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REVIEW ARTICLE

# Updates on artemisinin: an insight to mode of actions and strategies for enhanced global production

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**Abstract** Application of traditional Chinese drug, artemisinin, originally derived from *Artemisia annua* L., in malaria therapy has now been globally accepted. Artemisinin and its derivatives, with their established safety records, form the first line of malaria treatment via artemisinin combination therapies (ACTs). In addition to its antimalarial effects, artemisinin has recently been evaluated in terms of its antitumour, antibacterial, antiviral, antileishmanial, antischistosomiatic, herbicidal and other properties. However, low levels of artemisinin in plants have emerged various conventional, transgenic and nontransgenic approaches for enhanced production of the drug. According to WHO (2014), approximately 3.2 billion people are at risk of this disease. However, unfortunately, artemisinin availability is still facing its short supply. To fulfil artemisinin's global demand, no single method alone is reliable, and there is a need to collectively use conventional and advanced approaches for its higher production. Further, it is the unique structure of artemisinin that makes it a potential drug not only against malaria but to other diseases as well. Execution of its action through multiple mechanisms is probably the reason behind its wide spectrum of action. Unfortunately, due to clues for developing artemisinin resistance in malaria parasites, it has become desirable to explore all possible modes of action of artemisinin so that new generation antimalarial drugs can be developed in future. The present review provides a comprehensive updates on artemisinin modes of action and strategies for enhanced artemisinin production at global level.

**Keywords** *Artemisia annua* L · Artemisinin · Malaria · Cancer · Mode of action · Yield enhancement

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## Introduction

Artemisinins (artemisinin and its chemical derivatives), a family of unique sesquiterpene trioxane with endoperoxide bridge, is surely a natures' gift to the mankind. Artemisinins are derived from an Asteraceae member *Artemisia annua* and has long been used in traditional medication in the form of herbal preparations to cure intermittent fevers. With the rediscovery of artemisinin in 1970s, its tremendous potential to cure malaria infections have been established (Hsu 2006). In the present scenario, WHO recommended that artemisinin combination therapies (ACT) form the backbone of the global struggle to cure malaria. According to WHO (2014), 97 countries worldwide had ongoing malaria transmission, and approximately 3.2 billion people are at risk of this disease. Pharmacological action of artemisinin is now no more restricted to treat malaria but to other medical conditions such as various cancers, inflammatory diseases, viral (e.g. *Human cytomegalovirus*), protozoal (e.g. *Toxoplasma gondii*), helminths (*Schistosom* sp., *Fasciola hepatica*) and fungal (*Cryptococcus neoformans*) infections (Ho et al. 2013). Very recently, an artemisinin derivative, artesunate has been shown to inhibit autoimmune arthritis (Hou et al. 2014) and possess antihistaminic activity (Favero Fde et al. 2014). The uniqueness of artemisinin as potential drug for various diseases lies in its complex structure. Various studies have revealed that

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there is no single standard mode of action that exists for artemisinin. It executes its action through multiple mechanisms, and this could be the probable reason for its wide spectrum of action against many diseases. Being a frontline treatment for malaria and a promising drug for cancer treatment, there are continuous efforts to elucidate antimalarial and anticancer mechanisms of action for artemisinin. Therefore, there is a need to review different modes of action in order to carry forward the research of development and application of artemisinin in a better way.

Artemisinin has a low ratio of its production to demand. The various factors contributing to short falls in artemisinin availability are its low concentration in plants (less than 0.01 to 1.4 % of the plant dry weight) (Liu et al. 2006) and dependence of its yield on environmental variables such as temperature, humidity and soil types (White 2008). Owing to its complex structure, chemical synthesis is very uneconomical and low yielding (Geldre et al. 1997). All these factors cumulatively make artemisinin relatively expensive causing difficulty to fulfil demand of over 392 million courses of ACT (equivalent to 180 metric tonnes of artemisinin) each year (WHO 2014). Various efforts have been made, and research is still on to find out the most suitable way to enhance artemisinin production. However, any individual method is not enough to produce higher artemisinin at global level, and orchestrating various yield enhancing strategies is desirable to speed up the artemisinin supply to needy. The present review summarizes recent progress on artemisinin's mechanism of action and strategies for enhanced artemisinin production globally.

## Mode of action of artemisinin

No single mechanism exists for artemisinins' action. It functions through multiple modes. Despite of having plenty of biological properties, artemisinins' modes of action have been widely investigated in terms of its antimalarial and anticancer effects. There exist a number of reviews dealing with mechanism of antimalarial/anticancer individually. This review provides a compilation of latest advances in both action mechanisms together.

### Antimalarial modes of action

The exact mechanism of action of artemisinin is still a debated topic. Multiple antimalarial modes of action have been postulated for artemisinin (Fig. 1). Prior to its action, artemisinin needs to be activated that generate free radical species. The nature of 'artemisinin activator' is a bit controversial. Various groups have demonstrated a covalent reaction between artemisinin and haemoglobin-bound iron (Selmeczi et al. 2004; Kannan et al. 2005; Messori et al. 2006); however, its probability was denied due to steric hindrance caused by bulky artemisinin when approaching heme moiety of haemoglobin (Creek et al. 2009). Afterwards, it has been suggested that ferrous heme (heme-$Fe^{2+}$) is probably the most efficient activator of artemisinin (Zhang and Gerhard 2008).

Two most acceptable models for artemisinin activation are (i) reductive scission model and (ii) open peroxide model (O'Neill et al. 2010). In reductive scission, ferrous heme/non-heme exogenous $Fe^{2+}$ bind to artemisinin and via reductive scission of the peroxide bridge produce oxygen-centred radicals which self-arranges to give carbon-centred radicals. Further, iron-peroxide interaction occurs in different ways to form either primary carbon-centred radicals (via $C_3$-$C_4$ bond scission) or secondary carbon-centred radicals (via 1, 5 H-shifts). In contrast, open peroxide model suggests involvement of a Fanton reaction of $Fe^{2+}$ and the endoperoxide to produce secondary carbon-centred radicals (Haynes et al. 2007) that are short-lived and are damaging to parasite's vital biomolecules (Edikpo et al. 2013). This 'carbon-centred radical theory' may be strengthened by the fact that artemisinin activity greatly diminished in the presence of free radical scavengers and antioxidants (Hamacher-Brady et al. 2011) whereas pro-oxidants enhanced its activity (Krungkrai and Yuthawang 1987). A recent report by Gopalakrishnan and Kumar (2014) revealed that artesunate (an artemisinin derivative) induces DNA double-strand breaks in *P. falciparum* with simultaneous increase in intercellular ROS level in the parasite ultimately causing parasite death. In addition to oxidative stress-mediated parasite damage, artemisinins have been suggested to covalently interact with essential plasmodial biomolecules (Asawamahasakda et al. 1994). One such identified molecule is PfTCTP, a histamine releasing factor that is involved in regulation of cell cycle, histamine release, malignant transformation, immunological functions (Bommer and Thiele 2004) as well as a target molecule in tumour reversion and malaria treatment (Telerman and Amson 2009; Bhisutthibhan et al. 1998). Though artemisinin interacts covalently by alkylation of TCTP, it was unclear whether it might function as target for artemisinin; however, recently, Eichhorn et al. (2013) demonstrated a role of TCTP in mediating artemisinin effect owing to its very high *Kd* values (77–120 μM) that are several orders of magnitude higher than the artemisinin $IC_{50}$s for parasite growth inhibition.

