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Somatic embryogenesis and plant regeneration from zygotic embryo explants in mexican weeping bamboo, *Otatea acuminata aztecorum*

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Abstract

An efficient protocol has been developed for the *in vitro* propagation of Mexican Weeping Bamboo through somatic embryogenesis from zygotic embryo explants. Mature seeds and excised embryos were cultured in the light or in the dark on both Murashige and Skoog's and Gamborg's B5 basal media with various supplements. Optimal somatic embryogenesis and plant regeneration were obtained by culture in the dark on Murashige and Skoog's basal medium supplemented with 3 mg/l 2,4-dichlorophenoxyacetic acid, 0.5 mg/l 6-benzylaminopurine and 2.0% sucrose. More than 95% of the germinating somatic embryos developed shoots and roots, and were transferred to soil with 85% success.

Introduction

Bamboo, a term for more than 75 genera and 1250 species of the Gramineae (Soderstrom and Ellis 1988), provides food, raw material, shelter, and even medicine world wide. Bamboo is now threatened over a large area of the world due to its gregarious flowering habit and the human population pressure disrupting the natural cycle of reforestation. If reliable means of mass propagation were available, bamboo could become an important new multipurpose tree crop in many areas where it has not been traditionally used, along with increasing the economic potential and esthetic value of ornamental bamboos in landscapes (Reinhardt et al. 1989).

Efficient *in vitro* propagation could be a reliable and useful method for establishment of new bamboo plantations (Rao et al. 1985). The majority of bamboo tissue culture research to date has been aimed at the development of shoot multiplication systems (Banik 1987; Manzur 1988; Nadgauda et al. 1990; Nadgir et al. 1984; Saxena 1990). Plant regeneration via organogenesis from shoot apices was successful for four species (Huang et al. 1989). Reports which deal with somatic embryogenesis and plant regeneration of important economic bamboo species include two reports of plants regenerated from floral explants (Yeh and Chang 1986a, 1986b) and three reports of plant regeneration from seed explants (Yeh and Chang 1987; Mehta et al. 1982; Rao et al. 1985). However, only one of these reports (Rao et al. 1985) indicates potential as an efficient somatic embryogenesis system.

To date there have been no reports of successful tissue culture and plant regeneration in Mexican Weeping Bamboo, *Otatea acuminata aztecorum* (McClure and Smith) Claderon & Soderstrom, a highly prized ornamental species introduced to the U.S. from central western Mexico. This U.S. planting of this bamboo began flowering and seeding in 1987 and most clones are dead or dying. The aim of the present research was to develop reliable methods for regenerating plants via somatic embryogenesis from zygotic embryo explant tissue in Mexican Weeping Bamboo as a means of mass propagation.

Materials and Methods

Plant Material. Seeds of Mexican Weeping Bamboo were provided by the American Bamboo Society (Gerald Bol, Sebastopol, CA). The explant material for tissue culture included mature seeds and zygotic embryos excised from germinating seeds. Seeds were husked and washed for 10 min in water containing a few drops of detergent, rinsed in distilled water, dipped in 70% ethanol for 1 min, immersed for 15 min in 30% commercial bleach (1.6% NaClO final concentration) and rinsed 3 times in sterile water. The final rinse was with sterile water at a pH of 3.5 achieved by addition of HCl. Seeds were germinated in the dark at 26 °C on sterile filter paper wetted with the acid water. After 24 h, seeds or excised embryos were explanted as described below.

Experimental methods. Two basal media, MS salts (Murashige and Skoog 1962) with B5 vitamins, and Gamborg's B5 medium (Gamborg et al. 1968), were used in all experiments with various supplements as indicated (Tables 1, 2). Subcultures to fresh media were routinely performed at monthly intervals. The medium used for embryo germination and plant regeneration was B5 basal medium with no supplements. All media were adjusted to pH 5.8, gelled with 0.2% Gelrite™, and autoclaved for 25 min at 120 °C.

Three experiments were conducted. In Experiment 1, five replicates (plates) of seeds, four per plate, and five replicates (plates) of embryos, four per plate, were cultured on all media variations (Table 1) under a 23 h photoperiod of cool white fluorescent light at 25 °C. Five additional replicates of seeds and embryos on all callus induction

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media were cultured in the dark. MS-1, MS-2, and MS-3 were designed for shoot induction; MS-4, MS-5, B5-1, B5-2, and B5-3 were designed for callus induction. Each plate contained 25 ml of medium in 15 x 60 mm plastic dishes. The purpose of Experiment 1 was to determine the responsiveness of Mexican Weeping Bamboo zygotic embryos to different shoot and embryogenic callus induction media.

