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Development of an In Ovolo Embryo Culture Procedure for *Hydrangea*[1]

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Abstract

Panicle hydrangea (*Hydrangea paniculata*) is a potential source of cold hardiness that is needed for improvement of bigleaf hydrangea (*H. macrophylla*). Seeds have been produced from *H. macrophylla* x *H. paniculata* crosses, but seedlings derived from the few seed that germinated died at a very young age. The objective of this study was to investigate the use of in ovolo embryo culture for producing this interspecific hybrid. Six media, which differed in basal media formulation and sucrose concentration, were tested using *H. macrophylla* intraspecific ovules collected 3 to 4 and 5 to 6 weeks after pollination. The highest percent germination for both time periods was obtained on Gamborg's B-5 medium supplemented with 2% sucrose; therefore, this medium was chosen for culture of *H. macrophylla* x *H. paniculata* ovules. Plants germinated from interspecific ovules cultured 3 to 6 weeks after pollination, but all died before forming two sets of true leaves. A slow-growing bacterial contamination that was present in many of the interspecific cultures necessitated the elimination of cultures initiated 7 to 8 weeks after pollination, and may have been at least partially responsible for the death of the putative hybrids. A broad-spectrum biocide/fungicide was found to be 100% effective in controlling this endogenous contaminant, thereby allowing older ovules to be cultured. Putative *H. macrophylla* x *H. paniculata* hybrids have been obtained from ovules cultured 9 to 10 weeks after pollination.

Index words: interspecific hybridization, embryo rescue, in ovulo embryo culture, ovule culture, breeding, hydrangea.

Species used in this study: bigleaf hydrangea (*H. macrophylla* (Thunb. ex J.A. Murr.) Ser.); panicle hydrangea (*H. paniculata* Sieb.).

Significance to the Nursery Industry

Hybridization between bigleaf (*H. macrophylla*) and panicle (*H. paniculata*) hydrangea is being pursued for the purpose of producing a cold-hardy hydrangea with brightly colored flowers. Crosses between the two species have revealed that seed is produced if *H. macrophylla* is used as the maternal parent, but seedlings from the few seed that germinate die at a very early age. In vitro embryo rescue procedures have been successful in a number of genera for recovering interspecific hybrids that otherwise die due to embryo weakness or abortion. The purpose of this study was to evaluate the use of an embryo rescue procedure for recovering *H. macrophylla* x *H. paniculata* hybrids. Putative interspecific hybrids were obtained from *H. macrophylla* x *H. paniculata* ovules grown on a tissue culture media. If hybridity of these plants is verified, they will be used to produce a new type of hydrangea that combines cold hardiness with brightly colored flowers. Such a plant would have a high visual impact in landscapes, public and private gardens, and garden centers, and would benefit growers, landscape professionals and home gardeners.

Introduction

A lack of cold-hardiness, especially in floral buds, limits the growing range of bigleaf hydrangea (*H. macrophylla*). The inflorescences, which are set on the previous year's growth, are often damaged by unusually cold winters and late spring freezes. While rated as hardy to USDA hardiness zone 6, cold damage frequently results in diminished flowering in *H. macrophylla* in locations as warm as USDA zone 7 (2).

Development of an interspecific hybrid between *H. macrophylla* and *H. paniculata*, or panicle hydrangea, may serve as a means to combine cold-hardiness with floral color. While *H. paniculata* sets flower buds on new wood and is cold-hardy to zone 3, its white to pale-pink flowers lack the visual impact of the bright blue to pink inflorescences of *H. macrophylla*. Numerous reciprocal crosses between the two species have been made (6). Seed were produced only when *H. macrophylla* was used as the maternal parent; most of these seed did not germinate. The few seedlings that were obtained died either at the cotyledonary stage or while the first true leaves were expanding.

In vitro embryo rescue procedures have been used to facilitate the recovery of interspecific hybrids of many genera (1, 7), and have recently been used to recover a putative *H. macrophylla* x *H. arborescens* hybrid (4). Hybrid embryos often resume growth and develop into normal plants when removed from ovules and placed on an aseptic nutrient media. In some cases entire ovules have been placed into culture and hybrid plants recovered; this procedure is known as in ovolo embryo culture.

