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ORIGINAL PAPER

In vitro propagation of the neotropical giant bamboo, *Guadua angustifolia* Kunth, through axillary shoot proliferation

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Abstract *Guadua angustifolia* Kunth was successfully propagated in vitro from axillary buds. Culture initiation, bud sprouting, shoot and plant multiplication, rooting and acclimatization, were evaluated. Best results were obtained using explants from greenhouse-cultivated plants, following a disinfection procedure that comprised the sequential use of an alkaline detergent, a mixture of the fungicide Benomyl and the bactericide Agri-mycin, followed by immersion in sodium hypochlorite (1.5% w/v) for 10 min, and culturing on Murashige and Skoog medium containing 2 ml l^{-1} of Plant Preservative Mixture®. Highest bud sprouting in original explants was observed when 3 mg l^{-1} N^6-benzylaminopurine (BAP) was incorporated into the culture medium. Production of lateral shoots in in vitro growing plants increased with BAP concentration in culture medium, up to 5 mg l^{-1}, the highest concentration assessed. After six subcultures, clumps of 8–12 axes were obtained, and their division in groups of 3–5 axes allowed multiplication of the plants. Rooting occurred in vitro spontaneously in 100% of the explants that produced lateral shoots. Successful acclimatization of well-rooted clumps of 5–6 axes was achieved in the greenhouse under mist watering in a mixture of soil, sand and rice hulls (1:1:1).

Keywords Acclimatization · N^6-benzylaminopurine (BAP) · Micropropagation · Plant growth regulators · Plant Preservative Mixture (PPM) · Rooting

Abbreviations

BAP	N^6-benzylaminopurine
$Ca(OCl)_2$	calcium hypochlorite
PGR	plant growth regulator
PPM	Plant Preservative Mixture
NaOCl	sodium hypochlorite

Introduction

Guadua angustifolia Kunth, the giant bamboo of the neotropics, is native to Venezuela, Colombia and Ecuador, and has been widely introduced into other Andean and Central American countries (Judziewicz et al. 1999). In Colombia and Ecuador, it is extensively used as building material for housing, among other uses (Gutiérrez

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2000). Although large natural stands of this species are still present, there is some concern about preservation of these natural populations, as well as the need to develop propagation methodologies for new plantations and reestablishment of cleared stands (Judziewicz et al. 1999).

Taking into consideration the difficulties of conventional propagation of bamboo (reviewed by Gielis et al. 2001; Gielis and Oprins 2002), in vitro micropropagation constitutes a feasible alternative. Propagation using axillary buds has been implemented successfully in several, mainly Asian, bamboo species (Bag et al. 2000; Ramanayake et al. 2001 and references therein).

Only Marulanda et al. (2002) have reported in vitro regeneration in the genus *Guadua*. This was achieved through direct organogenesis from nodal explants. However, multiplication rates and rooting efficiency were not clearly described.

The aim of this work was to set up a protocol for the establishment, regeneration, multiplication, and rooting in vitro, as well as for the acclimatization of *G. angustifolia* Kunth from nodal explants.

Materials and methods

Culture initiation

Single-node segments (2.0–3.0 mm in diameter and 25–35 mm in length) were collected either from the lower healthy lateral branches of adult *G. angustifolia* plants growing at the field (Estación Experimental Los Diamantes, Ministerio de Agricultura y Ganadería, Guápiles, Costa Rica), or from young plants, originated from divisions of adult plants and maintained in a greenhouse.

In the case of plants growing in the greenhouse, watering was conducted only to the base of the plants. The following products were sprayed at weekly intervals during the rainy season (from May to November) and at monthly intervals during the dry season (from December to April) to the latter plants: Kilol L DF-100 (CitroBio, Costa Rica; 5 ml l^{-1}) or Agri-mycin (Pfizer, Mexico; 2 g l^{-1}), in combination with Benomyl (Piscis, Costa Rica; 2 g l^{-1}). When necessary, plants were sprayed with the insecticide Ambush 10 EC (Zeneca, Guatemala; 1 ml l^{-1}). Granular fertilizer (15-15-15) was applied every 120 d to the base of the plants.

