EFFECTS OF ARTIFICIAL POLYPLOIDY IN TRANSFORMED ROOTS OF *ARTEMISIA ANNUA* L.

By

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By

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ABSTRACT

In most plant species artificial polyploidy generally enhances the vigor of determinate plant parts and may be favorable where vegetative organs and biomass constitute the economic product. Furthermore, artificial polyploidy has been considered a method of increasing production potential of plants secondary metabolites. However, despite considerable research on polyploid plants, very few cases of polyploid medicinal plants have been reported.

Artemisia annua L. synthesizes artemisinin, an antimalarial sesquiterpene lactone. Artemisinin can be synthesized, but it is costly compared to the naturally derived product. Hairy root cultures of *Artemisia annua* L. (clone YUT16) show rapid growth and produce artemisinin. This culture offers a good model system for studying artemisinin production. Others have shown that tetraploid *Artemisia annua* L. plants produce more artemisinin/mg DW than diploids. These yields were offset, however, by decreases in biomass productivity. Little is known about how polyploidy may affect growth production of hairy roots. Using colchicine, we have produced four stable tetraploid clones of *Artemisia annua L.* from YUT16 hairy root clone. Compared to the diploid clone, these tetraploid clones showed major differences in growth and development. Nevertheless, artemisinin yields of these tetraploid clones were 2-5 times higher than the diploid and their production seemed to be by the age of the inoculum. This work will prove useful in furthering our understanding of the effects of artificial polyploidy on the growth and secondary metabolite production of hairy roots.

ACKNOWLEDGEMENTS

- This work is lovingly dedicated to my wife Julie and my unborn child –

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INTRODUCTION

Malaria is a serious disease that affects more than 275 million people worldwide and is the cause of at least 1 million deaths every year (Butler, 1997). The rise of multi-drug resistant *Plasmodium* species and the pesticide resistance of the infection vector, the *Anopheles* mosquito, have made the eradication of this disease very difficult (Winstanley *et al.*, 2002). Artemisinin is an antimalarial drug that has been used for centuries in China as a remedy for the symptoms of malaria (Meshnick *et al.*, 1991). This drug is a sesquiterpene secondary metabolite derived from the plant *Artemisia annua* and has shown effectiveness against multi-drug resistant strains of *Plasmodium* (Winstanley *et al.*, 2002). The primary problem with large-scale production of this and other secondary metabolites is the low concentration of product in the plant, usually for artemisinin, in the range of only 0.1-1.4% of the tissue dry weight (Delabays *et al.*, 2001; Jaziri *et al.*, 1995; Akhila *et al.*, 1987; Nair *et al.*, 1986). Furthermore, artemisinin is a complex molecule, which is difficult and expensive to prepare by organic synthesis (Delabays *et al.*, 2001; Dhingra *et al.*, 2000; Van Geldre *et al.*, 1997; Xu *et al.*, 1986; Zhou, 1986). The many reaction steps and low yields obtained by organic synthesis make it obvious that the *A. annua* plant is still the most economic source for producing cheap and large quantities of artemisinin (Wallaart *et al.*, 1999c). One of the shortcomings on the production of artemisinic compounds via whole plants is the long period of time (i.e. usually weeks or months) required to obtain appreciable yields. Unfortunately, all the problems associated with increasing artemisinin production are also characteristic of many of the secondary metabolites with more complex structures.

New technology is constantly being developed to meet the growing need for large-scale plant propagation and production of plant chemicals. One of these attempts is the development of genetically transformed roots. Several studies in tissue culture have shown the potential and the convenience of using roots transformed by *A. rhizogenes* for the biosynthesis of secondary metabolites in numerous species (Ramachandra-Rao and Ravishankar, 2002). These transformed (hairy) roots are easily cultured in a defined media such as Gamborg's B5 (Gamborg *et al.*, 1968), grow relatively fast, and tend to produce secondary metabolites characteristic of the species that was transformed. With the realization of the limitations associated with whole plant systems, the production of secondary metabolites can be enhanced using hairy roots.

Hairy root culture has become an active and important research area. Several studies have shown that genetically transformed roots of *A. annua* showed potentially higher production rates of artemisinic products in just a few days, providing at the same time cleaner extraction sources for these biochemical compounds (Paniego *et al.*, 1996; Jaziri *et al.*, 1995; Weathers *et al.*, 1994). Now that transformed roots of *A. annua* have been established, the next step is the optimization of culture growth and secondary metabolic production.

In most plant species artificial polyploidy has enhanced the vigor of determinate plant parts (Dhawan *et al.*, 1996). This may be favorable for vegetative organs, which are the source of many secondary metabolites. Thus, artificial polyploidy may prove useful in achieving rapid genetic improvement of plants (Dhawan *et al.*, 1996). Although the effects of polyploidy are not generally predictable, and each species must be generally examined individually, doubling the chromosome number of *A. annua* may offer a good

opportunity to obtain plants with increased productivity of artemisinic compounds. Indeed, Wallaart *et al.* (1999c), reported induced polyploidy of *A. annua* plants by using the mitotic inhibitor colchicine. In their study, they reported a polyploidy induction efficiency of 20% and an averaged artemisinin level in tetraploids that was 38% higher than the diploid plants. However, biomass accumulation of the tetraploid plants was lower than the diploid plants, so the yield of artemisinin per m^2 decreased by 25% (Wallaart *et al.*, 1999c). Although the results of their study were from whole *A. annua* plants, they suggested polyploid induction of hairy roots to increase artemisinin production.

The main goal of this research was to induce tetraploidy in *A. annua* hairy roots with the aim of increasing artemisinin yields. In this study the artemisinin and biomass production in tetraploid *A. annua* hairy root clones were compared with the diploid type clone. These results will prove useful in that little is known about polyploid induction in hairy roots and its subsequent effects on secondary metabolite production.

ORGANIZATION OF THESIS

This thesis is organized wherein the main investigative part consist of a manuscript already published by a peer reviewed journal. The first chapter consists of an expanded background to provide more information than is contained in the manuscript's introduction. Chapter 2 consists of hypothesis and objectives of this work. Chapter 3 consists of the manuscript while Chapter 4 includes data from experiments that are still in progress and were not included in the manuscript but support the data presented. Chapter 5 comprises a final conclusion section for the entire thesis and a section for suggestions for future experiments. Appendix A includes data from preliminary studies performed to analyze the effects of carbon source in the growth and artemisinin production of hairy root cultures of *A. annua*.

CHAPTER 1

1.0. BACKGROUND

1.1. OVERVIEW ON PLANT SECONDARY METABOLITES

1.1.1. Characteristic of plant secondary metabolites

A characteristic feature of plants is their capacity to synthesize an enormous variety of low molecular weight compounds, the so-called secondary metabolites. Many authors have discussed the problem of a proper definition of secondary metabolites (Verpoorte, 1999; Bennett and Bentley, 1989; Luckner, 1990). Bennett and Bentley (1989) extensively discussed the history of the term secondary metabolites, with special reference to microbial metabolites. They gave the following definition:

"General metabolites (hence general metabolism): A metabolic intermediate or product, found in most living systems, essential to growth and life, and biosynthesized by a limited number of biochemical pathways. Secondary metabolites (hence secondary metabolism): a metabolic intermediate or product, found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism, and biosynthesized from one or more general metabolites by a wider variety of pathways than is available in general metabolism."

The secondary metabolites are characterized by an enormous chemical diversity, every organism has its own characteristic set of secondary metabolites, some of which they may share with other related or totally unrelated organisms (Verpoorte, 1999). Although only 20-30% of higher plants have been investigated so far, tens of thousands

of secondary metabolites have already been isolated and identified (Table 1.1; Wink, 1999b).

Table 1.1. Number of known secondary metabolites from higher plants. (adapted from Wink, 1999b and Verpoorte, 1999)

Type of secondary metabolite	Number
Nitrogen-containing	
Alkaloids	15765
Nonprotein amino acids (NPAAs)	600
Amines	100
Cyanogenic glycosides	100
Glucosinolates	100
Without nitrogen	
Sesquiterpenes	8650
Monoterpenes	1946
Diterpenes	7834
Triterpenes, steroids, saponins	4952
Tetraterpenes	352
Flavonoids	8128
Polyacetylenes	1000
Polyketides	2442
Phenylpropanoids	500

Over the past decade, it has become evident that secondary metabolites are not just waste products or otherwise functionless molecules. In fact, the opposite is the case: most secondary metabolites have an important role in the plants producing them. Still our knowledge about the role of the secondary metabolites is limited, but now it is generally accepted that secondary metabolism is involved in the organism's interaction with its environment, e.g. in resistance against pests and diseases, as attractant of pollinators, or as a signal compound (Croteau *et al.*, 2000). Secondary metabolites are therefore ultimately important for the fitness of the plant producing them. Bennett and Bentley

(1989) do not say anything about the role of secondary metabolites, thus, their definition may not be fully satisfactory. However, the chemical diversity and the limited knowledge of the role secondary metabolites play in plants, hamper efforts to more sharply define the group. Verpoorte (1999) in his best effort offers the following definition:

"Secondary metabolites are compounds with a restricted occurrence in taxonomic groups that are not necessary for a cell (organism) to live, but play a role in the interaction of the cell (organism) with its environment, ensuring the survival of the organism in the ecosystem."

In this view secondary metabolites are essential for an organism to survive as a species in its ecosystem. Thus, secondary metabolites are closely related to the evolutionary and adaptative ability of a given plant in an endlessly changing environment (Facchini, 1999; Hartmann, 1996). Therefore, most plant species can be characterized by a unique and distinct metabolic fingerprint that explains, in part, the complexity of chemical structures, wide distribution, and variability of secondary metabolites in the plant kingdom (Souret, 2002; Memelink *et al.*, 2001; Rhodes, 1994). Individualized metabolite chemistry also implies the existence of both highly regulated basic pathways leading to the biosynthesis of key metabolites that can be suited for biochemical diversification, and specific biosynthetic enzymes that can catalyze the unlimited chemical modifications of these key metabolites, including glycosilation, sulphanation, hydroxylation, *o*-methylation, esterification, etc (Souret, 2002; Dixon, 1999).

1.1.2. Transport, storage and turnover of plant secondary metabolites

In plants the biosynthesis of secondary metabolites is compartmentalized and their accumulation, storage and release are almost exclusively associated with highly specialized organs or tissues (Croteau *et al.*, 2000). Water soluble compounds are usually stored in the vacuole (Wink, 1999a; Boller and Wiemken, 1986), whereas lipophilic substances are sequestered in resin ducts, lactifers, glandular hairs, trichomes, thylakoid membranes or the cuticle (Wiermann, 1981).

Most substances are synthesized in the cytoplasm, the ER or in organelles and, if hydrophilic, they are exported to the vacuole. They have to pass the tonoplast, which is impermeable to many of the polar secondary metabolites. For some alkaloids and flavonoids, a specific transporter has been described, which pumps the compounds into the vacuole (Deus-Neuman and Zenk, 1984). Alternatively, diverse trapping mechanisms (e.g. isoquinoline alkaloids by chelidonic acid, or meconic acid in the latex vesicles of *Papaver*) can also help to concentrate a particular compound in the vacuole (Wink, 1999a). Moreover, conjugation of secondary metabolites with glutathione in the cytoplasm and subsequent transportation by an ATP-dependent transporter into the vacuole has been proposed for some secondary metabolites that can be conjugated (Wink, 1997).

Lipophilic compounds will interfere not only with the biomembranes of microbes and herbivores but also with those of the producing plant (Wink, 1999a). To avoid autotoxicity, plants cannot store these compounds in the vacuole but usually sequester them on the cuticle, in dead resin ducts or cells, which are lined not by biomembrane but by an impermeable barrier. For example, the accumulation of monoterpene essential oils

in peppermint occurs specifically in glandular trichomes during the course of leaf development (Mc Garvey and Croteau, 1995).

In many cases, the site of biosynthesis is restricted to a single organ, such as roots, leaves or fruits, but processing and accumulation of the corresponding product can be detected in several other plant tissues. In these instances the xylem or phloem are likely transport routes for the translocation of secondary metabolites. For instance, while solanaceous roots produce the base compounds such as nicotine and hyoscyamine, which are then transported upwards via the xylem, the leaves not only accumulate these alkaloids but also transform them into a wide array of products (Pal Bais *et al.*, 2001). It is not unusual for a solanaceous leaf to contain between 50 and 100 alkaloids, all of which are derived from one compound synthesized in the root (Baldwin, 1998). In contrast, acotine and pyrrolidizidine alkaloids are examples of important plant organic compounds that are translocated by phloem to their site of storage (Wink and Roberts, 1998)

Storage can also be tissue- and cell-specific (Wink, 1999a). In a number of plants, secondary metabolites have been detected in idioblasts (St Pierre *et al.*, 1999) and more often in glandular hairs (terpenoids in *A. annua*; Ferreira *et al.*, 1997), stinging hairs (amines in Urticaceae), or the epidermis itself (Wiermann, 1981). Flowers, fruits and seeds are usually rich in secondary metabolites, especially in annual plants. In perennial species, high amounts of secondary metabolites are found in bulbs, roots, and the bark of roots and stems (Wink, 1999b).

Several other secondary metabolites are not end-products of metabolism but are turned over at a regular rate (Barz and Köster, 1981). During germination in particular,

N-containing secondary metabolites, such as alkaloids, non-protein amino acids, cyanogenic glycosides, and protease inhibitors are metabolized and serve as nitrogen source for the growing seedling (Wink, 1999b). Moreover, concentrations of some secondary metabolites such as quinolizidin alkaloids, nicotine, atropine, monoterpenes and phenylpropanoids, vary diurnally suggesting an active interplay between synthesis and turnover (Wink, 1997).

1.1.3. Classification of Plant Secondary Metabolites

Plants produce a broad spectrum of secondary metabolites that are often classified based on chemical characteristics, plant origin, or biosynthetic origin. The three major secondary metabolite groups are the terpenoids, alkaloids and phenolic compounds (Table 1.1).

1.1.3.1. Terpenoids

The largest class of plant secondary metabolites is undoubtedly that of terpenoids or isoprenoids from which more than 27,000 individual members of this class have been reported (Table 1.1). Terpenoids are not only numerous but extremely variable in structure (Figure 1.1), exhibiting hundreds of different carbon skeletons and a large assortment of functional groups. In spite of such diversity, all terpenoids are unified by a common mode of synthesis: the fusion of C_5 units with an isopentenoid structure (Banthorpe and Charlwood, 1980). The classification of terpenoids is based on the number of isoprenoid units present in their structure. The largest categories are those made up of compounds with two isoprenoid units (monoterpenes), three isoprenoid units

(sesquiterpenes), four isoprenoid units (diterpenes), six isoprenoid units (triterpenes) and eight isoprenoid units (tetraterpenes).

Monoterpenes occur in all higher plants and many of them are readily recognized by their characteristic flavors or odors like citronellal in lemon and menthol from peppermint. Other examples of terpenes in plants include sesquiterpenes such as zingiberene and the antimalarial, artemisinin, plant hormones like the diterpene, gibberellic acid, and sterols like the tetraterpene lanosterol (Gershenzon and Kreis, 1999)

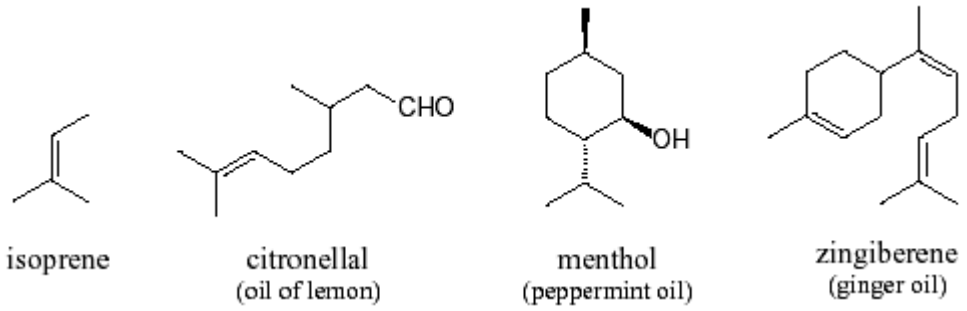


Figure 1.1. Examples of terpenoids that are of commercial importance or whose functional role has been investigated.

1.1.3.2. Alkaloids

The alkaloids include those natural products that contain nitrogen, usually as part of a cyclic system (Figure 1.2). Compounds of this type are numerous among plants and are perhaps best known for their often pharmacological properties (Herbert, 2001). Thus, many common drugs are alkaloid-based. Relatively mild examples include mescaline and nicotine. More potent examples include cocaine and morphine (Figure 1.2). Biosynthetically, natural drugs may be derived from amino acids, terpenes, or aromatics depending on the specific alkaloid structure. Because of this diversity, they are often

derived from the plant source rather than being produced synthetically (Heilmann and Bauer, 1999).

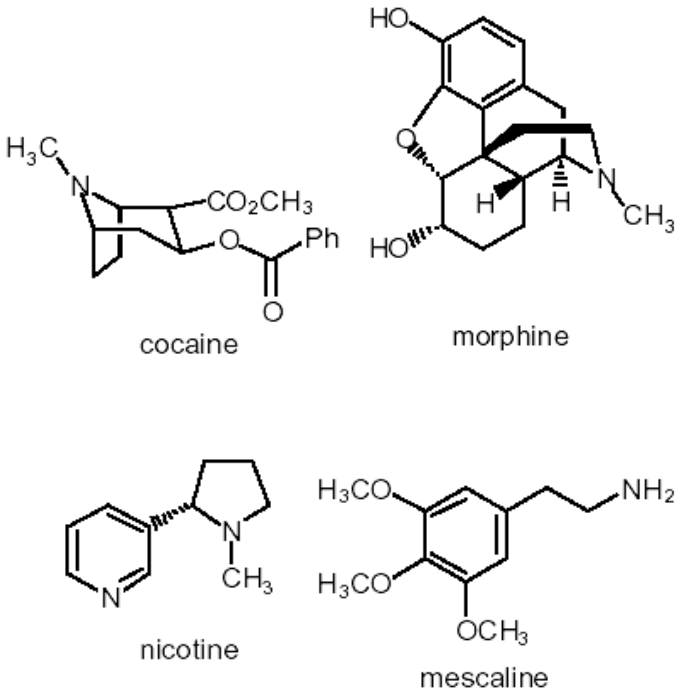


Figure 1.2. Examples of alkaloid natural products.

