Developing disease resistance in *Colocasia esculenta* L. Schott through *Agrobacterium tumefasciens*-mediated transformation with a stilbene synthase gene, *vst*1

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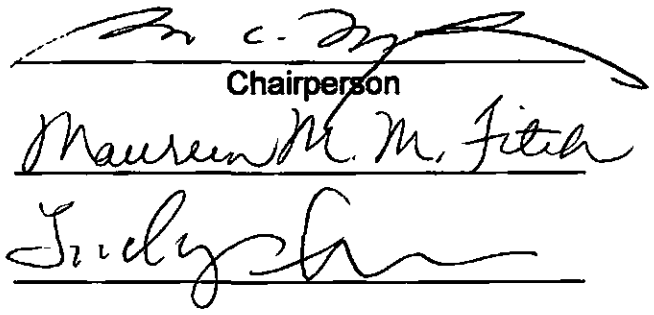

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1.1 Importance of Taro, Colocasia esculenta

1.1.1 Taro as an important food crop

Taro, *Colocasia esculenta* (L.) Schott, is a member of the Araceae family and is thought to be native to the lowland wetlands of Malaysia and estimated to have been in cultivation in India before 5000 BC. From India, taro was transported westward through India to Egypt, into East and West Africa and then to the Caribbean and America. It also spread eastward from its origin to Southeast Asia, eastern Asia, and the Pacific Islands, reaching Hawaii in 450 AD in the canoes of the earliest Polynesian settlers (Gibson, 1999; Lee, 1999). Today, taro is widely cultivated in tropical areas around the world such as Africa, the West Indies, the Pacific region and Asia and in many areas is depended on as a staple food crop (Lee, 1999).

Colocasia esculenta (L.) Schott is the most important edible species of *Colocasia.* Other edible aroids include *Xanthosoma sagittifolium*, *X. atrovirens*, *X. violaceum*, *Alocasia macrorrhiza*, *Cytosperma chamissonis*, and *Amorphophallus campanulatus* (Plucknett, 1970). All parts of the taro plant can be consumed and it is one of the few major staple foods where both leaf and the underground parts, or corms, are equally important to the human diet (Gibson, 1999; Lee, 1999). Throughout Melanesia and Polynesia, young taro leaves are used as a main vegetable and are boiled, steamed, or covered with coconut cream and wrapped in banana or breadfruit leaves and cooked on a hot stone (Lee, 1999). In Papua New Guinea, 'sepal,' a traditional fermented food is made by mixing cooked grated taro corm with coconut cream and allowing it to ferment at ambient temperature (Gubag et al., 1996). The Hawaiians make a fermented paste-like food from peeled, ground, and boiled corms called 'poi' and also use the leaves as a vegetable.

Both the leaves and the corms contain calcium oxalate crystals and must be cooked before being eaten; usually the corms are less acrid than the leaves (Gibson, 1999; Plucknett, 1970). Taro leaves are classified among the high protein leafy vegetables and contain 4% protein (Parkinson, 1984). In addition, the leaves have a high thiamine content, and are a source of folic acid, vitamin C, riboflavin, and vitamin A (Parkinson, 1984).

Taro corms are consumed for their high starch content and in recent years, the superiority of taro starch over other starches has been confirmed (Lee, 1999). Taro is easily digestible (97%) and has very small starch grains (0.05 – 0.08 μm) which are rich in amylose (28%) and amylopectin (72%) (Cambie and Ferguson, 2003; Sefa-Dedeh and Agvir-Sackey, 2002; Standal, 1984). Because of the high amylose content and its easy digestibility, taro is a good starch for people with digestive problems, allergies, or those sensitive to milk (Plucknett, 1970; Wang, 1983). In addition, it has been extensively used in baby formula and foods throughout the United States (Gibson, 1999; Lee, 1999). As well as being an excellent source of starch, taro corms are also rich in vitamins A, B1, B2, and C, are a good source of potassium, a significant source of dietary protein, and contain calcium and phosphorus (Gibson, 1999; Lee, 1999).

In addition to the value of taro as a nutritious food staple, it may have other health benefits as well. Cambie and Ferguson (2003) investigated the potential of functional foods in the traditional Maori diet and identified the protective chemical constituents of these foods. Taro corms contain three anthocyanins, cyanidin 3-glucoside, pelargonidin 3-glucoside, and cyanidin 3-rhamnoside, which are purported to have antioxidant and anti-inflammatory properties. In addition, they are reputed to improve blood circulation and eyesight, and inhibit cancer cell growth (Cambie and Ferguson, 2003). In Hawaii, where taro corms are consumed as the fermented paste 'poi,' researchers have linked lower incidence rates of colorectal cancer in native Hawaiian populations with their

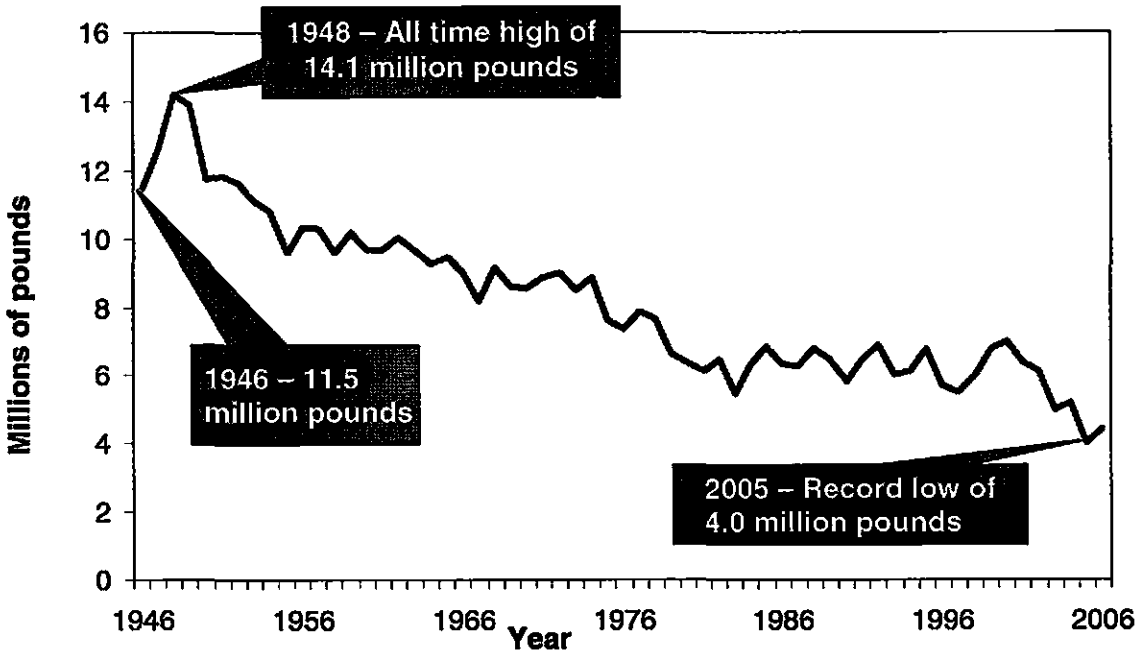
consumption of 'poi' (Brown et al., 2005). Brown et al. (2005) have identified two effects of soluble 'poi' extracts *in vitro* – apoptosis within colon cancer cells and non-specific activation of lymphocytes which can lyse cancer cells.

1.2 Need for Increased Disease Resistance in Taro

1.2.1 Overall effects of fungal and oomycete diseases

Taro production in Hawaii can be limited by numerous adverse conditions, including weather, apple snails, and disease (Hudson et al., 2006). While there are taro diseases caused by a number of pathogens, including viruses, bacteria, and pests, economically, fungal and oomycete diseases are the most significant (Brooks, 2000b; Fullerton and Tyson, 2003; Ooka, 1994; Philemon, 1994) Estimates show that between 25-50% of corm losses are a result of fungal and oomycete diseases (Miyasaka et al., 2001; Philemon, 1994).

Statistics on Hawaii taro production were first recorded in 1946, when low levels, only 11.5 million pounds, were produced as a result of a tidal wave that destroyed fields on Molokai and the Big Island (Figure 1.1) (Martin, 2004). Within two years, the taro industry had rebounded and an all-time high of 14.2 million pounds of taro were marketed in 1948 (Martin, 2004). Over the next 50 years, taro production gradually decreased due to urbanization and two hurricanes. In the mid-90's pests such as apple snails and diseases such as Taro Leaf Blight and Taro Pocket Rot became major problems for farmers (Martin, 2004). In 2005, taro production was estimated at 4.0 million pounds, the lowest total recorded in nearly 60 years (Figure 1.1) (Hudson et al., 2006). According to the Hawaii Agricultural Statistics Service, weather conditions ideal for the proliferation of fungal diseases, including Taro Leaf Blight and Taro Pocket Rot, were largely responsible for the decrease in production (Hudson et al., 2006). Production levels increased by 5% in 2006 to 4.5 million pounds despite inclement weather and pest problems; however production was still well below the five-year average of 5.1 million pounds (Hudson et al., 2007).



Data taken from the National Agriculture Statistics Service

Figure 1.1. Taro Production in Hawaii: 1946-2006.

Fungal and oomycete diseases of taro are currently controlled using several different methods, both chemical and cultural. There is only one fungicide, metalaxyl, that is registered for use in Hawaii for the control of fungal and oomycete diseases (Miyasaka et al., 2001; Ooka and Brennan, 2000). It is applied near the time of planting which limits its efficacy of controlling disease throughout the entire 9 to 11 month cropping cycle (Miyasaka et al., 2001). Cultural methods of control include exclusion – using only disease free planting materials to limit the introduction of disease, sanitation (i.e., removal of diseased plant materials to reduce the spread of disease), and the use of tolerant or resistant cultivars (Fullerton and Tyson, 2003; Ooka, 1994; Uchida et al., 2002).

Alternative methods for disease control, such as the use of composts, soil amendments, solarization, and mulches have been investigated by Miyasaka et al. (2001) for their effects on both yield and incidence of corm rots. They determined that while applications of mulches and organic fertilizers did increase

fresh and dry weight of corms and percentage dry matter of corms, there was also greater incidence of corm rots in those plots (Miyasaka et al., 2001). It was discovered that most of the corm rots were caused by opportunistic microorganisms rather than by corm rot pathogens such as *Pythium sp.* or *Phytophthora colocasiae.* Additionally, at the scale at which the experiment was conducted, the higher cost of organic inputs made their use unprofitable, despite higher corm yields (Miyasaka et al., 2001).

1.2.2 Major fungal and oomycete diseases

There are four major fungal and oomycete diseases that cause corm losses of taro in Hawaii (Ooka, 1994): Taro Leaf Blight, caused by *Phytophthora colocasiae* Rac.; Pythium rot, caused by *Pythium aphanidermatum*, *P. carolinianum*, *P. graminicola*, *P. irregulare*, *P. myriotylum*, or *P. ultimum*; Sclerotium blight, caused by *Sclerotium rolfsii* Sacc; and Pocket Rot of Taro caused by a newly identified species of *Phytophthora* (Uchida, 2003).

1.2.3 Taro Leaf Blight – *Phytophthora colocasiae*

Taro Leaf Blight (TLB) is the most commonly observed and most destructive oomycete disease of taro (Fullerton and Tyson, 2003; Ooka, 1994), capable of causing corm yield losses up to 30% (Miyasaka et al., 2001), leaf yield losses up to 95% (Brooks, 2005) and causing devastating epidemics (Trujillo, 1996). The first reporting of TLB was in Java, Indonesia in 1900 and it has since spread throughout Southeast Asia and the Pacific (Brooks, 2005). Prior to the arrival of TLB to the Hawaiian Islands in 1920, there were nearly 300 different taro cultivars in cultivation (Trujillo, 1996). Today, there are less than 70 of those remaining in cultivation (personal communications, S.C. Miyasaka). The introduction of TLB into American and Western Samoa in 1993 resulted in severe epidemics as a result of the high degree of susceptibility of the native taro

cultivars grown there. By 1996, taro production in the Samoan Islands was nearly nonexistent (Brooks and Utufiti, 2001; Trujillo, 1996).

Taro Leaf Blight is most common during wet, cool weather and is easily spread by water which is also necessary for sporangia germination (Fullerton and Tyson, 2003; Ooka, 1994; Uchida et al., 2002). Initial infection by *Phytophthora colocasiae* is characterized by small dark brown spots on the upper surface of the leaf (Figure 1.2). These spots can appear water soaked and a clear amber colored fluid exudes from their centers. As the spots rapidly enlarge, they take on a zonate appearance as a result of changes in the speed of growth which varies from the warmer days to the cooler nights (Figure 1.2). Sporangia appear along the lesion margins as powdery white fuzz. As the disease progresses, the lesions coalesce and collapse, destroying the entire leaf. The infection can also spread to the leaf petiole and down to the corm where it causes a rot (Brooks, 2005; Fullerton and Tyson, 2003; Ooka, 1994).

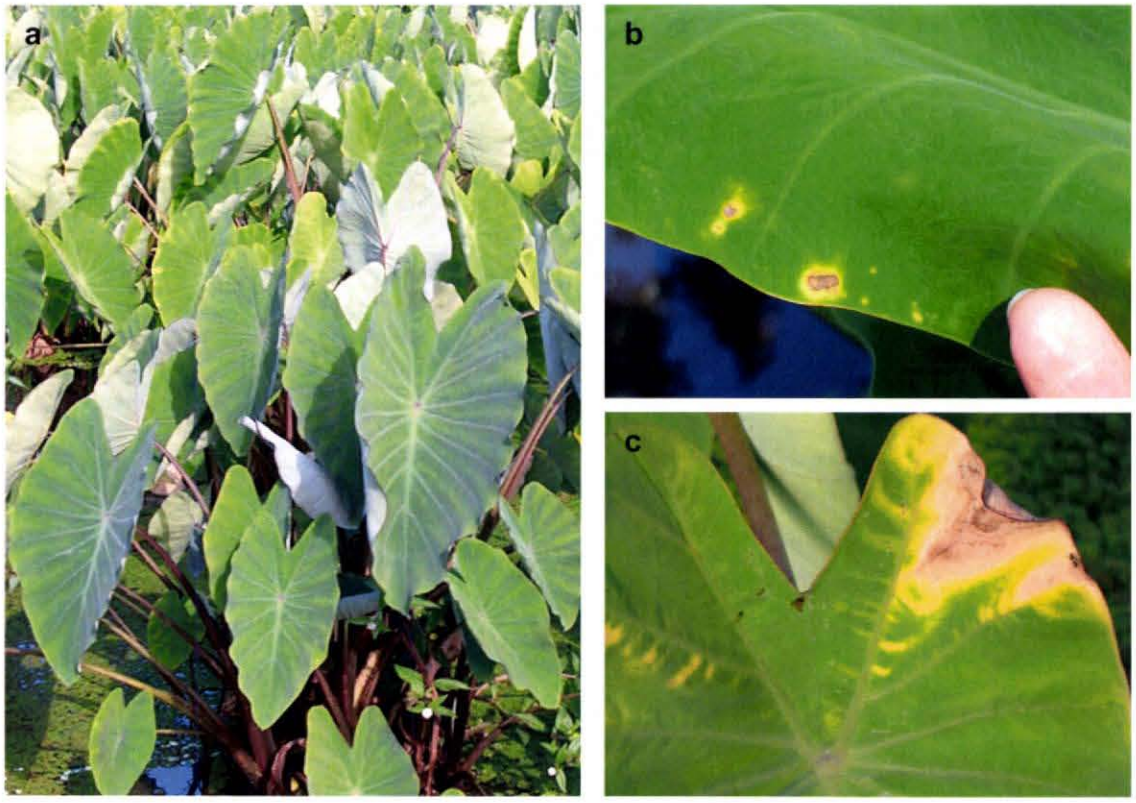


Figure 1.2. a. Healthy *Colocasia esculenta* cultivar 'Bun Long.' b. Initial infection of leaves with *Phytophthora colocasiae*. c. Zonate appearance of lesion as disease progresses.

1.2.4 Pythium rot – *Pythium spp.*

While Pythium rots are probably the most widely distributed diseases of taro, they are not nearly as destructive as TLB (Ooka, 1994). Several species of *Pythium*, including *P. aphanidermatum* Fitzpatrick; *P. graminicola* Subramaniam; *P. splendens* Braun; *P. irregulare* Buisman; *P. myriotylum* Dreschler; P. carolinianum Matthews; and *P. ultimum* Trow have been found on taro (Ooka, 1994). However, it is unknown if all these species are actually pathogenic or if they move in after infection by another pathogen. The disease, which turns the corm into a soft, mushy, and often bad smelling mass, is often a problem in acidic soils that are low in calcium (Trujillo et al., 2002). Additionally, warm, stagnant water and poor field sanitation can contribute to increased incidence of the disease (Ooka, 1994). In wetland taro systems, Pythium rots destroy nearly the entire root system, leaving only a fringe of roots at the top of the corm. Eventually, the main corm dies and lateral cormels develop. Diseased plants appear to be stunted, with short leaf stalks, and curled and crinkled leaf blades which are yellowish and spotted (Ooka, 1994).

1.2.5 Sclerotium blight – *Sclerotium rolfsii* Sacc.

Most commonly found in dryland fields, Sclerotium blight, caused by the soil-borne fungus *Sclerotium rolfsii*, can occur on wetland taro as well (Ooka, 1994; Uchida et al., 2002; Wall, 2000). Sclerotium blight is characterized by a pink rot of the corms which are covered with lots of white, thread-like growths of the pathogen and results in stunted plants (Uchida et al., 2002). The fungus produces numerous sclerotia that are roundish and dark brown to black when mature (Agrios, 1978). Sclerotia are capable of surviving for long periods and control is difficult, relying on deep-plowing to bury surface debris or crop rotation (Agrios, 1978).

1.2.6 Taro Pocket Rot – *Phytophthora sp.*

The causal agent of Taro Pocket rot has been causing problems for taro growers for many years and has been elusive to researchers. The disease is characterized by small to medium sized cavities which form on the taro corm, reducing both corm quality and yield. In 2002, a new species of *Phytophthora* was isolated from pocket rots on taro corms and used to reproduce the disease on healthy taro corms (Uchida, 2003).

1.2.7 Minor fungal pathogens

In addition to the four major fungal and oomycete pathogens of taro mentioned above, there are also several other diseases that affect taro, but usually not severely enough to warrant control measures. Phyllosticta Leaf Spot, caused by *Phyllosticta colocasiphila* Wheedon, is a foliar disease which, in its initial stage, causes small, irregularly shaped, buff to reddish brown spots on the leaves. As the lesions age, they darken and are surrounded by a chlorotic region (Ooka, 1994). A second leaf spot disease, Cladosporium Leaf Spot also infects taro. The pathogen *Cladosporium colocasiae* Sawada infects mainly the older leaves of both wetland and dryland taro. On the upper surface, infected areas are covered with diffuse, light yellow to copper spots, while on the lower surfaces the spots are darker brown (Ooka, 1994). Both of these diseases are relatively harmless to the health of the plant; however they do disfigure the leaves which may reduce their marketability for fresh use.

1.3 Crop Improvement of Taro

1.3.1 Conventional breeding of taro

Improvement in taro genetics through breeding or genetic engineering is necessary to increase resistance to stressful environmental conditions or pests and to increase taro vigor and yield (Cho, 2003). Additionally, there is a market for taros with different colors and tastes for use in the restaurant trade, as well as new ornamental varieties (Cho, 2003). As of 1990, there were only six programs worldwide that were breeding taro or studying its floral habits and genetics according to the Institute for Research, Extension and Training in Agriculture in Western Samoa (Wilson, 1990). In 1998, a breeding program was established at the Maui Agricultural Research Center to improve commercial taros by increasing pest resistance, plant vigor, and yield, and to develop new varieties for the restaurant and landscape trade (Cho, 2003). Additionally, projects to identify and breed taro cultivars with resistance to Taro Leaf Blight (*Phytophthora colocasiae*) are ongoing in American Samoa (Brooks, 2000a; Brooks, 2000b; Hamasaki et al., 2000; Trujillo et al., 2002).

