THE USE OF FUNGICIDAL SOLVENTS IN THE SURFACE STERILIZATION OF WOODY EXPLANTS OF PEACH AND PECAN

by

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CHAPTER I

INTRODUCTION

Most fruit trees are normally propagated by budding or grafting desirable scion cultivars onto a rootstock (28). This method of propagation is rather time consuming, labor intensive and expensive. Other methods which could reduce the cost of production and time spent in the nursery would be of value to the commercial propagator and grower. Rooted cuttings can be used as a method for the propagation of large numbers of plants with reductions in production costs and time spent in the nursery (32). However, rooting is often difficult to induce in many plants especially when mature cuttings are used (10, 74).

Tissue culture is another method which could be of tremendous value in the propagation of fruit trees (35). Tissue culture methods could be used to produce large numbers of plants within a relatively short period of time while using little space for the storage of stock plants (44). Tissue culture methods could provide plants for the study of own-rooted trees of species which are normally difficult to root (76). Tissue culture techniques could allow for the rapid propagation of new cultivars (22) or of seedling rootstocks which are adapted to adverse growing

conditions (46). Tissue culture techniques could be used to produce disease-free plants (69). The benefits of tissue culture methods in the propagation of fruit trees would be of great value to the commercial nursery industry.

However, tissue culture of fruit trees is not problem free. One problem associated with the in vitro propagation of fruit trees is microbial contamination of explants (22, 46). Source plants grown in the field are often infested with fungal and bacterial organisms. When the explant from a field grown plant is placed under the optimal growth conditions offered by the test tube environment, the contaminants will often overrun the explant causing death of the explant. Explants are often internally as well as externally infested by microbial organisms (45). Because of this inherent contamination, explants are often difficult to disinfest.

A method for the disinfestation of pecan shoot tips which are typically very difficult to disinfest involves the use of a saturated, aqueous benomyl soak in combination with ethanol and sodium hypochlorite soaks (9). However, benomyl, which is a systemic fungicide, is very insoluble in water (13), necessitating a relatively long exposure time in order for the benomyl to penetrate the explant tissue. Benomyl is soluble in many organic solvents and has been used in conjunction with organic solvents as a surface sterilization procedure for seeds (12, 53, 54). These solvents aid in the penetration of benomyl into the seed

coat and have aided in the increase of the germination percentage of treated seeds (12, 53).

Seven solvents which are used to surface sterilize seeds were chosen to find which solvent(s) were best suited as a fungicidal solvent for surface sterilization of field grown peach and pecan shoot tips. The objectives of this study were: 1) to determine the practicality of using benomyl and various organic solvents for surface sterilization of peach and pecan shoot tip explants, 2) to attempt to determine the best solvent treatment in terms of control of contamination, necrosis and survival of the explant as compared to a saturated, aqueous benomyl solution (9), 3) to determine the optimum duration of exposure to the benomyl solutions and 4) to determine the solvent-time combination which best controlled contamination with optimum survival of the explant.

CHAPTER II

LITERATURE REVIEW

Tissue Culture

Plant tissue culture is a general term commonly applied to the aseptic vegetative propagation of plants from plant cells, tissues or organs. Tissue culture involves excision of plant parts, placement of these tissues onto a nutrient medium and exposure of the culture to favorable environmental conditions in order to achieve a rapid multiplication of plants or plant cells in vitro (68). Any plant tissue can serve as an explant for tissue culture purposes because each plant cell is theoretically totipotent or capable of producing a new plant (68). Plants can be derived from tissue culture propagation of shoot tips, axillary buds, stem sections, leaf sections, pollen grains, embryos, ovules, seeds, spores, isolated cells or protoplasts among other sources (68).

Tissue culture methods have been employed in embryo culture (17, 25, 89), germplasm preservation (2), and the propagation of disease-indexed plants (34). Other applications include the production of useful secondary metabolites for medicinal and other purposes (78). The production of

haploid plants from pollen and anther culture may be of use in plant breeding (68). Plant tissue culture has also been used in plant growth and development studies (55).

The possibility of the propagation of plants from cells was suggested by the cellular theory stated by Schleiden (66) and Schwann (67) independently in the late 1830's. A portion of the cell theory states that cells are totipotent, the cells are capable of regenerating an entire plant from a single cell. It was on this theory that Haberlandt based his work. He attempted to culture isolated plant cells in a nutrient solution. He was able to keep the cells alive for up to 27 days with an increase in cell volume but with no cell division. From this and other work, he postulated that cell division was controlled by two substances (21). This prediction was later proved true with the discovery of auxins and cytokinins (47, 50).

A second major accomplishment in the new field of tissue culture was the report of the <u>in vitro</u> culture of excised tomato root tips over an unlimited period of time (86). By 1939, Nobecourt (59), Gautheret (18) and White (87) had achieved the successful culture of plant tissues over prolonged periods of time using tobacco and carrot tissues. These were the first reports of prolonged culture of unorganized cell masses.

The beginning of tissue culture micropropagation occurred in 1946 with the publication of a paper detailing the meristematic regions of shoots which would give rise

to new plants (19). Another important development was the proposal by Skoog and Miller (73) concerning the effects of a balance between auxin and cytokinin on the growth and differentiation of plant tissue cultures. In 1962, Murashige and Skoog (56) published a medium which has been used in the tissue culture of a wide range of plants.

Since the early 1960's, advances in tissue culture have become increasingly more numerous. Some of the major developments are listed below. Morel, 1960, stimulated commercial interest in the use of shoot apex culture as a method of propagation for orchids (52). Tissue culture propagation of orchids allowed for a more rapid multiplication of orchids than traditional propagation methods. Work by Vasil and Hildebrandt (84) showed that an isolated tobacco cell was capable of forming a colony which in turn regenerated a tobacco plantlet. Bourgin and Nitsch (3) in 1967 published results of an experiment in which haploid tobacco plants were produced from pollen grains.

Tissue culture is a fairly young field beginning with the first commercial applications in the propagation of orchids in the 1960's (11). More species are constantly being added to the list of plants which are being produced commercially by tissue culture methods and more are being propagated for the first time within the scientific community (11). Although there have been great improvements in tissue culture, much research has yet to be done.

Tissue Culture of Woody Plants

Micropropagation of woody plants has developed more slowly than tissue culture of herbaceous materials (69). Cultures of woody plants are typically more difficult to establish than cultures of herbaceous plants (69). Several reasons may explain the problems associated with *in vitro* propagation of woody plants. Woody plants generally have a prolonged life cycle. The genetic makeup of most woody plants is often very heterogeneous which affects their subsequent performance *in vitro* as well as their culture requirements (77). Adult tissues are generally less responsive to tissue culture techniques than are juvenile tissues (77). This can be a problem because in most situations, adult material is often the desired source of explants, especially in the propagation of scion cultivars that have been exclusively propagated vegetatively. Another problem in the tissue culture of woody plants is the microbial contamination normally associated with field grown plants (40, 46). Standard surface sterilization techniques applied to herbaceous materials are often not successful in the disinfestation of explants from woody plants (20, 37).

Microbial contamination of explants has often been a difficult problem for the tissue culture propagator. There are many instances in which the most widely used sterilization procedures are not successful in controlling contamination (20, 22, 37, 81). Among the orchids, incor-

poration of fungicides including benomyl and bactericides had no harmful effect on the germination of orchid seeds but was injurious to the development of orchid shoot tip cultures (4). Surface sterilization of flower stalk cuttings of Phalaenopsis using an antimicrobial solution containing 10 ppm Benlate in conjunction with other antimicrobials has proved effective in the control of microbial contamination. A longer immersion in the solution yielded better control of contaminants but also resulted in a higher death rate of the explants (81).

Among the woody plants, contamination of cultures becomes a more difficult problem to deal with especially when field grown plants are used as explant sources. An early study using apparently healthy buds of Fraxinus, Ginkgo, Magnolia, Populus and Robinia yielded cultures which were contaminated after standard surface sterilization treatments had been applied (40). A scanning electron microscope study has shown pecans to be internally infested with fungal organisms as well as with external contaminants (45). This can serve as an explanation for the difficulties associated with attaining aseptic cultures of pecan from field grown plants (46). The difficulties encountered in obtaining large numbers of clean cultures from field grown sources has been reported for citrus (20), apple, (37) and peach (22).

