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Transformation of Gossypium hirsutum and Gossypium barbadense by Rhizobium rhizogenes: Hairy root induction and gossypol production

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Transformation of *Gossypium hirsutum* and *Gossypium barbadense* by *Rhizobium rhizogenes*: Hairy root induction and gossypol production

A Thesis

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Entering into the Masters program in Biological Sciences was not my plan. I was working in Dr. Barbara Triplett's lab at USDA Southern Regional Research Center as an undergraduate when I discovered an interest in research. I changed my career path and was accepted in the University of New Orleans Graduate School where I have furthered my education in Biology and most importantly gained a wealth of experience. I have many people to thank for this opportunity.

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Abstract

Gossypol and its methylated derivatives are produced in the leaves, seeds, stems, and roots of cotton plants. Although gossypol is toxic to many animals, other uses for gossypol are actively being investigated. To develop an experimental model for gossypol biosynthesis, a project to produce hairy root cultures from cotton was initiated. Hairy root cultures from two cotton species, *Gossypium hirsutum* (DPL 90) and *Gossypium barbadense* (Sea Island) were developed in this study. Gossypol was synthesized and retained by hairy root tissue at levels similar to that found in cottonseed. Cultures originating from a single transformation event were more similar in their gossypol levels than cultures originating from different transformation events. The effects of media composition, temperature, and addition of elicitors and a signal transducer on culture growth rate and gossypol content were also monitored. The hairy root culture system developed by this project is a suitable model for studying gossypol biosynthesis and nematode resistance.

Introduction

Gossypol

Gossypol, a secondary metabolite, was first discovered by J. J. Longmore in 1886 and purified in crystalline form by L. Marchlewski in 1889 (Croteau *et al.*, 2000). Gossypol and at least 14 other related derivatives have been identified in cotton plant products (Buser *et al.*, 2001). The terpenoid aldehyde, gossypol or 1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl(2,2'-binaphthalene)-8,8'-dicarboxaldehyde is a naturally occurring, yellow, polyphenolic pigment localized in lysigenous glands located below the palisade cells of leaves and the hypodermal cells of stems and bolls in all cotton plant species (family Malvaceae, genus *Gossypium*) (Bell, 1986; Dowd, 2003). Lysigenous glands appear as small black specks (Figure 1-1, Rutgers *et al.*, 2004) and are composed of a large central cavity containing a yellow-orange oily substance surrounded by one layer of flattened epithelial cells. The oily substance within the gland cavity contains a high terpenoid aldehyde concentration (Bell, 1986).

Figure 1-1. Cotton leaf image. Gossypol-containing lysigenous glands in *Gossypium hirsutum* adaxial leaf surface. From www.anbg.gov.au/cpbr/program/sc/cotton_mole.htm Scale bar 10 mm.

Terpenoid aldehydes including gossypol are known to be involved in plant defense in cultivated cotton (Benedict *et al.*, 2004). Gossypol-containing lysigenous glands are found in many plant tissues but are particularly dense in the cotyledons of mature cotton seeds (Meng *et al.*, 1999). The glands are also prominent on cotton leaves and seeds, but are virtually absent on root tissues (Royce *et al.*, 1941; Smith, 1961), where gossypol is exuded to the rhizosphere by cotton roots (Hunter *et al.*, 1978). In addition to being found in glands, gossypol and its methyl ethers also accumulate in epidermal cells and a few cortical cells of young roots after they are a few days old and may accumulate later in root bark and xylem; however, gossypol is not found in the root tip zone (Bell, 1986; Mace *et al.*, 1974).

The biosynthesis of gossypol is not fully understood, although it has been concluded that cis-cis farnesyl pyrophosphate (FPP) is a precursor (Figure 1-2) (Bell, 1986; Heinstein *et al.*, 1970). The immediate precursors of gossypol are desoxyhemigossypol and hemigossypol. The first committed step and likely the control point from the conversion of FPP to gossypol and its precursors and methyl ethers is the catalysis of cis-trans-FPP to (+)-δ-cadinene by (+)-δ-cadinene synthase (cloned by Chen *et al.*, 1995) via a nerolidyl diphosphate intermediate (Chen *et al.*, 1995; Townsend *et al.*, 2005). (+)-δ-cadinene is then converted to 8-hydroxy-(+)-δ-cadinene by the enzyme (+)-δ-cadinene-8-hydroxylase (cloned by Luo *et al.*, 2001). There are two groups of cadinane-type sesquiterpenes in cotton, 7-hydroxylated cadinanes (leading, *e.g.* to the formation of lacinilene C) and 8-hydroxylated cadinanes (leading, *e.g.* to

the formation of gossypol and desoxyhemigossypol), that are differentiated by their positions of hydroxylation. Hydroxylation of (+)-δ-cadinene at C8 is a critical step for the formation of desoxyhemigossypol, a key precursor in the biosynthesis of gossypol and other sesquiterpene aldehydes of cotton (Wang *et al.*, 2003).

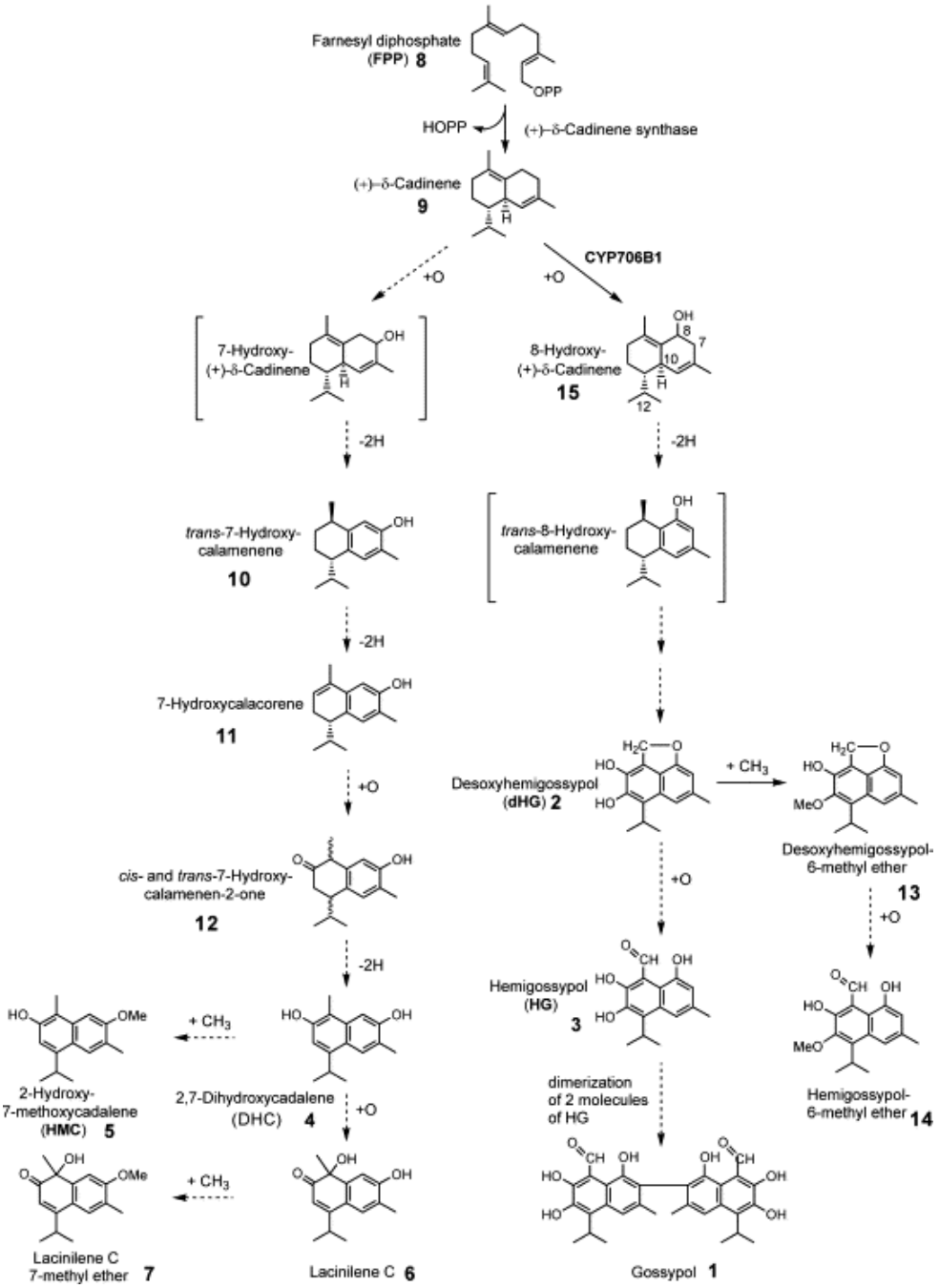

Figure 1-2. Gossypol biosynthetic pathway. This diagram shows the steps of catalysis leading to gossypol biosynthesis. From Wang et al. (2003).

Gossypol content is highest in cottonseeds, but the study of developing seeds is cumbersome and not subject to easy experimental manipulation. Undifferentiated cell suspension cultures initiated from cotton root and cotyledon callus tissue have been used to facilitate biosynthetic studies, but these cultures produce low levels of secondary metabolites and the level of production declines with time (Heinstein, 1981). Clearly, a culture system stably producing high levels of gossypol for long periods of time would be advantageous for investigations of the gossypol biosynthetic pathway.

Chemistry of Gossypol

Gossypol is a dimeric sesquiterpene (molecular weight 518.54) containing a total of 30 carbon atoms (Croteau *et al.*, 2000). The molecule is non-rotational at the interlinking C-C bond between the two naphthalene moieties enabling it to exist as a pair of optical isomers, (+)-gossypol and (-)-gossypol (Figure 1-3), that differ by being mirror images of each other. Both forms exist naturally within the cotton plant. The ratio of gossypol isomers varies within different *Gossypium* sp. (Gamboa *et al.*, 2001). The spectral characteristics and melting points of the isomers are identical.

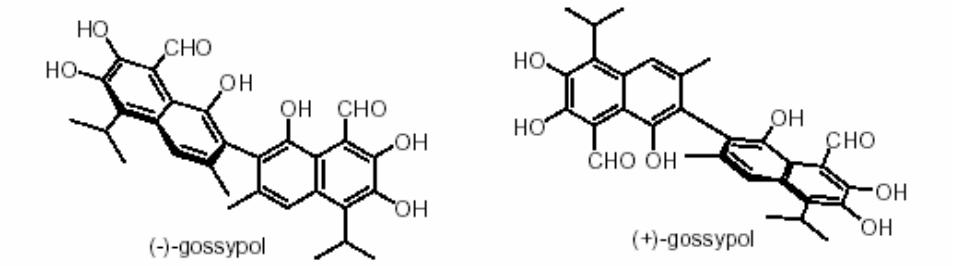

Figure 1-3. Gossypol isomers. (+) and (-)-gossypol are optical isomers or mirror images of each other. They have no rotation around the center C-C bond.

The quantity of gossypol can vary depending upon plant variety and environmental conditions; however, the gossypol content of commercial varieties grown throughout the Cotton Belt has not substantially changed in the last 40 years. Glandless varieties have been derived from a naturally occurring mutant that cannot form lysigenous glands (Buser *et al.*, 2001). Since gossypol is not able to accumulate in these genotypes, glandless varieties of cotton are less toxic and, therefore, are often preferentially damaged by insects and herbivores. The low gossypol content of glandless cotton is safer for ruminant and human consumption, but the productivity of these plants is lower than glanded cotton due to the low toxicity to cotton pests (Benedict *et al.*, 2004). No commercial glandless variety is currently being planted.

Naturally occurring gossypol derivatives include 6'-methoxy-gossypol, 6,6'-dimethoxy-gossypol, desoxyhemigossypol, desoxy-6-methylhemigossypol and hemigossypol (Figure 1-4). Gossypol analysis in different *Gossypium* species has shown that the unmethylated derivative is usually the predominant form in cottonseeds (Percy *et al.*, 1996). One exception is *G. barbadense* that for a few varieties shows elevated levels of methylated forms of gossypol compared to other species (Cass *et al.*, 2002; Percy *et al.*, 1996). The melting points of the corresponding derivatives of gossypol isomers are different (Dowd and Pelitire, 2006). This study focuses on gossypol, 6'-methoxy-gossypol and 6,6'-dimethoxy-gossypol because these compounds are known to be present in roots and lysigenous glands of the cotton varieties used in this study.

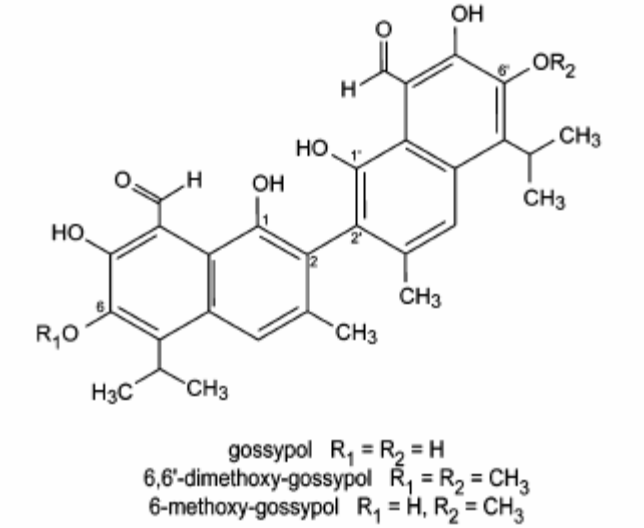

Figure 1-4. Structure of gossypol and methylated derivatives. (Dowd and Pelitire, 2006)

Biological Activities of Gossypol and Derivatives

Virtually all the gossypol in unruptured lysigenous glands is in a "free", *i.e.* not bound form (Buser *et al.*, 2001). Unbound gossypol tends to be more biologically active, and therefore tend to lead to greater toxicity. Gossypol reacts with amino groups of free amino acids and proteins, primarily at lysine residues (Medrano and Andreu, 1986). If the gossypol is bound to protein, biological activity and toxicity have been reported to decrease (Buser *et al.*, 2001). Gossypol is believed to provide a defense mechanism for the plant because it is toxic to insects and herbivores (Rudgers *et al.*, 2004, Bottger *et al.*, 1964; Kovacic, 2003; Meyer *et al.*, 2004).