After removal of the leaf sheath, individual nodes, each with a bud (explants without a visible bud were discarded at this point), were washed with running tap water for 15 min. Pretreatments prior to disinfection per se (Table 1) included immersion in an alkaline solution (0.05% w/v) of Extran® MA 01 (Merck, Darmstadt, Germany) for 10 min (as described by Bag et al. 2000), and a combination of the bactericide Agri-mycin (Pfizer, Mexico) and the fungicide Benomyl (Piscis, Costa Rica), at a concentration of 2 g l^{-1} each, also for 10 min. Disinfection as such was conducted with sodium hypochlorite (NaOCl) (1.0 or 1.5% w/v) for 10 min, or with calcium hypochlorite [$Ca(OCl)_2$] (10% w/v) for 40 min, supplemented with a drop of Tween 80 (Sigma, St. Louis, MO, USA) per 100 ml. Finally, the explants were washed with sterile distilled water 3–5 times.

Ends were trimmed and explants placed vertically into the culture medium so that the buds were at the level of the medium. The basal medium was Murashige and Skoog (1962) mineral salts, supplemented with thiamine–HCl (0.1 mg l^{-1}), pyridoxine–HCl (0.5 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), glycine (2 mg l^{-1}), myo-inositol (100 mg l^{-1}) and sucrose (3% w/v). Plant Preservative Mixture (PPM™, Plant Cell Technology, Washington DC, USA) (0.5 and 2.0 ml l^{-1}) was added to the culture medium in the corresponding treatments (Table 1). The pH was adjusted to 5.8 with KOH, and the medium gelled with 0.2% (w/v) Phytagel™ (Sigma, St. Louis, MO, USA), poured into 25 × 150 mm culture tubes, closed with plastic closures (Sigma, St. Louis, MO, USA) and autoclaved (1.05 kg cm^{-2}; 20 min). Explants were initially cultured in the dark at 26°C for 2 weeks.

Bud break and development of lateral shoots

Effect of N^6-benzylaminopurine (BAP) (0, 1, 3 and 5 mg l^{-1}) on bud sprouting was evaluated in the basal medium described above. In this experiment and the subsequent ones, the explants

Table 1 Effect of pre-disinfection as well as disinfection treatments, together with inclusion of PPM in the culture medium, on contamination, after 8 weeks, of nodal explants obtained from plants of *G. angustifolia* growing in the field and in the greenhouse

Source	Pre-disinfection[a]	Disinfection	PPM (ml l^{-1})	Contamination (%)
Field-grown	None	NaOCl (1.0%)	0	94
Field-grown	Extran	NaOCl (1.0%)	0	88
Field-grown	Extran + Agri-mycin + Benomyl	NaOCl (1.0%)	0	76
Field-grown	Extran + Agri-mycin + Benomyl	$Ca(OCl)_2$ (10%)	0	69
Field-grown	Extran + Agri-mycin + Benomyl	NaOCl (1.5%)	0	58
Field-grown	Extran + Agri-mycin + Benomyl	NaOCl (1.5%)	0.5	67
Field-grown	Extran + Agri-mycin + Benomyl	NaOCl (1.5%)	2	52
Greenhouse	Extran + Agri-mycin + Benomyl	NaOCl (1.5%)	0	63
Greenhouse	Extran + Agri-mycin + Benomyl	NaOCl (1.5%)	2	11

There were 12–30 explants per treatment

[a] Concentrations described in Materials and methods

with growing shoots that reached 1.5 cm in height were transferred to 170-ml flasks, containing 25 ml of the corresponding culture medium, closed with polyethylene food wrap (Glad, Costa Rica), and placed under a photoperiod of 12 h (10.9 μmol m^{-2} s^{-1}, Sylvania Supersaver Cool White, 32 W, F48T12/CW/SS) and at 26°C. When the explants reached 7 cm in height, they were transferred again, this time into larger culture flasks (17 cm in height and 9 cm in diameter), containing 100 ml of the chosen culture medium. Transfer of the active-growing explants to fresh medium was conducted every 3 weeks.

