP. It represents the rate-limiting step in cytokinin biosynthesis in tumorous plant tissue (Akiyoshi et al., 1983; 1984). The influence of over expression of isopentenyl transferase gene on the physiological and biochemical characteristics of A. annua L. plant was studied by Sa et al. (2001). The transgenic A. annua L. plants were found to accumulate more
cytokinins (2 to 3 folds), chlorophyll (20-60%) and artemisinin (30-70%), when compared with control plants (Sa et al., 2001).

The cDNAs encoding FPS have been isolated from a number of plant species, including *Arabidopsis thaliana* (Delourme et al., 1994) and *Lipinus albus* (Attucci et al., 1995). Since 15-carbon FPP can be catalyzed by sesquiterpene cyclases, such as ADS to form cyclic sesquiterpenoids (amorpha-4,11-diene in *A. annua* L.), over expressing foreign fps gene into *A. annua* L. plants holds the possibility of affecting the accumulation of artemisinin. A cDNA encoding cotton fps placed under CaMV 35S promoter was hence transferred into *A. annua* L. plants via *A. tumefaciens* strain LBA 4404 or *A. rhizogenes* strain ATCC 15834 mediated genetic transformation (Chen et al., 1999; 2000). In the transgenic plants, the concentration of artemisinin was approximately 8-10 mg g\(^{-1}\) DW, which was 2-3 folds higher than control plants. Han et al. (2006) achieved about 34.4% increase in artemisinin content by over expressing endogenous fps. It is quite interesting to compare this result with that of overexpressing heterogeneous (*G. arboreum*) fps in *A. annua* L., which caused a two fold increase in artemisinin content (Chen et al., 2000). Recently, Banyai et al. (2011) over-expressed fps in *Artemisia annua* L. plants and transgenic lines exhibited higher artemisinin content and yield of 2.5 and 3.6 fold, respectively, than that detected in wild type plants. A relatively high correlation was observed between the levels of expression of fps and artemisinin content. Ma et al. (2008) evaluated the effects of over-expressing fps in transgenic plants and terpenoid profiling of *A. annua* L. plants was detected by GC–MS. The results indicated that there was a clear difference in metabolic profiling between the control and transgenic plants in the late stage of development.

In our lab, we have over expressed the *hmgr* encoding one of the key regulatory enzymes of mevalonate pathway in low and high yielding *A. annua* L. strain via *A. tumefaciens* mediated transformation and achieved 22% and 38% enhancement in artemisinin content, respectively as compared to the wild type plants (Aquil et al., 2009; Nafis et al., 2011). Jing et al. (2008) simultaneously over-expressed *cyp71av1* and *cpr* genes in *A. annua* L. and
recorded 2.4 fold enhancement in artemisinin content. Amorpha-4,11-diene synthase is one of many different terpene cyclases in terpenoid metabolism. Recently, we have over expressed the *hmgr* and *ads* genes simultaneously in *A. annua* L. plants and found 7.68 fold enhancement in artemisinin content as compared to the wild type plants (Alam and Abdin, 2011). These results indicate that artemisinin biosynthesis is strictly regulated in the plant, and its production in the plant can be enhanced by over-expression of genes encoding enzymes of rate limiting steps in mevalonate and artemisinin biosynthetic pathways, respectively.