Even though most gossypol in whole cottonseed is considered to be in the free form, analyses for free and total gossypol will not necessarily result in the same values. This difference is due to the use of two different analytical procedures for free and total gossypol. In the 2005 National Cotton Variety

tests, total gossypol ranged from 0.80 to 1.13 % of whole seed dry weight within the tested varieties. One goal in cottonseed processing is to rupture the pigment glands containing gossypol, so that the gossypol binds to proteins, thus decreasing the free gossypol content. Once the free gossypol has bound to proteins, especially their lysine residues, the bound gossypol is considered to be less toxic to animals. The degree of binding is important, since the process reduces protein quality and amino acid availability. Lysine is an essential component in the diet of animals such as cattle that are fed cottonseed meal (Buser *et al.*, 2001).

A culture system that could provide a means to elucidate the gossypol biosynthetic pathway would potentially lead to a transgenic cotton plant without gossypol in the seed but still present in other tissues that pests attack. The ideal cotton plant would have significant levels of gossypol in the roots and aerial plant parts to protect the plant from pests and diseases and, therefore, maintain fiber yield and quality, but have low levels of gossypol and related compounds in the seed for improved cottonseed applications and reduced processing costs. The Australian native cotton species *Gossypium sturtianum* (Sturt desert rose) is a naturally occurring glanded plant with glandless seed, but its fiber is not worthy of commercial production. There are also fully glandless mutants of cultivated cotton which make them ideal for food and feed applications but more susceptible to a wider range of insects and diseases, and therefore they have a limited potential for commercial production (Figure 1-5).

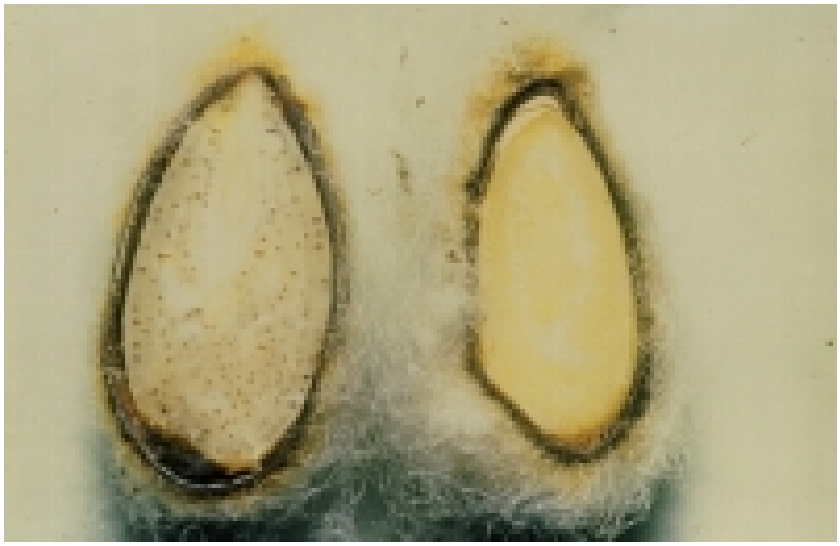

Figure 1-5. Glanded (left) and glandless (right) cottonseed.

Gossypol and related compounds are produced by the plant to provide a degree of natural protection against pests and diseases. Unfortunately, the beneficial aspects of gossypol are counteracted by disadvantages in the use of cottonseed products. Gossypol is toxic to humans and non-ruminant animals, can result in male sterility, discolors oil and lowers the nutritional value of cottonseed meal. Because of these problems, gossypol must be chemically removed from cottonseed oil before it can be considered for human consumption. Many years ago in China, gossypol consumption from crudely prepared cottonseed oil was linked to decreases in male fertility. Gossypol can bind to tubulin protein, inhibit microtubule assembly, and impair sperm motility (Medrano *et al.*, 1986). Liver toxicity and other undesirable side-effects have prevented the use of this plant secondary metabolite to be used as a male contraceptive.

In recent years, gossypol has been found to have many other pharmacological properties. Gossypol and related compounds, 6'-methoxy-gossypol, 6, 6'-dimethoxy-gossypol, desoxyhemigossypol, desoxy-6-methylhemigossypol and hemigossypol, have been reported to function as antifertility agents (Medrano *et al.,* 1986; Qian *et al.*, 1984), and to exhibit insecticidal (Bottger *et al.*, 1964), antimicrobial (Yildirim-Aksoy *et al.*, 2004), antifungal (Puckhaber *et al.*, 2002), antiviral (Lin *et al.*, 1989), antimitotic (Badria, *et al.*, 2001) and cytotoxic activity (Shelley *et al.*, 1999; Ligueros *et al.*, 1997). A study of gossypol's effects on human lung cancer cells indicated that "Gossypol has been shown to possess wide *in vitro* antineoplastic effects and good tolerability with little genotoxicity. Gossypol may be useful in combination with other therapeutic agents to manage primary lung cancer" (Chang *et al*, 2004). These important biological activities of gossypol and related compounds could greatly increase the demand for pure forms of these secondary metabolites. The (-)-gossypol isomer has been shown to be the more biologically active form in conferring some of the above properties (Stipanovic *et al.*, 2005; Puckhaber *et al.*, 2002). Currently, gossypol is recovered and purified from cottonseed but only by an expensive and inefficient method. Different preparations of gossypol may contain different proportions of (+) or (-)-gossypol or the methylated derivatives leading to unpredictable differences in the response when tested in bioassays. One of the goals of this research is to establish plant cell/tissue cultures that preferentially produce or are enriched in specific derivatives or isomers of gossypol. This achievement will lead to

more accurate testing of gossypol biological activity and, hopefully, discovery of a high value use for this by-product of cottonseed.

Rhizobium rhizogenes and Hairy Root Disease

Rhizobium rhizogenes (family Rhizobiaceae) often and erroneously called *Agrobacterium rhizogenes* (Young *et al.*, 2001), is a gram-negative soil bacterium that infects dicotyledonous plants causing root proliferation at the infection site (Nilsson and Olsson, 1997). This disease is known as hairy root disease because fine roots that resemble hair develop at the infection site. The roots are masses of differentiated, transformed cells. This phenotype is caused by genetic transformation in a manner similar to the development of crown gall disease by wild-type strains of *Agrobacterium tumefaciens (*renamed *Rhizobium radiobacter).* Infection of wound sites by *Rhizobium rhizogenes* is followed by the transfer, integration, and expression of T-DNA from the root-inducting (Ri) plasmid of the bacterium to the host plant genome and subsequent development of the hairy root phenotype (Christey, 2001). The appearance of hairy roots at the site of infection generally appear within days to weeks and the emergence of roots on *in vitro* cultures can take as little as a few days (Meyer *et al.*, 2000). Hairy root cultures maintain relative genetic and biochemical stability over long periods and, therefore, present an advantage over cell suspension cultures that are more prone to erratic metabolite production since they are undifferentiated (Flores *et al.*, 1999). Also, hairy root cultures often produce higher levels of secondary metabolites than their non-transformed counterparts.

Rhizobium rhizogenes strain 15834 contains three large plasmids: pAr15834a (107 X 10^6 daltons), pAr15834b (154 X 10^6 daltons) and pAr15834c (258 X 10^6 daltons). The root inducing (Ri) plasmid is pAr15834b (White *et al.*, 1980). The molecular basis of hairy root disease is the transfer and integration of a specific part of the Ri plasmid of *R. rhizogenes* called "transfer DNA" (T-DNA) into the genome of plant cells (Van de Velde *et al.*, 2003). T-DNA is transferred to wounded plant cells and becomes stably integrated into the host genome, thereby initiating the hairy root disease. Hairy root growth in liquid culture is fast, producing highly branched roots whose growth is independent of exogenous hormones.

In addition to the Ri T-DNA regions, a virulence (vir) region is also present on the Ri plasmid. The *vir* genes are necessary for T-DNA transfer to plant cells but do not get transferred into the plant genome. Phenolic compounds (e.g. acetosyringone) released by wounded plant cells induce *vir* genes that regulate the processing of the T-DNA as well as its transfer from the bacterium to the plant cell nucleus (Meyer *et al.*, 2000).

The expression of several root loci (*rol* genes) on the TL-DNA is implicated in the formation of transformed (hairy) roots. The TL-DNA has 18 open reading frames (ORFs) including the *rol* genes, *rolA*, *B*, *C* and *D* which correspond to ORFs 10, 11, 12 and 15 respectively (Michael and Spena, 1995; Tao and Li, 2006; Casanova *et al.*, 2005; Meyer *et al.*, 2000). The *rolD* gene is not needed to induce root formation (Meyer *et al.*, 2000).

Opines are carbon compounds produced in hairy roots that may be used as a growth substance for *R. rhizogenes.* *R. rhizogenes* strains are characterized by the opine they cause the plant to produce (Christey and Braun, 2005). The T-DNA of agropine strains (e.g. 15834, A4, TR105) consists of two separate regions, TL-DNA and TR-DNA (Michael and Spena, 1995) that can be integrated separately into the plant genome (Christey, 2001). TR-DNA is only found in agropine-type Ri plasmids of *R. rhizogenes*. Other strains such as mannopine (TR7, 8196), cucumopine (2588, 2657) or mikimopine (A5, A6), only transfer a single T-DNA fragment, homologous to the agropine TL-DNA but without the *rolD* gene and with genes involved in opine synthesis (Van de Velde *et al.*, 2003; Meyer *et al.*, 2000; Casanova *et al.*, 2005). Genes involved in agropine synthesis (*ags*) and auxin biosynthesis (*aux1* and *aux2*) are found on the TR-DNA (Christey, 2001). *ags* encodes agropine synthase, an enzyme responsible for catalyzing the production of agropine. *aux1* encodes an oxygenase that catalyzes the first step in the auxin biosynthetic pathway which is the conversion of tryptophan into indole-3-acetamide. *aux2* encodes a hydrolase that catalyzes the second step in the auxin biosynthetic pathway which is the conversion of indole-3-acetamide to 3-indoleacetic acid. The production of endogenous hormones by the expression of auxin biosynthetic genes is important for hairy root formation and continued growth. Expression of the auxin biosynthetic genes in the host plant eliminates the need to add hormones to media used for hairy root growth.

It is important to note that T-DNA segregation patterns in hairy root progeny indicate that each hairy root is a cellular clone and, thus, results from a single transformation event (Tempé and Casse-Delbart, 1989). Hairy roots that arise from the infection sites or *in vitro* infections are true roots, consisting of all the typical tissue types found in normal roots and are, therefore, highly organized. Hairy roots often exhibit plagiotrophic growth, i.e. the tendency to grow horizontally without any apparent influence of gravity. The biochemical basis for this response is not known. Hairy roots can be removed from the original explant or plant and established as long-term root clones capable of growing rapidly and maintaining their biosynthetic capacity. Hairy roots also stably express foreign genes, a trait that can be highly beneficial for analysis of transgenic traits (Cooke and Webb, 1997).

Hairy roots have been observed on a limited number of plants in nature including apple, cucumber and melon (Meyer et al., 2000). Artificially induced hairy roots have been produced *in vitro* from many plants including carrot, tomato (Shen *et al.*, 1990), soybean (Cho *et al.*, 2000), tobacco (Furner *et al.*, 1986), snow lotus (Fu *et al.*, 2005), pomelo (Yang *et al.,* 2006), henbane (Flores *et al.*, 1999), dandelion (Lee *et al.*, 2004), wishbone flower (Tao *et al.*, 2006), indigo woad (Xu *et al.*, 2004) and musk melon (Matsuda *et al.*, 2000).

Production and Elicitation of Secondary Metabolites in Hairy Roots

Plants naturally produce secondary metabolites and numerous studies have shown that hairy root cultures also produce these chemicals. Hairy root cultures have been initiated from a wide variety of plant species to produce a

range of secondary metabolites including alkaloids, anthraquinones, flavonoids, terpenoids, naphthoquinones and polyacetylenes (Table 1-1).

CHEMICAL CLASS	CHEMICAL COMPOUND PRODUCED BY HAIRY ROOTS	PLANT SPECIES	REFERENCES
ALKALOID	Scopolamine and hyoscyamine	Deadly nightshade (*Atropa belladonna)*	Bonhomme *et al.*, 2000
	Hyoscyamine	Egyptian henbane (*Hyoscyamus muticus*)	Flores *et al.*, 1999
ANTHRAQUINONE	Lucidin, alizarin and purpurin	Common madder (*Rubia tinctorum*)	Sato *et al.*, 1991
FLAVONOID	Puerarin	Tropical kudzu (*Pueraria phaseoloides)*	Shi *et al.*, 2003
	Syringin	Snow lotus (*Saussurea involucrata*)	Fu *et al.*, 2005
	Saponin	Ginseng root (*Panax ginseng*)	Yoshikawa and Furuya, 1987
TERPENOID	Artemisinin	Annual wormwood (*Artemisia annua*)	Weathers *et al.*, 2005
	Rishitin	Potato (*Solanum tuberosum*)	Komaraiah *et al.*, 2003
	Tanshinone	Red sage (*Salvia miltiorrhiza*)	Yan *et al.*, 2005
NAPHTHOQUINONE	Shikonin	Lithospermum (*Lithospermum erythrorhizon*)	Brigham *et al.*, 1999
POLYACETYLENE	Thiarubrine A	Common ragweed (*Ambrosia artemisiifolia*)	Bhagwath and Hjortso, 2000

Table 1-1. Hairy roots produce secondary metabolites. Hairy roots of various plant species and their respective secondary metabolites.