Growth and development of the sprouted buds, in terms of the number of shoots and roots formed per original explant, and the height of the tallest shoot, were recorded during 154 d in culture on medium containing the BAP concentration that stimulated the highest bud break.

Plant multiplication

Two approaches were evaluated to multiply the growing shoots using the culture medium that stimulated the highest bud break in the previous experiment. In one of them, single-node explants were dissected from the shoots growing in vitro when they reached 7–10 cm in height, and placed on fresh medium, in a similar manner to the original explants. In the other methodology, large clumps (with 8–10 shoots), obtained from growth of lateral buds, were divided in smaller clumps, with 3–5 shoots each, and placed on the same culture medium, after trimming off the roots. Multiplication rate (number of plantlets that originated from each initial explant), and the number of shoots and roots produced per explant were used to evaluate both procedures.

Once the best multiplication procedure was determined (i.e., division of clumps rather than use of single nodes), the effect of BAP concentration (0, 1, 3 and 5 mg l^{-1}) on shoot production was evaluated by subdividing clumps grown on medium with 3 mg l^{-1} BAP and recording the number of new lateral shoots after 6 weeks.

Rooting and acclimatization

Spontaneous rooting occurred in the various experiments. Acclimatization of explants was conducted by transferring rooted clumps to pots (10 cm in diameter and 15 cm in depth) containing a mixture of soil, sand and rice hulls (1:1:1). They were placed under mist irrigation (4 s watering every 15 min during the daylight hours). Plants were initially located under a 50% saran shade cloth, which was removed after 15–30 d. Thereafter plants were watered from the base and fertilized occasionally with Nitrofoska (12-12-17-2, BASF, Costa Rica, 1 g per plant).

Statistical analysis

The effect of BAP concentration on bud sprouting and on formation of new shoots was evaluated by regression analysis using Statistica 6.1 (StatSoft Inc., Tulsa, Oklahoma, USA).

Results

Culture initiation

Contamination was observed very early in culture, usually within the first 10 d, and although it was reduced through inclusion of pre-disinfection treatments (Extran, Agri-mycin and Benomyl) in field-grown plants (Table 1), more than half of the explants from field grown plants became rapidly contaminated. Inclusion of PPM did not give satisfactory results with the field-grown plants. Contamination in greenhouse plants without PPM was above 50% but when 2 ml l^{-1} PPM was added to the culture medium satisfactory disinfestation was achieved. $Ca(OCl)_2$ was discarded because it caused a decline in the green color of the explants and an increase in the flaccidity of the buds (data not shown).

Bud break and development of lateral shoots

In absence of BAP only 13% of the explants sprouted (Fig. 1), while highest sprouting rates (37.5%) were obtained with 3 mg l^{-1}. A further increase in the concentration of BAP caused a reduction in bud sprouting.

In a subsequent experiment, using 3 mg l^{-1} BAP, development of the explants was recorded during 5 months (Fig. 2). First, there was an increase in bud volume (Fig. 3A), followed by development of sheaths (starting usually 7 d after culture initiation; Fig. 3B). After 22 d in culture, the growing axes reached 3 cm in height (Figs. 2 and 3C). Development of lateral shoots started after 50 d in culture (Figs. 2 and 3D). When the growing axes reached 13 cm in height (approximately at day 90), they stopped growing, while the lateral shoots continued their growth (Figs. 2 and 3E). After six subcultures (approximately 2.5 months after culture initiation), clumps of 8–12 axes were observed.

Plant multiplication

Two methodologies were then evaluated for further plant multiplication on medium containing 3 mg l^{-1} BAP and showed that single nodal segments produced very few lateral shoots and roots, giving a multiplication rate close to one and after 71 d, all explants of this type either died or became chlorotic (data not shown). On the other hand, dividing the shoot clumps in groups of 3–5 shoots (Fig. 3E) yielded a more or less constant multiplication rate of 2.5 every 6 weeks (i.e., a mean of 2.5 individual new clusters, actively growing and with adequately number of roots, was produced constantly from each cluster after 6 weeks). The latter procedure could only be started after 150 d of culture of the original explants (with the corresponding subcultures, every 21 d), when clumps had produced enough lateral shoots to be divided.

