Sterilization and In Vitro Growth and Development of *Arundinaria*©

Alex Rajewski, Hazel Wetzstein and Donglin Zhang
Department of Horticulture, University of Georgia, Athens, Georgia 30602, USA
Email: Rajewski@uga.edu

INTRODUCTION

River cane, switch cane, and the newly identified hill cane, are the three species that make up the genus *Arundinaria* (Ohrnberger, 1999; Triplett, 2006). This genus of temperate woody bamboos is native only to North America and typically grows along waterways or in marshlands forming dense stands called canebrakes. These canebrakes have a dense system of rhizomes, which act as an effective riparian buffer to prevent excess nitrogen, in the form of agricultural runoff, from entering waterways. This dense network of rhizomes also helps to prevent erosion of river and stream banks. Beyond purely environmental benefits, ecologically, canebrakes form a unique habitat for many species of cane-obligate butterflies as well as many rare bird species (Platt et al., 2013).

Currently, the genus *Arundinaria* is sparsely distributed in 22 states of the southeastern United States. Although its distribution is wide, it is reported that the genus has suffered massive habitat loss due to altered burning regimes, conversion to farmland, and overgrazing. In fact, many historical accounts report canebrakes up to 20 miles long and ½ mile wide (Platt and Brantley, 1997); however, since European settlement of North America, the size of canebrakes has shrunken by an estimated 98% (Noss et al., 1995).

Successful large-scale propagation of *Arundinaria* would be of great interest to conservationists and to managers carrying out native plant restoration efforts, but large-scale propagation is fraught with many challenges. Biologically, seed-based bamboo propagation is not a viable option due to extremely long times to maturity and irregular flowering (Hughes, 1951; Janzen, 1976). Vegetative macropropagation is technically simple, but finding, harvesting, cleaning, transporting, and replanting rhizomes is extremely logistically difficult and very time sensitive. In vitro micropropagation would offer the possibility of generating large numbers of transplantable plants without the uncertainties in material acquisition, but bamboos are notoriously difficult to disinfest and any micropropagation system requires optimization. Our objective was to test procedures to successfully disinfest *Arundinaria* for in vitro micropropagation and to characterize its growth in vitro for later use in larger scale propagation experiments.

MATERIALS AND METHODS

Explant Material

Two types of propagation explant material were obtained for this experiment. In late January of 2014, rhizomes from a canebrake in Clarke County, Georgia were harvested, potted, and grown in a heated greenhouse on the University of Georgia campus. In March, the vigorously growing plants were treated with a systemic fungicide (Procure® 480SC, 8 oz/gal). These plants served as explant material for shoot disinfestation experiments conducted in May 2014 and July 2014. For these experiments, lateral shoots were disinfested either as multinodal branches or as single nodes. Additionally, the effect of leaf sheath removal to reduce contamination was investigated. For this, the sheath surrounding the stem was carefully removed with a scalpel down to the base of the node to expose the bud scale.

Unexpectedly, a second propagation material was provided. A local cane enthusiast and grower was able to provide seed from flowering plants he had scouted. The batch included several hundred seeds from a large canebrake in Arkansas. This larger batch was intended for nursery planting and unfortunately had been dusted with mycorrhizal spores beforehand. Due to the poor long-term viability of bamboo seeds, this batch served as the seed and embryo material for disinfestation experiments conducted only in May and early June. For these experiments, the seeds were disinfested as whole seeds with prophylls

(bracts) removed or as isolated embryos.

Sterilization Procedures

Sterilization procedures were improved iteratively over several trials. The initial procedure began with a rinse in soapy water to remove large debris, a tap water rinse, and a short dip in ethanol. During the ethanol dip, the explants were transferred to a laminar flow hood. A rinse in benzalkonium chloride (Lysol) and a subsequent rinse in bleach followed. Finally, the explants received three rinses with sterile water. A later series of treatments, adapted from Thakur (2006), were also tested. These treatments differed from the previous treatments in their handling of the explant material and had a long rinse with a combination antibiotic/fungicide solution (Rifampicin/Procure). Table 1 summarizes the lengths and concentrations of the rinses in the various sterilization treatments; the treatments are numbered in order of increasing harshness.