A decade ago, another potential target for artemisinin action was identified as PfATP6, the *P. falciparum* sarco-endoplasmic reticulum calcium ATPase (SERCA) (Eckstein-Ludwig et al. 2003). It reduces cytosolic free $Ca^{2+}$ by pumping two $Ca^{2+}$ into the endoplasmic reticulum, in exchange for $<4H^+$, a mechanism critical for parasite survival (Brini and Carafoli 2009). Of various $Ca^{2+}$-ATPases in *Plasmodium*, only one enzyme orthologous to SERCA;

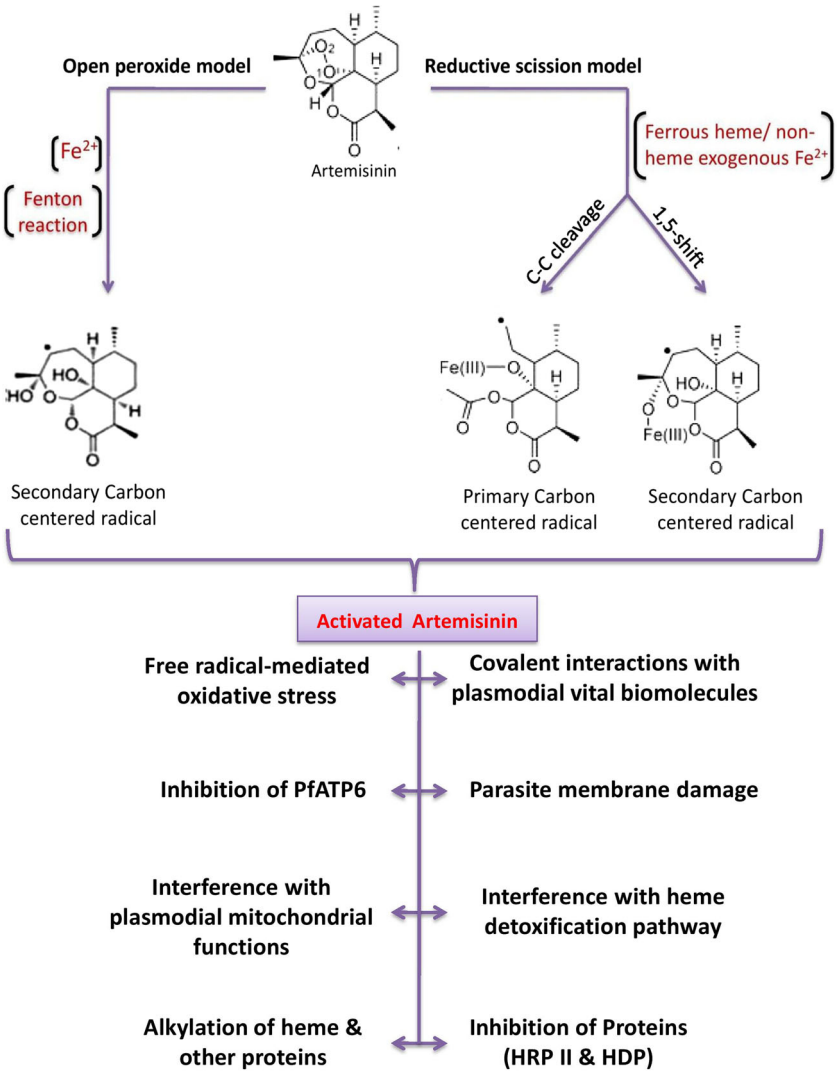


**Fig. 1** Different antimalarial modes of action of artemisinin

PfATP6 is found in *P. falciparum* (Eckstein-Ludwig et al. 2003) that consists of 10 transmembrane α-helix and three cytosolic domains—the effector (A) and the phosphorylation (P) that are connected to the M domain and third, the nucleotide-binding domain (N) that is connected to P domain (Brini and Carafoli 2009). Eckstein-Ludwig et al. (2003) have also suggested that mechanism of artemisinin-mediated PfATP6 inhibition is highly specific, not affecting any other malarial transporter including the non-SERCA $Ca^{2+}$ ATPase (PfATP4) and possibly function through allosteric mechanism (Shandilya et al. 2013). Further experiments dealing with mutation of a single residue (L263) that directly modulates PfATP6 sensitivity to artemisinin suggest a potential resistance mechanisms (Uhlemann et al. 2005). Artemisinin resistance is generally characterized by a delayed clearance of malarial parasite. Efforts are being implicated for using PfATP6 as a molecular marker for artemisinin resistance. However, mutations in the Kelch 13 (K13) propeller domain have recently been demonstrated as important determinants for artemisinin resistance both in vivo and in vitro (Ariey et al. 2014; Straimer et al. 2015). Mok et al. (2015) conducted population transcriptomics of 1043 human malaria parasites and revealed that elevated expression of unfolded protein response (UPR) pathways including the major PROSE and TRiC chaperon complexes is the possible underlying mechanism behind artemisinin resistance. Further, they provided the mechanistic evidence that artemisinin-resistant parasites upregulate UPR pathways in order to overcome the protein damage caused by artemisinin.

Recently, Hartwig et al. (2009) have demonstrated that artemisinin may cause parasite membrane damage by accumulating within its neutral lipids and suggested the role of endoperoxide moiety of artemisinin behind such effects. Experiments were conducted on *Sacchramyces cereviciae* where targeted deletion of genes encoding mitochondrial NADH dehydrogenases (NDE 1 or NDI 1) resulted in resistance to inhibitory effects of artemisinin whereas their overexpression increased sensitivity to artemisinin, suggesting a

mode of action associated with mitochondrial functions (Li et al. 2005). This idea was further attested by Wang et al. (2010), demonstrating artemisinin-induced mitochondrial membrane depolarization via locally generated reactive oxygen species (ROS) that lead to mitochondrial malfunctioning and ultimately parasite death. Additional mechanistic evidence was provided by the study conducted on mammalian cells with non-functional electron transport chain where actively respiring mitochondria have been shown to generate reactive oxygen species that plays central role in endoperoxide-induced cytotoxicity of artesunate (Mercer et al. 2011).

Within the host erythrocytes, parasite degrades host haemoglobin into 'heme' and 'globin' moiety through a series of proteases in to its food vacuole. Further, catabolism releases short peptides and amino acids which are needed for parasite nutrition. This releases a toxic by-product 'hematin' which undergoes bio-mineralization to form non-toxic 'hemozoin' (malaria pigment). It has been proposed that $Fe^{2+}$-heme-activated artemisinin interfere this catabolic pathway through heme alkylation at α, β and δ-carbon atoms that later create toxicity to parasite (Meunier et al. 2010; Cazelles et al. 2001). Two proteins related to this catabolic pathway—heme detoxification protein (HDP) and histidine-rich protein II (HRP II)—have been posited as targets of artemisinin action (Chugh et al. 2013).

An interesting demonstration regarding interaction of heme and mitochondria with artemisinin has recently been revealed (Sun et al. 2015). It has been suggested that heme and mitochondria play distinct roles for mediating artemisinin damage. In tumour cells, heme pathway has been shown to mediate artemisinin's killing whereas antimalarial action of artemisinin is driven by specific mitochondrial pathways.

To further uncover the artemisinins' mode of action, gene expression analysis was undertaken that revealed alterations in two genes (encoding heat shock protein, HSP70, and a hypoxanthine phosphoribosyltransferase, PF10_0121, related to purine biosynthesis) associated with decreased sensitivity to artemisinin. However, whether or not these proteins are directly a part of artemisinin mode of action is unclear.

## Anticancer modes of action

Similar to its antimalarial actions, artemisinins exert their anticancer effects through endoperoxide bridge-dependent ROS generation and subsequent oxidative damage to the target cell(s) (O'Neill et al. 2010). Many workers have established ROS-dependent artemisinin effects. Further boost to this theory has recently been given by Lu et al. (2014) where dihydroartemisinic acid (DHA) significantly reduced viability of human colorectal cancer HCT-116 cells in vitro through G1 cell arrest, apoptotic cell death and ROS accumulation. A consensus opinion is that artemisinin and its derivatives exert their therapeutic effects through multiple modes (Fig. 2). Though oxidative damage remains in the prime focus, it alone cannot describe everything about anticancer actions of artemisinins. This hypothesis was strengthened when DHA was demonstrated to activate p38 MAPK pathway in an ROS-independent way (Lu et al. 2008) through inhibiting vascular endothelial growth factor (VEGF)-induced endothelial cell migration (Guo et al. 2014). Further refinements were brought by workers who revealed an anticancer specific action of artemisinin through modulation of transferrin receptor-1 (TfR1), a type II transmembrane protein (Ba et al. 2012). TfR1 is required for iron import in to the cells by binding to diferric transferrin and subsequent internalization of the Tf-TfR1 complex through receptor-mediated endocytosis (Ponka and Lok 1999). For rapid proliferation of cancer cells, high iron concentration is required; therefore, TfR1 overexpresses in cancer. DHA deplete cellular iron levels via palmitoylation and subsequent lipid raft-mediated non-classical endocytosis of TfR1, leading to iron deficiency in cancer cells (Ba et al. 2012).