The objective of this study was to develop an embryo rescue procedure for the recovery of *H. macrophylla* x *H. paniculata* hybrids. Since the small size of *Hydrangea* seeds makes excision of embryos impractical, entire ovules were placed into culture. To ensure that sufficient viable embryos would be present to adequately evaluate media formations at different stages of embryo maturity, intraspecific crosses were used for the initial study. Information obtained from the intraspecific cultures was subsequently applied to interspecific ovules.

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Materials and Methods

Plant materials. The following cultivars were used in one or more of the experiments of this study: *H. macrophylla* f. *macrophylla* 'All Summer Beauty', 'Horben', Marechal Foch', 'Masja', 'Pia'; *H. macrophylla* f. *normalis* 'Blaumeise', 'Bluebird', 'Fasan', 'Kardinal', 'Taube'; *H. macrophylla* subsp. *serrata* 'Blue Billow', 'Tokyo Delight'; and *H. paniculata* 'Brussels Lace', 'Pink Diamond', 'Tardiva', and 'Unique'. Plants were grown in #7 or #15 containers outside under 60% shade. Just prior to making crosses, plants were brought into a greenhouse covered with 65% shadecloth. Plants remained in the greenhouse until all ovules were collected for culture.

Controlled pollinations. Sterile flowers were removed from inflorescences to be used as females prior to opening of the fertile flowers. Any open flowers, along with all extremely immature fertile flowers, were removed. After the petals and anthers of all remaining fertile flowers were removed using a pair of fine-tipped forceps, the inflorescence was securely covered with a breathable plastic bag.

Inflorescences to be used as males in crosses were also bagged prior to flower opening. A single pollination of each flower was made 1 to 4 days after emasculation. For pollinations, flowers with newly dehisced pollen were removed and used immediately. The bag was removed from the inflorescence of the maternal parent, and the dehiscing anthers touched directly to the exposed stigmas of the emasculated flowers. The bag was placed back over the inflorescence, where it remained for another two to three weeks.

Intraspecific experiments. The purpose of these experiments was to develop an in ovolo culture procedure and identify a medium that could be used for supporting the growth of immature *Hydrangea* ovules. Two experiments were conducted to determine the effectiveness of six media on germination of *Hydrangea* intraspecific ovules. Each experiment used the same explant sources and media, but differed in age of ovules cultured.

Eight sets of controlled pollinations were made between *H. macrophylla* cultivars during summer 1998. All of the crosses of each set were made on the same day. The following genotypes made up the eight sets of crosses: 'All Summer Beauty' x 'Bluebird'; 'Blaumeise' x 'Bluebird'; 'Bluebird' x 'Tokyo Delight'; 'Horben' x 'Blue Billow'; 'Marechal Foch' x 'All Summer Beauty'; 'Masja' x 'Bluebird'; 'Pia' x 'Blue Billow'; and, 'Pia' x 'Bluebird'. At least 50 crosses of each genotype were made to ensure that sufficient ovaries would be available for establishing the cultures of the intraspecific experiments.

The first experiment was designed as a randomized complete block, with eight genotypes, six media, and two replications. Twelve ovaries of each genotype were collected 3 to 4 weeks after pollination. Ovaries were surfaced disinfected for 1 minute in 70% ethanol, followed by 10 minutes in 1% sodium hypochlorite, and then rinsed twice in sterile distilled water. Ovaries were sliced open longitudinally under a stereomicroscope in a laminar flow hood. Using a scalpel and fine-tipped forceps, as many ovules as possible were removed from the ovary and placed onto the surface of a plate of medium. All cultured ovules from an individual ovary were placed on the same plate of media, which served as the experimental unit of the experiment. Each genotype was used to establish cultures on two plates of each of six media. Cultures were placed in a 25C (77F) incubator. To reduce the possibility of precocious germination, the cultures were kept in the dark for the first 2 weeks of culture, and then given a 16-hour light, 8-hour dark photoperiod. The experiment was repeated with ovules that were collected 5 to 6 weeks after pollination.

Six media formations, consisting of two basal media in combination with three concentrations of sucrose, were tested in both experiments. Basal media were Murashige and Skoog's (MS; 5) and Gamborg's B-5 (3). Sucrose concentrations tested were 2%, 8%, and 12%. Media were solidified with 0.9% agar and poured into 15 × 100 mm petri dishes.