1.3.2 Improvement of yield, vigor

To breed for improvement in yield or vigor of taro cultivars, information on the genetic variability of yield and yield attributing traits is necessary. Dwivedi and Sen (1997) estimated genetic variability of nine quantitative characters for thirty accessions of taro. These characters included length of main sucker, girth of main sucker, total number of side suckers per plant, total number of petioles per clump, number of side tubers per plant, weight of mother tuber per plant, mother tuber/side tubers ratio, average weight of side tuber, and side tubers yield (Dwivedi and Sen, 1997). Their estimates of genetic variability indicated that individual plant selection for three of these traits – total number of petioles per clump, total number of suckers per plant, and girth of main sucker – would be

effective for improvement of these characters (Dwivedi and Sen, 1997). These results agree with Singh and Singh (1985, in Dwivedi and Sen, 1997), who found high values of heritability for girth of main sucker and number of side tubers per plant.

1.3.3 Breeding for disease resistance

Testing to identify taro cultivars that are tolerant or resistant to Taro Leaf Blight (TLB) has been carried out in response to increased incidence of TLB in Hawaii and American Samoa. Cultivars from Palau, the Federated States of Micronesia, Hawaii, the Commonwealth of the Northern Mariana Islands, Guam, and Rota were evaluated for resistance to TLB between 1994 and 1996 in Hawaii (Brooks, 2000a; Brooks, 2000b; Trujillo, 1996; Trujillo et al., 2002). This evaluation indicated that Palau cultivars showed the most promising resistance to TLB and were multiplied using tissue culture (Brooks, 2000a; Trujillo et al., 2002). Disease resistance of these cultivars was attributed to the high water-repellent characteristics of the foliage as well as a hypersensitive reaction where infected leaf areas quickly died and formed a band of dead tissue around the infection site, reducing damage and spread (Trujillo, 1996). However, these cultivars also possess some undesirable characters such as developing suckers, and they have different eating qualities as compared to the traditional Hawaiian taros (Trujillo et al., 2002). These cultivars have also been evaluated in American Samoa and distributed to farmers there, as well as utilized for breeding programs in Hawaii (Brooks, 2000a; Cho, 2003; Hamasaki et al., 2000; Trujillo, 1996; Trujillo et al., 2002).

Trujillo et al. (2002) made crosses between 'Ngeruuch,' a Palauan taro and 'Maui Lehua,' a susceptible Polynesian taro, to combine the Palau taro's resistance to TLB with the desirable eating quality of the Polynesian taro. This breeding program resulted in the release of three promising new cultivars of taro with resistance to TLB. 'Pa'lehua' is a poi cultivar with twice the yield potential of

'Maui Lehua.' The two other cultivars, 'Pa'akala' and 'Pauakea,' may be suitable for production of 'table taro,' taro flour, or other products (Trujillo et al., 2002). These three varieties are the first successful attempt at breeding taro for disease resistance to *P. colocasiae*. However, based on further observations in the field, these varieties are more susceptible to *Pythium* rots, especially after reaching maturity (personal communications, R. Yamakawa).

Additionally, the Leaf Blight Tolerant Taro Variety Project was established in Hawaii to demonstrate to growers that TLB could be managed using disease tolerant or resistant cultivars (Hamasaki et al., 2000). Disease resistant Palauan taro varieties were introduced to growers and gained acceptance slowly but increasingly for uses as Polynesian table taro, moderate adoption as luau, and their use as a blend in poi processing is slowly but steadily increasing (Hamasaki et al., 2000).

1.4 Tissue culture and genetic transformation

An efficient and productive tissue culture system of taro is desirable for several reasons. Traditional taro production is done vegetatively by corms or cormels with 10% of the previous crop saved and used for the propagation of the next one (de la Pena, 1983; Malamug et al., 1992; Sabapathy and Nair, 1992). Diseases such as Taro Leaf Blight, Pythium corm rot, Sclerotium blight, Bacterial Soft Rot (*Erwinia carotovora*), and *Dasheen mosaic virus* (DsMV) are often spread through these planting materials, traditionally called *huli* (Ooka, 1994; Uchida et al., 2002). Tissue culture systems can provide a means of rapidly producing large quantities of healthy, genetically identical propagules for cultivation, eliminating diseases and allowing for complete harvests. Both fungal and bacterial diseases can be eliminated from *huli* through the use of sanitary cultural methods in the field, but viral diseases such as DsMV must be eliminated *in vitro* (Fukino et al., 2000). There are no taro varieties known to be immune to

DsMV (Ooka, 1994; Philemon, 1994) which is an aphid-vectored virus that decreases corm yields by up to 60% (Harding et al., 2003; Malamug et al., 1992; Ooka, 1994). Shoot tip culture has been used to eliminate the disease from taro, but the problem of quick re-infection in the field remains (Fukino et al., 2000; Hartman, 1974).

Establishment of effective tissue culture systems for taro has been achieved by several researchers by altering types and levels of cytokinins and auxins, and through the use of various media supplements. Cytokinins such as 6-benzyladenine (BA), N-3-methyl-2-butenyl-1H-purine-amine (2ip), and kinetin in combination with various auxins, including naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and trichlorophenoxyacetic acid (2,4,5-T), have been utilized to initiate and proliferate taro callus from shoot explants, axillary buds, and etiolated shoots (Fukino et al., 2000; Malamug et al., 1992; Murakami et al., 1995; Sabapathy and Nair, 1995; Yam et al., 1991; Yam et al., 1990a; Yam et al., 1990b). By altering the ratio of cytokinins to auxins, initiation of shoots from taro callus is possible. In addition, the use of various extracts has been found to enhance callus production and shoot initiation.

Establishing protocols for callus induction and plant regeneration *in vitro* is desirable for further research on transformation of taro with genes useful for improving yields, horticultural quality, or disease resistance. Reports of genetic transformation of taro in the literature are limited to a report by Fukino et al. (2000) of transformation via particle bombardment. They successfully transformed the triploid taro cultivar, 'Equimo,' with plasmid pREXHGUS containing a hygromycin resistance gene and the β-glucuronidase (GUS) reporter gene (Fukino et al., 2000). However, the efficiency of their transformation protocol was very low, yielding only 2 transformants from 96 bombardment attempts (Fukino et al., 2000). Recently, a rice chitinase gene was introduced into taro, also via particle bombardment (He, 2006). The efficiency of this transformation protocol was similarly low, with only 1 confirmed transformant from 30 attempts (He, 2006). Clearly, if genetic transformation is to be an

effective means of introducing positive characteristics to taro, then more efficient protocols for bombardment and use of other transformation methods need to be established. A means of efficiently transforming taro callus via *Agrobacterium tumefasciens* has been established by researchers at the University of Hawaii (He, 2006).

1.5 Engineering Fungal and Oomycete Resistance in Plants

1.5.1 Defense mechanisms

Plant defense mechanisms fall into two categories – constitutive, meaning that the mechanism is always there, such as physical barriers and constitutive compounds, and those that are induced or turned on upon exposure to the pathogen (Greenberg, 1997). Constitutive compounds shown to have antifungal activity include saponins, cyanogenic glycosides, and glucosinolates (Osbourn, 1996). Inducible resistance mechanisms are dependent on recognition of the pathogen by the host plant and its subsequent responses and are often exploited to engineer disease resistance in plants (Greenberg, 1997). These include the hypersensitive response (HR), production of active oxygen species, local production of pathogenesis-related (PR) proteins and other antifungal proteins, all of which lead to the final step of systemic acquired resistance (SAR) (Grover and Gowthaman, 2003).

The first step in a plant-pathogen interaction is the mutual recognition between the host plant and the pathogen involving interaction between their respective resistance and avirulence genes (Grover and Gowthaman, 2003). The "gene-for-gene" model of plant resistance was proposed by Flor in the 1940's based on his work on interactions between flax (*Linum usitatissiumum*) and the fungal rust pathogen *Melamspora lini* (Hammond-Kosack and Jones, 1997). This model proposes that complimentary pairs of dominant genes in both the host and the

pathogen are necessary for resistance to occur. Cases where no disease (incompatibility) occurs require a dominant resistance (R) gene in the host and a corresponding avirulence (Avr) gene in the pathogen. If either gene is lost, disease (compatibility) results. It is thought that the product of the host plant's R gene is a receptor that recognizes elicitors encoded by the pathogen's Avr genes (Staskawicz et al., 1995). Upon recognition, the host plant's first response to a pathogen is localized cell death at the site of the infection to restrict further pathogen growth. This is known as the hypersensitive response (HR) and can often be correlated with resistance (Grover and Gowthaman, 2003).

1.5.2 Engineering resistance

More than 30 R genes conferring resistance to a wide range of pathogens have been cloned. One method of creating resistance is by expressing an R gene from a resistant plant in a susceptible plant. For example, expression of the tomato R gene *pto* in tobacco conferred resistance to *Pseudomonas syringe* pv *tabaci* that expressed *avr pto* (Grover and Gowthaman, 2003). A second means, known as the gene-avirulence gene two-component system, involves expressing both the R gene and the Avr gene in the plant, both under pathogen-inducible promoters (Grover and Gowthaman, 2003). Upon infection, HR will be activated. This system would provide broader spectrum resistance because HR will be activated by any pathogen that induces the promoter (Grover and Gowthaman, 2003).

Development of transgenic plants that constitutively or on induction produce antifungal compounds, such as pathogenesis related (PR) proteins or phytoalexins, is another method for engineering fungal disease resistance in plants (Honee, 1999). Of the PR proteins, chitinases and glucanases are those which have been most widely used to develop transgenic resistant plants (Punja, 2004). They inhibit fungal growth by degrading chitins and glucans which make up a large part of fungal cell walls (Grover and Gowthaman, 2003; Punja, 2004).

Other PR proteins include ribosome-inactivating proteins, small cysteine-rich proteins, lipid transfer proteins, storage albumins, polygalacturonase inhibitor proteins (PGIPS), and antiviral proteins (Grover and Gowthaman, 2003). The production of phytoalexins, which are low molecular weight antimicrobial compounds produced at the infection site and thought to be part of plant defense, can also be utilized by developing plants that constitutively or inducibly express key regulatory genes in the phytoalexin biosynthesis pathways (Grover and Gowthaman, 2003; Punja, 2004). This approach has been met with success and will be discussed in greater detail below.

1.6 Phytoalexins

1.6.1 Introduction

The phytoalexin theory was first put forth by Muller and Borger (1940) who demonstrated that potato tubers that had been previously infected with an avirulent strain of *Phytophthora infestans* were resistant to subsequent infection with a virulent one. This induced resistance was hypothesized to be a result of accumulation of antifungal compounds in the tissue in response to the incompatible interaction (Hammerschmidt, 1999). These compounds, subsequently named phytoalexins, have been defined as antimicrobial, low-molecular-weight secondary metabolites that are synthesized *de novo* and accumulate in plants as a result of infection or stress (Hammerschmidt, 1999; Kuc, 1995; Purkayastha, 1995). The term phytoalexin is a combination of the Greek words *phyton*, meaning plant, and *alexin*, meaning warding off compound (Purkayastha, 1995).

Over 350 phytoalexins have been isolated and characterized from at least 30 plant families, including both monocots and dicots, and from all plant tissues (Kuc, 1995). While being diverse in both antimicrobial activity and structure,

phytoalexins are, in general, lipophilic and localized at and around infection sites (Kuc, 1995). Phytoalexin precursors arise from the three major biosynthetic pathways found in all plants; the shikimate acid, acetate-malonate, and acetate-mevalonate pathways, either from a single pathway or a combination of two or three (Kuc, 1995). This facilitates genetic engineering of plants with novel phytoalexin genes because the precursor molecules are already synthesized by the plant and insertion of a single gene, usually a phytoalexin synthase, results in production of the desired phytoalexin. There are several lines of evidence that support a role for phytoalexins in plant defense, rather than being merely a response to infection, including localization and timing studies, correlation between phytoalexin production and incompatible interactions, association of phytoalexin accumulation with resistance genes, metabolic inhibitor studies, relationship between pathogen virulence and tolerance of phytoalexins, and increases in resistance by stimulation of phytoalexin production prior to infection (Hammerschmidt, 1999). This review will focus on evidence related to phytoalexins from the *Vitaceae*, specifically resveratrol (*trans*-4,3',5'-trihydroxystilbene), in terms of its structure, synthesis, and role in plant defense mechanisms.

1.6.2 Phytoalexins from the *Vitaceae*

Phytoalexins from the *Vitaceae* belong to the stilbene family and are 14-carbon phenolic molecules based on a *trans*-resveratrol skeleton (Dercks et al., 1995; Hart, 1981; Jeandet et al., 2002). These include resveratrol (*trans*-4,3',5'-trihydroxystilbene), the predominant stilbene produced; two oligomers produced in the next highest amounts, α-viniferin and ε-viniferin; and pterostilbene (3,5-dimethoxy-4'-hydroxystilbene), produced in very small quantities (Figure 1.3) (Dercks et al., 1995). Recently, an isomer of ε-viniferin, called δ-viniferin was identified and shown to be one of the major stilbenes produced from resveratrol oxidation in grapes (Figure 1.3) (Pezet et al., 2003). In addition, resveratrol can be glycosylated into various forms such as piceid (5,4'-dihydroxystilbene-3-*O*-β-

glucopyranoside) (Figure 1.3) (Jeandet et al., 2002). Stilbenes can exist as both *trans-* and *cis-* isomers which have different chemical characteristics and biological activities (Hart, 1981). The *trans-* isomers are usually more stable and tend to dominate in plant tissues (Hart, 1981). Additionally, under ultraviolet (365 nm) light, stilbenes emit a characteristic bright blue fluorescence (Dercks et al., 1995; Hart, 1981; Langcake and Pryce, 1976).

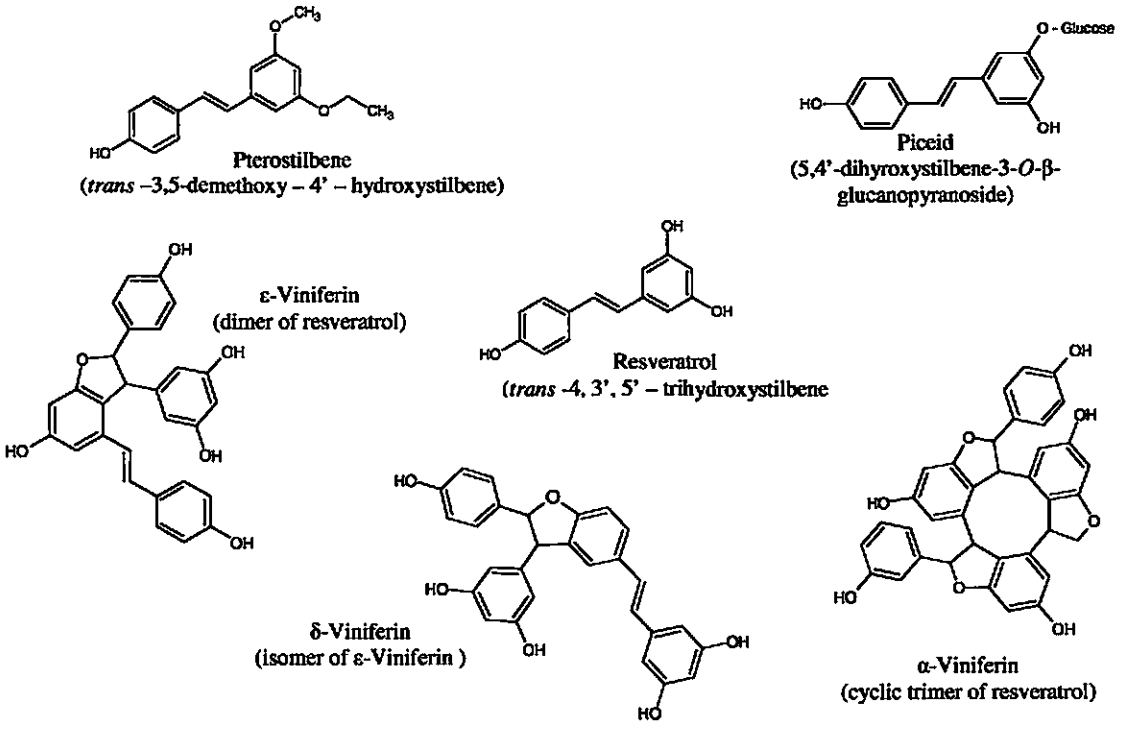


Figure 1.3. Stilbene compounds from the *Vitaceae*

The production of stilbenes is controlled by the enzyme stilbene synthase (EC 2.3.1.95), part of a multigenic family of genes that convert three molecules of malonyl-CoA and one molecule of *p*-coumaroyl-CoA to resveratrol (Figure 1.4) (Coutos-Thevenot et al., 2001; Jeandet et al., 2002). These precursors, found in all plants, arise from the shikimic acid pathway. Resveratrol derivatives, such as α-viniferin and ε-viniferin, are synthesized by oxidative dimerization of resveratrol (Jeandet et al., 2002). Pterostilbene is produced by dimethylation of resveratrol (Jeandet et al., 2002).

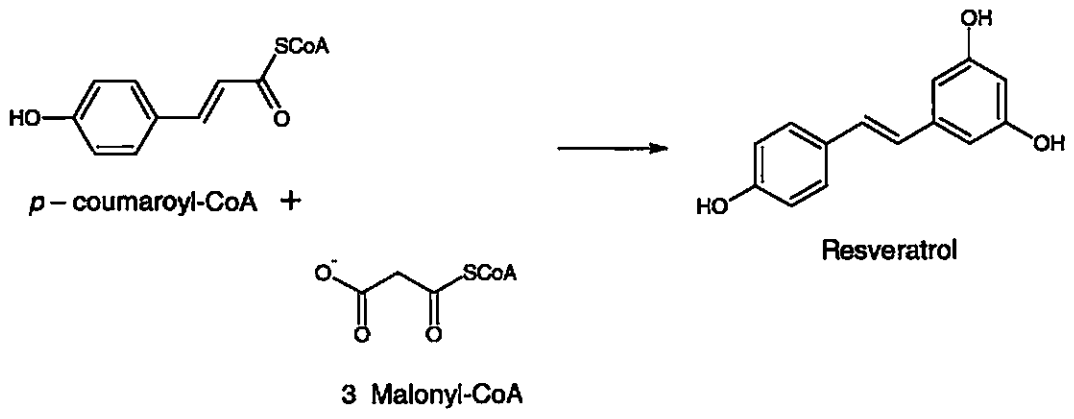


Figure 1.4. Reaction catalyzed by stilbene synthase to form resveratrol from 1 molecule of *p* – coumaroyl-CoA and 3 molecules of malonyl-CoA.