### 2.4.3.3 Suppression of endogenous genes

The mechanism of artemisinin biosynthesis has recently become much clearer (Abdin et al., 2003; Bertea et al., 2005; Liu et al., 2006). Ro et al. (2006) confirmed that down-regulation of *erg9* (ergosterolbiosynthesis-pathway gene9), a gene that encodes SQS in yeast, using a methionine-repressible promoter (PMET3), increased the production of amorpha-4,11-diene 2 fold in a yeast strain into which FPP-biosynthetic pathway genes and the *ads* (amorpha-4,11-diene synthase) gene from *A. annua* L. had been incorporated. Recently, the hpRNAi mediated technology has been used to suppress gene function in many plant species. Liu et al. (2002) reported that hpRNA mediated down regulation of *ghSAD*-1 and *ghFAD2*-1, two key enzymes in the fatty-acid-biosynthesis pathway in cotton (*Gossypium hirsutum*), elevated the steric acid content (44% compared to the normal level of 2%) and oleic acid content (77% compared with the normal level of 15%) in cotton seeds. In a study with opium poppy (*Papaver somniferum*), a hpRNA construct containing sequences from multiple cDNAs of genes in the codeine reductase gene family was used to silence several enzymes in the pathway. The similar strategy were used for enhanced artemisinin content up to 2-3 fold in transgenic *A. annua* L. plants (34 mg g⁻¹ DW as compared to 8-10 mg g⁻¹ DW in controlled plants) by suppressing the expression of *sqs* (squalene synthase gene), encoding SQS, a key enzyme of sterol pathway (a pathway competitive with that of artemisinin
biosynthesis) by means of hp-RNA-mediated RNAi (Zhang et al., 2009). The sterol content of transgenic plants was also reduced to 37-58% as compared to the wild type plants, but it had not affected the growth and development. In an attempt to increase artemisinin content of A. annua L. by suppressing the β-caryophyllene synthase gene encoding a sesquiterpene synthase, β-caryophyllene synthase (β-CPS) that competes artemisinin for its precursor FPS, the antisense fragment (750 bp) of β-caryophyllene synthase c-DNA (cps) was inserted into the plant expression vector, pbi121 and introduced into A. annua L. by Agrobacterium-mediated transformation. The expression of endogenous cps in the transgenic lines was significantly lower than that in the wild-type control A. annua L. plants, and β-caryophyllene content decreased sharply in the transgenic lines in comparison to the control. The artemisinin content of one of the transgenic lines showed an increase of 54.9 % compared with the wild-type control (Chen et al., 2011). This study along with others, therefore, demonstrates that the metabolic engineering strategy of suppressing sterol biosynthesis or other competing pathways using RNAi could be an effective and suitable strategy for increasing the artemisinin content in A. annua L. plants.

2.4.3.4 Artemisinin production from hairy root culture

The name “hairy root” was first introduced in the literature by Steward et al. (1900). Riker et al. (1930) later described and named the hairy-root-causing microorganism as Phytoponas rhizogenes, which was later renamed Agrobacterium rhizogenes. This conclusion was accepted and given wide recognition (White et al., 1982). A large number of small fine hairy roots covered with root hairs originated directly from the explants in response to A. rhizogenes infection (Tepfer, 1984) and, hence, the term hairy root was used. Porter (1991) reported that more than 450 species of many different genera and families are known to be susceptible to the infection by A. rhizogenes (Hamill and Lidgett, 1997). Since then many more additions have been made to the list. As they can be sub-cultured and indefinitely propagated on a
synthetic medium without phyto-hormones and have the capacity to form a profusion of lateral roots, the hairy roots received considerable attention from plant biotechnologists interested in the production of secondary compounds (Mishra and Ranjan, 2008). In addition, combining hairy roots cultivation to bacterial co-culture and elicitation strategies provide attractive resource for large-scale production of commercially valuable compounds (Shi et al., 2007; Wu et al., 2007; Mehrotra et al., 2010).

2.4.3.4.1 Mechanism of hairy root induction

Invasion of dicotyledonous plant tissues by A. rhizogenes soil bacteria usually occur at a wounded site and possibly caused by insect or mechanical damage. Wounded site produces phenolic compounds that attract A. rhizogenes by chemotaxis, which thereby infects the plant cell at the wounded site. This activity causes roots to proliferate rapidly at the infection site causing hairy root disease (large number of small roots protrudes as fine hairs directly from the infection site) (Banerjee et al., 1995). This phenotypic response (hairy root) results from the insertion into the plant genome of T-DNA (transfer DNA) carried on by the bacterial Ri-plasmid (root inducing plasmid), coding for auxin synthesis and other rhizogenic functions (Petit et al., 1983; Ambros et al., 1986). The transformed segment, T-DNA also contains genes for opine biosynthesis. Products of virulence (vir) genes located on non-transferred segment of the Ri plasmid are responsible for excision of the T-DNA for transfer into the plant cell, and possibly for chromosomal integration in the nucleus of the recipient cell (Giri and Narasu, 2000).