Plants produce secondary metabolites called phytoalexins in response to physical, biological, or chemical stress including elicitors. Plasma membrane receptors of plant cells appear to bind the elicitors. Then, intracellular signal transduction chains are initiated which finally result in the activation of plant defense genes including those that lead to the expression of phytoalexins such

as gossypol. Phytoalexins can also be induced in hairy root cultures by the addition of elicitors and/or signal transducers. Gossypol is considered to be a secondary metabolite and phytoalexin, *i.e.* a compound with general fungicidal or bacteriocidal action that is produced by a higher plant in response to pathogen attack or other stresses. Phytoalexins are low-M_r antimicrobial compounds that accumulate in the plant in response to infection or abiotic elicitors. The production of phytoalexins is very much like an antibody response since it is triggered by a foreign substance.

Elicitors. Elicitors signal the biosynthetic pathway either directly or indirectly to begin or increase production of phytoalexins. When the plant tissue comes in contact with an elicitor (either abiotic or biotic), the normal metabolism of the plant tissue changes and induces synthesis of signal transducers and enzymes that catalyze reactions in the defense-related pathways leading to phytoalexin production. Elicitor recognition may be a receptor-mediated process that involves the existence of cell surface receptors for specific elicitors (Garcia-Brugger *et al.*, 2006; Singh, 1997). The effect of elicitors depends on the concentration of elicitor, growth stage of the culture at the time of elicitation, period of contact between elicitor and plant tissue, and the time course of elicitation.

Many researchers have used elicitors to stimulate production of secondary metabolites in hairy root cultures (Table 1-2). Also, elicitors have stimulated the production of other sesquiterpenes, the terpenoid class that includes gossypol, in hairy roots (Table 1-3).

ELICITOR	CHEMICAL PRODUCED IN RESPONSE	PLANT SPECIES	REFERENCES
SALICYLIC ACID	Tropane alkaloids	*Brugmansia candida*	Pitta-Alvarez *et al.*, 2000
HEMICELLULASE	Tropane alkaloids	*Brugmansia candida*	Pitta-Alvarez *et al.*, 1999
COPPER SULFATE	Tropane alkaloids	*Brugmansia candida*	Pitta-Alvarez *et al.*, 1999
***PENICILLIUM* SP.**	Indole alkaloids	*Catharanthus roseus*	Sim *et al.*, 1994
JASMONIC ACID	Indole alkaloids	*Catharanthus roseus*	Rijhwani *et al.*, 1998
	Tropane alkaloids	*Brugmansia candida*	Spollansky *et al.*, 2000
	Terpenoid	*Panaz ginseng*	Yu *et al.*, 2000
ASPERGILLUS NIGER	Thiophene	*Tagetes patula*	Rajasekaran *et al.*, 1999
HAEMATOCOCCUS PLUVIALIS	Thiophene	*Tagetes patula*	Rao *et al.*, 2001
	Betalaines	*Beta vulgaris*	Rao *et al.*, 2001
RHIZOCTONIA SOLANI	Naphthoquinones	*Lithospermum erythrorhizon*	Brigham *et al.*, 1999
YEAST ELICITOR	Phenolic acids	*Salvia miltiorrhiza*	Chen *et al.*, 2001; Yan *et al.*, 2006
	Tanshinones	*Salvia miltiorrhiza*	Chen *et al.*, 2001; Yan *et al.*, 2005
VANADYL SULFATE	Polyacetylene (Bhagwath and Hjortso, 2000)	*Ambrosia artemisiifolia*	Bhagwath and Hjortso, 2000
BOTRYTIS CINEREAE	Polyacetylene (Bhagwath and Hjortso, 2000)	*Ambrosia artemisiifolia*	Bhagwath and Hjortso, 2000

Table 1-2. Elicitation of secondary metabolite production in hairy roots.

ELICITOR	CHEMICAL PRODUCED IN RESPONSE	PLANT SPECIES	REFERENCES
***Penicillium chysogenumn* 3446**	Artemisinin	*Artemisia annua*	Liu *et al.*, 1999
***Colletotrichum* sp.**	Artemisinin	*Artemisia annua*	Wang *et al.*, 2001
Pectinase	Lactuside A	*Lactuca virosa* L.	Malarz and Kisiel, 2000
Verticillium dahliae	Artemisinin	*Artemisia annua*	Wang *et al.*, 2000

Table 1-3. Elicitation of sesquiterpene production in hairy roots.

The two elicitors tested in this study are the fungus, *Trichoderma virens* and the bacterial protein, harpin. *T. virens* is a soil-borne filamentous fungus that secretes, Sm1, a small protein that induces the expression of defense-related genes both locally and systemically in cotton (Djonovic *et al.*, 2006;

Puckhaber *et al.*, 2002; Howell *et al.*, 2000). The data from Howell *et al.* (2000) indicate that terpene synthesis is in response to colonization and penetration of the epidermis and outer cortical tissues of the root and subsequent production of terpene stimulating factor or factors. Harpin is a 'hypersensitive response' elicitor. This bacterial protein is heat-stable, glycine-rich, cysteine-free and elicits a signal-transduction chain leading to a defense response or cell death in plants (El-Maarouf *et al.*, 2001). Harpin is sold commercially as a dry powder called Messenger® for the purpose of plant health regulation. Harpin and *T. virens* were readily available to our research group from collaborators and were tested for their ability to elicit a defense response in cotton hairy roots leading to increased gossypol production.

Signal transduction. Jasmonates (JAs) modulate various physiological events in plant cells such as resistance to pathogens and insects, fruit ripening, flowering, maturation of pollen, root growth, senescence through intracellular and intercellular signaling pathways, and regulation of gene expression (Sasaki *et al.*, 2001). JAs are synthesized by a pathway that involves enzymes located in two different subcellular compartments. The first part of the pathway takes place in the chloroplasts and involves the conversion of linolenic acid to 12-oxo-phytodienoic acid by the sequential catalysis of lipoxygenase, allene oxide synthase and allene oxide cyclase. The second part of the pathway takes place in the peroxisomes and involves the reduction of 12-oxo-phytodienoic acid by 12-oxo-phytodienoic acid reductase and three steps of β-oxidation (Li *et al.*, 2005; Sasaki *et al.*, 2001) (Figure 1-6). Jasmonic acid carboxyl

methyltransferase generates methyl jasmonate (MeJA). JA and MeJA accumulate when plants are wounded and subsequently activate the plant's defense response genes (Sasaki *et al.*, 2001).

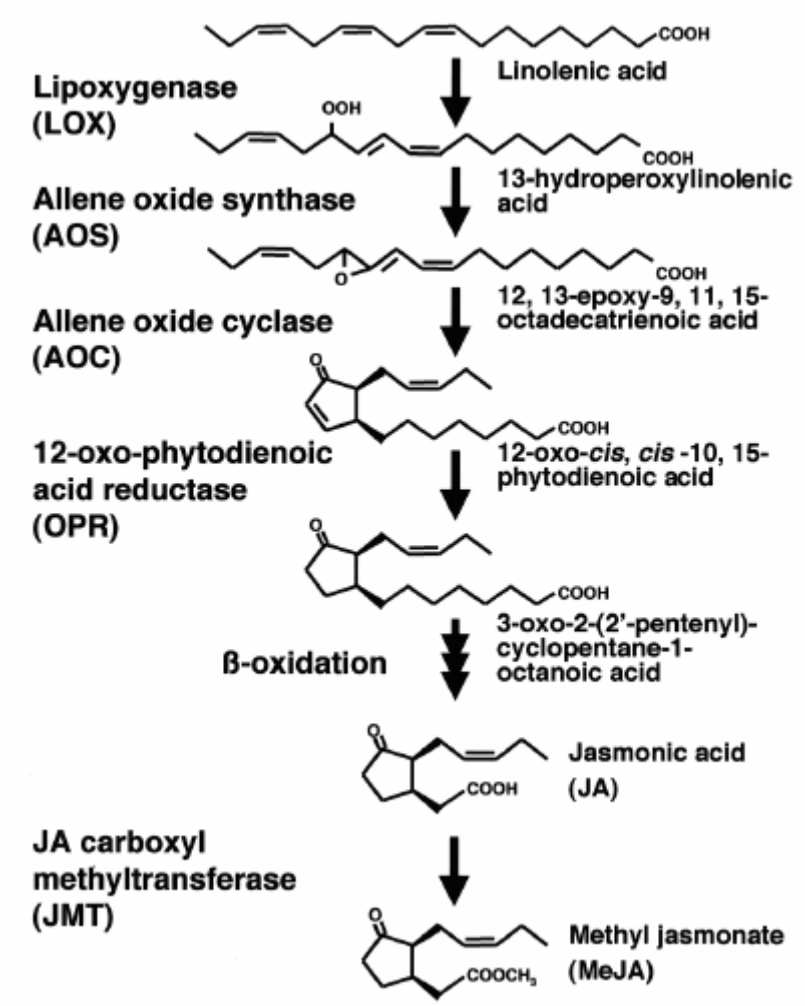

Figure 1-6. Biosynthetic pathway of jasmonic acid and methyl jasmonic acid. The steps of catalysis that lead to the synthesis of jasmonic acid. (Sasaki *et al.*, 2001)

Methyl jasmonate has been shown to increase phytoalexin production in various hairy root culture experiments. MeJA was shown to increase the production of valepotriates in *Rhizobium rhizogenes*-transformed *Valerianella locusta* hairy roots by Kittipongpatana *et al.* (2002). *R. rhizogenes*-transformed

hairy root cultures of *Taxus* x *media* var. *Hicksii* (European yew/Japanese yew hybrid cultivar) were elicited with MeJA subsequently increasing paclitaxel content after 1 week and with continued accumulation at an almost linear rate for 3 weeks when the maximum was reached (Furmanowa *et al.*, 2000). A study by Mandujano-Chavéz *et al.* (2000) indicated that MeJA induced the accumulation of capsidiol in tobacco cell suspension cultures. Kuroyanagi *et al.* (1998) showed that *Hyoscyamus albus* hairy roots treated with MeJa resulted in the production of seven new sesquiterpene phytoalexins. In a study by Stojakowska *et al.* (2002), maximum diacetylene accumulation resulted at 72 hours after the addition of 0.3 mM MeJa. Zabetakis *et al.* (1999) showed increased alkaloid concentrations in *Datura stratmonium* hairy root cultures treated with 0.1 mM MeJa.

Summary

Hairy root cultures have applications for many areas of plant biological research including root nodule research, artificial seed production and production of secondary metabolites. Experimental systems to study biochemical pathways and responses to chemicals and interactions with nematodes, mycorrhizal fungi and root pathogens have also been developed with hairy root cultures and have proven to be a useful tool in studying molecular and biochemical processes in order to control these damaging pests. Cho *et al.* (2000) demonstrated the efficiency of soybean hairy root cultures to study resistance genes for soybean cyst nematode infection. Root cultures established by *R. rhizogenes*-mediated transformation are widely used as a

source of useful compounds owing to their rapid growth in hormone-free media and the relatively high content of secondary metabolites compared with the starting plant material.

The goal of this study was to determine if hairy root cultures from cotton could be induced, and if so, whether the hairy roots produce gossypol and related compounds in culture. Two species of cotton were tested for hairy root induction, *Gossypium barbadense* St. Vincent Sea Island (SI) and *Gossypium hirsutum* Deltapine 90 (DPL 90), two cultivars that differ in their seed gossypol profiles. The *Gossypium barbadense* variety St. Vincent Sea Island used in this study was reported by Percy *et al.* (1996) to have a total seed gossypol content of 12.02 g gossypol per kg of seed kernel dry weight (1.2%). This SI variety was known to have a high degree of gossypol methylation in the seed (Dowd and Pelitire, 2006). In contrast, a *Gossypium hirsutum* variety was reported by Stipanovic *et al.* (2005) to have a total gossypol content of only 4.5 g/kg dry weight of seed (0.45%) but was chosen for this study as a representative of the commercial varieties used in the United States. During the course of this study, the levels and distributions of gossypol produced by hairy roots were compared with those of cotton roots. The variability of gossypol content in hairy root cultures originating from different transformation events was also measured. Additionally, the effects of media composition, temperature, and addition of elicitors on culture growth rate and gossypol content were evaluated. It was hoped that this project would generate an experimental model for studying gossypol biosynthesis that was as stable in growth and secondary metabolite

production as the Egyptian henbane hairy root cultures that have been stably producing hyoscyamine for over 20 years (Flores *et al.*, 1999).

Materials and Methods

Enzymes, Chemicals, Media and Kits

Primers for PCR amplification were purchased from Sigma-Genosys (The Woodlands, TX). SureStart® *Taq* DNA polymerase was purchased from Stratagene (La Jolla, CA). Molecular mass markers for DNA were purchased from Invitrogen (Carlsbad, CA). Agropine-type *Rhizobium rhizogenes* (ATTCC # 15834) was obtained from the American Type Tissue Collection (Manassas, VA) with USDA-APHIS permit #63268. Difco Nutrient Broth was purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ). Murashige-Skoog salts plus vitamins (MS) was purchased from Caisson Laboratories, Inc. (Sugar City, ID). Gamborg's B5 (B5) was purchased from PhytoTechnology Laboratories (Shawnee Mission, KS). Phytagel™ was purchased from Sigma-Aldrich (St. Louis, MO). Water used in the preparation of all buffers, media, and other reagents was purified using a Milli-Q water purification system (Millipore, Bedford, MA).

Plant Materials and Growth Conditions

Seeds of *Gossypium hirsutum* (DeltaPine 90) and *Gossypium barbadense* (St. Vincent Sea Island) were surface sterilized with 10% bleach for 20 min, washed with several changes of autoclaved Milli-Q water, and germinated in Magenta GA-7 boxes (Sigma-Aldrich, St. Louis, MO) containing Murashige-Skoog (MS) salts plus vitamins, pH 6.0 solidified with 2.5 g/L of Phytagel™. After radicle emergence, seedlings were germinated at room temperature (22-25ºC) with natural illumination from a window. Typically, seedling tissues were

inoculated with *R. rhizogenes* within two weeks after radicle emergence. In one experiment, older seedlings (46 days) were inoculated with *R. rhizogenes* to determine whether seedling age influenced hairy root induction.

