DISSERTATION

PROPAGATION, POLLEN STORAGE, AND *IN VITRO* LEMMATOXIN PRODUCTION OF ENDOD (*PHYTOLACCA DODECANDRA* L.)

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TIGST DEMEKE ENTITLED PROPAGATION, POLLEN STORAGE, AND *IN VITRO* LEMMATOXIN PRODUCTION OF ENDOD (*PHYTOLACCA DODECANDRA* L.) BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PROPAGATION, POLLEN STORAGE, AND *IN VITRO* LEMMATOXIN PRODUCTION OF ENDOD (*PHYTOLACCA DODECANDRA* L.)

Protocols for macropropagation and micropropagation of endod are described. High levels on IBA (19.7 and 39.4 mM) resulted in the greatest number of roots of semi-hardwood cuttings. Combinations of IBA and NAA did not improve rooting. Cuttings taken from the apical and medial regions of stem branches rooted faster than the basal ones, but there was no difference in rooting percentage at six weeks. Scarification of seeds resulted in faster germination. Over 71% seed germination was obtained under aseptic condition in the control, gibberellic acid treated and scarified seeds.

MS medium containing 0.44 μM BA induced 3.1 shoots per explant *in vitro* using shoot tips. Nodal explants produced up to 4.7 shoots per explant. Vertical and diagonal placement of nodal explants resulted in greater shoot proliferation than horizontal placement in strains 3 and 17. IBA at 0.49 μM induced 90% rooting of *in vitro* shoots with low callus production. Successful rooting was obtained at 1/2 and 1/4 MS basal salt concentrations with 0.49 μM IBA. Preculturing shoot tips in an MS medium containing IBA and transferring to medium without hormones did not reduce callus production at the explant base. Nodal explants gave 80 - 85% rooting with a low frequency of callus

formation (7.5%). *In vitro* rooted plantlets grew normally and flowered in a greenhouse.

Over 70% pollen germination was achieved on a medium containing 10% sucrose and 161.8 μM H_3BO_3. Pollen stored at 1 ± 1°C and -175°C maintained viability for six months, whereas pollen stored at 24 ± 2°C lost viability within one month. Pollen stored at -175°C for three months when used in pollination set normal fruits and seeds.

The greatest amount of callus was produced from shoot tip explants using IBA and 2iP. Flower bud and pericarp explants produced the least amount of callus. A hemolysis assay was found to be effective in analyzing saponin content of samples. No hemolytic activity was evident in the callus samples tested. Extracts from callus samples tested negatively for molluscicidal potency. Highest hemolytic activity of plant samples was recorded in the fully enlarged green berry stage. Seeds were found to be essential for hemolytic activity.

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CHAPTER 1

INTRODUCTION

Endod (*Phytolacca dodecandra* L' Herit) berries have the potential to be used as a molluscicide and/or detergent (Lemma *et al.,*1984). Schistosomiasis is one of the major parasitic diseases in many tropical and sub-tropical regions. The intermediate host snails that transmit schistosomiasis are susceptible to the lethal action of endod. The berries of endod have also been used to wash clothes for centuries in Africa. Thus, endod is also considered to have great potential as a component of a detergent.

The research on the chemistry, toxicology and extraction of endod is far ahead of the agrobotanical study (Lemma *et al.,* 1979; Lemma *et al.,* 1984). Thus, because of the lack of sufficient production of berries with known molluscicidal potency, endod has not been widely used for schistosomiasis control (Lugt, 1981).

Studies on endod growth characteristics, productivity and potency of berries began in the early 1970's on a small scale. In the late 1970's, 65 *Phytolacca dodecandra* strains were collected from various ecological regions of Ethiopia (Lugt, 1981). From these, three endod strains (strains 3, 17 and 44) were selected for their bushy, fast growing habit, high molluscicidal potency and high yield (Lugt, 1981). The selected strains produced up to 1.5 tons of dried berries per hectare per year.

Endod is a dioecious plant. In sexual propagation, both male and female plants have an equal chance of establishment but can only be distinguished during flowering. Since cross-pollination occurs, segregation may produce plants having different molluscicidal potencies as well as other agronomic attributes. Because of this, vegetative propagation by stem cuttings is considered valuable. In a previous experiment, only 20% of hardwood cuttings rooted when treated with rootone F and grown in a 2:1:1 mixture of sand: forest soil: soil media (Demeke, 1984). This low rooting percentage necessitates further studies on asexual propagation of endod.

No work has been done on the breeding and genetics of endod (Lemma *et al.,* 1984). Understanding the breeding and genetics of endod is a key to the improvement program to be launched. Because endod is dioecious, information on pollen germination and storage is considered important so as to more easily facilitate crossing between lines.

Plant cell cultures are potential alternatives to agriculture for the industrial production of biologically active compounds. Secondary metabolites have been produced *in vitro* in some plants (Fujita and Tabata, 1987; Tabata, 1977). As there are apparent constraints to field production of endod such as low yield, disease and insect susceptibility, the investigation of the potential for lemmatoxin production *in vitro* is considered worthwhile.

The present study was undertaken to:

1. develop a protocol for macropropagation and micropropagation of endod,
2. determine the germination and longevity of endod pollen under common storage

conditions, and

3. investigate *in vitro* biosynthesis of lemmatoxin in callus cultures.

CHAPTER 2

LITERATURE REVIEW

Phytolacca dodecandra

The family *Phytolaccaceae* consists of 17 genera and 110 species of herbs, shrubs or trees mostly native to tropical and subtropical America and Africa (Bailey, 1949). The genus *Phytolacca* comprises about 35 species. *Phytolacca dodecandra* L'Herit, commonly called endod, is reported to be indigenous to eastern, central and southern Africa (Dalziel, 1936). *Phytolacca dodecandra* has been found to be highly variable in its morphology in Africa (Adams *et al*., 1989). Endod is a sprawling woody climber with stems reaching a height of about 10 m, the average being 1.8 - 3.0 m. The plant is dioecious and produces flowers and berries on racemes. The ecological distribution and habitat of the plant is believed to be limited to highland areas (1800 - 3000 m above sea level) and is rarely reported to grow below 1400 m in Ethiopia (Takie and Yohannes, 1979).

Traditionally, endod has been used as purgative, soap, hirudinicide (leechicide), vermicide, taeniacide and abortifacient (Lemma *et al*., 1979). International attention was given to endod when its activity against schistosomiasis (bilharziasis) transmitting snails was discovered (Lemma, 1965).

Schistosomiasis is one of the most debilitating and pervasive diseases (Lemma *et al*., 1979; Lemma *et al*., 1984). An estimated 200-300 million people

in tropical and sub-tropical regions are affected by schistosomiasis, while another 400-500 million may be exposed. Two forms of schistosomiasis, namely the intestinal (*Schistosoma mansoni*) and the urinary (*Schistosoma haematobium*), are widely distributed in Ethiopia (Kloos *et al*., 1977; Kloos *et al*., 1978; Lemma, 1969). Another species, *Schistosoma japonicus,* is an intestinal schistosomiasis and is mainly found in Asia. The common schistosome transmitting snails belong to *Biomphalaria* spp., *Bulinus* spp. and *Oncomelania* spp. (Lemma and Yau, 1974). The capsulated eggs of the parasites are excreted with feces or urine. The eggs hatch upon coming in contact with water, and free swimming miracidiae enter the fresh water snail and develop into cercariae, the infective form of the parasite. The cercariae bore through the skin of people who come in contact with the infected water, enter the blood stream, and finally lodge in their preferred sites as male and female pairs. The disease causes considerable destruction of the liver or the bladder and ureter.

Chemical and plant molluscicides have been used to control the snails that transmit schistosomiasis (Lemma *et al*., 1984). The most commonly used chemical molluscicides are Bayluscide and Frescon (Bayer products). However, they are costly and, hence, are not affordable by developing countries. Several plant molluscicides such as seeds of *Croton* spp., seeds of *Jatropha curcas*, leaves and flowering tops of *Ambrosia maritima* and the berries of *Phytolacca dodecandra* have been studied for the control of schistosomiasis-transmitting snails. Endod is the most widely studied and

shows the greatest potential of the plant molluscicides (Kloos and McCullough, 1981; Lemma *et al.*, 1984).

The active molluscicidal compounds of endod are triterpenoids to which branched trisaccharides are linked at C_3 (Parkhurst *et al.,* 1974). Triterpenoids are built of isoprene or iso-pentane units linked together in various ways and with different types of ring closures,degree of unsaturation,and functional groups (Robinson, 1980). The isoprene units are derived from the mevalonic acid pathway. The aglycones of *Phytolacca dodecandra* consist of oleanolic acid (66.2%), hederogenin (8.9%), 2-hydroxyoleanolic acid (6.5%), and bayogenin (14.9%), all 28-carboxyoleonenes (Parkhurst, 1984). The structure of lemmatoxin is indicated in Appendix Fig. A. Lemmatoxin has a galactose and glucose unit attached to the 3 and 4 positions of the first glucose. *P. americana, P. dioica, P. rivinoides, P. octandra* and *P. esculenta* contain 28-30 dicarboxy and/or carbomethoxy oleanenes. Endod berries contain 25% or more saponins, whereas the other species contain 5% or less (e.g. *P. dioica*, about 1%) (Parkhurst, 1975).

Ten to twenty mg/L of sun dried, ground endod berries in water kills schistosome host snails within 24 hours of exposure (Lemma and Yohannes, 1979). The application of crude endod berries (collected from wild plants) in two small streams in Adwa (northern Ethiopia) has reduced the prevalence of *Schistosoma mansoni* in children (1-5 years of age) by 85%, and the overall prevalence of the disease dropped from 63% to 34% at the end of a five year study (Lemma *et al.*, 1978).

The active ingredient in the endod plant increases from the root to the leaves and from the flower buds to the green berries. Most of the saponins are concentrated in the berries. Parkhurst *et al*. (1989) reported that the potency of endod extract depends on the release of an enzyme (esterase) found only in the seed. Thus, breaking the seed is critical to the extraction process. The enzyme cleaves a glucose unit esterified at C(28),converting the inactive bidesmosidic saponin to active monodesmosidic saponin (Parkhurst *et al*., 1989).

The molluscicidal potency of endod is believed to be quite stable, and not affected by the pH of the water, by the concentration of organic matter or by irradiation with ultraviolet light. It is also easily biodegradable (Lemma, 1970; Lemma and Yau, 1974). Even though a carefully designed, long range toxicity study is required,the acute toxicity of endod to animals tested was low. Like all other molluscicides its toxicity to fish and tadpoles at molluscicidal concentration has been reported (Lemma, 1970). One disadvantage of endod is that repeated application is required, because the active ingredient is not lethal to the eggs of some snails. The membrane around *Biomphalaria* and *Bulinus* egg masses are not permeable to a material with a molecular weight over 500, whereas material with a molecular weight up to 3000 can permeate the membrane around *Lymnaea* eggs. The molecular weight of the bidesmosidic oleanolic acid of endod is 1104 (Dorsaz and Hostettmann, 1986).

When the berries of endod are crushed and suspended in water, a foaming solution is formed. This solution has been traditionally used in

Ethiopia, Zambia, and other African countries as a detergent for washing clothes. Based on this observation,the detergent and foaming properties of endod were tested (Lemma *et al*., 1984). Endod was found to be an effective cloth cleaning agent. Endod could thus be an effective substitute for dodecylbenzene sulfonic acid (DDBSA), a petrochemical by-product used as a surfactant in commercial detergent formulations (Addis Ababa University, Industrial Projects Service, and National Chemical Corporation, 1984). The extraction process used for the detergent preparation is basically the same as that used for the preparation of the molluscicides.

Other potential uses of endod include:

Larvicidal properties: Larvae of black fly (*Simulium* spp.), which transmits the river blindness disease onchocerciasis, and mosquito are susceptible to the lethal effect of endod (Assefa, 1979; Flemings, 1975). The active ingredient of endod appears to serve as a stomach poison to a number of feeding insects. Since snails and insects, such as malaria transmitting mosquitoes, could breed in the same type of environment, control of snails with endod provides potential control of mosquito populations.

Hirudinicidal properties: The aquatic leech (*Lymantis nilotica*), a major animal pest causing much damage to livestock in many tropical countries, has long been known to be susceptible to the lethal action of endod (Lemma *et al*.,1979). In Ethiopia endod has been used for centuries to control this pest.

Trematodicidal property: Schistosome cercariae and other trematode larvae are susceptible to the lethal action of endod (Lemma *et al*., 1984). The active

ingredient can also be prepared in an ointment form, for possible topical application on the exposed skin of workers in irrigation canals as a prophylaxis against cercarial penetration.

Abortifacient and spermicidal properties: Endod has long been used by traditional societies in Ethiopia and other parts of East Africa as an abortifacient. Intrauterine injection of an endod extract in pregnant mice caused sterile and apparently harmless abortion (Lemma *et al.*, 1979). The butanol extract of endod was found to be one of the most active biological agents against human sperm, suggesting its possible use as a birth control agent (Stolzenberg and Parkhurst, 1974).

Other snail killing properties: *Lymnae* spp.,the snail hosts of the important cattle and sheep liver fluke causing fascioliasis, were found to be susceptible to endod (Lemma *et al.*, 1984). Spraying pastures with relatively low concentrations of endod kills snails, eggs, and infective larvae of the parasites.

Fungicidal properties: Biological screening tests have shown that endod has a selective toxicity to dermatophytes, the fungi that cause a variety of skin diseases, such as athlete's foot and ringworm (Lemma *et al.*, 1979).

Physico-chemical or biological assays to determine the presence and relative amounts of bioactive saponins of endod are: molluscicidal activity (Lemma *et al.*, 1984), thin layer chromatography (Lugt, 1981), gas chromatography (Parkhurst *et al.*, 1990), high pressure liquid chromatography (Slacanin *et al.*, 1988), and hemolysis (Lugt, 1981; Monkiedje *et al.*, 1990; Thiilborg and Lemmich, 1990). Hemolysis has been used in saponin

determination and various other assays (Dacie and Vaughan, 1938; Fairley *et al.*, 1988; Koter and Laski, 1989; Ray and Noteboom, 1970; Reichert *et al.*, 1986).

In general, endod has many uses. Collaborative efforts of scientists from developing and developed countries have continued to effectively utilize this medicinal plant. Three international workshops have been convened on endod, namely in Zambia (1983), Swaziland (1986), and Ethiopia (1990).

Macropropagation

Rooting of stem cuttings

Rooting of stem cuttings is a complex phenomenon, influenced by physiological conditions, genotype, climatic conditions, treatment with growth regulators and the season in which the cuttings are taken (Hartmann and Kester, 1983). Each step of adventitious root initiation is controlled by a balance between plant growth regulators, rooting cofactors, carbohydrates, nutrients and various enzymes (Hartmann and Kester, 1983; Tukey, 1979). The lack of rooting in some species might result from the absence of necessary active enzymes or substrates that induce the meristematic state and thus the initiation of root primordia. Primordia may be initiated with certain growth stimulants.

Auxins such as IAA, IBA, and NAA have been used to induce rooting of stem cuttings (Thompson, 1986; Weaver, 1972). Auxins increase the plasticity and stimulate the growth of cell walls. During cell enlargement, the cell wall

stretches and also increases in thickness by deposition of new cell wall material. Auxins also stimulate cells to divide, and they influence the division of cambium leading to the formation of xylem and phloem vascular tissues (Wareing and Philips, 1970).

IBA and NAA are the most commonly used synthetic auxins for rooting (Hartmann and Kester, 1983). Weaver (1972) explained the probable success of IBA and NAA to be associated with their weak auxin activity, slow breakdown by IAA oxidase and their slow translocation, which results in their retention at the site of application.

Juvenile cuttings root better than mature cuttings under mist spray system. In *Ficus pumila* the differences between rooting of juvenile and mature cuttings were attributed to higher endogenous auxin levels in the juvenile cuttings, since lower IBA levels were required for optimal rooting of those cuttings (Davies and Joiner, 1980).

The season during which cuttings are collected affects rooting. The rooting percentage of adult olive cuttings varied markedly with season, the maximum occurring in summer and the minimum in autumn and winter (Porlingis and Therios, 1976). Juvenile olive cuttings rooted easily and relatively constant during the year, with some reduction in autumn. In *Telopea* spp., cuttings taken in the first half of spring, at the beginning of vegetative growth, gave the highest percentage of rooted cuttings and the greatest length of adventitious roots per rooted cutting (Worrall, 1976.)

Micropropagation

Growth regulators and shoot multiplication

Shoot multiplication occurs via axillary bud development and is regulated by apical dominance. Apical dominance is an aspect of the correlative events in a plant that alter plant form (Martin, 1987). Correlative inhibition involves nutritional or hormonal deficiencies in lateral buds of some essential factors required for their further development (Philips, 1975). So far there is no satisfactory explanation about the mechanism of apical dominance. Auxin from the young leaves of the apical bud is assumed to produce the principal correlative signal, influencing either cytokinin synthesis or utilization within lateral buds, or the distribution of root-synthesized cytokinins between meristems in the shoot. According to Tamas (1987) release of IAA from the apex and its subsequent transport is an essential component in axillary bud growth inhibition. Treatments that antagonize apical dominance such as cytokinins, excision of the apex and 2,3,5-triiodobenzoate (TIBA, an antiauxin) enhance shoot multiplication (Bressan *et al.*, 1982).

Cuttings grown in the absence of a root system must be provided a supply of cytokinin to ensure sustained development of shoots. Since cuttings which have a root system do not require the exogenous supply of cytokinin for shoot development it is likely that the root is a major source of cytokinin (Pool and Powell, 1975; Skene, 1975).

The development of axillary buds in several plant species has been reported to be stimulated by 6-benzylaminopurine (BA) (Bennett and Davies,

1986; Bressan *et al.*, 1982; Chee and Pool, 1985; Kitto and Young, 1981; Kyungchul and Stephens, 1987; Page and Visser, 1989; Passey and Jones, 1983; Reisch, 1986; Slabnik *et al.*, 1986; Stapfer and Heuser, 1985). Generally, the length of shoots decreases as BA concentration increases (Passey and Jones, 1983). The concentrations of BA that gave the best results generally ranged between 0.1 and 22 μM depending upon the plant species (Kitto and Young, 1981; Kyungchul and Stephens, 1987; Passey and Jones, 1983). The combination of BA and NAA also enhanced shoot multiplication (Lee and Thomas, 1985; Stapfer and Heuser, 1985).