Cultures were examined approximately every 2 weeks for the presence of plantlets. Number of germinated ovules, along with total number of ovules cultured, was recorded for each culture 13 weeks after pollination. All contaminated ovules were eliminated from the study, unless the contaminant did not interfere with germination. Data were converted to percent germination, and arcsin transformed when necessary to correct for variance heterogeneity.

Interspecific experiment #1. The objective of this experiment was to evaluate the germination of ovules obtained from *H. macrophylla* x *H. paniculata* crosses and cultured at different stages of development on the medium that produced the highest germination rate in intraspecific ovules. Interspecific crosses were made during summer 1998 and 1999, using the same procedure as the intraspecific crosses. *H. macrophylla* was used as the maternal parent in all crosses. Crosses were grouped into sets, with each set consisting of crosses that were made between the same parental cultivars on the same day. Two weeks after pollination, a count was made of number of ovaries that remained on the plants. Half of the ovaries of each set were collected for culture 3 to 4 weeks after pollination; the remaining ovaries were collected 5 to 6 weeks after pollination. The *H. macrophylla* x *H. paniculata* genotypes, along with the number of ovaries collected at each culture date, were: 'Pia' x 'Unique', 6; 'Pia' x 'Brussels Lace', 6; 'Fasan' x 'Pink Diamond', 4; 'Taube' x 'Brussels Lace', 24; and, 'Blaumeise' x 'Brussels Lace', 24. Ovules were cultured and data collected as described for the intraspecific hybridizations with the exception that only one medium, B-5 with 2% sucrose, was utilized. As many ovules as possible were cultured from the ovaries collected 3 to 4 weeks after pollination. For the ovules cultured 5 to 6 weeks after pollination, in which two types of ovules could be distinguished, all of the large opaque white ovules were placed into culture. Because it was often difficult to separate the small translucent ovules from the large opaque ovules, a few of the smaller ovules were also included in the cultures initiated 5 to 6 weeks after pollination. These were not included in the analysis of the number of ovules cultured.

Interspecific experiment #2. The objective of this experiment was to evaluate the use of a broad-spectrum biocide/fungicide for reducing or eliminating endogenous contamination in *Hydrangea* in ovolo embryo cultures. The experiment utilized the following genotypes: 'Blaumeise' x 'Pink Diamond'; 'Kardinal' x 'Brussels Lace'; 'Kardinal' x 'Pink Diamond'; 'Kardinal' x 'Unique'; and, 'Pia' x 'Tardiva'. These crosses were made during summer 1999, using the procedure described for the intraspecific crosses.

Table 1. Mean squares (MS) from analyses of variance for effects of media and genotype on percent germination of *H. macrophylla* intraspecific ovules cultured 3 to 4 and 5 to 6 weeks after pollination.

Source	df	MS	F	P
3 to 4 weeks after pollination:				
media	5	322	2.9	0.02
genotype	7	172	1.6	0.17
media × genotype	35	74	0.7	0.89
error	48	110		
5 to 6 weeks after pollination:				
media	5	5970	36.2	<0.01
genotype	7	1540	9.3	<0.01
media × genotype	35	227	1.4	0.15
error	48	165		

Ovaries were collected 62 to 70 days after pollination, surface disinfected and sliced open in a laminar flow hood. All large milky white ovules were excised and placed onto media. Twenty-five ovules from multiple ovaries of each genotype were placed onto each petri dish of media. Gamborg's B-5 medium with 2% sucrose was supplemented with 0, 0.05, and 0.1 % (v:v) Plant Preservation Medium (PPM™; Plant Cell Technology, Inc., Washington, DC). The experiment was replicated four times. Cultures were incubated as described for the intraspecific experiment. Cultures were scored for the presence of contaminated ovules six weeks after they were initiated. Number of germinated ovules in each petri dish was counted at the same time.

Statistical analyses. Data were analyzed using the general linear model of SigmaStat statistical software, version 2.03 (SPSS Inc., Chicago, IL). When significant (P = 0.05) differences among treatments were observed, treatment means were separated by Fisher's LSD test.