In Experiment II, 42 - 62 embryos for each media variation (Table 2), one per 15 x 60 mm plate, were cultured in the dark. Explants were grouped randomly into five replicates of 8 to 13 embryos each. The purpose of Experiment II was to determine the optimum medium for high efficiency somatic embryogenesis and plant recovery using fresh explant material.

In Experiment III, embryogenic callus from the two responsive media variations in Experiment I was cultured on all of the media variations utilized in Experiment II. Three clumps of callus per plate with four replicates (plates) per media variation were cultured in the dark. The purpose of Experiment III was to observe the effects of the media variations utilized in Experiment II on established embryogenic callus from Experiment I.

Evaluations and plant regeneration. Cultures in Experiments I and II were scored and subcultured at 30-d intervals for at least four passages. Cultures were evaluated as to size, color, callus type, root development, presence and frequency of somatic embryos, and shoot development. Cultures in Experiment III were maintained for two passages and evaluated for proliferation and maintenance of high quality somatic embryos.

For evaluation of plant regeneration, three clumps of embryos, 3-5 mm in diameter, were cultured per plate on germination medium (B5 with no supplements). Ten replicates each from two selected media were grown in the light for one month and scored for number of plantlets regenerated. When plantlets were 3 - 5 cm in height, five plants each from ten cultures were potted in soil and maintained in the greenhouse for observation.

Results and Discussion

Experiment I. Data obtained were utilized to design further experiments for optimizing protocols for somatic embryogenesis and plant recovery after evaluation of certain aspects of explant source, incubation conditions, and media composition as described below.

Whole seed vs. excised embryos. Following surface sterilization, high levels of contamination (more than 50%) resulted when whole seed were used as explant material, whereas contamination was very low (less than 5%) when excised embryo explants were used. Whole seed explants germinated and initiated callus more quickly than excised embryos, but callus development from excised embryos soon equaled or exceeded that from whole seed. Plant production on the two responsive media (Table 1) was equivalent for the whole seed and embryo explants, with about half of the plants recovered from cultures of each explant source.

It could not be verified in every case that the embryogenic callus from a culture was from one genotype because there were 4 seeds or embryos on a plate. In some cases only one explant survived, whereas in other cultures callus could have arisen from more than one genotype and may have been mixed. Because of the high contamination rate when seed were used, and for the purpose of identifying specific responsive genotypes, only one embryo per plate was used in Experiment II.