1.1.3.3. Plant Phenolics

The expression "phenolic compounds" embraces a considerable range of substances that posses an aromatic ring bearing a hydroxyl substituent (Figure 1.3), and while a significant number of such compounds occur in animals, most are of plant origin (Brielmann, 1999). Indeed, the presence of a "phenolic fraction" is a characteristic feature of all plant tissues. Among plant polyphenols, of which several thousand have now been described, the flavonoids form the largest group (Table 1.1). However, phenolic quinones, lignans, xanthones, coumarins, and other groups exist in considerable numbers, as well as monocyclic phenols (Croteau *et al.*, 2000).

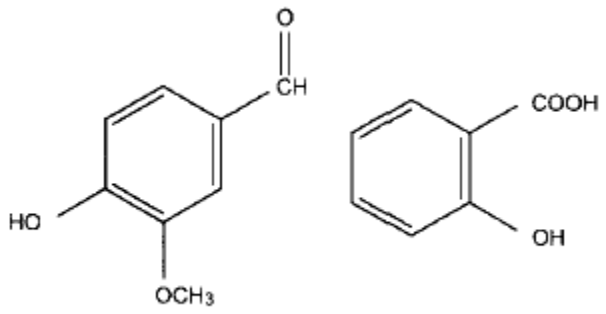


Figure 1.3 Some phenolic plant natural products.

1.1.4. Function of secondary metabolites

Although secondary metabolites have been used by humans for thousands of years (Wink and Roberts, 1998) as dyes (e.g. indigo, shikonin), flavours (e.g. vanillin, capsaicin), fragrances (e.g. rose and lavender oils), stimulants (e.g. caffeine, nicotine), hallucinogens (e.g. morphine, cocaine), insecticides (e.g. nicotine, piperine), poisons (e.g. coniine, strychnine) and even as therapeutic agents (e.g. atropine, quinine, codeine, etc.), their *in planta* functions have been a matter of controversy. It has often been argued that secondary metabolites are waste products or have no function at all. This hypothesis fails to explain several observations: 1) Waste products are characteristic and necessary for heterotrophic animals that cannot degrade their food completely for energy production (Wink, 1999b). These organisms excrete waste products that are often rich in nitrogen (i.e. urea). However, plants are essential autotrophs and therefore, do not need elaborate excretory mechanisms. Furthermore, nitrogen is a limiting nutrient for plants. Consequently, the production of nitrogen-containing excretions, such as alkaloids is often found in young or metabolically active tissues but not in dying or senescing cells, as would expected according to the waste product hypothesis. 2) Secondary metabolites are

often not inert end-products of metabolism (an expected trait of waste products) but many of them can be metabolized by plant cells (Wink, 1999b). For example, non-protein amino acids and cyanogenic glycosides are often stored in considerable quantities in leguminous seeds (Bell, 1980). During germination, degradation of these compounds can be seen, indicating that their nitrogen is reused by the seedling. 3) Secondary metabolites are often highly complex and regulated in a tissue- and developmentally-specific manner, which would be surprising for a waste product without a function (Wink, 1999b).

Alternatively, it was argued for years that secondary metabolites serve as defense compounds against herbivores. This hypothesis has been promulgated during past decades (Fraenkel, 1959; Ehrlich and Raven, 1964; Levin, 1976; Swain, 1977) and a large body of experimental evidence supports the concept that follows (Hartmann, 1996; Bernays and Chapman, 1994; Harborne, 1993). Several secondary metabolites have evolved against viruses, bacteria, fungi, competing plants and, importantly against herbivores (Wink, 1999b; Rhodes, 1994, Hahbrok and Scheel, 1989). For example, in *Lycopersicum*, the host defense against arthropod herbivores is mediated by the emission of various volatile metabolites, most of them derived from the degradation of carotenoids, that can elicit defensive responses in nearby plants (Colby *et al.*, 1998). Likewise, in conifers, insect attack is counteracted by the constitutive and inducible production of oleoresin, a complex mixture of mono-, sesqui-, and diterpenoids that accumulates at the wound site to kill invaders and both flush and seal the injury (Trap and Croteau, 2001).

In addition to plant defense, plant secondary metabolites also play essential biological roles in plant-environment interactions as well as in plant growth and development. They can serve as signal compounds to attract animals or insects for

pollination (e.g. fragrant monoterpenes, colored anthocyanins, or carotenoids) and for seed dispersal. (Cipollini and Levey, 1997; Crock *et al.*, 1997). Also, some secondary metabolites concomitantly exhibit physiological functions, for example they can serve as mobile and toxic nitrogen transport and storage compounds (Wink, 1999b) or UV-protectants (Holton and Cornish, 1995). Additionally, some of the larger terpenes, including sterols and carotenoids, are important membrane components, and are required for quinine electron transfer and photosynthesis (Faccini, 1999). Moreover, some secondary metabolites are used as metabolic precursors for the synthesis of important phytohormones including abscisic acid, brassinosteroids and gibberellic acid among others (Croteau *et al.*, 2000; Faccini, 1999).

1.1.5. Medicinal and commercial importance of plant secondary metabolites.

Natural products once served humankind as the source of all drugs, and higher plants provided most of these therapeutic agents. Today, natural products (and their derivatives and analogs) still represent over 50% of all drugs in clinical use, with higher plant-derived natural products representing 25% of the total (GEN, 1998; Balandrin *et al.*, 1993). The World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care, and about 85% of traditional medicine involves the use of plant extracts (WHO, 2002). This means that about 3.5 to 4 billion people in the world rely on plants as sources of drugs (De Smet, 1997). In the United States plant-derived drugs represent about 25% of the prescription drug market, and in the last decade this equated to a retail value of approximately $15.5 billion (Pezzuto, 1997). From 1983 to 1994 39%

of the New Approved Drugs were of natural origin, including original natural products, products derived semi synthetically from natural products, and synthetic products based on natural product models (Cragg *et al*., 1997).

Further evidence of the importance of natural products is provided by the fact that almost half of the world's 25 best selling pharmaceuticals are either natural products or their derivatives (Pezzuto, 1997). Conservative estimates suggest that there are more than 250,000 species of higher plants existing on this planet, and only a very small percentage of plants have been exhaustively studied for their potential value as a source of drugs. Obviously natural products will continue to be extremely important as sources of medicinal agents. In addition to the natural products which have found direct medicinal application as drug entities, many others can serve as chemical models or templates for the design, synthesis, and semi synthesis of novel substances for treating humankind's diseases. Such clinically useful drugs include the anticancer agents paclitaxel (Taxol®) and vincristine (Oncovin®) the sedative scopolamine, and the muscle relaxant (+)-tubocurarine (Faccini, 2001). These substances embrace some of the most exciting new therapeutic agents currently available for use in a clinical setting. Although there are some new approaches to drug discovery, such as combinatorial chemistry and computer-based molecular modeling design, none of them can replace the important role of natural products in drug discovery and development (Pezzuto, 1997).

1.1.6. General secondary metabolite pathways

Despite the enormous variety of secondary metabolites, the number of corresponding basic biosynthetic pathways is restricted and distinct. In plants three pathways are the source of most secondary metabolites: the shikimate pathway (i.e. major source of aromatic compounds), the isoprenoid (terpenoid) pathway, and the polyketide pathway. After the formation of the major basic skeletons, further modifications result in plant species specific compounds. For example hydroxyl, methoxy, aldehyde, and carboxyl groups and substitutions adding further carbon atoms to the molecule, such as prenyl-, malonyl-, and glucosyl-moieties create an almost endless variety of compounds (Verpoorte, 1999). Moreover, various oxidative reactions may result in loss of certain fragments of the molecule or rearrangements leading to new skeletons.

In the early 1900s, it was argued that secondary metabolites arise spontaneously (Wink, 1999a). Now from the ongoing functional analysis of the *Arabidopsis* and other plant genomes, there is evidence that approximately 15-25% of the genes present in each plant encode enzymes involved in secondary metabolism (Somerville and Somerville, 1999). Since the majority of secondary metabolites are plant- or species-specific (Wink and Waterman, 1999), and the biosynthetic enzymes are substrate-exclusive, it is likely that thousands of these enzymes remain to be elucidated (Wink, 1999b). A combination of genetic, molecular and biochemical studies has given considerable insight into the mechanisms underlying the process and overall regulation of some secondary metabolite pathways (De Luca and Laflamme, 2001; Memelink *et al.*, 2001; Croteau *et al.*, 2000; Dixon *et al.*, 1999; Lichtenthaler *et al.*, 1997a). In the following section, some details will

be given about the general parts of the terpenoid biosynthetic pathway which is most relevant to this work.

1.2. TERPENOIDS

1.2.1. Introduction

The largest class of plant secondary metabolites is undoubtedly that of the terpenoids or isoprenoids. As mentioned earlier, terpenoids are generally lipophilic substances derived from a simple five-carbon unit (Figure 1.1). Their large diversity arises from the number of basic units (C_5) in the chain and the various ways in which they are assembled. Formation of cyclic structures, addition of oxygen-containing functions, and conjugation with sugars or other molecules all add to the possible complexity (Gershenzon and Kreis, 1999). The enormous structural diversity of the terpenoids is almost matched by their functional variability.

Although considered as secondary metabolites, some terpenoids have well-established roles in almost all basic processes, including growth, development, reproduction and defense (Chappell, 1995). Among the best known terpenoids are the gibberellins and abscisic acid, plant hormones involved in developmental processes. In addition, important groups of plant compounds such as cytokinins, chlorophylls, and quinone-based electron carriers, have terpenoid side chains attached to non-terpenoid structures. These side chains facilitate anchoring to or movement within membranes (Gershenzon and Kreis, 1999). In the past decade, proteins have also been found to have terpenoid side chains attached as a result of post-translational modification. This

substantially increases protein hydrophobicity and serves to target proteins to membranes or to direct protein-protein interactions (Zhang and Casey, 1996).

Most of the thousands of terpenoids produced in plants have no discernible role in growth and development and are, therefore, often classified as secondary metabolites. Although, comparatively few of these substances have been investigated in depth, they are thought to serve primarily in ecological roles, providing defense against herbivores or pathogens, and acting as attractants for pollination and seed dispersal, or as inhibitors of the germination and growth of neighboring plants (Verpoorte, 1999).

1.2.2. Overview of Terpenoid Biosynthesis

Compartmentation is an important aspect of the terpenoid biosynthesis. It is now generally accepted that terpenoids are produced in the cytosol and in plastids (Mc Caskill and Croteau, 1995). While sesquiterpenes, triterpenes and steroids are generally synthesized in the cytoplasm from FFP, monoterpenes, diterpenes, carotenoids, plastoquinones and phytols are commonly synthesized in plastids from GPP or GGPP (Souret *et al.*, 2002; Croteau et al, 2000). In spite of their structural diversity, all terpenoids are derived from the C_5 isoprene unit isopentenyl pyrophosphate (IPP), through the mevalonate and the non-mevalonate pathway (Figure 1.4; Croteau, 2000).

1.2.2.1. Mevalonate (MVA) Pathway

The mevalonic acid pathway can be divided conveniently into several stages (Figure 1.4). In the first stage IPP is produced through the condensation of two molecules of acetyl Coenzyme A, which is then converted to β-hydroxy-β-methyl-glutaryl-CoA

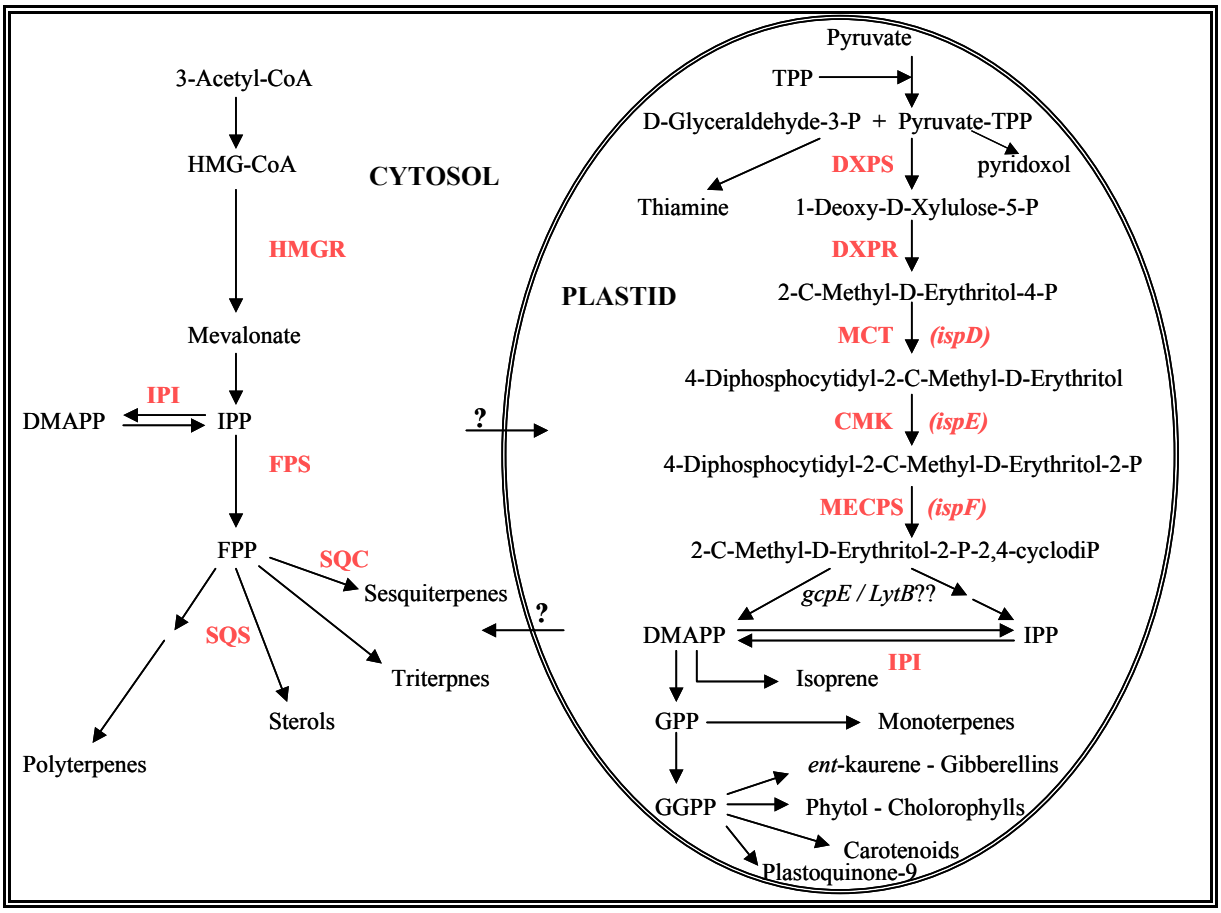


Figure 1.4. Mevalonate and non-mevalonate terpenoid biosynthetic pathway in plants. HMGR: 3-hydroxy-3-methylglutaryl coenzyme A reductase; DXPS: deoxy-D-xylulose-5- phosphate synthase; DXPR: deoxy-D-xylulose-5-phosphate reductoisomerase; MCT: 2-C-methyl-D-erythritol-4-phosphate synthase; CMK: 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol kinase; MECPS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; novel *E. coli* genes, *gcpE* and *LytB*, with unknown biochemical function but required for the mevalonate-independent pathway; IPI: isopentenyl diphosphate isomerase; FPS: farnesyl diphosphate synthase; SQC: sesquiterpene cyclase; SQS: Squalene synthase; IPP: Isopentyl pyrophosphate; FPP: farnesyl pyrophosphate; DMAPP: dimethylallyl diphosphate; GPP: C_{10} Geranyl diphosphate; GGPP: C_{20} Geranyl diphosphate. Arrows in and out indicate a potential but limited exchange of IPP pools between the plastid and the cytosol (Taken from Souret, 2002).

(HMG-CoA), which undergoes further modification to eventually produce isopentenyl pyrophosphate. The next stage encompasses head to tail addition of IPP molecules catalyzed by prenyltransferases to form prenyl diphosphate homolog precursors of various subclasses of terpenoids. In the first event of this stage, isopentenyl pyrophosphate (IPP) is isomerized into dimethylallyl diphosphate (DMAPP) by the enzyme IPP isomerase. The highly reactive DMAPP is the starter molecule of terpenoid biosynthesis (Lichtenthaler *et al.*, 1997b). The allylic phosphate group is an excellent leaving group, yielding a carbonium ion, stabilized by the allyl function (Verpoorte, 1999). This carbonium ion readily reacts with IPP, giving the C_{10} geranyl diphosphate (GPP). This molecule again has the active allylic phosphate group, and can thus further react with a molecule of IPP to give the C_{15} farnesyl diphosphate (FPP). A further reaction yields the C_{20} geranyl diphosphate (GGPP). These reactions catalyzed by prenyltranferases yield, respectively, monoterpenes (C_{10}), sesquiterpenes (C_{15}) and diterpenes (C_{20}) (Croteau *et al.*, 2000). The tail-to-tail coupling of two all-trans molecules of FPP results in the formation of the C_{40} squalene, the precursor for steroids and triterpenoids. These two important groups of terpenoids are both formed from squalene oxide, but from two different conformations of this precursor. Tail-to-tail coupling of two GGPP molecules results in the formation of phytoene (C_{40}), the precursor of the carotenoids (Figure 1.5; Verpoorte, 1999).

The final stage of terpenoid biosynthesis includes secondary enzymatic modifications and transformation to give rise to the wide diversity of terpenoids. The enormous diversity in mono-, sesqui-, and diterpene skeletons is first of all due to selective terpenoid synthases and cyclases, a large family of enzymes that catalyze the

cyclization of differently folded GPP-, FPP- or GGPP-molecules, which also may have undergone cis-trans-isomerizations. From the various basic skeletons further skeletal diversity can be introduced in subsequent biosynthetic steps in which, among others, various cytochrome P-450 enzymes play a role (McGarvey and Croteau, 1995).