In many instances, seedling or greenhouse grown plant materials are used as explant sources in order to eliminate

the contamination problems associated with the culture of field grown explants (46). With some plants, especially forest trees as well as other trees for which mature characteristics are needed as selection criteria, greenhouse culture may not be possible. In these situations, methods for the aseptic culture of adult buds are needed.

Various methods have been developed for aseptic culture of field grown explants. Giladi *et al.* (20) outlined a method for the aseptic culture of citrus bud explants. Ochatt and Caso (61) developed a method for the aseptic culture of dormant buds of peach. Cotten (9) used a 4.5 hour soak in a saturated, aqueous benomyl solution prior to an eight minute soak in 70% ethanol and a 15 minute soak in 0.525% sodium hypochlorite to reduce contamination of pecan shoot tip cultures collected in mid-December to 40% contamination.

Despite the limitations of woody plant culture, techniques for *in vitro* propagation have been and are rapidly being developed and used. A large number of woody plants have been propagated *in vitro* including *Bougainvillea* (6), *Rhododendron* (16), green and white ash (30), *Liquidambar styraciflua* (79), dwarf *Nandina* (75), Douglas fir (7), and several ornamental Rosaceous plants (60).

The application of mass *in vitro* propagation techniques to the propagation of fruit trees will be of tremendous benefit to the commercial nursery industry. Most scion cultivars of fruit trees are budded or grafted

onto a rootstock (28). With the development of tissue culture propagation methods, scion cultivars that are normally grafted onto rootstocks could be produced on their own root systems (76). This would allow for the testing of these cultivars on their own root system which has not yet been accomplished because of the difficulty of rooting cuttings of such plants. Tissue culture techniques could also allow for the rapid propagation of newly developed cultivars or rootstocks (22). Virus-free plants could also be made available (69).

Apple was one of the first fruit trees to be successfully propagated *in vitro*. Jones *et al.* (35) described a procedure for the production of M.26 apple rootstock shoots. The medium detailed in this report has been useful in the propagation of other fruit trees (36, 64). Several apple scion cultivars have also been propagated from shoot tips (1, 39).

Tissue Culture of *Prunus* spp.

Much tissue culture research has been done on the t techniques of propagation of various *Prunus* species (29, 36, 64, 65, 69. 76, 80). Early success produced cultures of *Prunus* spp. other than peach (*Prunus persica*). Peach cultures could not survive on the same media which is indicative of the differences in culture requirements among the many species of *Prunus*. Nekrasova (58), in a 1964 report, announced the successful culture of Bessey cherry

buds but was not as successful in the culture of apricot buds. The apricot buds produced leaflet expansion only, followed by a rapid yellowing. Differences in preference for sucrose or sorbitol as the carbon source for callus cultures of several species has been reported. Among the Prunus spp., callus cultures of sweet and sour cherry and plum produced a greater growth response on media supplemented with sucrose whereas peach callus cultures showed a greater growth response to sorbitol supplemented media (8). 'Montmorency' sour cherry shoot tip cultures required a lower amount of vitamins in the rooting media as compared to the requirements of 'Harbrite' peach on the same media (71). These examples illustrate a few of the differences in culture requirements found among the Prunus spp.

Micropropagation using shoot tips or bud explants has been the main method of tissue culture propagation of several Prunus species. Reports of successful cultures with some rooting from shoot tip or bud explants have been cited for almond (65, 80), apricot (72), cherry (36, 71, 72, 76) and plum (36, 64, 72) as well as peach (23, 38, 51, 63, 85).

Some work has been reported on the use of callus cultures among the Prunus species (24, 29, 65). From three Prunus spp. tested, only Prunus mahaleb was able to regenerate roots from callus cultures established from young leaf explants. There was no report of shoot differentiation (29). Progress has also been reported in the development of methods for the suspension culture of almond (65).

An area of study other than propagation which has benefited from tissue culture of the Prunus species has been the study of graft incompatibilities. Callus cultures of two cherry rootstocks proved more sensitive to cyanide than the callus culture of peach (31). Micrografting techniques have been used as methods to study graft incompatibilities as well as in the development of virus free plants. Improvements in the methods for micrografting were developed in a study utilizing shoot tips of two peach cultivars and seedling rootstocks of Prunus persica, P. armeniaca and P. cerasifera (33). The plum scion 'Victoria' and rootstock 'Pixy' have been grafted onto the peach rootstock GF305 with a success rate of 40-50%, a significant improvement over that of previous work with Prunus (57). Shoot tip and callus culture methods have been used to study non-infectious bud failure, a genetic disorder of almond (42). Tissue culture methods have been used to study a variety of subjects as well as serving as a means of propagation of many species of Prunus.

Tissue Culture of Peach

Peach (Prunus persica) trees for commercial production are normally propagated by budding onto clonal or seedling rootstocks (28). This process is time consuming and laborious. A more rapid method of propagation which would produce large numbers of plants is desirable. Rooted cuttings could be used as a method for the propagation of large numbers of

clones. Methods have been developed for the propagation of peaches from hardwood and semi-hardwood cuttings (10, 15, 27, 32, 41).

Another method which could be of use in the propagation of large numbers of peach trees is tissue culture. Tissue culture methods could provide a means for the rapid multiplication of new cultivars and for the establishment of disease free clones while utilizing a small amount of space for stock plants and a relatively short propagation period (22, 69).

Nekrasova (58) was one of the first to study the culture of peach buds. He was able to produce a rosette of leaves with no stem elongation from his cultures. In 1977, Skirvin and Chu (69) reported the first successful production of shoots from shoot tip cultures of 'Redhaven' peach. No rooting of the shoots was obtained. Also in 1977, Tabachnik and Kester (80) reported successful shoot culture of almond-peach hybrids. Only limited rooting was obtained in this study. In a 1978 report, Skirvin and Chu (70) announced the successful shoot tip culture of 'Redhaven' and 'Harbelle' peach.

Vertesy (85) was among the first to report successful rooting of peach shoot tips in research on the production of virus free plants utilizing a heat treatment of stock plants. Skirvin, Chu and Rukan (72) reported in 1980 a method for proliferation and rooting of shoot tips of peach, plum, apricot and cherry. In a 1981 report, Skirvin, Chu

and Kearnes (71) announced a success rate of 50% in the rooting of *in vitro* proliferated shoots of 'Harbrite' peach. Successful *in vitro* propagation of shoot apices of 'Nemaguard' peach rootstock was reported in 1982 with a rooting percentage of 95%. Seventy-three out of 120 plantlets survived transfer into the soil (51). In 1982, Hammerschlag (23) reported the culture of twelve cultivars of peach with variable success between cultivars.

The first successful culture of shoot segments of peach was reported by Rodriguez (63). A rooting percentage of 60% was obtained from stem segments from 5-7 month old greenhouse grown plants. A 1983 report on the effects of media and media pH stated that the best survival of 'Nemaguard' stem section explants was obtained on an agar media with a pH of 5.8 (62). Also noted was the gradual decline of the cultures with time. Out of 180 initial cultures, only ten shoots rooted with four surviving transfer to soil.

One of the difficulties associated with the successful *in vitro* propagation of peach has been the attainment of aseptic cultures. In a 1980 review on peach micropropagation, Hammerschlag (22) reported that culture of dormant buds was almost impossible because of microbial contamination. In a later article, Hammerschlag (23) described experiments which tested surface sterilization methods for dormant peach buds. When dormant buds were cultured, contamination ranged from 100% for untreated buds to a low of 70% for buds subjected to a five minute soak in 70% ethanol

followed by a 15-20 minute soak in a 0.525% sodium hypochlorite solution. Further reduction of contamination could be achieved with the use of forced shoot tips.

In 1983, Ochatt and Caso (61) reported the first successful *in vitro* propagation of Red-leaf peach from dormant buds. They noted an increase in fungal contamination of cultures as the growing season neared. Only limited rooting of the cultures was obtained.

Tissue Culture of Pecan

Another tree which could benefit from the application of tissue culture methods to its propagation is pecan (*Carya illinoensis*) (44). Pecans are normally propagated by grafting or budding desirable scion cultivars onto seedling rootstocks (46). Seedlings of pecans are highly heterogeneous in their genetic makeup which affects their subsequent performance in the field. It would be advantageous for the grower to be able to obtain rootstocks that are genetically alike and adapted to specific growing conditions. The selection of desirable rootstocks is complicated by the fact that the prospective clonal parent must be in the adult growth phase before evaluation of its characteristics can be made. Propagation of adult material has proved difficult for many woody plants including pecan. A method for the rapid clonal propagation of rootstocks or self-rooted scion cultivars is needed in order to provide large numbers of desirable clones to growers.

