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Development of Embryo Rescue and Shoot Regeneration Techniques in *Ilex*[1]

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Abstract

Studies were conducted on embryo rescue and shoot regeneration from juvenile leaves to develop a system to improve *Ilex* using in vitro techniques. Unlike previous reports for *Ilex*, embryo germination and growth in the eight species tested was not consistently affected by light. Gibberellic acid had little effect on excised embryo germination in August through October; however, embryo germination was completely inhibited by 3 µM GA in November and December.

Shoot regeneration was obtained from juvenile leaves of *Ilex myrtifolia* and *I. opaca*, the two species tested. Thidiazuron, at concentrations from 5 to 50 µM, consistently resulted in the largest percentage shoot regeneration, when compared to benzyl adenine or a lower rate of thidiazuron. Indole butyric acid or pretreatment of source shoots with cytokinin or auxin did not increase regeneration percentages. Colchicine treatment of source shoots had an effect on regeneration; 100 µM was slightly stimulatory while 5 µM was significantly inhibitory. This protocol has resulted in regeneration rates that appear suitable for production of polyploid or transformed plants.

Index words: holly, organogenesis, gibberellin, paclobutrazol, light, thidiazuron.

Species used in this study: *Ilex myrtifolia*; Winterberry (*I. verticillata* (L.) A. Gray); English holly (*I. aquifolium* L.); *I.* x *koehneana*; Yaupon holly (*I. vomitoria* Ait.); American holly (*I. opaca* Ait.); Japanese Winterberry (*I. serrata* Thunb.); and *I. pernyi* x *latifolia.*

Chemicals used in this study: Gibberellin A_3, A_4 and A_7; Paclobutrazol (Bonzi) ß-((4-chlorophenyl)methyl)α-(1,1,-dimethylethyl)-1*H*-triazole-1-ethanol as a 50 WP from Sandoz Crop Protection; Thidiazuron (Dropp), N-phenyl-N'-1,2,3-thiadiazol-5-ylurea) as a 99.6% technical grade sample from E.I. Lilly.

Significance to the Nursery Industry

Two techniques were developed to allow genetic improvement of hollies using both standard sexual hybridization and the tissue culture techniques of genetic engineering and mutagenesis. First, to reduce the time needed for holly seed germination, studies were conducted on embryos rescued from maturing seeds. Unlike previous studies, dark was not required for embryo growth. Gibberellic acid, normally used to promote embryo germination, completely inhibited holly embryo growth in November and December. Further study is required to determine if this GA effect is linked to the inhibition of embryo growth that results in prolonged seed dormancy in the field.

Second, an adventitious shoot regeneration technique was developed using juvenile in vitro-grown leaves. This technique allows researchers to recover novel and usable plants from cells that were intentionally altered. This clears a major obstacle for production of polyploid or mutant hollies or those with improved traits developed through genetic engineering.

Introduction

Until recently, tissue culture manipulation of *Ilex* (Aquifoliceae) was limited to excised embryo culture (10), which aids in reducing the period required for seed germination. In the past few years; propagation by shoot tip culture has been reported for *I. paraguariensis* St. Hillaire (15) and *I. vomitoria* Ait. 'Schillings Dwarf' (1). Woody Plant Medium with 4.4 µM benzyl adenine (BA), 0.05 (*I. myrtifolia* Walter) or 0.5 µM (*I. opaca* Aiton) indole butyric acid (IBA) and 0.22 µM adenine sulfate caused satisfactory prolifera-

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tion of adult *Ilex* shoots (12), This medium was similar to that used for *I. vomitoria* (1).

To date, however, information is lacking on the shoot regeneration techniques necessary for production of transgenic or mutagenized *Ilex*. Although initial regeneration experiments often utilize cotyledons as source tissue because of their enhanced ability to regenerate shoots (2, 7), *Ilex* embryos are very small (< 1 mm) and initial experiments were performed with juvenile shoots. *Ilex* sp. readily germinate in vitro in growth regulator free medium; however, light was found to strongly inhibit embryo germination and growth (6, 10). Therefore, experiments were designed to investigate the effects of plant growth regulators, mineral salts, environmental conditions and other factors on embryo development of several *Ilex* species and the effect of plant growth regulators on adventitious shoot regeneration from leaves of these proliferated juvenile shoots.