By definition, phytoalexins have antimicrobial properties, and stilbenes are no exception, being biologically active against various fungal pathogens of grapevine. Resveratrol is more active against spore germination than mycelial growth for the grapevine pathogens *Plasmopara viticola* and *Botrytis cinerea,* with EC_{50} values ranging from 71 to over 200 ug ml^{-1} (Dercks et al., 1995). Additionally, resistance to *P. viticola* has been positively correlated with grapevine's ability to synthesize resveratrol – the most resistant cultivar was able to accumulate five times the level of resveratrol as the susceptible cultivars (Dercks and Creasy, 1989). Recent work has shown that it is actually modification of resveratrol, via oxidation or glycosylation, which influences antifungal activity (Pezet et al., 2004). Resistant cultivars accumulated resveratrol initially, and then it was converted to δ-viniferin and ε-viniferin whereas in susceptible cultivars it was converted to piceid (Pezet et al., 2004). Both δ-viniferin and ε-viniferin have been shown to be highly toxic to both *P. viticola* and *B. cinerea* spores, with δ-viniferin being the more toxic of the two compounds (Pezet et al., 2004; Pezet et al., 2003).

1.6.3 Transformation with stilbene synthase genes

Stilbene synthase genes have been isolated from *Vitis vinifera* and *Arachis hypogea* and used to transform plants for the purpose of engineering fungal disease resistance (Coutos-Thevenot et al., 2001; Fettig and Hess, 1999; Hain et al., 1990; Hain et al., 1993; Hipskind and Paiva, 2000; Husken et al., 2005; Leckband and Lorz, 1998; Richter et al., 2006; Serazetdinova et al., 2005; Stark-Lorenzen et al., 1997; Szankowski et al., 2003; Thomzik et al., 1997; Zhu et al., 2004). Hain et al. (1990) were the first to show that tobacco plants transformed with a stilbene synthase gene from *A. hypogea* were able to synthesize resveratrol. Later, Hain et al. (1993) transformed tobacco with the stilbene synthase genes *vst*1 and *vst*2 with their own pathogen-inducible promoters isolated from *V. vinifera* and showed that resistance to *Botrytis cinerea* resulted from expression of these genes and subsequent resveratrol accumulation. Since then, stilbene synthase genes, under both constitutive and pathogen-inducible promoters have been transformed into tomato (Thomzik et al., 1997), rice (Stark-Lorenzen et al., 1997), barley (Leckband and Lorz, 1998), wheat (Fettig and Hess, 1999; Leckband and Lorz, 1998; Serazetdinova et al., 2005), alfalfa (Hipskind and Paiva, 2000), grapevine rootstock (Coutos-Thevenot et al., 2001), apple (Szankowski et al., 2003), white poplar (Giorcelli et al., 2004), papaya (Zhu et al., 2004), oilseed rape (Husken et al., 2005), and peas (Richter et al., 2006). These transformations have resulted in varying degrees of increased resistance to fungal pathogens.

Transgenic tomatoes expressing two stilbene synthase genes, *vst*1 and *vst2*, controlled by their own pathogen inducible promoters, showed increased resistance to *Phytophthora infestans*, but not to *Botrytis cinerea* or *Alternaria solani* (Thomzik et al., 1997). Interestingly, upon infection with both *B. cinerea* and *A. solani*, resveratrol was accumulated although it had no impact on the pathogens (Thomzik et al., 1997). Tobacco plants transformed with the same

genes and promoters, however, did show increased resistance to *B. cinerea* (Hain et al., 1993).

Monocots transformed with stilbene synthase genes include rice (Stark-Lorenzen et al., 1997), barley (Leckband and Lorz, 1998), and wheat (Fettig and Hess, 1999; Leckband and Lorz, 1998; Serazetdinova et al., 2005). The first researchers to do so, Stark-Lorenzen et al. (1997), transformed rice protoplasts with the *vst*1 gene and demonstrated that it, under the control of its own inducible promoter, behaved in transgenic rice as had been described in dicots (Hain et al., 1993; Thomzik et al., 1997). The transgenic rice plants also showed enhanced resistance to the rice blast fungus *Pyricularia oryzae* (Stark-Lorenzen et al., 1997). Barley was transformed via particle bombardment with two different constructs containing the *vst*1 gene, one including only the *vst*1 pathogen-inducible promoter, and the second with the same promoter preceded by a 4-fold enhancer under control of the CaMV 35S promoter (Leckband and Lorz, 1998). Only transgenic barley plants including the 4-fold enhancer accumulated resveratrol and showed increased resistance to *B. cinerea* (Leckband and Lorz, 1998).

Leckband and Lorz (1998) and Fettig and Hess (1999) transformed wheat with the *vst*1 gene with conflicting results. Despite also including the 4-fold enhancer sequence when transferring the *vst*1 gene to wheat, Leckband and Lorz (1998) did not report accumulation of resveratrol or increase in disease resistance. Later, Fettig and Hess (1999) were able to again transfer the *vst*1 gene, although this time using the maize ubiquitin promoter, and also to confirm expression via RT-PCR and to detect resveratrol using HPLC and mass spectrometry. Neither pair of researchers reported any increase in disease resistance in wheat (Fettig and Hess, 1999; Leckband and Lorz, 1998). Recently, Serazetdinova et al. (2005) transformed wheat with either *vst*1 or *vst*2 under control of their endogenous pathogen-inducible promoters. Transgenic plants expressing either gene accumulated unknown stilbene derivatives, one of which was very similar to

resveratrol but was more hydrophilic (Serazetdinova et al., 2005). The synthesis and accumulation of these compounds in the transgenic plants reduced the disease symptoms of two wheat pathogens – *Puccinia recondita* f.sp. *tritici* and *Septoria nodorum* Berk. (Serazetdinova et al., 2005).

Grapevine rootstock transformed with the *vst*1 gene under control of its own pathogen inducible promoter showed over-accumulation of resveratrol and *in vitro* tolerance to *Botrytis cinerea* (Coutos-Thevenot et al., 2001). In apple, transgenic lines carrying the *vst*1 gene and pathogen-inducible promoter were engineered and shown to accumulate the resveratrol glucoside, piceid (Ruhmann et al., 2006; Szankowski et al., 2003). The intention was to increase resistance to apple scab, caused by *Venturia inaequalis*, however there have been no reports thus far of increased resistance (Szankowski et al., 2003). However, *in vitro* studies have shown that both resveratrol and piceid inhibit *V. inaequalis* appressoria penetration and piceid also inhibits spore germination at levels that could realistically be produced *in vivo* (Schulze et al., 2005). Giorcelli et al. (2004) transformed white poplar with the *vst*1 gene under control of the CaMV 35s promoter. Transgenic white poplar plants accumulated both the *trans*- and *cis*-isomers of piceid but did not show increased resistance to the rust fungus *Melampsora pulcherrima* (Giorcelli et al., 2004). In papaya transformed with *vst*1 and its pathogen inducible promoter, a resveratrol-glycoside was produced and accumulated at a level which conferred increased resistance to *Phytophthora palmivora* (Zhu et al., 2004).

Overall, transformation with any combination of the two stilbene synthase genes from *V. vinifera* with either pathogen-inducible or constitutive promoters can sometimes result in increased disease resistance. Closely related tobacco and tomato plants transformed with the same construct (*vst*1 and *vst*2 with their own promoters) and accumulating resveratrol did not show increased resistance to the same pathogens. In fact, transgenic tobacco plants were more resistant to *B. cinerea*, whereas transgenic tomato plants showed no increase of resistance to

B. cinerea, but were more resistant to *Phytophthora infestans* (Hain et al., 1993; Thomzik et al., 1997). Among the monocots that have been transformed, rice plants transformed with *vst*1 under its own pathogen-inducible promoter showed increased resistance to *Pyricularia oryzae* (Stark-Lorenzen et al., 1997). Leckband and Lorz (1998) supplemented this construct with a 4-fold enhancer controlled by the CaMV 35S promoter and reported that transgenic barley plants were more resistant to *B. cinerea*. They also successfully transformed wheat, but did not report any accumulation of resveratrol or disease resistance (Leckband and Lorz, 1998). Further work on transforming wheat using the *vst*1 gene with the maize ubiquitin promoter resulted in production of resveratrol, but no resistance tests were performed (Fettig and Hess, 1999). Finally, Serazetdinova et al. (2005) transformed wheat with either *vst*1 or *vst*2 under their own pathogen-inducible promoters and reported accumulation of unknown stilbene compounds and reduced disease symptoms of *P. recondita* and *S. nodorum*.

In cases where resveratrol is the final product accumulated and resistance tests were performed, transgenic plants did show increased or enhanced resistance to their respective fungal pathogen. This is true for grapevine rootstock, tobacco, barley, rice, and tomato (Coutos-Thevenot et al., 2001; Hain et al., 1993; Leckband and Lorz, 1998; Stark-Lorenzen et al., 1997; Thomzik et al., 1997). However, the final product accumulated in some plants transformed with *Vitis* stilbene synthase genes is often not resveratrol, but a resveratrol-derivative, usually formed when resveratrol binds to a sugar, such as glucose or hexose (Szankowski et al., 2003). These resveratrol derivatives have varying effects of disease inhibition. Piceid was accumulated in white poplar, tomato, and kiwi plants transformed with stilbene synthase genes under control of the CaMV 35S promoter (Giorcelli et al., 2004; Giovinazzo et al., 2005; Kobayashi et al., 2000). Neither white poplar nor kiwi showed increased resistance to their respective fungal pathogens (Giorcelli et al., 2004; Kobayashi et al., 2000); the transgenic tomato plants were not subjected to testing (Giovinazzo et al., 2005). However,

alfalfa transformed with an *A. hypogea* stilbene synthase gene also accumulated piceid and showed significantly decreased symptoms caused by *Phoma medicaginis* (Hipskind and Paiva, 2000). Transgenic papaya plants accumulated a resveratrol-glycoside, likely piceid, and showed increased resistance to disease caused by *P. palmivora* (Zhu et al., 2004).

Stilbene synthase genes have also been used to create novel phenotypes or to increase the nutritive value of a crop. Overexpression of the *vst*1 gene under control of a duplicated CaMV 35S promoter in tobacco resulted in changes in flower color in the transgenic plants, indicating that stilbene synthase genes have potential for use in engineering cultivars with altered flower color (Fischer et al., 1997). By directing expression of *vst*1 to the anthers through use of a tapetum-specific promoter, Fischer et al. (1997) were able to engineer male sterility in tobacco plants. This technique has potential for development of a novel hybrid system. Giovinazzo et al. (2005) transformed tomato plants with *vst*1 and *vst*2 under control of the CaMV 35S promoter with the goal of improving overall antioxidant properties of the fruit. Regenerated transgenic plants were shown to accumulate resveratrol and resveratrol-glucopyranoside, as well as having an increase in levels of two naturally present antioxidant compounds, ascorbate and glutathione (Giovinazzo et al., 2005). Husken et al. (2005) transformed oilseed rape with the *vst*1 gene under control of the seed-specific napin promoter with the goal of increasing the nutrient value of the seeds. By creating a new metabolic sink with the addition of the *vst*1 gene, substrates were diverted from production of sinapate esters which reduce the quality of rapeseed meal to synthesis of piceid, which is considered beneficial to human health (Husken et al., 2005).

Overall, genetic transformation with stilbene synthase genes and the accumulation of resveratrol or one of its derivatives has shown to be an effective means to develop fungal disease resistance. In addition, expression of stilbene

synthase genes in transgenic plants has given rise to novel phenotypes other than disease resistance.

Plant Engineered	Gene(s) and promoter	Stilbene produced/Effect on Resistance	Reference
Apple (*Malus domestica*)	*Vst1*, native promoter	Piceid; no resistance tests	Ruhmann et al., 2006; Schulze et al., 2005; Szankowski et al., 2003
Barley (*Hordeum vulgare*)	*Vst1*, native promoter with 4x enhancer	Resveratrol; showed tolerance to *Botrytis cinerea*	Leckband and Lorz, 1997
Grapevine (*Vitis vinifera*)	*Vst1*, alfalfa PR-10 promoter	Resveratrol; reduced symptoms of *B. cinerea* and *Eutypa lata*	Coutos-Thevenot et al., 2001
Oilseed rape (*Brassica napus*)	*Vst1*, seed-specific napin promoter	Piceid; diverted substrates from sinapate ester biosynthetic pathway; no resistance tests	Husken et al., 2005
Papaya (*Carica papaya*)	*Vst1*, native promoter	Resveratrol-glycoside; increased resistance to *Phytophthora palmivora*	Zhu et al., 2004
Pea (*Pisum sativum*)	*Vst1*, native promoter	Piceid and an unknown resveratrol derivative; no resistance tests	Richter et al., 2006
Rice (*Oryzae sativa*)	*Vst1*, native promoter	*Vst1* mRNA accumulation; may enhance resistance to *Pyricularia oryzae*	Stark-Lorenzen et al., 1997
Tobacco (*N. tobacum*)	*Vst1* and *Vst1*, native promoter	Resveratrol; more resistant to infection by *B. cinerea*	Hain et al., 1993
Tobacco (*N. tobacum*)	*Vst1*, double CaMV 35s promoter or tapetum-specific *tap1* promoter	Resveratrol; altered flower color and male sterility	Fischer et al., 1997
Tomato (*Lycopersicon esculentum*)	*Vst1* and *Vst2*, native promoters	Resveratrol; increased resistance to *Phytophthora infestans*; no resistance to *B. cinerea* or *Alternaria solani*	Thomzik et al., 1997
Tomato (*L. esculentum*)	*Vst1* and *Vst2*, CaMV 35s promoter	Resveratrol and piceid; no resistance tests	Giovanazzo et al., 2005
Wheat (*Triticum aestivum*)	*Vst1*, ubiquitin promoter	Resveratrol; no resistance tests	Fettig and Hess, 1999
Wheat (*T. aestivum*)	*Vst1*, native promoter with 4x enhancer	No tests for expression or resistance	Leckband and Lorz, 1997
Wheat (*T. aestivum*)	*Vst1* and *Vst2*, native promoters	Unknown stilbene derivatives; reduced symptoms of *Puccinia recondita* f.sp. *tritici* and *Septoria nodorum*	Serazetdinova et al., 2006
White poplar (*Populus alba*)	*Vst1*, CaMV 35s promoter	Piceid; no increased resistance to *Melampsora pulcherrima*	Giorcelli et al., 2004

Table 1.1. Plant species engineered with *Vitis* stilbene synthase genes

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Chapter 2: Genetic transformation of *Colocasia esculenta* using *Agrobacterium tumefasciens* and regeneration of transgenic plants

2.1 Abstract

A system of *Agrobacterium tumefasciens*-mediated transformation and regeneration of taro, *Colocasia esculenta* cultivar 'Bun Long,' was developed. Beginning with micro-shoots isolated from taro corms, multiple shoots were generated and then induced to produce friable callus on medium supplemented with 2 mg l^{-1} 6-benzyladenine (BA) and 1 mg l^{-1}naphthalene acetic acid (NAA). These calli were co-cultivated for 5 time intervals with either of two strains of *A. tumefasciens* (EHA105 and LBA4404) both carrying the plasmid pCNL65. This plasmid contains the *gusA*/intron reporter gene, coding for β-glucuronidase (GUS), and the *nptII* selectable marker gene which confers resistance to the antibiotic geneticin (G418). Transient and stable GUS expression were measured in these calli throughout the selection period using both histochemical and fluorimetric assays. When assessed alone, neither assay was found to be an accurate indicator of transformation efficiency. The highest rate of transformation (22%) was achieved by co-cultivation for 4 days with EHA105. Calli co-cultivated with LBA4404 showed no GUS expression nor were any transgenic lines recovered. After the three month callus selection period, shoots were regenerated and plants proliferated on medium containing 4 mg l^{-1} BA. The presence of transgenes *nptII* and *gusA*/intron in transgenic lines was confirmed by enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) or Southern blot analysis. Development of a taro regeneration protocol and transformation method using *A. tumefasciens* is the first step toward realizing the overall goal of improving disease resistance in taro.

2.2 Introduction

Taro, *Colocasia esculenta* (L.) Schott, is the most important edible aroid species and is widely cultivated in tropical areas in Africa, Asia, and throughout the Pacific Islands (Wang, 1983). All parts of the taro plant can be consumed and it is one of the few major staple foods where both the leaf and the underground parts, or corms, are equally important to the human diet. In Hawaii, where taro production is a multi-million dollar industry, the leaves are used as a vegetable and the corms are made into 'poi.' Traditionally, taro is propagated vegetatively using corms or cormels and 10 percent of a year's crop is saved to plant the subsequent one (de la Pena, 1983). These propagules can harbor pests and diseases that can infect and potentially destroy the new crop. Development of a tissue culture system for taro can provide a means of rapidly producing large quantities of healthy, genetically identical propagules for cultivation, eliminating diseases and allowing for complete harvests. Additionally, establishment of such a system is the first step in development of a protocol for genetic transformation.

In 2005, overall taro production in Hawaii was estimated at 4.0 million pounds, the lowest total recorded in nearly 60 years (Hudson et al., 2006). Fungal and oomycete diseases play a significant role in overall production decreases seen in Hawaii's taro industry. Pathogens such as *Phytophthora colocasiae*, the causal agent of Taro Leaf Blight, cause diseases which stunt plant growth, damage foliage, and slow or halt corm production. Additionally, other corm rot diseases caused by *Pythium spp.* and *Sclerotium rolfsi* can reduce yields and corm quality. Estimates show that between 25 and 50 percent of corm losses are a result of fungal and oomycete diseases (Miyasaka et al., 2001; Philemon, 1994). Fungal and oomycete diseases of taro are currently controlled using several different methods, both chemical and cultural. There is only one fungicide, metalaxyl, that is registered for use in Hawaii for the control of fungal and oomycete diseases (Miyasaka et al., 2001). It is applied near the time of planting which limits its efficacy in controlling disease throughout the entire 9 to 11 month cropping cycle

(Miyasaka et al., 2001). Cultural methods of control include exclusion (i.e. using only disease free planting materials to limit the introduction of disease), sanitation (i.e. removal of diseased plant materials to reduce the spread of disease), and the use of tolerant or resistant cultivars (Fullerton and Tyson, 2003: Ooka, 1994; Uchida et al., 2002).

Establishment of effective tissue culture systems for taro has been achieved by several researchers through altering types and levels of cytokinins and auxins, and through the use of various media supplements. Cytokinins such as 6-benzyladenine (BA), N-3-methyl-2-butenyl-1H-purine-amine, and kinetin in combination with various auxins, including naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and trichlorophenoxyacetic acid (2,4,5-T), have been utilized to initiate and proliferate taro callus from shoot explants, axillary buds, and etiolated shoots (Fukino et al., 2000; Malamug et al., 1992; Murakami et al., 1995; Sabapathy and Nair, 1995; Yam et al., 1991; Yam et al., 1990a; Yam et al., 1990b). By altering the ratio of cytokinins to auxins, initiation of shoots from taro callus is possible. In addition, the use of taro extracts has been found to enhance callus production and shoot initiation (Yam et al., 1991; Yam et al., 1990a, Yam et al., 1990b).

Advances in genetic engineering of plants offer a means to ameliorate problems such as yield loss caused by fungal and oomycete pathogens through transformation with novel disease resistance genes. By developing an efficient transformation and regeneration system for taro, new opportunities to develop resistant cultivars arise. Fukino et al.'s (2000) transformation protocol using particle bombardment was the first cited effort, but resulted in only two transformants from 96 attempts. Attempts to use particle bombardment by researchers in our lab resulted in a low success rate – only 1 confirmed transformant from 30 attempts (He, 2006). Recently, a method for transforming taro callus via *Agrobacterium tumefasciens* was developed (He, 2006). Using friable callus generated from micro-shoot tip explants and co-cultivated with *A. tumefasciens* strain EHA105, transgenic taro plants were produced.

We report results from comparisons between two *A. tumefasciens* strains and time intervals for co-cultivation for the purpose of developing a highly efficient taro transformation protocol. Histochemical and fluorimetric assays for GUS expression were performed throughout the selection period to assess transient and stable GUS expression. Finally, polymerase chain reaction and Southern blotting were used to confirm the presence of the transgenes *gus*(*uida*) and *npt*II in the regenerated transgenic plants.