Growth of Rhizobium rhizogenes

The freeze-dried pellet containing the microbe was reconstituted by the addition of 400 μL of 8 g/L Difco Nutrient Broth, pH 6.8. The stock was streaked on solid Nutrient Broth plates and allowed to grow 48 hr at room temperature. A single colony was selected and used to inoculate 15 mL liquid cultures of *R. rhizogenes*.

Inoculation with Rhizobium rhizogenes

At selected times after germination, the cotyledonary leaves and hypocotyl segments of each seedling were placed on moist filter paper in a sterilized culture dish and inoculated with *R. rhizogenes*. Inoculation was carried out by stabbing the tissues with sterilized toothpicks that had been dipped in a 16 hr bacterial culture (Figure 2-1).

Figure 2-1. Cotyledonary leaves (left) and hypocotyl sections (right). Scale bars equal to 10 mm.

These primary cultures were placed in a plant growth chamber at 24-27 ºC with 16 hr days (photosynthetic photon flux, 46-51 μmol m^{-2} s^{-1}). Recently-inoculated

cultures were incubated under these conditions for 2-3 days and then placed on one of four different kinds of hairy root induction media (HRIM).

Establishing Cotton Hairy Root Cultures

Primary cultures. All four of the HRIM consisted of 1X MS salts plus vitamins, pH 5.8, 500 mg/L of carbenicillin (PhytoTechnology Laboratories, Shawnee Mission, KS), and 7 g/L agar (Becton, Dickinson, and Company, Franklin Lakes, NJ). HRIM-A had nothing else added. HRIM-B had 30 g/L of sucrose added. HRIM-C had 0.1 mg/L naphthalene acetic acid (NAA) hormone added. HRIM-D had both 30 g/L of sucrose and 0.1 mg/L NAA added. Four control cultures (two cotyledons and two hypocotyls) were mock-inoculated with toothpicks that were not dipped in *R. rhizogenes*. A total of 34 cotyledonary leaf pairs and 34 hypocotyl segments from *G. barbadense* (5 - 46 day old seedlings) were inoculated. For *G. hirsutum,* 13 cotyledonary leaf pairs and 13 hypocotyl segments (7 day old seedlings) were inoculated.

Solid culture media. Two to six weeks after inoculation, hairy roots (~1.3 cm sections) were transferred to HRIM-B plates containing 500 mg/L carbenicillin that were maintained at room temperature without illumination. Cultures were transferred several times to fresh HRIM-B with carbenicillin over a period of 3 months to ensure that *R. rhizogenes* was not growing in the cultures. The first root cultures harvested from cotyledonary leaf explants are referred to as the primary cultures. Secondary and tertiary cultures are the hairy roots that were transferred to HRIM-B plates with carbenicillin after two months and three months, respectively.

Liquid culture media. Hairy roots from the tertiary cultures were allowed to grow for 2 weeks. Young roots (~1.3 cm sections) were transferred to 100 x 25 mm culture dishes containing 10 to 15 mL of fresh liquid HRIM-B and gently shaken at 100 rpm in the dark at 21 – 25°C. The cultures have been transferred to fresh liquid HRIM-B or Gamborg's B5 medium every 3-5 weeks since September 2003. Some of the most rapidly growing cultures have been transferred to 1 L Erlenmeyer flasks containing 300 mL Gamborg's B5 (large-scale cultures).

Culture identification numbers. A numbering system was devised in order to track individual transformation events. Initially, the hairy roots were given a number that referenced the transformation event and the origin of the hairy root line including the seedling species, tissue type, and age of seedling tissue at inoculation. Roots from each transformation event were subcultured several times, especially after transfer to liquid HRIM-B. When cultures were divided, each subculture identification number received a lower-case letter after the transformation event number, *i.e.* 33a, 33b, 33c etc. With each subsequent transfer, a number was added to the identification number followed by a lower-case letter at the following transfer. For example, 33b1b1a would represent a hairy root culture originating from transformation event 33 that had been subcultured five times since there are five letters or numbers after 33.

Gossypol Quantification Using HPLC Analysis

Preparing hairy root samples for HPLC analysis. Complexing Reagent consisting of 2% 3-amino-1-propanol, 10% acetic acid and 88% N,N-

dimethylformamide (DMF) was prepared daily. Hairy root samples were lyophilized using a Virtis Genesis 25 XL freeze dryer (Gardiner, NY) and ground using a Wiley-mill (Thomas Scientific USA, Swedesboro, NJ). Approximately 50 mg of each sample was transferred into 20 x 125 mm Kimax glass vials with black phenolic PTFE-faced rubber lined screw-caps (VWR, West Chester, PA). Samples were first incubated with 2 mL of Complexing Reagent for 30 minutes in a 95˚C heat block. After cooling, the samples were diluted with 8 mL of filtered (0.45 micron Millipore filter, Fluoropore 47 mm, Billerica, MA) mobile phase (70% acetonitrile and 30% 0.01 M potassium phosphate buffer, adjusted to pH 3.0 with phosphoric acid).

Preparing culture media for HPLC analysis. Hairy roots were taken out of the culture dishes or flasks. Media were pooled. An equal volume of chloroform was added to 50 mL of media in a large polypropylene conical centrifuge tube. This mixture was shaken for two minutes and the phases were allowed to separate. The extraction was repeated with a fresh 50 mL aliquot of chloroform. A disposable pipette was used to extract the top aqueous layer and sludge leaving the chloroform extract. This chloroform extract was shaken again and allowed to separate for a final time. Any remaining aqueous layer and sludge was removed and the chloroform extract was saved for absorption spectrum readings using an Ultrospec 3000 UV/Visible spectrophotometer (Pharmacia Biotech, Piscataway, NJ) and finally HPLC analysis in which the extract was prepared as described in the section entitled *Preparing hairy root samples for HPLC analysis.*

HPLC analysis. HPLC was performed on a reverse-phase SGE Inertsil column (ODS-2, 100 x 4.0 mm) fitted to a high pressure liquid chromatograph composed of a Waters 2695 Separations Module (Milford, MA), a Waters 996 photodiode array detector (Milford, MA) and EmpowerPro software (Waters, Build #1154, 2002). The Mobile Phase was 70% acetonitrile and 30% 0.01 M KH_2PO_4 buffer (adjusted to pH 3.0 with H_3PO_4). Chromatography was performed at a flow rate of 1.0 mL/min and a pressure of 840 psi at room temperature. The absorbance at 254 nm was monitored, and peaks were compared to a gossypol acetic acid (GAA) standard curve. This 99+% pure GAA was isolated from cottonseed soapstock and purified by recrystallization (Dowd and Pelitire, 2001). The (+) and (-)–isomers of gossypol chromatograph as a single peak under these conditions.

Quantifying gossypol content. GAA standards were made by dissolving approximately 20 mg of GAA into 25 mL of Complexing Reagent. GAA is 89.62% gossypol (ratio of compounds' molecular weights with equimolar amounts of each); therefore, the gossypol concentration of the GAA standard was 0.72 mg/mL. Two-fold serial dilutions of the GAA standard stock were prepared with the addition of Complexing Reagent to a final volume of 2 mL. The samples were incubated at 95-100˚C for 30 minutes, allowed to cool to room temperature, and diluted with 8 mL of Mobile Phase. Approximately 1 mL of each sample was added to a centrifuge tube and centrifuged at 12,000 rpm for 3 min. The samples were poured directly into HPLC autoinjector vials

(Kimble, Vineland, NJ) and analyzed by HPLC. Single representative HPLC samples were analyzed for each experimental biological replicate.

PCR Amplification of rolB, rolC, and virG Genes

DNA isolation. Genomic DNA from approximately 0.1 g of SI hairy root lines 84Aa1 and 33b1b2b, DPL90 hairy root lines 28b1b1 and 28a2b, SI root tissue, DPL90 root tissue and *R. rhizogenes* 15834 was isolated by using a modified version of the protocol of Edwards *et al.*, 1991. All samples were ground in a mortar and pestle using liquid nitrogen and transferred to Eppendorf tubes. Extraction Buffer was 200 mM Tris-HCL pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS. To each microcentrifuge tube containing the ground samples, 400 µL of Extraction Buffer was added. The mixtures were vortexed for 4 seconds and left at room temperature for 1 hour. The samples were centrifuged at 8K for 1 minute and 300 µL of the supernatant liquid was transferred to a clean tube prefilled with 300 µL isopropanol. Samples were mixed and left at -20˚C for 1 hour and samples were centrifuged at 8K for 5 minutes to collect a pellet. The pellets were dried and dissolved in 100 µL 0.1X TE (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0) and absorbance measurements at 260, 280 and 360 nm were taken with a Ultrospec 3000 UV/Visible spectrophotometer (Pharmacia Biotech, Piscataway, NJ). The DNA concentration for each sample was determined by multiplying A_{260} x 50 µg/mL x 200 where 50 is the absorbance unit for double-stranded DNA at 260 nm and 200 is the dilution factor of the samples. Finally, the approximate amount of

DNA in the remaining 95 µL of each sample was calculated. Ratios of the absorbance measurements were taken to estimate the purity of the DNA.

Primer design. The primers used for amplification of *rolB* gene were 5'-GCTCTTGCAGTGCTAGATTT -3'/5'-GAAGGTGCAAGCTACCTCTC-3' and *rolC* 5'-CTCCTGACATCAAACTCGTC-3'/5'-TGCTTCGAGTTATGGGTACA -3' designed according to the sequence data of the entire *rolB* and *rolC* genes from the *R. rhizogenes* 15834 Ri-plasmid that is transferred to the host plant in the infection process. The primers used for amplification of *virG* gene were 5'-TTATCTGAGTGAAGTCGTCTCA-3'/5'-CGTCGCCTGAGATTAAGTGTC-3'.

Polymerase chain reaction. SureStart® Taq DNA polymerase (Stratagene, La Jolla, CA) was used with 100 ng genomic DNA and 10 µM primers in a reaction volume of 25 µL. Reactions were incubated in a GeneAmp 9700 Thermal Cycler (Applied Biosystems, Foster City, CA) at 95ºC for 5 minutes to denature the template and to activate the polymerase. Forty cycles of PCR were performed as follows: 95 ºC (30 seconds) to denature the double-stranded DNA template, 52 ºC (30 seconds) to allow annealing of primers to the template, 72 ºC (60 seconds) for amplification of template DNA and 72 ºC (10 minutes) for final extension. The three primer pairs for *rolB*, *rolC*, and *virG* were used to amplify fragments from genomic DNA from DPL90 hairy roots, SI hairy roots, seedlings from DPL90 and SI, and *R. rhizogenes.*

Gel electrophoresis of PCR amplification products. The PCR products were separated on a 0.8 % agarose gel in 0.5X Tris-Borate-EDTA (TBE) buffer and electrophoresed at 100 V at room temperature. The total volume in each gel

lane was 15 µL, 10 µL from each PCR reaction and 5 µL of 6X DNA gel loading buffer (0.25% bromophenol blue w/v, 0.25% xylene cyanol w/v and 15% Ficoll type 400 w/v adjusted to 10 mL with water). A 1kb DNA ladder was used as the molecular mass marker. The gel was stained with 0.5 µg/mL ethidium bromide solution by gently rocking for 30 minutes on a Hoefer Red Rocker (Hoefer Scientific Instruments, San Francisco, CA). After staining, the gel was washed with distilled water to remove unbound ethidium bromide. The DNA bands were visualized with a Gel Doc 1000 Imager (Bio-Rad, Hercules, CA) and analyzed with Bio-Rad Molecular Analyst version 1.5 software.

Media Composition

Under a laminar flow hood, a large mat of hairy root culture SI 3c1a was cut into 32 equal sections using flame-sterilized scalpels and forceps. The sections were drained of residual media, weighed, and transferred to the prepared culture dishes. The dishes were sealed with Parafilm (VWR, Batavia, IL) and placed in a New Brunswick environmental incubator shaker model G24 (New Brunswick Scientific, Inc., Edison, NJ) set at 100 rpm and 25 ˚C. Triplicate cultures were grown on MS media supplemented with 0, 10, 20, or 30 g/L of glucose or sucrose or B5 media supplemented with 0, 10, 20, or 30 g/L of glucose or sucrose for 21 days. The root masses were weighed at the end of the experiment as a measure of growth and prepared for HPLC analysis as described in the section entitled *Gossypol Quantification Using HPLC Analysis.*

Temperature

A large-scale culture, SI 61Bb, was divided into 9 approximately equal sections. One section was saved for continued sub-culturing. The other 8 sections were weighed and placed in sterile culture dishes containing B5 media, pH 5.8 and 30 g/L sucrose. Cultures were grown at 25, 28, 31, and 34 ˚C in duplicate for 14 days. The root masses were weighed at the end of the experiment and prepared for HPLC analysis as described in the section entitled *Gossypol Quantification Using HPLC Analysis.*

Treatment of Hairy Roots with Harpin

A large-scale culture, SI 33b1b2b was subcultured into 32 cultures of approximately 1.5 g of tissue each in 50 mL of B5, 30 g/L sucrose, pH 5.8. After 23 days of growth, 4 cultures were used as experimental cultures for the harpin treatment, 4 were used as the controls for the harpin treatment, and the remaining cultures were used for the *Trichoderma virens* experiment (see below) and other treatments. Old media as well as a 1/5 pie-shaped portion of the tissue mass was removed from each culture and replaced with 50 mL fresh media. Harpin is sold as a dry powder under the commercial name of Messenger®, (Eden Bioscience, Bothwell, WA). Harpin comprises 3% of the weight of the Messenger® formulation. To each of the replicate experimental cultures 50 µL of filter-sterilized harpin stock (final concentration 0.045 g/L) was added and 50 µL of filter-sterilized water was added to the control cultures. All eight cultures were placed on a shaker at 100 rpm at room temperature in the dark. At 12, 24, 48 and 72 hours, another 1/5 pie-shaped

wedge was removed from each replicate under a laminar flow hood to avoid contamination. All samples were placed in glass vials and stored in a -80˚C freezer. All samples were analyzed for gossypol content by the HPLC method presented in the section entitled *Gossypol Quantification Using HPLC Analysis.*

Treatment of Hairy Roots with Trichoderma virens

A Petri dish with sporulating *Trichoderma virens* was provided courtesy of Shannon Beltz (Food and Feed Safety Unit, USDA-ARS, Southern Regional Research Center). The spores were collected in sterile Inoculation Media (100 mL distilled water, 0.2 g agar, 0.05 mL Triton X-100 detergent). A hemocytometer was used to count the number of spores added to each experimental culture according to the protocol by Garvey (1977). Each experimental culture was inoculated with approximately 8.4×10^6 spores. All other growth conditions for hairy root cultures were identical to the protocol used for harpin treatment. Hairy root samples were removed at 12, 24, 48 and 72 hours and analyzed for gossypol content as described in the section entitled *Gossypol Quantification Using HPLC Analysis.*

Treatment of Hairy Roots with MeJA

A 10 µM stock of methyl jasmonate (MeJA) was prepared by diluting a 95% MeJA solution with 95% ethanol. The diluted solution was filter sterilized with a 0.22 µm Millex GV Filter Unit (Millipore, Billerica, MA). Methyl jasmonate was added to culture media at a final concentration of 100 µM. An equal volume of 95% ethanol (500 µL) was added to control cultures. Under a laminar flow hood, a large mat of hairy root culture SI 33b1b1a was cut into 8 equal

sections (1 subcultured, 1 for experiment starting material, 3 control and 3 treated with MeJA) and grown in an incubator shaker set at 100 rpm at 30˚C for 15 days. Samples were taken at 3, 6, 9, 12 and 15 days and analyzed for gossypol content as described in the section entitled *Gossypol Quantification Using HPLC Analysis*.