1.2.2.2. Non-mevalonate (MVA) Pathway

For many years it was thought that all terpenoids derive from mevalonate. Recently, however, there has been the discovery of a second, non-mevalonate pathway (Figure 1.4). Originally discovered in bacteria (Rohmer *et al.*, 1993) this pathway has also been shown to be involved in terpenoid biosynthesis in both higher and lower plants (Lichtenthaler *et al.*, 1997b). While the MVA pathway produces IPP through the condensation of two molecules of acetyl Coenzyme A, the alternative non-mevalonate pathway produces IPP through the condensation of pyruvate and glyceraldehyde-3-phosphate by the enzyme deoxy-D-xylulose-5-phosphate synthase (DXPS). The condensation of pyruvate and glyceraldehyde-3-phosphate, results in the formation of 1-deoxy-xylulose-5-phosphate. This molecule is further rearranged by the enzyme deoxy-D-xylulose-5-phosphate reducto-isomerase (DXPR) to produce 2-C-methyl-D-erythritol-4-phosphate (MEP), and finally through further rearrangements to IPP (Figure 1.4; Lichtenthaler *et al.*, 1997b).

1.3. *ARTEMISIA ANNUA* L.

1.3.1. Introduction

A. annua (Asteraceae), known in the United States as sweet Annie or annual wormwood, is an annual herb native of Asia, most probably China, where it is known as *quinghao* (Figure 1.5; Ferreira *et al.*, 1997). The plant has become naturalized in many countries including Argentina, Bulgaria, France, Hungary, Romania, Italy, Spain, United States, and the former Yugoslavia (Dhingra *et al.*, 2000). *A. annua* has been used for many centuries in traditional Chinese medicine for the treatment of fever and malaria. Its antimalarial application was first described in the "Chinese Handbook of Prescription for Emergency Treatments" edited around 340 A.D. (Sumner, 2000). In 1971, extraction of aerial parts of *A. annua* with low boiling solvents, such as diethyl ether, produced a compound mixture with antimalarial properties on infected mice and monkeys (Ferreira and Janick, 1996). The main active principle, artemisinin (formerly referred to as arteannuin and as *quinghaosu* in Chinese), was isolated and had its structure correctly defined in 1972 as a sesquiterpene lactone with an endoperoxide bridge (Fig. 1.5; Dhingra *et al.*, 2000; Ferreira *et al.*, 1997; Woerdenbag *et al.*, 1990).

In vitro trials conducted in China (WHO 1986) have shown artemisinin and its derivatives (artemether and artesunate) as being effective against the erythrocytic stages of chloroquine-resistant strains of *Plasmodium falciparum,* the malarial parasite, at lower minimum effective concentrations than chloroquine, the most commonly used drug. Artemisinin and its derivatives have effectively treated malaria and cerebral malaria in human subjects with no apparent adverse reactions or side effects (Van Geldre *et al.*,

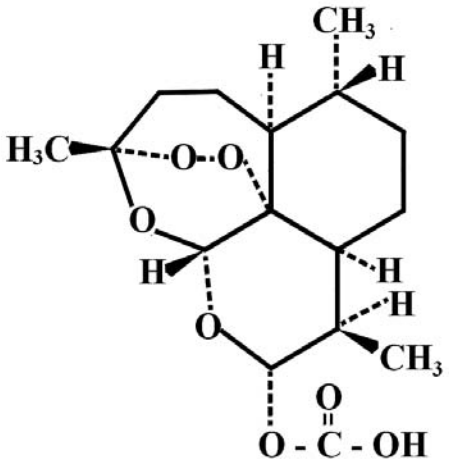


Figure 1.5. *A. annua* and chemical structure of artemisinin.

1997). A more recent study performed by Singh's group has shown the potential of artemisinin as a novel antitumor agent for certain types of cancer (Singh and Lai, 2001). They found that dihydroartemisinin, an analog of artemisinin, can selectively kill cancer cells in presence of holotransferrin which can increase intracellular iron concentrations, whereas normal breast cells (HTB 125) and lymphocytes were not affected under the same condition(Singh and Lai, 2001). Since it is relatively easy to increase the iron content inside cancer cells *in vivo*, administration of artemisinin-like drugs and intracellular iron-enhancing compounds may be a simple, effective, and economical treatment for cancer (Singh and Lai, 2001).

With *P. falciparum* developing resistance to chloroquine and pyrimethamine/sulfonamide (WHO, 1986), alternative treatments based on new compounds such as artemisinin and its derivatives are actively being sought. While artemisinin and its derivatives may be chemically synthesized (Xu *et al.* 1986; Zhou, 1986), theses synthetic compounds are unlikely to be economically competitive with the

naturally derived plant products (Dhingra *et al.*, 2000; Van Geldre *et al.*, 1997). Hence, at the present time, extraction from *A. annua* plants remains the only source of the drug.

In the past decades, the search for the concerned bioactive compounds synthesized in *A. annua* has led to the isolation of a broad range of compounds. In addition to artemisinin, *A. annua* synthesizes several sesquiterpenoids, flavonoids, coumarins, triterpenoids, sterols, phenolics, aliphatic compounds and monoterpenoids in different parts of the plant (Bhakuni *et al.*, 2001). Many of these compounds and their analogs display important pharmacological activities such as antiviral (e.g. fisetin), antifungal (e.g. polyacetylenes), antibacterial (e.g. artemisinic acid), anti-inflammatory (e.g. scopoletin) and antitulcerogenic (e.g. dehydroleucodin) (Bhakuni *et al.*, 2001; Tan *et al.*, 1998).

1.3.2. Biosynthesis of artemisinin

In the production of artemisinin as well other sesquiterpenes, there are a number of key regulatory enzymes involved. One is 3-hydroxy-3-methyl-CoA reductase (HMGR), which is a key enzyme under environmental control (e.g. light, wounding, pathogens; Learned, 1996; Nelson *et al.*, 1994). This enzyme catalyses the NADPH-dependent reduction of HMG CoA to mevalonic acid (MVA), which is the first committed step of the mevalonate biosynthetic pathway (Figure 1.4). In the nonmevalonate biosynthetic pathway, the key enzymes toward the formation of IPP are 1-deoxy-d-xylulose-5-phosphate (DXPS) and deoxy-D-xylulose-5-phosphate reducto-isomerase (DXPR) (Figure 1.4; Lange and Croteau, 1999). Recent findings have shown DXPS is upregulated by light, while DXPR appears to be unaffected (Souret, 2002).

Once IPP has been formed, farnesyl pyrophosphate synthase (FPS), a prenyltransferase, condenses two isoprene units (IPP) into farnesyl pyrophosphate (FPP) (Chen *et al*., 1999; Matsushita *et al*., 1996). FPS is another key point in regulation of isoprenoid fate and is affected by a number of factors including elicitation (Zook and Kuc, 1991), UV light (Back *et al*., 1998), wounding and insect attack (Steele *et al*., 1998). Subsequently, the FPP produced is converted to sesquiterpene by the action of a sesquiterpene cyclase (SQC). This has been considered the first important step in sesquiterpene production for two reasons. First, sesquiterpene cyclases divert FPP from entering sterol synthesis (Vögeli and Chappell, 1988). In addition, the cyclase reaction establishes an important stereochemical framework upon which all other chemical modifications take place (Van Geldre *et al.*, 2000). Therefore, the reactions catalyzed by sesquiterpene cyclases represent a potential committed step in the biosynthesis of these diverse compounds (Back *et al.*, 1994).

After the cyclization of FPP by sesquiterpene cyclase, little is known about the biosynthesis of artemisinin. The first probable step in artemisinin biosynthesis originates from the cyclization of FPP to generate amorpha-4,11-diene (Bouwmeester *et al.*, 1999; Wallaart *et al*., 2001), the central intermediate in the pathway (Figure 1.6). After this, it is likely that the immediate precursors of artemisinin are artemisinic acid and dihydroartemisinic acid (Van Geldre *et al.*, 2000; Merke *et al.*, 2000; Van Geldre *et al.*, 1997; Woerdenbag *et al.*, 1990). Dihydroartemisinic acid may then be converted to arteannuin B or non-enzymatically photooxidized to dihydroartemisinic acid peroxide that is apparently air oxidized to either artemisinin or artemisitene (Wallaart *et al.*, 1999a, 1999b; Figure 1.6).

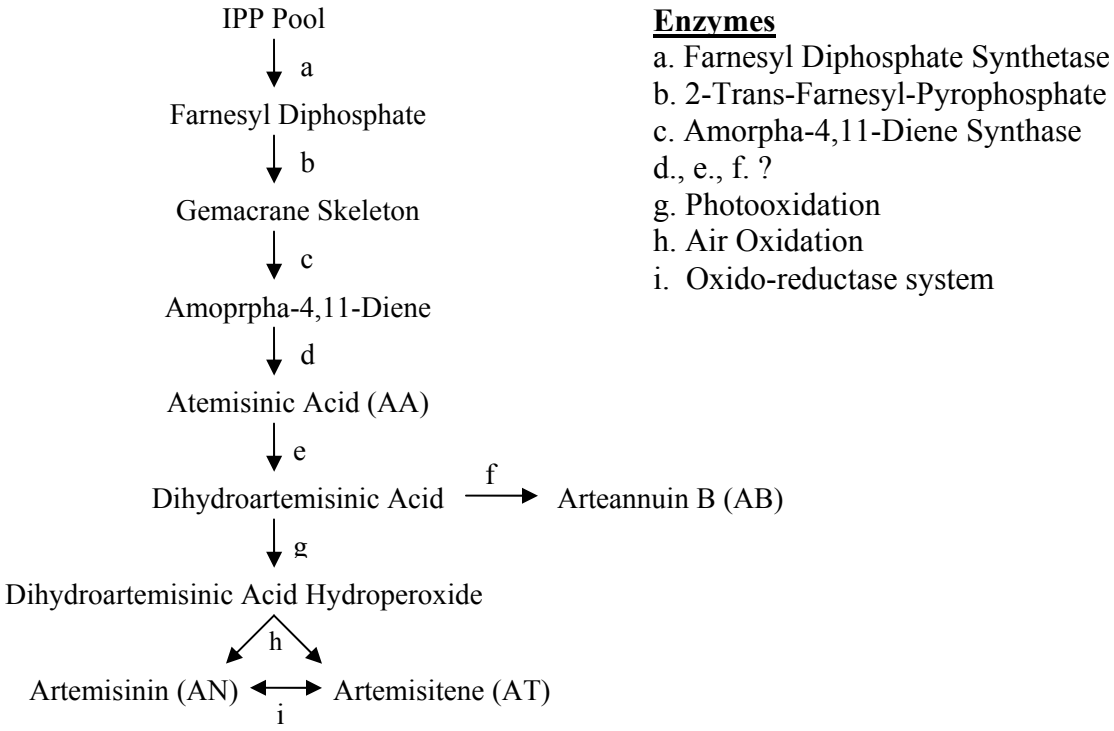


Figure 1.6. Proposed biosynthetic pathway of artemisinin in *A. annua*.

Also in this pathway, an interconversion between artemisinin and artemisitene can occur by an oxido-reductase system (Dhingra *et al.*, 2000).

In other reports, arteannuin B is considered to be another intermediate in the bioconversion of artemisinic acid to artemisinin (Dhingra *et al.*, 2000; Van Geldre *et al.*, 1997). Cell-free homogenates of *A. annua* leaves converted arteannuin B into artemisinin (Nair and Basile, 1993). While the purification and characterization of an enzyme involved in the biosynthesis *in vitro* of artemisinin from arteannuin B has been obtained, this step remains unclear and requires further study *in planta* (Dhingra and Lakshmi-Narasu, 2001).

Currently the artemisinin biosynthetic pathways postulated by various groups are contradictory (Dhingra *et al.*, 2000). Intermediate products or enzymes after FPP and before artemisinic acid, arteannuin B and artemisinin, have not been isolated *in vivo*. Therefore, further studies are required to clarify the pathway.

1.3.3. Production of artemisinin

Artemisinin is mainly localized in the aerial parts of the plant (Delabays *et al.*, 2001; Dhingra *et al.*, 2000; Van Geldre *et al.*, 1997) where it accumulates in the glandular trichomes present on the surface of the leaves as well as on the corolla and receptacle of the florets (Ferreira and Janick, 1996). Substantially variable contents in artemisinin have been observed in the leaves of different clones of *A. annua.* The yields in field- or greenhouse-grown plants range between 0.02 and 1.38 % in the dry leaves (Delabays *et al.*, 2001). These yields are low for commercial exploitation, where a full ton of leaves may be required to produce about 6 kg of artemisinin (Van Geldre *et al.*, 1997).

Several factors account for this variability, including the use of diverse methods for extraction and analysis and the collection and the preparation of the samples especially the separation of the leaves from the stems (Delabays *et al.*, 2001). Artemisinin content has been reported to be higher at the top of the plant in some clones (Charles *et al.* 1990; Laughlin, 1995), and equally distributed in others (Laughlin 1995). Moreover, seasonal variations and geographical origins also appear to be important factors affecting artemisinin production in adult plants (Wallaart *et al.*, 2000). Thus, it is likely that part of the variation reported in the literature in the artemisinin content of *A. annua* leaves results from different periods of harvest and plant variety. Furthermore, environmental stresses

(i.e. light, water, salt and temperature) or nutrient availability can also influence artemisinin yields in the plant (Delabays *et al.*, 2001; Van Geldre *et al.*, 1997). Therefore, alternative methods for improving artemisinin levels *in planta* have been sought, but with mixed results.

Classical approaches like hybridization between well characterized genotypes and seed selection are attractive options for establishing more productive strains of *A. annua*. However, despite the importance of artemisinin as an antimalarial and the necessity to grow the plant to produce it, studies devoted to the genetic transmission of artemisinin content in *A. annua* are very scarce (Delabays *et al.*, 2001). Such studies are difficult to realize because the flowers are miniscule (i.e. capitula, 2-3 mm diameter), and systems for controlled pollination have not been devised (Van Geldre *et al.*, 1997). In addition, *A. annua* is a determinate species; it dies after seed set (Ferreira *et al.*, 1997). Thus, plants with high artemisinin content cannot be maintained after flowering and their seed will be the result of open pollination (Ferreira *et al.*, *et al.*, 1997; Van Geldre *et al.*, 1997). Furthermore, seeds are rarely produced by self-fertilization, which infers self-incompatibility (Delabays *et al.*, 2001; Ferreira *et al.*, 1997; Van Geldre *et al.*, 1997).

Given that plants are the only reasonable source, a biotechnological approach has been considered as a viable alternative for producing artemisinin. This includes production by tissue cultures, suspension cell cultures, root cultures and intact plants.

Although callus and cell suspension cultures of *A. annua* have been successfully achieved, artemisinin yields have been disappointing (Van Geldre *et al.*, 1997; Brown, 1994). However, *in vitro* propagation of *A. annua* and artemisinin production via shoot cultures has been achieved (Ferreira and Janick 1996; Fulzele *et al.*, 1991; Martinez and

Staba 1988). Evidence suggests that artemisinin production in shoots is enhanced by the presence of roots (Ferreira and Janick 1996). These results together with the reports showing artemisinin accumulation in glandular trichomes suggest that a certain degree of differentiation is required for production (Dhingra *et al.*, 2000). Several laboratories, thus, developed hairy root cultures of *A. annua* by transformation with *Agrobacterium rhizogenes* (Paniego *et al.*, 1996; Jaziri *et al.*, 1995; Weathers *et al.*, 1994). In each particular case, the levels of artemisinin detected varied depending on the clone, culture conditions, and the culture age of the transformed roots, but were similar to the levels found in wild-type plants (Smith *et al*., 1997; Weathers *et al*., 1997; Jaziri *et al.*, 1995, Weathers *et al*., 1996; Weathers *et al*., 1994). The most attractive property of transformed hairy roots is their robustness which makes them suitable for use in large scale equipment (e.g. fermenters) offering potential commercial utility compared to traditional agricultural methods (Kim *et al*., 2002).

Finally, a more recent approach has been the use of metabolic engineering (Barkovich and Liao, 2001). Genetic engineering of a secondary metabolic pathway aims to modify (positively or negatively) the expression of single genes affecting the production of a certain compound or group of compounds (Verpoorte and Memelink, 2002). Recent interest has arisen to develop transgenic *Artemisia annua* plants or organs with increased accumulation of artemisinin, produced by introducing key genes encoding for enzymes regulating the biosynthetic pathway leading to the formation of artemisinin. The recent identification of the sesquiterpene cyclase, amorpha-4,11-diene synthase (Chang *et al*., 2000; Mercke *et al*., 2000; Wallaart *et al*., 2001), assumed responsible for catalyzing the committed step in the biosynthesis of artemisinin, represents a big step

forward in the overproduction of artemisinin through genetic manipulation of *A. annua*. In a different approach, Chen *et al*. (1999) developed a transgenic system for *A. annua* via *Agrobacterium rhizogenes*-mediated transformation. Using this system, a cDNA encoding farnesyl diphosphate synthase (FDS) placed under a CaMV35S promoter was transferred into *A. annua.* Their results showed three transgenic hairy roots clones with higher growth rates and 3-4 times more artemisinin production than the control hairy root clones (Chen *et al*., 1999). Additionally, using *Agrobacterium tumefaciens*, they established five transgenic plants overexpressing the same FDS transgene. Analysis of artemisinin content in such plants was about 2-3 times higher than that in the control plants (Chen *et al.,* 2000). Their work suggests that overexpressing enzymes regulating sesquiterpenoid formation may hold great potential for improving the production of artemisinin. However, attempts at increasing flux by manipulating the activity of single enzymes in plant secondary metabolite pathways have met with mixed success (Broun and Somerville, 2001). In addition, a major constraint in engineering plant secondary metabolite production, including artemisinin, is that only a few genes of these pathways are known (Dhingra *et al*., 2001).

1.4. PLANT "HAIRY" ROOT CULTURES

1.4.1. Introduction

The roots of higher plants comprise a metabolically active and largely unexplored biological frontier (Pal Bais *et al.*, 2001). Apart from the classical role of providing a mechanical anchor, water/nutrient uptake, and interacting with the rhizosphere, they are also the resource of a remarkably diverse group of secondary metabolites (Pal Bais *et al.*, 2001; Flores *et al.*, 1992). Numerous studies indicate that the biosynthetic and bioactive

capabilities of roots are as diverse and complex as those of any other part of the plant. The pharmacopoeia of traditional cultures throughout the world describes numerous uses of root extracts in the treatment of human diseases (Pal Bais *et al.*, 2001; Baker *et al.*, 1995). For instance, valuable phytochemicals such as the antimicrobial, shikonin, and the anti-tumorous compound, camptothecin, are some examples of the various molecules extracted from roots for medicinal purposes (Ramachandra-Rao and Ravishankar, 2002). In the past, the underground nature of roots and the lack of suitable experiments systems to study have hampered research on root biology. However, recent progress in growing roots in isolation with their elements has greatly facilitated the study of root-specific metabolism and contributed to our understanding of this organ.