Results and Discussion

Intraspecific experiments. Neither genotype nor media × genotype interaction had a significant effect on germination of *H. macrophylla* ovules cultured 3 to 4 weeks after pollination; however, there was a significant media effect (Table 1). B-5 medium with 2% sucrose was the most productive of the media tested, with 13% of the ovules cultured on this medium germinating (Table 2). There were no significant differences in germination among the other five media. Plants were first observed emerging from the cultured ovules 6 weeks after being placed into culture, which corresponds to 9 to 10 weeks after pollination. Plants were produced from all of the genotypes.

Hydrangea ovaries contain numerous small ovules. At 3 to 4 weeks after pollination, ovules were difficult to separate from surrounding maternal tissue and from each other. Identification of an embryo in these ovules was not possible using a stereomicroscope. A preliminary experiment indicated that excision of *H. macrophylla* ovules from ovaries sooner than 3 weeks after pollination was extremely difficult, and that the cultural conditions tested in this study were insufficient to allow development of ovules younger than 3 weeks of age.

Highly significant differences among media were observed for cultures initiated 5 to 6 weeks after pollination (Table 1). As with ovules cultured 3 to 4 weeks after pollination, germination was greatest among ovules cultured on B-5 medium with 2% sucrose (Table 2). Over one-third of the ovules cultured on this medium germinated. Germination was higher on media that contained 2% sucrose than on media with 8% or 12% sucrose. A few plants were observed in these cultures when they were removed from dark to light conditions, which was 2 weeks after the cultures were initiated, or 7 to 8 weeks after pollination.

Highly significant differences among genotypes were also present for percent germination of ovules cultured 5 to 6 weeks after pollination. The two genotypes that utilized 'Pia' as the maternal parent had significantly higher germination rates than did the other genotypes, with the exception of 'Bluebird' x 'Tokyo Delight' (Table 3). The genotypes responded similarly to the media, as evidenced by the lack of a significant media × genotype interaction.

By 5 to 6 weeks after pollination, ovules were larger and easier to excise from ovaries than those cultured 2 weeks earlier. Most of the ovules were an opaque milky white color, but a few translucent ovules were also observed. The translucent ovules were considerably smaller than the opaque white ovules. A few ovaries that were not needed for the cultures were collected 5 to 6 weeks after pollination and the two types of ovules examined for the presence of an embryo. When a small hole was made in one end of large white ovules and pressure applied to the other end, the embryo could be extracted from these older ovules. This embryo was milky-white in color, and could be identified by its color and opaque appearance while still inside the ovule. There was no evidence of an embryo in any of the small translucent ovules. Although none of the small translucent ovules that were

Table 2. Effect of media on germination of *H. macrophylla* intraspecific ovules cultured 3 to 4 and 5 to 6 weeks after pollination.

	Weeks after pollination			
	3 to 4		5 to 6	
Media	Number ovules cultured	Germination (%)[z]	Number ovules cultured	Germination (%)
B-5, 2% sucrose	774	13.1a	699	34.8a
B-5, 8% sucrose	837	3.7b	728	11.1c
B-5, 12% sucrose	845	1.9b	73	3.6de
MS, 2% sucrose	791	2.4b	724	21.1b
MS, 8% sucrose	797	2.4b	720	7.6cd
MS, 12% sucrose	773	1.0b	692	1.8e

[z]Mean separation within columns using Fisher's LSD test (P = 0.05).

Table 3. Effect of genotype on germination of *H. macrophylla* intraspecific ovules cultured 5 to 6 weeks after pollination.

Genotype	Number of ovules cultured	Germination of cultured ovules (%)[z]
'All Summer Beauty' x 'Bluebird'	544	14.2bc
'Blaumeise' x 'Bluebird'	474	5.3d
'Bluebird' x 'Tokyo Delight'	700	19.5ab
'Horben' x 'Blue Billow'	315	9.3cd
'Marechal Foch' x 'All Summer Beauty'	563	6.7d
'Masja' x 'Bluebird'	265	7.5d
'Pia' x 'Blue Billow'	899	22.9a
'Pia' x 'Bluebird'	536	21.1a

[z]Mean separation within columns using Fisher's LSD test (P = 0.05).

placed into culture germinated, they are included in the counts of total numbers of ovules cultured presented in Table 2. It is likely that these small ovules represent either unfertilized ovules or ovules in which the embryo died at a very early stage of development.