2.4.3.4.2 Advantages of hairy roots over conventional methods

Hairy roots are characterized by a high degree of lateral branching, profusion of root hairs and absence of geotropism (Tepfer, 1984). They often grow as fast as or faster than plant cell suspension cultures due to their extensive branching, resulting in the presence of many meristems (Charlwood and Charlwood, 1991; Flores et al., 1999) and do not require expensive hormones in the medium. The increase in the number of branches is approximately
logarithmic during the early stages of growth and thus overall pattern of growth is similar to cell suspension cultures (Flores and Filner, 1985; Flores, 1992). Owing to the highly organized and small celled region of the meristem in each lateral, cell cycle times for hairy roots averages less than 10 hour (Gould, 1982). Hairy roots do not require conditioning of the medium (Rhodes et al., 1987).

In addition, hairy root lines can be a promising source for the constant and standardized production of secondary metabolites. Numerous studies have indicated that hairy roots can produce secondary metabolites over successive generations without losing genetic or biosynthetic stability (Flores et al., 1999; Rao and Ravishankar, 2002). This can be related to the fact that the chromosome number remains the same as that of the parent plant and is not easily disrupted by somaclonal variation, as in common plant cell suspension cultures (Vivanco et al., 2002). For example, cultures of *Hyoscyamus muticus* hairy roots showed equal or higher levels of hyoscyamine synthesis compared to the roots of a whole plant (Flores and Filner, 1985) and have maintained the same biosynthetic capacity for more than 15 years (Flores et al., 1999) even after cultures are shifted back and forth between the more productive differentiated (roots) and the less productive undifferentiated (cells) state.

The efforts have been made in past on large scale production of artemisinin through optimization of hairy root system (Qin et al., 1994; Weathers et al., 1994; Cai et al., 1995; Banerjee et al., 1997; Giri et al., 2001; Xie et al., 2001; Liu et al., 2002) of the *Artemisia annua* L. plant, but the yield is very poor. Following stratagies were used to enhance artemisinin production in hairy root cultures of *A. annua* L.-
Fig. 6: Hairy root initiation from *Artemisia annua* L.
2.4.3.4.3 Strategies to increase artemisinin production in hairy root cultures of *A. annua* L.

2.4.3.4.3.1 Environmental factors

There is poor knowledge about the effects of environmental conditions like temperature, pH and light intensity on the artemisinin production. Better stability of artemisinin has been reported under mild acidic conditions (Jarvis et al., 1998). Alterations in the environmental factors such as light, and temperature have proved to be effective in increasing the product yield and productivity. The relative impact of these influences is usually species dependent (Bourgaud et al., 2001). Wang et al. (2001) investigated the dependence of biomass of hairy roots and artemisinin content on the light spectrum. They found that the highest biomass (5.73 g DW l⁻¹) and artemisinin content (31 mg g⁻¹) were obtained under red light at 660 nm which were 17 and 67% higher than those obtained under white light, respectively. The effects of light irradiation on growth and production of artemisinin were studied in hairy root cultures of *A. annua* L. by Liu et al. (2002). They found that when the hairy roots were cultured under illumination of 3,000 lx for 16 h using several cool-white fluorescent lamps, the dry weight and artemisinin concentration reached 13.8 g l⁻¹ and 244.5 mg l⁻¹, respectively (Liu et al., 2002).

Cai et al. (1995) studied the effect of temperature on hairy roots of *A. annua* L. and observed that the optimum pH value of the medium was 5.4. Temperature in the range of 15–35°C also affected growth and artemisinin biosynthesis in the cultured *A. annua* L. hairy roots. The maximum hairy root growth was found at 25°C. However, the highest artemisinin content in the root cultures was observed at 30°C (Guo et al., 2004).