Rooted cuttings could be used as a method for the rapid propagation of pecans. Attempts have been made to root cuttings from pecan rootstocks and cultivars (74). It has been difficult to stimulate the rooting of adult cuttings. In research by Smith and Chiu (74), adult cuttings produced fewer roots and a lower rooting percentage than juvenile cuttings. The greatest rooting percentage for juvenile cuttings was 71% whereas the largest rooting percentage for adult cuttings was 33%, indicating a difference in the ease of rooting for adult and juvenile cuttings.

Tissue culture could serve as a means for the rapid clonal propagation of pecan. Research has been conducted on the use of tissue culture methods in the propagation of pecan. Most of the tissue culture research on pecan has involved the use of juvenile seedlings as explant sources because of difficulties associated with the culture of adult explants. Knox and Smith (46) produced rooted plantlets from stem sections of 'Riverside' seedlings. Shoots have been produced from axillary buds on nodal explants of 'Stuart' seedlings (88). Plantlets produced from juvenile seedling pecans have been rooted and successfully transferred to soil in the greenhouse (26).

Little work has been done on the use of adult tissue for explants. This can be partially attributed to the difficulty of establishing cultures from adult sources. Establishment of cultures from adult sources is often difficult when compared with the establishment of juvenile

cultures in many woody species (77). Another reason for the few publications of work on micropropagation of pecan using adult explants is the difficulty encountered in obtaining sterile cultures from adult explants. Adult explants taken from field grown trees of the 'Riverside' cultivar often produced contaminated cultures whereas explants from seedling materials produced little contamination of cultures (46). Research with dormant pecan buds has shown internal fungal infestation of apparently healthy buds (45). The presence of internal fungal infestation of adult pecan buds makes the process of sterilization of adult pecan explants for tissue culture more difficult.

In work with shoot tip cultures of field grown 'Mohawk' pecans, Cotten (9) developed a method of surface sterilization for pecan shoot tips. Using dormant shoot tips collected in mid-December, she was able to reduce contamination to 40% by exposing the shoots to a 4.5 hour soak in a saturated, aqueous benomyl solution, followed by an eight minute treatment in 70% ethanol which was then followed by a 15 minute soak in a 0.525% solution of sodium hypochlorite.

Solvents

Microbial contamination has also been a source of problems in seed propagation. Germination percentages are often reduced due to death of the seedling caused by microbial agents. Seed treatments which have effectively controlled microbial contaminants have included fungicidal

treatments (5) as well as fungicide-organic solvent combinations (14, 53, 54).

Dichloromethane (DCM) has been extensively investigated as a seed treatment and as a means of introducing chemicals into the seed without the complications incurred from imbibition when aqueous solutions are used. Lettuce seeds soaked in DCM for 24 hours displayed no decrease in germination or in oxygen uptake (49). Lettuce seeds which normally require light for germination have been treated with gibberellic acid (GA_3) in DCM and have achieved almost 100% germination in the dark, which shows the effectiveness of DCM in the introduction of chemicals into seeds (43).

The use of DCM as a solvent for fungicides has proved effective with several seed species. The application of pentachloronitrobenzene (PCNB) in DCM was effective in increasing the germination of pea (Pisum sativum) seeds (83). Several fungicides including benomyl have been introduced into soybean seed in a DCM solution and have provided protection from fungal organisms as shown by a high percentage of germination following fungicide treatment (14, 54). Dichloromethane has also been used as a solvent for use in fungicide studies with snap beans and dry beans (12).

Another chemical which has been studied for its use as a solvent for compounds with low water solubility is dimethyl sulfoxide (DMSO). Lettuce seeds soaked for 24 hours in DMSO failed to germinate (49). This could be a result of the chemical's ability to penetrate the dry seed. It is

possible that a shorter soaking interval would have been a more appropriate test for DMSO. DMSO has also been used as a solvent for indolebutyric acid (IBA) in rooting studies. Cuttings of three juniper species were exposed to a five second dip in a DMSO-IBA solution. Best rooting occurred in two species when exposed to the DMSO-IBA solution. The third species which failed to root is considered difficult to root under established rooting procedures. DMSO used alone served to inhibit rooting slightly but was not toxic to the tissue (48).

Another chemical which has been used as a solvent for the introduction of substances into seeds is acetone (ACE). Lettuce seed soaked for 24 hours in ACE alone showed enhanced germination over that of the control (49). Gibberellic acid (GA_3) and abscisic acid have been introduced into lettuce seed using ACE solutions (43). Acetone has been used to introduce radioactive indoleacetic acid (IAA) into seeds of squash, pear, lima bean and lettuce (82). Acetone has also been used as a solvent for antibiotics in a study on the effects of the antibiotics on aging of lettuce seeds (83).

Ethanol has been studied for its use in seed treatments. Pea seeds soaked in ethanol for 24 hours achieved a germination percentage of 83% which was similar to the seeds which were not treated (49). This would seem to indicate that the ethanol was not toxic to the pea seed. Ethanol has been studied as a solvent for several fungicides

including benomyl, for use as a seed treatment of soybean seeds (53). Ethanol can effectively penetrate the seed coat allowing for accumulation of fungicide within the seed coat. Even after washing of the seed, the fungicide still effectively protects the seeds as evidenced by the higher germination percentage of those seeds which were treated with the fungicide over those that did not receive the fungicide treatment (53).

Other chemicals that have been studied for use as seed treatments include dimethyl formamide, dibromomethane and dichloroethane. These chemicals all reduced the germination percentage for lettuce and pea seeds as compared with the control (49). One possible reason for the reduction in germination could be that the chemicals were effectively penetrating the seed coat but were toxic over time. A shorter soaking time of less than 24 hours may have been a more appropriate test for these chemicals.

Because of the difficulties encountered in establishing aseptic cultures of peach and pecan explants, a new method of surface sterilization is needed which would allow for the year-long culture of these plants <u>in vitro</u> from field grown adult material. Based on the surface sterilization method which utilizes a 4.5 hour aqueous benomyl soak (9) and seed treatments using fungicides in organic solvents, it was proposed that a surface sterilization treatment utilizing a benomyl and organic solvent soak could be established which would be of a relatively short duration.

Thus, the aim of this research was to attempt to determine the optimal combination of solvent and time in regard to reducing contamination with reduced necrosis and good survival of the peach and pecan explants.

CHAPTER III

MATERIALS AND METHODS

Plant materials used in this research were collected from 6 year old 'Redhaven' peach trees at the Oklahoma State University Fruit Research Station near Perkins, Oklahoma and from 15 year old 'Mohawk' pecan trees at the Oklahoma Pecan Research Station near Sparks, Oklahoma. Stem cuttings approximately 5 cm long were collected, placed in plastic bags containing a damp cloth and were transported in a portable cooler to the laboratory facilities at the main campus in Stillwater, Oklahoma. After the cuttings arrived at the laboratory, the shoots were kept refrigerated until needed.

The cuttings were reduced in size to approximately 2 to 3 cm long. Under most circumstances, all buds surrounding the terminal bud were removed; however, with the pecan, axillary buds which were beginning to swell were also used as an explant source. The bud scales were then removed, leaving 4 to 6 leaf primordia to protect the apical meristem. The shoots were then placed in a beaker of distilled, deionized (DD) water.

The surface sterilization procedure began with soaks of varying length in a 1% weight to volume benomyl solution

using various organic solvents. All organic solvents were reagent grade and were used without further purification. For purposes of comparison, a soak in a saturated, aqueous benomyl solution was also performed. Following the benomyl soak, the shoot tips were rinsed 4 to 5 times with sterile DD water. The shoots were then placed in a 0.525% sodium hypochlorite (NaOCl) soak for 15 minutes during which time the beaker was transferred to a laminar air flow hood where all other operations occurred. Following the NaOCl soak, the shoot tips were rinsed once with sterile DD water and a few drops of Tween 20 and then rinsed 3 to 4 times with sterile DD water only. The shoot tips were then soaked in a 70% ethanol solution for 6 minutes for 'Redhaven' peach tissue or for 8 minutes for 'Mohawk' pecan shoot tips. Following the ethanol soak, the shoot segments were again rinsed with sterile DD water with Tween 20 followed by 3 to 4 rinses with sterile DD water only.