Results

Initiation and Optimization of Hairy Root Growth

Hairy root cultures were initiated from young seedlings of two cotton species, *Gossypium hirsutum* and *Gossypium barbadense*. *Gossypium hirsutum* DPL90 was selected because it is used routinely as a representative commercial variety in the Fiber Bioscience research program and contains seed gossypol levels that are typical for the species. *Gossypium barbadense* St. Vincent Sea Island was selected because it contains an unusually high level of methylated forms of gossypol in its seeds. Cotyledonary leaves and seedling stem segments of the two cotton species were inoculated at multiple sites with *Rhizobium rhizogenes* 15834. Two to six weeks later, prolific root growth was evident from some of the inoculation sites (Figure 3-1).

Figure 3-1. Hairy root growth from infection sites on cotyledonary leaves. Scale bar equal to 10 mm.

Root morphology (thickness, length, and degree of branching) varied among the primary cultures. After sub-culture of root tips (1.3 cm sections) through three serial transfers on HRIM-B plates, the cultures exhibited fewer morphological differences except for an occasional slight difference in thickness.

For both species of cotton evaluated in this study, hairy root initiation was higher when young cotyledonary leaves were used as the explant source compared with hypocotyl stem segments (Figure 3-2). Cotyledonary leaves of *G. barbadense* that were inoculated with *R. rhizogenes* produced 72% of the independently transformed hairy root lines suitable for primary transfer. For *G. hirsutum*, the difference in tissue response was greater with 93% of the cultures originating from cotyledonary leaves compared to hypocotyl stem segments.

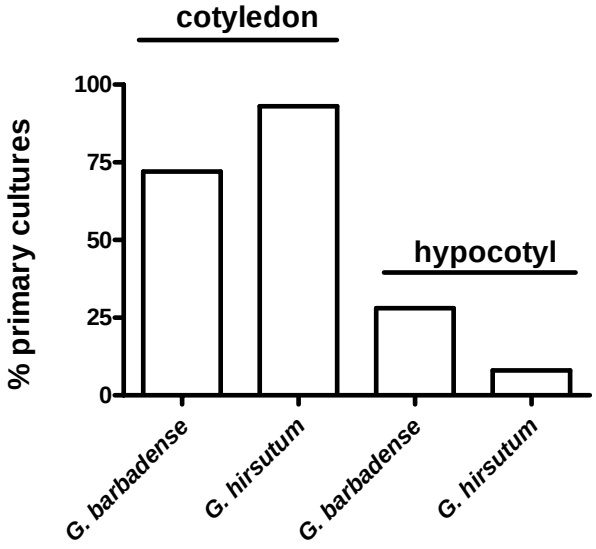

Figure 3-2. The percentage of primary hairy root cultures from cotyledonary leaves and hypocotyl segments for two species of cotton.

The age of cotyledonary leaves inoculated with *R. rhizogenes* also determined the success of transformation (Figure 3-3). Between 20-30% of the *G. barbadense* cotyledonary leaves younger than 13 days after germination produced hairy roots. In contrast, less than 2% of *G. barbadense* cotyledonary leaves harvested from seedlings left in Magenta boxes for 46 days after germination produced hairy roots under the same conditions.

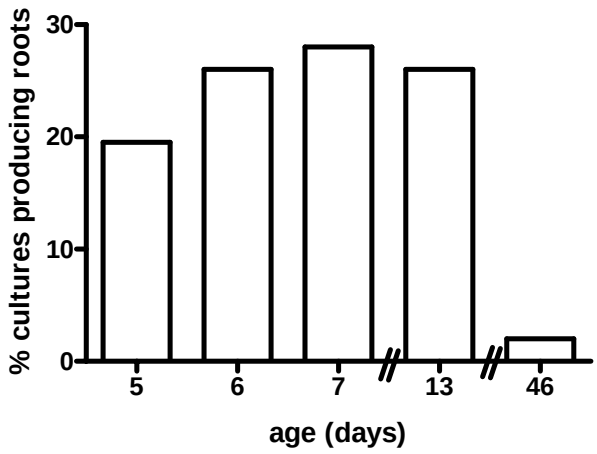

Figure 3-3. The percentage of inoculated *Gossypium barbadense* cotyledons of various ages that produced hairy roots.

Two to three days after inoculation, *G. barbadense* explants were transferred to four different Hairy Root Induction Media (HRIM) all containing 500 mg/L carbenicillin to inhibit further growth of *R. rhizogenes.* Basal medium or HRIM-A, contained Murashige-Skoog medium and vitamins, pH 5.8 solidified with 7 g/L agar. Based on several reports that sucrose and/or auxin supplementation could stimulate higher levels of hairy root production (Yoshikawa and Furuya, 1987; Sato *et al.*, 1991; Nilsson and Olsson, 1997) three other media were tested for their ability to induce cotton hairy root formation: HRIM-B, basal medium supplemented with 30 g/L sucrose; HRIM-C, basal medium supplemented with 0.1 mg/L naphthalene acetic acid; and HRIM-D, basal medium supplemented with 30 g/L sucrose and 0.1 mg/L naphthalene acetic acid. After four weeks incubation on the different media, cultures were evaluated for the presence of hairy roots (Figure 3-4). Media containing sucrose as the only supplement (HRIM-B) produced the greatest number of hairy roots. Cotyledonary leaves cultured on auxin-supplemented media (HRIM-C) produced

nearly the same number of hairy roots as the basal medium (HRIM-A). When auxin and sucrose were both added (HRIM-D), the total number of hairy roots was greater than the auxin-supplement treatment but less than the sucrose treatment. Based on this result, all subsequent cultures were transferred to HRIM-B at 2-3 days post-inoculation.

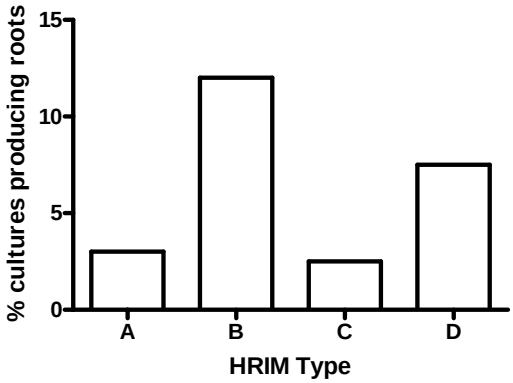

Figure 3-4. The percentage of inoculated *Gossypium barbadense* cotyledons producing hairy roots. Hairy Root Induction Media (HRIM): A=MS basal medium, B=MS with 30 g/L sucrose, C=MS with 0.1 mg/L NAA and D=MS with 30 g/L sucrose and NAA.

To ensure that the cultures were not still infected with *R. rhizogenes,* all cultures that produced hairy roots at least 1.3 cm in length after two weeks were serially transferred to fresh HRIM-B plates containing carbenicillin. A total of three transfers were made at two- to three-week intervals. During this time not all of the cultures continued to produce actively growing hairy roots (Table 3-1). Between 50-60% of the primary hairy root cultures initiated on *G. hirsutum* and *G. barbadense* cotyledonary leaves were still producing hairy roots suitable for the third transfer to solid media.

Cotyledonary Leaves	***Gossypium hirsutum* DPL90**	***Gossypium barbadense* St. Vincent Sea Island**
# primary cultures	73	109
# secondary cultures	42	70
# tertiary cultures	38	64
# primary liquid cultures	38	63
Hypocotyl Segments	***Gossypium hirsutum* DPL90**	***Gossypium barbadense* St. Vincent Sea Island**
# primary cultures	5	43
# secondary cultures	2	7
# tertiary cultures	1	5
# primary liquid cultures	0	5

Table 3-1. Summary of cotton hairy root production. Primary cultures resulted from *Rhizobium rhizogenes* infection of either cotyledonary leaves or hypocotyl segments from young cotton seedlings of *G. hirsutum* or *G. barbadense*. Primary cultures that grew roots longer than 1.3 cm were transferred to secondary culture plates. Secondary cultures that grew roots longer than 1.3 cm were transferred to tertiary culture plates. Tertiary cultures that grew roots longer than 1.3 cm were transferred to liquid culture media.

Gossypol Production in Hairy Root Cultures

After six weeks on carbenicillin-containing plates, hairy root cultures were transferred to small-scale (15 mL) liquid culture without antibiotic. Transfer to liquid culture greatly stimulated new root growth in most of the cultures. By the end of the first three week culture period, a brown material accumulated around the perimeter of the polystyrene culture dish and the media turned a light golden brown. When the hairy roots were transferred to fresh liquid media, the conditioned medium was extracted with an equal volume of chloroform. The chloroform-extracted material was analyzed by UV/Visible spectrophotometry and found to have an absorption spectrum very similar to authentic gossypol and related compounds (Figure 3-5).

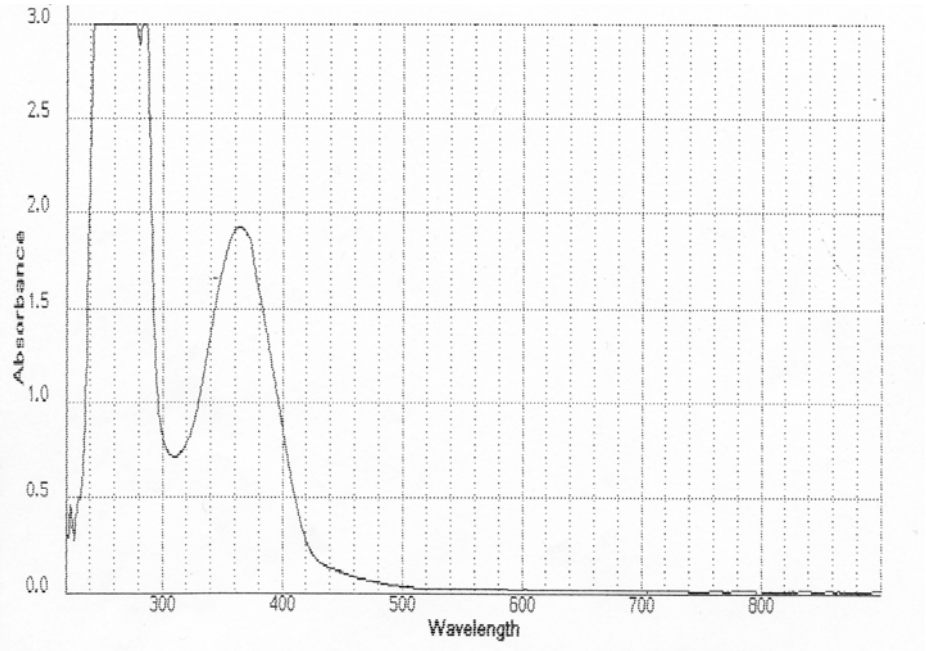

Figure 3-5. Ultraviolet/Visible absorption spectrum of chloroform-extracted culture media from *G. barbadense* St. Vincent Sea Island hairy root culture.

High performance liquid chromatography (Dowd, 2003) resolved the chloroform-extracted material into several peak fractions at 254 nm, including three peaks that co-eluted with the known peaks for gossypol, methoxy-gossypol, and dimethoxy-gossypol (Figure 3-6). The UV/Vis spectrum for each of the peak fractions was remarkably similar to gossypol standards isolated from *Gossypium barbadense* roots (Figure 3-6).