When the effect of the BAP concentration on formation of new shoots was evaluated there was a slight positive linear correlation i.e., higher BAP concentrations induced formation of more

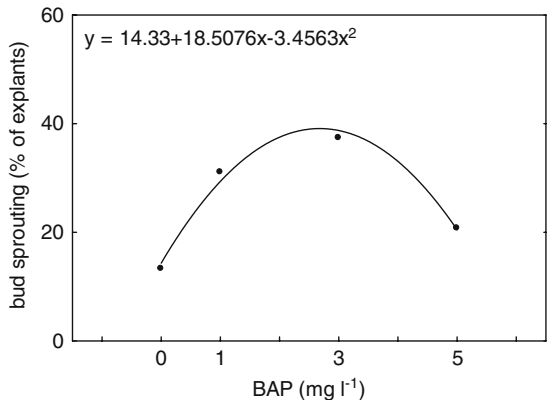

Fig. 1 Effect of the concentration of BAP in the culture medium on sprouting of axillary buds from *G. angustifolia* nodal explants, after 13 d in culture

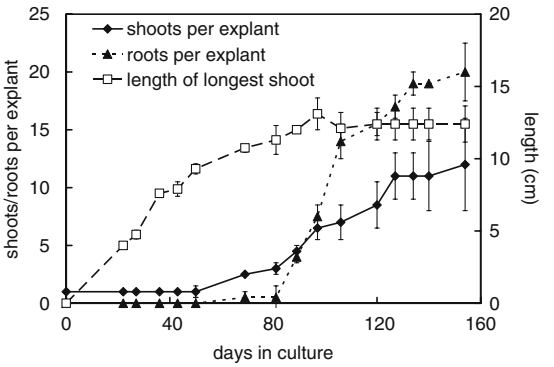

Fig. 2 Growth parameters, over 160 d of culture, of the sprouted nodal explants of *G. angustifolia* growing on medium containing 3 mg l^{-1} BAP