To uncover the probable molecular network governing anticancer mechanisms of artemisinins, recent efforts by Huang et al. (2013) have revealed key signalling pathways of artemisinin actions including Pi3k-akt, T cell receptor, toll-like receptor and TGF-beta signalling pathways. Further, different targets of artemisinins from these pathways were also predicted. Pi3k-akt (phosphatidylinositol-3 kinases–Akt), an intracellular signalling pathway, is activated in cancers and plays central role in cancer cell progression and survival (Courtney et al. 2010). However, a current report by Chen et al. (2014) suggests that dihydroartemisinin inhibits glioma cell proliferation and invasion possibly via suppressing disintegrin and metalloproteinase 17 (ADAM17) levels and downregulating epidermal growth factor receptor (EGFR)-Pi3k-akt signalling. Consequently, Pi3k-akt pathway is being developed as a potential drug target to cure cancers. Artemisinins, recently emerged potent anticancer drugs, target this pathway as a major mechanism of action and thereby efficiently regulate various cellular processes like cell growth, proliferation, survival and immunity probably through targeting several membrane receptors such as EGFR, EPOR, IGF1R, IL2RA, IL4R, ITGAV, KDR, KIT, MET, NGFR, PDGFRB and TLR4 (Huang et al. 2013). Additionally, artemisinins' derivatives such as DHA and artesunate were also postulated to induce apoptosis in prostate cancer cells and regulate immunological responses via modulating Pi3k-akt pathway (He et al. 2010; Xu et al. 2007).

In addition to Pi3k-akt, more targeted approaches for cancer treatment involve inhibition of molecular/signalling pathways that are crucial for tumour cell progression and maintenance, and hence, artemisinins are making their place for advance cancer therapies owing to its capability to target toll-like receptors (TLRs), TFG-beta and T cell signalling pathways.

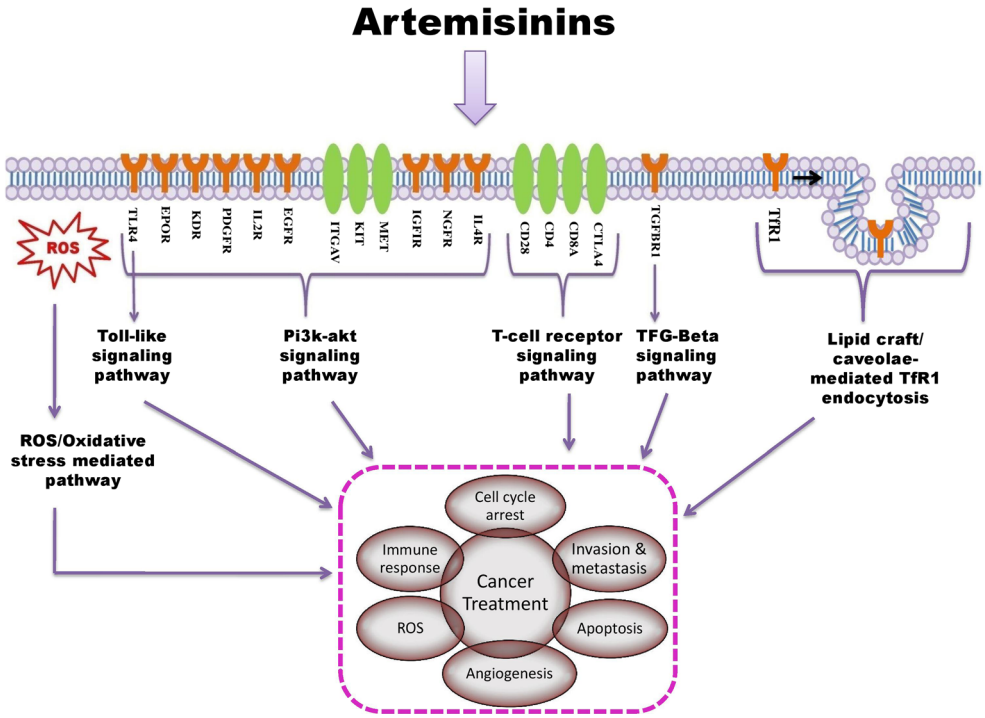


**Fig. 2** Proposed model for various anticancer modes of action of artemisinin

TLRs are integral membrane protein receptors which are believed to promote tumour growth and progression (Muccioli et al. 2012). Similarly, due to their growth promoting abilities, TFG-beta (transforming growth factor-beta) and T cell receptor signalling pathways are believed as excellent targets for treatment of cancers and other diseases (Akhurst and Hata 2012; Watanabe et al. 2014). Artemisinin-mediated targets for T cell receptor signalling pathways have been predicted as CD28, CD4, CD8A and CTLA4 whereas for toll-like receptor and TGF-beta signalling pathways, TLR4 and TGFBR1, respectively, are predicted targets (Huang et al. 2013). A recent report by Lee et al. (2014) suggests that artesunate, an artemisinin derivative, has a negative mitogenic effect on CD+ T cells as it inhibits CD4+ T cell proliferation and IL-2 (T cell growth factor) production and reduction in expression of cell surface protein CD25 (IL-2 receptor alpha chain) and CD69 on CD4+ T cells.

## Emerging resistance to artemisinin: an unfortunate condition

Although tremendous efforts have been implicated to elucidate possible mechanisms of action, many points are still controversial and unclear which are needed to be looked for. While attempts have been made and are still in process to eliminate malaria, unfortunately, spread of artemisinin resistance in malarial parasites has imperilled efforts for malaria control and elimination. As per the latest WHO report (February 2015), artemisinin resistance has been confirmed in Combodia, the Lao People's Democratic Republic, Myanmar, Thailand and Viet Nam, and many other regions are at high risk of emergence of multidrug resistance. Of various factors that have contributed towards evolution of artemisinin resistance, widely available oral artemisinin monotherapies and other substandard antimalarial drugs have played major roles. WHO along with other organizations are working with the strategy to remove oral artemisinin-based monotherapies and substandard antimalarial drugs from the market and emphasizing the use of artemisinin combination therapies to check further spread of drug resistance. However, this requires strong financial capital, long-term political commitments and cross-border cooperation. Besides various malaria elimination and control policies that is being developed, it is now highly desirable to uncover depth of every possible mechanism underlying artemisinin action so as to open new doors for development of accurate and potential new generation antimalarial drugs and modes of malaria treatment in case of further spreading of artemisinin resistance in future.

## Strategies for enhanced artemisinin production

Extensive efforts have been made to improve artemisinin synthesis through many approaches. The present review provides compilation of recent advancement in both conventional and modern approaches of yield enhancement such as (i) metabolic engineering of the plant using various genetic engineering tools; (ii) regulation of artemisinin biosynthesis through stress signals to plants, hairy root cultures and cell cultures and (iii) conventional, mutational and molecular breeding of *A. annua*.