After surface sterilization, the outer leaf primordia were removed, exposing the apical meristem surrounded by 2 to 3 leaf primordia. The apical meristem with leaf primordia was then excised and placed in a 25 mm x 150 mm test tube on an agar slant. The agar solidified media used in these experiments were variations of Murashige and Skoog's (56) high salts medium (Table I).

The medium used for 'Redhaven' peach explants consisted of one-half strength macronutrients with all other components at full strength (29). Additions to the medium

TABLE I

MEDIA COMPOSITION

Components	Peach[29] (mg/l)	Pecan[9] (mg/l)
Inorganic Constituents		
NH_4NO_3	825	1650
KNO_3	950	1900
$MgSO_4.7H_2O$	185	370
KH_2PO_4	85	170
Na_2-EDTA	18.65	37.3
$FeSO_4.7H_2O$	13.9	27.8
$CaCl_2.2H_2O$	150	150
H_3BO_3	6.2	6.2
$MnSO_4.4H_2O$	22.3	22.3
$ZnSO_4.4H_2O$	8.6	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25
KI	0.75	0.75
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
Organic Constituents		
Nicotinic Acid	1	1
Thiamine HCl	10	10
Pyridoxine HCl	1	1
Myo-inositol	100	100
Ascorbic acid	50	--
Sucrose	20,000	30,000
Agar	8,000	6,000
Activated Charcoal	3,000	3,000
Growth Regulators		
IBA	0.5	--
BA	0.8	1.0

included sucrose (20 g/l), Phytagar (GIBCO) (8 g/l), activated charcoal (3 g/l) and ascorbic acid (50 mg/l) (69). Growth regulators that were used included indolebutyric acid (IBA) at 0.5 mg/liter and 6-benzylamino-purine (BA) at 0.8 mg/liter. Distilled, deionized water was added to bring the medium up to volume.

The medium used for the 'Mohawk' pecan tissue consisted of the Murashige and Skoog (56) components at full strength with the following additions of sucrose at 30 g/liter, Phytagar (GIBCO) at 6 g/liter and activated charcoal at 3 g/liter. BA was used as the growth regulator at 1.0 mg/liter (9). Distilled, deionized water was used to bring the medium up to volume.

Growth regulators were dissolved in a small amount of 5N KOH and then brought up to volume with DD water. The growth regulators were dispensed into the medium as it was being prepared.

The pH of the medium was adjusted after all components of the medium were added with the exception of charcoal and agar. The pH was adjusted to 5.8 using 1.0N KOH or 1.0N HCl. After adjustment of the pH, the agar and charcoal were added and the medium was brought to a boil. Twenty-five milliliters of medium were then dispensed into 25 mm x 150 mm Pyrex culture tubes. The tubes were sealed with Bellco Kap-uts. The tubes were then autoclaved at 121° C. at 15 psi for 15 minutes. The agar medium was solidified at a 45° slant.

Once the explants were placed in the tubes, the test tubes were transferred to and maintained in a growth chamber for the duration of the experiment. Explants were exposed to a temperature of 25 to 28° C. and darkness for the first week of culture and then to 16 hours of 300-400 footcandles of illumination at 25 to 28° C. for the remainder of the experiment.

Explants were evaluated for % contamination, necrosis after one week of culture and survival over a period of eight weeks. Necrosis was evaluated by use of a visual rating index during the first week of culture:

0=No necrosis

1=1/5 of explant necrotic

2=2/5 of explant necrotic

3=3/5 of explant necrotic

4=4/5 of explant necrotic

5=Dead

A survival index was used in the visual rating of explants for weeks 2 to 8.

0=Dead

1=Browning of explant

2=Chlorosis (yellowing) of explant

3=Chlorosis beginning to develop (pale green)

4=Green explant

5=Green with growth of explant

The experiments were set up as a completely randomized design with factorial treatment combinations of solvents and

time. The experimental results were analyzed using analysis of variance tables and paired T-tests. The paired T-tests were used to indicate the significant differences between treatments and the controls. The least significant difference (LSD) was computed for comparisons between treatments. Numbers within the tables represent the least squares means for the data which consisted of 5 replications per treatment. Contaminated treatments were treated as lost replications.

Experiments

Peach

Experiment I - The Effect of Dichloromethane as a Fungicidal Solvent on 'Redhaven' Peach Explants. Dichloromethane (DCM) was used as a test solvent to determine the optimal duration of soaking time in regard to control of contamination and survival of the explant. DCM was selected for this experiment because of its wide usage as a fungicidal solvent for surface sterilization of seeds. A commercial preparation of benomyl was used to give a 1% weight to volume solution of benomyl in DCM. Shoot tips from 'Redhaven' peaches were used as explants. Tissues were collected on January 17, 1984. Following the benomyl soaks, the tissues were subjected to a "standard" sterilization treatment of a 15 minute soak in 0.525% NaOCl followed by a 6 minute soak in 70% ethanol which was a slight modifica-

tion of the procedure used by Cotten (9) in the micropropagation of pecan. Treatments were replicated 5 times. The treatments consisted of DCM soaks of 0.25, 0.50, 0.75, 1.0, 2.0, 4.0 and 5.0 minutes and a 4.0 hour saturated, aqueous benomyl soak.

Experiment II - The Effects of Various Fungicidal Solvents on 'Redhaven' Peach Explants. Several organic solvents were combined with benomyl to give a 1% weight to volume solution of benomyl. Explants were subjected to soaks of varying durations in an attempt to select the time and solvent combination which gave the best results in terms of control of contamination, necrosis and survival of the explant. The solvents that were tested included dichloromethane (DCM), dimethyl sulfoxide (DMSO), N,N-dimethyl formamide (DMF), 1,2-dichloroethane (DCE), dibromomethane (DBM), acetone (ACE) and 95% ethanol (ETOH). Treatments consisted of 0.25, 0.50, 0.75 and 1.00 minute soaks in each of the above benomyl-solvent combinations. 'Redhaven' peach trees were used as the source of explant material. Dormant shoots were collected on February 8, 1984. Two control treatments were included in this experiment. They consisted of a 4.0 hour exposure to a saturated, aqueous benomyl solution and a treatment given no soak prior to the NaOCl exposure. Following the benomyl soaks or no soak, the tissue was exposed to a 15 minute soak in 0.525% NaOCl followed by a 6 minute soak in 70% ethanol. There were 5 replicates of each treatment.

Pecan

Experiment III - The Effect of Dichloromethane as a Fungicidal Solvent on 'Mohawk' Pecan Explants. Shoot tips of 'Mohawk' pecans were exposed to a 1% weight to volume benomyl solution with DCM for various durations in an effort to find the soaking period which gave the best control of contamination and also, the best survival rating. Shoots were collected on March 6, 1984. Shoot segments received the "standard" surface sterilization procedure consisting of an 8 minute soak in 70% ethanol and a 15 minute soak in 0.525% NaOCl. Five replications were made of each treatment. The treatments consisted of 0.50, 0.75, 1.00, 2.00, 4.00 and 5.00 minute soaks in the 1% benomyl: DCM solution (w/v). A 4.5 hour soak in a saturated, aqueous benomyl solution served as a control.

Experiment IV - The Effects of Various Fungicidal Solvents on 'Mohawk' Pecan Explants. Several organic solvents and treatment times were combined in a factorial treatment combination in order to select the optimal time and solvent combination which best controlled contamination with minimal necrosis and optimal survival of the explant. The same solvents that were used in Experiment II with 'Redhaven' peach shoot tips were used. These included DCM, DMSO, DMF, DCE, DBM, ACE and ETOH. Explant tissues were soaked for 0.50, 0.75, 1.00 and 1.50 minutes in each of the above benomyl-solvent combinations at 1% w/v. Control

treatments consisted of a 4.5 hour saturated, aqueous benomyl soak and no soak of the pecan tips. All treatments including the controls were exposed to the "standard" sterilization procedure which consisted of an 8 minute soak in 70% ethanol followed by a 15 minute soak in 0.525% NaOCl. Apical meristems excised from 'Mohawk' pecan shoot tips collected on March 6, 1984 were used as the explants. Each treatment was replicated 5 times.