Material and Methods

Embryo rescue. Fruit was collected from specimens growing in the holly collection at the United States National Arboretum (Washington, DC). Seeds were removed from the pulp by rubbing on a < 60 mesh screen (1991–2) or in a blender (1992). Separated seeds were stored at 4C (39F) in the dark for less than 2 weeks before embryo excision in 1991–2 and less than a day in 1992. Seeds were subsequently surface disinfested for 15 min with 0.52% sodium hypochlorite with several drops of Tween 20 per liter. After three sterile water rinses, the embryos were excised under 8–10 × magnification in laboratory light (1991–2) or under dim green (Rosco Lux #90 filtered) safelight (1992). This involved scalpel cuts through the endocarp and seed coats to the endosperm, which occupies approximately 90% of the seed. The end of the scalpel blade was then used to pry the embryo out of the endosperm. Usually a very small amount of the endosperm was brought with the embryo; because of this, a 1 cm^3 area of the medium became cloudy for a week. Both embryo germination and weight were measured at 35 days. Embryo germination was defined as the greening and visible expansion of the cotyledons and elongation of the radical.

Media was purified agar solidified (A–7921 Sigma Co., St. Louis, MO) with one of two basal salt and vitamin formulations, Woody Plant Medium (WPM) (11) or a modified WPM, i.e., potassium reduced by eliminating K_2SO_4 from the medium and increasing KH_2PO_4 by only 0.6 mM. Calcium content was also increased by adding an additional 1 mM $Ca(NO_3)_2$ and 0.7 mM $CaCl_2$. Although these changes were made to reflect *Ilex* leaf mineral content (4); no differences were found in initial tests of this medium and later experiments consistently used WPM. Unless otherwise noted, supplements included in all media were 0.55 mM myo-inositol, 90 mM sucrose and 0.22 mM adenine sulfate. Malt extract at 100 mg/liter was tested in one initial embryo experiment, and since it had no effect, it was not used afterward. Media were adjusted to pH 5.4 with KOH and autoclaved at 121C (251F) at 15 psi for 15 minutes.

Cultures were kept at 23C (73F) in continuous light provided by cool white fluorescent lights at 50 μmol s^{-1} m^{-2}. Dark treatments were wrapped in aluminum foil and placed on the same shelves but shielded from light with black cloth.

Two embryo development experiments were conducted. The first study was designed to investigate the effects of light and basal media on germination of several holly species. From September 4, 1991, to March 4, 1992, fruit from eight species was harvested at 30-day intervals. Embryos were excised and placed on either modified or standard Woody Plant Medium with no added plant growth regulators. The cultures were then placed randomly in the light or dark. Five seeds were used for each treatment, each of the 8 months were used as replications. Thus 1280 seeds were cultured in this 8 species × 2 light treatments × 2 media × 8 monthly replications × 5 seeds per replication, 3-way randomized complete factorial.

A second embryo rescue experiment was conducted to test the effect of plant growth regulators on germination and to repeat the previous year test of the effect of light on seeds from three *Ilex* sp. Fruit was harvested five times at 28-day intervals from August 16 to December 6, 1992, and seeds were extracted. Embryos were excised and placed on WPM as above with either: no added plant growth regulator (control), 3 μM gibberellic acids $A_{3,4\&7}$ (GA) or 3 μM paclobutrazol (PAC), a GA and sterol biosynthesis inhibitor (3). Seven seeds per treatment were either placed in the light or dark as described above. Thus 630 seeds were used in this 3 plant growth regulator × 5 months × 2 light treatment × 3 species × 7 seeds per replicate, 3-way randomized complete factorial experiment. Arcsin transformation was used on analysis of all percent germination data; but non-transformed means are presented.

Shoot regeneration. Shoots from the above seed germination studies were placed on WPM (11) with B5 vitamins (8), 90 mM sucrose and solidified with 0.7% (w/v) Sigma purified agar at pH 5.4. Benzyl adenine at 5 to 20 μM and IBA at 0.5 μM were added for several subcultures to induce shoot proliferation. Leaves from these proliferating juvenile shoot cultures of *I. myrtifolia* and *I. opaca* (half the number of leaves as *I. myrtifolia*) were used for all experiments. In both experiments, only the apical most 2 or 3 leaves including half of the petiole were excised. Leaves were placed upside down on medium that had been poured into 100 mm diameter plastic petri dishes. Plates were placed in the dark for 7 days and returned to the same environmental conditions used for light grown seeds. Regeneration was noted as either buds with microscopically (10×) visible leaf primordia or growing shoots.