The use of N^6-(2-isopentenyl) adenine (2iP) was reported to stimulate shoot multiplication in some species (Economou and Spanoudaki, 1985; Norton and Norton, 1985), whereas it has resulted in poor shoot proliferation in other species (Bennett and Davies, 1986; Reisch, 1986). The differences in the activity of the cytokinins BA and 2iP is assumed to be caused by degradation of 2iP but not BA by the enzyme N^6 (-isopentenyl) adenosine oxidase. This enzyme degrades naturally occurring cytokinins by cleaving the isopentenyl side chain at the double bond, with resultant loss of cytokinin activity (McGaw and Horgan, 1983; Mok *et al.*, 1982). Based on this, it has been suggested that a high concentration of 2iP be used for the stimulation of shoot formation to compensate for the continuous breakdown. The concentration of 2iP that is typically used for shoot proliferation ranges between 25 and 98 μM (Economou and Spanoudaki, 1985; Norton and Norton, 1985).

The use of 6-furfurylaminopurine (kinetin) enhanced shoot multiplication in some plant species (Hampel *et al.*, 1985; Stapfer and Heuser, 1985; Stephens *et al.*, 1985). The mean number of shoots per shoot tip increased from 0.3 shoots at 1.39 μM to 8.5 shoots at 139.0 μM for Java x New Guinea T63-1 *Impatiens* hybrid. The effect of kinetin on shoot proliferation was reported to be intermediate between that of BA and 2iP (Passey and Jones, 1983).

The inhibitory effect of gibberellic acid (GA_3) on shoot multiplication has been documented (Hasegawa, 1980; Murashige, 1961). On the other hand, GA_3 has been observed to stimulate shoot multiplication when combined with auxins and cytokinins (Nair *et al.*, 1979; Thorpe, 1981). Gibberellin treatment of intact plants can cause enhanced elongation of existing internodal cells, and also increase the number of cells present in each internode, principally as a result of an increase in mitosis in the sub-apical region of the stem (Wareing and Philips, 1970).

Nodal explants and shoot multiplication

In *Euphorbia fulgens* nodal sections produced more shoots but fewer buds than did shoot tips on MS medium (Murashige and Skoog, 1962) containing zeatin (Zhang *et al.*, 1987). In French tarragon, removal of the apical bud significantly reduced the number of axillary shoots, but average shoot length was increased (Mackay and Kitto, 1987). One possibility for this reduction was attributed to the fact that the majority of proliferated tarragon shoots are generated at the apex, which was removed from the pinched shoots.

In the same study, horizontal placement of explants consistently gave more axillary shoots than vertical placement. The increase in proliferation associated with horizontal placement was assumed to result from better uptake of the medium constituents due to increased surface area in contact with the medium.

Auxins and rootings

The auxins IBA, NAA and IAA have been widely used for root initiation in tissue culture (Drew, 1987; Hartmann and Kester, 1983; Hasegawa, 1980; Murashige, 1974; Zimmerman, 1983). IBA and NAA have been widely used at concentrations of 0.5 to 10 μM to initiate roots (Drew, 1987; Zimmerman, 1983). IAA is the preferred auxin in tissue culture (Murashige, 1974) with minimum adversity on organ formation, but is the weakest auxin and is inactivated readily in tissue culture systems. IAA is readily destroyed by IAA oxidase, photo-oxidation and chemical oxidation. IBA and NAA are less sensitive to the above and thus are more effective (Weaver, 1972). Phenolic compounds, such as catechol, have been reported to protect IAA from destruction by IAA oxidase (King and Meyer, 1983).

As auxin concentration in the media increases, the number of roots produced increases, however, the average length of roots and main shoot length decreases (Mackay and Kitto, 1987). Levels of IBA and NAA at 4.9 to 10.8 μM induced excess callus formation at the cutting bases and inhibited root growth of apple and *Euphorbia fulgens* shoots (Zimmerman, 1983; Zhang *et al.*, 1987). The excess production of callus by IBA has been reduced in the desert milkweed by preculturing shoot tips for 48 hours on MS medium with IBA and

then subculturing on MS medium devoid of growth regulators (Lee and Thomas, 1985).

MS salt strength and rooting

Reduction in the concentration of MS mineral salts has resulted in induction of rooting (Feliciano and Assis, 1983; Hasegawa, 1980; Jaacov and Dax, 1981; Skirvin *et al.*, 1981). In rose shoot tips, reduction in the concentration of the MS mineral salt formulation to 1/4 or 1/2 of the original formulation stimulated both root formation and transplant survival. The shoot tips grown on 1/4 or 1/2 strength MS salts appeared normal (Hasegawa, 1980). This has also been observed in thornless trailing blackberries, where shoots were induced to root on medium consisting of MS mineral salts, myo-inositol, and thiamine hydrochloride diluted to 1/16 to 1/2 strength and supplemented with full strength sucrose and agar (Skirvin *et al.*, 1981).

Activated charcoal and rooting

Activated charcoal has been shown to give substantial benefit in tissue culture by adsorption of toxic metabolites such as phenolics, gases such as ethylene, excess auxins, cytokinins, vitamins, and other media constituents (Bressan *et al.*, 1982; Snir and Erez, 1980; Weatherhead *et al.*,1979; Zaghmout and Torello, 1988). Specific examples of the use of activated charcoal include stimulated shoot elongation in Mugo pine (Mudge, 1986), increased root length in apple rootstocks (Snir and Erez, 1980) and caused substantial root and shoot growth of *Phoenix dactylifera* embryos (Zaid, 1981).

Pollen viability and storage

Pollen viability

Assessment of pollen viability is important in breeding programs and in the use of pollen after storage (Sahar and Spiegel-Roy, 1980). The medium requirements for pollen germination of different plant species varies (Johri and Vasil, 1961). It has also been observed that pollen taken from different flowers (or even from different anthers of a flower) gives variable results (Kurtz and Liverman, 1958; Lee *et al.*, 1985; Vasil, 1960). Brewbaker and Kwack medium (1963), which consists of 10% sucrose, 1618.1 μM H_3BO_3, 1271.0 μM $Ca(NO_3)_2$. $4H_2O$, 813 μM $MgSO_4$. $7H_2O$ and 989.1 μM KNO_3, is the most widely used. Sucrose has been shown to be superior to dextrose, fructose, galactose, lactose and mannitol in pollen germination (Vasil, 1960). Sucrose acts both in regulating the osmotic pressure and as a carbon and energy source (Johri and Vasil, 1961; Vasil, 1960).

The essential role of boron in pollen germination has been well documented (Brewbaker and Kwack, 1963; James *et al.*, 1987; Pfahler, 1967; Vasil, 1963). However, pollen of most species appears to be naturally deficient in boron. Boron appears to be essential for the utilization of sugars (Gauch and Duggar, 1953) and in pectin synthesis (Stanley and Loewus, 1964). It was proposed that boron combines with sugar to form a sugar-borate complex which facilitates translocation of the sugar molecules (Gauch and Duggar, 1953). This idea, however, has lost support since boron was found to react weakly with sucrose and since the concentration of boron was particularly low

in the phloem (Pilbeam and Kirby, 1983). Boron is assumed to maintain the structural integrity of plant membranes. Boron is probably bound in cis-diol borate complexes with adjacent hydroxyl groups of mannose and certain other sugars in cell wall polysaccharides (Pilbeam and Kirby, 1983; Salisbury and Ross, 1985).

The importance of calcium in pollen germination has been confirmed in 86 species representing 39 plant families (Brewbaker and Kwack, 1963). Absence of calcium fully inhibits growth of the pollen tube. Pollen germination and growth has been enhanced whenever any soluble calcium salt (calcium chloride, nitrate, acetate and pantothenate as well as calcium EDTA) has been used over a wide range from 50 to 5000 ppm. The improvement in germination and growth of pollen due to calcium probably relates to the binding of calcium to pectate carboxyl groups in the pollen wall (Brewbaker and Kwack, 1963). Calcium is also essential for normal membrane functions in all cells, probably as a binder of phospholipids to each other or to membrane proteins (Salisbury and Ross, 1985). In maize pollen, a synergistic interaction (increased germination percentage and pollen tube length) occurred when calcium and boron were added to the medium (Pfahler, 1967). The probable reason was attributed to calcium influencing the rigidity and stability of the membrane and boron to facilitating translocation.

Magnesium and potassium also enhance pollen germination of some plant species (Brewbaker and Kwack, 1963; Sahar and Spiegel-Roy, 1984). However, no beneficial effect of potassium was observed for jojoba pollen (Lee

et al., 1985). Brewbaker and Kwack (1963) have attributed the beneficial role of potassium and magnesium to enhancing the calcium effect. Potassium and magnesium are suspected to serve in supporting roles to the uptake or binding of calcium.

Pollen Storage

Pollen storage is necessary to facilitate hybridization of plants which flower at different times, to supplement germplasm preservation and to conduct physiological and biochemical studies (Ganeshan, 1985; Sahar and Spiegel-Roy, 1980; Towill, 1985). Pollen is generally classified as either desiccation tolerant (binucleate pollen grains when shed; thicker exine) or desiccation intolerant (trinucleate pollen grains when shed; thinner exine) (Towill, 1985). Desiccation tolerant pollen usually can survive low temperature exposure if the pollen is first dried to a relatively low moisture content (less than 15 to 20% moisture). At temperatures below approximately -130°C no viability decline during storage is expected to occur due to low molecular kinetic energies, slow diffusion characteristics, and lack of liquid water to prevent most chemical reactions (Towill, 1985). The key to the success of low temperature storage is exposing the pollen to cryogenic temperature without viability loss due to ice crystallization.

Reducing the freezable water content of pollen below a threshold level is important for successful cryogenic storage. Pollen has been dried by placement over a desiccant, such as anhydrous $CaCl_2$, and by freeze drying and vacuum drying (Anderson *et al.*, 1978; Hanson and Campbell, 1972; Sahar

and Spiegel-Roy, 1980) before storage at low temperature. The desirable moisture content for pollen storage varies according to the plant species. Generally, low moisture content (0 - 15%) and low relative humidity (25 - 35%) is suitable for pollen storage of many plants. Immediate thawing (ca. 40°C water) and controlled rehydration has given successful germination of pollen stored under cryogenic temperature (Lee *et al.*, 1985). Saturated salt solutions such as $CuSO_4$ are used to rehydrate pollen.

Secondary plant products

A shortage of certain medicinal plants exists because of a drastic decrease in plant resources as a result of human disturbance of the natural environment, exploitation, increasing labor cost, and technical and/or economic difficulties in cultivating wild plants (Tabata, 1977). There is a possibility of reducing these difficulties through the use of cell culture systems for production of medicinal compounds. The plant tissue culture alternative to whole plants as a source of useful compounds arose as a possibility in the period from 1950-1955 as a result of demonstrations that plant cells could be grown suspended in liquid medium, as done with microorganisms to produce such compounds as penicillin (Dougall, 1979; Shuler, 1981). During normal *in vitro* development, plant cells produce two types of metabolites, primary and secondary. Primary metabolites are proteins, fats and vitamins that are essential to plant life. Secondary metabolites include dyes, fragrances and potential drugs and are produced as defense mechanisms for the survival of the plant and as an

adaptation and interaction with the environment (Butcher, 1977; Crocomo *et al.*, 1981; Diomande, 1984).

In order to be useful as an alternative source of secondary compounds, a cell culture must satisfy several requirements (Crocomo *et al.*, 1981; Tabata, 1977): 1. a high yield of the final product within a short time, 2. genetically stable cells which give a constant yield of the final product, 3. accumulation of the compound in the cell or release into the medium should be rapid in comparison to its degradation, and 4. production costs including culture medium, precursor, and chemical extraction should be low enough to be profitable. These requirements have been difficult to achieve in most circumstances.

The key problem that has to be overcome for *in vitro* production of useful compounds has generally been the selection of specific cells that produce high amounts of the desired compounds. Furthermore, there is a need for the development of an adequate culture medium for the production of such useful compounds (Yamada and Fujita, 1984; Yamada and Hashimoto, 1990).

Plant tissue culture is being used for the production of some pharmaceutically useful secondary compounds and for flavors, fragrances and food additives (Diomande, 1984; Misawa, 1977). Ginseng root (*Panax ginseng*), from which compounds such as saponins and sapogenins are extracted, is valued in some Asian countries as a sedative or stimulant. The plant requires from four to seven years to produce the two ingredients; however, the yield of saponins and sapogenins have been increased through tissue culture (Tabata,

1977). Shikonin from *Lithosphermum erythrorhizon* is used as a dye and pharmaceutical. It has been possible to produce shikonin commercially through tissue culture (Fujita and Tabata, 1987).

Phytohormones and callus production

Generally, a high concentration of auxin and a low concentration of cytokinin in the medium promotes abundant cell proliferation with the formation of callus (Thorpe, 1981). Conversely, a lower auxin concentration stimulated callus growth of *Persea americana* (Mooney and Van Staden, 1987). The auxins 2,4-D, IAA and NAA have hastened liverwort callus initiation and proliferation with an optimum at 10^{-5} M (Kumra and Chopra, 1987). In *Pisum sativum,* higher concentrations of BA and NAA were the most useful for callus induction, and low BA and NAA concentrations resulted in root differentiation (Tae and Narm, 1987). On the other hand, 2,4-D and kinetin were indicated to have no effect on callus formation and organogenesis in pea. The toxic effect of 2,4-D has been reported in *Panax ginseng* cultures (Furuya *et al.*, 1983).

Combinations of NAA and BA or NAA and kinetin gave maximum callus development for juvenile reversion stem derived callus and mature stem derived callus respectively in *Hedera helix* (Banks, 1979). In examples such as the use of stem segments of papaya seedlings with 25 μM NAA and 10 μM kinetin approximately 80% of the cultures produced callus (Pandey and Rajeevan, 1983).

Media requirements for secondary product formation

The components of the medium and the phytohormones used are controlled in order to produce secondary metabolites by cell culture. Media published by Gamborg *et al.* (1968), Linsmaier and Skoog (1965) and Murashige and Skoog (1962) have been used with various modifications (Fujita and Tabata, 1987; Yamada and Fujita, 1984). Secondary metabolites have been produced by reducing the concentration of the main medium components, particularly nitrate, ammonium and phosphate. Capsaicin, a spice compound, was produced when the concentration of nitrogen was reduced (Lindsey, 1985), while ajmalicine and serpentine were produced when the concentration of phosphate was lowered (Knobloch and Berlin, 1983). Rosmarinic acid was produced when both nitrogen and phosphate concentrations were lowered (De-Eknamukl and Ellis, 1985).

Media that promote rapid cell growth are unfavorable for secondary metabolites production. Therefore, a suitable production medium for each metabolite or plant species must be developed. A two stage culture, growth medium and a production medium are recommended for the culture of higher plants (Fujita *et al.*, 1981b). For shikonin, the MG-5 growth medium was developed from Linsmaier and Skoog medium and the M-9 production medium from White's medium by optimizing the concentration of nutrients in each medium (Fujita and Tabata, 1987; Yamada and Fujita, 1984). The production medium has a lower content of nitrate or phosphate or both. A lower sugar

content or a less commonly utilizable carbon source can enhance secondary compound production (Becker, 1987)

Phytohormones are important in the production of secondary metabolites. The auxin and cytokinin concentrations alter both cell growth and yield of specific compounds. An increase in the auxin level often leads to dedifferentiation via a stimulation of cell division and consequently diminishes the level of secondary metabolites (Becker, 1987). Another study has shown that high doses of growth regulators increase the content of secondary compounds in *Dioscorea deltoidea* (diosgenin) and *Mucuna pruriens* (L-DOPA) (Misawa, 1985). The saponin synthesis in *Panax ginseng* callus was promoted by 2,4-D and inhibited by IAA (Furuya *et al.*, 1983). On the other hand, 2,4-D suppressed the production of nicotine, antabine and anabasine in callus cultures, whereas IAA promoted the production of these alkaloids (Furuya *et al.*, 1971). IAA gave both the highest cell and serpentine alkaloid yields in suspension cultures of *Catharanthus roseus* (Zenk *et al.*, 1977). Gibberellins often have negative effects on the production of secondary metabolites, but berberine production by *Coptis japonica* cells was increased substantially by gibberellin, whereas shikonin production was inhibited (Fujita and Tabata, 1987). Overall, growth regulators affect the production of secondary metabolites differently and thus the most suitable combinations have to be developed for each plant species.

An exogenous supplement of biosynthetic precursors and elicitors to the culture medium may increase the yield of the end product (Eilert *et al.*, 1985;

Zenk *et al.*, 1977). But, getting the most efficient compound and the cost of it may pose problems. In one example, the screening of a variety of elicitor preparations for dose response for sanguinarine (antimicrobial compound) production resulted in a 1% *Botrytis* culture homogenate to be most effective (Eilert *et al.*, 1985).

Secondary metabolites are mostly stored within the vacuoles of the producing cells (Brodelius, 1990). The release of metabolites from vacuole by permeabilization of the plasma membrane and tonoplast without killing the cells has been difficult.

Secondary product formation is always positively correlated with the activity of the enzymes linking primary and secondary metabolism, and these enzymes are considered a bottleneck for optimal productivity (Andersen *et al.*, 1985; Berlin *et al.*, 1985). So far, there is little knowledge about the enzymes involved in the synthesis of many secondary products and their compartmentation in the cell. According to Luckner and Diettrich (1990), only small amounts of secondary metabolic enzymes are formed due to limited expression of secondary metabolic genes. They suggested that higher rates may be obtained after fusion of the structural part of the genes with strong promoters. Genetic engineering will supposedly raise the rate of biosynthesis of plant products in the future.

In general, the principles integrating expression of secondary metabolism in the differentiation programs of plant cells are nearly unknown (Luckner and

Diettrich, 1987). Investigating the pathways of secondary metabolism is the key to understanding the triggering of the desired products.

CHAPTER 3

PROPAGATION BY STEM CUTTINGS AND SEED

Introduction

Phytolacca dodecandra L'Herit, Phytolaccaceae, commonly called endod, has great potential as a molluscicide for the control of schistosomiasis transmitting snails (Lemma *et al.,* 1984). Schistosomiasis is one of the most pervasive parasitic diseases affecting 200-300 million people in tropical and subtropical regions (Lemma *et al.,* 1979). Traditionally, endod berries have been used as a soap, emetic, leechicide and abortifacient. At present, research is ongoing in the chemistry, toxicology and agronomy of endod in Ethiopia, Zambia, Swaziland, Zimbabwe, The Netherlands, Canada, U.S.A. and other countries (Lemma, personal communication).