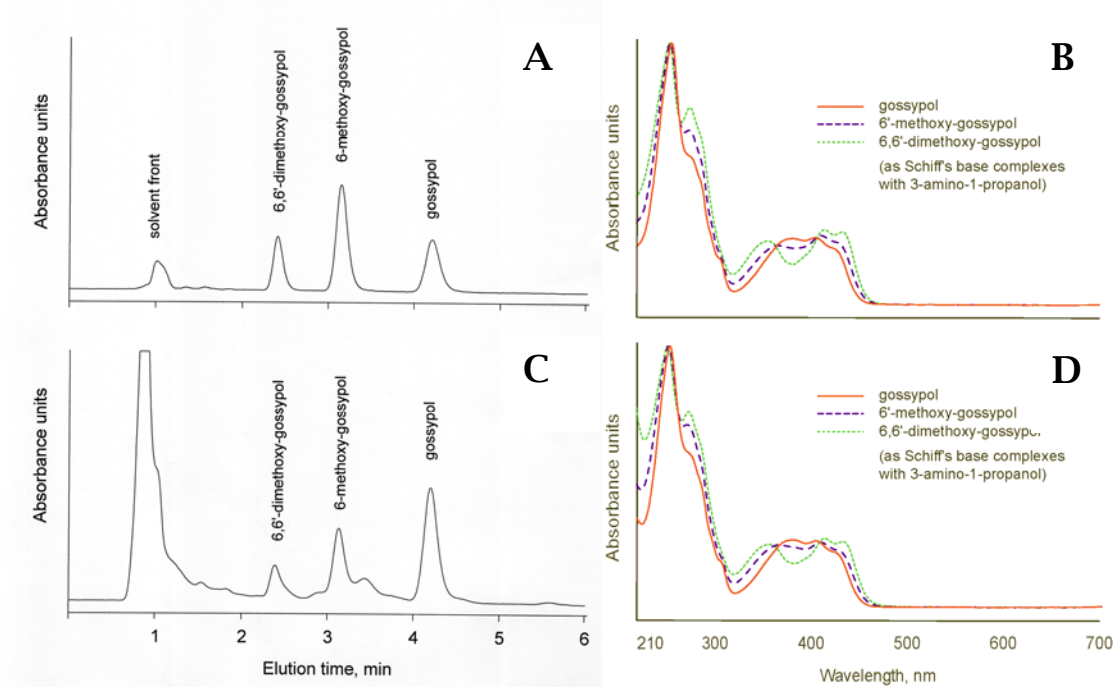

Figure 3-6. HPLC chromatogram of gossypol standards isolated from native *Gossypium barbadense roots* (A), UV/Visible spectrum from each of the peak fractions from Figure A (B), HPLC chromatogram of gossypol and related compounds from chloroform extracted hairy root culture media (C), and UV/Visible spectrum from each of the peak fraction from Figure C (D).

The relative proportions of gossypol in hairy root tissue and culture media were estimated by freeze-drying a mass of hairy roots from five 3-week old cultures, grinding the samples with a Wiley-mill and analyzing the gossypol content by HPLC (Kim *et al.*, 1996). Media from the cultures was chloroform-extracted three times, concentrated, and analyzed for gossypol content following the same procedure used for hairy root tissue. Approximately 90% of the gossypol produced was retained in the hairy root tissue and 10% was secreted into the media.

When hairy root mass completely filled a 100 x 25 mm culture dish, some of the cultures were transferred to 1L Erlenmeyer flasks containing 300 mL media (large-scale cultures). Before August 29, 2005, 18 independently

transformed lines were moved to large-scale culture. Three of these "large-scale" lines were initiated from *G. hirsutum* cotyledonary leaves and 15 were from *G. barbadense*. Due to limitations in shaker space in the environmental chamber, not all cultures were advanced to the larger flasks. Lines that were selected for additional study were chosen based on their total level of gossypol content and/or their distribution of methylated forms of gossypol.

Growth and gossypol content for each of the lines were measured 21 days after sub-culturing to fresh media. Within subcultures of a single transformant there was variability in culture growth rate, but the gossypol content and degree of methylation were similar among the cultures (Table 3-2).

	Dry culture Mass, g	**Total gossypol, %**	**% Methylated**
Minimum	0.083	1.07	39.0
Maximum	0.309	1.78	70.0
Average	0.192	1.48	53.6
Std. deviation	0.069	0.23	8.0
CV, %	36.4	15.5	14.9

Table 3-2. Single transformant variability. Twenty-six subcultures from SI transformant #3 were grown in fresh media for 21 days and then analyzed for growth and gossypol content.

In comparison with the low variability found within subcultures of a single transformation event, high variability was found among the entire population of cultures (96 cultures from 26 distinct transformants). Growth rates also varied greatly (Table 3-3).

	Dry culture mass, g	Total gossypol, %	% Methylated
Minimum	0.003	0.18	26.8
Maximum	1.28	4.17	75.8
Average	0.238	1.45	48.9
Std. Deviation	0.272	0.82	11.4
CV, %	114	56.6	23.3

Table 3-3. Multiple transformant variability. Ninety-six cultures originating from 26 transformants were grown in fresh media for 21 days and then analyzed for growth and gossypol content.

On August 29, 2005, the liquid cultures were lost due to extended power failure in the facility. Fortunately many of the cultures were rescued from solid plates that were maintained as back-up cultures. After repeated culture to remove fungal contaminants, 35 cultures are being maintained representing 8 original transformants.

Media Composition

As seen in Table 3-4, a carbohydrate source is essential for hairy root growth with the only statistically significant difference being that of the control (no sugar source) and the experimental cultures whose carbohydrate levels ranged from 10-30 g/L. Control cultures that did not contain either glucose or sucrose failed to increase in mass during the experiment. There was no significant difference in hairy root growth between any of the different treatments. Hairy root growth rates were similar on the two media, but Gamborg's B5 medium supported significantly higher gossypol content. All cultures grown on B5 medium had significantly higher gossypol content than B5 control cultures. None of the cultures grown on MS media supplemented with either glucose or sucrose had total gossypol levels significantly different from MS control cultures that did not contain sugar.

		Cell mass g (fresh weight)		Total gossypol % (dry weight)	
Sugar:		**B5**	**MS**	**B5**	**MS**
Control	**0**	0.38 ± 0.18	0.64 ± 0.21	0.68 ± 0.18	0.77 ± 0.46
Glucose (g/L)	**10**	2.48 ± 0.64*	2.31 ± 0.22*	1.46 ± 0.15*	1.13 ± 0.31
	20	2.54 ± 0.90*	2.69 ± 0.64*	1.36 ± 0.19*	1.24 ± 0.48
	30	3.14 ± 1.21*	2.87 ± 0.31*	1.22 ± 0.25*°	0.69 ± 0.13
Sucrose (g/L)	**10**	1.95 ± 0.37*	2.76 ± 0.77*	1.32 ± 0.12*°	0.88 ± 0.23
	20	3.14 ± 1.21*	2.01 ± 0.89*	1.70 ± 0.12*°	0.80 ± 0.24
	30	2.89 ± 0.06*	2.80 ± 0.94*	1.36 ± 0.28*°	0.60 ± 0.15

Table 3-4. Effect of carbon source and media composition on culture growth and gossypol content by hairy roots. B5 is Gamborg's B5 media, pH 5.8 and MS is Murashige-Skoog + vitamins media, pH 5.8. Statistical analysis was performed by GraphPad Prism 4 using an unpaired two-tailed t-test, 95% confidence interval. * represents a treatment value statistically higher than control, ° represents a treatment value statistically higher than corresponding treatment in different medium. All P-values were less than 0.05 to be considered statistically different.

Optimum Temperature

The optimum temperature for hairy root growth was 28ºC (Figure 3-7). Hairy root cultures grown at 31ºC had the highest level of total gossypol content, but the degree of gossypol methylation increased with increasing temperature.

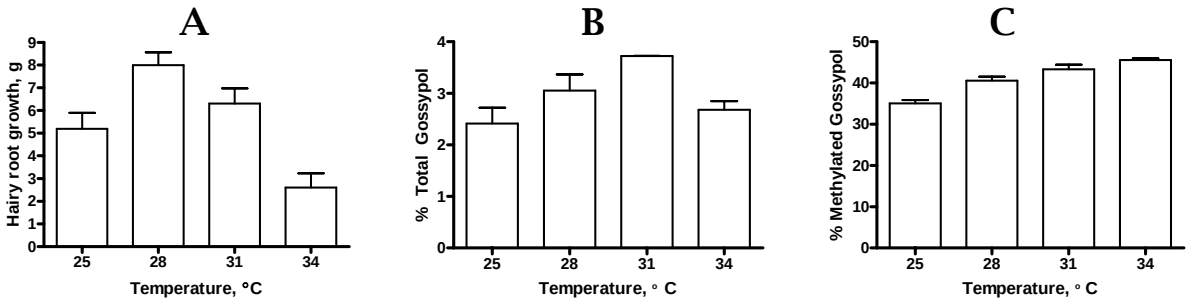

Figure 3-7. Hairy root growth and gossypol content at various temperatures. (A) Hairy root growth (g fresh weight) (B) Total gossypol content (% dry weight), (C) methylated gossypol content (% dry weight).

This was the first and only successful temperature experiment out of a total of four temperature experiments that were started. For various reasons such as fungal contamination and temperature control problems, the temperature experiment was not successfully replicated.

PCR Confirmation of R. rhizogenes Transformation

Transformation of cotton hairy root tissues by the *R. rhizogenes* Ri plasmid was demonstrated by PCR amplification of the *rolB* (422 bp) and *rolC* (625 bp) genes from hairy root DNA and lack of amplification from cotton seedling root DNA (Figure 3-8). Furner *et al.* (1986) showed that the *rolB* and *C* genes from agropine strain A4 Ri plasmid are 422 bp and 625 bp respectively.

Also, lack of amplification of *virG* (1000 bp) in hairy roots and seedling roots whereas the control using *R. rhizogenes* DNA had an amplified band shows that *rolB* and *rolC* amplification did not result from cultures contaminated with *R. rhizogenes*.

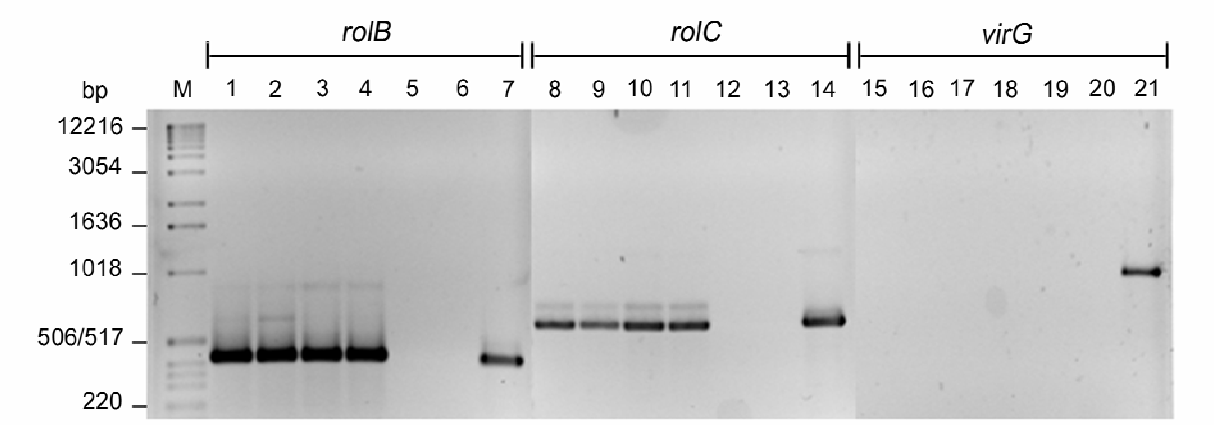

Figure 3-8. PCR amplification of *rolB*, *rolC* and *virG* genes in SI and DPL 90 roots and hairy roots. Lane M is a 1KB ladder. DNA sources for amplification were: lanes 1, 8, and 15: SI hairy root (# 84Aa1); lanes 2, 9, and 16: SI hairy root (# 33b1b2b); lanes 3, 10, 17: DPL90 hairy root (# 28b1b1); lanes 4, 11, 18: DPL90 hairy root (# 28a2b); lanes 5, 12, 19: SI seedling root; lanes 6, 13, 20: DPL90 seedling root; lanes 7, 14, 21: *R. rhizogenes* 15834. Primers sets for amplification were: Lanes 1-7 *rolB*; lanes 8-14 *rolC*; lanes 15-21 *virG*.

Harpin Treatment

Gossypol content began to increase at 12 hours after treatment with harpin (Figure 3-9). The gossypol content continued to increase up to the last sampling time of 72 hours. The harpin-treated cultures at 12 hours after treatment had significantly higher gossypol content than the starting material but the untreated cultures did not. The treated cultures at 48 and 72 hours after treatment had significantly higher gossypol content than the untreated cultures at 48 and 72 hours (P values are 0.0284 and 0.0014 respectively). There was a 44% increase in gossypol content for the treated cultures at 72 hours versus the 72 hour control cultures.

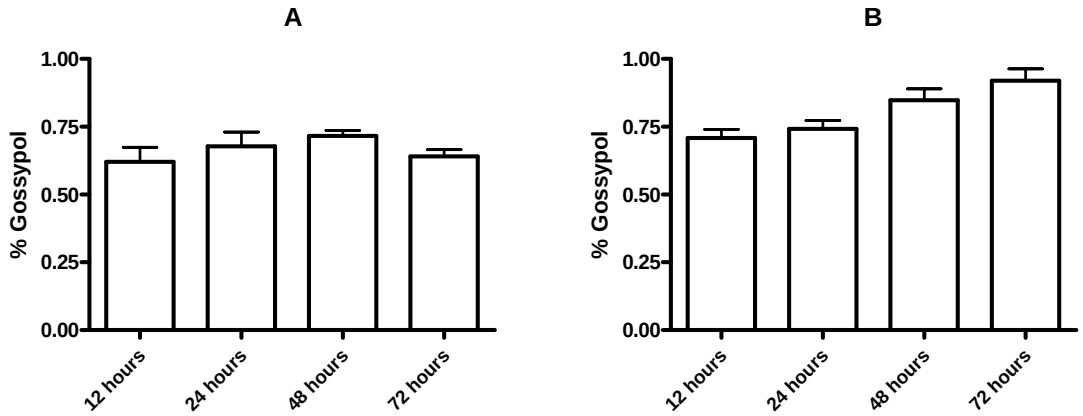

Figure 3-9. Gossypol content with harpin treatment. % gossypol in (A) control cultures and (B) harpin treated cultures at 12, 24, 48 and 72 hours. Eight 23 day old SI 33b1b2b subcultures were selected based on closeness of weight. 4 control cultures were treated with 50 µL of filter-sterile water. 4 cultures were treated with 50 µL harpin stock solution (final concentration: 0.045 g/L). Cultures were maintained at room temperature. % Gossypol in control starting material was 0.55 ± 0.09. % Gossypol in treated starting material was 0.69 ± 0.17.