Regeneration experiment one was a 640 leaf (for *I. myrtifolia*) randomized complete factorial design with 4 pretreatments × 5 cytokinin treatments × 2 IBA treatments × 16 leaves per cell. This experiment was repeated. Proliferating shoots were placed in one of four pre-leaf excision (pretreatment) media: no plant growth regulators, 2 μM thidiazuron (TDZ), 2 μM indole acetic acid (IAA)(filter sterilized) or a combination of the same concentrations of TDZ and IAA. After three weeks, leaves were excised and placed on organogenesis medium containing various concentrations of cytokinins (0, 0.5, 5 and 50 μM TDZ and 5 or 50 μM BA) and IBA (0 or 1 μM).

Regeneration experiment 2 was designed as a randomized complete factorial design in which the treatments were given in three of four consecutive cultures; first shoots were cultured for 3 weeks on 0.5 μM TDZ or 5 μM BA. Second, shoots were transferred into one of three concentrations of colchicine (0, 100 or 5000 μM) in 100 rpm swirled liquid WPM culture devoid of plant growth regulators for 20 hours.

Shoots from these treatments were all cultured for 3 weeks on agar solidified WPM containing 2 μM TDZ and 0.2 μM IBA, i.e., no treatments were applied in this subculture. Finally, leaves from shoots experiencing these six treatment combinations were placed on medium containing 5 μM TDZ and either 0, 0.1, 1 or 10 μM IBA. This experiment was repeated, with 360 *I. myrtifolia* leaves used in the first replication (15 leaves per cell); 240 leaves in the second (10 leaves per cell).

Results and Discussion

Seed germination—1991–2 experiment. ANOVA of percentage germination of *Ilex* embryos indicated two primary effects; light (P = 1%) and species (p = 1%), and their interaction (p = 5%) were significantly different. Light was inhibitory to germination of the 6 evergreen species investigated; but, this effect was not significant for the two deciduous species investigated, *I. verticillata* L. Gray and *I. serrata* Thunberg ex. J.A. Murray (Table 1). The medium effect and other interactions were not significant.

Seed germination—1992 experiment. Contrary to the previous year, light had a positive effect on the germination of *I. opaca* and no effect on *I. pernyi* Franchet x *latifolia* Thunberg ex. J.A. Murray (Table 1). Germination of *I. serrata* seedlings was not affected by light in either year.

Seeds from several *Ilex* species produced viable embryos that could be germinated in the light or dark, contrary to previous reports that indicated light was totally inhibitory (6, 10). The reasons for these differences in results, or the year to year variability in our results are unknown and few plausible explanations exist. The effects of medium salts were not significant in our experiments, and one treatment was almost identical to that used previously. Since myo-inositol and adenine were added in our experiments but were not listed in previous attempts, the effect of these components on the sensitivity of *Ilex* to light during germination remains unknown; however, these compounds generally do not have growth regulating properties. The addition of malt extract had no effect on germination. Embryos were stored at 4C (39F) for up to 2 weeks in our 1991–2 experiment; however, only some embryos were stored for one day at 4C (39F) in the 1992 experiment. The rest used were not chilled. As

Table 1. Percentage germination of *Ilex* embryos in light and dark on medium devoid of plant growth regulators for two dormant seasons, September 1991 through March 1992 and August 1992 through December 1992.

		Percentage germination[z]	
Species	Year	Light	Dark
I. myrtifolia	1991–2	48	84
I. verticillata	1991–2	80	95
I. aquifolium	1991–2	53	82
I. x *koehneana*	1991–2	62	96
I. vomitoria	1991–2	54	80
I. opaca	1991–2	16	45
	1992	78	51
I. serrata	1991–2	83	86
	1992	78	79
I. pernyi x *I. latifolia*	1991–2	30	73
	1992	76	77

[z]Least Significant Difference p = 5% was 22%.

Table 2. Percentage germination of three *Ilex* species embryos harvested from August to December 1992, excised and placed on medium with no plant growth regulators (control), with paclobutrazol or with a mixture of GAs.