Table 1. Summary of sterilization treatments. Times are given in minutes or seconds. Bleach concentrations are given as concentration of sodium hypochlorite (NaOCl), and the ethanol used was 70%.

Treatment	Soapy water (min.)	Ethanol (s)	Antibiotic/fungicide (min.)	Bleach (conc.) (min.)
1	20 + rinse	30	-	10 (0.53%)
2	20 + rinse	30	-	10 (1.05%)
3	20 + rinse	30	-	10 (2.63%)
4	20 + rinse	30	-	10 (2.63%) + 10 (0.53%)
5	20 + rinse	30	10	10 (0.53%)
6	20 + rinse	30	10	20 (0.53%)
7	5	-	60 + rinse	5 (4%)

Growth Conditions

Based on the promising preliminary work of Baldwin et al. (2009), Murashige and Skoog (MS) media were shown as being comparable to WPM and superior to DKW media for river cane growth. Therefore, all media recipes used were based on MS media. The media were all made with 3% sucrose, gelled with 0.4% Gelzan, and brought to pH 5.8 before autoclaving. For initial growth conditions, the media was supplemented with 1.98 $mg \cdot L^{-1}$ BAP (6-benzylaminopurine) for seed-based explants and 3 $mg \cdot L^{-1}$ BAP for shoot-based explants.

Data Collection

For the first week after disinfestation, the explants were examined daily for signs of bacterial or fungal contamination. Thereafter, the explants were examined every other day. Additionally, the growth of the plants was observed. Times to root and shoot emergence were recorded as well as the timing of any lateral shoots that developed. For shoot-based disinfestations, results are presented for only the first six weeks, corresponding to the duration since the most recent trial; seed-based trials, however, have data for approximately 100 days.

RESULTS AND DISCUSSION

Shoot Disinfestation and Development

The initial shoot sterilization procedure was conducted on single nodes with the leaf sheaths removed. Contamination was moderate. Treatment 5 showed 55% disinfested shoots after 6 weeks, but the harsher Treatment 6 showed only 33% disinfested shoots (Fig. 1A). A second round of treatments on the same type of explant material included Treatments 1, 5, and 7. Treatment 5 again showed similar results, 57% disinfestation,

suggesting the ‘baseline’ contamination of the plants had not changed between the two times. Treatment 1, which generates less waste material than Treatments 5, 6, and 7, showed an improvement with 76% disinfested shoots. Finally, Treatment 7 was conducted on single nodes with leaf sheaths removed as well as on multimodal segments with and without leaf sheaths. The multimodal segments showed similar results to the single nodes under Treatment 1 between 75% and 80% disinfestation. The best treatment by far, however, was the combination of Treatment 7, the single nodes, and the removal of the leaf sheaths; this treatment showed 100% disinfestation. It should be noted however, that for the shoot disinfestation experiments latent contamination was a severe problem. Many nodes did not show signs of contamination until 2 months after being treated; so long term evaluation will be necessary.