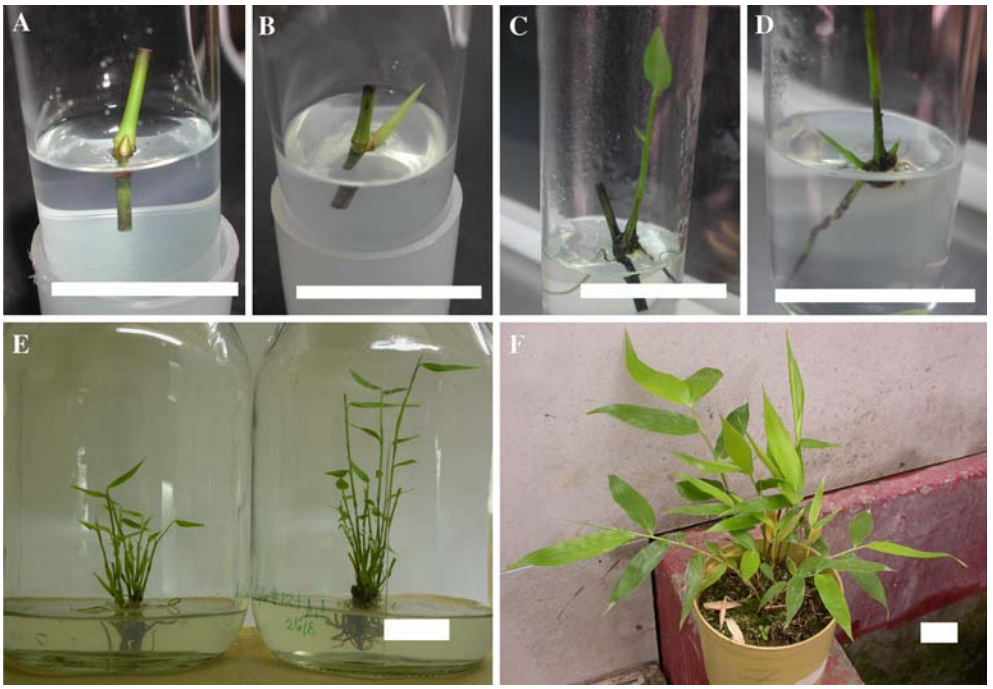

Fig. 3 Stages in development of *G. angustifolia* cultured in vitro. (**A**) Explant with the sheath removed, showing a healthy axillary bud increasing its volume (first day after culture initiation). (**B**) Development of first leaves (ca. 7 d after culture initiation). (**C**) Development of first expanded leaf and lateral roots (ca. 22 d after culture initiation). (**D**) Lateral shoots (ca. 50 d after culture initiation). (**E**) Cessation of growth of the main axis with continued growth of the lateral axes and initiation of new laterals. (**F**) Acclimatized plants in the greenhouse (ca. 2 months after transfer to the greenhouse). Bar: 2.5 cm

lateral shoots (Fig. 4), up to the highest concentration tested (5 mg l^{-1}).

Rooting and acclimatization

Rooting occurred spontaneously in 100% of the explants that developed lateral shoots. From approximately 69 d after culture initiation, a continuous and exponential development of new roots was observed (Figs. 2 and 3E). All roots were trimmed off during subculture and new ones developed very rapidly. When well-developed and rooted clumps of 5–6 axes were selected, over 85% of clumps survived acclimatization (Fig. 3F).

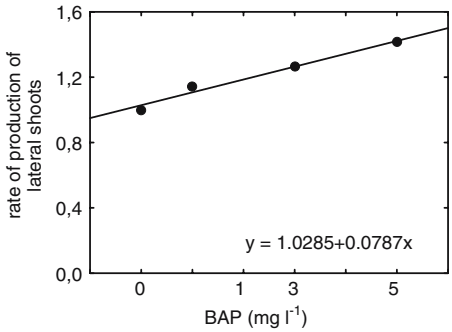

Fig. 4 Effect of BAP concentration on production of lateral shoots in in vitro-growing plantlets of *G. angustifolia*, 21 d after the second subculture on the corresponding medium

Discussion

Culture initiation

To reduce initial contamination of explants it was necessary to combine standard disinfection procedures involving the use of NaOCl (Yeh and Chang 1986; Prutpongse and Gavinlertvatana 1992) with pre-treatments containing Extran, Agri-mycin and Benomyl. Extran, a product commonly used to disinfect laboratory instruments and surfaces, was previously used in com-

bination with mercuric chloride, to disinfect seeds of the Asian bamboo *Thamnocalamus spathiflorus* (Bag et al. 2000). The combination of Agrimycin and Benomyl is frequently used as disinfection pre-treatment for explants that show high contamination rates in our lab (Dalsaso and Guevara 1989).

In contrast, to work on *Dendrocalamus giganteus*, in which contamination of explants was not observed for 3 weeks (Ramanayake and Yakandawala 1997), in our experiments contamination was observed within the first 10 d in culture, coinciding with the moment in which the buds started growing and the sheaths separate. This fact suggests that the contaminants were enclosed within the sheaths that cover the bud, and were not reached by the disinfection treatments.

PPM is a biocide compound, heat stable and effective against a wide spectrum of common in vitro contaminants (Guri and Patel 1998), especially those that appear some time after tissue inoculation (Niedz 1998). PPM at 2.0 mg l^{-1}, effectively reduced contamination of the explants taken from plants growing in the greenhouse but was not effective on field plants (Table 1). Use of the pretreatments, NaOCl, and PPM constituted an efficient way of establishing in vitro explants of *G. angustifolia* cultivated in the greenhouse without the need of the highly toxic mercuric chloride used by other authors (Saxena 1990; Ramanayake and Yakandawala 1997; Ramanayake et al. 2001; Marulanda et al. 2002).