Experiment V - The Effects of Selected Fungicidal Solvents at Longer Time Intervals on 'Mohawk' Pecan Explants. Several of the solvents used in Experiment IV were retained in this experiment to test the effects of longer soaking periods on % contamination, necrosis and survival of the explants. Those solvents that were discarded appeared to be phytotoxic to the tissue. Shoot segments from 'Mohawk' pecan trees were collected on April 4, 1984. Shoot tips were subjected to the "standard" surface sterilization procedure for pecans following the benomyl-solvent soak. Each treatment consisted of 5 replications. Treatments consisted of 1.0, 2.0, 3.0, 4.0, 5.0, 7.0 and 10.0 minute soaks in 1% benomyl:DMSO, DMF, DBM, ACE and ETOH solutions. The control consisted of a 4.5 hour soak in a saturated, aqueous benomyl solution.

CHAPTER IV

RESULTS AND DISCUSSION

Peach

Experiment I - The Effects of Dichloromethane as a Fungicidal Solvent on 'Redhaven' Peach Explants

All treatments using DCM with the exception of the 0.5 minute treatment controlled contamination comparably to the 4.0 hour benomyl soak (Table II). The 0.25 and 0.75 minute soaks did not differ significantly in necrosis from the 4.0 hour aqueous benomyl soak. All other soaking times gave a significantly higher necrosis rating after one week of culture (Table III). After two weeks in culture, the explants were given a rating of survival in which all treatments other than the 0.75 minute soak had a highly significantly lower survival rating than the control. The 0.75 minute soak again was not significantly different from the 4.0 hour benomyl soak (Table IV). This trend continued throughout the experiment and was still apparent after eight weeks of culture at the termination of the experiment (Table V). All of the explants with the exception of the 0.75 minute treatment were dead or dying. Those explants

TABLE II

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN DICHLOROMETHANE ON TOTAL CONTAMINATION OF 'REDHAVEN' PEACH EXPLANTS

Exposure Time (Minutes)	Contamination (%)
0.25	0[z]
0.50	60*
0.75	20
1.00	40
2.00	40
4.00	20
5.00	0

[z]Mean separation of rates, $LSD_{.05}=52$, $LSD_{.01}=70$.

* Significant at 5% from 4.0 hour aqueous benomyl soak (0% contamination).

TABLE III

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN DICHLOROMETHANE ON NECROSIS OF 'REDHAVEN' PEACH EXPLANTS

Exposure Time (Minutes)	Necrosis[z] (Index)
0.25	2.2[y]
0.50	3.0**
0.75	1.0
1.00	3.0**
2.00	2.5*
4.00	3.2**
5.00	3.0**

[z]After one week of culture.

[y]Mean separation of rates, $LSD_{.05}=1.2$, $LSD_{.01}=1.6$.

*,** Significant at 5% (*) or 1% (**) from 4.0 hour aqueous benomyl soak (1.0-necrosis).

TABLE IV

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN DICHLOROMETHANE ON SURVIVAL OF 'REDHAVEN' PEACH EXPLANTS

Exposure Time (Minutes)	Survival[z] (Index)
0.25	2.4**[y]
0.50	1.0**
0.75	4.0
1.00	1.0***
2.00	1.3***
4.00	1.2***
5.00	1.4***

[z]After two weeks of culture.

[y]Mean separation of rates, $LSD_{.05}$=1.5, $LSD_{.01}$=2.1.

,* Significant at 1% (**) or 0.1% (***) from 4.0 hour aqueous benomyl soak (4.6-survival).

TABLE V

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN DICHLOROMETHANE ON SURVIVAL OF 'REDHAVEN' PEACH EXPLANTS

Exposure Time (Minutes)	Survival[z] (Index)
0.25	0.6***[y]
0.50	0.0***
0.75	2.2
1.00	0.0***
2.00	0.0***
4.00	0.0***
5.00	0.0***

[z]After eight weeks of culture.

[y]Mean separation of rates, $LSD_{.05}=1.0$, $LSD_{.01}=1.4$.

*** Significant at 0.1% from 4.0 hour aqueous benomyl soak (2.8-survival).

receiving the 0.75 minute soak were beginning to decline but were not significantly different from those explants subjected to the 4.0 hour saturated, aqueous benomyl soak.

Contamination was controlled with increases in the duration of the soaks. However, with the increase in soaking time, there was an increase in necrosis and a decrease in the survival of the explants. Dichloromethane produced adequate control of contamination but appears to be phytotoxic to the tissue especially under conditions of long soaking time.

The best treatment time was 0.75 minutes which yielded control of contamination, low necrosis and good explant survival comparable to the 4.0 hour aqueous benomyl soak. This would indicate that fungicidal solvents can be used to treat explant tissues with good results.

Experiment II - The Effects of Various Fungicidal Solvents on 'Redhaven' Peach Explants

All treatment times for the solvents DMF, ACE and ETOH gave significantly better control of contamination than the controls. Other solvents yielded better control of contamination at various times throughout the experiment. This variation in times was unpredictable with some shorter time period treatments producing less contamination than longer time treatments within a solvent (Table VI).

TABLE VI

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN VARIOUS SOLVENTS ON TOTAL CONTAMINATION OF 'REDHAVEN' PEACH EXPLANTS

	Contamination (%)			
	Exposure Time (Minutes)			
Solvent	0.25	0.50	0.75	1.00
DCM	40^{z}	0_{*}^{**}	40	20^{*}
DMSO	40	20^{*}	75	40
DMF	20^{*}	20^{*}	20^{*}	0_{*}^{**}
DCE	0_{*}^{**}	40	20^{*}	60
DBM	40	20^{*}	40	100
ACE	20^{*}	20^{*}	20^{*}	20^{*}
ETOH	20^{*}	0_{*}^{**}	20^{*}	0_{*}^{**}

[z]Mean separation of rates within solvents or solvents within rates are $LSD_{.05}=57$, $LSD_{.01}=75$.

*,** Significant at 5% (*) or 1% (**). Superscript indicates significance from 4.0 hour aqueous benomyl soak (67% contamination) and subscript indicates significance from no benomyl soak (60% contamination).

When the effects of solvent and time interval are analyzed in respect to necrosis of the explant, it appears that treatments of DCM at all times except for 1.0 minute, DCE over all times, and ETOH at 1.0 minute produced significantly more necrosis of the explant than did the 4.0 hour benomyl soak and no fungicidal soak treatments. The ACE-benomyl soak at 0.25 minutes gave significantly less necrosis of the explants as compared to the 4.0 hour benomyl soak. All other treatments were not significantly different from the 4.0 hour benomyl soak or from the no soak controls (Table VII).

Survival of the explants was evaluated by the use of a visual rating system based on a survival index of 0 to 5. A high rating indicated growth and survival whereas a low number indicated necrosis and death of the explant. DCM at 0.25 and 0.5 minutes and DCE at 0.25, 0.5 and 1.0 minutes produced significantly lower ratings than the two controls at the end of two weeks of culture. The solvents, DMSO, DMF, DBM, ACE and ETOH, all produced higher survival ratings at most times tested; however, these ratings were not significantly different from the controls (Table VIII). These results remained fairly consistent throughout the experiment.

The time component for this experiment was difficult to analyze because of the similarity of the means within a particular solvent. It appears that there is for some solvents, DBM and ETOH, a decrease in the survival of the

TABLE VII

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN VARIOUS SOLVENTS ON NECROSIS OF 'REDHAVEN' PEACH EXPLANTS

	Necrosis[z] (Index)			
	Exposure Time (Minutes)			
Solvent	0.25	0.50	0.75	1.00
DCM	3.0**[y]	3.0**	2.8*	2.0
DMSO	1.5	1.8	1.0	2.0
DMF	2.0	2.5	2.2	2.0
DCE	2.6*	3.0**	2.6*	3.0**
DBM	1.7	2.0	1.8	1.0
ACE	1.0*	2.0	2.2	1.8
ETOH	1.8	2.0	1.5	2.8*

[z]After one week of culture.

[y]Mean separation of rates within solvents or solvents within rates, $LSD_{.05}=0.8$, $LSD_{.01}=1.0$.

*,** Significant at 5% (*) or 1% (**) from 4.0 hour aqueous benomyl soak (1.8-necrosis). Treatments were not significant from the no benomyl soak (2.0-necrosis).