Trichoderma virens Treatment

Gossypol content began to increase at 12 hours after treatment and continued to increase until 24 hours after which the gossypol content began to decrease (Figure 3-10). The treated cultures at 24 hours after treatment had significantly higher gossypol content than the untreated cultures at 24 hours. The percent increase in gossypol content at 24 hours compared with that of the untreated cultures at 24 hours was 84%. At 72 hours post-elicitation the cultures were heavily contaminated with *T. virens*.

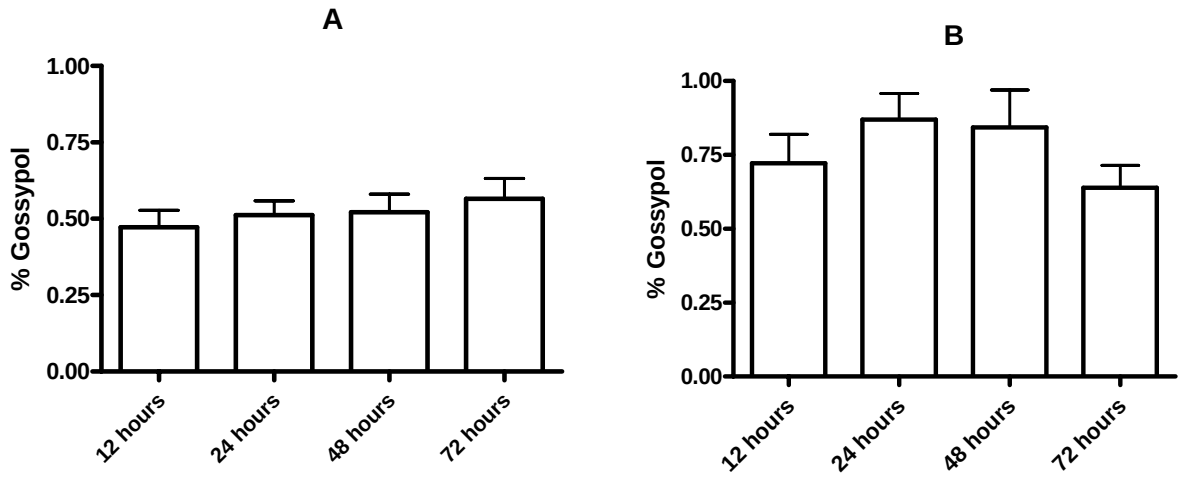

Figure 3-10. Gossypol content with *Trichoderma virens* treatment. % gossypol in (A) control cultures and (B) *T. virens* treated cultures at 12, 24, 48 and 72 hours. Eight 23 day old SI 33b1b2b subcultures were selected based on closeness of weight. 4 control cultures were treated with 100 µL of inoculation media. 4 cultures were treated with 100 µL trichoderma solution (approx. 8.4×10^7 *T. virens* cells in 100 µL inoculation media). Cultures were maintained at room temperature. % Gossypol in control starting material was 0.42 ± 0.07. % Gossypol in starting material used for treatment was not recorded.

Methyl Jasmonate Treatment

Gossypol content increased in cultures treated with 100 µM MeJA by day 3 and continued to increase until 9 days after the treatment where the total level of gossypol produced by the culture began to plateau (Figure 3-11). On each sampling day, the treated cultures had higher gossypol contents than the untreated culture samples. The treated cultures sampled on day 9 and 15 were significantly higher than the untreated cultures sampled on those days with P-values of 0.03 and 0.02 respectively. There was a 57% increase in gossypol content for the treated cultures at 15 days versus the control cultures at 15 days.

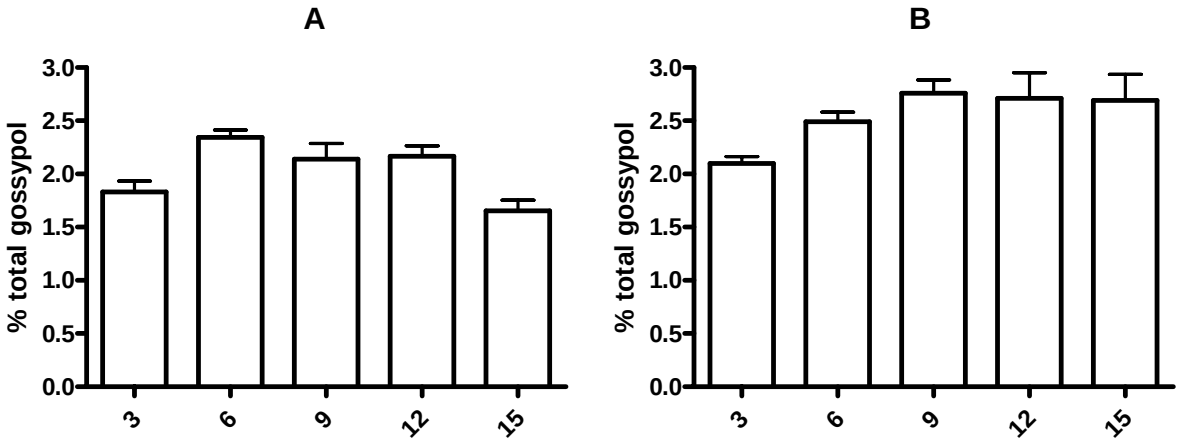

Figure 3-11. Gossypol content with methyl jasmonate treatment. % total gossypol in (A) control cultures and (B) MeJA treated cultures at 3, 5, 9, 12 and 15 days. A three week old culture, SI 33b1b1a was divided into 8 equal pie-shaped pieces. One piece was subcultured in fresh B5 with 20 g/L sucrose, pH 5.8. One piece was analyzed as starting material. Three pieces were subcultured into the same fresh media and treated as controls with 500 µL 95% ethanol added to each 50 mL culture. The last three pieces were set up with the same culture conditions and treated as experimental with 500 µL 10mM MeJA (final concentration: 100µM). Cultures were maintained at a temperature between 28 and 32°C. % Gossypol of starting material was 1.71.

Comparison of Hairy Root Gossypol Content with Seed and Root Standards

The gossypol content and distribution of methylated forms was determined for 18 large-scale hairy root cultures. The culture data were compared with those of SI seed and root standards (Table 3-5). The hairy root culture with the highest total gossypol content (3.97%) was *Gossypium hirsutum* DPL90 28b1b1. The same culture had the highest distribution of methylated forms (58.13%). The average distribution of methylated forms in the SI cultures was 38.33%. Analysis of seed and root from cotton varieties has shown *Gossypium barbadense* to have higher methylated forms of gossypol than *Gossypium hirsutum*, but the same pattern is not seen in hairy root cultures.

Tissue	Compound	% Gossypol	% Distribution
Seed	di-methyl gossypol	0.135	7.78
	methyl gossypol	0.700	40.28
	gossypol	0.903	51.95
	total	1.738	
Root	di-methyl gossypol	0.376	27.64
	methyl gossypol	0.643	47.22
	gossypol	0.432	25.14
	total	1.362	
Hairy roots	di-methyl gossypol	0.047-0.478*	4.19-24.70**
	methyl gossypol	0.108-0.927*	9.75-42.95**
	gossypol	0.386-2.016*	34.92-86.06**
	total	0.7810-2.804*	

Table 3-5. *Gossypium barbadense* Sea Island seed, root, and hairy root gossypol content and distribution of methylated forms. * range of % gossypol, n=64. ** range of % distribution, n=64. n=the number of hairy root samples taken from 15 SI cultures over a period of subculturing.

Discussion

Hairy root cultures have been produced from a variety of dicotyledonous plants in the last 30 years to produce specific plant secondary metabolites (Bonhomme *et al.*, 2000; Flores *et al.*, 1999; Sato *et al.*, 1991; Shi *et al.*, 2003; Fu *et al.*, 2005; Yoshikawa and Furuya, 1987; Weathers *et al.*, 2005, Komaraiah *et al.*, 2003; Yan *et al.*, 2005; Brigham *et al.*, 1999; and Bhagwath and Hjortso, 2000). Although previous workers reported that cotton cell suspension cultures could produce gossypol *in vitro*, production was neither high nor stable with time (Heinstein, 1981). This thesis project was initiated to determine (1) if cotton hairy roots could produce gossypol and, if so, whether the production was stable with time and (2) if the profile of gossypol produced by hairy roots could be predicted by the seed gossypol content.

Gossypol, a terpenoid aldehyde, is found naturally in plants from the genus *Gossypium,* and has a wide array of biological activities (Medrano *et al.*, 1986; Qian *et al.*, 1984; Lin *et al.*, 1989; Badria *et al.*, 2001; Shelley *et al.*, 1999; Ligueros *et al.*, 1997; and Chang *et al.*, 2004). Because cotton is a dicotyledonous plant, the hypothesis that *R. rhizogenes* infection could initiate gossypol-producing hairy roots seemed promising.

The response of the two cotton species, *G. barbadense* and *G. hirsutum* and infection parameters for the induction of hairy roots obtained through *R. rhizogenes* 15834 mediated transformation were observed in order to identify conditions leading to optimal hairy root growth. The cotyledonary leaves of

both cotton species that were infected with *R. rhizogenes* 15834 produced more hairy roots per inoculation than hypocotyl segments. This tissue preference was also the case in a study by Matsuda *et al.* (2000) that used cotyledons to successfully induce hairy roots after inoculation with *R. rhizogenes* MAFF 03-01724. Another observation of cotton hairy root induction was that older cotyledonary leaf tissues were less likely to produce hairy roots than younger cotyledons. To date, no other studies were found that directly examined the optimal tissue age for inoculation with *R. rhizogenes* to produce hairy roots. However, studies by Cho *et al.* (2000), Fu *et al.* (2005) and Lee *et al.* (2004) used cotyledons two weeks old or less to efficiently induce hairy roots. Another observation was that excised hairy root growth increased on MS basal medium containing only 30 g/L sucrose pH 5.8. The importance of sugar in hairy root induction and secondary metabolite production has been shown by Weathers *et al.* (2004) and Sivakumar *et al.* (2005). Auxin in the induction media did not promote increased hairy root growth. The absence of an affect of auxin on the induction process can be explained by the presence of auxin biosynthesis genes located on the TR-DNA from the *R. rhizogenes* plasmid. Most likely, the tissue had enough endogenous auxin before any was added to the culture medium. From these observations, the optimal infection parameters were established for the next round of *R. rhizogenes* inoculations.

Two to three weeks after advancing the hairy roots to liquid culture, the media turned dark brown and particulate material accumulated around the edge of the culture dish. This observation led to a spectrophotometric analysis

of the media and particulate material. The UV/Visible absorption spectrum of the chloroform-extracted media and particulate material from a SI hairy root culture was compared with that of gossypol extracted from *G. barbadense* roots. This analysis confirmed that the hairy roots produced gossypol and methylated derivatives. Culture media were then prepared for HPLC analysis to compare elution times of gossypol extracted from hairy root media and particulates with that of gossypol standards extracted from *G. barbadense* roots. The hairy root samples had peaks at 254 nm that eluted at the same time as the gossypol standard peaks. The absorbance units for each peak were recorded at 210-700 nm creating a UV/Visible spectrum profile for hairy root media and particulate that was nearly identical to that of gossypol standards.

Indeed, cotton hairy root cultures were easily induced by infecting cotton cotyledonary leaves with *Rhizobium rhizogenes* 15834. Moreover, these cotton hairy roots do produce gossypol and related compounds. Over 100 independently transformed lines (64 from *G. barbadense* and 38 from *G. hirsutum*) have been advanced to liquid culture over the past 3 years and are still producing gossypol and related compounds.

R. rhizogenes-mediated transformation was confirmed by PCR analysis of cotton hairy root tissue and appropriate controls. By using primers designed from the sequences of the *rolB* and *rolC* genes in the TL-DNA of *R. rhizogenes* 15834, *rolB* and *rolC* genes from the genomes of hairy root lines were successfully amplified. PCR analysis using primers specific to *virG*, a nontransferred region of the Ri plasmid showed amplification only in the

positive control (bacterial DNA) and not in DNA isolated from the untransformed or transformed roots. This result shows that the *rol* gene amplification was not due to bacterial contamination and that the *rol* genes were successfully integrated into the plant genome.

Once cultures were initiated and *R. rhizogenes* transformation was confirmed, the next step was to optimize the culture conditions for increased tissue growth and gossypol production. Experiments designed to define these optimal conditions revealed several key variables. Gossypol levels were not greatly influenced by the carbohydrate source added to the medium for cotton hairy roots. A study by Weathers *et al.* (2004) presented evidence that sugars such as glucose and sucrose can be signaling molecules and not just carbon sources. Furthermore, Weathers *et al.* (2004) suggested that autoclaving media causes variable hydrolysis of sugars, and filter sterilization preserves a greater proportion of the sugar molecules. These results further suggest that artemisinin, a sesquiterpene from *Artemisia annua* hairy roots, is produced at significantly higher levels when sucrose is added to the medium than when glucose was used. In contrast, the results of this thesis study indicate that the carbon source of the medium did not have a major influence on the levels of gossypol produced by cotton hairy root cultures. In all cases, the media for cotton hairy root cultures was autoclaved. Repeating this experiment with filter-sterilized media may more closely replicate the study of the Weathers group.

Gossypol production was affected by the culture medium composition. Cultures grown on Gamborg's B5 media produced significantly higher levels of gossypol and related compounds than cultures grown on Murashige-Skoog media with the most significant difference (P=0.0044) being between Gamborg's B5 with 20 g/L sucrose and MS with 20 g/L sucrose. The cultures grown in Gamborg's B5 with 20 g/L sucrose produced the highest average level of gossypol at 1.70%. The lower ammonium to nitrate ratio of Gamborg's B5 could be responsible for the higher gossypol levels. Gamborg's B5 has a lower total nitrogen content of 2634 mg/L in comparison to MS (3550 mg/L). Nitrogen is a major limiting nutrient for most plants, but a low ammonium:nitrate ratio favors the development of hairy roots and secondary metabolite production (Huang *et al.*, 2006; Lourenço *et al.*, 2002; Wang and Tan, 2002). MS has a higher ammonium:nitrate ratio (0.30) than B5 (0.24). Ammonium decreases medium pH which is detrimental for sesquiterpene biosynthesis because pH can alter the regulation of secondary metabolism (Wang and Tan, 2002; Morgan *et al.*, 2000).