	Percentage germination[z]		
Month and species	Control	Gibberellins	Paclobutrazol
August			
I. opaca	68	80	0
I. serrata	73	76	2
I. pernyi x *I. latifolia*	56	94	4
September			
I. opaca	90	74	0
I. serrata	64	74	6
I. pernyi x *I. latifolia*	80	84	0
October			
I. opaca	52	40	0
I. serrata	80	80	24
I. pernyi x *I. latifolia*	78	62	18
November			
I. opaca	60	0	0
I. serrata	78	0	24
I. pernyi x *I. latifolia*	80	0	4
December			
I. opaca	48	0	0
I. serrata	80	0	20
I. pernyi x *I. latifolia*	84	0	36

[z]Least Significant Differences at p = 5%: Aug = 17; Sept = 19; Oct = 19; Nov = 26; Dec = 28.

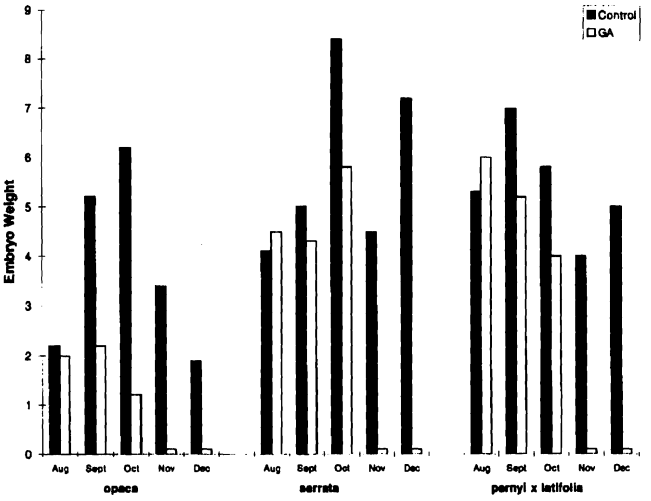

Fig 1. Weight (in mg) of *Ilex* embryos grown on medium devoid of plant growth regulators (control) or containing a mixture of gibberellic acids. Embryos were from fruit harvested August 1992 through December 1992 and cultured in the dark or under continuous light. All PAC treatments embryos were less than 2 mg. Least significant difference (p = 5%) was 2.5 mg.

chilling naturally occurs after early October at the fruit collection site, it is unlikely that less than 2 weeks of artificial chilling was responsible for the yearly variation in light germinated seeds.

The effect of plant growth regulators on embryo germination and growth and its interaction with month of sampling were highly significant (P = 1%) (Table 2, Fig. 1). Control (no plant growth regulators added to the medium) treated embryos germinated throughout the period of sampling; however, both GA and PAC had significant effects on germination and embryo weight. GA had little consistent effect on *I. serrata* and *I. pernyi* x *latifolia* germination in August through October. In *I. opaca*, GA reduced embryo weight in September and October but did not prevent germination. In November and December, GA totally inhibited germination in both light and dark grown seeds of all species. PAC generally inhibited seed germination throughout the experiment, regardless of light treatment or species. The inhibition was strongest in the earlier months. In a single month repeat of the experiment with *I. myrtifolia* in January 1995, 72% of the control embryos germinated. At 1 μM GA_3, 37% germinated; at 10 μM, 8% germinated. In an extra set of replicates, abscisic acid (0.3 μM) had no effect on light and dark grown seeds in December, 1993 (data not shown).

The physiological basis for GA inhibition of germination is unknown. Since the endosperm is thought to act as a potent inhibitor of seed germination in *Ilex*, and endosperm produces GA in other woody species (9), it would be reasonable to speculate that endosperm-derived GA may play a critical role in arresting embryo development and delaying germination through the winter. In annual species, GA content of embryos decreases significantly during embryo maturation (5, 13, 16) and the addition of GA in somatic embryogenesis medium inhibits embryo development (14).

Paclobutrazol does not improve germination during the period of GA sensitivity, contrary to what would be expected if it were to act on GA biosynthesis alone. Since paclobutrazol also inhibits germination when embryos are not sensitive to GA, paclobutrazol evidently exerts effects not associated with this physiological response.

Ilex embryo development at the time of the onset of sensitivity to added GA ranged from heart in *I. opaca* (0.5 mm length) and *I. pernyi x latifolia* (0.6 mm length) to torpedo stages (1 mm length) in *I. serrata*. Therefore in *Ilex*, GA sensitivity seems to be more related to an environmental stimulus than to a developmental stage or embryo size.