2.3 Materials and Methods

Initiation of embryogenic callus in tissue culture

Shoot tip explants of *Colocasia esculenta* 'Bun Long' and 'Maui Lehua' were treated according to the methods of Hartman (1974) to eliminate *Dasheen mosaic virus* (DsMV). Under a dissecting microscope, micro-shoot explants between 0.5 and 1.5 mm in size were excised from taro corms. Explants were subsequently surface-sterilized with 100 ml of a 1.05% sodium hypochlorite solution containing 1 drop of Tween for 16 seconds, rinsed in sterile water, and then transferred to test tubes containing M15 medium. M15 medium is Murashige and Skoog (MS) (1962) medium supplemented with 4 mg l^{-1} 6-benzyladenine (BA). DsMV-free shoots were verified by enzyme-linked immunosorbent assay (ELISA) (Agdia, Elkhart, IN). Callus was induced from shoot tips cultured on M5 medium (MS containing 2 mg l^{-1} 6-benzyladenine (BA) and 1 mg l^{-1} naphthalene acetic acid) in the dark for 60 days. Further multiplication of friable callus was carried on M5 medium in the dark.

Agrobacterium tumefasciens strains

Two strains of *Agrobacterium tumefasciens*, EHA105 and LBA4404, both harboring the binary vector pCNL65 were used. This plasmid has a T-DNA region that contains a selection gene, neomycin phosphotransferase (*nptII*) that confers resistance to both kanamycin and geneticin (G418 sulfate) and a reporter gene, β-glucuronidase (*gusA*/intron) with an intron (GUS) (Figure 2.1) (Liu et al., 1992). Both strains were grown at 28 °C on solid Luria-Bertani (LB) medium containing 50 mg l^{-1} kanamycin (Agri-bio) and 25 mg l^{-1} rifampicin (Agri-bio).

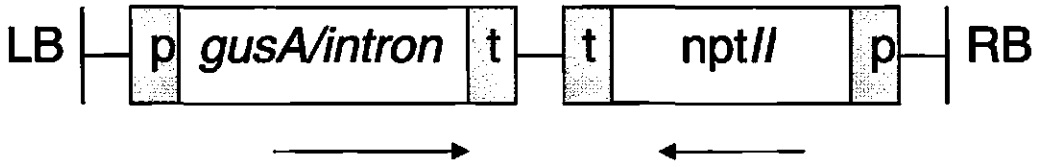

Figure 2.1. Map of T-DNA region of plasmid pCNL65 that contains *gusA*/intron with *CaMV* 35S promoter and *ocs* terminator and the *nptII* gene with *nos* promoter and terminator.

Tolerance of non-transformed *C. esculenta* callus to G418

To determine G418 sulfate selection levels for transformed taro callus, a kill curve experiment was set up testing 4 concentrations (0, 25, 50, and 100 mg l^{-1}) of G418 sulfate (Agri-bio) in M5 medium. The four concentrations were tested in 3 replicates, each of which was a plate containing medium with one of the G418 sulfate concentrations and 6 pieces of 3 mm diameter callus. The plates were incubated at 22 °C in the dark. After 1 month, callus diameter and weight measurements were taken. Diameter measurement was the average of three different directional measurements. Analysis of variance (ANOVA) was conducted and means were separated by the Student's t test at the $p < 0.05$ level (JMP, Version 6, SAS Institute, Cary, NC).

Transformation procedure

A single colony of the desired strain of *Agrobacterium* was inoculated into a 3 ml liquid culture of LB medium containing 50 mg l^{-1} kanamycin and 25 mg l^{-1} rifampicin. Cultures were grown overnight on a 250 rpm shaker at 28 °C until the bacterial suspension reached an OD_{600} value of 0.5-1.0. On the day of co-cultivation, 2.0 µl of 0.3 M acetosyringone was added to the bacterial suspension and allowed to incubate for 30 minutes at room temperature. The suspension was diluted to a total volume of 30 ml with LB medium (final concentration of acetosyringone = 20 µM). Callus pieces were chopped to approximately 1-3 mm in diameter and submersed into the bacterial suspension for 10 minutes. After submersion, callus pieces were blotted on sterile paper towels and transferred to MS medium (Murashige and Skoog, 1962) for the desired co-cultivation period.

Following co-cultivation, callus pieces were washed 3 times in sterile double distilled (dd) H_2O, soaked in 3 PPM™ (Plant Preservative Mixture; Plant Cell Technology, Inc., Washington, DC) for 2 hours, and blotted on sterile paper towels before being transferred to selection medium (S1), M5 medium containing 50 mg l^{-1} G418 and 250 mg l^{-1} cefotaxime. Calli were monitored for *A. tumefasciens* regrowth and overgrown callus pieces were rewashed as described above and re-plated on selection medium. After one month, calli were transferred to fresh S1 medium. A second transfer to fresh S1 medium was done at two months and after three months regeneration of shoots was initiated. Surviving calli were transferred to S3 medium (M15 containing 50 mg l^{-1} G418 and 125 mg l^{-1} cefotaxime) for two weeks and then to S4 medium (M5 containing 50 mg l^{-1} G418 and 125 mg l^{-1} cefotaxime) for two weeks. These transfers were repeated once and regenerated shoots were proliferated on MS medium.

Histochemical and fluorimetric assays of GUS activity

For evaluation of transient GUS expression callus pieces were incubated overnight at 37 °C in 100 µl of phosphate buffer solution containing X-Glucuronide (5-bromo-4-chloro-3-indoyl-beta-D-glucuronide) (Jefferson, 1987). Data on GUS expression were collected as number of pieces of callus showing blue areas and expressed as a percentage of total callus tested. GUS assays were performed for callus from each co-cultivation period at 1, 2, 4, 8, and 12 weeks after transfer to selection medium; assays were repeated three times. To quantify GUS expression, fluorimetric assays using 4-methylumbelliferyl β-D-glucoronide (MUG) as a substrate were carried out according to the procedure of Jefferson (1987). Three replicate assays were performed for callus co-cultivated for 2, 4, and 7 days at 1, 2, and 4 weeks after selection. Fluorescence was measured using a fluorimeter (Fluorolite 1000, Dynex Technologies, Chantilly, VA) after 1 hour of incubation at 37 °C. Measurements made at time points later than 1 hour showed that GUS activity remained linear (data not shown). Protein concentrations were determined according to Bradford (1976). GUS activity was expressed as pmol of 4-methylumbelliferone (MU) produced per minute per mg of protein. Statistical analysis using ANOVA and mean separations by the Student's t test at the $p < 0.05$ level were conducted using JMP software for statistical analysis (JMP, Version 6, SAS Institute, Cary, NC).

ELISA, PCR and Southern blot analysis

To confirm expression of the *nptII* gene, enzyme linked immunosorbent assays (ELISA) were accomplished using the Agdia kit for *nptII* (Elkhart, IN). For DNA analysis, whole genomic DNA was extracted from taro shoots using a modified version of the Lin et al. (2001) extraction protocol. Extraction buffer (EB) was pre-heated to 65 °C for 5 minutes prior to grinding of tissues. Plant tissues were frozen with liquid nitrogen and ground to a fine white powder using a mortar and pestle; pre-heated EB was added to the powder, mixed, and the mixture was

transferred to a 1.5 ml microcentrifuge tube. Tubes were vortexed briefly and incubated at 65 °C for 1 h. Remaining steps in the extraction procedure were followed according to the Lin et al. (2001) protocol with the exception that all centrifuge times were increased by 2 minutes and DNA was resuspended in 35 µl TE [10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8)] buffer.

Primers (50F 5' – AGA GGC TAT TCG GCT ATG AC – 3'; and 783R 5' – GTC AAG AAG GCG ATA GAA GG – 3') specific to the *nptII* gene amplifying a 723 bp fragment were used for polymerase chain reaction (PCR). The PCR reactions were carried out in a 25 µl volume consisting of 1 µl template DNA, 1 ul each primer (20 µm), 2 µl dNTPs (2.5 mM each), 2.5 µl 10x PCR buffer, 1.5 µl $MgCl_2$, 0.5 µl Taq polymerase (5 units/µl) (Promega Corporation, Madison, WI), and 15.5 µl sterile water. The PCR reaction conditions were 5 minutes at 95 °C followed by 30 cycles of 15 s denaturation at 95° C, 20 s primer annealing at 60 °C, and 30 s primer extension at 72 °C. A final 10 minute incubation at 72 °C was allowed for complementation of amplified products. For detection of the *gusA*/intron gene primers (gusF 5' – GCT GTC GGC TTT AAC CTC TC – 3'; and gusR 5' – CCG GTT CGT TGG CAA TAC TC – 3') amplifying a 200 bp fragment were used for PCR. The PCR reaction mixture volumes and conditions were the same as for *nptII*.

For Southern blots, restriction digest reactions were set up using 25 µg genomic DNA, 4 µl *Nco*I enzyme (Promega Corporation), 40 µl buffer, and sterile water to make up a total reaction volume of 400 µl and incubated overnight at 37 °C. To precipitate DNA, 800 µl 100% ethanol and 0.3 M sodium acetate were added to the digests and the mixture was stored at -20 °C for 2 hours. Samples were centrifuged at 12,000 rpm at 4 °C for 1 hour. The supernatant was discarded and samples were washed with 1 ml of 70% ethanol, centrifuged at 12,000 rpm at 4 °C for 10 minutes. The supernatant was discarded and samples were dried in a laminar flow hood for 30 minutes. The DNA samples were resuspended in

22.5 µl TE (pH 8) and 2.5 µl 10x loading dye and incubated at 37 °C for 30 minutes.

Samples were loaded in a 1% agarose gel and run overnight at 23 V. The gel was transferred to a glass dish and depurinated in 2% (w/v) hydrochloric acid for 10 minutes at room temperature and shaken gently. The gel was subsequently rinsed with distilled water and placed in neutralization solution (0.4 *M* NaOH and 1.5 *M* NaCl) and gently shaken for 30 minutes. The gel was rinsed again with distilled water before being blotted onto Hybond+ membrane overnight at room temperature (Amersham Biosciences, San Francisco, CA). The transfer buffer contained 0.25 *M* NaOH and 1.5 *M* NaCl. After transfer, the DNA was crosslinked to the membrane, then soaked in 100% ethanol for 10 minutes and rinsed twice in 2X SSC. The blot was hybridized using the AlkPhos Direct™ Labeling and Detection System with CDP-*Star* (Ambersham Biosciences). The probe was a 723 bp *npt*II PCR product .

2.4 Results and Discussion

Sensitivity of *C. esculenta* callus to G418 sulfate

In order to determine an optimal concentration for the selection of transformed cells, the sensitivity of *C. esculenta* callus to G418 was established prior to transformation experiments. After one month, calli grown on medium containing 0 mg l^{-1} G418 sulfate had an average weight of 0.17 g and an average diameter of 0.90 cm (Figures 2.2 a and b). Increasing concentrations of G418 decreased average callus weight and diameter.

Callus diameter is a quality that can be assessed visually whereas measuring callus weight requires additional equipment, such as scales, is more labor intensive, and could potentially lead to contamination of precious materials.

Therefore, initial selection medium concentrations for transformed callus were chosen based on the average diameter measurements rather than average weight. Medium M5 supplemented with 50 mg l^{-1} G418 sulfate was used, because this level significantly ($p < 0.05$) reduced callus diameter from the control by approximately 25% after one month. Subsequent regeneration and proliferation of transgenic taro plants was done using M15 medium supplemented with 50 mg l^{-1} G418 sulfate.

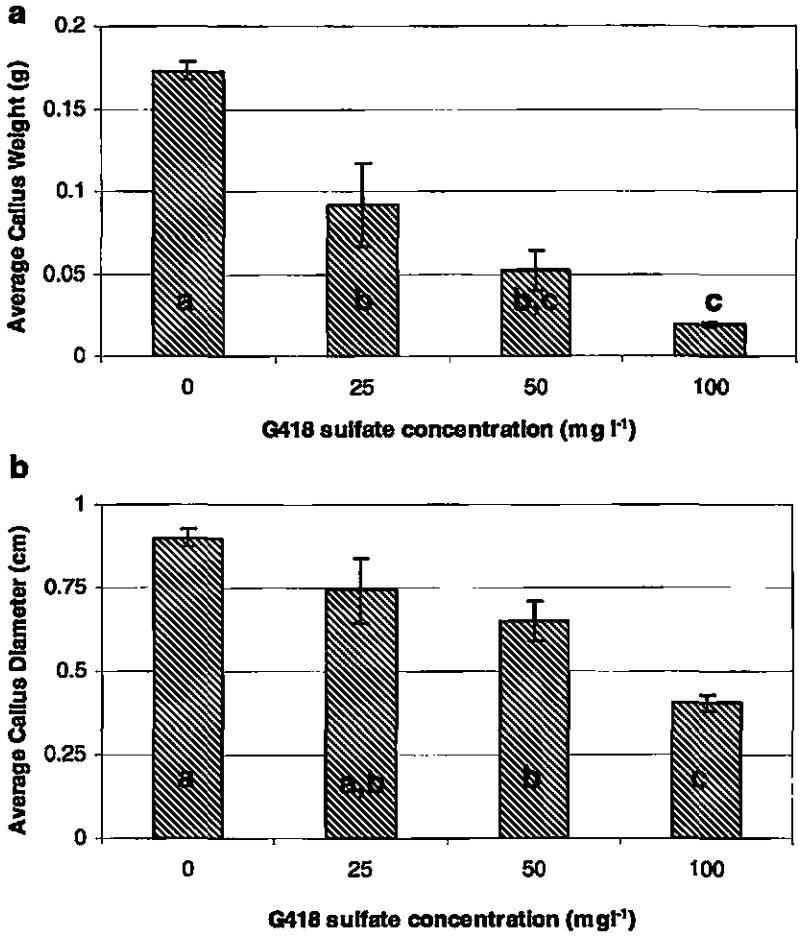

Figure 2.2. Effects of G418 sulfate concentration on a) average taro callus weight and b) average taro callus diameter after one month. Treatments with the same letter are not different ($p < 0.05$) using Student's t test. Error bars are standard errors of the mean.

GUS transient activity in *C. esculenta* callus

Calli co-cultivated with *A. tumefasciens* strain LBA4404 pCNL65 did not show GUS expression at any point throughout the experiments. Additionally, no calli survived on selection medium nor were any transgenic lines recovered. For future work transforming taro with disease resistance genes, *A. tumefasciens* strain EHA105 should be used.

Calli showing transient GUS expression in the X-Glucuronide assay had few well-defined blue areas (Figure 2.3a) whereas calli showing stable GUS expression were completely blue (Figure 2.3b). Overall, transient expression was highest in callus tested 1 week after transfer to selection medium with 87% of co-cultivated callus pieces showing blue spots (Figure 2.4). Expression significantly declined ($p < 0.05$) over the next 11 weeks, and at 12 weeks only 47% GUS expression was measured. There was no significant difference between the 1, 2, 4, and 8 week treatments ($p < 0.05$); but these four treatments were all significantly higher than the 12 day assay. There was also no effect of co-cultivation day on GUS expression, meaning that transient expression was not dependent on the duration that the callus was co-cultivated. However, transgenic lines expressing both the *gusA*/intron gene and *nptII* were only obtained from calli co-cultivated for either 4 or 7 days. Plants were only regenerated from calli co-cultivated for 4 days with EHA105 pCNL65.

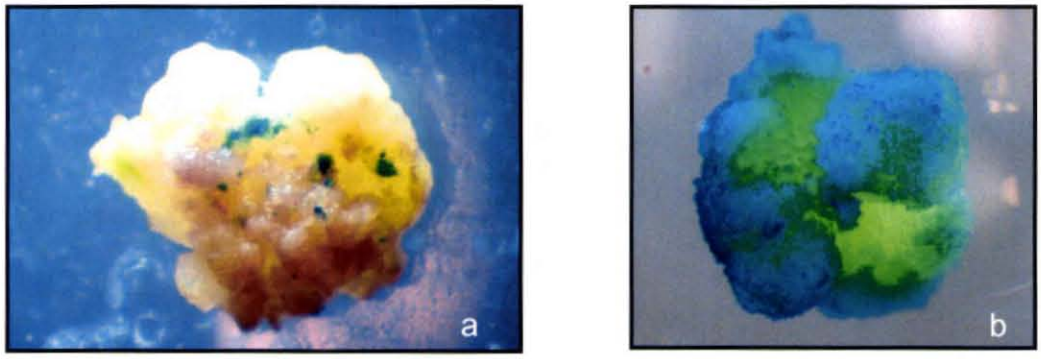

Figure 2.3a. Transient GUS expression 1 week after transfer to selection medium. 2.3b. Stable GUS expression after 6 months of selection.

Using the MUG assay to quantify GUS expression, there was no difference ($p < 0.05$) between the three co-cultivation days tested or the three assay times (data not shown).

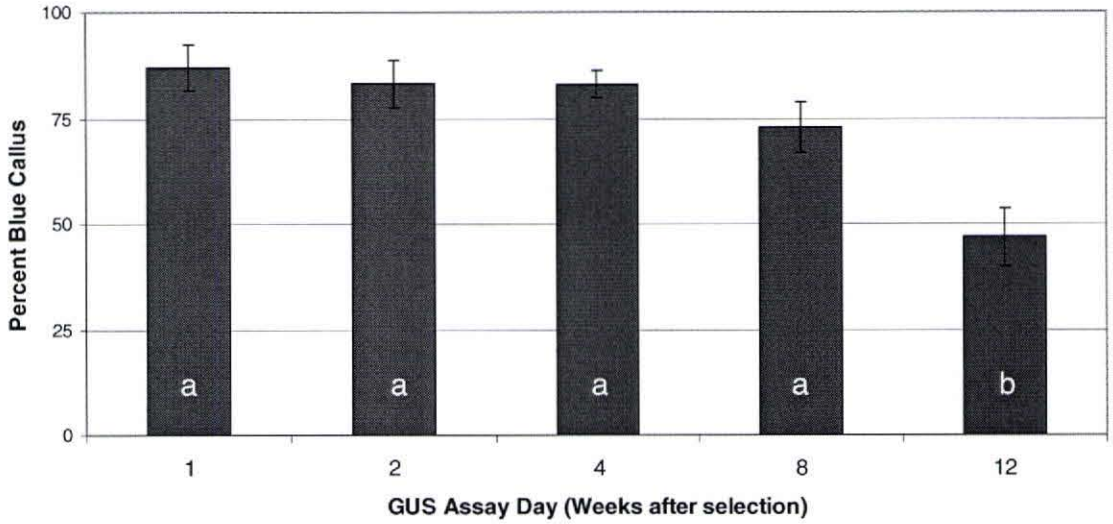

Figure 2.4. GUS expression in *Colocasia esculenta* callus after transformation with EHA105 pCNL65. Percent blue callus calculated as number of pieces showing blue areas divided by total number of callus tested. Gus assays were performed 1, 2, 4, 8, and 12 weeks after co-cultivated callus was transferred to selection medium. Treatments with the same letter are not different at $p < 0.05$ according to the Tukey-Kramer HSD test.

Confirming presence of *gus* (*uida*) and *npt*II

Calli surviving three months of G418 selection were screened using *nptII* ELISA before regeneration of transgenic plants. Thirteen lines were positive for *nptII* and were used to regenerate plants. Of these lines, seven produced shoots after 2 months of induction and produced small plantlets after 3 months on MS medium. Additionally, these lines were positive for expression of the *gus* gene using the X-Glucuronidase assay. PCR amplification of a bands specific to the *nptII* was also successful for all seven lines (Figure 2.5).