Based on preliminary experimentation, optimal hairy root growth occurred at 28ºC, optimal gossypol production occurred at 31ºC and optimal production of methylated forms of gossypol occurred at 34ºC. Despite multiple attempts, these results were never successfully repeated due to several uncontrolled variables such as problems with temperature regulation and culture contamination. Nevertheless, this preliminary experiment suggests that there may be an optimal temperature for hairy root growth and, once a critical

level of biomass has developed, the cultures could be shifted to another temperature to stimulate production of the methylated forms of gossypol.

Harpin is known to elicit a natural defense mechanism in plants by inducing early response components of a signal transduction pathway leading to plant defense responses. This thesis study showed an increase in gossypol content after the addition of harpin to hairy root cultures. This result suggests that the same response to harpin shown by *in planta* tissues occurs in *in vitro* hairy roots as well. Not until 48 hours post-elicitation did gossypol content become significantly higher than the control. This delay may be due to the fact that harpin elicits early response components, and it takes some time before the phytoalexin production increases in response to the preliminary chain of events that must first occur.

T. virens treated cultures resulted in significantly higher levels of gossypol at 24 hours post-elicitation after which a decrease in the gossypol content was observed. This study is the first to elicit gossypol production from hairy roots using *T. virens.* A study by Howell *et al.* (2000) shows that *T. virens* induced terpene biosynthesis and Djonovic *et al.* (2006) showed that a secreted protein, Sm1, from *T. virens* induces the expression of defense-related genes including *CAD1* that encodes an enzyme essential for gossypol biosynthesis. Similarly, gossypol content increased in *T. virens*-inoculated cotton hairy root cultures. Unfortunately, *T. virens* is resistant to gossypol, so its growth will continue uninterrupted (Howell *et al.*, 2000). In fact, the treated cultures did become contaminated with sporulating fungi by 72 hours. As a result,

treatment of hairy root cultures with *T. virens* is not as well-defined a system for inducing gossypol synthesis as harpin or MeJA treatment.

Treatment of the hairy roots with methyl jasmonate (MeJA) proved successful in increasing gossypol levels. Xu *et al.* (2004) found that adding methyl jasmonate to cotton cell suspension cultures strongly induces the transcription of *GaWRKY1* and *CAD1-A,* two genes involved in gossypol biosynthesis, and coordinately increases the production of gossypol and related sesquiterpene aldehydes in *G. arboreum* cells (Xu *et al.*, 2004). *CAD1-A* is a target gene of the transcription factor, GaWRKY1 that participates in the regulation of sesquiterpene biosynthesis in cotton seedlings. The increase in gossypol levels in cotton hairy roots after treatment with MeJA could be explained by this evidence. MeJA has also been suggested to be involved in the up-regulation of enzymes involved in the biosynthesis of other secondary metabolite classes such as alkaloids and phenylpropanoids (Zabetakis *et al.*, 1999). MeJA has previously been used to elicit other phytoalexins (Kittipongpatana *et al.*, 2002; Stojakowska *et al.*, 2002; Furmanowa *et al.*, 2000; Mandujano-Chavéz *et al.*, 2000; Zabetakis *et al.*, 1999; and Kuroyanagi *et al.*, 1998), but this study shows the first evidence for MeJA exerting an influence on gossypol production in cotton hairy root cultures. However, according to simple observation (no data were taken) there appeared to be a slight inhibitory effect on biomass when MeJA was added to cultures. Similar results were also shown in a study of MeJA elicitation of *Beta vulgaris* (common beet) hairy roots

resulted in increased betalaine production despite the poor growth of the culture (Suresh *et al.*, 2004).

In many cases with longer term cultures, gossypol levels decreased in older hairy roots. This phenomenon has been reported in other studies, but an explanation has not been established. There are many factors that could be the cause of this decline in gossypol content after reaching a plateau. These factors could be tested in individual experiments to further explain the findings. Gossypol production could stop due to down-regulation of enzymes in the biosynthetic pathway due to gossypol abundance, nutrient resource depletion, or lowered pH levels in the media, but these explanations do not explain an apparent decrease in gossypol content. A potential explanation could be gossypol biodegradation for use as a carbon source after nutrients are depleted from the media. The gossypol content in the MeJA treated cultures continued to increase which may indicate MeJA preventing gossypol degradation. Another explanation could be release of gossypol into the media. Since gossypol is not water soluble, free gossypol would accumulate in media. On occasion a layer of particles containing gossypol accumulated on the periphery of plastic culture dishes. Quantitative recovery and analysis of this material should reveal whether measurements of total gossypol content should include these materials released from the roots, especially with older cultures.

Investigating the individual cultures for gossypol production showed that some cultures may be better gossypol producers than others and can be useful for future studies that need lower or higher gossypol content. Variability of

gossypol content within 26 cultures of a single transformant was low. This was expected since all 26 cultures are from the same transformation event indicating that they have the same genotype because of the site of integration of T-DNA. Variability of gossypol content within 96 cultures representing 26 distinct transformants was high. This result was expected because there are 26 different transformation events where the TL and TR-DNA of *R. rhizogenes* plasmid could have integrated into the host genome in different places. A study by Baíza *et al.* (1998) reported a difference in alkaloid accumulation between different hairy root lines, but they did not examine hairy root lines from the same transformation events. The Baíza *et al.* study considers the variability of different hairy root lines to be due to chromosomal positional effects of the inserted Ri T-DNA, the length and number of copies stably integrated into the genome, or to genotypic differences of the original plant material. This study did not go into a lot of detail about the origins of the different hairy root lines, they simply referred to them as "different". To date, no other studies have been found in the literature that directly compare secondary metabolite production or growth of hairy root cultures of the same transformation event with those from different transformation events.

Future Work

Now that lines producing high levels of gossypol and related compounds have been identified, bioreactor-scale cultures could be established. These larger-scale cultures could potentially result in large amounts of biomass and yield very large quantities of gossypol. A study by Kim *et al.* (2003) found that

hairy roots grown in mist reactors produce more artemisinin, but bubble column reactors produce more biomass. The gossypol-producing hairy roots of this study are suitable for large-scale culture, but the optimal bioreactor type and exact culture conditions will have to be determined.

Also, future studies could investigate the effect of using different strains of *R. rhizogenes* on cotton hairy root initiation. Ionkova *et al.* (1997) infected *Astragalus mongholicus* (Milk-Vetch) with four strains of *R. rhizogenes*. This work showed differences in the levels of hairy root induction, growth rate of hairy roots, and secondary metabolite production among plants infected with the different strains. The same strain used in the cotton hairy root study, ATCC 15834, as well as strain LBA 9402 resulted in faster growing and larger *Astragalus mongholicus* cultures than those resulting from the other two strains, R 1601 and TR 105. Maximum secondary metabolite production was achieved with strain LBA 9402. Giri *et al.* (2001) used strains A4, 15834, K599, LBA 9402, LBA 9365 and LBA 9340 to induce *Artemisia annua* hairy roots. In their study, strain LBA 9402 resulted in the highest frequency of transformation, *i.e.* hairy roots emerged from each LBA 9402 inoculation site. LBA 9365 produced the highest level of artemisinin, also a sesquiterpene. Vanhala *et al.* (1995) used strains LBA 9402, A4 and 15834 to induce *Hyoscyamus muticus* (Egyptian Henbane) hairy roots. Strain LBA 9402 and 15834 resulted in the highest frequency of transformation and strain A4 resulted in the highest levels of alkaloid accumulation. Tao and Li (2006) used agropine strains R1000, R1601, A4 and R1205 to induce *Torenia fournieri*

(Wishbone Flower) hairy roots and found that strain R1000 resulted in the highest transformation frequency. It may prove beneficial to try additional *R. rhizogenes* strains to induce cotton hairy root formation and potentially increase gossypol production. This thesis project only used strain 15834, but based on the results from other groups, strains LBA 9402, LBA 9365 and A4 might be good candidates to further optimize a gossypol-producing, cotton hairy root system.

Another approach that might be beneficial would be to induce transcription of the *vir* region by pre-treating *R. rhizogenes* with acetosyringone (Vanhala *et al.*, 1995; Giri *et al.*, 2001). Acetosyringone is a phenolic compound that induces transcription of the virulence genes. Giri *et al.* (2001) showed that for all the *R. rhizogenes* strains tested for *A. annua* transformation, the addition of acetosyringone during co-cultivation reduced the time required for hairy root induction. In contrast, Vanhala *et al.* (1995) showed that the co-cultivation with acetosyringone did not affect hairy root induction rate for any *R. rhizogenes* strains tested for *H. muticus* transformation. These reports have different conclusions, but the plant species is probably a key factor in the difference. *H. muticus* may produce sufficient acetosyringone for *virG* activation, whereas *A. annua* may produce less acetosyringone when infected and an exogenous source assists the process. This line of investigation would be worth examining in order to more quickly induce hairy root formation.

Observing the effect of different hormones on cotton hairy roots could result in further optimization of the culture system for gossypol production.

Many studies including that of Weathers *et al.* (2005) reported increased secondary metabolite levels in hairy roots stimulated with various phytohormones. The study by Weathers *et al.* (2005) examined the effect of auxins, cytokinins, ethylene, gibberellins (GA) and abscisic acid (ABA) on growth and sesquiterpene production in *A. annua* hairy roots. Root elongation was stimulated by GA, ABA resulted in increased biomass and the cytokinin, 2iP, stimulated sesquiterpene production. Since gossypol is also a sesquiterpene, evaluating the effects of cytokinins on gossypol production by cotton hairy roots should be given a high priority for future experiments.

A study by Baíza *et al.* (1998) reported optimal alkaloid accumulation in Jimsonweed (*Datura stramonium*) hairy roots that had advanced to a slow growth stage. The methods used in the this study could also be used for the cotton hairy root culture system in order to determine to optimal time for extraction from the hairy roots that would yield the highest levels of gossypol and methylated derivatives.

In addition to PCR amplification of *rol* genes to verify *R. rhizogenes* transformation, some studies examine opine synthesis as yet another means of verification. In this thesis project, an agropine strain of *R. rhizogenes*, 15834, was used. Agropine strains contain both TL and TR-DNA. The TR-DNA does not always get transferred into the plant genome with the TL-DNA resulting in hairy roots that are not as prolific as those resulting from TL and TR-DNA transfer. Hairy roots that do not contain the TR-DNA do not synthesize opines

or auxins (Tempé and Casse-Delbart, 1989). Screening for the opine, agropine, would be another reliable marker for transformation in this study.

Methyl and di-methyl gossypol standards have been prepared recently (Dowd and Pelitire, 2006). To date, analyses of gossypol content assumed that the gossypol standard curve was an appropriate measure of methyl gossypol and di-methyl gossypol contents. Recent results indicate that the extinction coefficients of methylated forms vary slightly from gossypol (Dowd and Pelitire, 2006). As a result, the levels of the methylated forms of gossypol reported in this thesis may be slightly different. Furthermore, a slightly varied HPLC analysis using a Schiff's base resulting from the reaction of 2-amino-1-propanol and gossypol instead of 3-amino-1-propanol could separate (+) and (-) gossypol isomers giving a chromatogram with six peak elution times instead of three (Recommended Practice BA 8a-99, Sampling and Analysis of Oilseed By-Products).

Conclusions

Establishing optimal growing conditions and gossypol levels produced by cotton hairy root cultures under these conditions will benefit future investigations of gossypol-producing cotton hairy roots. The cotton hairy root culture system actively produces gossypol and related compounds and is easily maintained in the laboratory without the need to grow plants in the greenhouse or field. According to the HPLC analysis of 18 large-scale cultures, cottonseed gossypol content of SI and DPL90 varieties do not act as predictors of gossypol content or distribution in the hairy root cultures. Gossypol production was

highly variable among cultures from the different transformation events even though they originated from the same species of cotton. Also, large-scale cultures originating from *Gossypium barbadense* Sea Island did not produce the highest levels of total gossypol or methylated gossypol. This finding was not expected based on the HPLC analysis of Sea Island seed gossypol content which has a higher methylated gossypol production than other varieties. Differences between hairy root gossypol production and cotton root and seed gossypol content will have to be examined further.

Using biotechnology to investigate the gossypol biosynthetic pathway could lead to manipulation of genes to alter the amount and types of gossypol produced by cotton in different parts of the plant. This novel approach could help to create a transgenic cotton plant with gossypol-free seed and normal glanded foliage. This culture system also produces gossypol and related compounds in quantities sufficient for medicinal uses. As mentioned above, if the system could be scaled-up and cultures selected that produce predominately one form of gossypol, even larger quantities of purified (+) or (-) gossypol and the methylated derivatives of gossypol could be provided for biomedical research. Finding a high-value use for these secondary metabolites will drive research to find a cost-effective method to recover gossypol from cottonseed, currently a low-value product that is fed to cattle.

Finally, during the course of this investigation we learned of the interest of several other investigators to use cotton hairy root cultures as a tool for studying cotton resistance to nematode infection. Reniform nematode, root-

knot nematode and soybean cyst nematode cost growers a significant percentage of their cotton crop each year. Data from the National Cotton Council show that the average annual yield loss of cotton due to nematodes has been 2.13% over the last 50 years. A recent article in *The Delta Farm Press* indicated that "nematodes continue to cost cotton producers more in chemical control, direct damage, and indirect losses than any other plant disease." The estimated losses due to nematode damage in 2004 were over $400 million, or greater than one million bales of cotton. The molecular basis for nematode resistance can be studied more easily in axenic culture of cotton hairy roots than in plants grown in soil. Better control on the number of nematodes infecting the plant tissue and fewer complications from other soil organisms are two of the advantages for using cotton hairy root cultures. This biotechnological approach may result in transgenic cotton plants that can withstand nematode infection in the field in the near future.

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