Bud sprouting and shoot multiplication

BAP (1–12 mg l^{-1}) has been very effective in inducing sprouting of axillary buds in several bamboo species belonging to different genera (Prutpongse and Gavinlertvatana 1992; Ramanayake and Yakandawala 1997; Ravikumar et al. 1998; Bag et al. 2000). Marulanda et al. (2002) obtained best results in *G. angustifolia* with 1.0 mg l^{-1} BAP (7.16% of the explants sprouted), but they did not evaluate higher concentrations. The higher sprouting rates obtained in our investigation (37.5%; Fig. 1), could be the consequence of the use of BAP at a higher level (3 mg l^{-1}) and/or employment of a less aggressive disinfection procedure (NaOCl 1.5% rather than mercuric chloride), which might have caused less damage to the explants.

Bag et al. (2000) observed in *Thamnocalamus spathiflorus* a lag phase of 25–30 d before development of lateral shoots while in *D. giganteus* this phase lasted 65 d (Ramanayake and Yakandawala 1997). In our case, it took *G. angustifolia* explants about 50 d for their lateral shoots to start developing (Fig. 2). An increase in the formation of lateral shoots was observed after 90 d in culture, which coincided with the cessation in growth of the main shoot. This reduction in apical dominance may have stimulated development of new lateral shoots (Bangerth 1994).

Plant multiplication

Higher multiplication rates occurred when multiplication was conducted through division of shoot clumps, rather than cutting shoots into individual nodes. Most reports indicate division of clumps to be the preferred method to multiply bamboo plants obtained through axillary shoot proliferation in vitro (Saxena 1990; Prutpongse and Gavinlertvatana 1992; Ramanayake and Yakandawala 1997; Ravikumar et al. 1998; Bag et al. 2000; Ramanayake et al. 2001); there are few in which nodes were employed (Sood et al. 1992; Marulanda et al. 2002). In previous work on *Guadua*, Marulanda et al. (2002) did not state the efficiency of the multiplication procedure

Multiplication rate in vitro for bamboo varies according to the species (Ramanayake and Yakandawala 1997; Bag et al. 2000; Gielis et al. 2001), and the size of the explant (Prutpongse and Gavinlertvatana 1992). We report in this work a multiplication rate of 2.5 new clusters per original cluster every 42 d. It gives a potential multiplication rate of ca. 2900 new clumps per clump per year. However, further research has to be conducted to evaluate whether this multiplication rate can be maintained. Ramanayake et al. (2001) reported changes in multiplication rate of *D. giganteus* with subculture. Moreover, if this procedure is going to be implemented for large-scale propagation of *G. angustifolia*, the amount of somaclonal variation, an important source of variability in vitro (Kaeppler et al. 2000), has to be assessed.

Rooting and acclimatization

In other bamboo genera inclusion of indole butyric acid or naphthalene acetic acid was necessary to induce rooting in vitro (Ramanayake and Yakandawala 1997; Prutpongse and Gavinlertvatana 1992; Bag et al. 2000) or ex vitro (Ravikumar et al. 1998). *Guadua* plantlets rooted spontaneously after 69 d in culture (Fig. 2) and formed new roots very quickly after roots were pruned during subculture. *Guadua* plantlets obtained in vitro by Marulanda et al. (2002) apparently rooted spontaneously; however, it is not clear whether rooting occurred in vitro or ex vitro. They acclimatized 100% of the plants (a total of 14 plants) in their experiment using compost as potting medium. Ramanayake and Yakandawala (1997) included a hardening phase prior transfer of the plants to the potting mix. In that phase, rooted shoots were first transferred to Murashige and Skoog (1962) medium devoid of PGRs with macronutrients at half strength for 1 week, and then to sucrose-free medium. At this latter point, the caps of the tubes were removed periodically. They reported 30% survival during this hardening phase, while 100% of the hardened plants survived acclimatization to the greenhouse. The simpler method of acclimatization reported here gave 100% survival when healthy well developed clumps were selected.

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