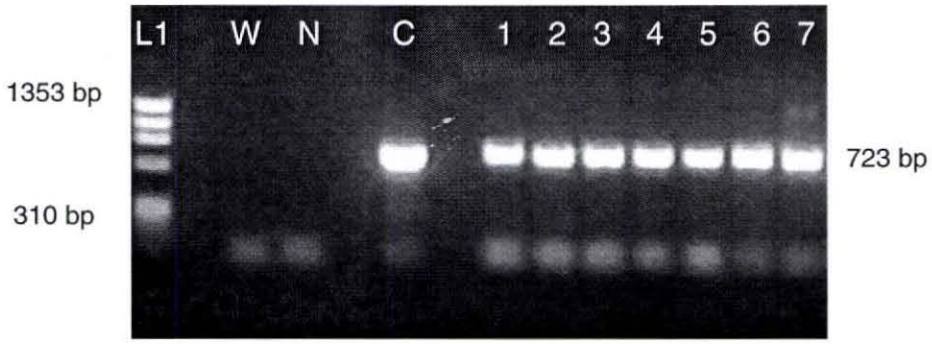

Figure 2.5. PCR amplification of a 723 bp fragment with primers specific to the *nptII* gene.
Key:
L1 – *HindIII* Lamda ladder
N – nontransformed control
C – pCNL65
W – water control
1-7 – lines 6-21, 6-22, 6-24, 6-25, 6-26, 6-27, and 6-28, respectively

Southern analysis of the *nptII* gene showed that four lines (6-21, 6-22, 6-24, and 6-25) that had been treated as independent lines had the same 4 fragment banding pattern (Figure 2.6). These four lines were co-cultivated in the same plate but were derived from different callus pieces. The restriction enzyme *NcoI* cuts at only one site in the plasmid pCNL65, therefore each band represents a single insertion of the T-DNA. It is unlikely that these same four T-DNA insertions into the taro genome would occur independently in all four different lines, implying that these lines are, in fact, the same.

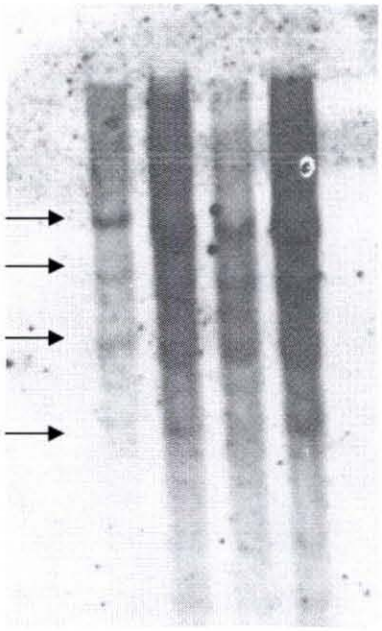

Figure 2.6. Southern blot analysis of transgenic taro lines for the *nptII* selectable marker gene.

Conclusions

Agrobacterium tumefasciens strain EHA105 is an effective means of transforming friable taro callus yielding ~1 positive transgenic line per gram of callus co-cultivated. Following a 4 day co-cultivation period, three months of cultivation on 50 mg^{-1} G418 medium is sufficient to select for transgenic callus lines. Throughout the selection period, transient expression decreased from 87% at 1 week after the initial transfer to selection medium to 47% after 12 weeks. Four transgenic plant lines were regenerated and transgene integration was confirmed by PCR and Southern analysis. Overall, the transformation procedure took between 10-13 months, from initiation of friable callus to regeneration of transgenic plants suitable for bioassay. Establishment of an effective transformation system for taro using *Agrobacterium tumefasciens* is an important step in developing disease resistance in taro using novel disease resistance genes.

2.5 References

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Chapter 3: *Agrobacterium tumefasciens* transformed *Colocasia esculenta* 'Bun long' expressing the stilbene synthase gene *vst*1

3.1 Abstract

Taro is the most important edible aroid species in the Pacific Islands. It is highly susceptible to fungal and oomycete diseases that can reduce yield by up to 50%. Other plant species previously susceptible to fungal disease have been made more resistant through insertion of a stilbene synthase (*vst*1) gene from grapevine and the subsequent production of the phytoalexin resveratrol (*trans*-4, 3', 5' –trihydroxystilbene) or one of its derivatives. Phytoalexins are plant antimicrobial compounds that are synthesized *de novo* at the site of pathogen infection. Resveratrol occurs naturally in grapevine and peanut and is an important component of their respective defenses against fungal pathogen invasion. Herein we report the successful integration of the *vst*1 gene with its inducible promoter into the taro genome. Multiple lines were produced with *vst*1 gene copy numbers ranging from 1 to 3. Upon induction with UV light, mRNA transcripts were produced and detected using RT-PCR. The transformed plants appear to be normal and will be clonally propagated for additional disease resistance testing using the taro pathogen *Phytophthora colocasiae* and other important pathogens.

3.2 Introduction

Taro, *Colocasia esculenta* (L.) Schott, is an important staple food crop in Hawaii and throughout the Pacific. The corm is prized for its easily digestible starch. In Hawaii, it is used to make a fermented paste called "poi," and its leaves are eaten as a vegetable (Plucknett, 1970). In Hawaii, taro production for 2006 was worth $2.6 million with 4.5 million pounds of taro produced, a level approximately 15% less than the five year average (Hudson et al., 2007). Disease problems as well as inclement weather were cited as reasons for the decrease in production (Hudson, 2007).

Diseases caused by oomycete pathogens *Phytophthora colocasiae* and *Pythium spp.* are the most economically significant (Miyasaka et al., 2001; Philemon, 1994). Taro Leaf Blight (TLB), caused by *P. colocasiae*, is the most commonly observed and destructive oomycete disease of taro, capable of reducing corm yields up to 50% (Miyasaka et al., 2001; Philemon, 1994) and leaf yield losses up to 95% (Brooks, 2005). Devastating epidemics can be caused by TLB (Trujillo, 1996). *Pythium* rot, while as widespread as TLB, is not nearly as destructive and causes a mushy, malodorous rot that destroys the corm (Ooka, 1994). Currently, these diseases are controlled using both chemical and cultural methods. Metalaxyl is the only fungicide registered for use in Hawaii and is applied near the time of planting which limits its efficacy of controlling disease throughout the 9 to 11 month cropping cycle (Miyasaka et al., 2001). Cultural methods of control include exclusion (i.e., using only disease free planting materials to limit the introduction of disease); sanitation (i.e. removal of diseased plant materials to reduce the spread of disease); and the use of tolerant or resistant cultivars (Fullerton and Tyson, 2003; Ooka, 1994; Uchida et al., 2002).

Reports of genetic transformation of taro in the literature are limited to a report by Fukino et al. (2000) of transformation via particle bombardment. They successfully transformed taro with a plasmid containing a hygromycin resistance

gene and the β-glucoronidase (GUS) reporter gene (Fukino et al., 2000). However, the efficiency of their transformation protocol was very low, yielding only two transformants from 96 bombardment attempts (Fukino et al., 2000). Particle bombardment was used to transform taro with a rice chitinase gene with similarly low efficiency, yielding only one positive transformant line from 30 attempts (He, 2006). Recently, *Agrobacterium tumefasciens*-mediated transformation was used to generate taro lines expressing a rice chitinase gene or an oxalate oxidase gene. Lines transformed with an oxalate oxidase gene showed promising resistance to *Phytophthora colocasiae* (He, 2006).

Phytoalexins are low-molecular-weight antimicrobial compounds that accumulate in plants as a result of infection or stress (Kuc, 1995). The *vst*1 gene encodes the enzyme stilbene synthase which synthesizes resveratrol (trans-3,5,4'-trihydroxystilbene), a phytoalexin, from precursors that are naturally occurring in all plants (Rupprich and Kindl, 1978). Several crops, such as *Arachis hypogaea* (peanut) and *Vitis spp.* (grapevine), synthesize resveratrol as part of the hypersensitive response to fungal pathogen infection (Langcake and Pryce, 1977; Zhu et al., 2004). Genetic engineering of several crop species, such as *Carica papaya* (papaya) and *Nicotiana tobacum* (tobacco) with the stilbene synthase gene, *vst*1, has resulted in increased resistance to fungal pathogens *Phytophthora palmivora* and *Botrytis cinerea*, respectively (Hain et al., 1993; Zhu et al., 2004). Other crops that have been engineered with stilbene synthase genes include wheat, tomato, kiwi, rice, and apple (Fettig and Hess, 1999; Giovinazzo et al., 2005; Kobayashi et al., 200; Stark-Lorenzen et al., 1997; Thomzik et al., 1997). The production of resveratrol in taro should offer no negative side effects for human consumption, as resveratrol is naturally occurring in grapevine. Additionally, resveratrol has been shown to be an antioxidant and anti-cancer agent, as well as being responsible for many of the health benefits attributed to drinking red wine (Jang et al., 1997)

The significance of taro as a staple food crop throughout the Pacific, coupled with its high susceptibility to fungal disease make it an ideal candidate for investigating new methods of engineering disease resistance. The use of the stilbene synthase gene *vst*1 for transformation of several other plant species with the result of increased resistance to fungal pathogens has been well documented. Further work to increase the effectiveness of our protocol for *Agrobacterium*-mediated transformation of taro callus will enhance our ability to efficiently generate transformant lines expressing the stilbene synthase gene.

3.3 Materials and Methods

Transformation vector construction

Standard gene cloning methods (Sambrook and Russell, 2001) were used to construct the binary transformation vector pBI*vst*1 (Figure 3.1). The T-DNA region of backbone binary vector pBI121 contains the reporter gene *gus* driven by the CaMV 35S promoter and the selectable marker gene neomycin phosphotransferase II (*nptII*, Chen et al., 2003) driven by the NOS promoter. The stilbene synthase gene *vst*1 and its pathogen-inducible promoter were isolated from plasmid p*vst*1 (Hain et al., 1993) by an *Eco*RI (Promega Corporation, Madison, WI) restriction enzyme digest and subcloned into pBI121 (Clontech, Palo Alto, CA). The subsequently named vector pBI*vst*1 was introduced into the disarmed *A. tumefasciens* strain EHA105 using the freeze-thaw method (Sambrook and Russell, 2001).

Tissue culture and genetic transformation

Friable callus was induced from shoot tip explants of *Colocasia esculenta* 'Bun long' cultured on M5 medium (Murashige and Skoog medium supplemented with 2 mg/L BA and 1 mg/L NAA in the dark for 1 month (Murashige and Skoog, 1962). Callus was multiplied with monthly subculture on M5 medium until suitable

quantities for transformation were produced. Three transformation experiments using 10 g friable callus per transformation were performed.

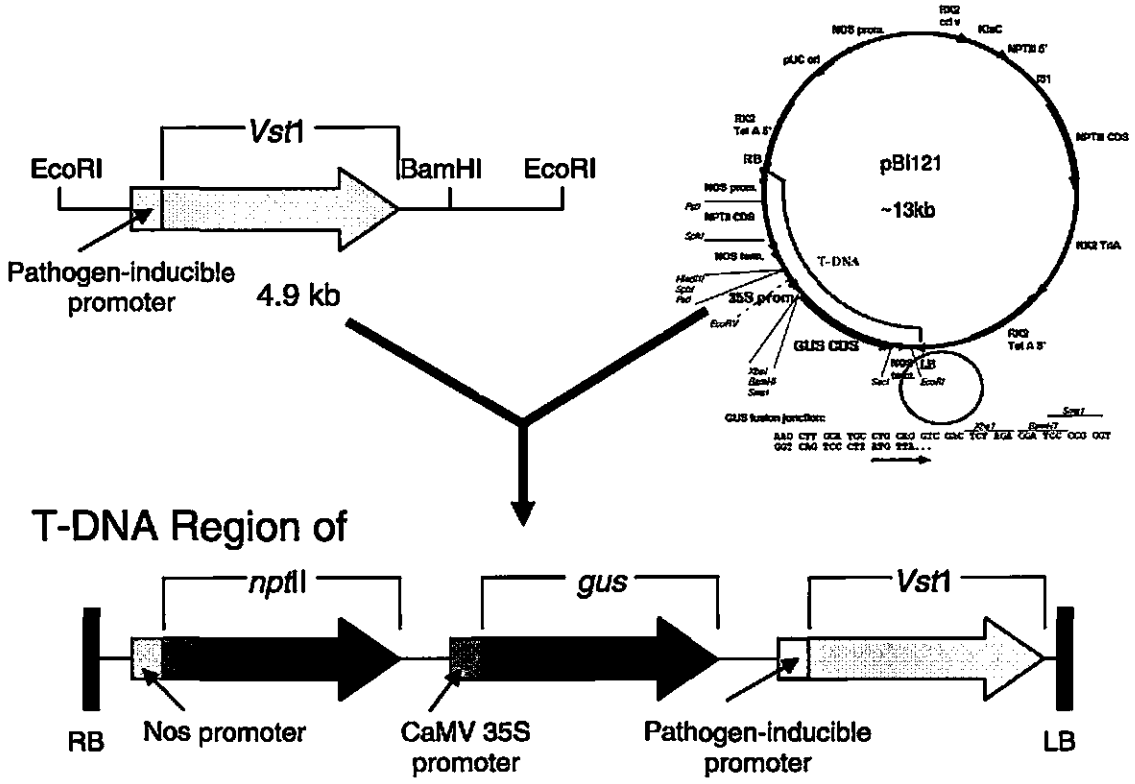

Figure 3.1. Schematic drawing of subcloning of pBI*Vst1*.

A single colony of *Agrobacterium* strain EHA105 pBI*vst1* was inoculated to a 3 ml liquid culture of YEB medium containing 50 mg l^{-1} kanamycin and 25 mg l^{-1} rifampicin. Cultures were grown overnight on a 250 rpm shaker at 28 °C until the bacterial suspension reached an OD_{600} of 0.5-1.0. On the day of co-cultivation, 2.0 µl of 0.3 M acetosyringone was added to the bacterial suspension and allowed to incubate for 30 minutes at room temperature. The suspension was diluted to a total volume of 30 ml with YEB medium (final concentration of acetosyringone = 20 µ*M*). Callus pieces were chopped to approximately 1-3 mm in diameter and submersed into the bacterial suspension for 10 minutes. After

submersion, callus pieces were blotted on sterile paper towels and transferred to MS medium for the desired co-cultivation period (Murashige and Skoog, 1962).

Following co-cultivation, callus pieces were washed 3 times in sterile ddH_2O, soaked in 3 PPM™ (Plant Preservative Mixture; Plant Cell Technology, Inc., Washington, DC) for 2 hours, and blotted on sterile paper towels before being transferred to selection medium (S1), M5 medium containing 50 mg l^{-1} G418 and 250 mg l^{-1} cefotaxime. Calli were monitored for *A. tumefasciens* regrowth and overgrown callus pieces were rewashed as described above and re-plated on selection medium. After one month, calli were transferred to fresh S1 medium. A second transfer to fresh S1 medium was conducted at two months and regeneration of shoots was initiated after three months. Surviving calli were transferred to S3 medium (M15 containing 50 mg l^{-1} G418 and 125 mg l^{-1} cefotaxime) for two weeks and then to S4 medium (M5 containing 50 mg l^{-1} G418 and 125 mg l^{-1} cefotaxime) for two weeks. These transfers were repeated once and regenerated shoots were proliferated on MS medium.

PCR and Southern analysis

Genomic plant DNA was isolated using the methods of Lin et al. (2001). To detect the transgenes in the taro genome, polymerase chain reaction (PCR) analyses were performed. Primers (50F 5' – AGA GGC TAT TCG GCT ATG AC – 3'; and 783R 5' – GTC AAG AAG GCG ATA GAA GG – 3') specific to the *nptII* gene amplifying a 723 bp fragment were used. For detection of the *gusA*/intron gene primers (gusF 5' – GCT GTC GGC TTT AAC CTC TC – 3'; and gusR 5' – CCG GTT CGT TGG CAA TAC TC – 3') amplifying a 200 bp fragment were used. The PCR reactions were carried out in a 25 µl volume consisting of 1 µl template DNA, 1 ul each primer (20 µm), 2 µl dNTPs (2.5 mM each), 2.5 µl 10x PCR buffer, 1.5 µl $MgCl_2$, 0.5 µl Taq polymerase (5 units/µl) (Promega Corporation, Madison, WI), and 15.5 µl sterile water. The PCR reaction

conditions were 5 minutes at 95 °C followed by 30 cycles of 15 s denaturation at 95°C, 20 s primer annealing at 60 °C, and 30 s primer extension at 72 °C. A final 10 minute incubation at 72 °C was allowed for complementation of amplified products. Forward (5' GAC AGT TCC ACC TGC ATA G 3') and reverse (5' GAG GAA ATT AGA AAC GCT 3') PCR primers were designed to amplify an 850 bp fragment specific to the *vst*1 gene (Zhu et al., 2004). The PCR reactions were carried out in a 25 µl volume consisting of 1 µl template DNA, 1 ul each primer (20 µm), 2 µl dNTPs (2.5 mM each), 2.5 µl 10x PCR buffer, 1.5 µl $MgCl_2$, 0.5 µl Taq polymerase (5 units/µl), and 11.5 µl water. The PCR reaction conditions were 30 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 58 °C, and 1 minute primer extension at 72 °C. A final 5 minute incubation at 72 °C was allowed for complementation of amplified products.

Southern blot analyses were performed to verify the integration of the transgenes in the taro genome and to determine copy number in the respective lines. Twenty five micrograms of genomic DNA was digested with 4 units Fastdigest™ SmaI (Fermentas, Hanover, MD) at 37 °C overnight . DNA was separated on a 1% agarose gel and blotted onto Hybond N+ membrane (Amersham Biosciences, San Francisco, CA). The blot was hybridized using the AlkPhos Direct™ Labeling and Detection System with CDP-*Star* (Amersham Biosciences). The probe was a 850 bp *vst*1 PCR product. All steps were performed according to the supplier's specifications (Amersham Biosciences).

Expression Analysis by RT-PCR

Reverse transcriptase (RT)-PCR was used to check for mRNAs specific to the *nptII* and *vst*1 genes in transgenic taro leaves. Because the *vst*1 gene is controlled by in the inducible *vst*1 promoter, transgenic plants were treated with UV light (254 nm, 1200 J cm^{-2}) in a crosslinker (Stratagene, Germany) for 10 minutes. Induced leaves were collected at 0, 5, and 24 hours after induction for expression analysis.

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) and DNase (Promega) treated. Generation of cDNAs was done as follows: 4 µl (250 ng) of DNase treated total RNA and 1 µl oligo dT-primer (20 µM) were mixed and incubated for 5 minutes at 70 °C, then chilled on ice for 5 minutes. Reverse transcription mix containing 4 µl of ImProm-II 5x reaction buffer (Promega), 0.5 µl Recombinant RNasin (Promega), 2.4 µl $MgCl_2$ (25mM; final concentration 3 mM), 1 µl dNTPs (10mM each dNTP), 6.1 µl DEPC-H_2O, and 1 µl ImProm-II Reverse Transcriptase was added to each reaction. Reverse transcription conditions were 5 minutes at 25 °C, 60 minutes at 42 °C, and 15 minutes at 70 °C. Primer sequences for PCR and reaction conditions for detecting the transgenes were the same as previously mentioned.