Covello (2008) had suggested the implementation of mix mode production system involving both biological and chemical synthesis, as a good option for artemisinin production. Besides various approaches for improved artemisinin synthesis, recent success has been achieved in its semi-synthetic production with the completion of The Bill and Melinda Gates Foundation funded 'The semi-synthetic Artemisinin Project' in a partnership between University of California (Berkeley, USA), Amyris Inc. and the Institute for One World Health (a non-profit pharmaceutical company and now known as PATH Drug Solutions). The project involved combined use of metabolic engineering of microorganisms and synthetic biology to produce semi-synthetic artemisinin. They achieved 25 gm per litre artemisinic acid production from engineered *S. cerevisiae* (Baker's yeast) as well as 40–45 % conversion of artemisinic acid into artemisinin (Paddon et al. 2013). Details of the complete scheme and approaches adopted for semi-synthetic artemisinin production have been very recently reviewed by the project leaders themselves (Paddon and Keasling 2014). Even though successful production of semi-synthetic artemisinin to supplement the plant-derived production, research is still on to produce WHO recommended potent artemisinin derivatives at large scale to be used in ACTs. Therefore, it is still highly desirable not to be dependent solely on semi-synthetic artemisinin, and efforts should be implemented to enhance drug production at global level by other means as well.

It is interesting to note that dried leaves of *A. annua* containing artemisinin and artemisinin synergistic flavonoids are more effective to cure malaria than a comparable dose of pure artemisinin as ACT oral therapy (Weathers et al. 2014). As discussed earlier in this review, use of combination therapy (as ACT) was promoted to overcome the increasing resistance to existing antimalarial drugs. However, very recently, consumption of dried *A. annua* leaves has been demonstrated to overcome existing resistance to pure artemisinin in *Plasmodium yoelii* (Elfawal et al. 2015). These recent reports promote the idea of increasing artemisinin synthesis through *in-planta* yield enhancement over the semi-synthetic production of pure artemisinin for better and more effective cure of human malaria.

## Artemisinin biosynthetic pathway and overexpression of concerned pathway gene(s)

Like other terpenes, artemisinin biosynthesis involves two pathways, the cytosolic 'mevalonate (MVA) pathway' stemming from acetyl co-A and the plastidic 'non-mevalonte/MEP pathway' originating from glyceraldehyde-3-phosphate and pyruvate. These two pathways result in the formation of two isoprenoid precursors, isopentenyl diphosphate (IPP) and dimethyl allyl diphosphte (DMAPP). These two precursors derived from two pathways do not lead rest of the pathway separately and independently, rather it has been demonstrated that cytosolic DMAPP (mevalonate origin) is transferred to the plastid and it is the plastidic IPP unit that form the central isoprenoid unit of majority of fernesyl diphosphate (FPP) engaged in artemisinin biosynthesis (Schramek et al. 2009). Two stages of artemisinin biosynthesis have now been completely elucidated: first stage involves the formation of FPP while second is the cyclization of FPP to form amorpha-4,11-diene, which ultimately lead to the synthesis of artemisinin and its various derivatives, all steps catalyzed by a series of enzymes. Key enzymes involved are as follows: HMGS, HMGR, DXS, DXR, FPS, ADS, CYP71AV1, DBR2 and ALDH1 (Fig. 3). All the biosynthetic pathway genes have been shown to be differentially expressed in various plant parts. Glandular trichomes of *A. annua* have been well characterized as the site of artemisinin biosynthesis and tissues with high density of glandular trichomes such as flower buds and young leaves show relatively higher expression of biosynthetic pathway genes (Olofsson et al. 2011). In contrast to the all enzyme-catalyzed steps of artemisinin biosynthesis, the conversion of dihydroartemisinic acid to artemisinin is, however, a non-enzymatic photooxidative step. It has been hypothesized that in *A. annua*, there are many oxygenated sesquiterpenoids including artemisinin, which are formed by spontaneous autoxidation of terpene precursors via highly reactive allyllic hydroperoxide intermediate (Brown 2010).

Manipulations of biosynthetic pathways to channelize the carbon flux for enhanced synthesis of a particular metabolite involves either upregulation of desired pathway or downregulation of competing pathways through overexpression and suppression of their respective pathway genes, respectively.

Overexpression of key biosynthetic genes for hyperproduction of precursors of different stages and hence greater synthesis of product (artemisinin) is an excellent idea. Much success has been achieved in engineering almost all the key genes from artemisinin biosynthetic pathway. Basic chemical skeleton of artemisinin involves fusion of three C-5 isoprenoid unit (IPP or DMAPP). Two important key enzymes leading to C-5 isoprenoid unit are HMGR and DXR. HMGR shunts HMG Co-A into the mevalonate pathway, leading to the synthesis of isoprenoid unit in cytosol, whereas in plastidic non-mevalonate pathway DXR catalyzes the first committed step of isoprenoid (C5) synthesis by converting 1-deoxy-D-xylulose-5-phosphate in to 2-*C*-methyl-D-erythritol-4-phosphate.

These two key genes have been overexpressed in *A. annua* by many workers. *Agrobacterium*-mediated *HMGR* gene transfer from *Catheranthus roseus* to *A. annua* was first performed by Aquil et al. (2009), and this resulted in 38.9 % higher artemisinin level in one of the transgenic line. However, when *HMGR* was co-expressed along with *ADS* gene, artemisinin enhanced up to 7.65-folds in one of the