Endod is dioecious, and propagation by seeds produces both male and female plants. Propagation by stem cuttings was attempted by Demeke (1984) with little success under conditions used. There is little information about the germination of endod seeds. The present study was carried out to determine the effect of IBA, NAA and shoot position on the rooting of endod semi-hardwood cuttings. Observations were also made on the seed germination of strains 3, 17 and 44. These strains are high yielding and partially pest resistant endod types selected in Ethiopia (Lugt, 1981).

Materials and methods

Rooting of cuttings

Plants were grown in the greenhouse from endod strain 17 seeds obtained from Ethiopia. Semi-hardwood cuttings of 10-month old seedlings from male and female plants were used. Cuttings consisted of 2-4 nodal sections cut immediately below and above the nodes. The basal leaves were removed. The larger terminal leaves were cut in half. Indole-3-butyric acid and naphthaleneacetic acid were applied to the basal one cm of the cuttings by the concentrated solution dip method for five seconds. The IBA and NAA solutions were prepared by dissolving in 50% ethanol. After dipping, the cuttings were immediately inserted 4-5 cm deep into a medium containing equal proportions of perlite and vermiculite in flats 28 cm wide x 51 cm long x 6 cm deep.

The first experiment was conducted to determine the effect of different IBA concentrations (0, 50% ethanol, 50% ethanol containing 1.2, 2.5, 4.9, 9.8, 19.7 and 39.4 mM IBA) on the rooting of semi-hardwood cuttings. The second experiment evaluated the effect of IBA and NAA treatments on promoting rooting of semi-hardwood cuttings. The third experiment was conducted to determine the effect of shoot position (apical, medial and basal) on rooting of cuttings. The relative characteristics of those cuttings used for the position experiment are noted in Table 3.1. Succulent tips and basal portions of shoots were discarded.

Table 3.1. Characteristics of cuttings used for determining the influence of shoot position on rooting.

Parameter	Shoot positions		
	Apical	Medial	Basal
Number of nodes (basipetal)	5-9	10-17	18-26
Diameter (mm)	3.5-5	5.1-6.5	6.6-7.5
Length (cm)	5-10	4-8	4-6

Cuttings were rooted under intermittent mist at a rate of 10 seconds every 4 minutes for the first 15 days and 10 seconds every 8 minutes thereafter. The day temperature of the greenhouse was $24 \pm 2^{\circ}C$ while the night temperature was $21 \pm 2^{\circ}C$. The cuttings received day light. A completely randomized design was used with three replications of 20 cuttings per treatment. The experiment was terminated at 6 weeks. Data recorded included number of roots, longest root length (cm), rooting percentage and longest shoot length (mm). Each cutting having one or more visible roots was classified as rooted. Duncan's New Multiple Range Test was used at the 5% level to separate significant mean differences. Square root transformation was used for number of roots, root length and shoot length data, whereas arcsine transformation was used for the rooting percentage data (Gomez and Gomez, 1976). Actual means are presented in the tables.

Seed germination

One-year old dried berries of endod strains 3, 17 and 44 were soaked in deionized water overnight for the seed germination experiment. The pericarp

was removed and floating seeds were discarded. Seeds were surface sterilized by a quick dip in 70% ethanol followed by a 5-minute soak in 1.05% sodium hypochlorite and three rinses in deionized sterile water. The seed coat was slightly cut with a razor blade in the scarification treatment. In the gibberellic acid treatment, seeds were soaked in 1.37 mM GA_3 for one hour prior to germination test. Seeds were germinated under aseptic conditions in petri dishes on blotting paper at 25$\pm$3°C. Germination percentage was recorded at 1 and 4 weeks. The data were transformed by the arcsine method prior to statistical analysis.

Results and discussion

Rooting of cuttings

The IBA treatment increased rooting percentage (Table 3.2). The rooting percentage of the control cuttings was lower than those exposed to the high levels of IBA (19.7 and 39.4 mM), but was similar to the low levels of IBA. Greater numbers of roots were observed at high concentrations of IBA, whereas longer and thicker roots were observed with low concentrations of IBA (Fig. 3.1). Greater root numbers per cutting associated with high concentration of IBA have been documented in other species (Bennel and Barth, 1986; Porlingis and Therios, 1976). Although many of the cuttings did not root after 2 weeks under mist, most rooted after 4 weeks. There was no substantial difference in shoot length among the treatments (Table 3.2).

Table 3.2. Effect of IBA on root and shoot development of *Phytolacca dodecandra* semi-hardwood cuttings (strain 17) after 6 weeks of planting.

Treatment	Root			Shoot
	No.	length (cm)	%	length (mm)
Control	5.8c[x]	8.7ab	63c	11.2a
50% ETOH	6.4c	11.4a	68bc	11.5a
1.2 mM IBA[y]	7.5c	7.8bc	72abc	10.2ab
2.5 mM IBA	10.1b	7.9b	77abc	11.8a
4.9 mM IBA	10.2b	8.6ab	78ab	10.5ab
9.8 mM IBA	11.2b	7.2bc	75abc	11.3a
19.7 mM IBA	12.7ab	6.7bc	83a	8.6b
39.4 mM IBA	14.1a	5.5c	83a	9.6ab

[x] Sixty cuttings per treatment. Within column, means followed by different letters are significantly different at the 5% level.
[y] All IBA solutions are in 50% ethanol.

The synergistic effect of IBA and NAA on rooting capacity has been reported for other species (Caldwell *et al.*, 1988; Rauch and Yamakawa, 1980), whereas little synergistic effect was documented by others (Khatamian and Abuelgasim, 1986; Lo, 1985). Combinations of low and high concentrations of IBA and NAA were used to determine if a synergistic effect could be induced. Combinations of IBA and NAA did not improve the rooting percentage of endod cuttings (Table 3.3). Since the control cuttings resulted in a high rooting percentage (78%) endod cuttings might have sufficient endogenous root promoting substances without the need for exogenous growth regulators.

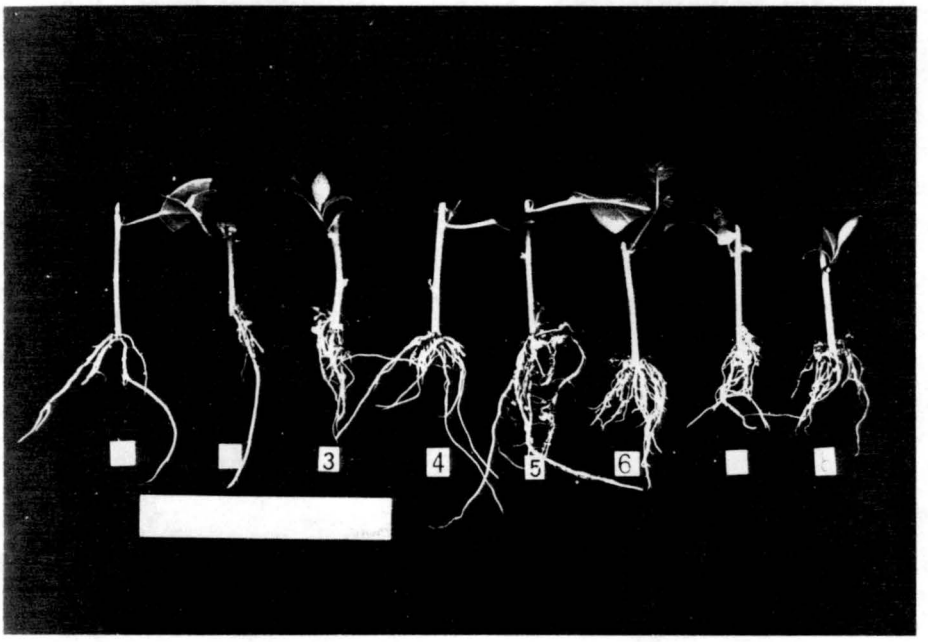

Figure 3.1. Rooting of *Phytolacca dodecandra* cuttings at different IBA concentrations: 1 (control), 2 (50% ethanol), 3 (1.2 mM), 4 (2.5 mM), 5 (4.9 mM), 6 (9.8 mM), 7 (19.7 mM) and 8 (39.4 mM) IBA.

Table 3.3. Effect of IBA and NAA on rooting of *Phytolacca dodecandra* semi-hardwood cuttings (strain 17) after 6 weeks of planting.

Treatment	Root No.	Root Length (cm)	Root %
Control	5.9b[x]	7.5a	78b
IBA 4.9 mM	11.4a	6.2ab	95a
IBA 19.7 mM	12.5a	5.4bc	85ab
NAA 5.4 mM	9.1b	7.1a	80b
NAA 21.5 mM	11.4a	4.9bcd	87ab
IBA and NAA (4.9 and 5.4 mM)	11.7a	4.6cd	82ab
IBA and NAA (19.7 and 21.5 mM)	13.0a	4.1d	85ab

[x] Sixty cuttings per treatment. Within column, means followed by different letters are significantly different at the 5% level.

Callus production at the base of the cuttings was greater with increasing levels of IBA and NAA combinations.

Variations in rooting among cuttings taken from different shoot positions are reported in 'Hayward' Kiwifruit cuttings (Caldwell *et al.*, 1988). No differences in root numbers were associated with position of cutting when 2.5 mM of IBA was used; however, when no hormone was used, significantly greater numbers of roots were produced on basal cuttings (Table 3.4). Apical and medial cuttings produced longer roots than basal cuttings. Apical, medial and basal cuttings gave an average of 76%, 62% and 48% rooting respectively after 2 weeks under mist (Fig. 3.2). Similar results were reported with *Banksia*

coccinea cuttings in that the terminal semi-hardwood cuttings rooted faster than the basal semi-hardwood cuttings (Bennel and Barth, 1986). However, the basal endod cuttings gave 90-93% rooting percentage after 6 weeks in mist. Increased rooting was not observed from basal cuttings in 'Hayward' kiwifruit cuttings (Caldwell *et al.*, 1988), but was observed in *Epacris impressa* cuttings (Thompson, 1986). A tendency for longer shoots on basal endod cuttings was observed when no hormone was applied.

Table 3.4. Effect of shoot position on rooting of *Phytolacca dodecandra* semi-hardwood cuttings (strain 17) after 6 weeks of planting.

Shoot Position	Root number A	Root number B	Root length (cm) A	Root length (cm) B	Shoot length (mm) A	Shoot length (mm) B
Apical	14.1a[x]	7.6b	10.0a	5.2a	16.6a	13.7b
Medial	13.0a	7.0b	8.2a	5.3a	18.5a	13.5b
Basal	12.1a	9.4a	7.5b	5.1a	17.1a	20.8a

A = First experiment conducted in April 1989. Cuttings were treated with 2.5 mM IBA.
B = Second experiment conducted in August 1989. Growth regulator was not used.

[x] Sixty cuttings per treatment. Within column, means followed by different letters are significantly different at the 5% level.

Seed germination

Information on endod seed germination is limited. This work was carried out to determine the germination of endod seeds under aseptic conditions. Significant differences were achieved among the treatments in seed germination at one week from planting (Table 3.5). Scarification resulted in a faster

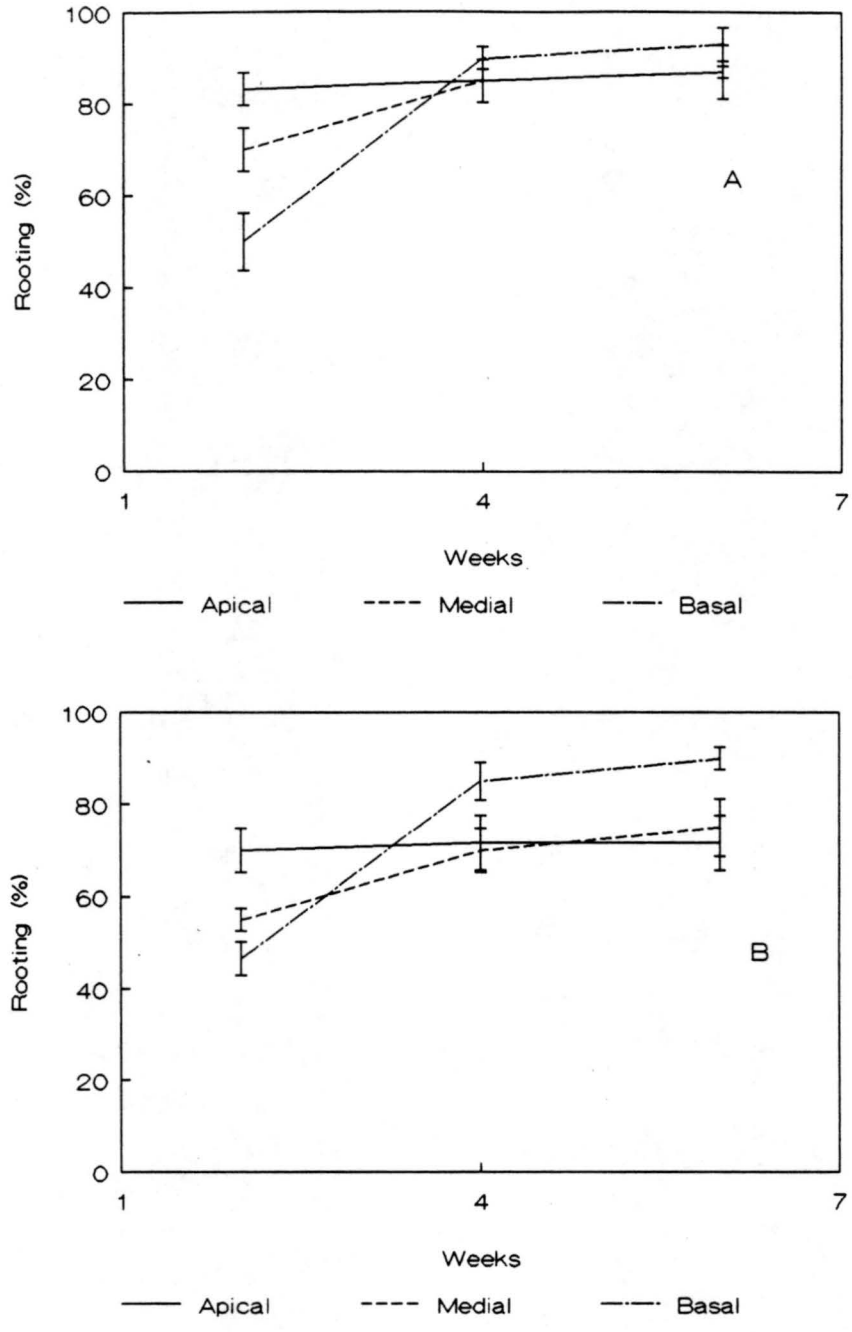

Figure 3.2. Rooting percentage of apical, medial and basal cuttings of *Phytolacca dodecandra*. Vertical bars indicate $\pm$ S.E.

A = First experiment conducted in April 1989. Cuttings were treated with 2.5 mM IBA.

B = Second experiment conducted in August 1989. Growth regulator was not used.

germination rate than the control and gibberellic acid treatment. The faster germination of scarified seeds could be due to faster water imbibition and oxygen uptake. The germination percentage for the scarification treatment for strains 3, 17 and 44 was 68.7, 68.5 and 82.4 respectively at one week from planting. The gibberellic acid and control treatments gave less than 45% germination. At 4 weeks the germination of the control and GA_3 treated seeds increased substantially and over 71% germination was recorded in all the treatments.

Table 3.5. Effect of scarification and gibberellic acid on the germination percentage of *Phytolacca dodecandra* seeds.

Strain	Treatment	Germination %	
		1 wk	4 wk
3	Control	20.0cd[x]	73.3c
	Scarification	68.7ab	97.8a
	GA_3	35.6bcd	80.0bc
17	Control	17.0cd	91.3ab
	Scarification	68.5ab	87.3ab
	GA_3	8.0d	71.8c
44	Control	40.0bc	97.8a
	Scarification	82.4a	95.5a
	GA_3	43.6bc	93.2ab

[x] Three petri dishes of 15 seeds each per treatment. Within column, means followed by different letters are significantly different at the 5% level.

In general, endod could be readily propagated by semi-hardwood cuttings under mist, with or without rooting hormones. A greater rooting

percentage could be obtained with rooting hormones. Cuttings taken from the apical and medial regions of stem branches root faster than the basal ones. The poor rooting percentage (20%) recorded in a previous experiment (Demeke, 1984) was probably associated with wilting and desiccation of cuttings as a result of high temperature and low relative humidity. Endod seeds could be easily germinated under aseptic conditions. The scarification of seeds enhanced germination rate.

CHAPTER 4

MICROPROPAGATION

Introduction

Endod, *Phytolacca dodecandra* L'Herit (Phytolaccaceae), is indigenous to eastern, central and southern Africa (Dalziel, 1936). The berries of this plant have been traditionally used in Ethiopia, Zambia and other African countries for washing clothes. The uses of endod berries as both medicine and poison (purgative, leechicide, abortifacient, etc.) have been well documented (Kloos, 1976). The discovery of endod as a potent molluscicide led to its international recognition as a means of controlling schistosomiasis-transmitting snails (Lemma, 1965). Schistosomiasis is a debilitating disease affecting millions of people in tropical and subtropical regions (Lemma *et al.*, 1984).

Endod is a dioecious plant in which vegetative propagation by stem cuttings has been attempted, but with little success (Demeke, 1984). Micropropagation is a rapid means of multiplying clonal materials (Murashige, 1974). Adams and Balandrin (1984) suggested the possibility of *in vitro* propagation of endod, but results have not been reported. The development of a protocol for micropropagation of endod is thought to facilitate the rapid production of the plant on a large scale. The present study was undertaken to

determine the feasibility of tissue culture propagation of endod by shoot-tip and nodal culture.

Materials and methods

Seeds of *Phytolacca dodecandra* L'Herit, strains 3, 17 and 44 (highly productive Ethiopian cultivars obtained from the Institute of Pathobiology, Addis Ababa), were grown in a greenhouse. Terminal and lateral shoot tips 1.5 to 2.0 cm were obtained from nine-month old seedlings, surface sterilized in 70% ethanol (2-5 S), immersed in 1.05% sodium hypochlorite containing 1 ml/L of Tween 20 for 5 minutes, and then rinsed with autoclaved distilled water. This disinfestation procedure yielded a high percentage of microorganism-free cultures (>95%).