TABLE VIII

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN VARIOUS SOLVENTS ON SURVIVAL OF 'REDHAVEN' PEACH EXPLANTS

	Survival[z] (Index)			
	Exposure Time (Minutes)			
Solvent	0.25	0.50	0.75	1.00
DCM	1.2^{***y}_{***}	1.0^{***}_{***}	2.7	3.2
DMSO	4.0	3.5	4.0	3.5
DMF	4.0	3.8	2.8	4.0
DCE	1.6^{***}_{**}	2.0^{*}_{*}	2.5	1.3^{***}_{**}
DBM	4.0	3.5	3.3	---
ACE	4.0	4.0	3.5	3.8
ETOH	4.0	3.8	3.8	3.0

[z]After two weeks of culture.

[y]Mean separation of rates within solvents or solvents within rates, $LSD_{.05}=1.0$, $LSD_{.01}=1.3$.

*,**,*** Significant at 5% (*), 1% (**) or 0.1% (***). Superscript indicates significance from 4.0 hour aqueous benomyl soak (3.3-survival) and subscript indicates significance from no benomyl soak (3.5-survival).

explants with an increase in the time of exposure to the solvent-benomyl solution (Table VIII).

The main effects for solvents are more easily isolated (Table IX). Those solvents which produced a significantly lower amount of contamination of the explants in comparison to the controls included DCM, DMF, DCE, ACE and ETOH. The remaining solvents, DMSO and DBM, did not differ significantly from the controls. When the necrosis and survival ratings are taken into account, it appears that DCM and DCE may be toxic to the explants while the other solvents show no significant differences from the controls in terms of necrosis or survival.

From this experiment, it appears that the use of fungicidal solvents may be a viable alternative method for the surface sterilization of peach explants, ACE, ETOH and DMF are likely candidates for use in the surface sterilization of peach explants taken from field grown trees.

Pecan

Experiment III - The Effect of Dichloromethane as a Fungicidal Solvent on 'Mohawk' Pecan Explants

At all time levels tested, the DCM soaks gave significantly improved control of contamination over the no soak treatment. The 0.75, 2.0 and 5.0 minute treatments gave significantly better control of contamination than the

TABLE IX

THE INFLUENCE OF VARIOUS SOLVENTS ON TOTAL CONTAMINATION, NECROSIS AND SURVIVAL OF 'REDHAVEN' PEACH EXPLANTS

Solvent	Contamination (%)	Necrosis[z] (Index)	Survival[y] (Index)	Survival[x] (Index)
DCM	25^{**}	2.7^{**}	2.0^{***}_{*}	0.7^{**}
DMSO	44	1.6	3.8	2.0
DMF	15^{***}_{*}	2.2	3.6	1.7
DCE	30^{*}	2.8^{**}	1.8^{***}_{**}	0.8^{*}
DBM	50	1.6	3.6	2.2
ACE	20^{**}	1.7	3.8	2.4
ETOH	10^{***}_{*}	2.0	3.6	2.0
4.0 hour soak	67	1.8	3.3	2.2
No benomyl soak	60	2.0	3.5	2.0

[z]After one week of culture.

[y]After two weeks of culture.

[x]After eight weeks of culture.

*,**,*** Significant at 5% (*), 1% (**) or 0.1% (***). Superscript indicates significance from 4.0 hour aqueous benomyl soak and subscript indicates significance from no benomyl soak.

4.5 hour benomyl soak. However, for all time levels, DCM appears to be toxic to the tissue producing high necrosis ratings and low survival ratings throughout the experiment. Because of the severe toxicity to the explants, this data has been omitted.

Experiment IV - The Effects of Various Fungicidal Solvents on 'Mohawk' Pecan Explants

When compared with the no soak control, significantly better results were gained in regard to the control of contamination of 'Mohawk' pecan shoot apices with the following time and solvent combinations: all time levels of DCM, 0.75 minute soak in DMSO, 0.5 minute exposure to DMF, 0.5 and 1.0 minute soaks in DCE, 0.75 and 1.5 minute treatments with DBM, a 1.0 minute exposure to ACE and 0.5 and 1.0 minute soaks in ETOH. When compared with the 4.5 hour saturated benomyl soak, only the 0.75 minute soak in DCM was significantly better in the control of contamination. The 0.50 minute treatment with ACE resulted in 100% contamination of the cultures, which was significantly more contamination than received with the 4.5 hour saturated benomyl soak. All other treatments did not differ significantly from the controls (Table X).

The solvents, DCM, DCE and DBM at all time levels and ACE at all time levels except 1.0 minute, produced significantly higher necrosis ratings when compared with the

TABLE X

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN VARIOUS SOLVENTS ON TOTAL CONTAMINATION OF 'MOHAWK' PECAN EXPLANTS

	Contamination (%)			
	Exposure Time (Minutes)			
Solvent	0.50	0.75	1.00	1.50
DCM	30^{Z}_{*}	10^{*}_{**}	30_{*}	20_{**}
DMSO	68	28_{*}	88	82
DMF	28_{*}	88	48	88
DCE	28_{*}	48	8_{**}	68
DBM	48	8_{**}	68	8_{**}
ACE	100^{**}	68	28_{*}	58
ETOH	28_{*}	48	28_{*}	48

[Z]Mean separation of rates within solvents or solvents within rates, $LSD_{.05}=57$, $LSD_{.01}=75$.

*,** Significant at 5% (*) or 1% (**). Superscript indicates significance from 4.5 hour aqueous benomyl soak (51% contamination) and subscript indicates significance from no benomyl soak (92% contamination).

4.5 hour aqueous benomyl soak (Table XI). DMSO produced significantly less necrosis than the controls at 1.0 and 1.5 minute exposure times. All other results did not differ significantly from the 4.5 hour soak or the no soak controls.

From the week two survival ratings, it can be seen that DCM, DCE and DBM soaks produced significantly lower survival ratings when compared with the 4.5 hour benomyl soak (Table XII). The longer duration of DMSO and DMF at 1.00 minute resulted in significantly higher survival of the explants. These trends continued into the final week of culture (Table XIII). Three treatments produced a highly significantly better survival rating than the 4.5 hour soak. These treatments were the 1.0 and 1.5 minute soaks in DMSO and the 1.0 minute soak in the DMF-benomyl solution. All other treatments did not differ significantly from the controls. These results were fairly consistent from week two through week eight of culture, although other treatments may have given significantly better survival ratings at isolated weeks of culture.

Again, it was difficult to analyze the time component of this experiment because of the similarity of the means within a solvent.

An analysis of the main effects for the solvents showed that DCM, DCE, DBM and ETOH gave significantly better control of contamination in comparison with the no soak control and DCM gave better control in comparison with the

TABLE XI

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN VARIOUS SOLVENTS ON NECROSIS OF 'MOHAWK' PECAN EXPLANTS

	Necrosis[z] (Index)			
	Exposure Time (Minutes)			
Solvent	0.50	0.75	1.00	1.50
DCM	3.9^{***y}_{***}	4.0^{***}_{***}	4.0^{***}_{***}	3.9^{***}_{***}
DMSO	1.6	1.4	0.9^{*}	0.9^{**}_{*}
DMF	2.4	1.4	1.7	1.4
DCE	2.7^{*}	2.9^{**}_{*}	2.9^{***}_{*}	3.3^{***}_{**}
DBM	3.7^{***}_{***}	3.9^{***}_{***}	2.7^{*}	2.9^{***}_{*}
ACE	2.9^{*}	2.6^{*}	2.1	2.6^{*}
ETOH	2.4	1.6	1.5	2.2

[z]After one week of culture.

[y]Mean separation of rates within solvents or solvents within rates, $LSD_{.05}=0.7$, $LSD_{.01}=0.9$.

*,**,*** Significant at 5% (*), 1% (**) or 0.1% (***). Superscript indicates significance from 4.5 hour aqueous benomyl soak (2.0-necrosis) and subscript indicates significance from no benomyl soak (2.0-necrosis).

TABLE XII

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN VARIOUS SOLVENTS ON SURVIVAL OF 'MOHAWK' PECAN EXPLANTS

	Survival[z] (Index)			
	Exposure Time (Minutes)			
Solvent	0.50	0.75	1.00	1.50
DCM	1.1^{***}_{***}[y]	1.2^{***}_{***}	1.2^{***}_{***}	1.1^{***}_{***}
DMSO	3.4	3.3	4.0	4.0^{*}
DMF	2.8	3.5	3.7^{*}	4.0
DCE	1.5^{***}_{***}	2.0^{*}_{**}	1.4^{***}_{***}	1.7^{**}_{***}
DBM	1.0^{***}_{***}	1.0^{***}_{***}	1.0^{***}_{***}	2.2^{*}_{**}
ACE	3.0	2.7	3.4	2.7
ETOH	3.0	3.4	3.0	3.0

[z]After two weeks of culture.