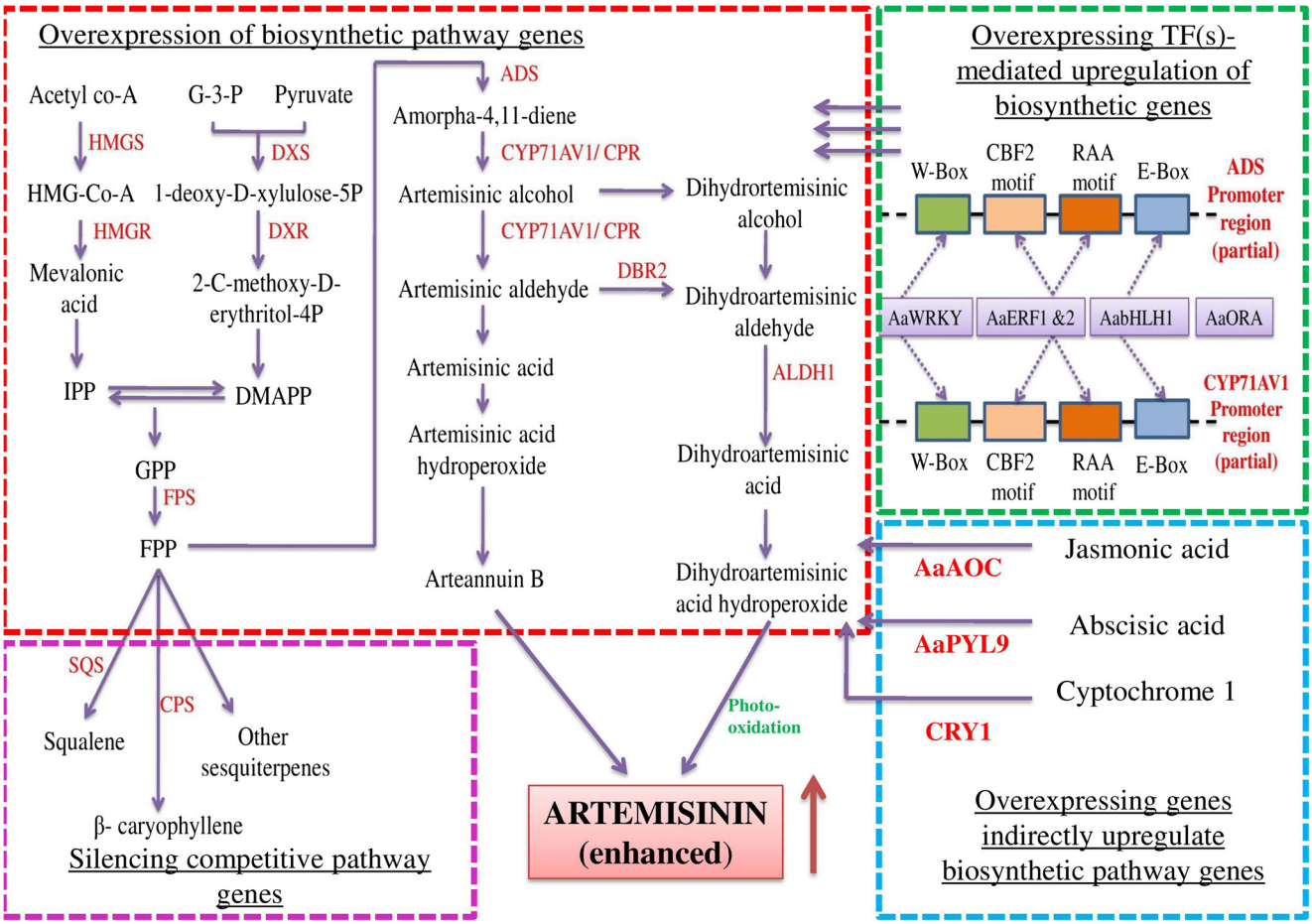


**Fig. 3** Transgenic approaches for enhanced artemisinin biosynthesis in *A. annua*. (3-hydroxy-3-methylglutaryl-CoA synthase: HMGS, 3-hydroxy-3-methylglutaryl-CoA reductase: HMGR, 1-deoxyxylulose 5-phosphate synthase: DXS, 1-deoxyxylulouse 5-phosphate reductoisomerase: DXR, amorpha-4,11-diene synthaase: ADS, cytochrome P 450 CYP71AV1: CYP, double bond reductase 2: DBR2, dihydroartemisinic aldehyde reductase 1: RED1, aldehyde dehydrogenase 1: ALDH1, farnesyl diphosphate synthase: FPS, squalene synthase: SQS, caryophyllene synthase: CPS)

transgenic line as compared to non-transgenic plants (Alam and Abdin 2011).

Isotopologue ($^{13}CO_2$) profiling (Schramek et al. 2009) and fosmidomycin (MEP pathway blocker) treatment (Towler and Weathers 2007) confirmed DXR to be an important regulator of artemisinin biosynthesis. Further, genetic map (Graham et al. 2010) itself did not bring direct evidence for DXR to be regulator of artemisinin biosynthesis; however, it pointed the fact that DXR locus co-localizes with QTL for artemisinin yield and concentration. Recently, CaMV 35S promoter driven overexpression of *DXR* gene in *A. annua* depicted 1.21–2.35-folds higher artemisinin in transgenic plants (Xiang et al. 2012).

C5 isoprenoid unit thus produced (via actions of key enzymes HMGR and DXR) undergo two sequential 1-4 condensations catalyzed by fernesyl diphosphate synthase (FPS) to give **C15** fernesyl diphosphate (FPP), which is further utilized to produce variety of sesquiterpenoids and triterpenoids including artemisinin. Thus, overexpression of *FPS* gene may probably contribute to greater metabolic flux towards artemisinin synthesis and both heterologous (from *Gossypium arboreum*) and homologous (from *A. annua*) *FPS* gene overexpression resulted in 2–3-folds higher artemisinin content in transgenic lines in comparison to wild-type plants (Chen et al. 2000; Banyai et al. 2010). Further, when *FPS* gene was coexpressed with *HMGR* gene (both were homologous), they boosted artemisinin content 1.8-fold in transgenic plants.

The first committed step of artemisinin biosynthesis is the cyclization of FPP in to amorpha-4,11-diene catalyzed by amorpha-4,11-diene synthase (ADS). Thus, the overexpression of *ADS* is a promising approach for upregulated artemisinin biosynthesis in *A. annua*. Tang et al. (2008) cloned and expressed *ADS* gene in *A. annua* and observed 2.3-folds more artemisinin as compared to control. However, spraying of artemisinic acid to *A. annua* plants reduced *ADS* transcript level suggesting a possibility of some feedback inhibition on artemisinin biosynthesis (Arsenault et al. 2010a) Further,

diversion of amorpha-4,11-diene towards artemisinin synthesis is carried out by cytochrome P450 monooxygenase (CYP71AV1) that catalyzes three-step sequential conversion of amorpha-4,11-diene to artemisinin alcohol, then artemisinic aldehyde, and ultimately to artemisinic acid. The activity of CYP71AV1 also requires a reducing companion cytochrome P450 oxidoreductase (CPR), which is considered to be co-expressed with CYP71AV1 (Ro et al. 2006). In addition, Chen et al. (2012) tried to co-overexpress *CYP71AV1* and *CRP* with *FPS* gene. This led to 3.6-fold greater accumulation of artemisinin in *FPS+CYP71AV1+CPR* co-overexpressing transgenic lines in comparison to wild-type plants. In a similar approach, *CYP71AV1* and *CPR* were co-overexpressed with *ADS* and resulted in 2.4-fold higher artemisinin in one of the transgenic line as compared to control plants. Further in the artemisinin biosynthetic pathway, reduction of artemisinic aldehyde in to dihydroartemisinic aldehyde has been proposed to be an important step which is catalyzed by artemisinic aldehyde Δ11(13) reductase (DBR2). Very recently, Yuan et al. (2014) made an effort to overexpress *DBR2* gene driven by the CaMV 35S promoter and observed remarkable increase in artemisinin and its direct precursor DHA, as confirmed by HPLC analysis. In addition, *DBR2*-overexpressing lines also produced more arteannuin B and its direct precursor artemisinic acid.

All the important genes encoding key enzymes of upstream and downstream artemisinin biosynthetic pathway have been cloned and transgenic lines overexpressing single or multiple genes have so far resulted in 1.8–to 7.6-folds elevated artemisinin production, suggesting overexpression of pathway genes as a promising approach to effectively improve artemisinin synthesis.

### Indirect upregulation of artemisinin biosynthetic pathway genes

The expression of genes is largely regulated by specific transcription factors (TFs). Therefore, overexpression of TFs offers an alternative and complementary strategy to upregulate biosynthetic pathway genes (Fig. 3). Promoters of three key genes of artemisinin biosynthesis, i.e. *ADS*, *CYP71AV1* and *DBR2*, have been characterized till date. The first ever transcription factor of *A. annua* that has been characterized is AaWRKY1 (a WRKY type transcription factor) and is proposed to bind to the W-boxes in *ADS* and *CYP71AV1* promoters. Transient expression of AaWRKY cDNA in leaves of *A. annua* was shown to increase the transcript level of the majority of artemisinin biosynthetic genes (Ma et al. 2009) and AaWRKY-overexpressing transgenic lines produced 4.4-folds higher artemisinin as compared to control lines (Tang et al. 2012). Recently, in a more targeted approach, trichome-specific overexpression of AaWRKY1 resulted in more effective activation of *CYP71AV1* transcription as compared to the constitutive overexpression of AaWRKY (Han et al. 2014). However, this did not lead to any significant improvement in transcript level of other key genes such as *FDS*, *ADS* and *DBR2* and promoted artemisinin content 1.8 times as compared to wild-type plants. Further, CRTD REHVCBF2 (CBF2) and RAV1AAT (RAA) motifs in promoters of both *ADS* and *CYP71AV1* have been proposed to be the binding sites of two TFs, AaERF1 and AaERF2, which belong to JA-responsive AP2 family of TF. Both the TFs were cloned from *A. annua* and overexpression of either of the two promoted *ADS* and *CYP71AV1* transcripts level and artemisinin as well. The fourth TF that has been successfully cloned and overexpressed in *A. annua* is AaORA which is a trichome-specific APETALA2/ethylene response factor (AP2/ERF) family TF. Its overexpression in *A. annua* significantly boosted transcription of *ADS*, *CYP71AV1*, *DBR2* and *AaERF1*. AaORA-overexpressing and AaORA-RNAi transgenic lines showed significant increase (53 %) and decrease in artemisinin content, respectively, as compared to control (Lu et al. 2013). Recently, a fifth TF, AabHLH1, a bHLH TF has been successfully isolated from a cDNA library of glandular secretary trichomes (GSTs) of *A. annua* and has been demonstrated to be able to bind to the E-box-*cis* element in the promoter of *ADS* and *CYP71AV1*. Transient expression of AabHLH1 in the leaves of *A. annua* significantly enhanced transcript levels of *ADS*, *CYP71AV1* and *HMGR* (Ji et al. 2014).