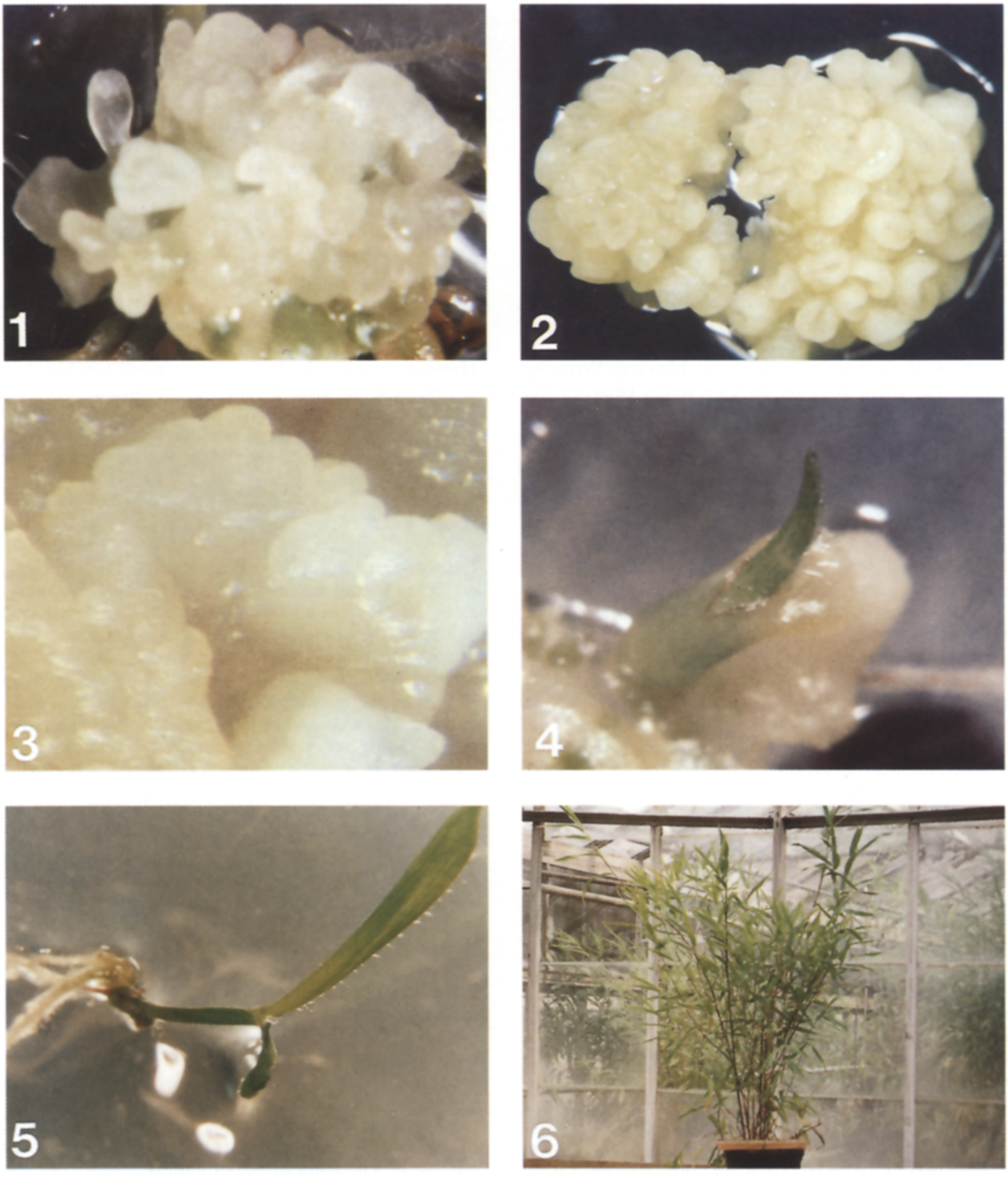
Dark vs. light incubation. Almost all regeneration responses occurred from cultures incubated in the dark. However, two normal plants and several albino plants were produced from callus incubated in the light. Embryogenic callus generated in the light was moved to the dark for two passages, and two additional cultures then produced several somatic embryos which germinated and formed normal-appearing plants. Previous reports in bamboo indicated that somatic embryogenesis occurred under both light (Yeh and Chang 1986b; Rao et al. 1985) and dark (Yeh and Chang, 1987, 1986b) conditions, but no data was given to indicate whether there was a difference between the two conditions.

Influence of hormones. Shoot induction media (MS-1, MS-2, MS-3) produced a low incidence of multiple shoots (3-5 shoots) in early stages but this did not continue with subculture, and these cultures developed many roots (rooty). All callus induction media (MS-4, MS-5, B5-1, B5-2, and B5-3) produced callus of four basic types: (1) soft creamy smooth; (2) cream to white friable; (3) rooty; (4) creamy compact nodular. Cultures were generally a mixture of these callus types but only the compact nodular callus developed somatic embryos. Two media produced embryogenic callus and plants, namely, B5-3 and MS-5 (Table 1). Somatic embryogenesis occurred only on media containing 2,4-D, whereas NAA and picloram were ineffective auxin sources for this response. These results are consistent with a previous report (Yeh and Chang, 1987) which indicated that 2,4-D was essential for bamboo somatic embryogenesis, and NAA and indole-3-acetic acid could not substitute for this effect. In our study, somatic embryos were smooth, compact, shiny globular structures which developed into three basic types: an elongated club-shape (Fig. 1), an indented vase-shape (Fig. 2), and a convoluted disc-shaped (Fig. 3). The majority of plants developed from the vase-shaped embryos (Fig. 4).

Conversion of Somatic Embryos into Plants. Plants grew and rooted efficiently (Fig. 5) on the germination medium (95%). Rooted plants were transferred to a soilless mix in covered containers for hardening, and subsequently moved to the greenhouse (Fig. 6). Survival in the greenhouse after six months was 85% when plants were adequately hardened. This technique for plantlet development from somatic embryos and re-establishment of plants is simpler than that reported previously for bamboo (Rao et al. 1985), and apparently more efficient.

Experiment II

Only one embryo per plate was used in Experiment II in order to keep individual genotypes identified and to avoid contamination problems from whole seed. A very low contamination rate of 1.3% of the cultures from excised embryo explants was obtained. All media variations produced embryogenic callus (Table 2), with callus and embryo types much like those described for Experiment I. Somatic embryos became visible after three weeks of culture and continued to develop for four monthly passages. After four passages, secondary somatic embryos continued to develop, but initiation of new embryogenic cultures was infrequent. The highest number of embryogenic cultures occurred during the second passage. Several factors in the media composition were critically evaluated, as described



Figures 1-6: In vitro somatic embryogenesis and plant regeneration of Mexican Weeping Bamboo.