Plants obtained from both intraspecific experiments were normal in appearance. Shortly after germination, root systems developed in the medium. Twenty-four plants from each of the intraspecific experiments were removed from culture two to three weeks after the cultures were scored for germination, and planted into potting soil in a greenhouse. All of these plants were obtained from petri dishes in which there was no contamination. All of these intraspecific plants survived and developed into normal-appearing mature plants. Most of the plants that were not removed from culture continued to grow until the medium was exhausted, eventually filling the medium with roots and often breaking the seal on the petri dish.

The only major contaminant that was observed in either of the intraspecific experiments was a slow-growing pink-colored bacterium that appeared to be associated with the surface of the ovules. Many cultures had one to a few ovules that were contaminated with this organism, but it did not spread to other ovules on the same petri dish. None of the ovules that were heavily covered with this organism germinated; however, seedlings emerged from ovules in which a lower level of contamination appeared to be present. These seedlings quickly became covered with the bacterium, and did not appear to grow as vigorously as those seedlings that were not contaminated. Experiments with ovules collected 7 to 8 weeks after pollination were originally planned as part of this study, but were eliminated because of excessive contamination. Methods for controlling endogenous contaminants in *Hydrangea* cultured ovules may be needed if this contamination problem persists and/or if there is a need to culture interspecific ovules later than 5 to 6 weeks after pollination.

Based on these results of the intraspecific experiments, B-5 medium with 2% sucrose was chosen for culture of *H. macrophylla* x *H. paniculata* ovules collected 3 to 6 weeks after pollination. This was the most productive of the six media tested for intraspecific ovules cultured 3 to 4 and 5 to 6 weeks after pollination. However, improvements in this media may be needed for interspecific ovules, especially if there is a need to rescue embryos at a very early stage of development. Young embryos generally require culture media with high sucrose levels (1, 7). At early stages of embryo development, the sucrose serves not only as a carbon source, but also as an osmotic stabilizer. For *H. macrophylla* ovules placed into culture 3 to 4 weeks after pollination, media with 8 and 12% sucrose were not superior to those with 2% sucrose. It is possible that these young ovules had an initial need for high levels of sucrose, but that high sucrose concentrations in the medium became detrimental to the embryo as it matured in culture. A two-stage media procedure, in which ovules are initially cultured on high levels of sucrose, but later transferred onto a medium with low sucrose levels, may increase germination of ovules cultured 3 to 4 weeks after pollination. Addition of natural extracts, such as coconut milk and casein hydrolysate, to the medium may also be beneficial to the rearing of young *Hydrangea* ovules. Manipulation of nitrate-to-ammonium levels in the medium may result in increased ovule germination, not only of very young ovules, but also of ovules cultured 5 to 6 weeks after pollination.

Interspecific experiment #1. Germination occurred in all five *H. macrophylla* x *H. paniculata* genotypes cultured both

Table 4. Germination of *H. macrophylla* x *H. paniculata* ovules cultured 3 to 4 weeks and 5 to 6 weeks after pollination.

Genotype (*H. macrophylla* x *H. paniculata*)	3 to 4 weeks after pollination				5 to 6 weeks after pollination			
	No. ovules cultured	Germination (%)		No. ovules germinated	No. ovules cultured	Germination (%)		No. ovules germinated
		Mean ± s.e.	Range			Mean ± s.e.	Range	
'Blaumeise' x 'Brussels Lace'	562	6.9 ± 1.6	0 – 33.3	28	270	11.5 ± 2.4	0 – 33.3	27
'Fasan' x 'Pink Diamond'	53	18.1 ± 4.8	9.1 – 30.8	9	19	16.1 ±11.8	0 – 50.0	2
'Pia' x 'Brussels Lace'	235	5.4 ± 1.4	0 – 10.0	14	129	6.6 ± 1.6	0 – 12.0	9
'Pia' x 'Unique'	239	4.3 ± 1.5	0 – 10.3	10	123	8.6 ± 2.4	0 – 16.0	12
'Taube' x 'Brussels Lace'	525	8.6 ± 1.3	0 – 22.7	43	262	15.1 ± 2.9	0 – 50.0	42

Table 5. Mean squares (MS) from analyses of variance of effects of biocide concentration and genotype on contamination and germination of *H. macrophylla* x *H. paniculata* ovules cultured 9 to 10 weeks after pollination.