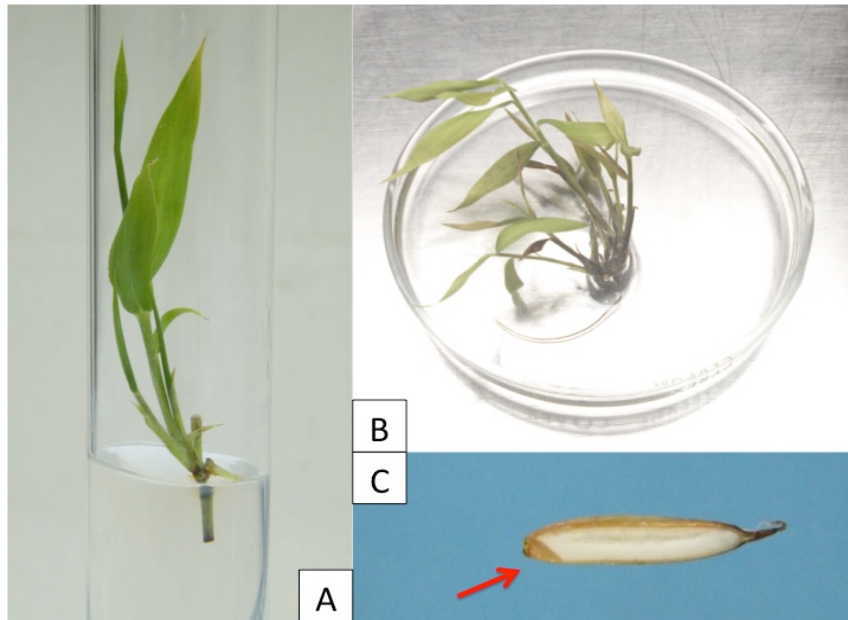

Fig. 1. (A) A sterilized nodal segment growing in vitro with lateral branches; (B) A developing plantlet with several later shoots and roots derived from a sterilized embryo; (C) A longitudinally dissected seed of *Arundinaria* showing the starchy endosperm and embryo (arrow).

Because of the prolonged fungicide rinse in Treatment 7, which has been shown in other plants to affect proliferation (Ruzić et al., 2009; Werbrouck and Debergh, 1996), the fold multiplication of the shoots and the time to first lateral shoot emergence were calculated. After 6 weeks, the shoots showed 1.41-fold (±0.75) multiplication. This rate was statistically equal for all dates and sterilization treatments. The average time to emergence of the first lateral shoot was 34 days (±7.7) days post sterilizations (DPS), however Treatment 5 showed a significant difference between trials. The explants showed lateral shoot emergence at 39 DPS versus 26 DPS for the earlier and later trials, respectively. The results for contamination percentages and growth are summarized in Table 2. Overall, these results show that Treatment 7 has a very high success for disinfestation of shoot material and that it has not been shown to significantly alter growth as compared to other methods tested.

Table 2. Results of the contamination and growth of the explant material. Shoot emergence times for nodes are note given because all growth from nodes is lateral growth. Numbers in parenthesis are times in days ± the standard deviation.

Treatment	Explant	Disinfestation (%)	Shoot emergence (%) (days ± s.d.)	Lateral shoot emergence (%) (days ± s.d.)
1	Single node	76	-	100 (33±7)
2	Embryo	50	50 (35±28)	45 (58±28)
3	Embryo	28	46 (24±24)	30 (52±26)
4	Embryo	95	18 (29±20)	21 (59±26)
5	Single node	55	-	100 (32±6)
6	Single node	33	-	100 (33±9)
7	Single node	100	-	100 (34±6)
7	Multinodes	75	-	100 (34±11)
7	Multinodes (w/ sheath)	80	-	100 (38±9)

Seed-Based Disinfestation and Development

Although over 200 whole seeds were sterilized, their response in vitro was limited to a small number of seeds, which failed to develop past the stage of root emergence. Because of this lack of response, experiments using the isolated embryos were carried out. In these experiments, the seeds were surface-sterilized using Treatments 2 or 3, and the embryos were aseptically dissected out and placed in culture vessels (Fig. 1C). There was an initial burst of contamination in the first five days after sterilization, and overall contamination was high. Treatment 2 had an overall success rate of 50%, with 61% of the contamination being attributable to bacteria. Treatment 3 had lower success; only 28% of embryos were successfully sterilized. In Treatment 3, 75% of the contamination was bacterial, the same trend as with Treatment 2. Subsequently, a harsher procedure, Treatment 4, was undertaken to surface-sterilize the seeds and then to sterilize the isolated embryos. This treatment successfully resulted in contamination rates of only 5%, stemming from a defective vessel, but unfortunately depressed development.