Fig. 1. Embryo cluster showing elongated clubshaped somatic embryo.

Fig. 2. Embryo cluster showing indented vase-shaped somatic embryo.

Fig. 3. Cluster of convoluted disk-shaped somatic embryos.

Fig. 4. Shoot emerging from germinating vase-shaped somatic embryo.

Fig. 5. Rooted shoot regenerated from somatic embryo.

Fig. 6. One year old regenerated plant in the greenhouse.

below.

Effect of BA. The presence or absence of BA in the culture medium was tested for its influence on the initiation of embryogenic callus using four direct comparisons: MS-6 contrasted with MS-7; MS-5 with MS-8; B5-3 with B5-4; and B5-5 with B5-6 (Table 2). The influence of BA was tested in the presence or absence of casein hydrolysate in both basal media. Both comparisons involving MS basal media, and the comparison involving B5 basal medium in the absence of CH, indicated that the presence of BA strongly promoted the initiation of embryogenic callus. The comparison involving B5 basal medium in the presence of CH did not show any significant influence from the presence or absence of BA. These results provide evidence that BA is important for promoting the production of embryogenic callus in Mexican Weeping Bamboo, but there may be an interaction between the B5 basal medium, BA and CH. Data were not provided in previous reports to document the influence of the presence vs absence of a cytokinin on bamboo somatic embryogenesis.

Effect of CH. The influence of CH on the initiation of embryogenic callus was studied using four contrasting pairs of media: MS-6 compared with MS-8; MS-5 with MS-7; B5-3 with B5-5; and B5-4 with B5-6 (Table 2). The effect of CH was tested in the presence or absence of BA in both basal media. Three of these four comparisons showed no significant effect of CH on initiation of embryogenic callus. However, the omission of CH resulted in enhanced somatic embryogenesis when BA was included in the B5 basal medium. These results indicated that CH was not an important factor for somatic embryogenesis in Mexican Weeping Bamboo, however, further evidence was obtained that an interaction might be occurring between the B5 basal medium, BA and CH.

Effect of Sucrose Concentration. A direct comparison of the influence of 2% sucrose vs 5% sucrose on somatic embryogenesis was made with each basal medium: MS-5 contrasted with MS-9; and B5-3 with B5-7 (Table 2). In both basal media, the 2% sucrose concentration was more conducive to the initiation of embryogenic callus in Mexican Weeping Bamboo, than was the 5% sucrose concentration. Sucrose concentrations had not been compared in previous reports on bamboo somatic embryogenesis.

Effect of Basal Medium. The B5 and MS basal media were contrasted for their influence on somatic embryogenesis in two direct comparisons: MS-6 vs B5-7; and MS-9 vs B5-6 (Table 2). When BA and CH were absent and the sucrose level was 5% (MS-6 vs B5-7), the B5 basal medium resulted in more embryogenic callus than the MS basal medium. But, in the presence of both BA and CH and using 2% sucrose, the MS basal medium was superior to B5. However, the absence of BA and CH, and the use of 5% sucrose, clearly resulted in suboptimal frequencies of somatic embryogenesis in Mexican Weeping Bamboo. Thus, we conclude that the MS basal medium is superior to B5 when other factors in the medium (such as BA and sucrose) are present in optimal combinations. This evidence is supported by observations of callus which has been cultured for more than six passages. Comparison of older cultures grown on all media variations showed that the cultures on the MS basal media consistently maintained embryogenic capacity and continued to produce more somatic embryos than did the cultures on the B5 basal media variations. Further evidence is provided by these results that a complex interaction between basal medium, BA and CH may be influencing somatic embryogenesis in Mexican Weeping Bamboo. Previous reports did not compare different basal media directly for bamboo somatic embryogenesis. However, Saxena (1990) found MS basal medium to be superior to B5 basal medium for bamboo shoot multiplication.

The best results were obtained in Experiment II using MS-9 and B5-4 media, which were equivalent in their effectiveness for promoting somatic embryogenesis. However, the results from all treatment combinations in Experiment II suggest that the optimal medium for Mexican Weeping Bamboo somatic embryogenesis would consist of MS basal medium with 2% sucrose, plus 3 mg/L 2,4-D and 0.5 mg/L BA, without the addition of CH.