[y]Mean separation of rates within solvents or solvents within rates, $LSD_{.05}=0.8$, $LSD_{.01}=1.0$.

*,**,*** Significant at 5% (*), 1% (**) or 0.1% (***). Superscript indicates significance from 4.5 hour aqueous benomyl soak (3.0-survival) and subscript indicates significance from no benomyl soak (3.5-survival).

TABLE XIII

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN VARIOUS SOLVENTS ON SURVIVAL OF 'MOHAWK' PECAN EXPLANTS

	Survival[z] (Index)			
	Exposure Time (Minutes)			
Solvent	0.50	0.75	1.00	1.50
DCM	0.0^{***}_{***}[y]	0.0^{***}_{***}	0.0^{***}_{***}	0.0^{***}_{***}
DMSO	1.0	2.0^{*}	3.0^{**}	3.0^{**}
DMF	1.8	1.0	2.7^{***}	2.0
DCE	0.0^{***}_{**}	0.0^{***}_{**}	0.4^{**}_{**}	0.0^{**}_{**}
DBM	0.0^{***}_{**}	0.0^{***}_{***}	0.0^{**}_{**}	0.4^{**}_{**}
ACE	---	1.0	1.8	1.0
ETOH	0.5^{*}_{*}	0.3^{**}_{**}	1.5	2.3^{**}

[z]After eight weeks of culture.

[y]Mean separation of rates within solvents or solvents within rates, $LSD_{.05}=0.7$, $LSD_{.01}=0.9$.

*,**,*** Significant at 5% (*), 1% (**) or 0.1% (***). Superscript indicates significance from 4.5 hour aqueous benomyl soak (1.3-survival) and subscript indicates significance from no benomyl soak (2.0-survival).

4.5 hour benomyl soak (Table XIV). Other treatments did not differ significantly from the controls in the control of contamination. The DCM, DCE and DBM treatments produced significantly more necrosis of the explants than both controls. In regard to necrosis, the DMSO treatment produced significantly less necrosis and the ACE soak produced more necrosis when compared with the 4.5 hour benomyl soak. When survival ratings are compared for weeks two and eight, it appears that the soaks with DCM, DCE and DBM consistently produced lower survival ratings that are statistically different from the controls. It also appears that the DMSO soak produced a consistently high survival rating.

There appears to be a correlation between control of contaminants and survival of the explant. In most cases, DCM, DCE and DBM are effective in the control of contaminants but are phytotoxic to the tissue. Other solvents control contamination to a degree similar to that of the 4.5 hour benomyl soak and yet do not appear to be phytotoxic to the explant. Ethanol appears promising due to its effectiveness in the control of contamination (as compared with the no soak control) and to its relatively low toxicity to tissues.

Experiment V - The Effects of Selected Fungicidal Solvents on 'Mohawk' Pecan Explants

Because of the apparent phytotoxicity of DCM and DCE

TABLE XIV

THE INFLUENCE OF VARIOUS SOLVENTS ON TOTAL CONTAMINATION, NECROSIS AND SURVIVAL OF 'MOHAWK' PECAN EXPLANTS

Solvent	Contamination (%)	Necrosis[z] (Index)	Survival[y] (Index)	Survival[x] (Index)
DCM	22^{**}_{***}	3.9^{***}_{***}	1.2^{***}_{***}	0.0^{***}_{***}
DMSO	66	1.2^{***}	3.7^{**}	2.2^{***}
DMF	62	1.8	3.5^{*}	1.8^{*}
DCE	38_{*}	3.0^{***}_{*}	1.7^{***}_{***}	0.1^{***}_{***}
DBM	32_{*}	3.3^{***}_{***}	1.3^{***}_{***}	0.1^{***}_{***}
ACE	65	2.6^{**}	3.0	---
ETOH	38_{*}	2.0	3.1	1.2
4.5 hour soak	51	2.0	3.0	1.3
No benomyl soak	92	2.0	3.5	2.0

[z]After one week of culture.

[y]After two weeks of culture.

[x]After eight weeks of culture.

*,**,*** Significant at 5% (*), 1% (**) or 0.1% (***). Superscript indicates significance from 4.5 hour aqueous benomyl soak and subscript indicates significance from no benomyl soak.

(Tables IX and XIV), these solvents were discarded. All other solvents were retained for further testing at longer exposure times. The solvents, DBM at all times except 3.0 minutes and ACE at all times except 3.0 and 4.0 minutes, produced highly significantly greater control of contamination as compared with the 4.5 hour benomyl soak. All other treatments were not significantly different from the control (Table XV).

Examination of the necrosis ratings showed that, at most time levels, the two solvents which gave the best control of contamination, DBM and ACE, also produced significantly higher necrosis ratings than did the 4.5 hour control (Table XVI). The 3.0 minute treatment with DMSO also produced a significantly higher necrosis rating than the control. After one week of culture, the 3.0 and 4.0 minute treatments with DMF gave a significantly lower necrosis rating than the control. All other treatments were not significantly different from the control.

The data for the second week's survival ratings in Table XVII shows the apparent toxicity of DBM and ACE to the pecan explant over most times tested. All times tested for DMSO excluding those that were highly contaminated, produced significantly higher survival ratings when compared with the control. This may indicate a relatively low toxicity to the tissue. The 4.0 minute treatment with ETOH also produced a highly significantly better survival rating than the control. All other times and solvents tested,

TABLE XV

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN VARIOUS SOLVENTS ON TOTAL CONTAMINATION OF 'MOHAWK' PECAN EXPLANTS

	Contamination (%)						
	Exposure Time (Minutes)						
Solvent	1.0	2.0	3.0	4.0	5.0	7.0	10.0
DMSO	92[z]	72	92	92	72	52	92
DMF	88	100	100	100	100	88	100
DBM	8***	8***	68	48**	28***	8***	28***
ACE	48**	32**	68	88	8***	8***	8***
ETOH	100	88	88	100	88	88	100

[z]Mean separation of rates within solvents or solvents within rates, $LSD_{.05}=41$, $LSD_{.01}=54$.

,* Significant at 1% (**) or 0.1% (***) from 4.5 hour aqueous benomyl soak (92% contamination).

TABLE XVIII

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN VARIOUS SOLVENTS ON SURVIVAL OF 'MOHAWK' PECAN EXPLANTS

	Survival[z] (Index)						
	Exposure Time (Minutes)						
Solvent	1.0	2.0	3.0	4.0	5.0	7.0	10.0
DMSO	---	0.0[y]	---	---	3.0**	2.5**	---
DMF	0.0	---	---	---	---	1.0	---
DBM	0.2	0.0	0.0	0.0	0.3	0.4	0.0
ACE	1.0	0.0	0.0	0.0	0.0	0.0	0.0
ETOH	---	2.0	2.0	---	2.0	2.0	---

[z]After five weeks of culture.

[y]Mean separation of rates within solvents or solvents within rates, $LSD_{.05}=0.7$, $LSD_{.01}=1.0$.

** Significant at 1% from 4.5 hour aqueous benomyl soak (0.7-survival).

TABLE XVII

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN VARIOUS SOLVENTS ON SURVIVAL OF 'MOHAWK' PECAN EXPLANTS

	Survival[z] (Index)						
	Exposure Time (Minutes)						
Solvent	1.0	2.0	3.0	4.0	5.0	7.0	10.0
DMSO	---	3.0*[y]	---	---	4.0***	3.5***	4.0***
DMF	2.0	---	---	3.0	3.0	2.0	---
DBM	1.2*	1.0**	1.0*	1.0**	1.0**	1.0**	1.0**
ACE	2.0	1.0**	1.0*	2.5	1.0**	1.0**	1.2*
ETOH	---	3.0	2.0	4.0***	3.0	3.0	---

[z]After two weeks of culture.

[y]Mean separation of rates within solvents or solvents within rates, $LSD_{.05}=0.6$, $LSD_{.01}=0.8$.