The embryo-based methods showed much higher response than the whole seeds. Development between Treatments 2 and 3 was similar but highly variable. The embryos showed shoot emergence as early as 6 DPS but had an average shoot emergence time of 26.4 DPS. Development of lateral shoots was similarly variable and occurred as early as 19 DPS, but averaged 54 DPS (Fig. 1B). For both treatments there was no significant difference in the percentage of embryos that showed shoot emergence, which averaged 47.2%; however, the percentage of those embryos showing lateral shoot growth was higher in Treatment 2, 45.2% compared to 29.8% ($p=0.062$). For Treatment 4, the embryos showed almost no response; less than 20% showed shoot emergence. Of the embryos that showed shoot emergence, 21% also showed lateral shoot growth, but this corresponded to a very small number of embryos ($n=3/14$).

Unfortunately, Treatment 7, the most effective shoot treatment, could not be evaluated on the embryos, due to the low viability of the seeds long term. Overall, these results show that the gentler Treatment 2 resulted in lower contamination than the harsher treatments and produced embryos with greater development of lateral shoots.

Further research investigating latent contamination rates as well as long-term growth responses will be necessary. In this report, each explant material received only one concentration of BAP, but further experiments will be necessary to determine the optimum concentrations and ratios of hormones and growth regulators for use in in vitro proliferation methods. As interest grows in large-scale native plant restoration in the southeast United States, a reliable supply of these plants will be necessary. The results presented here demonstrate that *Arundinaria* embryos and shoots can be successfully

disinfested and that these materials are amenable to growth in vitro.

ACKNOWLEDGEMENTS

Thanks are given to the State Botanical Garden of Georgia and Athens-Clarke County, Georgia for the use of their lands. Additionally, Justin Porter provided much help with the sterilizations of the material mentioned in this report. Finally, the bulk of this work would not have been possible without the advising, expertise, and generous donation of seeds provided by Thomas Peters.

Literature Cited

Baldwin, B.S., Cirtain, M., Horton, D.S., Ouellette, J., Franklin, S.B. and Preece, J.E. 2009. Propagation methods for rivercane [*Arundinaria gigantea* L. (Walter) Muhl.]. Castanea 74(3):300-316.

Hughes, R.H. 1951. Observations of Cane (*Arundinaria*) Flowers, Seed, and Seedlings in the North Carolina Coastal Plain. Bulletin of the Torrey Botanical Club (2), 113. doi: 10.2307/2482044.

Janzen, D.H. 1976. Why Bamboos Wait So Long to Flower. Annual Review of Ecology and Systematics 7:347-391.

Noss, R.F., Scott, J.M. and LaRoe, E.T. 1995. Endangered ecosystems of the United States: a preliminary assessment of loss and degradation. Biol Rept 28. USDI Natl Biol. Service. Washington, DC.

Ohrnberger, D. 1999. The bamboos of the world. Annotated nomenclature and literature of the species and the higher and lower taxa The bamboos of the world. Annotated nomenclature and literature of the species and the higher and lower taxa. Amsterdam; Netherlands: Elsevier Science Ltd.

Platt, S.G. and Brantley, C.G. 1997. Canebrakes: An Ecological and Historical Perspective. Castanea(1), 8. doi: 10.2307/4034098

Platt, S.G., Rainwater, T.R., Elsey, R.M. and Brantley, C.G. 2013. Canebrake fauna revisited: additional records of species diversity in a critically endangered ecosystem. Bamboo Science & Culture 26:1-12.

Ruzić, D., Vujović, T., Cerović, R. and Kuzmanović, M. 2009. The influence of imidazole fungicides on multiplication in vitro of low vigorous pear and cherry rootstocks. Acta Hort. 839:79-86.

Thakur, R.S. 2006. An efficient method for explant sterilization for reduced contamination. Plant Cell, Tissue and Organ Culture. 84:369-371. doi: 10.1007/s11240-005-9034-6.

Triplett, J.K., Weakley, A.S. and Clark, L.G. 2006. Hill cane (*Arundinaria appalachiana*), a new species of bamboo from the southern appalachian mountains. SIDA, Contributions to Botany 22:79-95.

Werbrouck, S.P.O. and Debergh, P.C. 1996. Imidazole fungicides and paclobutrazol enhance cytokinin-induced adventitious shoot proliferation in Araceae. Journal of Plant Growth Regulation 15(2):81-85.