		Contamination			Germination		
Source	df	MS	F	P	MS	F	P
biocide	2	4.267	76.80	<0.01	34.67	10.89	<0.01
genotype	4	0.0583	1.05	0.39	3.892	1.22	0.31
biocide × genotype	8	0.0583	1.05	0.41	2.367	0.74	0.65
error	45	0.0556			3.178		

3 to 4 and 5 to 6 weeks after pollination (Table 4). Mean germination for ovules cultured 3 to 4 weeks after pollination ranged from 4.3% in 'Pia' x 'Unique' to 18.1% in 'Fasan' x 'Pink Diamond'. For ovules cultured 5 to 6 weeks after pollination, germination ranged from 6.6% in 'Pia' x 'Brussels Lace' to 16.1% in 'Fasan' x 'Pink Diamond'.

Ovules collected from interspecific crosses 3 to 4 weeks after pollination were very small and difficult to excise from the ovary. It was not possible to determine if an embryo was present in any of the 3- to 4-week old interspecific ovules. Two types of ovules were observed in ovaries collected 5 to 6 weeks after pollination. One was larger, with an opaque milky-white color, while the other was smaller and translucent. As discussed earlier, both types of ovules were also observed in intraspecific ovaries, but the small translucent form occurred at a higher frequency in the interspecific ovaries. As with the intraspecific ovules, an embryo was observed inside the large white ovules. None of the small translucent ovules that were inadvertently included in the cultures germinated; as mentioned earlier, these were not included in the counts of total number of ovules cultured presented in Table 4. The ability to distinguish between these two types of ovules in ovaries collected 5 to 6 weeks after pollination, and to selectively culture primarily the large white ovules, resulted in lesser numbers of ovules being placed into culture 5 to 6 weeks after pollination than at 3 to 4 weeks after pollination. For this reason, a comparison of germination rates between the two time periods may not be valid.

A contamination problem was encountered in this experiment. Over 50% of the petri dishes contained one or more ovules that were contaminated with the pink bacterium observed in intraspecific cultures. Many of the cultures contained only one to a few contaminated ovules, and the contamination did not appear to move quickly throughout the medium. In addition, many of the lightly contaminated ovules germinated; therefore, cultures contaminated with the pink bacterium were discarded from the study only if they were heavily contaminated. Plants that germinated from contaminated ovules became covered with the bacterium. Plants germinated from non-contaminated ovules in petri dishes in which the bacterium was present were transferred to fresh media in an effort to prevent their contamination; however, in most cases the bacterium was soon observed on the fresh medium. These plants did not develop root systems and eventually died. A few plants from the contaminated cultures were transferred to soil and placed in a growth chamber. These plants were small and lacked roots; they either died immediately, or remained green for a few weeks and then died. It is not known what part, if any, the contaminant played in the death of the contaminated plants. While the bacterium was not lethal to contaminated plants in the intraspecific experiments, it seemed to negatively affect their vigor. The plants obtained from the interspecific ovules may have been much weaker than those from the intraspecific ovules, and thus more susceptible to damage from the contaminant.

Survival problems were also encountered among plants germinated from non-contaminated cultures. Many of the plants died shortly after germination. Others developed one to two sets of true leaves before either dying or forming callus tissue on stems and leaves. A few plants were removed from culture prior to callus formation, planted in soil, and placed in a growth chamber. Similarly to plants transferred to soil from contaminated cultures, these either died immediately or grew very slowly for several weeks and then turned brown and died. None of the plants obtained from any of these interspecific cultures developed more than two full sets of leaves before dying.

Table 6. Effect of Plant Preservation Media (PPM™) on contamination and germination of *H. macrophylla* x *H. paniculata* ovules cultured 9 to 10 weeks after pollination.

PPM concentation (%, v:v)	Contaminated cultures (%)[z]	Number plants germinated per 25 ovules cultured
0	80a	4.0a
0.05	0b	1.6b
0.10	0b	1.8b

[z]Mean separation within columns using Fisher's LSD test (P = 0.05)

Interspecific experiment #2. PPM™ is marketed as a broad-spectrum biocide/fungicide specifically for use in plant tissue cultures. Manufacturer's recommendations for concentration of this biocide needed in media for controlling endogenous contaminants range from 0.05 to 0.2% v:v. A preliminary experiment indicated that rates as low as 0.1% are effective in controlling contamination in *Hydrangea* cultured ovules; therefore 0.05 and 0.1% concentrations were evaluated for their effect on endogenous contamination and germination of in ovolo embryo cultures of *H. macrophylla* x *H. paniculata*.

