BIOLOGIA PLANTARUM 39 (4): 515-522, 1997

# *In vitro* plant regeneration *via* callogenesis and organogenesis in *Bambusa vulgaris*

G.R. ROUT and P. DAS*

*Plant Tissue Culture Laboratory, Regional Plant Resource Centre, Bhubaneswar - 751015, India*

## Abstract

Friable calli were induced from mature excised shoots of *Bambusa vulgaris* on Murashige and Skoog's (MS) medium supplemented with 2.2 μM 6-benzylaminopurine (BAP), 9.04 μM 2,4-dichlorophenoxyacetic acid and 14.76 μM indole-3-butyric acid (IBA) with 3 % (m/v) saccharose. Adventitious shoots with root hairs were achieved from calli on MS medium supplemented with 13.33 μM BAP and 1.23 2.46 μM IBA within 4 weeks of subculture. The frequency of shoot bud regeneration was better in the light incubated cultures than in the dark incubated cultures. Isolated shoots were rooted on liquid half-strength MS basal medium supplemented with 0.49 μM IBA and 2 % (m/v) saccharose. Histological observations confirmed the regeneration of shoot buds from calli. The rooted plantlets were successfully transferred to greenhouse.

*Additional key words*: 2,4-D, mature culm, shoot bud regeneration, tissue culture.

## Introduction

Bamboo is one of the important natural and renewable resources in the tropical and sub-tropical regions of the world. It is used for building of houses and production of paper. Indiscriminate and unplanned exploitation has resulted in rapid depletion of bamboo. The conventional method of seed propagation is unreliable due to the rapid loss of viability and infection of seeds by pests and diseases during storage and the uncertain flowering cycle that may take 40 - 60 years in *Bambusa vulgaris*. Multiplication by vegetative methods such as separation of culms, rhizomes or rooting of stem cuttings is rather slow. There are many reports of micropropagation

*Received* 15 January 1996, *accepted* 10 April 1996.

*Abbreviations*: Ads - adenine sulphate; BAP - 6-benzylaminopurine; Kn - kinetin; NAA - 1-naphthaleneacetic acid; IBA - indole-3-butyric acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; MS - Murashige and Skoog's medium.

*Acknowledgement*: Financial assistance provided by Department of Biotechnology, Government of India, New Delhi is gratefully acknowledged.

* To whom correspondence should be sent; fax: (+91) 0674 410274

on different bamboo species using seedling explants (Nadgir *et al.* 1984, Saxena 1990, Das and Rout 1991, 1994). These include propagation *via* somatic embryogenesis through callus culture, using nodal explants derived from *in vitro* grown seedlings and excised zygotic embryos (Rao *et al.* 1985, Yeh and Chang 1986, Woods *et al.* 1992, Rout and Das 1994). To date, no report of bamboo micropropagation from mature explants is known. We report for the first time, *in vitro* plant regeneration from callus derived from mature culm tissue of *Bambusa vulgaris*.

## Materials and methods

**Explant source:** Twigs about 20 - 25 cm long consisting of 10 - 12 nodes were collected from an elite culm of *Bambusa vulgaris* in the Bambusetum of the Regional Plant Resource Centre. Nodal segments (1.0 - 2.0 cm) with axillary buds were dipped in a fungicide solution (*Bavistin*) 1.0 g $dm^{-3}$ (m/v), constantly agitated for 45 min by a rotary shaker, and were washed with slow running tap water for 10 - 20 min. The segments then were surface disinfected with a 0.2 % (m/v) aqueous mercuric chloride solution for 30 min, then thoroughly washed 3 - 4 times with sterile distilled water. The segments were trimmed on both ends to 0.5 - 1.5 cm size and were cultured aseptically on MS (Murashige and Skoog 1962) medium with 555 μM *m*-inositol, 4.06 μM nicotinic acid, 2.43 μM pyridoxine-HCl, 0.296 μM thiamine-HCl, 3 % (m/v) saccharose and 0.8 % (m/v) agar (*Qualigen*, Bombay, India) for bud sprouting. The pH of all culture media was adjusted to 5.8 with 0.1 M HCl or 0.1 M NaOH prior to autoclaving at 104 kPa for 15 min. Bud break and shoot proliferation were initiated by placing nodal segments in 25 × 150 mm culture tubes (one nodal explant per tube) containing 15 $cm^3$ of the culture medium and plugged with non-absorbent cotton wrapped in one layer of cheese-cloth. The cultures were incubated at 25 ± 2 °C under both 16-h photoperiod (cool, white fluorescent light, irradiance 55 μmol $m^{-2}$ $s^{-1}$). After 10 - 12 d of culture, the sprouted shoots were excised and used as explants for callus production.