The ability of *Agrobacterium rhizogenes* to induce hairy roots in a range of host plants has offered a promising system for the study of secondary metabolite production (Ramachandra-Rao and Ravishankar, 2002; Giri and Lakshmi, 2000; Shanks and Morgan, 1999). Tepfer (1990) summarized 166 plants belonging to 30 dicotyledons families wherein hairy roots are induced. Hairy roots are induced by transfer of T-DNA from the plasmid of *A. rhizogenes* (Ramachandra-Rao and Ravishankar, 2002; Gelvin, 2000; Giri and Lakshmi, 2000) to the host plant genome resulting in root formation by virtue of auxin synthesis genes encoded by bacterial DNA (see Figure 1.7 and 1.8). The Ri (root inducing) plasmid of *A. rhizogenes* also elicits the synthesis of opines such as agropine and mannopine. The integration of the T-DNA into the plant genome is a random process and other critical factors encoded by the *vir* genes in the plasmid T-DNA are also known to play a key role in triggering the transfer process (Hamill and Lidgett,

1997). The transformed nature of the roots is genetically validated by opine detection or Southern analysis (Ramachandra-Rao and Ravishankar, 2002).

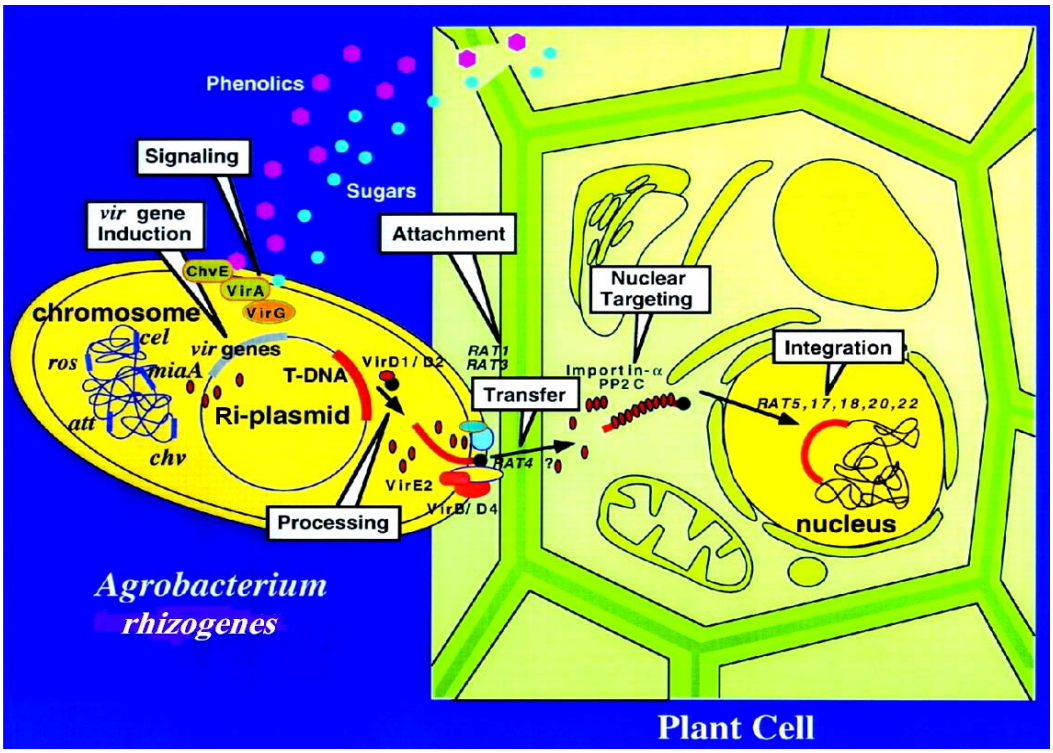


Figure 1.7. Schematic diagram of the *Agrobacterium* infection process. Critical steps that occur to or within the bacterium (chemical signaling, *vir* gene induction, and T-DNA processing) and within the plant cell (bacterial attachment, T-DNA transfer, nuclear targeting, and T-DNA integration) are highlighted, along with genes and/or proteins known to mediate these events (Adapted from Gelvin, 2000).

1.4.2 Characteristics of Plant "Hairy" Roots

"Hairy" roots (Figure 1.8) have several properties that have promoted their use in plant biotechnology. Often these roots grow rapidly with mass doubling times resembling those of disorganized cell suspensions, but unlike the latter they are fully differentiated tissues that tend to produce secondary metabolites characteristic of the species that has been transformed (Hamill and Lidgett, 1997). Even in cases where secondary

metabolites accumulate only in the aerial part of an intact plant, hairy root cultures have been shown to accumulate the same metabolites (Liu *et al.*, 1999; Bakkali *et al.*, 1997; Jaziri *et al.*, 1995; Weathers *et al.*, 1994). Their ease of maintenance and ability to synthesize a range of chemical compounds offers an additional advantage as a continuous source for the production of valuable secondary metabolites. Since production of secondary metabolites is generally higher and genetically more stable in differentiated tissue, hairy root cultures represent a technical alternative to the use of plant cell suspension cultures for the production of secondary metabolites (Shanks and Morgan, 1999).

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 (Ramachandra-Rao and Ravishankar, 2002; Giri and Lakshmi, 2000; Flores *et al.*, 1999). 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; Baiza *et al.*, 1999; Aird *et al.*, 1998).

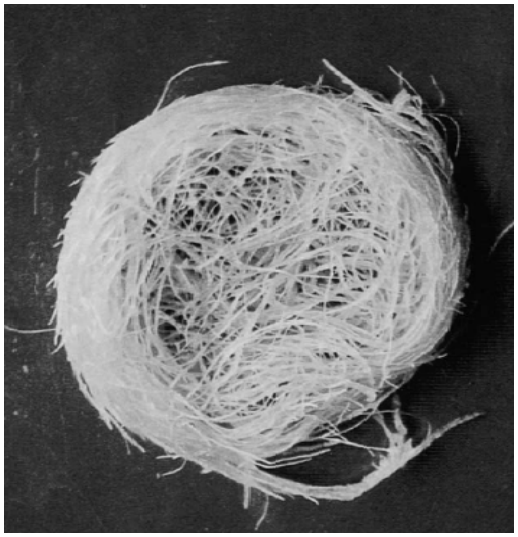


Figure 1.8. Hairy root culture of *A. annua* L.

1.4.3. Hairy root cultures as source of secondary metabolites

The potential of hairy root cultures for studies of plant-specific bioactive compounds has been confirmed by numerous research groups (for references see Hamill and Lidgett, 1997). In addition to tropane alkaloids and nicotine, hairy root cultures have been shown to produce cinchona alkaloids, terpenoids, polyacetylenes, thiopenes and shikonins among others (Vivanco *et al.*, 2002; Pal Bais *et al.*, 2001; Flores *et al.*, 1999). New root clones and root specific metabolites are frequently reported and span an ever-increasing range of plant families (Vivanco *et al.*, 2002; Porter, 1991).

In addition to low-molecular-weight compounds, roots can accumulate polymers such as starch, fructans and proteins. Among these polymers, proteins represent one of the most biologically puzzling molecules produced and secreted by roots (Vivanco *et al.*, 2002). The production of industrial and therapeutic proteins by plants is an area of intense commercial interest and one that is also being investigated in hairy roots from the bioprocess view point (Shanks and Morgan, 1999). Indeed, transformed hairy roots have

been implemented to study the production of bioactive proteins such as glucanases, chitinases, and ribosome-inactivating proteins (Vivanco *et al.*, 2002; Pal Bais *et al.*, 2001; Flores *et al.*, 1999).

1.4.3.1. Strategies to increase secondary metabolite production in plant hairy root cultures

During the past decade, considerable progress has been made to stimulate formation and accumulation of secondary metabolites using hairy root cultures. Factors that affect the increase or decrease in the biosynthetic activity of hairy root cultures are of interest to both those trying to understand variation as well as to workers looking for high yields of secondary metabolites. In plants, the expression of many secondary metabolite pathways is easily altered by environmental factors such as nutrient availability, stress factors, light, and growth regulators (Giri and Lakshmi, 2000). Thus, it is not surprising that the production of secondary metabolites in hairy roots is also greatly affected by culture conditions (Kim *et al*., 2002). In this section, some of the adopted strategies for enhancing the secondary metabolites of hairy root cultures are briefly described.

1.4.3.1.1. Screening and selection of highly productive root lines

To obtain high-producing hairy root lines, strain improvement begins with the choice of a parent plant with a high content of the desired product. Different strains of *Agrobacterium rhizogenes* can also affect transformation. For example, Giri *et al.* (2001) showed that in contrast to the A_4, 15834, K_{599}, LBA 9402 and 9340 strains of *A. rhizogenes*, the LBA 9365 strain of was found to induce hairy roots of *A. annua* with

higher concentrations of artemisinin. However, other plant species may respond better to other *A. rhizogenes* strains (Kim *et al*., 2002).

1.4.3.1.2. Manipulation of nutrients and culture environment

Many constituents of hairy root culture media are important determinants of growth and secondary metabolite production (Giri and Lakshmi, 2000). Optimization of the phytohormone concentration and combinations are often effective (Kim *et al*., 2002). Alterations in environmental factors such as nutrient levels, light, and carbon sources may also be effective in increasing productivity (Kim *et al*., 2002; Rothe *et al*., 2001; Weathers *et al*., 1997; Taya *et al*., 1994). For example, reduced phosphate levels often stimulate product accumulation (Kim *et al*., 2002; Dunlop and Curtis, 1991). Nitrogen sources can also play an important role in product yields (Bensaddek et al., 2001; Wang and Tan, 2002). For example, reduced levels of NH_4^+ and increased levels of NO_3^- promoted the production artemisinin in hairy roots of *A. annua* and overall high levels of total nitrogen have been shown to inhibit growth and production (Wang and Tan, 2002; Weathers *et al*., 1997; Weathers *et al*., 1996).

1.4.3.1.3. Elicitation

Plants produce secondary metabolites in nature as a defense mechanism against attack by pathogens (Croteau *et al.*, 2000). Plants have been found to elicit the same response as the pathogen itself when challenged by compounds of pathogenic origin (elicitors) (Pal Bais *et al*., 2001). 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 (Ramachandra-Rao and Ravinshankar, 2002). Elicitors of fungal, bacterial and yeast origin, viz. polysaccharides, glycoproteins, inactivated enzymes, purified curdlan, xanthan and chitosan, and salts of heavy metals have been shown to increase production of various secondary metabolites (Kim *et al*., 2002; Ramachandra-Rao and Ravinshankar, 2002; Robbins *et al*., 1991). For example, treatment of *A. annua* hairy roots with a homogenate of *Colletotrichum* sp. mycelium resulted in an accumulation of artemisinin of up to 44% over the control (Wang *et al*., 2001). The treatment of root cultures of *Datura stramonium* with copper and cadmium salts has been found to induce the rapid accumulation of high levels of sesquiterpenoid defensive compounds (Furze *et al*., 1991).

1.4.3.1.4. Avoiding sequestration of the secondary metabolites

In most cases, the products formed by plant cell cultures are stored in the vacuoles (Wink, 1997). In order to release the products from vacuoles of plant cells, two membrane barriers the plasma membrane and the tonoplast have to be penetrated. Attempts have been made to permeabilize the plant cells transiently, to maintain the cell viability and to have short time periods of increased mass transfer of substrate and metabolites to and from the cell (Ramachandra-Rao and Ravinshankar, 2002). Cell permeabilization depends on the formation of pores in one or more of the membrane systems of the plant cell, enabling the passage of various molecules into and out of the cell.

The permeability of the cells can be monitored by measuring the activity of enzymes of the primary metabolism, viz. hexokinase, glucose 6-phosphate dehydrogenase, isocitrate dehydrogenase, and malic and citrate synthetase (Ramachandra-Rao and Ravinshankar, 2002). A wide variety of permeabilizing agents are used to enhance the accessibility to provoke release of intracellular stored product into the culture medium. For example, in hairy root cultures of *Datura innoxia* Mill, the surfactant Tween 20 has been shown to be an effective permeabilizing agent on the release of tropane alkaloids into the culture medium (Boitel-Conti *et al.,* 1996). Also, organic solvents such as isopropanol, dimethylsulfoxide (DMSO), salts, and polysaccharides like chitosan have been used as permeabilizing agents (Di Iorio, 1991; Brodelius, 1988). Other permeabilization methods include ultrasonication, electroporation and ionophoretic release, in which the cells are subjected to a low current in a specially designed device (Ramachandra-Rao and Ravinshankar, 2002).

1.4.3.1.5. Metabolic engineering

The ability to alter the genomic DNA of hairy roots through use of engineered Ri plasmids has also been of tremendous use for the enhancement of plant metabolic pathways (Ramachandra-Rao and Ravishankar, 2002). *A. rhizogenes* mediated transformation has the advantage that any foreign gene of interest placed in a binary vector can be transferred to the transformed hairy root clone (Giri and Lakshmi, 2000). Some examples of genes of interest with regard to secondary metabolism that were introduced into hairy roots are the ornithine decarboxylase gene from yeast in *Nicotiana rubica* roots (Hamill *et al.*, 1990), and the tryptophan decarboxylase gene from

Catharanthus roseus introduced in *Peganum harmala* roots (Berlin *et al.*, 1993). These engineered hairy roots have been shown to produce increased amounts of the secondary metabolite nicotine and serotonin, respectively, when expressing the transgene.

In any design process to overproduce a given metabolite, however, a key obstacle for the metabolic engineer is assessment of the pathway steps that need to be altered (Shanks and Morgan, 1999). Attempts at increasing production by genetic engineering have met with mixed success since many metabolic pathways remain to be fully characterized (Broun and Somerville, 2001).

1.4.3.1.6. Artificial polyploidy

Artificial polyploidy is an alternative technique for increasing productivity of secondary metabolites in a variety of plant species (Dhawan and Lavania, 1996). Often the polyploid plants are bigger due to increased complement of the chromosomes (Gao *et al.,* 1996; Lavania, 1988), the phenomenon which may enhance the accumulation of commercially important bioactive compounds. This was the case when artificial autotetraploids were produced by colchicine treatment in the essential-oil-bearing vetiver grass (*Vetiveria zizanioides* L. Nash). Tetraploids were vigorous with thicker and longer roots, and the percent of essential oil increased from 0.98% to 1.4% in freshly harvested roots of tetraploid plants compared to the control (Lavania, 1988). Similarly, roots of a diploid medicinal plant, *Salvia miltiorrhiza,* contained higher amounts of tanshinones as a result of colchicine treatment due to the autotetraploid chromosome complement (Gao *et al.,* 1996). In section 1.6, a more detailed overview will be given about the effects of polyploidy in secondary metabolite production, which is especially relevant to this work.

1.4.4. Biotechnological opportunities with hairy root cultures.

Most of the studies using hairy roots have been concerned with the formation of secondary compounds of pharmaceutical value and during the past 18 years have provided a powerful tool for the elucidation of metabolic pathways, enzymatic steps, and regulation of secondary metabolism. However, the use of hairy roots is not limited to the production of secondary products. Hairy root systems have also been applied for the isolation of novel natural products with pharmaceutical activity (Asada *et al.*, 1998; Fuki *et al.*, 1998; Kwon *et al.*, 1997), the regeneration of transgenic plants exhibiting beneficial traits (Torregrosa and Bouquet, 1997), and more recently as a model system in the emerging area of phytoremediation (Pletsch *et al.*, 1999; Kas *et al.*, 1997). Additionally, hairy roots have also been implemented for the production of valuable compounds by biotransformation. An innovative approach using coculture of hairy roots of *Atropa belladona* with shooty teratomas of scopolamine-rich *Duboisia* plants showed that hyoscyamine released by the former was bioconverted to scopolamine by the latter tissues (Subroto *et al.*, 1996). However, these systems need to be studied carefully within the parameters of uptake mechanisms, biosynthetic potential of the tissue, and if possible, the extracellular release of the final product (Ramachandra-Rao and Ravishankar, 2002). Thus, hairy root technology will be useful in not only producing root-derived compounds but in producing novel compounds by biotransformation.

In addition to secondary metabolite production, hairy root cultures have been also engineered to express pharmaceutical proteins. For example, Wongsamuth and Doran (1997) reported production of monoclonal antibodies by hairy roots. They initiated hairy roots from transgenic tobacco plants expressing the full length IgG monoclonal antibody.

The total antibody titers observed in the root cultures were significantly higher than the yields of antibodies reported for other heterologous systems, including plant systems. However, most of the antibody remained associated with the root tissue rather than being secreted extracellularly.

The large-scale production of recombinant proteins in plants is limited by relatively low yields and difficulties in extraction and purification (Vivanco *et al.*, 2002). To address this problem, tobacco plants were engineered to continuously secrete recombinant proteins from their roots to hydroponic medium (Borisjuk *et al.*, 1999). The biological activity of the proteins secreted by this plant was retained and the accumulation levels reached higher amounts in the medium than in the root tissue. This approach could be extrapolated to hairy root culture for protein yield enhancement. Retransformation of hairy roots with *Agrobacterium tumefaciens* has been achieved and may open new opportunities for adding multiple genes in a stepwise fashion (Merritt *et al.*, 1999; Shanks and Morgan, 1999).

1.5. POLYPLOIDY AND SECONDARY METABOLITE PRODUCTION IN PLANTS

1.5.1. Introduction

The most widespread cellular process affecting plant evolution is polyploidy (Materson, 1994). Organisms with more than two sets of chromosomes are polyploids and result from aberrant chromosomal separation during cell division. Domesticated grains such as durum wheat (tetraploid), oats (hexaploid) and rye (hexaploid) are polyploids, as are cotton, tobacco and potato. Polyploids also include garden flowers such as chrysanthemums, pansies, and daylilies. Polyploidy is far rarer in animals than in plants, yet there are hundreds of examples of recent and ancient polyploidization events throughout the animal kingdom (Otto and Whitton, 2000). The rarity among animals may be related to their having distinct sex chromosomes. Polyploid sets of sex chromosomes in animals may fatally disrupt hormonal and sexual development (Griffiths *et al.*, 1996).