3.4 Results and Discussion

Subcloning

Successful ligation and transformation products were verified using restriction endonuclease digestion as well as by polymerase chain reaction (PCR). Digestion of both p*vst*1 and pBI*vst*1 with EcoRI yields a 4.9 kb fragment which contains the stilbene synthase gene and its promoter (Figure 3.2a). Digestion of both pBI121 and pBI*vst*1 with BamHI yields 1 band and 2 bands, respectively, also confirming the presence of the *vst*1 gene in pBI*vst*1, which contains the additional BamHI restriction site after cloning (Figure 3.2a, lanes P-B and VP-B). The presence of bands of approximately 13.5 kb and 6 kb after digestion of pBI*vst*1 with BamHI also indicates that the insert was cloned in the same direction as the *nptII* gene and β-glucuronidase genes already present in the pBI121 backbone. Had the insert been inverted, digestion would have yielded a 3.2 kb fragment and a 16.3 kb fragment (not shown). PCR amplification of an 850 bp fragment confirmed the presence of the *vst*1 gene in pBI*vst*1 isolated both from DH5α and EHA105 (Figure 3.2b).

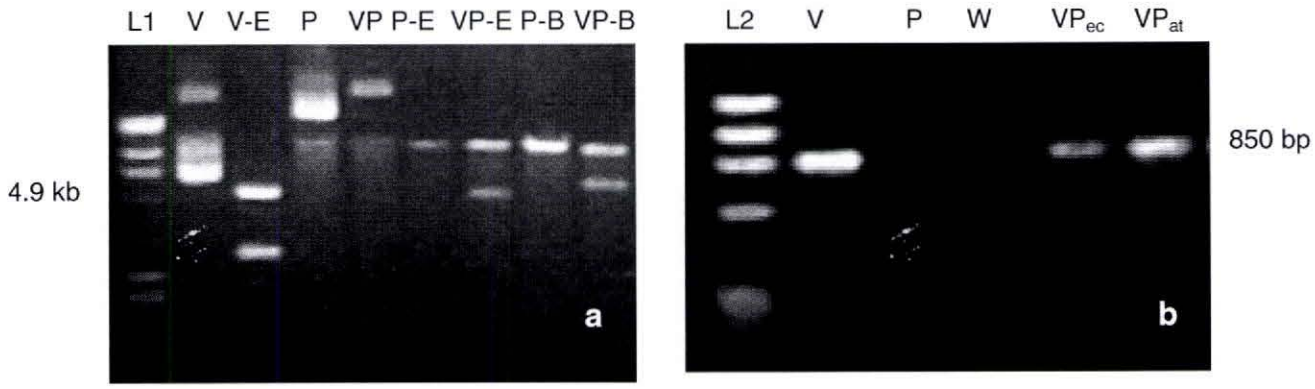

Figure 3.2. Verification of subcloning of pBI*Vst*1 using restriction enzyme digest (a) and PCR analysis (b).
Key:

L1 – *Hind*III Lamda ladder	L2 – ΦX174 ladder
V – p*Vst*1 P – pBI121	VP – pBI*Vst*1
E – *EcoR*I B – *BamH*I	VP$_{ec}$ - extracted from *E.coli* DH5α
W – water	VP$_{at}$ – extracted from *A. tumefasciens*

Transformation with the v*st*1 gene

From the initial transformation experiment, 8 callus pieces out of the 10 g co-cultivated survived 3 months of G418 selection. These 8 lines were analyzed using PCR for presence of the *nptII* and *vst*1 genes (Figure 3.3). The 723 bp band specific to the *npt*II gene was amplified in all 8 lines, but only 7 lines were positive for *vst*1. T-DNA transfer from *Agrobacterium tumefasciens* to the plant cell proceeds from the right border to the left border (Hellens, 2000). Therefore, in pBI*vst*1, the *npt*II gene which is closest to the right border would be inserted first into the taro genome, followed by the *gus* gene, and then finally, the *vst*1 gene. Interruption of the T-DNA transfer process can result in transgenic plants containing only the selectable marker gene and no other genes of interest (Hellens, 2000). This phenomenon could explain why line 9-16 is capable of surviving on medium supplemented with G418 and is PCR positive for the *npt*II gene, but negative for the gene of interest, *vst*1. Following induction of shoots, 5 out of 8 lines, including 9-16, produced plantlets after 3 months on MS medium.

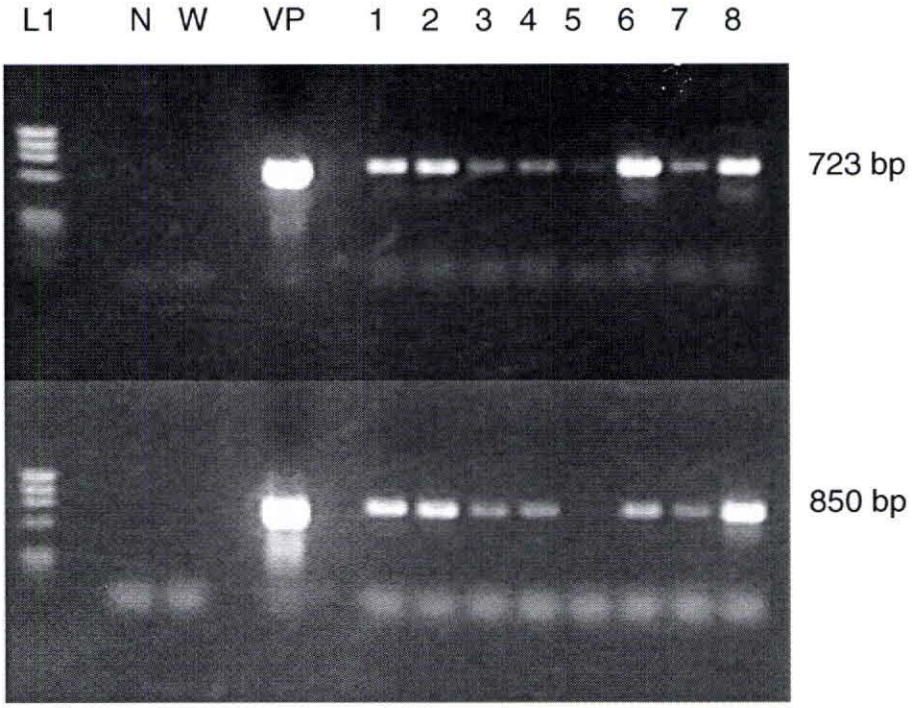

Figure 3.3. PCR amplification of a 723 bp band specific to the *npt*II gene and a 850 bp band specific to the *Vst*1 gene.

Key:

L1 – *Hind*III Lamda ladder
N – nontransformed control
VP – pBI*Vst*1
W – water control
1-8 – lines 9-11, 9-12, 9-13, 9-15, 9-16, 6-1, 8-11, and 8-12, respectively

The second transformation experiment using 10 g of callus yielded 26 lines that survived the initial three months of callus selection on G418 medium. Of these lines, 14 lines were *vst*1 PCR positive (data not shown). Additionally, transgenic plants were regenerated from 5 of these lines. The third co-cultivation experiment yielded 8 callus lines that were *vst*1 PCR positive (data not shown) and these lines all also yielded plants. In total, from 30 grams of co-cultivated callus, 42 callus lines survived the G418 selection process, of which 29 were found to be PCR positive for the *vst*1 gene indicating a callus transformation rate of 1.0 line per gram of callus co-cultivated. However, plantlets were only

regenerated from 18 of these callus lines, yielding a transformation success rate of 0.6 lines per gram of callus co-cultivated.

Southern blot analysis of 10 of the *vst1* PCR positive lines showed that they each possessed a distinct banding pattern and insert copy numbers ranging from 1 to 3 (Table 3.1). From the initial round of co-cultivation, Southern analysis of the *vst1* gene showed that lines 9-11, 9-12, and 9-13 arose from independent T-DNA transfer events and each line possessed one copy of the *vst1* gene (Table 3.1). The second round of co-cultivation yielded three lines, 5-33, 5-37, and 8-13 with 2, 3, and 2 gene insertions, respectively. All taro lines regenerated from the third round of co-cultivation possessed one copy of *vst1*.

Co-cultivation	Line	Copy Number
1	9-11	1
1	9-12	1
1	9-13	1
2	5-33	2
2	5-37	3
2	8-13	2
3	4-18	1
3	4-22	1
3	4-25	1
3	4-31	1

Table 3.1. Copy Number of *Vst1* transgene inserts in transgenic taro lines as assessed by Southern analysis.

Induction of the *vst1* gene promoter by UV light and production of mRNA transcripts at 0, 5, and 24 hours post-induction was analyzed using RT-PCR. The *vst1* specific primers amplify a gene fragment containing an intron that is 357 bp in length. The resulting 493 bp cDNA fragment from samples taken from *vst1* line 4-31 (Figure 4.3c) indicate that this intron was spliced from the 850 bp

genomic DNA sequence (Figure 3.3c). No fragment was amplified from the nontransformed control (ntc) plants. The appropriate fragment was amplified in samples collected at 5 hours after induction, showing that UV light is an effective stimulator of the *vst*1 promoter. The v*st*1 specific band was not amplified in the sample collected immediately after induction (0 hr), indicating that there is no basal rate of v*st*1 expression in the transgenic line. The v*st1* band was not amplified in the sample from 24 hours post-induction, indicating that there is a limit to the time in which the gene is expressed. This is reasonable because phytoalexins are synthesized *de novo* after pathogen invasion occurs and are broken down when the threat has past. In this instance, the stilbene synthase promoter was induced by UV light rather than a pathogen, however gene expression followed the same pattern. Transgenic line 4-31 was also assessed using RT-PCR for expression of the *npt*II gene which is constitutively expressed, therefore induction is not required. A 723 bp band specific to the *npt*II gene was amplified from the 0, 5, and 24 hour post-induction samples as was expected (Figure 3.4b). No bands were amplified in the ntc samples or in the –RT control samples (Figure 3.4a).

Transformation using *Agrobacterium tumefasciens* resulted in 10 lines containing the v*st*1 gene as verified by PCR and Southern blot. Expression analysis has shown that these transgenic lines expressed the gene. It is of interest to see if resveratrol or one of its glycosides is produced from these transcripts. High pressure liquid chromatography (HPLC) has been used to quantify resveratrol production in grapevine (Jeandet et al., 2002) and will be the next step in analyzing the transgenic v*st*1 lines. In addition, transgenic taro plants expressing the v*st*1 gene will be tested for increased resistance to the taro pathogens *Phytophthora colocasiae* and *Pythium* spp. in laboratory bioassays. Promising lines that accumulate resveratrol or one of its glycosides and show resistance to taro pathogens will subsequently be multiplied for future greenhouse and field experiments.

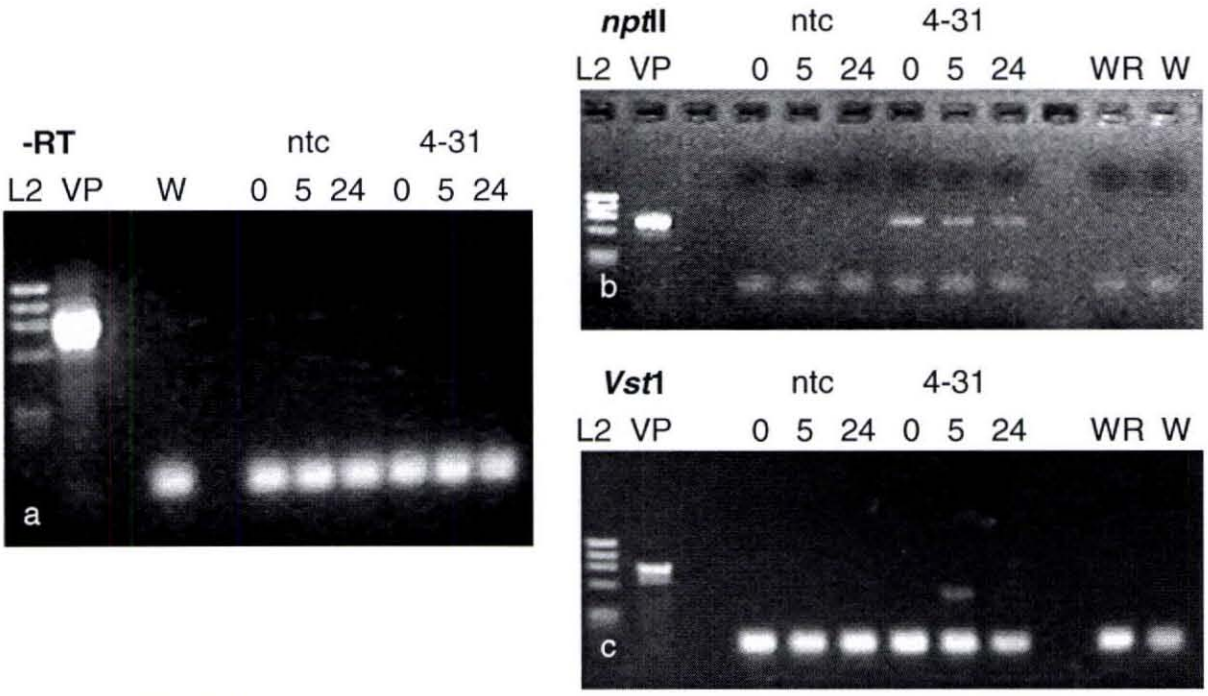

Figure 3.4. RT-PCR of non-transformed control (ntc) plants and transgenic line 4-31. Control (- RT, panel a) PCR was performed to check for DNA contamination of RNA samples. In panel b, a 723 bp fragment specific to the *npt*II gene was amplified from transgenic line 4-31. RT-PCR gene product of the *Vst*1 (493 bp) gene was amplified with intron-flanking *Vst*1 primers at 5 hours after UV light induction. Genomic fragments of the *npt*II (723 bp) and *Vst*1 (850 bp) were amplified from pBI*Vst*1 (VP). Water samples W for PCR and WR for reverse transcription were included as controls.

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Chapter 4: Fungal and oomycete pathogens of *Colocasia esculenta*: pathogenicity and response to the phytoalexin resveratrol

4.1 Abstract

Fungal and oomycete pathogens of taro, *Colocasia esculenta* (L.) Schott, can reduce the yields of this important staple aroid crop by nearly 50% when disease conditions are optimum. Genetic engineering with disease resistance genes has been used to develop crops that are resistant to fungal and oomycete pathogens. One example of such a gene is *vst*1, a stilbene synthase gene from *Vitis vinifera* that when expressed makes the phytoalexin resveratrol. Resveratrol has been shown to have antifungal activity and is a component of *Vitis* defense response. The purpose of this study was to isolate and identify fungal and oomycete pathogens of taro and determine if resveratrol inhibited their growth *in vitro* as a preliminary step to developing a genetically engineered variety of taro expressing the *vst*1 gene. Four oomycete pathogens and one fungal pathogen were isolated and identified from diseased taro corms or leaves. Mycelium growth of three *Pythium* species was inhibited by 500 µM resveratrol after 2 days of culture ($p < 0.05$). *Phytophthora colocasiae*, the causal agent of Taro Leaf Blight, was inhibited after 7 days of culture by 200 µM ($p < 0.05$). The fungal pathogen *Sclerotium rolfsii* was inhibited by resveratrol in experiments using both single sclerotia and mycelium as inoculum. *In vitro* inhibition of mycelium growth of 5 taro pathogens tested indicates that a taro plant expressing the *vst*1 gene and accumulating resveratrol might show increased resistance to the diseases caused by these pathogens.

4.2 Introduction

Taro, *Colocasia esculenta* (L.) Schott, is an important staple food crop in Hawaii and throughout the Pacific. The corm is prized as an easily digestible starch and in Hawaii it is used to make a fermented paste called "poi." Additionally, taro is grown for fresh use as a vegetable (Plucknett et al., 1970). In 2005, overall taro production in Hawaii was estimated at 4.0 million pounds, the lowest harvest total recorded since 1946 (Hudson et al., 2006). This 19% decrease from 2004 production was attributed to both inclement weather and high incidence of Taro Leaf Blight (TLB) caused by *Phytophthora colocasiae* (Hudson et al., 2006).

While there are taro diseases caused by a number of pathogens, including viruses, bacteria, and nematodes, fungal and oomycete diseases are the most economically significant (Brooks, 2000; Fullerton and Tyson, 2003; Ooka, 1994; Philemon, 1994). Estimates show that between 25-50% of corm losses are a result of fungal and oomycete diseases (Erwin and Ribeiro, 1996; Miyasaka et al., 2001; Philemon, 1994). The main fungal and oomycete pathogens that affect taro are *Phytophthora spp.*, *Pythium spp.*, and *Sclerotium rolfsii.* Taro Leaf Blight (TLB), caused by *P. colocasiae*, is the most commonly observed and destructive oomycete disease of taro (Fullerton and Tyson, 2003; Ooka, 1994). Under optimum conditions, TLB is capable of reducing corm yields up to 50% (Erwin and Ribeiro, 1996; Miyasaka et al., 2001; Philemon, 1994), decreasing leaf yields by up to 95% (Brooks, 2005), and has been responsible for devastating epidemics (Trujillo, 1996). Invasion by a second, newly discovered *Phytophthora sp.* results in Taro Pocket Rot (Uchida, 2003). *Pythium* rot, although as widespread as TLB, is not nearly as destructive and causes a mushy, malodorous rot that destroys the corm (Ooka, 1994). Sclerotium blight caused by *Sclerotium rolfsii* is characterized by a pink corm rot and results in stunted plants and decreased yields (Uchida et al., 2002).

According to the literature, there are many species of *Pythium*, including *P. aphanidermatum* Fitzpatrick; *P. graminicola* Subramaniam; *P. splendens* Braun;

P. irregulare Buisman; *P. myriotylum* Dreschler; P. carolinianum Matthews; and *P. ultimum* Trow that have been found on taro (Ooka, 1994). However, it is unknown if all these species are actually pathogenic or if they are secondary pests that move in after infection by another pathogen. It is important to determine which of these species are in fact causing taro corm rots and to obtain cultures of them in order to better understand the epidemiology of *Pythium* rots on taro and to control them in the fields.

Currently, fungal and oomycete diseases of taro are controlled using both chemical and cultural methods. Metalaxyl is the only fungicide registered for use in Hawaii for the control of fungal and oomycete diseases, however, it is only applicable in dryland taro operations (Miyasaka et al., 2001; Uchida et al., 2002). It is applied near the time of planting which limits its efficacy of controlling disease throughout the 9 to 11 month cropping cycle (Miyasaka et al., 2001). Cultural methods of control include exclusion (i.e., using only disease free planting materials to limit the introduction of disease); sanitation (i.e., removal of diseased plant materials to reduce the spread of disease); and the use of tolerant or resistant cultivars (Fullerton and Tyson, 2003; Ooka 1994; Uchida et al., 2002).

Research on the use of genetic engineering to produce tolerant or resistant cultivars is ongoing. Recently, taro was transformed by particle bombardment with a rice chitinase gene, but subsequent lines did not show increased resistance to *P. colocasiae* (He, 2006). Taro transformed with an oxalate oxidase gene (*gf*2.8) via *Agrobacterium tumefasciens* showed promising resistance to both *Sclerotium rolfsii* and *P. colocasiae* in laboratory bioassays (He, 2006). Work has been initiated to develop disease resistance in taro through genetic transformation with the stilbene synthase gene *vst*1. The enzyme stilbene synthase synthesizes the phytoalexin resveratrol (*trans*-4,3',5'–trihydroxystilbene) from molecules found in all plants. Phytoalexins are plant antimicrobial compounds that are synthesized *de novo* at the site of pathogen

infection. Resveratrol occurs naturally in grapevine and peanut and is an important component of their respective defenses against fungal pathogen invasion. Additionally, plants such as tobacco and papaya have been transformed with *vst*1 and through subsequent gene expression and accumulation of resveratrol, have been shown to be more resistant to their respective fungal pathogens (Hain et al., 1993; Zhu et al., 2004).