The medium for micropropagation consisted of MS (Murashige and Skoog, 1962) basal salts with 2.43 μM pyridoxine HCl; 1.2 μM thiamine HCl; 4.06 μM nicotinic acid; 555.1 μM myoinositol and 26.6 μM glycine. Sucrose and agar were used at 2.0% and 0.8%, respectively. The pH of the medium was adjusted with either 0.1 N NaOH or HCl to 5.7$\pm$ 0.1 prior to the addition of agar. All growth regulators were added prior to autoclaving. Twenty ml of medium were dispensed into 25x150 mm test tubes capped with Bellco kaputs. Media were autoclaved for 15 minutes at 121°C and 15 PSI. Cultures were maintained in a growth room at 25$\pm$3°C with a 16-hour photoperiod at 90-95 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps. Twenty cultures were used per treatment in all experiments. In a preliminary experiment BA at

0.44 μM produced about 1.9 shoots per explant and was used for culture maintenance. All experiments were repeated.

Shoot multiplication

In the first experiment, strain 17 explants subcultured twice on MS medium with 0.44 μM BA were used. The shoot tips were trimmed to ca. 5 mm and cultured on the basal medium supplemented with 0.44, 4.4 or 22 μM BA; 0.46, 4.6 or 23.2 μM kinetin and 0.49, 4.90 or 24.6 μM 2iP. In the second experiment, the effect of removal of the shoot apex was investigated using strain 3 and 17 nodal explants cultured with 0.44 μM BA and 0.27 μM GA_3. The nodal explants were 1.0 to 1.5 cm in length with 2 to 4 nodes. In the third experiment, the effect of placement of nodal explants (vertical, diagonal and horizontal) on shoot multiplication was investigated using explants of strains 3, 17 and 44. The nodal explants were cultured on MS medium with 0.44 μM BA. The nodal explants consisted of 1.0 to 3.0 cm length and 2 to 5 nodes with the shoot apex removed. At 6 weeks, number of shoots per explant and main shoot length were recorded.

Root initiation

In vitro proliferated explants subcultured two to three times as described above were used for the root initiation experiment.

Auxins

Shoot tips (ca. 5 mm) of strain 17 were incubated on the basal MS medium using the auxins IAA (0.57, 2.85 or 5.70 μM), IBA (0.49, 2.45 or 4.90 μM) or NAA (0.54, 2.70 or 5.40 μM).

Pulsing

The objective of this study was to reduce callus production at the base of the explants. Shoot tips of strain 17 proliferated on MS basal salt medium with 0.44 μM BA were used for the experiment. The shoot tips were precultured on MS basal salt medium with 0, 0.49, 49.2, or 492.1 μM IBA for 2,4 and 6 days. The shoot tips were then subcultured on hormone free MS medium.

MS salt strength

The effects of different concentrations of the MS mineral salt formulation on rooting of cultured shoot tips were investigated at 1, 1/2 and 1/4 x of the original concentration. Explants of strains 3 and 17 were used. IBA (0.49 μM) was used in all the concentrations.

Explant type and charcoal

Nodal explants of 1.0 to 1.5 cm length with 2 to 4 nodes and shoot tips of ca. 5 mm length were cultured on hormone-free MS medium. Activated charcoal was used at 0.5 g/L.

Number of roots, longest root length, rooting percentage, longest shoot length, callus diameter and callus percentage were recorded at 6 weeks.

Data within each experiment was subjected to ANOVA with a Duncan's Multiple Range Test at the 5% level. Where appropriate, data was transformed by square root and arcsine method before statistical analysis was performed. For the data in which there were no significant differences, the mean and standard error of the mean are presented.

Eighty of the rooted explants were transferred to superfine germinating mix and kept in the greenhouse with or without mist. After 4 weeks they were transferred to 15-cm pots and placed into the greenhouse for further observation.

Results and discussion

Shoot multiplication

The best shoot production (3.1 shoots per explant) was obtained with 0.44 μM BA (Table 4.1). The main shoot length decreased as the level of cytokinins was increased. The value of BA for shoot proliferation has been well documented for various species (Chee and Pool, 1985; Han and Stephens, 1987). Kinetin was only slightly effective in shoot proliferation, and 2iP was ineffective. The use of 2iP has increased shoot proliferation in some species (Economou and Spanoudaki, 1985; Norton and Norton, 1985) but has resulted in no increase or poor growth in other species (Bennett and Davies, 1986).

Apical dominance leads to the suppression of growth of lateral buds. Removal of the terminal bud may enhance the growth of lateral buds (Bressan *et al.*, 1982). Significant differences were not obtained in terms of number of shoots by removal of the shoot apex and addition of growth regulators using nodal explants (Table 4.2). However, when BA (0.44 μM) and GA_3 (0.27 μM) were combined, 4.7 shoots per explant were obtained without the removal of apex, which was better than that obtained using shoot tips (3.1). As seen in Fig. 4.1A, large shoots were obtained from nodal explants.

Vertical and diagonal placement of nodal explants resulted in more number of shoot proliferation (4.5 to 5.3 shoots per explant) than horizontal placement (2.9 to 3.2 shoots per explant) in strains 3 and 17 explants (Table 4.3). Explants from strain 44 produced fewer shoots than strains 3 and 17 in vertical and diagonal placement. Horizontal placement produced the least number of shoots in all the three strains. Horizontal placement of explants increased shoot proliferation in tarragon (Mackay and Kitt0, 1987) unlike this experiment. The increase in shooot proliferation was assumed to be because of better uptake of medium constituents.

Table 4.1. Influence of cytokinins on in vitro shoot proliferation of *Phytolacca dodecandra* (strain 17) after 6 weeks in culture.

CK	Conc (μM)	No. Shoots/ explant	Main shoot length (mm)
Control	0.00	1.0d[x,y]	0.9bc
BA	0.44	3.1a	10.8b
	4.4	2.6b	6.1cd
	22.0	2.3bc	3.7d
KIN	0.46	1.0d	23.4a
	4.6	1.8c	10.5bc
	23.2	2.1c	9.1bc
2iP	0.49	1.0d	9.8bc
	4.9	1.0d	8.9bc
	24.6	1.0d	8.4bc

x - Square root transformation was used.

[y] Within column, means followed by different letters are significantly different at the 5% level.

Table 4.2. Effect of growth regulators and removal of shoot apex on *in vitro* shoot proliferation of *Phytolacca dodecandra* after 6 weeks in culture (Mean ± S.E.).

Strain	Medium[x]	No. shoots	
		Apex removed	Apex present
3	BA	3.4 ± 0.3	3.2 ± 0.3
	BA + GA_3	3.4 ± 0.4	4.2 ± 0.3
17	BA	4.3 ± 0.4	3.9 ± 0.4
	BA + GA_3	4.5 ± 0.4	4.7 ± 0.5

[x] BA = 0.44 μM and GA_3 = 0.27 μM.

Table 4.3. Effect of placement of nodal explants on shoot proliferation of *Phytolacca dodecandra* after 6 weeks in culture (MS medium with 0.44 μM BA).

Strain	Placement	No. Shoots/ explant
3	Vertical	4.5 ab[x]
	Diagonal	5.3 a
	Horizontal	2.9 d
17	Vertical	5.2 a
	Diagonal	5.2 a
	Horizontal	3.2 cd
44	Vertical	3.8 bcd
	Diagonal	4.0 bc
	Horizontal	3.2 cd

[x] Within column, means followed by different letters are significantly different at the 5% level.

Root initiation

Auxins

The auxins IAA, IBA and NAA enhanced root production of *in vitro* proliferated shoot tips (Table 4.4). A high rooting percentage was obtained in most of the treatments with the exception of the control and IAA at 0.57 μM; however, callus was evident in most of the treatments. IBA at 0.49 μM caused a high rooting percentage with minimal callus (Fig. 4.1B). In most of the treatments there was a direct association between high rooting capacity and high callus production (Table 4.4). At low auxin levels, thin and long roots were observed. Increasing the auxin concentration resulted in the production of greater amounts of callus and thick and short roots. Zimmerman (1983) reported a similar observation. Distorted and thick leaves were also produced at the highest auxin concentrations.

Pulsing

Preculturing shoot tips (ca. 5 mm) on an MS medium containing IBA and transferring to hormoneless medium did not result in a reduction of callus at the explant base (Table 4.5). The lowest concentration of IBA (0.49 μM) produced relatively low amount of callus at the explant base, irrespective of the number of days of pulsing. The highest concentration of IBA was inhibitory to shoot and root development. In desert milkweed preculturing shoot tips on an MS medium containing IBA and transferring to hormoneless medium reduced callus production at the base of the explants (Lee and Thomas, 1985). This did not work with endod (Table 4.5). The effect of concentration was significant, while

there was no significant interaction between concentration and time (Appendix Table A). The IBA concentrations had the same effect irrespective of the number of days of pulsing.

Table 4.4. Influence of auxins on *in vitro* rooting and callusing of proliferated shoot tips of *Phytolacca dodecandra* (strain 17) after 6 weeks in culture.

		Root			Callus	
Auxins	Conc (μM)	No	length (mm)	%	diam. (mm)	%
Control	0.0	0.3e[x,z]	7.1c[x]	26c[y]	0.0e[x]	0.0e[y]
IAA	0.57	2.1d	16.1a	35bc	2.3d	35d
	2.85	2.4cd	14.6a	60b	6.9c	70bc
	5.70	4.7a	14.6a	90a	11.0ab	95ab
IBA	0.49	2.2cd	16.2a	90a	4.3d	45cd
	2.45	4.0ab	13.6a	90a	14.8a	100a
	4.90	3.7ab	9.8a	100a	12.1ab	100a
NAA	0.54	2.5bcd	15.7a	90a	3.9d	50cd
	2.70	4.8a	8.5ab	100a	10.4b	95ab
	5.40	3.3abc	4.4bc	95a	15.6a	95ab

[x,y] Square root and arcsine transformation used respectively.

[z] Within column, means followed by different letters are significantly different at the 5% level.

Table 4.5. Effect of 2, 4 and 6 days pulsing on *in vitro* rooting, shooting and callusing of *Phytolacca dodecandra* explants (strain 17) after 6 weeks in culture.

Days of pulsing	IBA conc (μM)	No. roots	Root length (mm)	Rooting (%)	Callus diam.(mm)	Callus (%)	Shoot length (mm)
2	0.0	2.7 fg[x]	15.1 de	45c	2.0c	27d	15.7f
	0.49	7.1 cde	28.9 ab	93 a	3.7 c	40 cd	24.7 bcde
	4.9	9.3 abc	30.1 a	100 a	9.1 b	87 b	29.5 abc
	49.2	10.9 abc	21.9 abcd	93 a	16.2 a	100 a	23.2 bcdef
	492.1	5.6 de	14.9 cde	93 a	12.4 a	100 a	2.2 g
4	0.49	5.3 def	23.9 abc	100 a	3.9 c	47 c	18.3 def
	4.9	12.8 a	28.7 a	100 a	12.7 a	93 a	31.7 ab
	49.2	8.5 bcd	26.5 ab	93 a	14.8 a	100 a	26.3 bcd
	492.1	4.0 efg	7.9 ef	93a	14.1 a	100 a	2.9 g
6	0.49	7.8 cde	24.9 abc	87 ab	4.1 c	47 c	21.3 bcdef
	4.9	11.8 ab	31.3 a	100 a	14.5 a	100 a	35.7 a
	49.2	8.1 cd	17.8 bcde	80 ab	16.1 a	100 a	17.3 ef
	492.1	1.9 g	5.8 f	60 bc	7.9 b	100 a	0.7 g

[x] Within column, means followed by different letters are significantly different at the 5% level.

MS salt strength

There was no significant difference in the rooting percentage of the shoot tips at the different MS salt concentrations (Table 4.6). The number of roots and root length of the explants was not severely affected by the reduction of the salt concentration. There was a tendency of producing less callus in the reduced salt concentration. However, this was not substantial because the callus percentage was over 45%. Reducing MS mineral salt concentration stimulated rooting in rose (Hasegawa, 1980) and thornless trailing blackberries (Skirvin *et al.*, 1981).

Table 4.6. *In vitro* rooting of *Phytolacca dodecandra* shoot tip explants as affected by MS salt concentration (0.49 μM IBA).

Strain	MS salt conc	No. roots	Root length (mm)	Rooting (%)	Callusing (%)
3	1x	8.1a[x]	41.0a[x]	95a[y]	90a[y,z]
	1/2x	5.5ab	38.9ab	85a	80ab
	1/4x	4.6b	35.8ab	80a	45c
17	1x	4.7b	35.8ab	90a	95a
	1/2x	3.1b	24.3b	80a	55bc
	1/4x	5.3ab	31.2ab	80a	60bc

[x,y] - square root and arcsine transformation used respectively.

[z] Within column, means followed by different letters are significantly different at the 5% level.

Explant type and charcoal

The rooting percentage of nodal explants was significantly higher than that of shoot tips (Table 4.7). Over 50% of the nodal explants rooted 15 days after planting, whereas less than 5% of the shoot tips rooted (data not shown). In a preceding experiment, *in vitro* proliferated shoot tips treated with auxins produced 90 - 100% rooting; however, the callus association with the roots was also high, making them undesirable for planting. In this experiment, the nodal explants gave 80 - 85% rooting with low callus association (7.5%), which is more desirable for transplanting. Shoot tips cultured on a medium containing activated charcoal initiated significantly larger number of roots and longer roots than shoot tips cultured without charcoal. Activated charcoal also increased the root length of Malling Merton apple root stocks (Snir and Erez, 1980) similar to results in this experiment. Nodal explants produced longer shoots than did shoot tips. In general, there was a tendency of producing fewer roots in nodal and shoot tip explants compared to previous experiments presumably due to the absence of auxins.

The rooted plantlets that were transplanted directly into the greenhouse wilted during the first week. About 55% survived and developed into normal plants. Those under mist had 100% survival after 4 weeks. These plants derived from staminate clones showed a growth habit similar to plants from seed (Fig. 4.1C), and some produced staminate flowers after 6 months in the greenhouse.

Table 4.7. Effect of charcoal and explant type on rooting of *Phytolacca dodecandra* (strain 17) after 6 weeks in culture.

Explant	Char. (g/l)	No. roots	Root length (mm)	Rooting (%)	Shoot length (mm)	Callus (%)
Nodal	0.0	1.7a[x]	24.7a[x]	80ab[y]	11.1a	7.5ab[y,z]
	0.5	1.8a	37.5a	85a	14.2a	7.5ab
Shoot tip	0.0	0.3b	5.3b	20c	3.0b	0.0b
	0.5	0.8a	26.4a	50bc	5.7b	20.0a

[x,y] Square root and arcsine transformation used respectively.
[z] Within column means folllowed by different letters are significantly different at the 5% level. Plant growth regulator was not used.

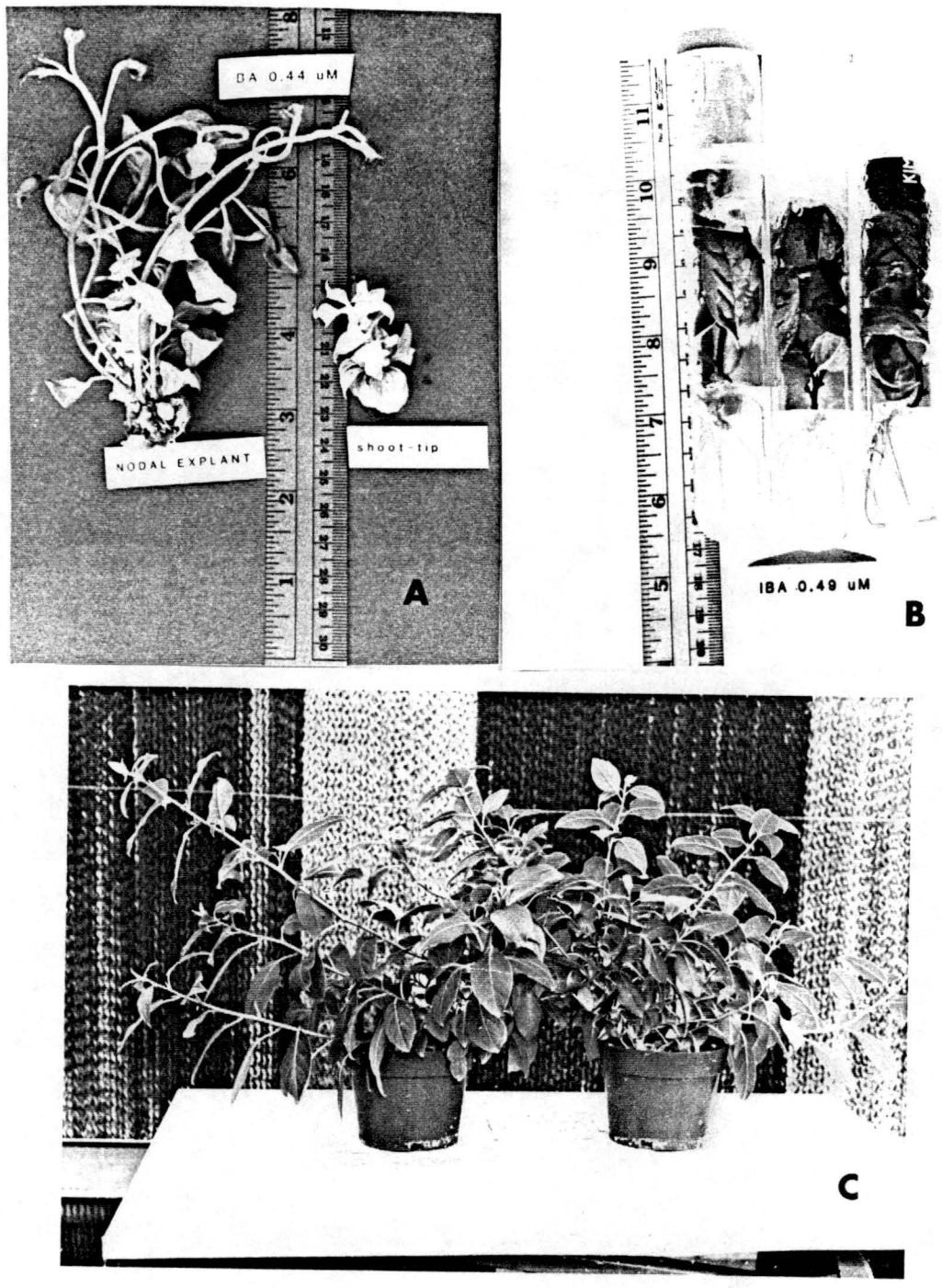

Fig. 4.1. Micropropagation of endod. A. Shoot proliferation of nodal explant (in 50x95 mm culture vessel) and shoot tip (in 25x150 mm test tube) on MS medium plus 0.44 μM BA after 6 weeks. B. Rooting of shoots on MS medium plus 0.49 μM IBA after 6 weeks. C. Plants derived from *in vitro* cultures after 3 months in the greenhouse (11.5 x 15 cm pots).