The evolutionary significance of polyploidy remains a mystery. To date, two diametrically opposing views exist, one assigning polyploidy a marginal role in evolution and the other granting it a primary creative role (Otto and Whitton, 2000). On the one hand, polyploids may represent a relatively frequent class of mutation, one that occasionally establishes within populations when its phenotypic effects are relatively mild. Simply put, polyploidy may be widespread because it arises repeatedly, without playing a significant role in evolution. Conversely, polyploidy may be common because polyploid species evolve faster or in more novel directions than related diploid species. Under this view, polyploidy promotes adaptive evolutionary change.

In the realm of polyploids, a distinction must be made between autopolyploids that are composed of multiple sets from within one species, and allopolyploids, that are composed of sets from different species. A brief explanation of these chromosomal conditions follows.

1.5.2. Autopolyploids

Autopolyploid plants usually form within a single species when chromosomes are duplicated but not separated. When spinder fibers are disrupted the cell may not divide cleanly; therefore the resulting cell has twice the original number of chromosomes. This non disjunction of chromosomes (Figure, 1.9) can arise naturally by the spontaneous accidental doubling of a *2x* (diploid) genome to a *4x* (tetraploid) genome, and also can be induced artificially through the use of colchicine (Gao *et al.*, 1996; Chen and Goeden-Kallmeyn, 1979), a drug derived from the autumn crocus (*Colchicum autumnale*). Colchicine disrupts mitotic spindle formation and prevents chromosome separation during mitosis.

Autopolyploids occur naturally in many genera, but sometimes plants have been induced to become autopolyploids to promote desired characteristics (Figure 1.10). For example, polyploids often have larger cells, thicker leaves, increased water retention, slower growth, delayed flowering, and extended seasonal flowering (Kondorosi *et al.*, 2000; Otto and Whitton, 2000; Brochmann, 1993; von Well, 1998).

Cold tolerance is also enhanced and polyploid plants frequently occur at higher altitudes and more polar latitudes than their diploid counterparts (Otto and Whitton, 2000). For these reasons, natural autopolyploids are most common in harsh environments where selective pressures are intense. Enhanced tolerance to harsh environmental factors

is often the competitive edge that allows a species to establish a permanent population. Increased heterozygosity provides metabolic flexibility to cope with a broader array of conditions (Levin, 1983).

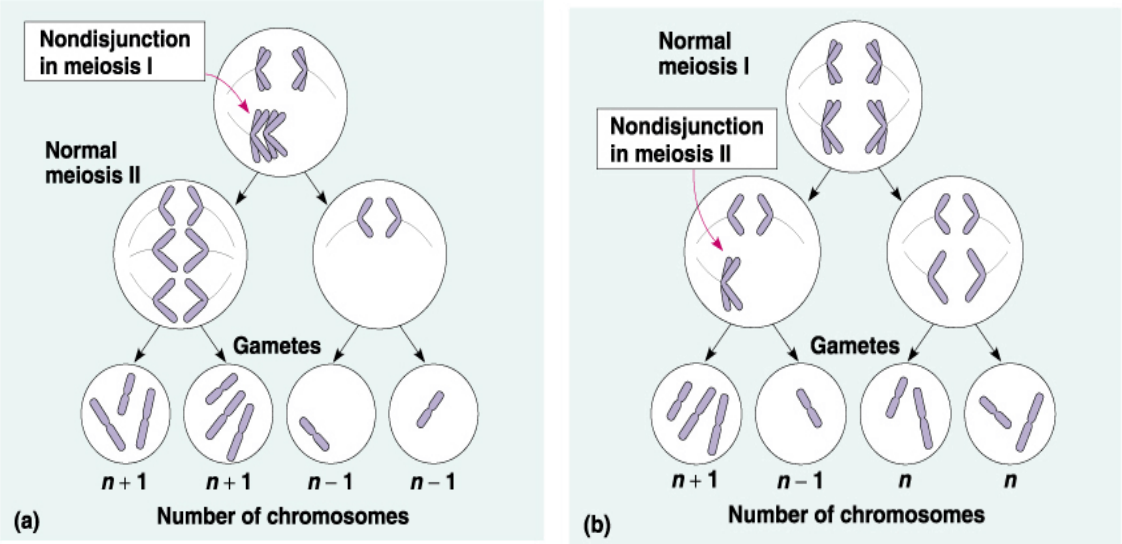


Figure 1.9. Meiotic nondisjunction leads to polyploidy. Homologues may fail to separate during anaphase of meiosis I, or chromatids may fail to separate during anaphase of meiosis II. Either type of meiotic error will produce gametes with an abnormal chromosome number. If chromosome numbers are multiplied within a species, it is autopolyploid. If multiple sets of chromosomes form by polyploidy of a hybrid or by fusion of gametes from different species, it is allopolyploidy (Adapted from Fosket, 1994).

1.5.3. Allopolyploids

Allopolyploids have two or more distinct sets of chromosomes typically derived by duplication and nondisjunction in hybrids already having mixed chromosome sets. The combination of polyploidy with hybridization has served as a natural prominent driving force in plant evolution to facilitate the establishment of populations with wider adaptability and superior growth potential compared to their parents (Dhawan and

Lavania, 1996). Such potential of allopolyploids has, therefore, been suitably exploited for introducing useful alien genes. Most plant hybrids are considered allopolyploids, including cultured hybrids that have been artificially selected for their large cells, plump plant parts, high water content, and drought resistance (Ramsey and Schemske, 1998).

Polyploids, especially allopolyploids, can restore sexual viability to an otherwise sterile hybrid. For example, the commercial tobacco plant *Nicotiana tobacum* arose as a result of a sexual cross between two different tobacco species, *N. tomentosiformis* X *N. sylvestris*, both of which have a haploid chromosome number of 12 (Fosket, 1994). This hybrid will contain one set of chromosomes from each parent, but it is sterile because these chromosomes are not homologous and cannot synapse. If, however the chromosomes are first doubled (artificially) so that the somatic cells contain two sets of chromosomes from each parent, then meiosis will be normal and the F1 hybrid will be fertile. In addition, the new fertile plant will be an allotetraploid. This also can be an intermediate mechanism of sympatric speciation in higher plants. Polyploidy restores homologues and fertility; consequently the resulting allopolyploid cannot readily reproduce with either parent and immediately becomes a new species.

Despite several inherent problems generally encountered in interspecific gene transfer, artificial allopolyploidy has played a significant role in restoring fertility, overcoming cross incompatibility by furnishing compatible ploidy status in the prospective parents serving as a genetic bridge, and regulating chromosome pairing to effect incorporation of specific chromosomes.

1.5.4. Polyploidy and chemical changes

Artificial polyploidy generally enhances the vigor of determinate plant parts and may be favorable when determinate plant organs and/or biomass constitute the economic product. (Dhawan and Lavania, 1996). Polyploidy is considered a method of increasing plant production potential. For example, it is known that genomic multiplication can confer enhanced production and or/qualitative improvement in the biochemical profile of secondary metabolites (Table 1.2).

The evidence derived from chemical analysis of induced allopolyploids indicates that such polyploids are richer in phenolic compounds and also express greater enzymatic diversity than either parent (Dhawan and Lavania, 1996). These polyploid hybrids often have distinct chemical advantages favoring natural selection, and are more likely the result of intergenomic complementation of the blockages/limiting factors attendant in the biosynthetic pathway of one parent by the other (Otto and Whitton, 2000; Dhawan and Lavania, 1996). The production of an esterase isozyme in an allooctoploid Triticale, not present in either parent, is an example of the chemical modifications obtained through allopolyploidy (Schwartz and Laughner, 1969).

Since autopolyploids arise as a consequence to direct genomic multiplication, the basic genetic material remains the same with multiplied gene dosage (Levin, 1983). Therefore, an enhanced production of metabolites is expected in the autopolyploids (Dhawan and Lavania, 1996). Some examples are given in Table 1.2. Interestingly, an altered chemical profile of the metabolic products is frequently encountered. Chromosome doubling is accompanied by conspicuous changes in the secondary metabolism of plants as well as the primary metabolism (Levin, 1983).

Table 1.2. Some examples of the influence of polyploidization on the concentration of useful secondary metabolites (Adapted from Dhawan and Lavania, 1996).

Plant Species	Ploidy Level	Influence of the polyploid over the diploid
Atropa belladonna	4x	68% increase in the yield of tropane alkaloids
Acorus calamus	4x	300% increase in volatile oil content
Camellia sinensis	4x	Increase in the concentration of polyphenols, catechins, extractines and caffeine in two leaf shoots
Capsicum species	4x	Significant increase in ascorbic acid concentration
Cichona succiruba	4x	100% increase in quinine concentration
Cympogon flexuosus	4x	Significant increase in essential oil content
Datura innoxia	4x	Decrease in hyoscine and atropine content
Hyocyamus niger	4x	35% increase in tropane alkaloids
Mentha spicata	4x	Decrease in essential oil concentration
Papaver somniferum	4x	Significant increase in morphine concentration
Solanum khasianum	4x	30-50% increase in solasodine content

Autopolyploids in many drug plants including species such as *Atropa*, *Camellia*, *Hyocyamus* and *Solanum*, have sharply increased quantities of useful secondary metabolites per unit dry weight whereas species such as *Datura* and *Mentha* have a decreased production of these compounds (Dhawan and Lavania, 1996). Chromosome doubling also may alter the secondary chemistry of a plant in a qualitative manner. For example, qualitative differences were found in glycoflavone profiles of 14 of 15 synthetic autotetraploid populations as compared to their diploid prototypes, including 14 instances of flavonoids present in the tetraploids but not the diploid, and eight instances of flavonoids present in the diploid but not in the tetraploids (Levin, 1983).

The changes in a metabolite profile accruing in the autopolyploids on mere multiplication of the basic genome have been interpreted as a disturbance of metabolic mechanisms that regulate the biosynthesis of individual compounds (Dhawan and

Lavania, 1996). The loss of diploid compounds in the corresponding autopolyploids is explained as functional repression of existing structural genes and the gain in the autopolyploids is explained as functional derepression of previously silent structural genes (Leitch and Bennett, 1997). The multiplicity of the allelic combinations that is possible in the polyploids offers the advantage of producing hybrid enzymes and enzymatic diversity thus providing greater versatility and homeostasis (Dhawan and Lavania, 1996).

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

In many plant species, chromosome doubling is accompanied by conspicuous changes in the secondary metabolism of plants as well as the primary metabolism (Levin, 1983). Some of these changes include an overall increase in enzymatic activity, isozyme diversity, and alteration in plant phytochemicals etc., which may lead to enhanced production and certain qualitative changes in the biosynthesis of secondary metabolites (Dhawan and Lavania, 1996; Levin, 1983). Such inherent potential could be exploited by resorting to experimental manipulations to achieve commercial gains with respect to the production of useful secondary metabolites.

Wallaart's (1999c) creation of *A. annua* tetraploids plants improved the production of secondary metabolites although not biomass accumulation, therefore, offsetting the total yields of these compounds. Based on these findings a working hypothesis is that the same approach may be used for the production of these biochemicals in fast growing *A. annua* hairy roots, provided that biomass accumulation is not affected. For years, the fast growth and stable production capacity of hairy roots has been exploited to harness the production of many important bioactive compounds. This production efficiency offers additional advantages that may accrue at a polyploid level.

The purpose of this study is to achieve artificial polyploidy in *A. annua* hairy roots with the aim of increasing the yield of artemisinin. The main objectives of this project are:

- To assess adequate colchicine concentration and exposure times for tetraploidy induction of *A. annua* hairy roots.
- To verify that tetraploid *A. annua* hairy roots have been obtained.
- To measure the yield of artemisinin in tetraploid *A. annua* hairy roots.
- To measure root biomass in tetraploid *A. annua* hairy roots.
- To compare growth rate, biomass production, and artemisinin yields of tetraploid *A. annua* hairy roots with the diploid parent.

CHAPTER 3

TETRAPLOID ARTEMISIA ANNUA HAIRY ROOTS PRODUCE MORE ARTEMISININ THAN DIPLOIDS[1]

3.0 Summary

Hairy root cultures of diploid *Artemisia annua* L. (clone YUT16) grow rapidly and produce the antimalarial sesquiterpene artemisinin. Polyploidy has been shown to increase yields of secondary metabolites in whole plants. However, little is known about how polyploidy may affect the growth of transformed hairy roots and the production of secondary metabolites. Using colchicine, we have produced at least 4 stable tetraploid clones of *Artemisia annua* L. from the YUT16 hairy root clone. Analysis of these tetraploid clones showed major differences in growth and artemisinin production compared to the diploid clone. All of the tetraploid clones produced as much as 3 to 6 times more artemisinin than the diploid parent. This study provides an initial step in increasing our understanding of the role of polyploidy in secondary metabolite production especially in hairy roots.

3.1 Introduction

In most plant species, artificial polyploidy has increased the size of cells leading to larger reproductive and vegetative plant organs (Adiniya and Shira, 2001; Watrous and Wimber, 1988; Chen and Goeden-Kallemeyn, 1979). Vegetative plant organs such as

[1] The content of this chapter has been published in Plant Cell Reports (2003) 21: 809-813.

roots are the source of many commercially important secondary metabolites (Pal Bais *et al.*, 2001). Widely known compounds such as the ginkgolides of *Ginkgo biloba* and forskolin from the Indian herb *Coleus forskohlii,* both traditionally used to treat heart and respiratory diseases, are just some of many root-specific secondary metabolites that have medicinal applications. Tropane alkaloids, terpenoids and isoflavonoids are among the most important medicinal compounds found in plant roots. Thus, induction of artificial polyploidy may prove useful in increasing the production of these important medicinal compounds (Dhawan and Lavania, 1996).

Indeed, artificial polyploidy has been shown to increase the production of secondary metabolites in many plants compared to their diploid parent. For instance, it has been reported that tetraploids *Chamomilla recutita*, Petunia "Mitchell" and *Salvia miltiorrhiza Bge* produce more flavonoids and terpenoids per gram of tissue than their diploid counterparts (Švehlíková and Repčák, 2000; Griesbach and Kamo, 1996; Gao *et al.*, 1996). Although the effects of polyploidy are not generally predictable, and each species must be examined individually, doubling the chromosome number of a plant species that produces useful compounds can enhance overall secondary metabolism.

A. annua L. produces, along with many other terpenoids, the sesquiterpene antimalarial drug artemisinin (Meshnick *et al.*, 1991). Our laboratory group and others have also shown that transformation of *A. annua* plants with *Agrobacterium rhizogenes* results in formation of transformed (hairy) roots that produce artemisinin (Weathers *et al.*, 1994; Jaziri *et al.*, 1995). Thus, *A. annua* offers a useful model system for studying the role of artificial polyploidy on terpenoid production.

Previously, Wallaart *et al.* (1999c), reported induced polyploidy of *A. annua* plants (i.e., from 18 to 36 chromosomes) by using the mitotic inhibitor colchicine. In their study, they reported a polyploidy induction efficiency of 20% and an average artemisinin level in the resulting tetraploids that was 38% higher than the diploid plants. However, biomass accumulation of the tetraploid plants was lower than the diploid plants, so the net yield of artemisinin per m^2 of field-grown plants decreased by 25% (Wallaart *et al.*, 1999c). Even though their results were disappointing in whole plants, they suggested the possibility of using hairy root cultures that are then optimized for growth and artemisinin production. Here we report the growth and artemisinin productions of tetraploid *A. annua* hairy roots, and compare the results with the diploid parent.

3.2 Experimental Procedures

3.2.1. Plant material

The transformed root culture used in this study was the *A. annua* YUT16 clone described by Weathers *et al.*, (1994). Cultures were maintained by subculturing 0.5 g fresh weight of roots every two weeks into a 125 mL Erlenmeyer flask containing 50 mL of Gamborg's B5 medium (pH 5.7) (Gamborg *et al.*, 1968) supplemented with 3% sucrose and grown at 25º C, at 100 rpm under cool white fluorescent light (5 $\mu E\ m^{-2}\ s^{-1}$).

3.2.2. Induction of polyploidy in *Artemisia annua* hairy roots

Filtered sterilized (0.2 μm) colchicine (Sigma-Aldrich, St. Louis, MO) at concentrations of 0, 0.5, 0.25, 0.1, or 0.05% (w/v) in water, was added to filter sterilized

culture medium (Gamborg's B5 + 3% sucrose, pH 5.7). To induce polyploidy, single 2 cm root tips from 14-day-old liquid cultures, grown as described above, were placed in six-well plates containing 5 mL per well of colchicine-containing media and incubated at room temperature for 1-7 days. After colchicine treatment, the root tips were rinsed in fresh B5 medium, transferred to fresh B5 semi-solid medium (0.2 % w/v GelRite, Sigma-Aldrich, St. Louis, MO) and incubated at 25°C in the dark for 30 days. For determination of relative survival, roots were removed from colchicine medium every other day for 7 days. Subsequent root lines were initiated from any lateral root tip growing from the original colchicine-treated roots. These new root lines were examined on a monthly basis for ploidy level stability. The diploid *A. annua* YUT-16 clone was grown the same way as the colchicine-treated root tips, but without colchicine.

3.2.3. Preparation of chromosomes for ploidy level determination

Before inducing polyploidy, the YUT16 clone of *A. annua* was cytologically examined to confirm its diploid number of chromosomes (2n=18). Samples of single hairy roots from all cultures were pretreated in a saturated α-bromonaphthalene solution for 24 h at 4°C to accumulate cells in metaphase. A few roots were then fixed overnight in cold Carnoy's solution (3:1, 100% ethanol: glacial acetic acid). After rinsing twice with deionized water, root tips were then hydrolyzed with 1N HCl at 60°C for 8 minutes and then rinsed in deionized water. Excess water was removed by blotting, and the roots were stained for 2 h in Feulgen solution (Singh, 1993) at room temperature. Stained root tip meristems were removed and placed on a clean slide, and squashed in 1% (w/v) aceto-carmine (Singh, 1993). The preparations were observed with an optical microscope at a magnification of 1,000x. The best metaphase views were observed with a Nikon Coolpix

digital camera (Nikon, Japan). For each clone at least twenty meristems were analyzed. Each putative tetraploid clone was validated as polyploid at least 20 times over 40 subcultures.