Isolating, identifying, and characterizing the pathogenicity of fungi and oomycetes found infecting taro is important because of the significance of taro as a staple food crop throughout the Pacific. Yield losses in recent years attributed to fungal and oomycete diseases also substantiate the need for additional research on this subject as well as the need to develop taro varieties that are resistant to these diseases.

4.3 Materials & Methods

Isolation of Pathogens

Phytophthora colocasiae was isolated and identified by Dr. Susan Schenck of the Hawaii Agriculture Research Center from symptomatic 'Bun long' taro leaves. *P. colocasiae* mycelium is tube-like, coenocytic, and diploid (Brooks, 2005). Sporangia are ovoid to elliptical (40-70 µm x 17-28 µm) and formed on the end of short and usually unbranched sporangiophores (Brooks, 2005; Erwin and Ribeiro, 1996). They are semipapillate and have a length-breadth ratio of 1:6:1 (Brooks, 2005; Erwin and Ribeiro, 1996). Additionally, sporangia are caducous with a pedicel length between 3.5 and 10 µm (Brooks, 2005; Erwin and Ribeiro, 1996). Germination of sporangia and zoospore production was induced from 3- to 7-day-old cultures on 10% V8 agar by covering mycelia with sterile distilled water and incubating plates at 4 °C for 30 minutes. Zoospores are reniform with 2 flagellae on the concave side. Chlamydospores were not observed in this isolate.

Sclerotium rolfsii was isolated and identified by Xiaoling He of the Department of Tropical Plant and Soil Science, University of Hawaii at Manoa, from 'Bun long' corms displaying symptoms of Sclerotium blight. *S. rolfsii* has coarse white mycelia that are abundant on infected host tissues. The main branch hyphae (5-9 µm in diameter) are hyaline with infrequent cross walls (Mullen, 2001). Smaller (2-4 µm) "feeding branch" hyphae are found penetrating the plant tissue (Mullen, 2001). After 1 week on 10% V8 agar, hyphae start to form fuzzy spherical sclerotia that eventually turn black-brown. Sclerotia have a thickened outer rind that surrounds a cortex of thin walled cells at the center of which are filamentous hyphae (Mullen, 2001).

Pythium splendens was obtained from Anthony Ortiz of the Department of Plant and Environmental Protection Sciences, University of Hawaii at Manoa. Hyphae are branched and measure 3.5-9.2 µm in diameter (Matthews, 1931; Middleton, 1943). Sporangia are large, spherical (average diameter 36.2 µm), with a thin wall surrounding dense, dark in color, globules (Middleton, 1943). *P. splendens* rarely produces sexual stages in culture and none were observed.

Pythium monospermum was isolated from taro corms showing disease symptoms of *Pythium* rot. Oogonia are apleuoritc; antheridia monoclinous. To induce sporangia production and zoospore release, agar blocks from 3 day grown cultures on 10% V8 medium were transferred to sterile Petri plates and covered with 30 ml sterile distilled water. Plates were incubated overnight at room temperature.

Pythium myriotylum was isolated from greenhouse grown 'Bun long' plants that were showing symptoms of root rot. Hyphae were rapidly growing and flocculent, measuring 2.5 to 8 µm in diameter (Middleton, 1943). Sporangia production was induced from 2-day-old cultures grown on 10% V8 medium using the same procedure as above, substituting 0.01 M Ca^{2+} solution for water (Nyochembeng et al., 2002). Sporangia are terminal or intercalary with an inflated lobulate

element (Middleton, 1943). Zoospores are broadly reniform, biciliate, and germinate usually by a single germ tube (Matthews, 1931; Middleton, 1943).

Resveratrol Bioassays

Phytophthora bioassays

In a preliminary experiment, mycelial growth of *P. colocasiae* was evaluated in 10% V8 agar medium containing different concentrations of resveratrol according to procedures reported by Coutos-Thevenot (2001) and Zhu et al. (2004). As a control, *Phytophthora palmivora* also was evaluated. It has been published that 1000 µM resveratrol inhibits growth of *P. palmivora* to about 50% of growth in control medium (Zhu et al., 2004). Resveratrol (Sigma-Aldrich, St. Louis, MI) was solubilized in ethanol, filter-sterilized, and added to 10% V8 agar at 50 °C to give final resveratrol concentrations of 0, 500, and 1000 µM and a final ethanol concentration of 1%. Media was poured into Petri plates and cooled overnight at room temperature. Agar plugs (2 mm x 2 mm) from 7-day-old pathogen cultures were placed in the center of the solidified resveratrol plates. After 4 days, the diameter of the pathogen mycelium mass was recorded as the average of three directional diameter measurements. Relative mycelium growth was calculated using the 0 mM resveratrol control as 100%. Each resveratrol concentration was evaluated in three plates. Statistical analysis of the data was performed using ANOVA (JMP, Version 6, SAS Institute, Cary, NC).

A second assay using only *Phytophthora colocasiae* and 0, 100, 200, 300, 400 and 500 µM resveratrol media (final ethanol concentration, 0.5%) was performed as described above and measurements were taken at 2, 4, and 7 days after inoculation.

Pythium bioassays

Mycelial growth of the three *Pythium spp.* was evaluated in two independent experiments using water agar containing 0 or 500 µM resveratrol. Resveratrol was solubilized in 100% ethanol, filter sterilized, and added to water agar at 50 °C. The medium was poured into 100 x 15 mm Petri plates and cooled overnight at room temperature. Agar plugs (4 mm diameter) from 2-day-old pathogen cultures were placed in the center of each plate. There were three replicates of each concentration per species. Three directional diameter measurements of mycelium growth were taken at 1 day and 2 days after inoculation. These measurements were averaged to obtain an overall measure of mycelium growth and analyzed using ANOVA (JMP, Version 6, SAS Institute, Cary, NC).

Bioassays to determine mycelial growth inhibition by resveratrol were repeated using lower concentrations and more replicates to achieve an accurate estimate of its effect on growth. Agar containing 10% V8 was used in place of water agar to facilitate data collection. Resveratrol was treated as described above before adding to 10% V8 agar medium to final concentrations of 0, 100, 200, 300, 400, and 500 µM resveratrol. All treatments had a final ethanol concentration of 0.5%. Agar plugs (4 mm diameter) from 2-day-grown pathogen cultures were placed in the center of each 60 x 15 mm agar plate. Three replicate plates for each resveratrol concentration per species were used per experiment and the experiment was repeated once. Plates were observed daily, with mycelium growth measurements taken at 2, 4, and 7 days after inoculation or until mycelial growth covered the entire plate.

Sclerotium rolfsii bioassays

Growth of *S. rolfsii* was evaluated in the same manner as the *Pythium spp.* previously mentioned. Either one sclerotium or a 4-mm-diameter agar plug containing mycelium was used as inoculum.

4.4 Results & Discussion

In preliminary bioassays, resveratrol was shown to reduce mycelial growth of both *P. colocasiae* and *P. palmivora* at the 500 µM concentration to 40% and 76% of their controls, respectively (Figure 4.1 and 4.2). Mycelium growth of *P. palmivora* was inhibited to 51.5% of the growth in control medium by 1000 µM resveratrol, similar to results obtained by Zhu et al. (2004). In 1000 µM resveratrol, growth of *P. colocasiae* was inhibited 86% compared to the control (Figure 4.2). According to Student's t test, the 0 µM and 1000 µM, and the 0 µM and 500 µM treatments for *P. colocasiae* were significantly different ($p < 0.05$) (Figure 4.2). There was no difference between the 500 µM and the 1000 µM treatments ($p < 0.05$). Growth inhibition of *P. palmivora* to about 50% of the control in 1.0 mM resveratrol was considered by Zhu et al. (2004) to be sufficient for justification of transforming *Carica papaya* with the stilbene synthase (*vst*1) gene. Resveratrol inhibits *in vitro* growth of *P. colocasiae* to a much greater extent (86% vs. 50%) than *P. palmivora*. This, combined with a highly significant difference between control and 1000 µM or 500 µM treatments indicates that engineering taro to produce resveratrol *in vivo* may be an effective means of increasing disease resistance against *P. colocasiae*, providing that sufficient levels are produced in the plant.

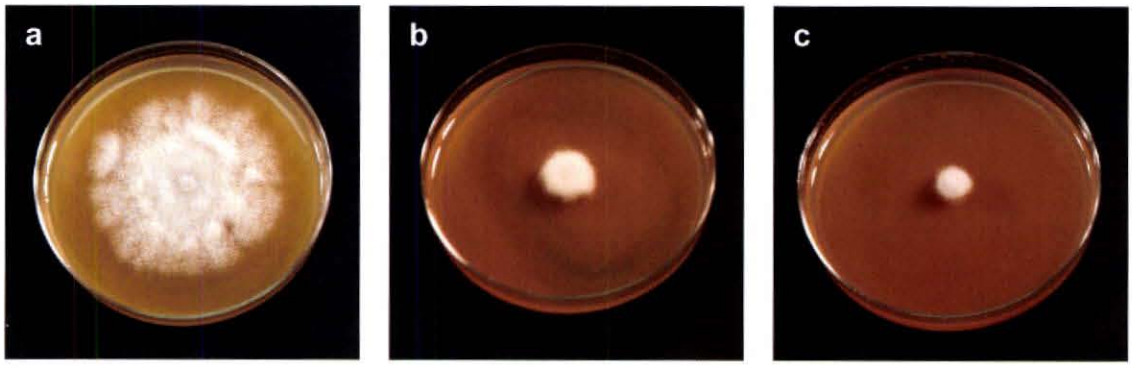


Figure 4.1. Mycelium growth of *Phytophthora colocasiae* after 4 days on 10% V8 agar supplemented with resveratrol. a. Medium supplemented with 0 µM resveratrol. b. Medium supplemented with 500 µM resveratrol. c. Medium supplemented with 1000 µM resveratrol.

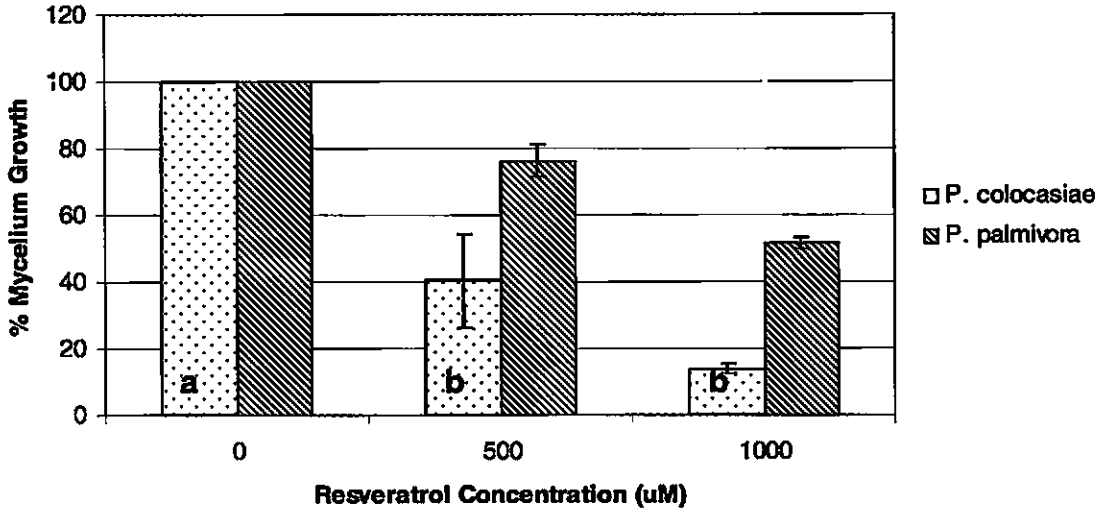


Figure 4.2. Effect of resveratrol concentration on mycelium growth of two species of *Phytophthora* 4 days after inoculation. Statistical comparisons were made using Student's t test ($p < 0.05$). Error bars are standard error of the mean.

Resveratrol in its *trans-* form is more stable and more biologically active than its *cis-* form (Hart, 1981). However, light can cause spontaneous conversion from the *trans-* to *cis-* form, potentially decreasing its fungitoxicity (Hart, 1981). In bioassays using resveratrol concentrations ranging from 0 to 500 µM with growth measurements taken at 2, 4, and 7 days after inoculation, a more accurate estimate of the effect of resveratrol on mycelium growth of *P. colocasiae* was found. Two days after inoculation, mycelium growth was inhibited completely by 400 and 500 µM resveratrol, nearly completely by 300 µM resveratrol (0.17 mm), and slowed to less than half the growth of the control by 200 and 100 µM resveratrol (Figure 4.3 and 4.4). After 4 days, 100 µM resveratrol still significantly decreased mycelium growth ($p < 0.05$). Seven days after inoculation, the 100 µM resveratrol treatment was not different from the control ($p < 0.05$), however all other treatments (200 to 500 µM resveratrol) were different and 500 uM resveratrol inhibited growth by 80%, similar to data obtained in the preliminary bioassays above. These results indicate that resveratrol is still effective at inhibiting growth despite 7 days of exposure to light, implying that either there is was very little *trans-* to *cis-* conversion, or that *cis-*resveratrol is still an effective inhibitor of *P. colocasiae*.

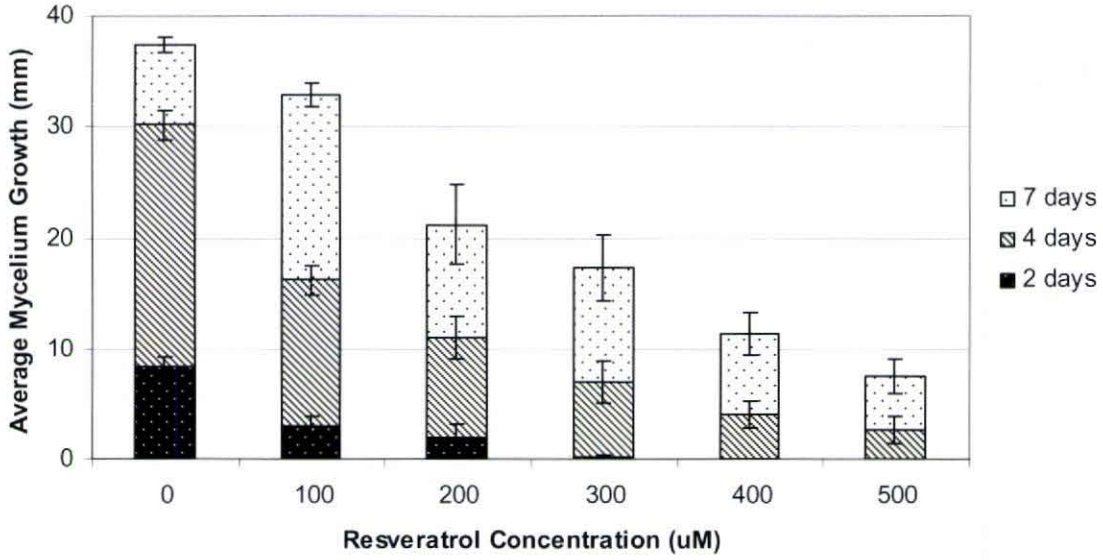


Figure 4.3. Effect of resveratrol concentration on average mycelium growth of *Phytophthora colocasiae* 2, 4, and 7 days after inoculation. Error bars are standard error of the mean.

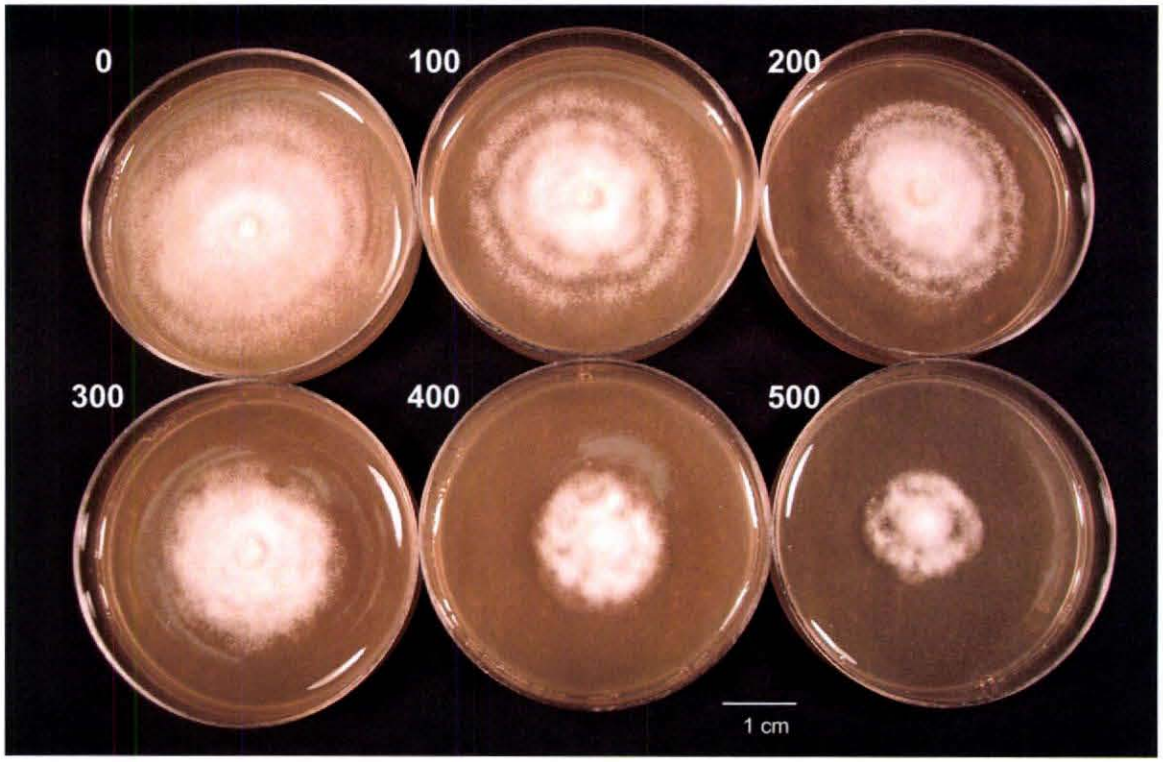


Figure 4.4. Effect of resveratrol concentration on mycelium growth of *Phytophthora colocasiae* 7 days after inoculation.

Resveratrol at 500 µM had an effect ($p < 0.05$) on mycelial growth of all three *Pythium spp.* tested; however there were no differences between the species. *P. myriotylum* growth was inhibited nearly 80% compared to the control by 500 µM resveratrol (5 cm vs. 1.2 cm, respectively) (Figure 4.5), the largest inhibition of the three species. Mycelial growth of both *P. monospermum* and *P. splendens* was inhibited by 40% compared to the growth in the 0 mM resveratrol control plate by 500 µM resveratrol, but *P. monospermum* exhibited overall slower growth compared to *P. splendens* (Figure 4.5).