*,**,*** Significant at 5% (*), 1% (**) or 0.1% (***) from 4.5 hour aqueous benomyl soak (2.0-survival).

excluding those treatments with high contamination rates, did not differ significantly from the 4.5 hour benomyl soak.

Analysis of this experiment was terminated with the data of the fifth week of *in vitro* culture because of the high contamination rate and large amount of necrosis in many treatments including the control. Results from week five show that the 5.0 and 7.0 minute treatments with DMSO produced highly significantly better survival ratings than the control. All other treatments did not differ significantly from the control; the ETOH treatments, however, displayed a relatively high survival rating in comparison with the other treatments. DBM and ACE tended to produce relatively low survival ratings when compared to the other treatments (Table XVIII).

Analysis of the main effect of the solvent alone shows that DBM and ACE were effective in the control of contamination when compared to the control treatment of a 4.5 hour aqueous benomyl soak (Table XIX). Although these chemicals were effective in the control of contamination, they also produced a high degree of necrosis and a low survival of explants in the following weeks. The other treatments which were not different from the 4.5 hour benomyl soak in terms of the control of contamination and necrosis but produced better survival than the control, were DMSO and ETOH. The treatments with DMF were similar to the control in terms of control of contamination and survival of the explants.

Analysis of the results of this experiment was made

TABLE XVI

THE INFLUENCE OF EXPOSURE TIME TO A 1% BENOMYL SOLUTION IN VARIOUS SOLVENTS ON NECROSIS OF 'MOHAWK' PECAN EXPLANTS

	Necrosis[z] (Index)						
	Exposure Time (Minutes)						
Solvent	1.0	2.0	3.0	4.0	5.0	7.0	10.0
DMSO	3.2[y]	3.2	4.2*	3.2	3.7	2.7	3.7
DMF	2.8	2.8	2.5*	2.5*	2.8	3.1	3.3
DBM	3.8*	3.6	3.8	3.8*	3.8*	3.8*	3.8*
ACE	3.5	3.8*	3.8*	3.8*	3.8*	3.8*	3.8*
ETOH	3.8	3.8	3.3	3.1	3.3	3.3	3.5

[z]After one week of culture.

[y]Mean separation of rates within solvents or solvents within rates, $LSD_{.05}=0.5$, $LSD_{.01}=0.7$.

* Significant at 5% from 4.5 hour aqueous benomyl soak (3.2-necrosis).

TABLE XIX

THE INFLUENCE OF VARIOUS SOLVENTS ON TOTAL CONTAMINATION, NECROSIS AND SURVIVAL OF 'MOHAWK' PECAN EXPLANTS

Solvent	Contamination (%)	Necrosis[z] (Index)	Survival[y] (Index)	Survival[x] (Index)
DMSO	81	3.4	3.6***	1.8*
DMF	100	2.8	2.5	0.5
DBM	28***	3.8***	1.0***	0.1
ACE	37***	3.8**	1.4*	0.1
ETOH	96	3.4	3.0**	2.0**
4.5 hour soak	92	3.2	2.0	0.7

[z]After one week of culture.

[y]After two weeks of culture.

[x]After five weeks of culture.

*,**,*** Significant at 5% (*), 1% (**) or 0.1% (***) from 4.5 hour aqueous benomyl soak.

more difficult because of the high rate of contamination of cultures. The high contamination rate can be expected during the spring season with pecan when the buds are beginning to expand and grow. This phenomenon of high contamination during this season has been documented among species of Ginkgo, Magnolia, Populus and Robinia (40).

CHAPTER V

SUMMARY AND CONCLUSIONS

It is often difficult to obtain clean cultures from field grown adult woody plant materials with standard surface sterilization techniques. Such tissues may be internally infested with microbial organisms (45). Specialized surface sterilization techniques are needed in order to obtain clean cultures from field grown woody plant materials. Cotten (9) developed one such method which utilizes an aqueous benomyl soak. This research represents an effort to modify this method by the use of organic solvents and benomyl solutions.

Dichloromethane was used as a test solvent in the determination of the length of time intervals to be used in subsequent experiments. Only the 0.75 minute treatment gave adequate control of contamination with little necrosis of peach explants only. The other time treatments produced significantly higher necrosis and lower survival ratings indicating the possibility of DCM being phytotoxic to both peach and pecan explants.

The next experiment tested the effects of all seven organic solvents at four time intervals on % contamination, necrosis and survival of shoot apices of 'Redhaven' peach

and 'Mohawk' pecan. DCM (peach and pecan) and DCE (peach only) were effective in the control of contamination but were toxic to the tissues as shown by high necrosis and low survival ratings. DBM and DCE were also toxic to the pecan explants but were similar to the control in contamination control. DBM (peach only) was similar to the aqueous benomyl soak in control of contaminants as well as survival of the explants. ACE produced high necrosis but was similar to the control in terms of survival and contamination control of pecan explants. DMF, ETOH and ACE (peach only) were similar to the control in terms of necrosis and survival but were superior to the controls in controlling contamination when used on peach explants. DMF was similar to the control in controlling contamination of pecan explants but produced improved survival while ETOH was similar to the control in terms of the control of contamination and survival. DMSO produced lower necrosis and higher survival than the control for pecan but was similar to the control for peach explants with control of contamination being similar for both peach and pecan explants.

Because of the high necrosis and low survival ratings of DCM and DCE treated explants in earlier experiments, these solvents were eliminated from the last experiment. Time intervals tested with the remaining solvents were lengthened in an attempt to find the time and solvent combination which best controlled contaminants of pecan with the least amount of necrosis. DBM and ACE both produced better

control of contamination but lower survival ratings when compared to the 4.5 hour benomyl soak. DMSO, DMF and ETOH produced results similar to the control in the areas of % contamination, necrosis and survival of the explants.

The time portion of these experiments is difficult to analyze because, in many cases, there is not enough difference between the time interval results within a solvent to differentiate the effects of time on survival and contamination of the explant. There are many instances in which there is higher contamination or better survival with longer exposures to a solvent, which is contrary to that which would be expected. One would expect lower contamination and lower survival with an increase in length of exposure to the solvent.

There is a trend, in some cases, toward lower contamination with higher necrosis as the time interval increases. Because of the similarities of responses to time intervals within a solvent and the unpredictable behavior of some responses, it would be difficult to suggest a time interval which gives the most desirable results. However, it does appear likely that a short soak time of a few minutes or less in a benomyl-organic solvent solution could be an effective method for the surface sterilization of woody explants.

It is difficult to suggest a solvent which gives the best results in terms of the surface sterilization of woody tissue explants. Several factors need to be investigated

in such a selection. Within these experiments, it was apparent that there existed a difference between peach and pecan explants in their tolerance of DBM and ACE. Treatment of peach shoot tips with DBM and ACE resulted in similar or better control of contamination, necrosis and survival ratings when compared with the 4.5 hour control. Treatment of pecan shoot tips with these solvents resulted in similar or better control of contamination but lower survival ratings in a comparison with the control.

For the peach explants, the solvents which produced the best results were DMF, ACE and ETOH with improved control of contamination and similar necrosis and survival ratings when compared with the control. The best response of pecan explants was to DMSO and ETOH in combination with benomyl which produced comparable contamination control and necrosis but better survival than the control. The differences in responses between peach and pecan explants to a solvent suggests that effective surface sterilization methods using organic solvents may need to be established for each species.

It is also possible that the solvent or time interval may need to be changed in accordance with the time of year or physiological state of the tissue. Contamination of cultures was high for the control treatment as well as for other treatments of pecan shoots in April. All solvents were better or similar to the control in % contamination. Separation of solvents should be based on toxicity to the

explant in such cases.

Under the conditions of these experiments, DCM and DCE are probably not suitable for use in the surface sterilization of shoot apices because of an apparent phytotoxicity to explants. DBM and ACE could possibly be suitable as solvents for use in the surface sterilization of explants from woody plants. Of all the solvents tested, DMSO, DMF and ETOH appear to be the most promising because of their low toxicity to the tissue as well as their effectiveness in the control of contamination.

From these experiments, it appears that soaks of a relatively short duration in a solution of 1% weight to volume benomyl to organic solvent can be effective as a surface sterilization procedure for woody explants. The relatively short time intervals which can be used can serve as a time-saving alternative to the 4.5 hour saturated, aqueous benomyl soak. With further research, it is hoped that more definitive combinations of time and solvent can be established and proven useful in the commercial tissue culture industry.

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