**Culture media and culture conditions:** Excised shoots were transferred to MS basal medium supplemented with various concentrations of BAP, Kn, IBA and 2,4-D alone or in combination for induction of callus. The pH of media was adjusted to 5.8 using 0.1 M HCl or 0.1 M NaOH before autoclaving. Routinely, 15 $cm^3$ of the molten medium gelled with 0.8 % (m/v) agar was dispensed into culture tubes (25 × 150 mm) and plugged with non-absorbent cotton wrapped in one layer of cheese cloth. The culture tubes were then steam sterilised at 121 °C at 104 kPa for 15 min. After 8 weeks, the friable calli (300 mg ± 50 mg) were transferred to different regeneration media containing various concentration of auxins and cytokinins. The cultures were incubated at 25 ± 2 °C in the dark or in the light (16-h photoperiod, irradiance 55 μmol $m^{-2}$ $s^{-1}$) for both callus culture and regeneration of shoot buds. After the initial 8 weeks of shoot culture, the induced calli were subcultured at 4-week

intervals. Each treatment consisted of 20 replicates per treatment and each experiment was repeated three times.

Visual observations were taken every 4-week and the effects of different treatments were quantified on the basis of the percentage of cultures showing response. The number of regenerants per culture was recorded after 4 weeks of initial incubation. Data were statistically analysed by Post Hoc Multiple comparisons test (Marascuilo and McSweeney 1977).

**Induction of rooting and field acclimatization:** Regenerated shoots were cultured into full, 1/2 and 1/4 strength MS media supplemented with various concentrations of IBA (0, 0.49, 0.98, 1.74 and 2.46 μM) or NAA (0, 0.53, 1.06, 1.59 and 2.68 μM) and 2 % (m/v) saccharose for induction of roots. The cultures were incubated at 25 ± 2 °C at 16 h photoperiod. The rooted micropropagules were thoroughly washed to remove the nutrient media and planted in 2.5 cm earthern pots containing a mixture of soil:sand:manure in the ratio of 1:1:1 (v/v) and kept in a greenhouse for about 30 d.

**Histology:** For histological studies, the organogenic calli were fixed in FAA (formalin/glacial acetic acid/ethanol, 5:5:90, v/v) for 48 h, dehydrated through graded ethanol-xylol series and embedded in paraffin wax. The tissues were sectioned at 10 μm thickness, double stained with 1 % (m/v) safranin, toluidine blue (0.5 %, v/v) and examined under a light microscope.

## Results

**Callus culture:** Calli were initiated in excised shoots derived from mature culms of *B. vulgaris* within 18 - 20 d of inoculation onto MS basal medium supplemented with auxins and cytokinins (Table 1). Addition of BAP or Kn + 2,4-D + IBA in the medium showed higher response than BAP or Kn + IBA. The combinations of 2,4-D at higher concentration with cytokinin at lower concentration were effective in the induction and proliferation of callus. In the absence of growth regulators, there was no sign of callus formation. Inclusion of BA, Kn, NAA, 2,4-D or IBA alone did not help in the induction of callus (data not shown). The percentage of cultures showing callus initiation in darkness was less than that observed with a light period. Initially, slow-growing friable, yellowish white calli developed on the basal region of the excised shoots which subsequently developed into large callus masses (Fig. 1*A*). Incubation of the cultures in the light induced faster growth of calli than in the dark.