2.4.3.4.3.2 Media compositions and culture conditions

Growth of plant cells and production of secondary metabolites in cell cultures depend on concentration and interaction of nutrients present in cultivation medium (Mairet et al., 2009). The productivity of any tissue culture system including hairy roots is greatly influenced by the cultivation conditions (Rao and
Ravishankar, 2002). Many constituents of hairy root culture media are important determinants of growth and secondary metabolite production (Giri and Narasu, 2000). Wang and Tan (2002) reported the influence of the ratio of NO$_3$/NH$_4^+$ and total initial nitrogen concentration on the artemisinin yield in hairy roots. With the ratio of NO$_3$/NH$_4$ at 5:1 (w/w), the optimum concentration of total nitrogen for artemisinin production was 20 mM. Under this concentration, artemisinin production was 57% higher than that in the standard MS medium. Fast growth of the hairy roots and maximal production of artemisinin was observed in the presence of 3% sucrose (Cai et al., 1995). Weathers research group investigated the effects of media sterilization method and types of sugar on growth and artemisinin accumulation of *A. annua* hairy roots (Weathers et al., 2004). They found that biomass from filter-sterilized medium was greater than that from autoclaved medium, but artemisinin accumulation from filter-sterilized medium was less than that from autoclaved medium. Growth of hairy roots in the medium with sucrose (3.99 g DW l$^{-1}$) was equivalent to the growth in the medium with fructose (3.75 g DW l$^{-1}$) and significantly better than in the medium with glucose (2.16 g DW l$^{-1}$), while the roots that grew in glucose showed a dramatic stimulation in artemisinin content which is three and two fold higher than that in medium with sucrose and fructose, respectively (Weathers et al., 2004).

2.4.3.4.3.3 Application of elicitors, precursors and permeabilizing agents

Plants produce secondary metabolites in nature as a defense mechanism against attack by pathogens (Croteau et al., 2000). Elicitors are signals triggering the formation of secondary metabolites. Secondary pathways are activated in response to stress (Croteau et al., 2000). Biotic and abiotic elicitors are used to stimulate secondary metabolite product formation in hairy cultures, thereby reducing the process time to attain high product concentrations and increased culture volumes (Rao and Ravinshankar, 2002). Biological elicitors have been reported to be very effective to this purpose (Galletti et al., 2009); they can be derived from other organisms such as fungi,
bacteria, viruses or herbivores, and include plant-derived chemicals that are released at the attack site or accumulate systemically upon pathogen or herbivore attack. The treatment of plant cells with biotic and/or abiotic stimuli is a useful strategy to overcome the generally inherent low yield of plant secondary metabolites (Weathers et al., 2011). The artemisinin accumulation in the hairy root cultures of *Artemisia annua* L. was enhanced via treatment of three fungal elicitors separately (*Verticillium dahliae*, *Rhizopus stolonifer* Vuill and *Colletotrichum dematium*). The artemisinin content of the hairy root cultures treated with *V. dahliae* elicitor was 1.12 mg g\(^{-1}\) DW, which was 45% higher than the control (Hong et al., 2000). When (22S, 23S)-homobrassinolide (SSHB) was added at 1 μg l\(^{-1}\) to hairy root cultures of *Artemisia annua* L., the production of artemisinin reached to 14 mg l\(^{-1}\). SSHB treatment led concomitantly to an increased biomass production of 15 g l\(^{-1}\). A stimulatory activity of SSHB on nucleic acids and soluble protein content in hairy roots was also observed at the growth stage (Wang et al., 2004). The different components of crude mycelium of the predominant endophytic *Colletotrichum gloeosporioides* of *Artemisia annua* L. have been extracted and effect of the isolated components on artemisinin biosynthesis in hairy root cultures was compared. When hairy roots of 23 days old cultures were exposed to the elicitor at 0.4 mg ml\(^{-1}\) for 4 days, the maximum production of artemisinin reached to 13.51 mg l\(^{-1}\) (Wang et al., 2004). Artemisinin production by hairy roots of *Artemisia annua* L. was increased 6 fold to 1.8 μg mg\(^{-1}\) DW over 6 days by adding 150 mg chitosan l\(^{-1}\) (Putalun et al., 2007). Similar treatment of hairy roots with methyl jasmonate (0.2 mM) or yeast extract (2 mg ml\(^{-1}\)) increased artemisinin production to 1.5 and 0.9 μg mg\(^{-1}\) DW, respectively. Pathogen-secreted hydrolytic enzymes that degrade host cell wall polymers, i.e. endopolygalacturonases (PGs; EC 3.2.1.15), are also able to induce defense responses in plants and their involvement has been demonstrated in many pathosystems (Ferrari et al., 2008).