CHAPTER 5

GERMINATION AND STORAGE OF POLLEN

Introduction

Phytolacca dodecandra L'Herit (endod, *Phytolaccaceae*), produces a plant molluscicide that potentially could control schistosomiasis-transmitting snails (Lemma *et al*., 1984). Schistosomiasis is a debilitating disease that threatens about 200-300 million people in tropical and subtropical regions. Endod fruit also has other potential properties such as detergent, hirudinicide, leechicide, larvicide and fungicide (Lemma *et al.,* 1984). Endod is dioecious and, thus, propagation by seed results in both male and female plants. The proportion of male to female plants has not been determined, but observation indicates approximately a 1:1 ratio.

Studies of pollen viability, fertility, and storage are important for an endod breeding program. The medium components required for pollen germination of different plant species varies. Modifications of the medium developed by Brewbaker and Kwack (1963) have been widely used for germinating pollen of various species. Pollen availability could be ensured by storage under low temperature (Ganeshan, 1985; Lee *et al*., 1985; Sahar and Spiegel-Roy, 1980). The objective of this study was to determine the optimum medium requirements for germination of *Phytolacca dodecandra* pollen grains and to assess the

storability and fertilizing capacity of stored pollen. This study is the first report to our knowledge on pollen germination and storage of *Phytolacca dodecandra*.

Materials and methods

Flowers were harvested betweem 10 - 12 a.m. from *Phytolacca dodecandra* strain 17 plants. Pollen was collected from dehiscing anthers and used within four to five hours for germination studies. Since the germination percentage of pollen varies among plants of the same species and even among flowers of the same plant, pollen for each experiment was taken from the anthers of a single flower and thoroughly mixed.

Pollen germination

The following modification of Brewbaker and Kwack medium (1963) was used as a basal medium in liquid form: 10% sucrose (w/v); 989.1 μM KNO_3; 161.8 μM H_3BO_3; 847.5 μM $Ca(NO_3)_2.4H_2O$; and 406 μM $MgSO_4.7H_2O$, pH 5.7$\pm$0.1. The medium was autoclaved for 15 min at 121°C. Pollen was cultured in 12x75 mm vials containing 400-500 μl of the medium and incubated on a water bath shaker (100-120 RPM) at 25$\pm$1°C. Three vials were used for each treatment. One of the above described medium components was varied in each test while keeping the other components constant. The optimum of that component was taken and the next component was varied in turn. The concentrations investigated were as follows: 0, 5, 10, 15, 20, 25 and 30% sucrose (w/v); 0, 1.6, 16.2, 161.8, 809.1 and 1618.1 μM H_3BO_3; 0, .99, 9.9, 98.9, 494.5 and 989.1 μM KNO_3; 0, 211.9, 423.7, 847.5, 1271.0 and 1694.9 μM

$Ca(NO_3)_2.4H_2O$; and 0, 203.0, 406.0, 609.0, 812.0, 1015.0 and 1218.0 μM $MgSO_4.7H_20$. Germination was determined after 16 hours of incubation by counting the number of germinating and non germinating grains within a 10 μl drop under a stereoscope (63x). Twenty counts were made for each treatment. Pollen grains with tubes longer than the diameter of the grains were considered germinated. Pollen grain density in the medium varied from 2 to 5 per μl. All experiments were repeated at least once. The germination data in the form of absolute percentage of germinated grains were analyzed for variance using arcsine transformation; however, actual means are listed.

Pollen storage

The storage temperatures investigated were $24\pm2^{\circ}C$ (room), $1\pm1^{\circ}C$ (refrigerator) and $-175^{\circ}C$ (vapor phase above liquid nitrogen in a storage vat). Pollen was collected from flowers of one plant and placed in a desiccator over anhydrous $CaCl_2$ for 24 hours. Different storage treatments began after this initial treatment. The moisture content of fresh, desiccated and rehydrated pollen was determined. Desiccated pollen was placed in gelatin capsules which were inserted into cryogenic plastic vials and stored at the above temperatures. For the germination test, vials from cryogenic storage were thawed in a $40^{\circ}C$ water bath (5 min). The pollen were rehydrated over a saturated $CuSO_4.5H_2O$ solution for two hours before incubation for germination. The medium used for germination consisted of: 10% sucrose; 161.8 μM H_3BO_3; 847.5 μM $Ca(NO_3)_2.4H_2O$; 406.0 μM $MgSO_4.7H_2O$ and 989.1 μM KNO_3. The storage experiment started before the germination experiment was over, and thus the

optimum medium was not used. This was necessary in order to make efficient use of the available flowers at the beginning of the experiment. Data were analyzed as described above. Pollen stored at -175°C for three months was used to determine fruit setting capacity. Glassine bags were used for the controlled pollinations.

Results and discussion

Pollen germination

Sucrose has been reported to be essential for pollen germination of angiosperm plant species (Brewbaker and Kwack, 1963; James *et al.,* 1987; Lee *et al*., 1985). The optimum concentration of sucrose for endod pollen germination was 10% (Fig. 5.1). Pollen germination was suppressed at 0% and 30% sucrose. Boron was observed to be critical for the germination of endod pollen (Fig. 5.2). Low concentrations of H_3BO_3 (1.6 and 16 μM) resulted in poor germination. Those which did germinate in the low boron medium had very short pollen tubes. The highest pollen germination was achieved at 162 and 809 μM H_3BO_3. Boron plays a key role in pollen germination of many plant species (Brewbaker and Kwack, 1963; James *et al*., 1987; Pfahler, 1967; Vasil, 1963) and is assumed to maintain the structural integrity of plant membranes (Pilbeam and Kirby, 1983).

Potassium nitrate enhanced pollen germination of several plant species (Brewbaker and Kwack, 1963; Sahar and Spiegel-Roy, 1984), whereas no beneficial effect was recorded for jojoba pollen (Lee *et al*., 1985). Here

potassium nitrate did not promote the germination of endod pollen (Fig. 5.3). High concentrations of KNO_3 decreased the germination percentage of pollen grains. Calcium nitrate did not enhance the overall germination percentage of endod pollen grains (Fig. 5.4). High levels of $Ca(NO_3)_2.4H_2O$ suppressed pollen germination. Calcium has been indicated to promote pollen tube length of pollen grains (Brewbaker and Kwack, 1963; James *et al.*, 1987). In this experiment the pollen tube length was not affected. Thus, endod pollen grains might have sufficient endogenous calcium for germination. Magnesium sulphate did not promote the germination of the pollens (Fig. 5.5). The essential role of magnesium sulphate for pollen germination has been documented (Brewbaker and Kwack, 1963); however, it did not enhance the germination of endod pollen.

Pollen storage

Over 60% germination was observed at all the three storage temperatures (24 ± 2, 1 ± 1 and -175 °C) after one week of storage. However, germination percentage of pollen grains at room temperature declined to 25.2% at two weeks and no germination was observed at 4 weeks (Fig. 5.6). Pollen grains at 1 ± 1°C and -175°C were still viable for six months. Pollen stored at 1 ± 1°C resulted in 78% and 79% germination at two weeks and four weeks from storage and then declined to 59% at four months and increased slightly at five and six months. The germination percentage of cryopreserved pollen remained high (over 60%) and increased slightly with time. The reason for the increase in the germination percentage of cryopreserved pollen with time is not known.

The increase in germination of pollen stored at low temperatures for short periods was speculated to be either because of after-ripening processes going on after shedding or because of release of some needed nutrients (Stanley and Linskens, 1974). Normally, pollen stored under cryogenic temperature is assumed to maintain viability indefinitely (Towill, 1985).

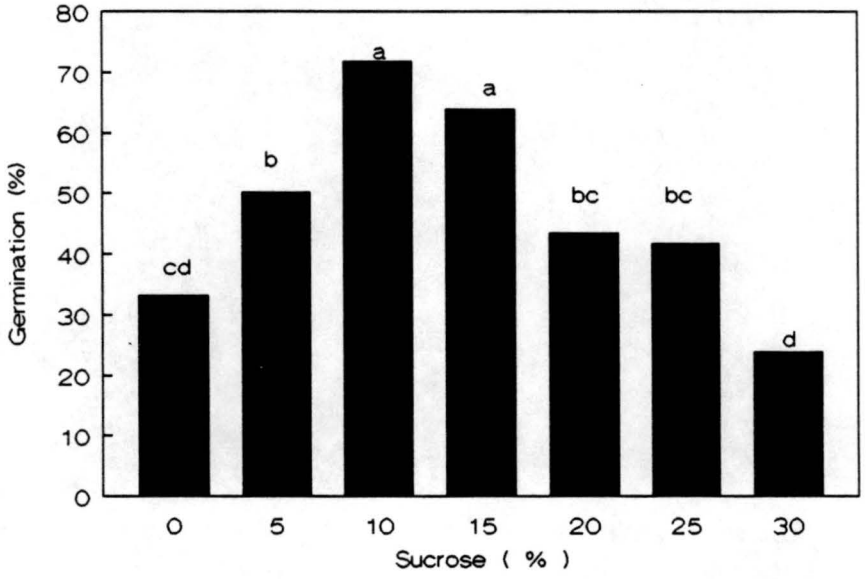

Fig. 5.1. Effect of sucrose on endod pollen germination. Bars having different letters are significantly different at the 5% level.

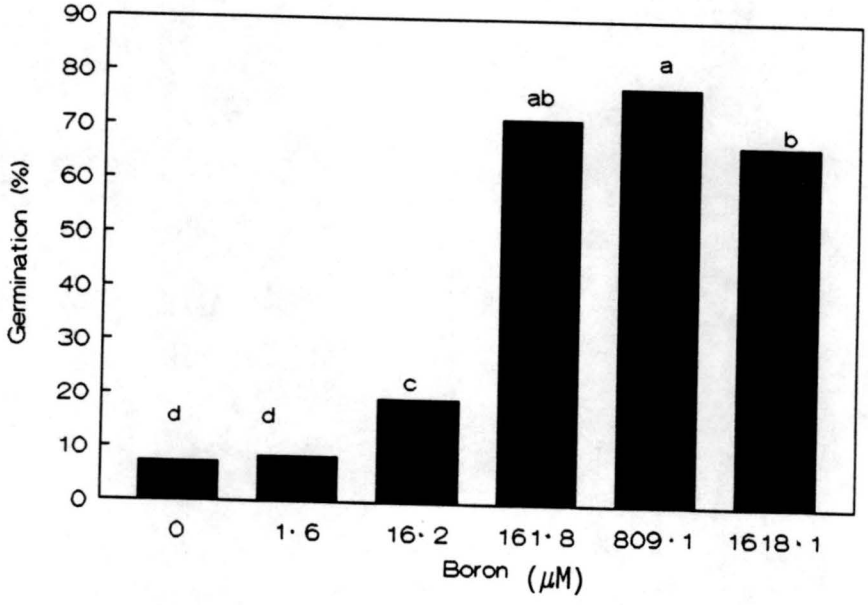

Fig. 5.2. Effect of boron on endod pollen germination. Bars having different letters are significantly different at the 5% level.

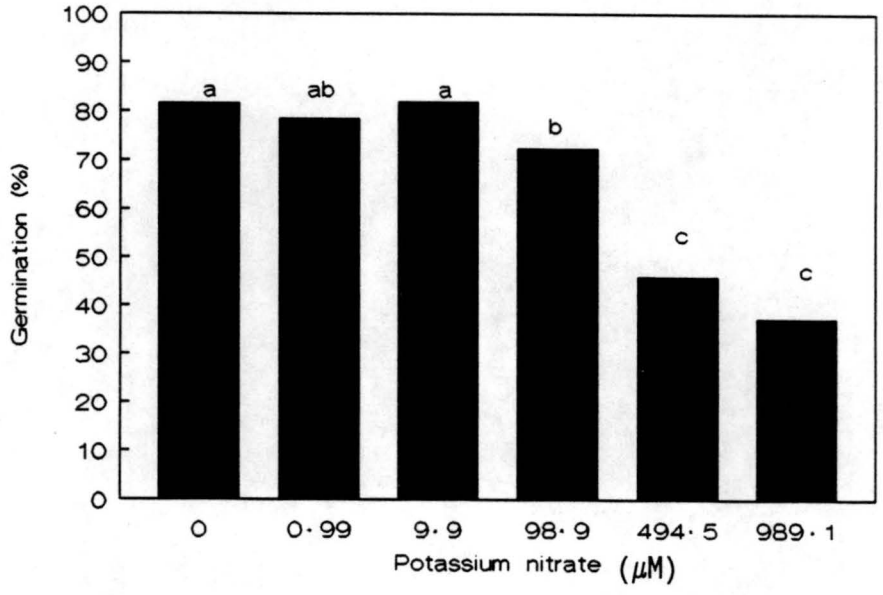

Fig. 5.3. Effect of potassium nitrate on endod pollen germination. Bars having different letters are significantl different at the 5% level.

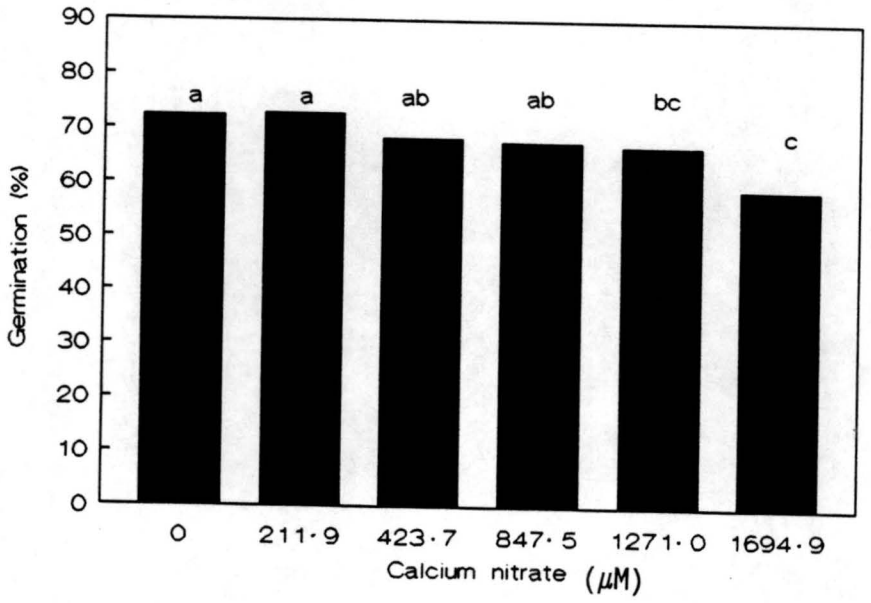

Fig. 5.4. Effect of calcium nitrate on endod pollen germination. Bars having different letters are significantly different at the 5% level.

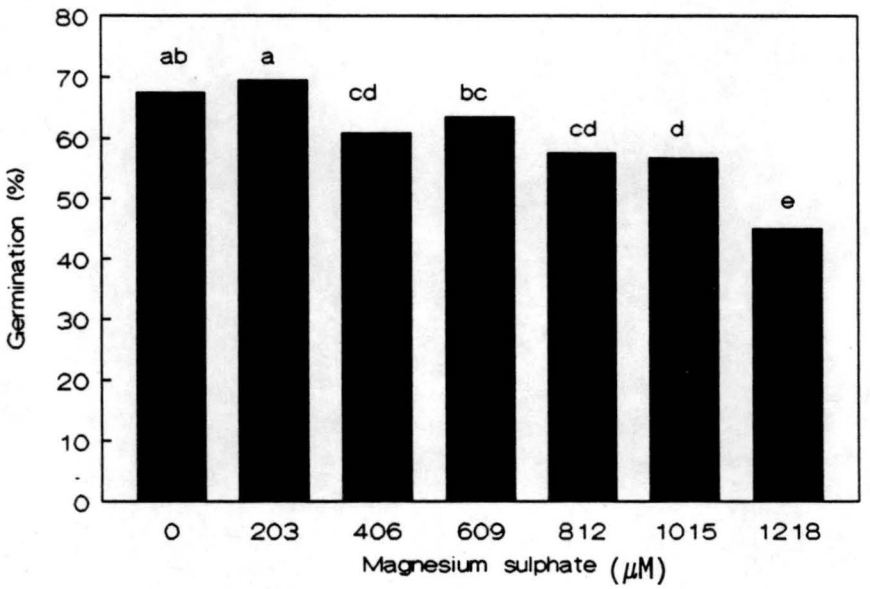

Fig. 5.5. Effect of magnesium sulphate on endod pollen germination. Bars having different letters are significantly different at the 5% level.

Low moisture content is necessary for survival of pollen at cryogenic temperature (Towill, 1985). The moisture content of endod pollen grains at harvest was low (7.8% f.wt basis), and dehydration over $CaCl_2$ further reduced the moisture content to 1·4%, which was the moisture content of the stored pollen (Table 5.1). Thus edod pollen collected from dehiscing anthers has low moisture content.

Rehydration of pollen stored under low temperature and low relative humidity prior to germination test gave high percentage of *in vitro* germination (Towill, 1985). Rehydration of the dehydrated and stored endod pollen over saturated $CuSO_4.5H_2O$ solution raised the moisture content to 18% within two hours. Rehydration for 96 hours raised the moisture content to 41% (Table 5.1).

Pollen stored under cryogenic temperature for three months produced normal fruit set when used in pollination (Table 5.2). The pollen was rehydrated before pollination. The control (bagged and not pollinated) flowers gave no fruit set. The open pollinated, fresh pollen applied and cryo-preserved pollen applied to pistillate flowers produced a similar percentage of fruit set (85-88%). The seeds obtained were also viable. Endod fruit contains 4-5 seeds.

In conclusion, endod pollen could germinate on a medium containing sucrose and boron. A concentration of 10% sucrose and 162 μM H_3BO_3 resulted in greater than 70% pollen germination. The pollen was preserved at $1\pm1^{\circ}C$ and -175°C for at least six months at a moisture content of about 1.4%. Storage at -175°C will help to maintain pollen viability for a long time. Dehydrated pollen could be kept at $24\pm2^{\circ}C$ for approximately 15 days and still

retain satisfactory viability. Endod is a dioecious plant. So far no breeding work has been undertaken on this species. One of the important steps in a breeding program is to have viable pollen available for crossing. This study has demonstrated that endod pollen could be held for later use in crossing.