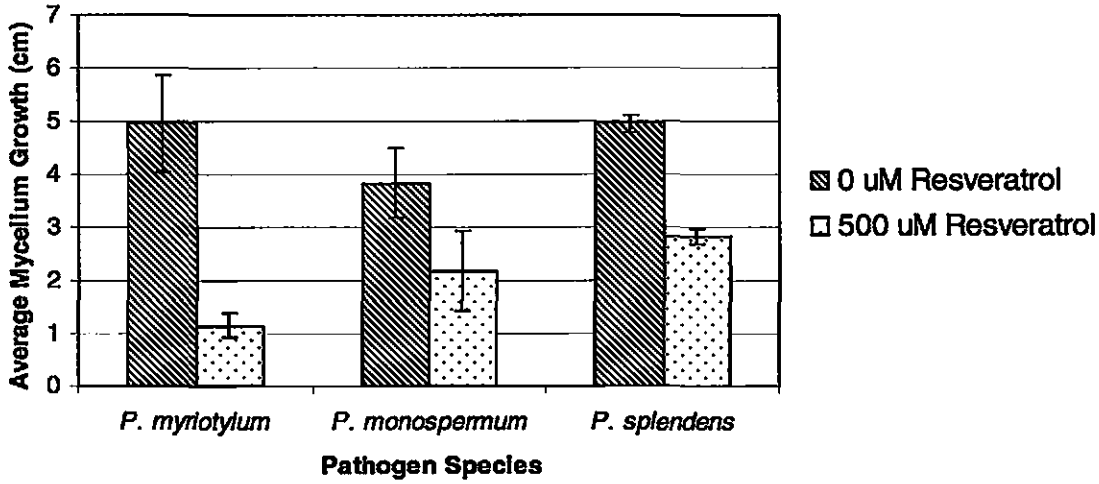


Figure 4.5. Effect of resveratrol concentration on mycelium growth of three species of *Pythium* four days after inoculation. Data are combined from two independent experiments. Error bars are standard error of the mean.

Further examination of resveratrol concentration effects on *P. myriotylum* mycelium growth showed that 200 µM resveratrol significantly decreased growth compared to the control (Figure 4.6). By 4 days post-inoculation, mycelium growth in the 0, 100 µM, and 200 µM treatments completely covered the Petri plates (Figure 4.7) and there was no longer a significant inhibition by 200 µM resveratrol. However, 300 µM significantly inhibited growth by 24% after 7 days (Figure 4.7).

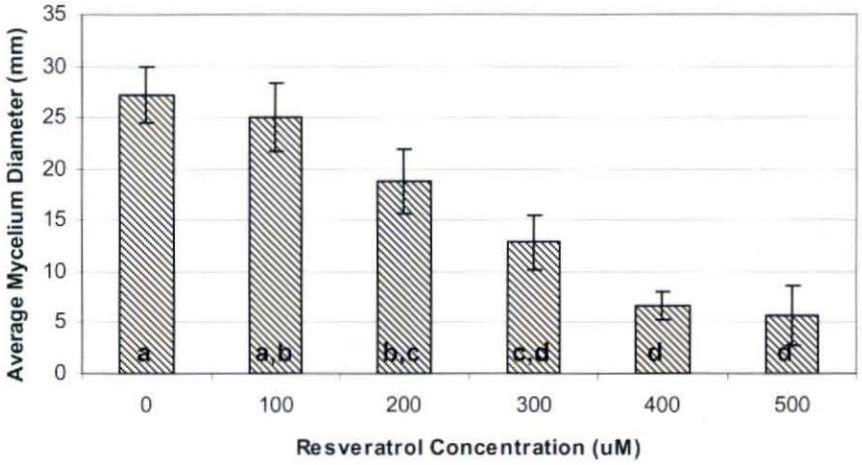


Figure 4.6. Effect of resveratrol concentration on mycelium growth of *Pyhtium myriotylum* 2 days after inoculation. Data are combined from two independent experiments. Means are separated using Student's t-test ($p < 0.05$). Error bars are standard error of the mean.

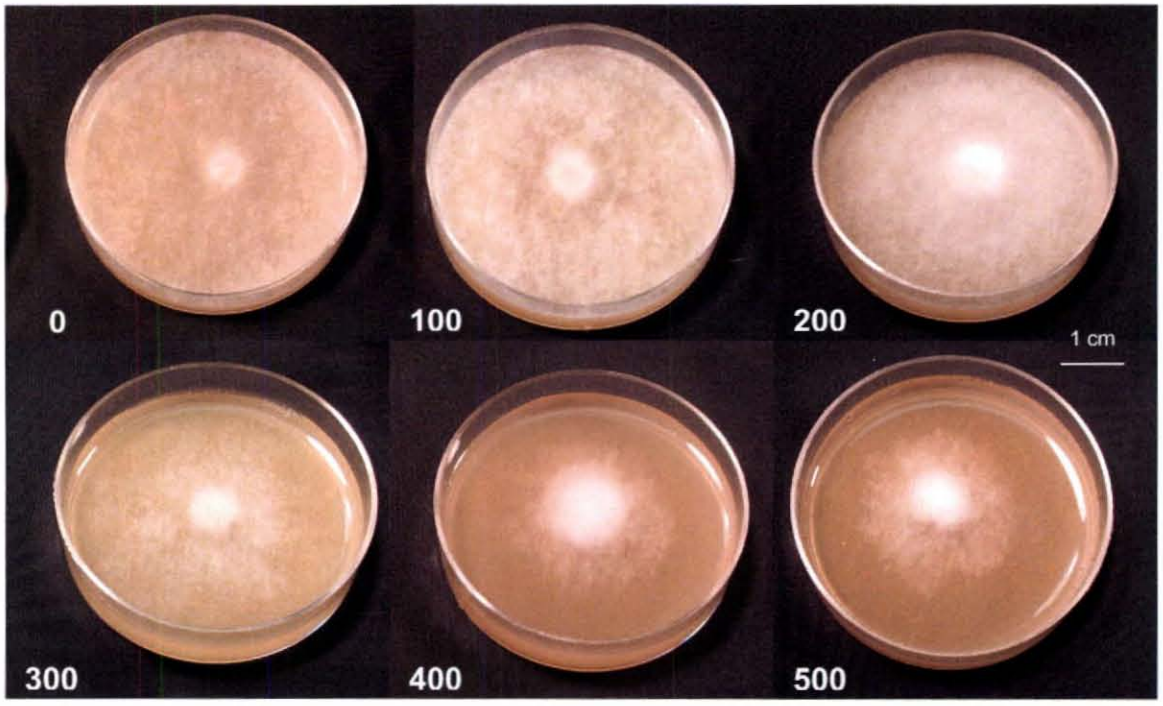


Figure 4.7. Effect of resveratrol concentration on mycelium growth of *Pythium myriotylum* 4 days after inoculation.

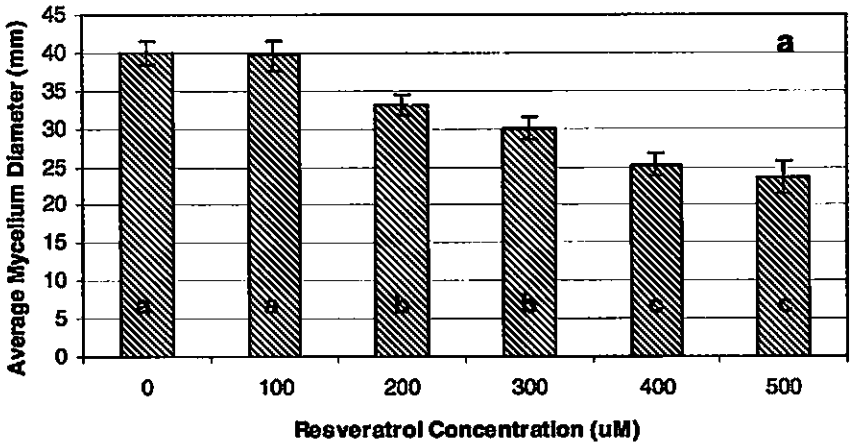
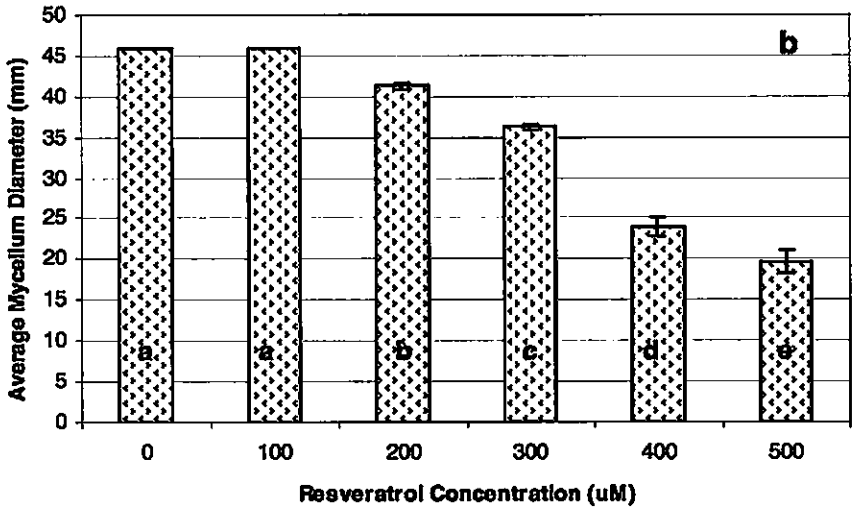


Figure 4.8. Effect of resveratrol concentration on mycelium growth of *Pythium monospermum* (a) and *Pythium splendens* (b) 2 days after inoculation. Data are combined from two independent experiments. Error bars are standard error of the mean.



Two days post-inoculation, 200 µM resveratrol significantly reduced the growth of both *P. monospermum* and *P. splendens* compared to the control (Figure 4.8 a & b). However, as with *P. myriotylum*, after 4 days this level of resveratrol no longer inhibited growth (data not shown). In fact, for both *P. monospermum*, and *P. splendens* only 500 µM resveratrol was able to inhibit growth after 4 days (Figure 4.5). This is the first time that the effects of resveratrol on mycelium growth of *Pythium spp.* have been examined and high concentrations (i.e., 500 µM) seem to have similar inhibitory effects of their mycelium growth as has been shown in *Phytophthora spp* (this paper; Zhu et al., 2004). However, at lower concentrations and over longer periods growth inhibition effects are less impressive. Inhibition effects by 200 µM resveratrol at 2 days post-inoculation

are no longer seen at 4 days post-inoculation for all three *Pythium spp.* and for *P. splendens*, only 500 µM resveratrol remains effective. This could be due to isomeric conversion of resveratrol resulting in the *cis-* form, which is generally less biologically active (Hart, 1981).

Sclerotia, the storage bodies of *S. rolfsii,* are capable of long-term survival in the soil which makes control of Sclerotium blight difficult (Agrios, 1978). Inhibition of sclerotia germination could be an important means of limiting the spread of disease from long-dormant inoculum. Four days post-inoculation with a single sclerotium the 0 and 100 µM treatments were not different from each other (Figure 4.9). However, while it is not significant, average mycelial growth on the 100 µM medium was greater than on the 0 µM medium. The 200 µM and 300 µM treatments were not different from the 0 µM treatment, but were different from the 100 µM treatment. It is possible that a low level of resveratrol stimulated germination of the sclerotium, while increasing levels inhibit mycelial growth. 400 µM and 500 µM resveratrol significantly reduced the growth of *S. rolfsii* mycelial growth compared to both the 0 and 100 µM treatments. In additional

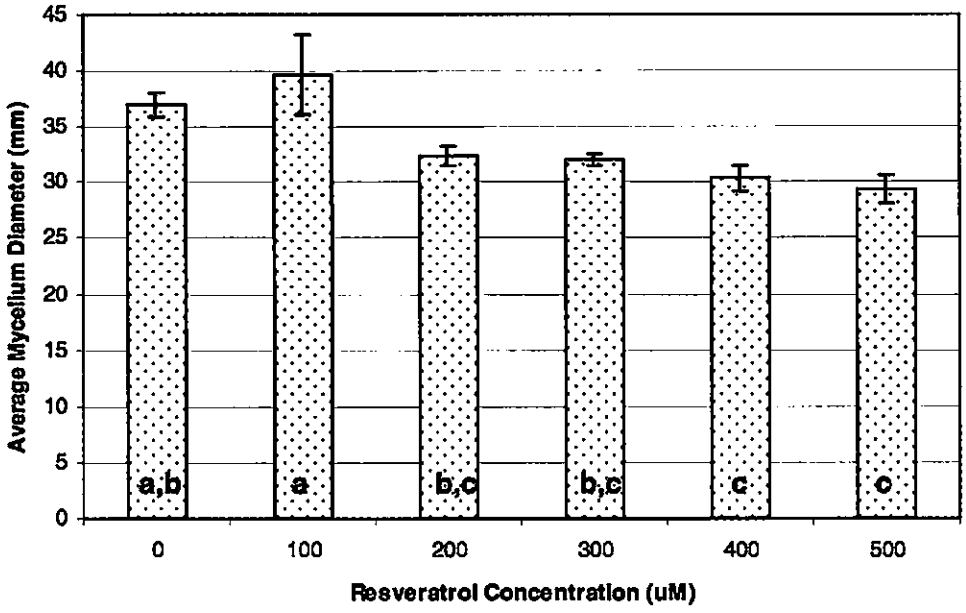


Figure 4.9. Effect of resveratrol concentration on growth of *Sclerotium rolfsii* from single sclerotium inoculations. Means are separated using Student's t test ($p < 0.5$). Error bars are standard errors of the means.

experiments using mycelial plugs as inoculum, 300 µM resveratrol was the lowest concentration at which mycelial growth was significantly reduced compared to the 0 µM control (Figure 4.10).

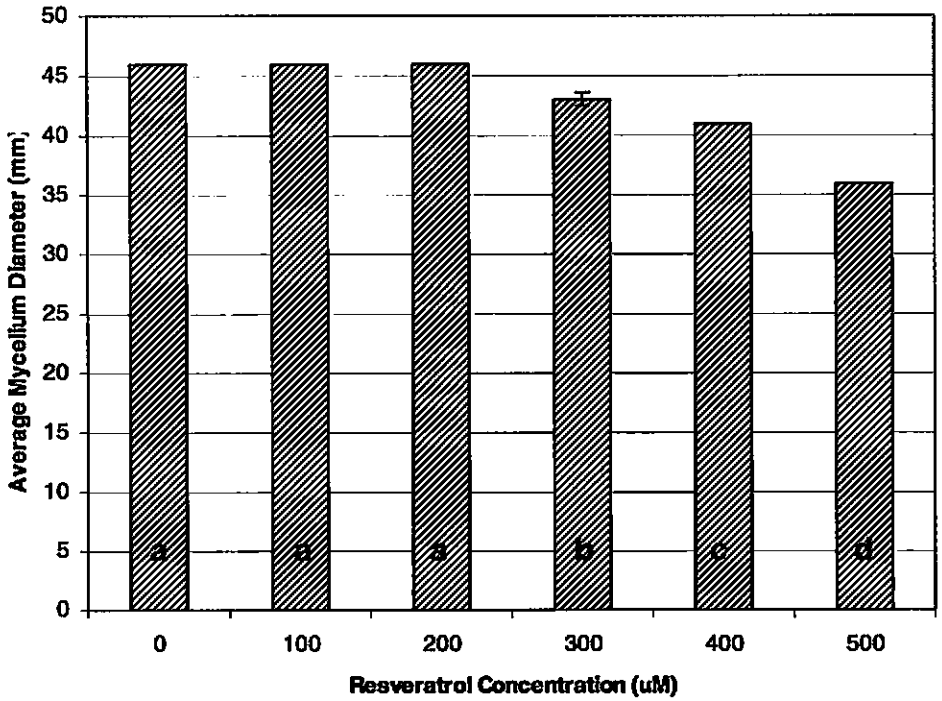


Figure 4.10. Effect of resverarol concentration on growth of *Sclerotium rolfsii* from mycelium inoculation. Means are separated using Student's t-test ($p < 0.05$). Error bars are standard error of the mean.

In transgenic plants expressing the *vst*1 gene, resveratrol levels ranging from 54.5 to 400 µM fresh weight have been measured (Hain et al, 1993; Zhu et al., 2004). Thus, *in vitro* inhibition results that lie in this range are promising indicators that transgenic *Colocasia esculenta* expressing the *vst*1 gene could be more resistant to these pathogens. In these experiments, concentrations ranging from 200 to 500 µM resveratrol were found to inhibit pathogen growth. While there are reports of monocot species such as rice and wheat being transformed with the *vst*1 gene, there are no reports of *vst*1 transformation of aroids (Fettig and Hess, 1999; Leckband and Lorz, 1998; Serazetdinova et al, 2005; Stark-Lorenzen et al., 1997). Additionally, reports of successful transformation of *C.*

esculenta are few (Fukino et al., 2000; He, 2006) and transgene expression data is minimal (He, 2006). *In vitro* inhibition of pathogen growth by resveratrol suggests that taro plants expressing the *vst*1 gene could be more resistant to these pathogens. However, resistance depends on accumulation of significant amounts of resveratrol *in planta*.

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Chapter 5: Conclusions and Future Work

The first objective of my research was to obtain data on transient GUS expression in co-cultivated taro callus to determine effective transformation conditions via *Agrobacterium tumefasciens*. Taro callus was co-cultivated with two strains of *A. tumefasciens*, LBA4404 and EHA105, both carrying plasmid pCNL65 that has the *gusA*/intron reporter gene and the *npt*II selectable marker gene. Transient GUS expression was measured using both histochemical and fluorometric assays at various time points during the selection process. *A. tumefasciens* strain LBA4404 was not effective at transforming taro callus, with no GUS expression observed or transgenic lines regenerated. Callus transformed using EHA105 showed high transient expression within the first 2 weeks after co-cultivation with expression decreasing as the selection period progressed. Transgenic taro lines expressing both the *gusA* and *npt*II genes were regenerated from callus co-cultivated for 4 days with EHA105 pCNL65 and confirmed using ELISA, PCR, and Southern analysis.

The second main objective of my work was to use this *Agrobacterium tumefasciens* mediated transformation protocol to develop transgenic taro lines that express the stilbene synthase gene *vst*1 with the intent of increasing resistance to fungal and oomycete pathogens. This gene synthesizes the phytoalexin resveratrol from the precursor molecules 4-coumaroyl CoA and malonyl CoA which are common to all plants. The *vst*1 gene under control of its native pathogen inducible promoter along with the selection gene *nptII* and marker gene *gus* were transformed into the taro genome via *A. tumefasciens*.

Molecular techniques were used to detect stable gene insertion into the taro genome. Polymerase chain reaction identified 29 transformed callus lines containing the *vst*1, *nptII* and *gus* genes after 5 months of selection. Of these,

plants were regenerated from 18 lines and analyzed using Southern hybridization. Southern blot analysis for the *vst*1 gene confirmed the insertion of the *vst*1 gene into 10 transgenic taro lines with copy numbers ranging from 1 to 3. Analysis using RT-PCR showed that mRNA transcripts of the *Vst*1 gene were produced at 5 hours after UV induction. Ongoing studies will determine if these mRNA transcripts are subsequently translated into the stilbene synthase enzyme and high pressure liquid chromatography (HPLC) will be used to quantify production of resveratrol.

A third objective of my research was to isolate and identify fungal and oomycete taro pathogens and determine their sensitivity *in vitro* to the phytoalexin, resveratrol. *Phytophthora colocasiae*, *Pythium monospermum*, *Pythium myriotylum*, *Pythium splendens*, and *Sclerotium rolfsii* were isolated from symptomatic taro corms or leaves and identified based on their respective known characteristics. Mycelial growth of the 4 oomycete pathogens *P. colocasiae*, *P. monospermum*, *P. myriotylum*, and *P. splendens* were inhibited by 500 µ*M* resveratrol, indicating that transgenic taro plants producing resveratrol might be more resistant to the diseases caused by these pathogens. Mycelial growth inhibition was also observed for *S. rolfsii*, the only true fungus tested.

Future studies should concentrate on testing the existing transgenic taro lines expressing the *vst*1 gene against the aforementioned oomycete pathogens in laboratory bioassays. Promising lines should be multiplied and grown to suitable size for greenhouse based resistance tests.