In addition to TF engineering, artemisinin biosynthesis may also be improved by blocking/silencing artemisinin competitive pathway/genes and hence ensuring diversion of common isoprenoid precursors more towards artemisinin biosynthetic pathway (Fig. 3). So far, two competitive pathway enzymes have been engineered in *A. annua*: squalene synthase (SQS) and β-caryophyllene synthase (CPS). RNA interference (RNAi) offers a potential 'reverse genetics' approach to knock down the expression of particular gene(s) and in *A. annua* Zhang et al. (2009) applied RNAi technology for the first time to knock down the expression of *SQS*. SQS catalyzes the first committed step in sterol biosynthetic pathway by converting FPP into squalene and thus competes with ADS for utilizing FPP in the biosynthesis of sterols. Suppression of *SQS* expression in *A. annua* through hairpin RNA-mediated RNAi technique reported a 3.14-fold higher artemisinin content along with significantly lower sterol level in transgenic plants as compared to control. Another gene that has been artificially modulated for its expression in *A. annua* is *CPS*. CPS, being a sesquiterpene synthase, competes with ADS for utilizing FPP and converts FPP into β-caryophyllene. Using antisense RNA technology, Chen et al. (2011) tried to suppress the expression of *CPS* gene by *Agrobacterium*-mediated insertion of 750-bp antisense fragment of *CPS* cDNA into *A. annua via* pBI121 plant expression vector. This lowered the endogenous *CPS* expression and elevated

artemisinin content by 54.9 % in one of the transgenic line as compared to wild-type plants.

## Regulation of artemisinin biosynthesis through stress signals

Various environmental stresses in a moderate dose and duration can act as a potential elicitor for enhanced secondary metabolite production. When plants recognize stress signals at cellular level, a stress response is induced. A number of researchers have exposed *A. annua* to a number of stress signals and had reported alterations in artemisinin content along with transcript level of its biosynthetic genes which is summarized in Table 1. Environmental stress signals such as temperature (low or high), salinity, water (drought or flooding), radiations, chemical, mechanical and various kinds of biotic stresses often result in induced accumulation of phenylpropanoids (Dixon and Paiva 1995). In recent years, many of them have been explored in terms of their potential to boost artemisinin level in *A. annua*. Chilling has been demonstrated to affect artemisinin content in *A. annua*. Yang et al. (2010) had revealed chilling-mediated induction in artemisinin level along with its biosynthetic genes such as *ADS*, *CYP* and *DXS*. Similarly, night frost also increased artemisinin content (Wallaart et al. 2000). Influence of salinity on artemisinin accumulation has been investigated by different groups. Qureshi et al. (2005) showed that salt treatment (0–160 mM) increased artemisinin content during the early phase of plant growth, possibly due to sudden non-enzymatic conversion of artemisinic acid/DHA (artemisinin precursors) in to artemisinin under increasing oxidative stress. However, at later stages, it reduced artemisinin. In contrast, when *A. annua* seedlings were subjected to salinity stress (4–6 g/l NaCl) significantly boosted artemisinin (2–3 % dry weight) compared to non-treated plants (1 % dry weight) (Qian et al. 2007). Role of nutrient deficiency in artemisinin production was first reported by Ferreira (2007). Higher level of artemisinin was found in plants grown in potassium deficiency as compared to plants grown with full supplementation of macronutrients and had suggested that artemisinin production per hectare may be enhanced by growing *A. annua* under a mild potassium deficiency. In contrast, supplementation of few elements reportedly enhances artemisinin content. Mild boron stress (1.0 mM) enhanced artemisinin as compared to control (Aftab et al. 2010). Similarly, arsenic (As) (3000 μg/l) for 7 days and cadmium (Cd) treatment (20 and 100 μmol/l) to

**Table 1** Different stress signals for enhanced artemisinin biosynthesis *in-planta*

| Stress signals for artemisinin enhancement | Experiments conducted on | Reference |
|---|---|---|
| Chilling | Soil-grown plants/In vitro propagated plants | Yang et al. 2010; Lulu et al. 2008 |
| Night frost | Soil-grown plants | Wallaart et al. 2000 |
| Salinity | Soil-grown plants | Qian et al. 2007; Qureshi et al. 2005 |
| Potassium deficiency | Soil-grown plants | Ferreira 2007 |
| Boron | Soil-grown plants | Aftab et al. 2010 |
| Arsenic | Soil grown plants/hydroponic culture | Rai et al. 2011a; Paul and Shakya 2013 |
| Phosphorus | Soil grown plants | Kapoor et al. 2007 |
| Cadmium | Hydroponic culture | Li et al. 2012 |
| Drought | Soil-grown plants | Marchese et al. 2010 |
| Post harvest drying | Soil grown plants | Laughlin 2002 |
| Miconazole | Hydroponic culture | Towler and Weathers 2007 |
| UV-B | In vitro propagated plants | Pandey and Pandey-Rai 2014a |
| UV-C | Soil-grown plants | Rai et al. 2011b |
| Jasmonic acid | Exogenous application to soil-grown plants | Wang et al. 2009; Maes et al. 2011 |
| Salicylic acid | Exogenous application to soil-grown plants | Pu et al. 2009; Guo et al. 2010 |
| Phytohormones (GA3+BAP+ ABA) | Soil-grown plants | Maes et al. 2011 |
| Abscisic acid (alone) | Pot culture of in vitro germinated seedlings | Jing et al. 2009 |
| Sugars | In-vitro propagated plants | Wang and Weathers; Arsenault et al. 2010b |
| Methyl jasmonate (MeJA)+Miconzole | Cell suspension culture | Caretto et al. 2011 |
| MeJA (alone) | Hairy root culture | Patra et al. 2013 |
| Oligosaccharide elicitor (OE) of fungal origin | Hairy root culture | Zheng et al. 2008; Wang et al. 2006; Wang et al. 2009 |
| Yeast extract+MeJA+Chitosan | Hairy root culture | Putalun et al. 2007 |
| MeJA+cell homogenate of *Piriformospora indica* | Hairy root culture | Ahlawat et al. 2014 |

hydroponically grown *A. annua* promoted synthesis and accumulation of artemisinin (Rai et al. 2011a; Li et al. 2012). RT-PCR analysis revealed upregulated transcript levels of *HMGR*, *FDS*, *ADS* and *CYP71AV1* genes under arsenic stress (Rai et al. 2011a). In tune with boron and As, phosphorus also enhanced artemisinin level (Kapoor et al. 2007). Not all the metal stress signals affect artemisinin positively. A recent study by Paul and Shakya (2013) has demonstrated that As(III) (at 5 and 7.5 μg/ml) and NaCl favour growth of *A. annua* and artemisinin biosynthesis while higher doses of As(III) (10 μg/ml) and Cr(VI) even at mild doses have adverse effects on growth and artemisinin level (Paul and Shakya 2013).

There are reports in support of drought stress affecting artemisinin accumulation (Marchese et al. 2010). Water deficit of 38 h (W=−1.39 MPa) significantly increased both leaf and plant artemisinin up to 29 % without causing any serious harm to biomass production. This suggests that pre-harvest water deficit may reduce time and costs in crop drying along with gain in artemisinin content. Further, different ways of post-harvest drying of crops also play role in overall artemisinin availability. Field crop drying is beneficial over artificial drying (such as oven-drying) and gives higher artemisinin; however, mode of post-harvest drying does not seem to affect artemisinic acid level (Laughlin 2002). The exact mechanism behind such abiotic factor-mediated elevation in artemisinin is still not clear. However, a common factor behind these stresses is the ROS-mediated oxidative stress which may probably be one of the reasons for greater artemisinin accumulation.