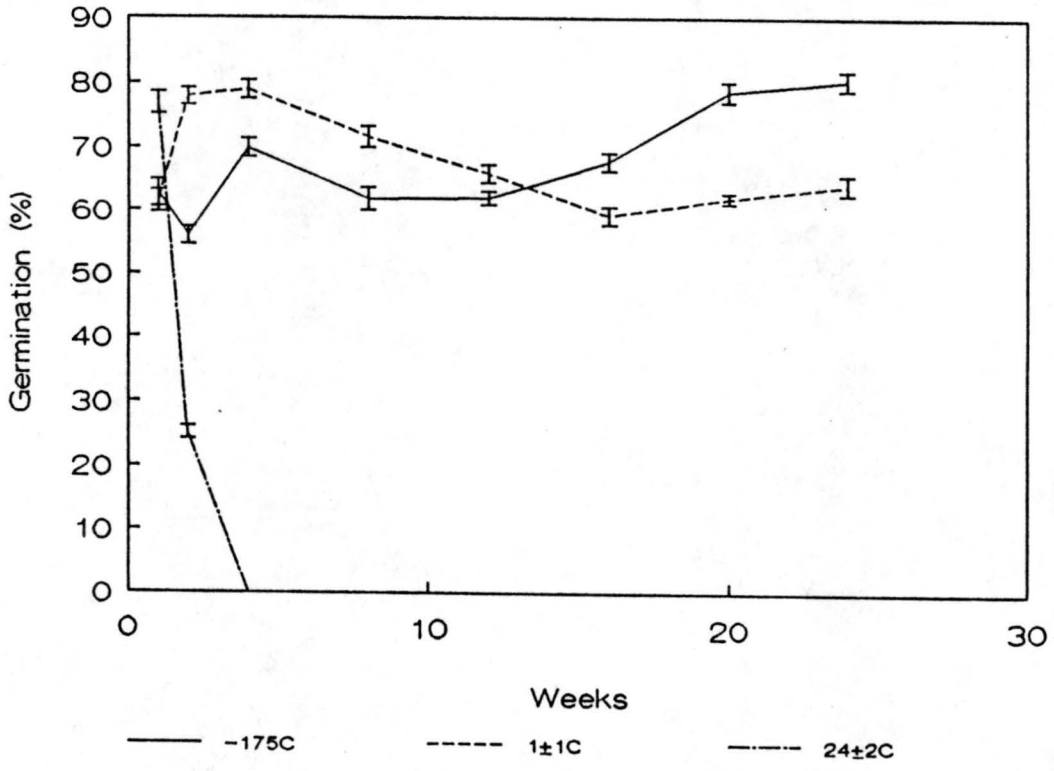

Fig. 5.6. Effect of storage temperature on endod pollen germination. Vertical bars indicate ± S.E.

Table 5.1. Relative moisture content of fresh endod pollen as compared to dehydrated and rehydrated with time (Mean ± S.E.).

Pollen condition	Moisture content (%)
Fresh	7.8 ± 0.6
Dehydrated for 24 h ($CaCl_2$)	1.4 ± 0.7
Rehydrated for 2 h	18.2 ± 0.7
Rehydrated for 24 h	35.2 ± 0.1
Rehydrated for 72 h	41.1 ± 0.5
Rehydrated for 96 h	41.2 ± 0.7
Rehydrated for 120 h	38.7 ± 1.2

Table 5.2. Effect of mode of pollination on endod fruit setting.

Pollination	No. flowers pollinated	% Fruit set
Control (bagged)	128	0.0
Open Pollinated	132	88.2
Fresh Pollen Applied (bagged)	129	85.5
Cryopollen Applied (bagged)	298	88.0

CHAPTER 6

POTENTIAL OF CALLUS AS A SOURCE OF LEMMATOXIN

Introduction

In vitro biosynthesis of secondary compounds may be used to enhance the production of some economically important metabolites such as shikonin (Tabata, 1977). In some cases, the plants producing these metabolites are difficult to grow under natural or field conditions necessitating other means of producing the metabolites. Secondary compounds could be produced under controlled environmental conditions free of microbes and insects. Endod produces triterpenoid saponins in leaves, flowers and berries in increasing amounts. Field production of endod has constraints such as low yield, disease and insect susceptibility. Thus producing the active ingredient of endod (lemmatoxin) *in vitro* might enhance productivity. The objective of this study was to determine the feasibility of producing lemmatoxin in callus cultures.

Materials and methods

Callus production

Shoot tips, immature flower buds and pericarps from endod strain 17 were cultured on an MS medium containing 26.8 μM NAA, 4.9 μM IBA, 5.7 μM IAA, 24.6 μM 2iP, 4.6 μM kinetin or 4.5 μM 2,4-D. The sterilization procedure used was: rinsing the explants in running cold water for 2 hours, surface

sterilizing in 70% ethanol, immersing in 1.05% sodium hypochlorite containing 1 ml/L of Tween 20 for 5 minutes, followed by rinsing with autoclaved distilled water three times. With the exception of 2,4-D, the above concentrations were selected on the basis of high callus proliferation obtained during experimentation for shoot and root production. Callus was also produced from hypocotyl and cotyledon. The cultures were subcultured every 4 weeks. A completely randomized design with 15 replications was used. The fresh weight of callus produced was recorded.

ABA was used at the rate of 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M to try to enhance lemmatoxin production. The ABA solution was filter sterilized and added to the autoclaved medium. Calli obtained from shoot tip, flower bud and pericarp that were maintained on 4.9 μM IBA were cultured on the above ABA concentrations. Lemmatoxin activity was determined after two passages.

Reducing the concentration of inorganic mineral salts such as phosphate and nitrate has been reported to enhance the accumulation of secondary metabolites. Callus obtained from shoot tip and maintained on an MS medium with 4.9 μM IBA was cultured on an MS medium at concentrations of 1, 1/2 and 1/4x of the original concentration. The concentrations of agar, sucrose, vitamins and IBA were the same for all.

Sample preparation

Fresh callus and plant samples were freeze dried and ground to a fine powder for analysis. The samples were held in a cold room (ca. 2-4°C) until used.

Methods used for the determination of lemmatoxin activity

1. Hemolysis

Hemolytic activity of saponins was determined according to Kawai *et al.* (1988). The dilution ratio used was 1:200 (e.g. 0.02 ml sheep blood was added to 3.98 ml sample solution). The pH of all solutions was adjusted to 7.4 before the addition of citrated sheep blood. The citrated sheep blood was obtained from Colorado Serum Corporation (Denver, Colorado) and was used within one month of purchase. The blood was kept in a refrigerator. The normal saline solution consisted of 0.9% NaCl (sterile, nonpyrogenic). After the addition of sheep blood, the samples were incubated at room temperature for 20 minutes. Then they were centrifuged at 2500 RPM for 5 minutes. The absorbance of the supernatant was measured with spectronic 20 at 540 nm against a water blank set at zero absorbance. One cuvette (1 cm) was used for all the measurements. Three measurements were taken for each sample.

Hemolytic activity in each supernatant was determined using the following formula:

$$\text{hemolytic activity (\%)} = \frac{a - b}{c - b} \times 100, \text{ where}$$

a = absorbance of sample solution
b = absorbance of normal saline solution
c = absorbance of 100% hemolysis (sheep blood + distilled water)

2. Molluscicidal test

Molluscicidal activity of callus samples was tested at the Institute of Pathobiology, Addis Ababa, Ethiopia. Letter codes were given for the samples

to avoid bias in analysis. Medium sized *Biomphalaria pfeifferi* snails with 8 - 10 mm shell diameter obtained from a highland stream (about 70 km east of Addis Ababa) were used for bioassay. The stock solution was prepared by dissolving 0.1 g of the sample in 1000 ml of tap water. The stock solution was kept for 16 hours before serial dilution. The pH of the solution was adjusted to 7.2 and the room temperature was about 22°C. For each test 6 replications of 5 snails each were exposed. The snails were left in the solutions for 24 hours. All snails were allowed to recover for 48 hours in tap water before mortality was determined by response or lack of response after pricking the head/foot with a probe and if necessary by dissection. Two replications of 5 snails each were used as controls.

Results and discussion

Callus production

The greatest callus production was obtained from shoot tip explants (Table 6.1) with little callus production from flower bud or pericarp explants. Overall, IBA and 2iP stimulated callus production. Callus production was apparently suppressed by 2,4-D and kinetin. The color of the callus ranged from white to brown and the texture ranged from soft to friable. The relative dry weight percentage after freeze drying of the callus ranged from 4.0 to 6.9.

Table 6.1. Effect of growth regulators on callus production of *Phytolacca dodecandra* (strain 17) (4th subculture).

Growth regulator	Fresh wt. of callus (g) shoot tip	flower bud	peri-carp	Dry wt. % shoot tip callus[x]	Shoot tip callus characteristics
NAA (26.8 μM)	11.51c[y]	0.11b	0.05c	6.2±2.1	soft; brown
IBA (4.9 μM)	19.56a	0.49a	0.16ab	6.1±0.8	soft, white-brown
IAA (5.7 μM)	13.42bc	0.22b	0.09bc	4.0±0.9	semi-friable; white brown
2iP (24.6 μM)	15.71ab	0.72a	0.22a	6.9±0.7	friable; white-brown
Kin (4.6 μM)	1.14d	0.18b	0.10bc	6.6±0.1	soft; white
2,4-D (4.5 μM)	0.58d	0.07b	0.07bc	-	very soft; white

[x] The values indicate mean ± S.E. The dash line indicates that data was not available.
[y] Within column, means followed by different letters are significantly different at the 5% level.

Production of lemmatoxin in plant and callus samples

From different endod plant samples, hemolytic activity was high in the reproductive organs with unopened female flowers showing greater hemolytic activity than opened and unopened male flowers (Table 6.2). The greatest hemolytic activity was found in the berries. The fully enlarged and green berry stage had the greatest hemolytic activity as compared to the immature and ripe berry stages. The molluscicidal activity of endod extract increased from leaves to the green berries with no activity in the roots (Lemma *et al.*, 1979) which was similar to the results of the hemolysis assay. The rachis (part of a stem that supports berries) showed low hemolytic activity and is generally separated from the berries for analysis. The hemolysis assay proved to be a simple

method for detection of the active ingredient in endod samples. The practical use of this assay in the determination of the active ingredient of endod has been recently reported (Monkiedje *et al.*, 1990; Thiilborg *et al.*, 1990).

Table 6.2. Hemolytic activity of samples from different plant parts (Mean± S.E.).

Plant sample	Hemolysis %
Roots	1.7±0.5[x]
Young stem branches	30.0±1.4[x]
Old leaves	0.00[x]
Young leaves	0.00[x]
Rachis	17.5±0.6[x]
Male flower (unopened)	37.2±2.1
Male flower (opened)	13.5±1.7
Female flower (unopened)	65.0±2.5
Immature berry	52.5±0.5
Fully grown berry	96.3±0.1
Ripe berry	40.8±0.8

[x] Values with 'x' indicate hemolysis percentage from an extract of 500 mg/L samples, whereas the other values indicate hemolysis percentage from 100 mg/L of the samples.

Callus samples showed little hemolysis activity (Table 6.3). Even a high concentration of the callus samples (5000 mg/L) resulted in minimal hemolysis. Callus obtained from cotyledon and hypocotyl explants did not show hemolytic activity. Abscisic acid did not enhance the production of lemmatoxin. Callus obtained from shoot tip, flower bud and pericarp incubated on an MS medium

containing 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M ABA showed little hemolytic activity at 500 mg/L. Similarly, reducing the MS mineral salt concentration to 1/2 and 1/4 of the original concentration did not have an impact on the production of lemmatoxin. Data for these are not shown as the results are zero or nearly so. However, ground whole berry and spray dried samples produced high hemolytic activity (Table 6.4). A concentration of 100 mg/L of these samples resulted in over 85% hemolysis.

Table 6.3. Hemolytic activity of callus samples from various plant parts and grown with various hormone levels (Mean±S.E.).

	Hemolysis (%) Relative concentration of samples		
Callus source	5000 mg/L	2500 mg/L	1250 mg/L
Flower bud callus (IBA)	9.3±.09	5.1±.17	2.5±.17
Pericarp callus (IBA)	9.6±.08	2.6±.05	2.0±.05
Shoot tip callus (NAA)	10.9±.10	5.2±.05	1.0±.05
Shoot tip callus (IAA)	8.0±.27	3.2±.15	2.5±.12
Shoot tip callus (2ip)	8.8±.11	5.9±.09	2.6±.23

Table 6.4. Hemolytic activity of sample derived from *Saponaria molina,* spray dried and whole berry samples (Mean±S.E.).

Conc (mg/l)	Hemolysis (%)				
	Spray[x] dried	*Saponaria*[y] *molina*	Freeze dried samples of strain 3	17	44
100	109.0±0.5	100.0±0.5	95.0±0.4	101.6±0.7	86.8±0.5
50	102.3±0.3	73.6±1.7	92.2±1.6	94.1±0.2	81.7±0.7
25	97.7±0.6	25.8±1.7	45.5±1.4	81.8±1.2	63.7±1.0
12.5	46.6±0.8	2.9±0.1	9.5±0.2	18.2±0.5	23.2±1.8
6.25	10.9±0.2	0.9±0.2	3.5±0.1	0.5±0.1	1.5±0.4

[x]Water extracted and spray dried sample of strain 44 berries obtained from Dr. Lugt (the Netherlands).
[y]Saponin sample derived from *Saponaria molina* and obtained from Kodak company (Denver, Colorado).

According to Parkhurst *et al*. (1989), inactive saponins are contained in the pericarp and are converted into active saponins by enzymes contained in the seed. Known amounts of callus and other samples were mixed with known amounts of ground seed and incubated overnight in a saline solution and checked for hemolysis. As seen in Table 6.5, the callus samples showed weak hemolytic activity. The seed by itself produced some hemolytic activity. The whole ground berry samples showed extensive hemolytic activity, as expected.

In a further observation, berries were soaked in saline solution and incubated overnight. Seeds, pericarps and the remaining solution (whole berry extract) were separated and freeze dried for analysis. Separately, each of the three components had minimal hemolytic activity. However, mixing ground seeds with either whole berry extract or pericarp gave high hemolytic activity

(Table 6.6). This confirms the work of Parkhurst *et al.* (1989) in that seeds and pericarp or whole berry extract are needed for the production of lemmatoxin. However, callus samples failed to show much hemolytic activity when ground seeds were added. Thus it is evident that the callus lacked the necessary substrate(s) for the enzyme(s) to act upon.

Table 6.5. Effect of seed extract on the activity of different samples (Mean±S.E.)[x].

Samples	Hemolysis (%)
50 mg seed + 950 mg callus	13.8±.09
100 mg seed + 900 mg callus	14.6±.11
200 mg seed + 800 mg callus	14.1±.05
300 mg seed + 700 mg callus	16.3±.08
400 mg seed + 600 mg callus	16.3±.08
500 mg seed + 500 mg callus	11.1±.03
1000 mg seed	14.1±.40
1000 mg callus	2.4±.16
100 mg strain 44 ground whole berry	94.3±.05
100 mg spray dried sample	85.9±.08
100 mg strain 44 freeze dried ground whole berry	87.7±.13
10 mg strain 44 freeze dried ground whole berry + 990 mg callus	2.2±.23

[x] All samples were kept in saline solution overnight before testing for hemolysis. Final volume was brought to one litre. The callus used was obtained from pericarp.

Table 6.6. Stimulation of hemolytic activity of ground whole berry extract (WBE) and pericarp by ground seed (strain 44)[x].

Unripe ground seed (mg)	WBE or pericarp (mg)	Hemolysis (%) seed + WBE	Hemolysis (%) seed + pericarp
10	90	99.6±0.02	94.1±0.6
20	80	99.6±0.02	96.8±0.1
30	70	97.5±0.40	96.1±0.4
40	60	97.9±1.40	89.1±0.9
50	50	97.9±1.40	64.8±1.7
100	0	0.00	0.00
0	100	8.03±.24	6.9±0.2

[x] Ground seeds were mixed with either ground whole berry extract or pericarp and incubated overnight in saline solution prior to hemolysis test. Final volume was brought to one litre.

Molluscicidal test

The six callus samples (shoot tip callus proliferated on IBA, IAA, NAA and 2iP as well as pericarp and flower bud callus proliferated on IBA) did not show any snail killing potency at 100 mg/L within 48 hours exposure and were excluded from further analysis. Normally, if activity is not achieved at this concentration and time, the material is not considered promising as a molluscicide. The other samples (whole berry samples of strains 3, 17, and 44 and spray dried endod sample) showed molluscicidal activity as noted in Table 6.7.

The molluscicidal test result agrees with the hemolysis data. The proliferated calli evidently did not accumulate the active material. In addition, an

Table 6.7. Molluscicidal activity of whole berries and freeze dried endod samples.

Samples	Concentration (mg/L)	Mortality (%)[x] after 48 hrs recovery
Strain 3	15	100.0
whole berry	14	100.0
	13	96.6
	12	66.6
	11	26.6
Strain 17	15	100.0
whole berry	14	100.0
	12	100.0
	10	93.3
	8	73.3
Strain 44	15	100.0
whole berry	14	100.0
	12	100.0
	10	93.3
	8	73.3
Spray dried	10	100.0
endod sample	8	100.0
	6	93.3
	4	53.3

[x] 30 snails (6 tests of 5 snails each) were exposed for 24 h.

HPLC analysis was carried out on endod callus and berry samples (sent from here) at the Royal Danish School of Pharmacy (Lemmich, personal communication). The callus samples showed a different pattern of peaks as compared to berry samples. It was concluded that the callus samples did not contain saponins.

Suggestions for future research:

1. Develop a suitable medium for consistent callus proliferation from flower and pericarp. Identify the suitable growth medium and production medium.

2. Establishment of cell suspension cultures. Cell suspension systems provide the potential of large-scale culturing of cells from which saponins could be extracted.

3. Investigate the role of elicitors or precursors for enhancing the production of lemmatoxin. Squalene ($C_{30}H_{50}$), a hydrocarbon compound, is supposed to be the precursor of oleanolic acid (Parkhurst, personal communication).

4. Determine the biochemical pathway for lemmatoxin production. This should aid in the production of lemmatoxin *in vitro*. As described in other studies, the enzymes and substrates involved in the metabolism of secondary products need to be known to understand the triggering mechanism for production of the desired compound.

CHAPTER 7

SUMMARY

Macropropagation

Semi-hardwood endod stem cuttings gave the highest number of roots at 19.7 and 39.4 mM IBA using a concentrated solution dip method under mist. Satisfactory rooting ($\geq$ 63%) was also obtained without rooting hormones. Combinations of IBA and NAA did not improve rooting. Cuttings taken from the apical and medial regions of stem branches rooted faster than the basal ones, but there was no difference in rooting percentage at 6 weeks.