There were highly significant differences among biocide concentrations for number of dishes of media in which contamination was present, but neither genotype nor biocide × genotype interaction was significant (Table 5). Eighty percent of the petri dishes containing media without the biocide were contaminated with a pink bacterium (Table 6). In contrast, no contamination was observed in any of the ovules cultured on media containing either 0.05 or 0.1% concentrations of the biocide.

Within the contaminated cultures, the number of contaminated ovules ranged from 1 to 21, with a mean of 8 contaminated ovules per contaminated culture. Plants obtained from non-contaminated ovules that were grown in petri dishes in which some of the other ovules were contaminated were transferred to fresh media. While there was no evidence of contamination on these plants while they were in the original dish of media, the pink bacterium quickly became noticeable on the new medium. Either the contaminant was spread from contaminated to non-contaminated ovules during the transfer or it was present, but not noticeable, before the transfer to fresh media. None of the contaminated plants have died, but appear less vigorous than those that are not contaminated. Plants were also transferred from biocide-containing media to fresh Gamborg's B-5 medium without the biocide. No contamination was observed on these plants; therefore, it appears that PPM™ may have been effective in eliminating, rather than just suppressing, the bacterial contaminant. Other antibiotics may also be effective in controlling endogenous contamination of *Hydrangea* cultured ovules, but have not been tested.

Biocide level had a significant effect on number of plants germinated from cultured ovules, but neither significant genotype nor biocide × genotype interaction effects were observed (Table 5). Over twice as many ovules germinated on media without PPM™ as on media that contained either 0.05 or 0.1% PPM™ (Table 6). Although the use of PPM™ resulted in a reduction in germination of the cultured ovules, the plants that germinated in the presence of PPM™ did not exhibit any obvious symptoms of phytotoxicity. Concentrations of PPM™ less than 0.05% should be tested for effect on contamination and germination. Use of PPM™-containing media for a short, initial culture period, followed by a transfer to PPM™-free media may also provide a means of controlling endogenous contamination without reducing germination of *Hydrangea* cultured ovules.

Even with the reduction of germination that was encountered in this study, PPM™ is a very valuable addition to the *Hydrangea* in ovolo embryo culture protocol. The availability of contaminant-free cultures will allow for a more efficient evaluation of culture media developed specifically for interspecific hybrids. It will also allow putative hybrids to be micro-grafted onto *H. macrophylla* scions in culture if necessary to ensure their survival. In addition, in ovolo embryo culture can now be tested for purposes other than recovery of interspecific hybrids. Self-pollinations of *H. macrophylla* have been found to produce primarily weak seedlings that die shortly after germination (unpublished data). Crosses between diploid and tetrapoid *H. macrophylla* cultivars have been observed to suffer from a similar fate due to endosperm inbalance (R. J. Griesbach, personal communication). It may be possible to rescue these weak seedlings through the use of in ovolo embryo culture.

The abnormalities that were common among plants germinated from *H. macrophylla* x *H. paniculata* ovules cultured 3 to 6 weeks after pollination were rare among the plants obtained from ovules cultured 9 to 10 weeks after pollination. This was true for plants obtained from both contaminated and non-contaminated cultures, and for those grown in the presence or absence of the biocide. Of the 148 plants germinated in this experiment, only five have developed callus tissue, and none have died in culture. It appears that embryos produced from *H. macrophylla* x *H. paniculata* crosses continue to develop in vivo for at least 9 to 10 weeks after pollination, and that the medium provided to the 3 to 6 week-old ovules in the first interspecific experiment was inferior to in vivo conditions for the growth and development of the ovules. Further media modifications and/or the culture of ovules collected later than 10 weeks after pollination may increase the number and vigor of plants obtained from *Hydrangea* in ovolo embryo cultures.

Plants with up to four sets of true leaves and small root systems are presently growing in culture. There are no gross morphological differences between seedlings of *H. macrophylla* and *H. paniculata* that can be used as evidence of hybridity in these seedlings; however work has been initiated on the development of molecular markers that will assist in the analysis of the hybrid status of plants obtained from in ovolo embryo cultures of *H. macrophylla* x *H. paniculata*.

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