Though there are many reports of deciphering roles of abiotic stress signals in influencing artemisinin content in *A. annua*, relatively fewer work have been conducted to depict artemisinin regulation through biotic stress signals. A study by Kapoor et al. (2007) showed that inoculation by two arbuscular mycorrhizal fungi, *Glomus macrocarpum* and *Glomus fassiculatum*, significantly enhanced artemisinin concentration density of leaf glandular trichomes (GT) which are the accumulation sites of artemisinin. They also observed a strong positive linear correlation between leaf GT density and artemisinin, suggesting that certain biotic stress signals may be potential tools to improve *in-planta* improvement of artemisinin synthesis and accumulation. Few more biotic elicitors have been demonstrated to improve artemisinin but in hairy root culture which will be discussed later in this section.

Exogenous application of signalling molecules such as jasmonic acid (Wang et al. 2009; Maes et al. 2011) and salicylic acid (Pu et al. 2009; Guo et al. 2010) also has role in boosting biosynthesis of artemisinin probably due to induced burst of ROS and subsequent conversion of DHA in to artemisinin as well as upregulation of key artemisinin biosynthetic genes. In addition, few phytohormones such as gibberellic acid cytokinins (BAP) and abscisic acid also boost artemisinin level (Maes et al. 2011; Jing et al. 2009).

## Elicitation and plant cell/tissue culture

Plants respond quickly to even a small fluctuation in its physical or chemical environment. These fluctuations are very well known to elicit the plant's secondary metabolism and hence are exploited to get the desired secondary metabolite. Plant tissue culture offers an opportunity to grow plants in vitro in a controlled environment and manipulation of plant physical and chemical environment to get the desired product. Rapidly increasing world population is putting more and more pressure on availability of cultivation land. Therefore, in the present scenario, there is a need to explore plant cell/tissue culture technique in integration with elicitation to get higher secondary metabolite production with minimum land usage. This will reduce the land use by medicinal plants which are largely grown for production of pharmaceuticals and other phytochemicals and better utilization of lands for various other motives.

There are established standard protocols for in vitro propagation of *A. annua*. It can easily be propagated through micro-cuttings in MS media. Since accumulation of secondary metabolites in plants in response to abiotic factors is a common phenomenon, manipulation of abiotic environment of in vitro propagated *A. annua* plantlets may lead to differential accumulation of artemisinin. We applied this hypothesis in our recent work and had demonstrated that UV-B radiation exposure (3 h) to in vitro propagated *A. annua* plantlets can double the artemisinin content (Pandey and Pandey-Rai 2014a) without causing any serious damage to physiology including photosynthesis and hence survival (Pandey and Pandey-Rai 2014b). Chilling (4 °C) treatment for 30 min also affected artemisinin content as well as transcript level of *ADS* (11-fold) and *CYP* (7-fold) genes in in vitro cultured plants as revealed by HPLC and qPCR analysis, respectively (Lulu et al. 2008). Different sugar supplementation during in vitro plantlet development also affects artemisinin content in plants. Seedlings were inoculated in Gamborg's B5 medium containing 3 % (*w*/*v*) sucrose, glucose or fructose. Fructose inhibited artemisinin synthesis while seedlings growing in 3 % glucose produced more artemisinin (Wang and Weathers 2007). These results were further confirmed by Arsenault et al. (2010) through expression analysis of biosynthetic genes as well as quantification of artemisinin and its various derivatives.

Various efforts have been made to get higher artemisinin through integrated approach of elicitation with hairy root or cell suspension culture in *A. annua*. Among these, hairy root culture seems advantageous over cell suspension culture in terms of high growth rate, better genetic stability and independency of hormone supplemented media requirement for growth (Guillon et al. 2006). Plant signalling compounds, precursors, inhibitors and various other kinds of molecules have been explored in terms of their potential to elicit artemisinin biosynthesis. Caretto et al. (2011) investigated

efficacy of methyl jasmonate (MeJA) and miconazole on artemisinin biosynthesis in cell suspension cultures of *A. annua*. Three-fold increment in just 30 min of MeJA (22 μmol) treatment and 2.5-fold after 24 h of miconazole treatment were observed. However, miconazole treatment severely affected cell viability. Earlier, this antifungal compound miconazole significantly induced artemisinin level in *A. annua* seedlings (Towler and Weathers 2007). Miconazole-mediated induction in artemisinin is due to its ability to inhibit sterol biosynthesis and redirection of carbon flux towards terpenoid pathway (Zarn et al. 2003). Using MeJA alone (40 μg/l, 15 days) as elicitor, 3.45 mg/g artemisinin was produced (Patra et al. 2013). Nitric oxide (NO) also stimulates artemisinin synthesis in hairy root culture. Zheng et al. (2008) studied that NO generation induced by an oligosaccharide elicitor (OE) from *Fusarium oxysporum* mycelium elevated artemisinin from 0.7 to 1.3 mg/g DW by treating 20-day-old hairy roots with OE for 4 days. Further, combined treatment of OE with NO donor sodium nitroprusside increased artemisinin from 1.2 to 2.2 mg/g DW. Various other elicitors have been reported to stimulate artemisinin biosynthesis in hairy root culture such as OE from *Colletotrichum gloeosporioides* (Wang et al. 2006), cerebroside (Wang et al. 2009), yeast extract, methyl jasmonate and chitosan (Putalun et al. 2007). Very recently, in an interesting approach of combined application of abiotic and biotic elicitor (MeJA and cell homogenate of *Piriformospora indica*) to hairy root culture, 2.44 times greater artemisinin was obtained (Ahlawat et al. 2014). This increase was probably due to upregulation of biosynthetic genes. They also reported for the first time a positive correlation between elicitor application and expression of biosynthetic pathway genes viz. *HMGR*, *ADS*, *CYP71V1*, *ALDH1*, *DXR*, *DXS* and *DBR2* in hairy root cultures of *A. annua*.

Combined approaches utilizing in vitro techniques and elicitation is a promising idea for large-scale production of artemisinin through bioreactor cultivation technology.

## Breeding approaches for enhanced artemisinin production

According to the factsheets on the World Malaria Report 2014, issued by WHO (December 2014), in the year 2013, there were an estimated 198 million cases of malaria (uncertainty range 124–283 million) and an estimated 584,000 deaths (uncertainty range 367,000–755,000) in the world, of which 90 % deaths occur in Africa. Further, the second most affected part of the world is South-East Asia where India has the highest malaria burden, followed by Indonesia and Myanmar. These facts suggest that major risk and burden of this disease is in the developing countries which are generally with insufficient advanced infrastructure/techniques. Therefore, despite of various efforts to produce high artemisinin by diverse means, there is still a need to think on ground level as well because agricultural production is not a problem or limiting factor in developing countries. Integrating cultivation of *A. annua* with different breeding techniques may be a viable alternative to produce more artemisinin thereby aiding in overall enhanced production of artemisinin globally. By bringing *Artemisia annua* into cultivation, conventional and new biotechnological plant-breeding techniques can be applied at the genetic level to improve yield and uniformity with stable high performing phenotypes across adverse/variable environments and to modify desired valuable compounds. Conventional plant breeding practices include collection/creation of population or germplasm with useful desired genetic variation, identification of superior individuals and development of improved variety from selected individuals. Success of conventional breeding is dependent on the selection process. Numerous selection methods can be adopted for *A. annua*. Mass selection is dependent mainly on selection of plants according to their phenotypes and performance and can be used to improve the overall population of *A. annua* by positive or negative mass selection. One drawback of mass selection is the influence of the environment on the development, phenotype and performance of single plants. Another method is the recurrent selection, which is more suitable for the *A. annua* as it is cross-pollinating species. In this method, the seed from selected plants is not added together but is kept apart and used to perform offspring tests. Through different selection strategies, certain high yielding varieties of *A. annua* such as 'CIM-Arogya' Jeevan Raksha' and Asha were released by Central Institute for Medicinal and Aromatic Plants (CIMAP), India, as superior lines rich in artemisinin (Patra and Kumar 2005). Recently, Townsend et al. (2013) have reported that selection of material for breeding using combining ability analysis of a diallele cross can be used for the identification of elite parents to produce improved *A. annua* hybrids. This selection method was reportedly consistent with advanced QTL-based molecular breeding approaches.