Endod seeds readily germinated under aseptic condition. Faster germination of seeds was obtained by scarification. Over 71% germination was recorded at 4 weeks in the control, gibberellic acid treated and scarified seeds.

Micropropagation

Among the cytokinins used for shoot proliferation, BA at 0.44 μM resulted in the greatest shoot production (3.1 shoots/explant) from shoot tips. Kinetin was only slightly effective in shoot proliferation and 2iP was ineffective. The combination of BA (0.44 μM) and GA_3 (0.27 μM) produced 4.7 shoots per explant in nodal explants without the removal of the shoot apex, which was better than shoot tips. Vertical and diagonal placement of nodal explants resulted in greater numbers of shoots than horizontal placement in strains 3 and 17.

Rooting of *in vitro* proliferated shoot tips was enhanced by the auxins IAA, IBA and NAA. Callus production at the base of the explants was a problem in most of the treatments with high concentration of auxins. IBA at 0.49 μM caused a high rooting percentage with relatively low callus association (45%). At low auxin concentrations, thin and long roots were observed, whereas at high auxin concentrations thick and short roots were observed. Preculturing shoot tips in an MS medium containing IBA and transferring to hormoneless medium did not result in a reduction of callus at the explant base.

Reducing the MS salt concentration did not affect the rooting percentage of shoot tips. Over 80% rooting was obtained at 1/2 and 1/4 MS salt concentrations. Nodal explants rooted significantly better than shoot tips with no auxins in either case. Nodal explants gave 80 - 85% rooting with low callus association (7.5%). Shoot tips cultured on a medium containing activated charcoal initiated a significantly greater number of roots and at 6 weeks were greater in length than shoot tips cultured without charcoal.

In vitro rooted plantlets that were directly transferred to the greenhouse under mist showed 100% survival after four weeks. These plants derived from staminate clones showed a similar growth habit to plants from seed and some produced staminate flowers after 6 months in the greenhouse.

Germination and storage of pollen

Successful endod pollen germination (over 70% germination) was achieved on a medium containing 10% sucrose and 161.8 μM H_3BO_3. Germination of pollen was not enhanced by $Ca(NO_3)_2.4H_2O$, KNO_3 and

$MgSO_4.7H_2O$. Dehydration of pollen over $CaCl_2$ reduced the moisture content to about 1.4%. Rehydration of dehydrated and stored pollen over $CuSO_4.5H_2O$ raised the moisture content to about 18% within two hours. Placing dehydrated pollen in gelatin capsules and inserting those into cryogenic vials that were stored at -175°C, 1±1°C and 24 ±2°C resulted in over 60% germination after one week of storage. However, germination percentage of pollen stored at 24±2°C declined to 25.2% at two weeks, and no germination was observed at four weeks. Pollen grains at 1±1°C and -175°C maintained viability for six months. The germination percentage of cryopreserved pollen remained high (over 60%). Pollen stored under cryogenic temperature for three months produced normal fruits and seeds when used in pollination.

Potential of callus as a source of lemmatoxin

The greatest amount of callus was produced from shoot tip explants using 4.9 μM IBA and 24.6 μM 2iP. Flower bud and pericarp explants produced little callus. Callus production was suppressed by 2,4-D and kinetin. Callus samples obtained from shoot tips, flower buds, pericarps, cotyledons and hypocotyls did not show hemolytic activity. Callus derived from shoot tip, flower bud and pericarp and incubated on an MS medium containing 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M ABA showed no hemolytic activity when 500 mg/L samples were used. Similarly, reducing the MS mineral salt concentration to 1/2 and 1/4 of the full concentration did not enhance lemmatoxin production. On the other hand, ground whole berry and spray-dried samples produced a high hemolytic activity. The callus samples showed no molluscicidal activity. Whole berry

samples of strains 3, 17 and 44 and spray dried endod samples gave high molluscicidal activity. These results were similar to the hemolysis test.

From the different endod plant samples tested, the fully grown and green berry stage showed the greatest hemolytic activity. The hemolytic activity of the samples in ascending order was leaves, roots, rachis, young stem branches, flowers and berries. The hemolysis test was, thus, a simple method for checking saponin content of endod samples.

Inactive saponins are supposed to be contained in pericarp and to be converted into active saponins by enzymes in the seed. Mixing known amounts of callus with ground seed samples and incubating overnight in saline solution did not result in hemolytic activity. Seeds, pericarps and whole berry extracts have low hemolytic activity when tested separately. However, mixing ground seeds with either whole berry extract or pericarp resulted in a high hemolytic activity unlike the callus samples. From this it is assumed that the callus samples failed to produce the necessary substrate(s) required for activity.

This study focused on endod propagation, pollen longevity and *in vitro* lemmatoxin production. Successful propagation methods had not been developed in previous experiments. This study lays the foundation for the development of commercially successful macropropagation and micropropagation procedures for endod. The research on *in vitro* lemmatoxin production, however, failed to give positive results. Further work is necessary although production of bioactive compounds via callus has only rarely proven successful. However, an increase in the productivity of endod by selection and

breeding may be helpful. The existence of variability among endod populations in Africa has been reported (Adams *et al*., 1989). The selection and breeding work that is being planned with the existing endod populations should result in superior genotypes with greater yields and disease resistance. Developing successful pollen storage procedures facilitates breeding programs. This has been accomplished in this work with storage under low and cryogenic temperatures.

REFERENCES

Adams, R.P. and M.F. Balandrin. 1984. Application of biotechnological methods for the *in vitro* biosynthesis of bioactive saponins and propagation of endod, p. 133-137. *In*: A. Lemma, D. Heyneman, and S.M. Silangwa (eds.). *Phytolacca dodecandra* (endod). Tycooly International Publishing Limited, Dublin.

Adams, R.P., K.R. Neisess, R.M. Parkhurst, L.P. Makhubu and L.W. Yohannes. 1989. *Phytolacca dodecandra* (Phytolaccaceae) in Africa: Geographical variation in morphology. Taxon 38 (1):17-26.

Addis Ababa University, Industrial Projects Service and National Chemical Corporation. 1984. Findings on the viability of utilizing endod (*Phytolacca dodecandra*) in detergent production. Unpublished Report.

Andersen, L.A., J.D. Phillipson, and M.D. Roberts. 1985. Biosynthesis of secondary products by cell cultures of higher plants, P. 1-36. *In*: A. Fiechter (ed.). Advances in biochemical engineering/biotechnology. 31 Plant cell culture. Springer-Verlag, Berlin.

Anderson, J.O., J. Nath, and E.J. Harner. 1978. Effects of freeze preservation on some pollen enzymes. II. Freezing and freeze-drying stresses. Cryobiology. 15:469-477.

Assefa, G 1979. Studies on the insecticidal property of endod, p. 203-205. *In*: A. Lemma, D. Heyneman, and H. Kloos (eds.). Studies on the molluscicidal and other properties of the endod plant, *Phytolacca dodecandra*. Special report, San Francisco.

Bailey, L.H. 1949. Manual of cultivated plants. MacMillan Publ. Co., New York.

Banks, M.S. 1979. Plant regeneration from callus from two growth phases of English ivy, *Hedera helix*, L. Z. Pflanzenphysiol. Bd. 92.S. 349-353.

Becker, H. 1987. Regulation of secondary metabolism in plant cell cultures, p. 199-212. *In*: C.E. Green, D.A. Somers, W.P. Hackett, and D.D. Biesober (eds.) Plant tissue and cell culture. Alan, R. Liss, Inc., New York.

Bennel, M. and G. Barth. 1986. Propagation of *Banksia coccinea* by cuttings and seed. Comb. Proc. intl. Plant Prop. Soc. 36: 148-152.

Bennett, L.K. and F.T. Davies, Jr. 1986. *In Vitro* Propagation of *Quercus shumardii* seedlings. HortScience. 21(4):1045-1047.

Berlin, J., H. Beier, L. Fecker, E. Forche, W. Noe, F. Sasse, O. Schiel, and V. Wray. 1985. Conventional and new approaches to increase the alkaloid production of plant cell cultures, P. 272-280. *In*: K.H. Neumann, W. Barz, and E. Reinhard (eds.). Primary and secondary metabolism of plant cell cultures. Springer-Verlag, Berlin.

Bressan, P.H., Y.J. Kim, S.E. Hyndman, P.M. Hasegawa, and R.A. Bressan. 1982. Factors affecting *in vitro* propagation of rose. J.Amer. Soc. Hort. Sci. 107(6):979-990.

Brewbaker, J.L. and B.H. Kwack. 1963. The essential role of calcium in pollen germination and pollen tube growth. Amer. J. Bot. 50(9):859-865.

Brodelius, P.E. 1990. Transport and accumulation of secondary metabolites, p. 567-575. *In*: H.J.J. Nijkamp, L.H.W. Vanderplas, and J. Vanaartrijk (eds.). Progress in plant cellular and molecular biology. Kluwer Academic Publishers, Dordrecht.

Butcher, D.N. 1977. Secondary products in tissue culture, p. 668-693. *In*: J. Reinhert and Y.P.S. Bajaj (eds.). Plant cell, tissue and organ culture. Springer-Verlag, Berlin.

Caldwell, J.D., C.D. Coston, and K.H. Brock. 1988. Rooting of semi-hardwood 'Hayward' kiwifruit cuttings. HortScience. 23(4):714-717.

Chee, R. and R.M. Pool. 1985. *In vitro* propagation of Vitis: The effects of organic substances on shoot multiplication. Vitis. 24:106-118.

Crocomo, O.J., E.Aquarone, and O.R. Gottlieb. 1981. Biosynthesis of secondary products *in vitro*, p. 359-372. *In*: T.A. Thorpe (ed.). Plant tissue culture methods and applications in agriculture. Academic Press, Inc., Orlando.

Dacie, J.V. and J.M. Vaughan. 1938. The fragility of the red blood cells: its measurement and significance. J.Path. Bact. 46:341-356.

Dalziel, J.M. 1936. The useful plants of west tropcal Africa. Crown Agents, London.

Davies, F.T. and J.N. Joiner. 1980. Growth regulator effects on adventitious root formation in leaf bud cuttings of juvenile and mature *Ficus pumila*. J. Amer. Soc. Hort. Sci. 105:91-95.

De-Eknamukl, W. and B.E. Ellis. 1985. Effects of macronutrients on growth and rosmarinic acid formation in cell suspension cultures of *Anchusa officinalis*. Plant Cell Rep. 4:46-49.

Demeke, T. 1984. The influence and interactions of growth media, rooting hormones, and cutting types on root and sprout development of endod (*Phytolacca dodecandra*, L.). M.S. Thesis, Addis Ababa University, Ethiopia.

Diomande, M. 1984. Tissue culture technology and development. Applications for industrial plants. Advance Technology Alert Systems (ATAS) Bulletin. United Nations, New York.

Dorsaz, A.C. and K. Hostettmann. 1986. Further saponins from *Phytolacca dodecandra* L. Herit. Helv. Chim. Acta. 69:2038-2047.

Dougall, D.K. 1979. Factors affecting the yield of secondary products in plant tissue cultures, p. 727-743. *In*: W.R. Sharp, P.O. Larsen, E.F. Paddock, and V. Raghavan (eds.). Plant cell and tissue culture. Principles and applications. Ohio State University Press, Columbus.

Drew, R.A. 1987. The effects of medium compostion and cultural conditions on *in vitro* root initiation and growth of papaya (*Carica papaya*, L.). J. Hort. Sci. 62(4):551-556.

Economou, A.S. and M.J. Spanoudaki. 1985. *In vitro* propagation of gardenia. HortScience. 20(2):213.

Eilert, U., W.G.W. Kurz, and F. Constabel. 1985. Stimulation of sanguinarine accumulation in *Papaver somniferum* cell cultures by fungal elicitors. J. Plant Physiol. 119:65-76.

Fairley, N.M., G.S. Price, and D.J. Meuten. 1988. Evaluation of red blood cell fragility in pygmy goats. Am.J. Vet. Res. 49(9):1598-1600.

Feliciano, A.J. and M.D. Assis. 1983. *In vitro* rooting of shoots from embryo cultured peach seedlings. HortScience 18(5):705-706.

Flemings, M.B. 1975. Effects of endod on the perispiracular glands in larvae of mosquitoes. Unpublished report. C.W. Post College, Long Island University, Brookville, N.Y. Ref. No. 5 p. 198-202.

Fujita, Y., Y. Hara, C. Suga, and M. Morimoto. 1981b. Production of shikonin derivatives by suspension cultures of *Lithospermum erythrorhizon*. II. A new medium for the production of shikonin derivatives. Plant Cell Rep. 1:61-63.

Fujita, Y. and M. Tabata. 1987. Secondary metabolites from plant cells. Pharmaceutical applications and progress in commercial production, p. 169-185. *In:* Green, C.E., D.A. Somers, W.P. Hackett, and D.D. Biesboer (eds.). Plant tissue and cell culture. Alan R. Liss, Inc., New York.

Furuya, T., H. Kojima, and K. Synono. 1971. Regulation of nicotine biosynthesis by auxins in tobacco callus tissues. Phytochemistry. 10:1529-1532.

Furuya, T., T. Yoshikawa, T. Ishii, and K. Kajii. 1983. Effects of auxins on growth and saponin production in callus cultures of *Panax ginseng*. Planta medica. 47:183-187.

Ganeshan, S. 1985. Viability and fertilizing capacity of onion pollen (*Allium cepa* L.) stored in liquid nitrogen (-196°c). Trop. Agric. (Tri.) 63(1):46-48.

Gamborg, O.L., R.A. Miller, and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151-158.

Gauch, H.G. and W.M. Duggar, Jr. 1953. The role of boron in the translocation of sucrose. Plant Physiol. 28:457-466.

Gomez, K.A. and A.A. Gomez. 1976. Statistical procedures for agricultural research. 2nd ed. John Wiley and Sons, New York.

Hampel, M., B.P. Witkowska, and J. Tymoszuk. 1985. The influence of cytokinins on multiplication and subsequent rooting of Gerbera *in vitro*. Acta Hort. 167:301-305.

Han, K. and L.C. Stephens. 1987. Growth regulators affect *in vitro* propagation of two interspecific Impatiens hybrids. Scientia Hort. 32:307-313.

Hanson, C.H. and T.A. Campbell. 1972. Vacuum-dried pollen of alfalfa (*Medicago sativa* L.) viable after 11 years. Crop Sci. 12:874.

Hartmann, H.T. and D.E. Kester. 1983. Plant propagation. Principles and practices. 4th ed. Prentice-Hall Inc., Englewood Cliffs, New Jersey.

Hasegawa, P.M. 1980. Factors affecting shoot and root initiation from cultured rose shoot tips. J. Amer. Soc. Hort. Sci. 105(2):216-220.

Jaacov, J.B. and E. Dax. 1981. *In vitro* propagation of *Grevillea rosmarinifolia*. HortScience. 16(3):309-310.

James, D., R.P. Ariyanayagam, and E.J. Duncan. 1987. Comparative studies of *in vitro* germination of pollen of pigeon pea (*Cajanus cajan*, (L.) Millsp and *Atylosia platycarpa* Benth. Trop. Agric. (Tri.) 64(4):343-346

Johri, B.M. and I.K. Vasil. 1961. Physiology of pollen . Botan. Rev. 27(3):325-381.

Kawai, H., M. Kuroyanagi, K. Umehara, A. Ueno, and M. Satake. 1988. Studies on the saponins of *Lonicera japonica* Thunb. Chem. Phar. Bull. 36(12):4769-4775.

Khatamian, H. and Z. Abuelgasim. 1986. Auxins aid western soapberry cuttings. American Nurseryman. 16(4):65-68, 70-73.

King, G.J. and M.M. Meyer, Jr. 1983. Effects of phenolic compounds and indoleacetic acid on adventitious root initiation in cuttings of *Phaseolus aureus, Acer saccharinum,* and *Acer griseum.* HortScience. 18(3):352-354.

Kitto, S.L. and M.J. Young. 1981. In vitro propagation of *Carrizo citrange*. Hort Science. 16(3):305-306

Kloos, H. 1976. Preliminary studies of medicinal plants and plant products in markets in central Ethiopia. Ethnomedizin 4:63-103.

Kloos, H., A. Lemma, and G. Desole. 1978. *Schistosoma mansoni* distribution in Ethiopia, p. 463-469. *In*: A. Lemma, D. Heyneman, and H. Kloos (eds.). Studies on the molluscicidal and other properties of the endod plant, *Phytolacca dodecandra*. Special report, San Francisco.

Kloos, H. and F.S. McCullough. 1981. Plant molluscicides: A review, WHO/Schisto/. 81:59 (Unpublished WHO document).

Kloos, H., A.M. Polderman, G. Desole, and A. Lemma. 1977. *Schistosomiasis haematobium* in seminomadic and agricultural Afar in Ethiopia. Trop. Geogr. Med. 29:339-406.

Knobloch, K.H. and J. Berlin. 1983. Influence of phosphate on the formation of the indole alkaloids and phenolic compounds in cell suspension cultures of *Catharanthus roseus*. I. Comparison of enzyme activities and product accumulation. Plant Cell Tissue Organ Culture. 2:333-340.

Koter, M. and J. Laski. 1989. Does thermotolerance occur in human red cells? Intl J. Radiat. Biol. 56(1):59-66.

Kumra, S. and R.N. Chopra. 1987. Callus initiation, its growth and differentiation in the liverwort, *Asterella wallichiana* (Lechm. ET Lindenb.) Grolle. I. Effects of auxins and cytokinins. Journ. Hattori Bot. Lab. 63:237-245.

Kurtz, E.B., Jr. and J.L. Liverman. 1958. some effects of temperature on pollen characters. Bull. Torrey Bot. Club. 85:136-138.

Kyungchul, H. and L.C. Stephens. 1987. Growth regulators affect *in vitro* propagation of two interspecific Impatiens hybrids. Scientia Hort. 32:307-313.

Lee, C.W. and J.C. Thomas. 1985. Tissue culture propagation of Buffalo gourd. HortScience 20(2):218-219.

Lee, C.W. and J.C. Thomas. 1985. Propagation of desert milkweed by shoot tip culture. HortScience. 20(2): 263-264.

Lee. C.W., J.C. Thomas, and S.L. Buchmann. 1985. Factors affecting *in vitro* germination and storage of jojoba pollen. J. Amer. Soc. Hort. Sci. 110(5):671-676.