Any intermediate compound endogenous or exogenous, which can be converted into desired secondary metabolite, is known as a precursor.
Metabolic precursors have been commonly applied to the culture medium to enhance the production of secondary metabolites (Ciddi and Kokate, 1997). The addition of precursor may influence spatial orientation of enzymes, compartmentation of enzymes and substrate accumulation for secondary metabolite biosynthesis. Scaled-up hairy root culture of *Artemisia annua* L. was established in three-liter Erlenmeyer flask (Xie et al., 2000). Both artemisinin and stigmasterol that derive from the common precursors of isopentenyl diphosphate and farnesyl pyrophosphate were isolated from hairy roots. The production rate of artemisinin isolated by column chromatography from hairy root cultures was 0.54% (mg g\(^{-1}\) DW). The production of stigmasterol isolated by column chromatography from hairy root cultures was 108.3% (mg g\(^{-1}\) DW).

Permeabilizing agents are those compounds which have the ability to reversibly increase the pore size in one or more of the membrane systems of the plant cell, enabling the passage of various molecules into and out of the cell. Successful attempts have been made in the literature to reversibly permeabilize the plant cells for a shorter period of time to leach out the bioactive compound and increase mass transfer to substrate and metabolites across the cell membrane with little or no significant effect on cell viability (Brodelius and Nilsson, 1983; Parr et al., 1984; Dornenburg and Knorr, 1995).

Optimization of the phytohormone concentration and combinations are often effective (Kim et al., 2002). Endogenously produced in a variety of plant tissues, plant hormones are present in trace amounts and are responsible for an array of developmental processes that involve abiotic as well as biotic factors (Crozier et al., 2000). Optimization of the hormone concentration and their combinations are also found to be highly effective in increasing the metabolite yields (Dornenburg and Knorr, 1995). Low concentration of naphthylacetic acid (0.025 mg l\(^{-1}\)) enhanced the growth of the roots but inhibited the production of artemisinin. The growth and artemisinin production in hairy root cultures were greatly promoted by the addition of gibberellin (4.8 mg l\(^{-1}\)) to the medium (Cai et al. 1995). Liu et al. (1997) observed that the addition of GA\(_3\) to the growth medium increased artemisinin content in hairy
roots of *A. annua* L. Smith et al. (1997) reported that GA$_3$ (0.01 mg l$^{-1}$) increased the growth rate of hairy roots of *A. annua* L. by 25% with a slight increase in artemisinin levels as compared to control. Growth, development, and production of the artemisinin was measured in *Artemisia annua* L. hairy roots in response to the five hormones (Weathers et al., 2005): auxins, cytokinins, ethylene, gibberellin (GA$_3$), and abscisic acid (ABA). GA$_3$ produced the highest values overall in terms of the number of lateral roots, length of the primary root, lateral root tip density, total lateral root length, and total root length. When the total root lengths are compared, the best conditions for stimulating elongation appear to be: GA 0.01 mg l$^{-1}$ (0.029 µM) > ABA 1.0 mg l$^{-1}$ (3.78 µM) = GA 0.02 mg l$^{-1}$ (0.058µM). Bulk yields of biomass were inversely proportional to the concentration of each hormone tested. All cultures provided with ABA yielded the highest amount of biomass. Both 6-benzylaminopurine and 2-isopentenyladenine inhibited root growth. Only 2-isopentenyladenine, however, stimulated artemisinin production, more than twice that of the B5 controls, and more than any other hormone studied.