Modernization of plant breeding using different biotechnological tools is opening new avenues for crop improvement. Two such tools are mutation and molecular breeding (Fig. 4). Mutation breeding involves induced mutation through chemicals or radiation with the advantage of improving one or two characters without modifying the rest of genotypes.

A successful breeding effort was made by Mediplant (a swiss not-for-profit organization) by developing high-yielding F1 hybrid populations of *A. annua* known as 'Artemis' with mean annual artemisinin production of about 32 kg/ha. Further, they also created new high yielding hybrids with 40.5–52.0 kg/ha artemisinin (Simonnet et al. 2008).

There has been significant progress in the development of biotechnological plant-breeding techniques using various molecular tools. Different DNA markers (for example, RFLP, RAPD, AFLP, SSR, SNP, STMS etc) and functional markers (such as EST, microarray and qRT-PCR) can be variously

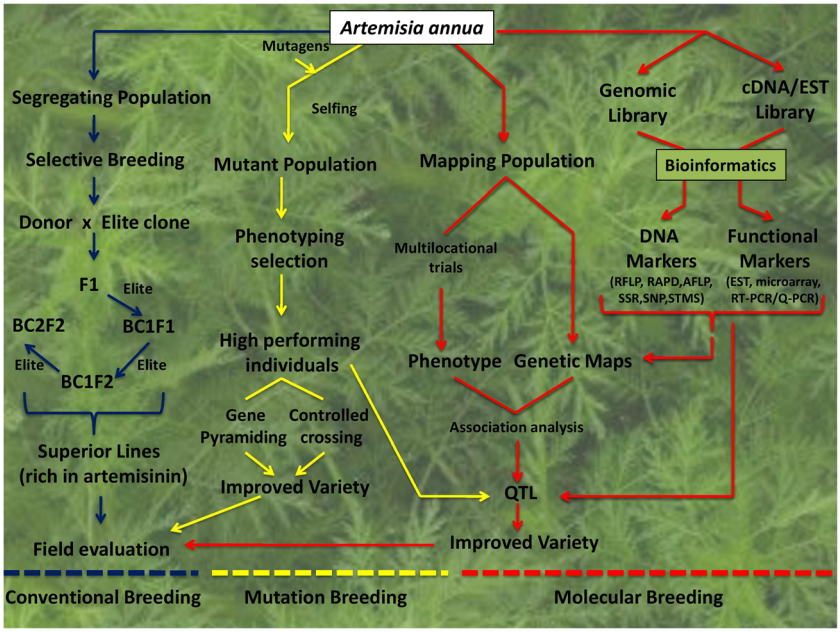


**Fig. 4** Different proposed breeding approaches in *A. annua* for enhanced production of artemisinin

used to speed up the selection for recognition of desired genotypes at an early stage. Although these marker-assisted selections are being applied in various crops, there are relatively few studies of molecular marker-based approaches in medicinal plants. One such effort was made by CIMAP (India) for developing high-artemisinin variety CIM-arogya, through marker-assisted selection breeding.

For the production of high-yield varieties of *A. annua*, a fast track molecular breeding project known as 'The CNAP Artemisia Research Project' has been initiated at the Centre for Novel Agricultural Products (CNAP) which is a part of university of York. This project led by CNAP's Director Dianna Bowles and Deputy Director Ian Graham was funded by Bill & Melinda Gates Foundation. With the aim to produce high yielding non-GM variety of *A. annua*, over 23,000 parental lines were screened for desired traits and 768 different hybrid crosses were made, of which 268 most promising hybrids were forwarded to field trials. After rigorous selection procedure, two best performing hybrids viz. Hyb1209r (Shennong) and Hyb8001r (Zenith) with 36.3 and 54.5 kg/ha artemisinin have been commercially released. Using various molecular markers, they also developed the genetic map of *A. annua* with nine linkage groups (Graham et al. 2010). For this, they used the Artemis (high yielding variety) pedigree and established genetic linkage and QTL maps. Positive QTL related to artemisinin yield were also independently validated.

## Conclusion and future directions

Various approaches have been adopted for improvement in the yield of artemisinin. However, it is still a challenge for any individual approach alone to meet the global demand of artemisinin. Though recent achievement of semi-synthetic production of artemisinin by Keasling's group is a major breakthrough in bringing artemisinin production at industrial level, there still exist many hurdles for sustainable production of this drug to be used in ACTs. Its insecure supply leads to price hike and to reduce the drug cost there is a need to speed up the artemisinin production worldwide.

Therefore, *in-planta* yield enhancement through various ways as discussed in this review remains the prime focus for sustainable drug supply. It is worth mentioning that the all

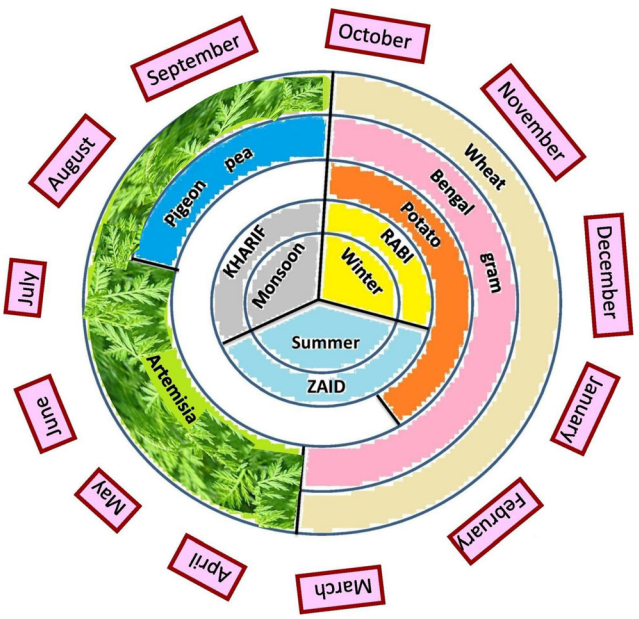


**Fig. 5** Intercropping model with special reference to Indian cropping for growing *A. annua*

transgenic or non-transgenic tools for enhancement of artemisinin biosynthesis can be more fruitful if implemented on high yielding chemotypes/hybrids of *A. annua*. Integration of conventional methods with advanced techniques will serve to extend and enhance the continued production of drug. As discussed earlier, more than 90 % burden of malaria is in African and South-East Asian developing countries. As majority of these countries have agricultural economies, agri-based enhancement of artemisinin production *in-planta* can be a promising approach for overall improved production.

Various agricultural strategies can be posited depending on the local scenario of agricultural practices in different countries. Deficiency in the supply chain of artemisinin can be remediated by integrating agri-based technologies with molecular tools for affordable drug production. As for example in India, we hope that intercropping of high yielding varieties of *A. annua* (developed through molecular/mutation breeding) with staple food crops can be good option for its cultivation (Fig. 5) without engaging additional cultivable land and meantime execution of multiharvesting and advanced drug extraction practices may result in greater artemisinin production with lower production cost. Similar approaches may also be applied in other countries as well which can give a further boost to artemisinin production at the same time bettering life standards of their farmers.

Keeping in mind, benefits and limitations associated with every yield enhancement strategies, we emphasize that to bring artemisinin supply up to the level of its demand requires its production collectively through semi-synthesis, *in-planta* yield enhancement by both transgenic and non-transgenic methods as well as modern breeding.

**Conflict of interest** The authors (NP and SPR) of the review article entitled "Updates on Artemisinin: an insight to mode of actions and strategies for enhanced global production" have no conflict of interest.

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