Table 3. Effect of TDZ, BA and IBA in the regeneration media on the percentage adventitious shoot regeneration from *Ilex myrtifolia* leaves. Percentages are averaged over the four pretreatments for each of the two replicate experiments.

Cytokinin and IBA concentration	0 μM IBA		1 μM IBA		
	Rep. 1	Rep. 2	Rep. 1	Rep. 2	DMRt
none	0	0	0	0	a[z]
0.5 μM TDZ	15	8	35	20	ab
5 μM TDZ	34	21	40	19	b
50 μM TDZ	44	11	43	25	b
5 μM BA	nd	0	nd	6	
50 μM BA	0	nd	0	nd	

[z]Letters in the right hand column indicate significant differences between TDZ and control treatments as determined by the Duncan's Multiple Range test (p = 5%); nd = not determined.

Table 4. Percentage shoot regeneration for *I. myrtifolia* leaves taken from shoots pretreated with various levels of colchicine and cultured on proliferation medium for 3 weeks. Leaves were then placed on medium containing 5 μM TDZ and various levels of IBA as indicated below.

Colchicine in μM	Percentage regeneration		
	Replicate 1	Replicate 2	DMRt
0	34	39	ab[z]
100	47	56	b
5000	12	19	a

Indolebutyric acid in μM	Percentage regeneration		
	Replicate 1	Replicate 2	DMRt
0	25	37	a
0.01	29	29	a
0.1	30	25	a
1	41	25	a

[z]Letters in the right hand column indicate significant differences between colchicine or IBA and control treatments as determined by the Duncan's Multiple Range test (DMRt) (p = 5%).

Shoot regeneration—Experiment 1. For both *I. myrtifolia* and *I. opaca*, percentage regeneration was significantly (p = 5%) affected only by the concentration of cytokinin used. For *I. myrtifolia* (Table 3), cytokinin was necessary for regeneration and thidiazuron was more effective than benzyl adenine (although BA data were not included in the analysis). Pretreatment of source shoots and IBA in the regeneration medium treatment means varied by less than 7% when averaged over the two experiment replications and were not significant. Over both experiment replications and IBA levels, *I. opaca* regeneration was: 2% on no cytokinin; 9% on 0.5 μM TDZ; 25% on 5 μM TDZ and 43% on 50 μM TDZ. Tens of shoots were obtained from each regenerating leaf of both species.

Shoot regeneration—Experiment 2. Of the pretreatments and regeneration medium treatments attempted on *I. myrtifolia*, only colchicine had a significant inhibition (p = 5%) on regeneration percentage (Table 4). Auxin concentration during regeneration and cytokinin pretreatments of source shoots with BA (31%) vs. TDZ (21%) in the subculture before application of colchicine had no effect on regeneration rates, nor was any interaction obtained. *I. opaca* regeneration were unaffected by auxin concentrations (average regeneration = 7%). Similar to *I. myrtifolia*, *I. opaca* regeneration was inhibited by pretreatment with 5000 μM colchicine (8% for no colchicine; 11% for 100 μM colchicine and 0% for 5000 μM colchicine).

Juvenile shoots were used for regeneration experiments as they were easier to culture and presumably were more organogenic (2, 7). These shoots were easily proliferated on medium containing moderate amounts (i.e., 5 to 10 μM) of BA. In contrast, TDZ promoted more adventitious regeneration than BA, as in other woody species (7, 17, 18). Although cytokinin pretreatments did not increase regenera-

tion percentages as in some woody plants (17), it should be noted that these seedlings were actively growing. Later experiences with non-growing shoots on a plant growth regulator free medium indicated proliferating shoots are preferred sources of leaves for regeneration (data not shown).

The highest regeneration rates for *I. myrtifolia* and *I. opaca*, obtained with 100 µM pretreatment of shoots with colchicine and the use of 5 µM thidiazuron to induce organogenesis was 56%. Although initial shoot size and number made it impossible to count, regenerating *Ilex* produce several tens of usable shoots from each regenerating leaf. These numbers of shoots per leaf are greater than those obtained for other woody species, where transformation and mutagenesis have been successful (18). Initial screening of adventitious regenerated shoots of *I. myrtifolia* treated with 5 µM colchicine indicates this technique can be used to produce polyploid plants.

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