3.2.4. Biomass analysis

Fresh weight was measured after roots were carefully washed with deionized water and blotted dry. Dry weight was measured after roots were oven-dried at 60°C for at least 24 h or until weight no longer fluctuated.

3.2.5. Extraction and analysis of artemisinin

Artemisinin was extracted and assayed at 260 nm using HPLC according to the method described by Smith *et al.*, (1997) with the following modifications. Two gram of roots (blotted FW) was extracted twice with 3 mL of scintanalyzed toluene in an ultrasonic bath for 30 min in ice-cold water. The extracts were centrifuged at 4390 x g for 10 min and the supernatants were decanted, pooled, dried under nitrogen and stored at – 20ºC for later analysis by HPLC.

For HPLC analysis the artemisinin samples were first converted to their Q260 derivatives (Smith *et al.*, 1997), and then applied to a 15 cm Microsorb-MV C-18 column with a 4.6 mm i.d., containing 5 μm silica beads with 10 nm pore size (Varian, Walnut Creek, CA). The mobile phase was 0.01M sodium phosphate buffer: methanol, 55:45 (v/v) pH 7.0, at 1.0 mL/min. A linear calibration curve of artemisinin (Sigma-Aldrich, St. Louis, MO) was measured in the 0.1-0.5 μg/mL range. The retention time of artemisinin

under these conditions was about 12.0 min. Each sample was co-injected a second time with a known amount of authentic artemisinin to validate peak identification.

3.2.6. Single-root growth studies

Single hairy roots, 2 cm in length, from the established diploid (YUT16) and the tetraploid clones (Figure 3.1) were inoculated into six-well polystyrene plates (i.e. one root per well) and grown as described above for cultures in shake flasks (Kim et el., 2002; Srinivasan *et al.*, 1997). Root lengths, number of laterals and general appearance of roots were recorded. To compare results from different tetraploid clones directly, the fractional increase in length of each root was calculated by scaling the total length of the primary root by the number of root tips (Wyslouzil *et al.*, 2000; Yu and Doran, 1994). This is defined as the root growth unit (RGU):

(RGU) = (Σ length of all laterals + length of primary root) (Number of root tips)$^{-1}$

For root diameter measurements, single roots from 14 day-old shake flask cultures were mounted on microscope slides and their diameter was measured 1 cm from the root tip using an optical microscope with a 40x objective plus a micrometer in a 10x ocular; 10 measurements per clone were obtained.

3.2.7. Statistical analysis

The data from all experiments were analyzed using standard statistical methods of analysis including analysis of variance (ANOVA). All experiments had at least 3-6 replicates and each experiment was conducted at least twice.

3.3. Results and Discussion

3.3.1. Confirmation and stability of polyploidy

Cytological examination of root tips of the YUT16 parent clone was verified as being diploid with 18 chromosomes (Figure 1A) as previously determined for *A. annua* by others (Wallaart *et al.*, 1999c; Bennet, 1982). During colchicine treatment about 25% of the root tips died regardless of the concentration of colchicine used. The surviving colchicine-treated roots developed new lateral root tips that were used for the creation of subsequent root lines. In total, more than 40 root lines were generated by this method. These roots were prepared for microscopic examination of chromosomes and at least 20 well-defined metaphase displays per root tip were counted (Figure 3.1). Colchicine concentrations of 0.25 % or 0.5 % (w/v) for 7 days were the most effective in producing these tetraploid hairy root clones. The efficiency of polyploidy induction by using colchicine in this method was low; of 40 root lines screened, only 4 were determined to be stable tetraploids with n=36: YUT16-9Lb, YUT16-8LT, YUT16-6P and YUT16-7P (Figure 1, B-E).

The tetraploid clones and the diploid parent were cytologically examined for 18 months to validate ploidy stability (Figure 1, A). Although no obvious morphological differences were observed between the diploid and the tetraploid clones (Figures 1, G-J), one clone (not shown), YUT16-7LT, produced very thick curled roots and required weekly sub-culturing, compared to the semi-weekly culturing required by the other clones. The poor growth of this unusual clone made it difficult to study; consequently chromosome analyses were not done (data not shown).

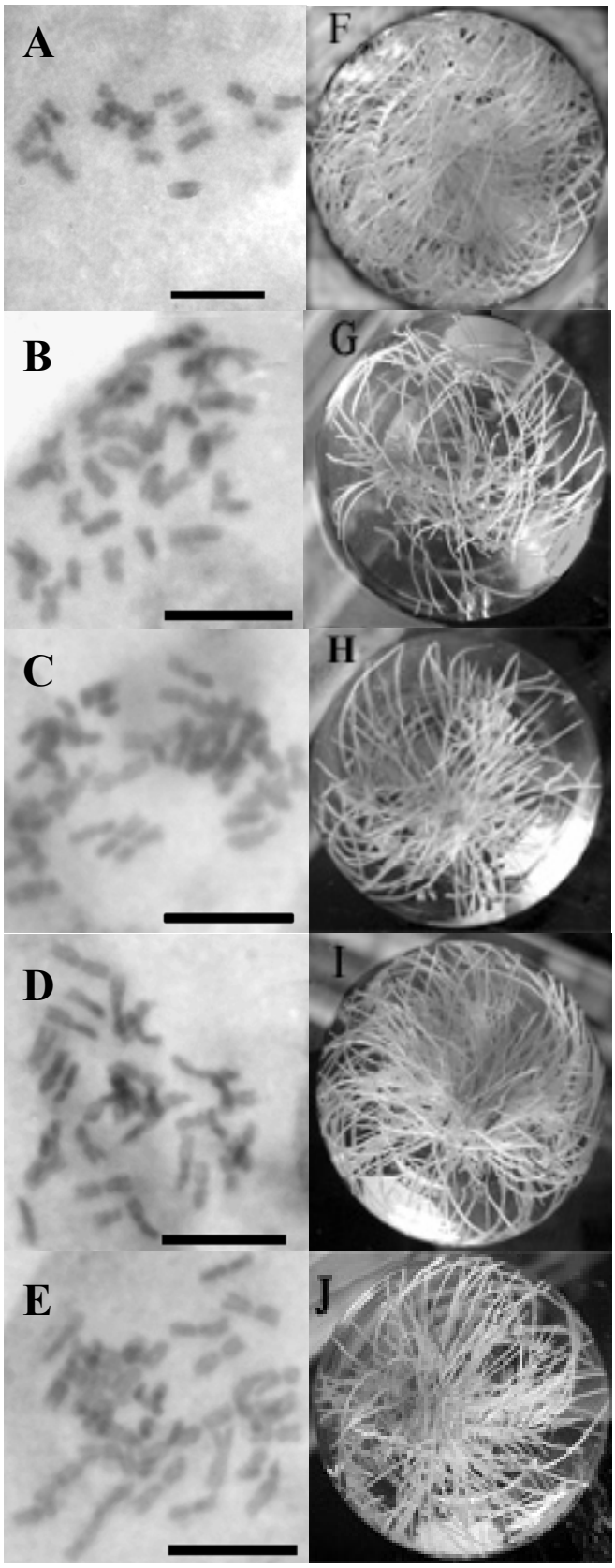


Figure 3.1. Root morphology (seen from the bottom of a 125 mL shake flask) and chromosome number of diploid and tetraploid clones of *Artemisia annua*. (**A**) Cells from YUT16 showing normal diploid (2n=18) chromosomes. (**B-E**) Cells from clones YUT16-7P, YUT16-8LT, YUT16-6P and YUT16-9Lb, respectively. (**F**) Root morphology of diploid YUT16. (**G-J**) Root morphology of tetraploid clones YUT16-7P, YUT16-8LT, YUT16-6P and YUT16-9Lb, respectively. Bar = 10 μm

3.3.2. Growth of diploid and tetraploid clones

To compare the growth of the tetraploid clones with their diploid parent, roots were grown in flasks or singly in 6-well plates. Initially we used inoculum from late log cultures (14 d) to compare biomass yields in flasks. Table 3.1 shows that all tetraploid clones produced significantly less biomass (i.e. FW, DW and %DW) than the diploid YUT16 when the cultures were initiated with a 14 day inoculum. A common result of polyploidy in plants is a reduced number of cell divisions during growth and development (Otto and Whitton, 2000; Adaniya and Shirai, 2001), so overall reduced growth is not surprising. However, previous studies with hairy root cultures have shown that the age of the inoculum culture may have an effect on root growth as well as on secondary metabolite production (Weathers *et al.*, 1996, Weathers *et al.*, 1997, Takahashi *et al.*, 2001). Since increasing growth rate is just as important in achieving high productivity as product yield, we therefore decided to use an older inoculum (20 d) to see if there was an effect on biomass accumulation. When a 20 day inoculum was used, the diploid YUT16 still grew better than the tetraploids (Table 3.1). Thus, inoculum age does not appear to alter the growth yields of the tetraploids. Even though the tetraploid clones all accumulate less biomass than the diploid, one clone, YUT16-6P, consistently grew better than the other 3 tetraploids. Indeed, the growth yield of this clone was only 12-20% less than the diploid YUT16 (Table 3.1).

Although the total biomass and metabolite production of a particular root clone is routinely determined in shake flask experiments, measuring growth of individual roots is difficult. A better method for understanding the root growth behavior in a liquid environment is to follow elongation and branching of individual roots (Wyslouzil *et al.*,

Table 3.1. Comparative biomass accumulation and artemisinin production of diploid and tetraploid clones of *A. annua* from cultures initiated with a 14- or a 20-day-old inoculum.

Clone	*Biomass (g FW)*	*Biomass (g DW)*	*% Dry Weight*	*Biomass Ratio*	*[AN] (µg/g DW)*	*[AN] Ratio*	*Total [AN] (µg/L)*	*Total [AN] Ratio*
14 Day-Old Inoculum								
YUT16	3.1a	0.3a	9.4a	1.0	0.4a	1.0	2.0a	1.0
YUT16-9Lb	2.0b	0.2b	8.0b	0.7	0.9b	2.1	2.9b	1.4
YUT16-8LT	2.1b	0.2b	7.6b,c	0.7	2.1b,c	4.8	6.3b,c	3.1
YUT16-6P	2.3b,c	0.2b,c	8.3b	0.8	1.0b	2.3	3.3b	1.6
YUT16-7P	1.6b,c	0.1b,c	7.5b,c	0.5	1.2b	2.8	2.8b	1.4
20 Day-Old Inoculum								
YUT16	2.9a	0.3a	9.3a	1.0	0.4a	1.0	2.5a	1.0
YUT16-9Lb	2.2b,c	0.2b,c	8.2b	0.8	0.6a	1.2	1.5b	0.6
YUT16-8LT	1.9b,c	0.1b,c	7.4b,c	0.7	0.4a	0.7	1.1b	0.4
YUT16-6P	2.5b,c	0.2b,c	8.0b	0.9	0.3a	0.7	1.2b	0.5
YUT16-7P	1.5b,c	0.1b,c	8.0b	0.5	3.2b,c	6.0	7.4b,c	3.0

Cultures were harvested after 14 days growth in B5 medium + 3% sucrose. Biomass and total [AN] ratios are in comparison to the diploid YUT16 culture from each group. Each data value represents an average of two experiments with three replicates each. AN, artemisinin. Letters after data indicate statistical differences at a 95% level after ANOVA and for $P<0.05$; b, statistically different when compared to the diploid clone; c, statistically different when compared only to the other tetraploids.

2000; Kim *et al.*, 2002). This allows a more detailed understanding of root growth kinetics (Yu and Doran, 1994), and variation in morphology may be related to root characteristics such as growth rate and secondary metabolite production level among other factors (Bhadra *et al.*, 1993). The use of six-well plates has proved useful for such a study (Kim *et al.*, 2002; Srinivasan *et al.*, 1997). Each six-well plate provides six replicate experiments with only minimal use of medium and space and can provide a considerable amount of information (Table 3.2).

Our comparative single-root growth study showed that all the tetraploid clones had considerably larger root diameter compared to the diploid YUT-16 (Table 3.2). This was not a surprise since larger plant organs are often the result of polyploidy (Otto and Whitton, 2000). Interestingly, all the tetraploid clones showed lower growth values for most of the other characteristics measured when compared to the diploid YUT16 (Table 3.2). However, when compared to the other tetraploids, clone YUT16-9Lb produced the highest number of lateral roots and lateral roots per cm (i.e. lateral density). Likewise, clone YUT16-6P had the greatest total lateral length and clone YUT16-8LT yielded the greatest total root length (Table 3.2).

When root growth unit (RGU) values were compared, a major difference was observed between the diploid parent and the tetraploids. Three tetraploid clones, YUT16-9Lb, YUT16-8LT and YUT16-7P showed final RGU values higher than the diploid YUT16. Clone YUT16-6P was the only clone with a final RGU value equivalent to that of the diploid clone. The RGU unit is a morphological parameter indicating the branching activity of the root system. YUT16-6P grew with a mean length per root tip of about 1 cm, which is a typical value for roots grown in shake flasks (Wyslouzil *et al.*, 2000)

Table 3.2. Comparative growth of *A. annua* diploid and tetraploid single roots after 14 days growth in six-well plates. Roots were grown in B5 medium + 3% sucrose. Each data value represents an average of six replicates.

Root characteristic	YUT16	YUT16-9Lb	YUT16-6P	YUT16-8LT	YUT16-7P
Number of laterals per root	10.1a	6.8a,c	6.4a	4.8b	2.8b
Lateral density (# laterals/cm)	2.5a	1.9a,c	1.6b	1.2b	1.0b
Length of primary root (cm)	4.0a	4.1a	4.3a	4.8a,c	3.7a
Length of laterals (cm)	1.5a	1.0b	1.2a	1.3a	1.1a
Total length (laterals + primary) (cm)	11.6a	6.7b	7.0b	10.0a,c	5.5b
Total lateral length (cm)	14.6a	6.9b	8.3b,c	5.8b	3.4b
Root growth unit (cm per root tip)	1.1a	1.8a	1.0a	2.2b,c	2.0b
Root diameter (mm)	3.4a	4.4b	4.0b	4.2b	4.0b

Lateral density equals the number of laterals divided by the length of the primary root. Letters after data indicate statistical differences at a 95% level after ANOVA and for $P<0.05$; b, statistically different when compared to the diploid clone; c, statistically different when compared only to the other tetraploids. Likewise, clone YUT16-6P had the greatest total lateral length and clone YUT16-8LT yielded the greatest total root length (Table 3.2).

suggesting that it grows nearly as fast as the diploid YUT16 (Table 3.2). This is consistent with the biomass data (Table 3.1) that showed that YUT16-6P had the highest biomass accumulation of the tetraploid tested.

3.3.3. Artemisinin production of diploid and tetraploid clones

When the concentration of artemisinin was analyzed in the clones (Table 3.1), it was found that all tetraploid cultures initiated with a 14-day-old inoculum produced more artemisinin per gram of fresh weight than the diploid YUT16 even though they produced less biomass. Clone YUT16-8LT, in particular, produced nearly 5 times the artemisinin per gram of dry weight than the diploid YUT16 and was about twice as productive as the other tetraploid clones similarly inoculated. The overall productivity per culture for YUT16-8LT was about three times that of the diploid clone (Table 3.1). Similar increases in artemisinin levels were also reported by Wallaart *et al.* (1999c) in tetraploid whole *A. annua* plants. Unfortunately, in their case, the overall yield of artemisinin per tetraploid plant was effectively the same as the diploid because tetraploid plants were proportionately smaller.

Surprisingly, the artemisinin production profile was quite different with the tetraploid cultures started from a 20-day-old inoculum. In this case a different tetraploid clone, YUT16-7P, was the highest artemisinin producer when compared to results from experiment using a 14-day-old inoculum. Indeed YUT16-7P produced six times the artemisinin per gram of dry weight yielded by the diploid (Table1). Overall, the YUT16-7P tetraploid was about four times as productive as the diploid (Table 1). These results

suggest a possible effect of inoculum age on secondary metabolite production in two of the tetraploid clones, YUT16-7P and YUT16-8LT.

Unfortunately, the increased yields of the tetraploid clones have not reached commercially useful quantities of artemisinin (Table1). Since YUT16 was first isolated in the early 1990s we have noticed a steady decline in overall productivity (Kim *et al.*, 2001). It is not clear what selective pressure would be needed to recover the original high production (>250 μg/g DW) of the diploid YUT16 clone (Weathers *et al.*, 1994).

3.4. Conclusions

This is, to our knowledge, the first report of the formation of tetraploid hairy roots. Similar to polyploid whole plants, polyploid hairy roots produced significantly more artemisinin than their diploid counterparts. Although the artemisinin yields obtained from these tetraploid clones were not as high as previously reported from their parent clone when it was isolated 10 years ago (Weathers *et al.*, 1994), some of them produced more artemisinin than the diploid. These results show that although careful screening is still required of polyploids induced from an elite clone, there appears to be advantages in selecting for high yielding polyploids artificially produced from hairy root cultures.

CHAPTER 4

GROWTH AND DEVELOPMENT OF SINGLE TETRAPLOID HAIRY ROOTS OF *ARTEMISIA ANNUA* IN LIQUID CULTURE[1]

4.0. Introduction

Hairy roots are a potential means for *in vitro* manufacture of plant secondary metabolites and foreign proteins (Hamill and Lidgett, 1997). The complex fibrous structure of the roots, however, makes analyzing their growth and development a challenge (Wyslouzil *et al*., 2000). Compared to microbial cultures that contain mostly identical cells that pass repeatedly through identical cell division cycles, roots contain a wide range of different types of cells. From a purely kinetic standpoint, roots contain two kinds of cells: meristematic cells that are actively dividing, and the remaining cells that are not dividing (Hjortso, 1997). The non-dividing cells arise from tip cells, but they elongate until they are at least an order of magnitude larger than their original size and differentiate into the various tissues of the root (Wyslouzil *et al*., 2000; Lloret and Casero, 2002). Just behind the zone of elongation, lateral meristematic cells divide to form new tips that result in the formation of lateral branches as cells within them start the terminal differentiation process. It is this branching process whereby new tips and lateral branches are formed that determines the overall growth rate of the root biomass (Hjortso, 1997; Lloret and Casero, 2002). From the standpoint of metabolite production, different hairy root morphologies can result in dissimilar levels of secondary metabolite production (Berzin et al., 1997). Moreover, evidence suggests that root tips may produce more secondary products than the mature root, and lateral roots may produce more

[1] This chapter in preparation for publication

products than primary roots (Wobbe *et al*., 1997; Woo *et al*., 1997). Furthermore, the resulting morphology of roots is quite plastic as they respond to changes in their local environment (Wyslouzil *et al*., 2000). Changes in morphology may, thus, influence the ability to scale-up root cultures along with their secondary metabolite production (Carvalho *et al*., 1997).