Lemma, A. 1965. A preliminary report on the molluscicidal property of endod (*Phytolacca dodecandra*). Eth. Med. J. 3:187-190.

Lemma, A. 1969. Bilharziasis in the Awash Valley. I. Epidemiological study with special emphasis on its possible future economic and public health importance. Eth. Med. J. 7:147-176.

Lemma, A. 1970. Laboratory and field evaluation of the molluscicidal properties of *Phytolacca dodecandra*. Bull. WHO. 42:597-617.

Lemma, A., P.H. Goll, J. Duncan, and B. Mazengia. 1978. Control of schistosomiasis with the use of endod in Adwa, Ethiopia: result of a five year study. Proc. Int. Conf. in Sch. (Cairo). 415-430.

Lemma, A., D. Heyneman, and H. Kloos. 1979. Studies on the molluscicidal and other properties of the endod plant, *Phytolacca dodecandra*. Special report, San Francisco.

Lemma, A., D. Heyneman, and S.M. Silangwa. 1984. *Phytolacca dodecandra* (endod). Tycooly International Publishing Limited, Dublin.

Lemma, A. and P. Yau. 1974. Studies on the molluscicidal properties of endod (*Phytolacca dodecandra*). II. Comparative toxicity of various molluscicides to fish and snails. Eth. Med. J. 12:109-114.

Lemma, T. and L.W. Yohannes. 1979. Selected stage for endod berry harvesting, p. 241-246. *In*: A. Lemma, D. Heyneman, and H. Kloos (eds.). Studies on the molluscicidal and other properties of the endod plant, *Phytolacca dodecandra*. Special report, San Francisco.

Lindsey, K. 1985. Manipulation by nutrient limitation of the biosynthetic activity of immobolized cells of *Capsicum frutescens* Mill cv. Annum. planta. 165:126-133.

Linsmaier, E.F. and F. Skoog. 1965. Organic growth requirements for tobacco tissue cultures. Physiol. Plant. 18:100-127.

Lo, Y.N. 1985. Root initiation of *Shorea macrophylla* cuttings. Effects of node position, growth regulators and misting regime. For. Ecol. Manage. 12:43-52.

Luckner, M. and B. Diettrich. 1987. Biosynthesis of cardenolides in cell cultures of *Digitalis lantana* - the result of a new strategy, p. 187-197. *In*. C. E. Green, D. A. Somers, W.P. Hackett, and D.D. Biesboer (eds). Plant tissue and cell culture. Alan R. Liss, Inc., New York.

Luckner, M. and B. Diettrich. 1990. Principles regulating formation and activity of secondary metabolic enzymes in plant tissue and cell cultures, p. 745-753. *In*: H.J.J. Nijkamp, L.H.W. Vanderplas, and J. Vanaartrijk (eds.) Progress in plant cellular and molecular biology. Kluwer Academic Publishers, Dordrecht.

Lugt, C.B. 1981. *Phytolacca dodecandra* berries as a means of controlling bilharzia transmitting snails. Institute of Pathobiology. Addis Ababa University.

Mackay, W.A. and S.L. Kitto. 1987. Rapid propagation of French tarragon using *in vitro* techniques. Acta Hort. 208:251-257.

Martin, G.C. 1987. Apical dominance. HortScience 22(5): 824-833.

McGaw, B.A. and R. Horgan. 1983. Cytokinin oxidase from *Zea mays* kernels and *Vinca rosea* crown-gall tissue. Planta. 159:30-37.

Misawa, M. 1977. Production of natural substances by plant cell cultures described in Japanese patents, p. 17-26. *In*: W. Braz, E. Reinhard, and M.H. Zenk (eds.). Plant tissue culture and its biotechnological application. Springer-Verlag, Berlin.

Misawa, M. 1985. Production of useful plant metabolites, p. 59-88. *In*: A. Fiechter (ed.). Advances in Biochemical Engineering/Biotechnology. 31 Plant cell culture. Springer-Verlag, Berlin.

Mok, M.C., D.W.S. Mok, S.C. Dixon, D.J. Armstrong, and G. Shaw. 1982. Cytokinin structure - activity relationships and the metabolism of N^6 - (isopentenyl) adenosine 8-^{14}C in Phaseolus callus tissues. Plant Physiol. 70:173-178.

Monkiedje, A., J.H. Wall, and A.J. Englande. 1990. An in-field method for determining concentrations of the plant molluscicide *Phytolacca dodecandra* (Endod - S) in water. A Paper Presented at the Third International Workshop on Endod. October 15-19, Addis Ababa, Ethiopia.

Mooney, P.A. and J. Van Staden. 1987. Effects of auxins, cytokinins, and inhibitors on growth of stem callus of *Persea americana* cv. Duke 7 and Fuerte. S. Afr. J. Sci. 83:52-54.

Mudge, K.W. 1986. Micropropagation of Mugo pine from embryonic and seedling explants. HortScience. 21(2):298-299.

Murashige, T. 1961. Suppression of shoot formation in cultured tobacco cells by gibberellic acid. Science. 134:280.

Murashige, T. 1974. Plant propagation through tissue cultures. Ann. Rev. Plant Physiol. 25:135-166.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nair, N.G., K.K. Kartha, and O.L. Gamborg. 1979. Effect of growth regulators on plant regeneration from shoot apical meristems of cassava (*Manihot esculenta* Crantz) and on the culture of internodes *in vitro*. Z. Pflanzenphysiol. Bd. 95.S.51-56.

Norton, M.E. and C.R. Norton. 1985. *In vitro* propagation of Ericaceae: A comparison of the activity of the cytokinins N^6-benzyladenine and N^6-isopentyladenine in shoot proliferation. Scientia Hort. 27:335-340.

Page, Y.M. and J.H. Visser. 1989. *In vitro* propagation of Geraldton wax: initiation, proliferation, and rooting. HortScience. 24(2):381-382.

Pandey, R.M. and M.S. Rajeevan. 1983. Callus initiation and regeneration in tissue culture of papaya, p. 427-430. *In:* S. K. Sen and K. L. Giles (eds.). Plant cell culture in crop improvement. Plenum Press, New York.

Parkhurst, R.M. 1975. The chemotaxonomy of *Phytolacca species.* Indian J. Chem. 13:757-758.

Parkhurst, R.M. 1984. The chemistry of Phytolacca dodecandra, p. 138-144. *In*: A. Lemma, D. Heyneman, and S. M. Silangwa (eds.). *Phytolacca dodecandra* (endod). Tycooly International Publishing Limited, Dublin.

Parkhurst, R.M., B.M. Mthupha, Y.S. Liang, J.I. Bruce, J.D.H. Lambert, T.L. Collier, J.W. ApSimon, L.W. Yohannes, G.E. Heath, W.O. Jones, J.K. Stobaeus,

and L.P. Makhubu. 1989. The molluscicidal activity of *Phytolacca dodecandra*. I. Location of the activating esterase. Biochem. Biophs. Res. Comm. 158(2):436-439.

Parkhurst, R.M., D.W. Thomas, R.P. Adams, L.P. Makhubu, B.M. Mthupha, L. W. Yohannes, E. Mamo, G.E. Heath, J.K. Stobaeus, and W.O. Jones. 1990. Triterpene aglycones from various *Phytolacca dodecandra* populations. Phytochemistry. 29(4):1171-1174.

Parkhurst, R.M., D.W. Thomas, W.A. Skinner, and L.W. Cary. 1974. Molluscicidal saponins of *Phytolacca dodecandra*: Lemmatoxin. Can. J. Chem. 52:702-705.

Passey, A.J. and O.P. Jones. 1983. Shoot proliferation and rooting *in vitro* of *Theobroma cacao* L. type Amelonado. J. Hort. Sci. 58(4):589-592.

Pfahler, P.L. 1967. *In vitro* germination and pollen tube growth of maize (*Zea mays*, L.) pollen. I. Calcium and boron effects. Can. J. Bot. 45:839-845.

Philips, I.D.J. 1975. Apical dominance. Ann. Rev. Plant Physiol. 26:341-367.

Pilbeam, D.J. and E.A. Kirby. 1983. The physiological role of boron in plants. J. Plant Nutr. 6(7):563-582.

Pool, R.M. and L.E. Powell. 1975. The influence of cytokinins on *in vitro* shoot development of 'concord grape'. J. Amer. Soc. Hort. Sci. 100(2):200-202.

Porlingis, I.C. and I. Therios. 1976. Rooting response of juvenile and adult leafy olive cuttings to various factors. J. Hort. Sci. 51:31-39.

Rauch, F.D. and R.Y. Yamakawa. 1980. Effects of auxin on rooting of *Ixora acuminata*. HortScience. 15(1):97.

Ray, M. and G. Noteboom. 1970. A modification of the erythrocyte osmotic fragility test. Amer. J. Clin. Path. 54:711-715.

Reichert, R.D., J.T. Tatarynovich, and R.T. Tyler. 1986. Abrasive dehulling of Quinoa (*Chenopodium quinoa*): Effect on saponin content as determined by an adapted hemolytic assay. Cereal Chem. 63(6):471-475.

Reisch, B.I. 1986. Influence of genotype and cytokinins on *in vitro* shoot proliferation of grapes. J.Amer.Soc.Hort.Sci. 111(1):138-141.

Robinson, T. 1980. The organic constituents of higher plants. Cordus Press, North Amherst, Massachusetts.

Sahar, N. and P. Spiegel-Roy. 1980. Citrus pollen storage. HortScience. 15(1):81-82.

Sahar, N. and P. Spiegel-Roy. 1984. *In vitro* germination of avocado pollen. HortScience. 19(6):886-888.

Salisbury, F.B. and C.W. Ross. 1985. Plant Physiology. 3rd.ed. Wadsworth Publishing Company, Belmont, California.

Shuler, M.L. 1981. Production of secondary metabolites from plant tissue culture-problems and prospects. Ann. N.Y.Acad.Sci. 369:65-79.

Skene, K.G.M. 1975. Cytokinin production by roots as a factor in the control of plant growth, p. 365-369. *In*: J.G.Torrey and D.T. Clarkson (eds.). The development and function of roots. Academic Press, New York.

Skirvin, R.M., M.C. Chu, and E. Gomez. 1981. *In vitro* propagation of thornless trailing blackberries. HortScience. 16(3):310-312.

Slabnik, E., O.H. Caso, M.C. Pomar, and H. Diaz. 1986. Auxin-cytokinin interaction and morphogenic responses from leaf sections and shoot meristem cultures of *Taraxacum officinale*. J. Plant Physiol. 22:227-233.

Slacanin, I., A. Marston, and K. Hostettmann. 1988. High performance liquid chromatographic determination of molluscicidal saponins from *Phytolacca dodecandra*(Phytolaccaceae). J. Chromatogr. 448(2):265-274.

Snir, I. and A. Erez. 1980. *In vitro* propagation of Malling Merton apple rootstocks. HortScience. 15(5):597-598.

Stanley, R.G. and H.F. Linskens. 1974. Pollen: Biology, biochemistry and management. Springer-Verlag, Berlin.

Stanley, R.G. and F.A. Loewus. 1964. Boron and myoinositol in pollen pectin biosynthesis, p. 128-136. *In*: J. F. Linskens (ed.). Pollen physiology and fertilization. Amsterdam, North Holland, Pub. Co.

Stapfer, R.E. and C.W. Heuser. 1985. *In vitro* propagation of Periwinkle. HortScience. 20(1):141-142.

Stephens, L.C., S.L. Krell, and J.L. Weigle. 1985. *In vitro* propagation of Java, New Guinea, and Java x New Guinea Impatiens. HortScience. 20(3):362-363.

Stolzenberg, S.J. and R.M. Parkhurst. 1974. Spermicidal actions of extracts and compounds from *Phytolacca dodecandra*. Contraception. 10(2):136-143.

Tabata, M. 1977. Recent advances in the production of medicinal substances by plant cell cultures, p. 1-16. *In*: W. Braz, E. Reinhard, and M. H. Zenk (eds.). Plant tissue culture and its biotechnological application. Springer-Verlag, Berlin.

Tae, L.H. and S.Y. Narm. 1987. Factors affecting callus formation and organogenesis from *in vitro* culture of pea (*Pisum sativum* L.). J. Kor. Soc. Hort. Sci. 28(1):18-23.

Takie, Y. and L.W. Yohannes. 1979. The endod research project: progress report and plan of work, p. 256-259. *In*: A. Lemma, D. Heyneman, and H. Kloos (ed.). Studies on the molluscicidal and other properties of the endod plant, *Phytolacca dodecandra*. Special report, San Francisco.

Tamas, I.A. 1987. Hormonal regulation of apical dominance, p. 393-410. *In*: P.J. Davies (eds.). Plant hormones and their role in plant growth and development. Martinus Nijhoff Publishers, Dordrecht.

Thiilborg, S. and E. Lemmich. 1990. The hemolytic activity of endod saponins. A Paper Presented at the Third International Workshop on Endod. October 15-19, Addis Ababa, Ethiopia.

Thompson, W.K. 1986. Effects of origin, time of collection, auxins, and planting media on rooting of cuttings of *Epacris impressa* Labill. Scientia Hort. 30:127-134.

Thorpe, T.A. 1981. Plant tissue culture. Methods and applications in agriculture. Academic Press, Inc., New York.

Towill, L.E. 1985. Low temperature and freeze/vacuum drying preservation of pollen, p. 171-198. *In*: K. K. Kartha (ed.). Cryopreservation of plant cells and organs. CRC Press, Boca Raton.

Tukey, H.B. Jr. 1979. Back to basics of rooting. Proc. Int. Plant Prop. Soc. 29:422-427.

Vasil, I.K. 1960. Studies on pollen germination of certain Cucurbittaceae. Amer. J. Bot. 47(4):239-247.

Vasil, I.K. 1963. Effect of boron on pollen germination and pollen tube growth, p. 107-119. *In*: J.F. Linskens (ed.). Pollen physiology and fertilization. North Holland Pub. Co., Amsterdam.

Wareing, P.F. and I.D.J. Philips. 1970. The control of growth and differentiation in plants. Pergamon Press, New York.

Weatherhead, M.A., J. Burdon, and G.G. Henshaw. 1979. Effects of activated charcoal as an additive plant tissue culture media. Part 2. Pflanzenphysiol. Bd. 94.S.399-405.

Weaver, R.J. 1972. Plant growth substances in Agriculture. W. H. Freeman Company, San Francisco.

Worrall, R.J. 1976. Effects of time of collection, growing conditions of mother plants, and growth regulators on rooting of cuttings of *Telopea speciosissima* (Proteaceae). Scientia Hort. 5:153-160.

Yamada, Y. and T. Hashimoto. 1990. Possibilities for improving yields of secondary metabolites in plant cell cultures, p. 547-556. *In*: H. J. J. Nijkamp, L. H. W. Vanderplas, and J. Vanaartrijk (eds.). Progress in plant cellular and molecular biology. Kluwer Academic Publishers, Dordrecht.

Yamada, Y. and Y. Fujita. 1984. Production of useful compounds in culture, p. 717-728. *In*: D.A. Evans, W.R. Sharp, P.V. Ammirato, and Y. Yamada (eds). Handbook of plant cell culture. I. Techniques for propagation and breeding. MacMillan Publishing Company, New York.

Zaghmout, O.M.F. and W.A. Torello. 1988. Enhanced regeneration in long term callus cultures of red fescue by pretreatment with activated charcoal. HortScience. 23(3):615-616.

Zaid, A. 1981. Rapid propagation of date palm through tissue culture. M.Sc. Thesis. University of California, Davies.

Zenk, M.H., H. El-Shagi, H. Arens, J. Stockigt, E.W. Weiler, and B. Deus. 1977. Formation of the indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus*, p. 27-43. *In*: W. Barz, E. Reinhard, and M. H. Zenk (eds.). Plant tissue culture and its biotechnological application. Springer-Verlag, Berlin.

Zhang, B., L.P. Stoltz, and J.C. Snyder. 1987. *In vitro* propagation of *Euphorbia fulgens*. HortScience. 22(3):486-488.

Zimmerman, R.H. 1983. Factors affecting *in vitro* propagation of apple cultivars. Acta Hort. 131:171-179.

APPENDIX

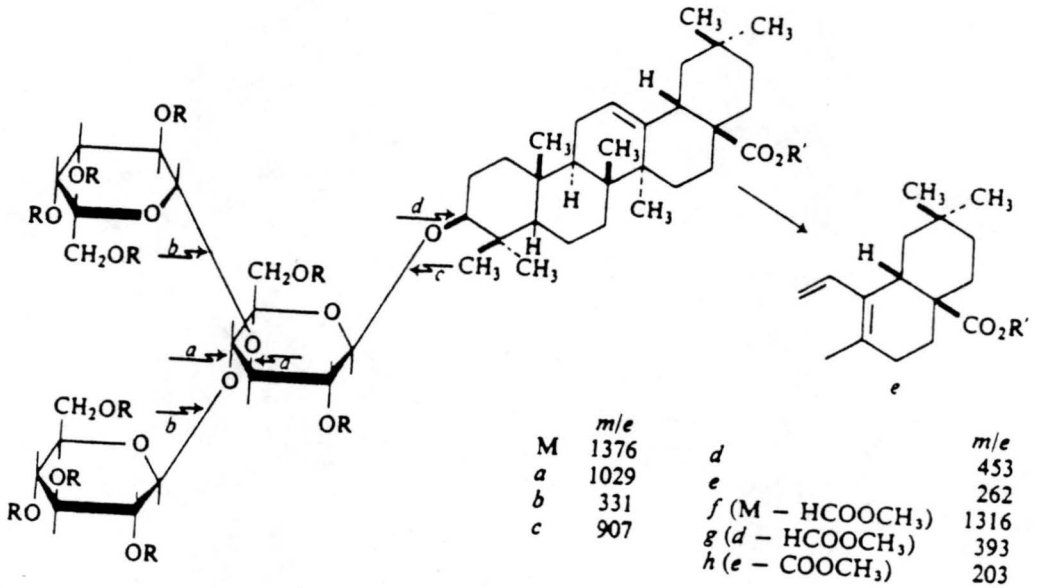

Fig. A. Structure of lemmatoxin acetate methyl ester. R = Ac; R' = CH_3. Parkhurst, *et al.* (1974).

Table A. Analysis of variance table for pulsing experiment.

Source of variation	Df	Variables		
		No of roots	Root length	Callus diameter
Constant	1	NS	NS	NS
Conc	4	***	***	***
Time	2	NS	NS	NS
Concx time	8	NS	NS	NS
Error	210			

*** = significant at the .001 level
NS = not significant at the .05 level