Experiment III

Embryogenic callus from Experiment I that originated on B5-3 medium continued to proliferate callus and embryos on all media variations used in Experiment 3, although the number of high quality embryos was consistently lower on the B5 media variations. The optimal B5 media variation was B5-5 with an average of 3.67 clusters of somatic embryos per plate, and the optimal MS media variation was MS-9 with an average of 4.25 clusters of somatic embryos per plate.

Embryogenic callus from Experiment I that originated on MS-5 medium continued to proliferate somatic embryos on all MS media variations used in Experiment III. The optimal medium was MS-9 which produced an average of 10.7 clusters of well formed somatic embryos per plate. However, when the MS-5 derived callus was cultured on B5 media variations, callus proliferated but somatic embryo development averaged less than one cluster of embryos per plate.

The results of Experiment III support the evidence in Experiment II that the MS basal medium is superior to the B5 basal medium, and that MS-9 is the optimal medium of the variations tested. This medium yielded an average of 10.7 clusters of somatic embryos per culture as scored in this study, with each cluster consisting of at least three somatic embryos. The only previous report that quantified the efficiency of bamboo somatic embryogenesis (Rao et al., 1985) documented an average of 8.45 embryoids per culture. Thus, our results for Mexican Weeping Bamboo are at least three times as efficient as that previously documented for bamboo.

Recovery of Plants. Plants were regenerated from somatic embryos produced on all media variations. The two media variations selected for more extensive regeneration evaluation were MS-9, which produced a total of 194 plants or an average of 17.6 plants per plate, and B5-6, which produced a total of 118 plants or an average of 12.1 plants per plate after transfer to

Table 1. Composition of Media Tested for the Initiation of Embryogenic Callus and Plant Regeneration from Zygotic Embryo Explants and Whole Seeds of Mexican Weeping Bamboo in Experiment I.

	Media components (mg/L)				% Cultures producing embryogenic callus and plants	
Basal* media	2,4-D	K	P	NAA	callus	plants
MS-1	--	0.5	--	--	0	0
MS-2	--	0.5	0.5	--	0	0
MS-3	--	0.5	--	0.5	0	0
MS-4	--	--	0.5	--	0	0
MS-5	3.0	--	--	--	32	22
B5-1	--	--	--	5.0	0	0
B5-2	--	--	0.5	--	0	0
B5-3	3.0	--	--	--	38	22

* All MS basal media contained 5% sucrose, 250 mg/l casein hydrolysate (CH), 100 mg/l ascorbic acid, 0.5 mg/l 6-benzylaminopurine (BA), plus other supplements as indicated. All B5 basal media contained 2% sucrose plus supplements as indicated. 2,4-D = 2,4-dichlorophenoxyacetic acid; K = kinetin; P = picloram, 4-amino-3,5,6-trichloropicolinic acid; NAA = 1-naphthaleneacetic acid.

Table 2. Composition of Media Tested for the Initiation and Maintenance of Embryogenic Callus from Zygotic Embryo Explants of Mexican Weeping Bamboo in Experiment II. (Cumulative Responses over Four Culture Passages.)

	Media components (mg/L)			% Cultures producing embryogenic callus(mean ± S.E.)
Basal* media	BA	CH	Sucrose	
MS-5	0.5	250	5%	26.1 ± 6.4
MS-6	--	--	5%	6.7 ± 2.8
MS-7	0.5	--	5%	30.0 ± 2.8
MS-8	--	250	5%	10.2 ± 2.8
MS-9	0.5	250	2%	51.3 ± 7.7
B5-3	--	--	2%	27.5 ± 5.0
B5-4	0.5	--	2%	52.0 ± 6.6
B5-5	--	250	2%	29.3 ± 6.1
B5-6	0.5	250	2%	34.5 ± 3.1
B5-7	--	--	5%	16.7 ± 4.7

* All MS basal media contained 3 mg/l 2,4-D, 100 mg/l ascorbic acid, plus other supplements as indicated. All B5 basal media contained 3 mg/l 2,4-D plus other supplements as indicated.

Conclusions

High efficiency somatic embryogenesis can be readily obtained from zygotic embryo explants of Mexican Weeping Bamboo. Optimal results were obtained when excised zygotic embryos were cultured in the dark on MS basal medium supplemented with 2,4-D (3 mg/l), BA (0.5 mg/l), and 2% sucrose. Plant regeneration and recovery were reliable and efficient and all plants recovered are normal in appearance. The embryogenic callus appeared to arise from the mesocotyl area of the zygotic embryo, although histological examination is necessary to confirm this observation. Callus has been maintained for two years and continues to be embryogenic. This system should be useful for developing a mass propagation system for Mexican Weeping Bamboo and other species of elite bamboos.

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