Tetraploid hairy root cultures of *A. annua* that produce the sesquiterpene artemisinin have been established (De Jesus-Gonzalez and Weathers, 2003). Although some of the tetraploid *A. annua* cultures produced more artemisinin than the diploid parent, their growth was less. Visual observation of the tetraploids showed a degree of root branching quite different from the diploids. While total biomass and metabolite production for a particular clone can be determined in shake flasks, detailed measurements of individual root growth and development are difficult. To better understand the development and growth of these tetraploid clones in a liquid environment, single-root growth studies were carried out to follow their elongation and branching through a culture period. The main objective here was to determine how the growth kinetics of *A. annua* hairy roots was affected by polyploidy.

4.1 Experimental Procedures

4.1.1. Plant Material

The diploid hairy root clone YUT16 originated from a Yugoslavian strain of *A. annua* L. (Weathers *et al*., 1994) and methods used to establish tetraploid hairy root clones YUT16-9Lb, YUT16-8LT, YUT16-6P, and YUT16-7P are described in detail in De Jesus-Gonzalez and Weathers (2003). Details about subculturing and maintenance

procedures of these clones can be found in Chapter 3. Roots grown in filtered sterilized B5 medium in shake flasks for 14 days were used as inoculum for the root growth experiments.

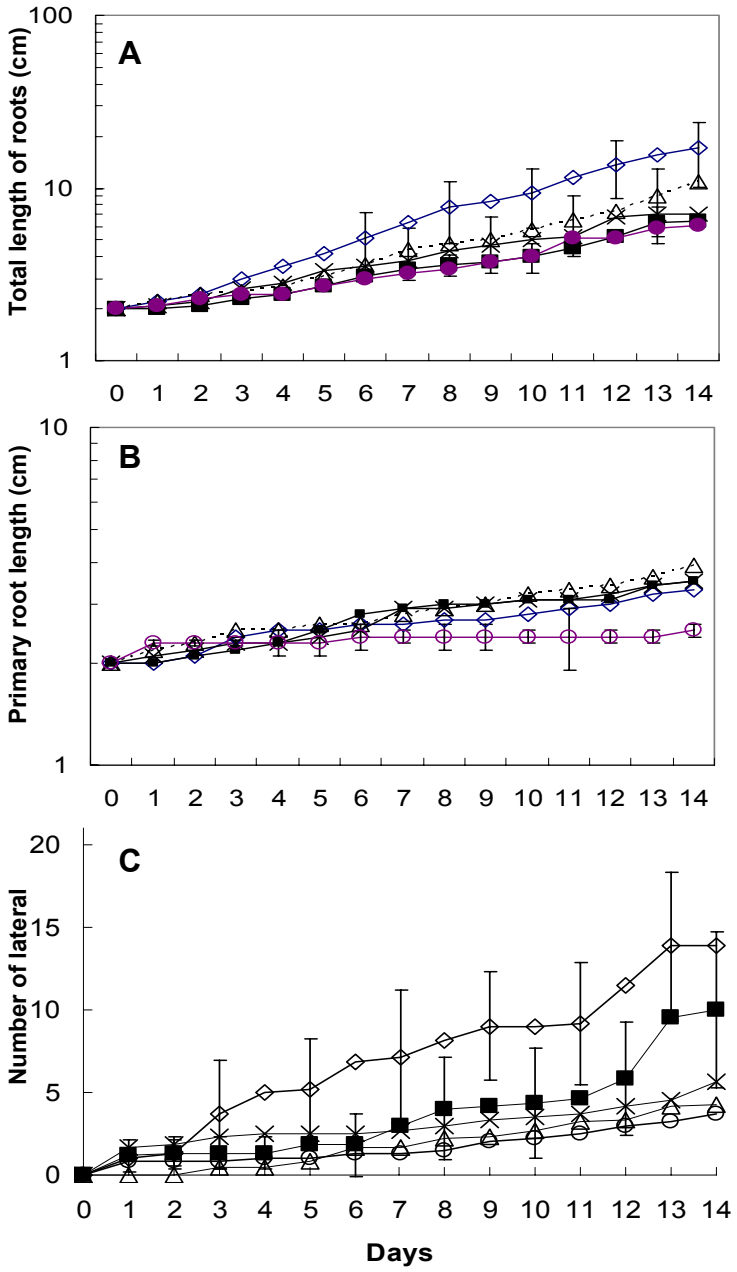
4.1.2. Single-Root Growth Kinetics

Single hairy roots, 2 cm in length, from the established diploid YUT16 and the tetraploid clones YUT16-9Lb, YUT16-8LT, YUT16-6P, and YUT16-7P, were each inoculated into six-well polystyrene plates (i.e. one root per well) and grown as described before (Chapter 3). The length of each hairy root, and the number and length of laterals were measured every 24 h for 14 days. Data for hairy root growth and development were analyzed at the end of the experiment by averaging each day's measurements. Six replicate experiments per clone were conducted. The growth kinetic data gathered from the hairy roots grown in six well plates also included the change in lateral branching with time, and is defined as the Root Growth Unit (Chapter 3).

4.2. RESULTS AND DISCUSSION

4.2.1. Total and Primary Root Growth

When increases in total root length for single diploid and tetraploid *A. annua* hairy roots were measured, all clones exhibited exponential growth with short lag phases (~ 2 d), although each clone grew at slightly different rates (Figure 4.1A). All the tetraploid clones had specific growth rates lower than their diploid parent, except for primary root length (Table 4.1). This is not surprising since polyploids usually have lower growth rates than their related diploids (Levin, 1983). However, of all the



Figures 4.1 A, B & C: The sum of root lengths, primary root lengths, and the number of lateral roots for diploid and tetraploid single hairy roots grown in six-well plates. (A) Sum of primary and all lateral lengths of roots. (B) The length of the primary root. (C) The number of lateral roots. Legend: (◊) YUT16; (■) YUT16-9Lb; (∆) YUT16-8LT; (x) YUT16-6P; (○) YUT16-7P. Data are the average of 6 roots; means ± 1 SD are shown for critical divergence points.

tetraploids, the specific growth rate of the clone YUT16-8LT was consistently higher by about 20-40 %. Indeed, the elongation rate of the primary root of this clone was consistently the highest and about 50 % greater than the diploid (Table 4.1).

Table 4.1. Average specific growth rates of the diploid and tetraploid *A. annua* hairy roots.

Clone	Specific Growth Rate			
ploid	Total Root (cm/day)	Primary Root (cm/day)	Lateral Root (cm/day)	# Lateral (#/day)
YUT16	1.30 ±0.3 a	0.10 ±0.1a	0.072 ±0.03 a	1.40 ±0.6 a
Tetraploid				
YUT16-9Lb	0.32 ±0.04 b,c	0.13 ±0.05 a	0.059 ±0.07 b	1.10 ±0.6 a, c
YUT16-8LT	0.84 ±0.6 a, c	0.15 ±0.1 a	0.067 ±0.03 b	0.40 ±0.2 b
YUT16-6P	0.75 ±0.6 b, c	0.11 ±0.1 a	0.062 ±0.05 b	0.88 ±0.5 b, c
YUT16-7P	0.59 ±0.6 b, c	0.08 ±0.03 a	0.037 ±0.07 b, c	0.34 ±0.2 b

Letters after data indicate statistical differences at a 95% level after ANOVA and for $P<0.05$; b, statistically different when compared to the diploid clone; c, statistically different when compared only to the other tetraploids.

Root systems grow by a simple branching process, with laterals emerging from main (primary) roots some distance behind the tip. The root system consists of a population of meristems that varies greatly in duration and activity (Fitter, 2002). A better understanding of the growth behavior of a root system can, thus, be obtained by analyzing the growth of the primary root and the production of its laterals (Figures 4.1B and C). This can change considerably along the total root length.

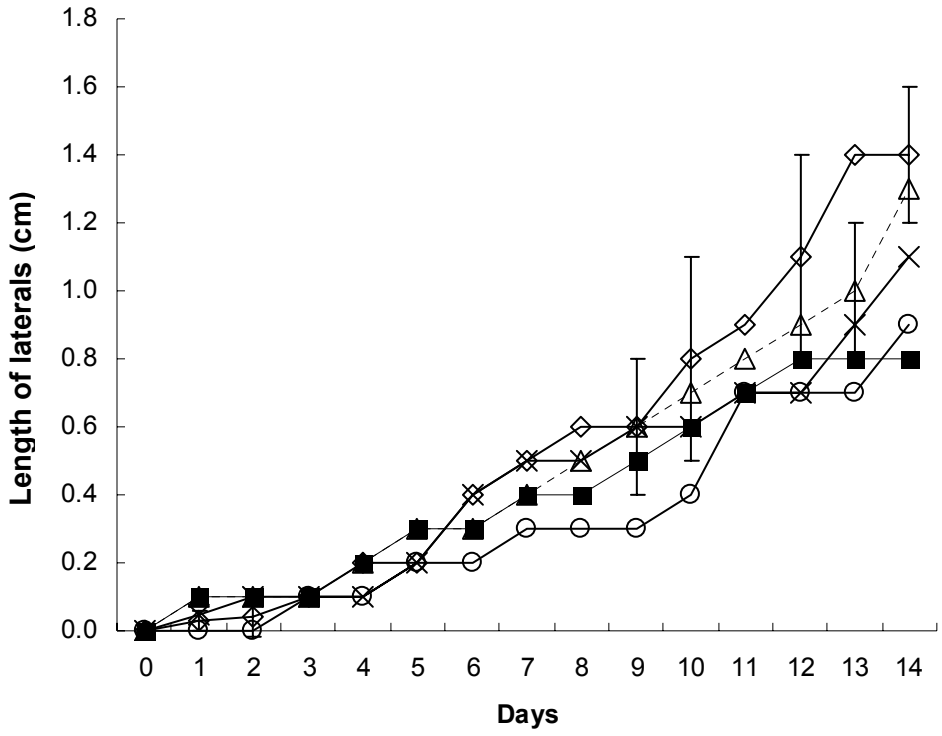
In a comparison of the growth of the primary roots of diploid and tetraploid clones, three of the tetraploids grew faster than the diploid with decreasing order of growth as follows: YUT16-8LT > YUT16-6P > YUT16-9Lb > YUT16 > YUT16-7P (Table 4.1). Exponential growth of the primary root of YUT16-8LT and YUT16-6P began immediately after subculture, whereas both the diploid YUT16 and tetraploid

YUT16-9Lb had a short (~1d) lag phase (Figure 4.1B). Interestingly, clone YUT16-7P showed very low growth of its primary root compared to the other clones; it took 14 days for this clone to increase its primary root length by 0.5 cm (Figure 4.1B).

4.2.2. Lateral Root Growth

The production of lateral roots in all the clones was discontinuous. Furthermore, the number of lateral roots increased only after two to three branches had been formed (Figure 4.1C). For some clones the specific growth rates based on the number of lateral roots were roughly the same as those calculated from total root length (Table 4.1). All tetraploids, however, produced significantly fewer lateral roots than the diploid. Of the tetraploids, clone YUT16-9Lb produced up to 3 times as many lateral roots as the other three tetraploids. In contrast, clone YUT16-7P produced only about two lateral roots per primary root during the entire culture period (Figure 4.1C). All clones, except YUT16-8LT, produced lateral roots within 24 hours (Figure 4.1C). It took YUT16-8LT 3 days to produce its first lateral root.

Throughout the culture period all clones were characterized by the development of relatively short unbranched laterals arising from the primary root (Figure 4.2). The laterals grew 0.8-1.4 cm in length without, for the most part, forming secondary branches. It was not until day 14 that small secondary laterals began to appear on some clones, particularly the diploid YUT16. In all the clones, the average lateral length increased within 1-2 days except for YUT16-7P, where lateral elongation did not begin until day 3 (Figure 4.2). The growth rate of the average lateral length in most of the tetraploid clones was somewhat lower compared to the diploid YUT16 (Table 4.1). Although most of the



Figures 4.2: The average length of lateral roots from single diploid and tetraploid hairy roots grown in six-well plates. Legend: (◊) YUT16; (■) YUT16-9Lb; (∆) YUT16-8LT; (x) YUT16-6P; (○) YUT16-7P. Data are the average of six roots; means ± 1 SD are shown for critical divergence points.

tetraploids produced fewer lateral roots than the diploid (Figure 4.1B), clone YUT16-8LT yielded a final lateral length close to that of the diploid (Figure 4.2).

4.2.3. Relationship between Lateral and Primary Root

"Highly branched" is a term that is almost universally used to describe transformed root cultures. The degree of root branching is dependent on the frequency of branch formation as compared to the rate of elongation (Carvalho *et al*., 1997). This morphological parameter is usually described with the Root Growth Unit (RGU) and it could be an important factor in design of reactors for large-scale hairy root cultures (Yu and Doran, 1994). In hairy roots, changes in the RGU are influenced by the formation and/or elongation of new tips. When the formation of new tips in a root is faster than the overall elongation of roots, the RGU is decreased, while when the overall elongation of root tips is faster than the formation of new tips, the RGU is increased. It is only when the overall elongation of roots and formation of new tips are constant that the RGU remains steady.

The effect of induced polyploidy in the branching (RGU) of *A. annua* diploid and tetraploid hairy roots is illustrated in Figure 4.3. Each clone displayed a different lateral root formation sequence. The diploid YUT16 showed a dramatic decrease in the RGU after one day, as new tip formation dominated over the elongation of the primary root (Figure 4.3). Then, the RGU of this clone remained fairly constant for a period of three days as neither lateral root tip formation nor elongation dominated growth. After day 4 the RGU of YUT16 decreased once more as new root tips developed for two days and then increased once more as the primary and lateral roots elongated.

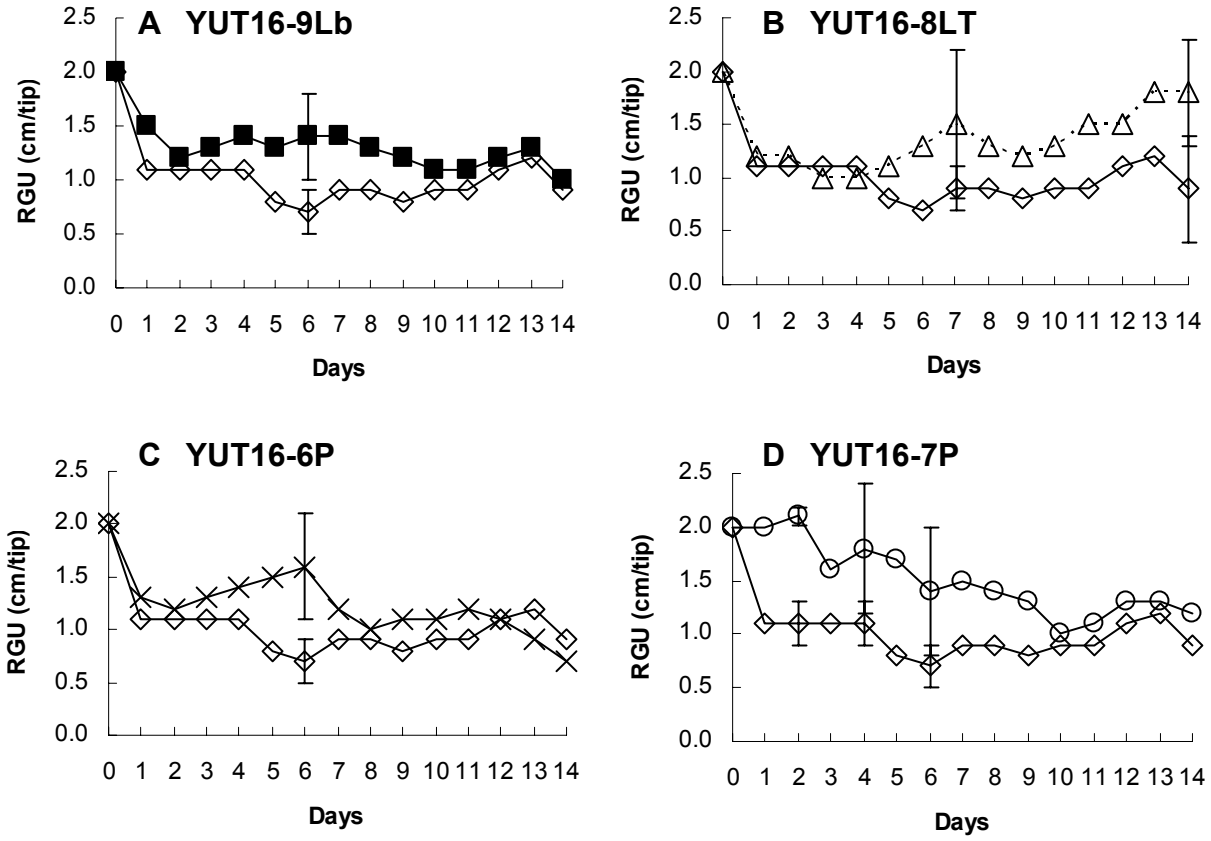


Figure 4.3 A, B, C and D: Value of the Root Growth Unit from single diploid and tetraploid hairy roots grown in six-well plates. (A) Clone YUT16-9Lb. (B) Clone YUT16-8LT. (C) Clone YUT16-6P. (D) Clone YUT16-7P. Legend: (◊) YUT16; (■) YUT16-9Lb; (∆) YUT16-8LT; (x) YUT16-6P; (○) YUT16-7P. Data are the average of 6 roots; means ± 1 SD are shown for critical divergence points.

Then the RGU of this clone remained fairly constant once again from days 8 to11 as neither tip formation nor elongation dominated growth. Then from day 11 the primary and lateral roots elongated for 2 additional days making the RGU increase once more. Finally, the formation of new lateral root tips on YUT16 resulted in an RGU decrease for the rest of the culture period (Figure 4.3).

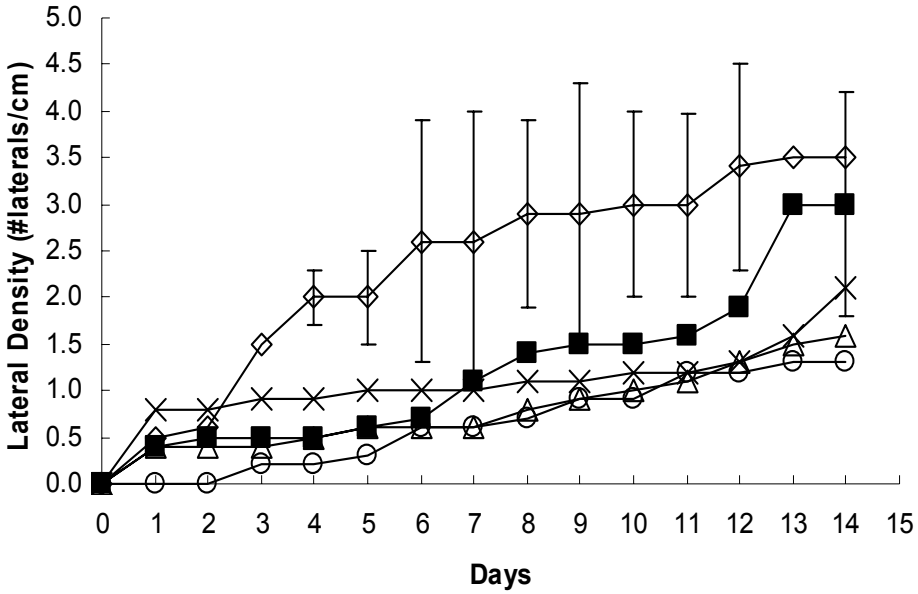
With the exception of clone YUT16-7P most of the tetraploid clones, had a dramatic decreased of the RGU through the first 1-2 days indicating that the number of lateral root tips was increasing in all of them (Figure 4.3A-C). Clone YUT16-7P showed a subtle initial increase in the RGU as the primary root lengthens through the first two days. As the number of root tips slowly increased in this clone, the value of the RGU decreased steadily for the rest of the culture period (Figure 4.3D). On clone YUT16-9Lb, the RGU increased after day 2, remaining fairly constant for 4 days as neither tip formation or elongation dominated growth (Figure 4.3A). By day 8 the RGU of this clone decreased as new lateral tips began to emerge. Lateral formation on this clone stopped at day 10, and the RGU increased again for 3 days as both the primary and lateral roots elongated. By day 13 the RGU of clone YUT16-9Lb started to decrease again indicating the formation of new tips (Figure 4.3A). On clone YUT16-8LT, the RGU increased after day 3 as the primary and newly formed lateral roots lengthen for four days (Figure 4.3B). After day 7 the RGU of this clone decreased again as new root tips developed for two days and finally increasing once more as the primary and lateral roots elongated for the last 5 days of the culture period (Figure 4.3B). As with clone YUT16-9Lb, the RGU profile of clone YUT16-6P was also defined by two cycles of root tip formation and then elongation (Figure 4.3A). After day 2 the RGU value of this clone dramatically increased

as the primary and laterals grew for 4 days. Later, after day 6, as new root tips were forming, the RGU decreased again for 2 days (Figure 4.3C). Then at day 8 the primary and lateral roots elongated for 3 additional days making the RGU increase once more. Finally, the formation of new lateral root tips resulted in an RGU decrease for the rest of the culture period (Figure 4.3C).

When roots grow under controlled environmental conditions they tend to form lateral roots at a regular rate (Lloret and Casero, 2002). Consequently, the total number of lateral roots is positively correlated with the total root length (Lloret *et al*., 1988) and similar number of roots per centimeter (lateral density) are observed along extended regions of primary roots (Lloret and Pulgarín, 1992). In an effort to provide some insight on the arrangement of lateral roots along the parent root on these tetraploid clones, the lateral density (# laterals/cm) was also followed for 14 days. Although clone YUT16-9Lb produced the largest number of lateral roots per cm (~3.0) of all the tetraploids, it was still less than the diploid YUT16 (Figure 4.4).

4.3. CONCLUSIONS

This work compared the development and growth kinetics of four tetraploid hairy root clones of *A. annua* with their diploid parent. Compared to the diploid, all the tetraploid clones showed lower specific growth rates for most of the characteristics measured. However many developmental differences were found even among thetetraploid clones. For instance, tetraploids YUT16-8LT, YUT16-9Lb and YUT16-6P all produced larger primary root lengths compared to the diploid YUT16.



Figures 4.4: Growth kinetics data showing the density of lateral roots of single diploid and tetraploid hairy roots grown in six-well plates. Legend: (◊) YUT16; (■) YUT16-9Lb; (∆) YUT16-8LT; (x) YUT16-6P; (○) YUT16-7P. Data are the average of six roots; means ± 1 SD are shown for critical divergence points.

Also clone YUT16-8LT had longer lateral roots than the diploid, although it's density along the primary root was low. Likewise, of all tetraploids, clone YUT16-9Lb showed the greatest lateral density along its long primary root. Also, another significant difference between the diploid and tetraploid clones was the cyclic nature of the branching rate as described by the RGU. The wide variation in root branching and lateral root density between diploid and tetraploid clones suggests that polyploidy has possibly altered some aspect of root tip initiation out of the pericycle.

Interestingly, the correlation between the developmental characteristics of these tetraploid clones and artemisinin production seemed to be different than that of the diploid clone (data not shown). Wobbe *et al.* (1997) showed that tips from the YUT16 produced more artemisinin per dry mass than mature roots, suggesting that production of more root tips would yield more artemisinin. However none of the lateral root parameters (i.e. tips/day, lateral length and lateral density) correlated with the highest artemisinin producing clone, YUT16-8LT (De Jesus-Gonzalez and Weathers, 2003). Indeed the only correlation between artemisinin production and growth seems to be in the primary root length (data not shown). Clone YUT16-8LT had the highest primary root growth rate of both diploid and tetraploid clones (Figure 4.1B).

This is to my knowledge the first description of the development and growth of tetraploid hairy roots. Root morphology is a major factor in nutrient uptake efficiency (Bar-Tal *et al*., 1997) and its relationship with culture performance may be important in the development of large-scale processes using hairy roots (Falk and Doran, 1996).

CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE RESEARCH

5.1. Summary and Conclusions

The overall goal of this study was to further our fundamental understanding of the effects of artificial polyploidy on the growth and secondary metabolite production of hairy roots using cultures of *A. annua* L. (strain YUT16) as a model. Four tetraploid clones were established and their growth and secondary metabolite production characteristic were examined. These clones were: YUT16-9Lb, YUT16-8LT, YUT16-6P, and YUT16-7P, each showing particular biomass production and artemisinin accumulation levels, and also ploidy stability. Detailed analysis of the tetraploid clones showed that although they grew less than the diploid parent, their artemisinin production was 2-5 times higher, and that this production can be affected (i.e. positively or negatively) by the age of the inoculum.

This work was then extended to analyze the development and growth kinetics of tetraploid *A. annua* hairy roots. Since detailed measurements of individual root growth and development are often difficult to perform in shake flasks, single-root growth studies were carried out to follow their elongation and branching through a culture period. Compared to the diploid, all the tetraploid clones showed lower specific growth rates for most of the root characteristics measured. However, most of the tetraploids clones were characterized by having larger primary root lengths than the diploid, YUT16. There was also a wide variation in lateral root density and the cyclic branching rate (RGU) among

the tetraploids suggesting that polyploidy has possibly affected some aspect of root tip initiation.

The results from this study show that although careful screening and optimization is still required of polyploids induced from an elite clone, there appears to be advantages in selecting for high yielding polyploids artificially produced from hairy root cultures. Increasing artemisinin production is of interest since high levels of this sesquiterpene in *A. annua* transformed roots are needed to make its production more economical. Therefore, artificial polyploidy may serve as a useful tool with respect to the productivity of natural plant products *in vitro*.

5.2. Future Research

The data provided herein highlights the inherent potential of induced polyploidy approach as a rapid means for attaining enhanced production of secondary metabolites. In the case of *A. annua* hairy roots, induced polyploidy proved to increase artemisinin accumulation although not biomass production. The fact that the developmental characteristics of the tetraploid clones are different from the diploid suggests that different culture conditions may be required for their growth. Since high biomass production is just as important in achieving high overall secondary metabolite productivity as is specific product yield, it is clearly important to optimize culture conditions for these tetraploid cultures.

Nutrients in the culture medium can be easily manipulated and have a great impact on growth and secondary metabolite production (Kim *et al*., 2001). Therefore, we should thoroughly explore which components in the culture media formulation may

affect the biomass and productivity of artemisinin in tetraploid *A. annua* hairy roots. For example, growth hormones have a regulatory influence on most plant functions and the effect of chromosome doubling upon them is an important consideration (Hopkins, 1999). Studies have shown that hormone content of tetraploids averaged about 30% less than diploids for several cultivated species (Levin, 1983). Therefore, studies involving exogenous addition of phytohormones to these cultures would be particularly informative. Alternatively, considering the recent evidence suggesting sugars as regulators of various developmental processes (Sheen *et al*., 1999; Smeekens, 2000) and the altered production of artemisinin by different sugars (Appendix A), we should also evaluate the role of sugars in the regulation of growth and terpenoid biosynthesis. Additional research can also be done to evaluate the level of gases which must be provided to the tetraploid *A. annua* hairy roots for optimum growth and secondary metabolite production. For example, transport of oxygen within roots is dependent upon the availability of oxygen at the surface and its movement within the root by diffusion (Carvalho *et al*., 1997). Due to the thicker nature of the tetraploid *A. annua* hairy roots and their somewhat slower growth it is possible that their respiration is lower than the diploid.

Finally, manipulation of branching pattern in root cultures is important since branching can influence growth rate and production of metabolites (Wobbe *et al*., 1997; Woo *et al*., 1997). The low lateral density (#laterals/cm) nature of these tetraploid clones suggests the possibility of apical dominance. By definition, apical dominance implies that the 'dominant' terminal meristems exert an influence over the laterals, thus affecting their growth potential (Carvalho et al., 1997; Falk and Doran, 1996). For root systems,

branching patterns are critically important in determining nutrient and water uptake (Falk and Doran, 1996). A study to measure the effects of excision of the terminal meristems in the formation of lateral branches and the total root length might also be informative.

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APPENDIX A

ALTERATION OF BIOMASS AND ARTEMISININ PRODUCTION BY MEDIA PREPARATION AND SUGARS

In the recent efforts to elucidate the effects of artificial polyploidy in the growth and secondary metabolite production of *A. annua* hairy roots, it was observed that using filter-sterilized medium instead of autoclaved medium, produced consistent but dramatically different results. For instance, the same amounts of biomass and artemisinin accumulation compared to controls (i.e. diploid clone) were observed in tetraploid *A. annua* roots grown in autoclaved medium (Figure A1 and A2). When the same experiments were performed using filter-sterilized medium, the biomass production of these tetraploids was lower but their artemisinin levels were higher than the diploid. Furthermore, results from replicate cultures grown in autoclaved medium varied widely, while data from replicates grown in filter-sterilized medium were statistically "tight" (Figure A1 and A2).

Since it is known that autoclaving the culture medium can lead to the partial hydrolysis of sucrose to its monomers, glucose and fructose, that can change the original composition of the media and that filter-sterilized medium only has sucrose (Table A1), it was hypothesized based on recent compelling evidence that sugars might be serving not only as carbon sources but also as a regulator. (for review: Sheen *et al*., 1999; Smeekens, 2000).

Experiments in shake flasks using combinations of filtered-sterilized sugars at a constant total carbon level in the medium showed stimulation of growth by glucose and inhibition of growth by fructose, but only when fructose was provided at a ratio in excess of glucose (Table A2). Subsequent extraction and analysis of each culture showed that

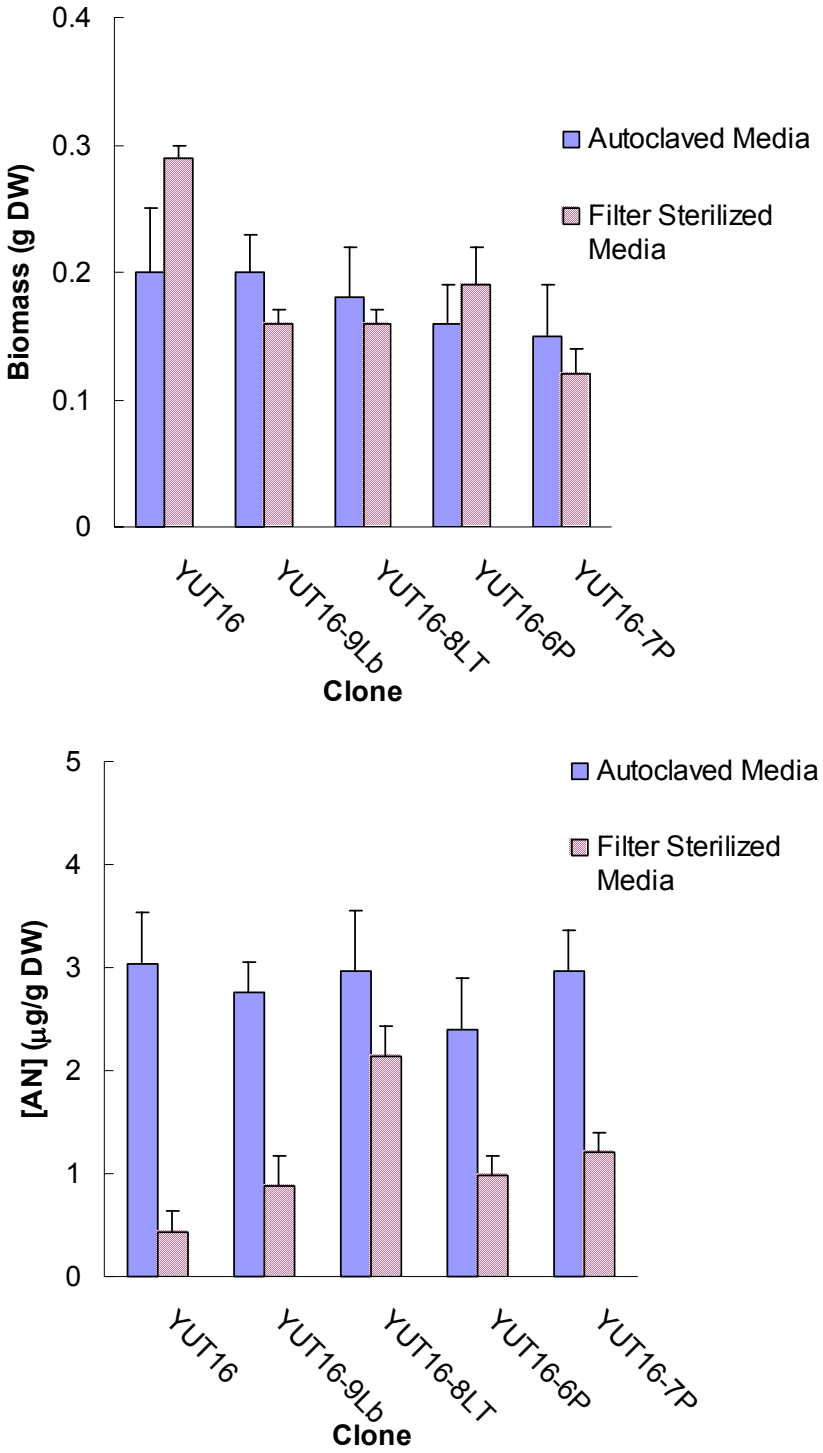

Figure A. (1) Comparison of biomass accumulation in diploid and tetraploid *A. annua* hairy root cultures. (2)Comparison of artemisinin production in diploid and tetraploid *A. annua* hairy root cultures. Each data value represents the mean of three replicates.

Table A1. Amount of sucrose hydrolysis and sugar composition in autoclaved and filter-sterilized media (Data from Kim, 2001).

Sugar Characteristics	Autoclaved Media	Filter-sterilized Media
% sucrose hydrolyzed	9-25	0
Range of hexose/sucrose ratio at inoculation	0.16-0.4 (g L^{-1}/g L^{-1})	0 (100% sucrose)

Table A2. Effects of different sugars on the artemisinin levels and root growth of diploid YUT16.

Culture Condition	Carbon moles L^{-1}	Biomass (g FW)	[AN] (μg/g DW)
B5 + Sucrose (control)	0.21	1.79 a	1.64 a
B5 + Sucrose + Glucose	0.174+0.036	2.10 b	1.19 a
B5 + Sucrose + Fructose	0.174+0.036	1.21 c	0.00 b
B5 + Sucrose + Glucose + Fructose	0.07+0.07+0.07	2.23 b	1.14 a

Cultures were harvested after 14 days growth in the different culture conditions. Each data value represents an average of four replicates. Inoculum was 0.5 g from 14 d cultures grown in filter-sterilized B5 medium. AN, artemisinin. Letters after data indicate statistical differences at a 95% level after ANOVA and for $P<0.05$; b, statistically different when compared to the control condition; c statistically different when compared only to the other sugar conditions.

significantly more artemisinin was produced in the cultures where the glucose to fructose ratio was ≥ 1.0 either through the natural action of invertase on sucrose, or artificially by addition of glucose or glucose plus fructose to the medium (Table A2). More important cultures with a fructose to glucose ratio > 1.0 showed an apparent inhibition of artemisinin production (Table A2). Since the amount of total carbon in all flasks was kept constant, the response is not merely the result of an increased carbon source. Previous experiments showed that at 12 days, the sucrose in the medium is all inverted, but that glucose is definitely not all consumed (Kim, 2001). However, since glucose is consumed in preference to fructose, the ratio of fructose to glucose increases and correlates with decreasing artemisinin levels (μg/g DW) as the culture ages (Weathers *et al*., 1996).

Taken together these results suggest that sugars play a key role in regulating the biosynthesis of artemisinin, and likely other secondary metabolites. Indeed, there is some evidence suggesting that fructose has a role in the production of the monoterpene (-)-carvone in *Mentha spicata* (Maffei *et al*., 2001). Furthermore, Rothe *et al* (2001) suggested that the alkaloid clystegines are under a sucrose control as neither glucose nor fructose were able to induce production. In the case of *Artemisia annua* hairy roots, terpenoid biosynthesis may be inhibited by fructose and possibly stimulated by glucose and/or sucrose. The exact effects of the available carbon sources in secondary metabolite production in plants are not well understood. These interesting results deserve further investigation.