**Induction of shoot bud regeneration:** After first subculture, the friable calli were transferred to various MS media with different concentrations of BAP, Kn, NAA and IBA in combination for shoot bud regeneration. Calli differentiated both shoot buds and roots with root hairs after 30 d of culture on the media supplemented with BA + IBA or BAP + NAA (Fig. 1*B*). The highest regeneration (78.4 %) was observed from calli on medium containing 13.33 μM BAP + 2.46 μM IBA when the cultures were

incubated in the light for 4-week (Table 2). The frequency of regenerated shoots per culture (300 ± 50 mg fresh mass) varied from 1.36 to 9.72 in case of the light and 0.56 to 3.21 in case of the dark incubation (Table 2).

Significant differences were observed among the treatments for both the percent of cultures initiating shoot buds and the mean number of shoots per culture. The calli with small regenerated shoot buds turned brown and subsequently dried up when the cultures were incubated in the dark. Histological observations revealed development of shoot primordia from calli after 30 d of transfer, which subsequently developed into shoot buds (Fig. 1*C*). Calli subcultured for 3 - 4 passages lost their regeneration ability.

Table 1. Effect of different hormonal constitution in MS medium with 3 % (m/v) saccharose and of culture conditions on callus growth in excised shoots of *Bambusa vulgaris* (20 replicates per treatment; repeated three times; results after 4 weeks of culture).

| Growth regulators [μM] | | | | Explants producing callus [%] | |
|---|---|---|---|---|---|
| BA | Kn | IBA | 2,4-D | Light | Dark |
| 0 | 0 | 0 | 0 | 0 | 0 |
| 2.22 | 0 | 4.92 | 0 | 0 | 0 |
| 2.22 | 0 | 9.84 | 0 | 14.6 | 10.8 |
| 2.22 | 0 | 14.76 | 0 | 26.8 | 18.2 |
| 4.44 | 0 | 19.68 | 0 | 32.4 | 22.4 |
| 0 | 2.32 | 14.76 | 0 | 24.2 | 16.8 |
| 2.22 | 0 | 0 | 9.04 | 4.8 | 8.6 |
| 2.22 | 0 | 0 | 13.57 | 18.4 | 12.6 |
| 0 | 2.32 | 0 | 9.04 | 0 | 0 |
| 0 | 4.64 | 0 | 9.04 | 0 | 0 |
| 2.22 | 0 | 9.84 | 9.04 | 48.6 | 36.4 |
| 2.22 | 0 | 14.76 | 9.04 | 86.4 | 54.8 |
| 2.22 | 0 | 19.68 | 9.04 | 68.2 | 46.4 |
| 2.22 | 0 | 9.84 | 13.57 | 74.2 | 54.6 |
| 4.44 | 0 | 14.76 | 9.04 | 72.6 | 52.2 |
| 0 | 2.32 | 14.76 | 9.04 | 44.2 | 36.8 |
| 0 | 2.32 | 9.84 | 13.57 | 48.6 | 30.2 |
| 0 | 0 | 9.84 | 13.57 | 46.7 | 32.8 |
| 0 | 0 | 4.92 | 13.57 | 58.2 | 40.6 |

**Induction of rooting and field establishment:** Growing shoots (1 cm in length) derived from callus were excised and cultured on full, 1/2 and 1/4 strength MS liquid or solid medium supplemented with various concentrations of NAA or IBA. Rooting was noticed with solid media containing either NAA or IBA; high percentage of shoots (82.4 %) rooted within 10 d of culture on 1/2 MS + 0.49 μM IBA (Table 3). The root became thin, long and well spread in the culture media (Fig. 1*D*). With increased IBA or NAA concentration in the culture media, the rooting was affected and a small amount of callus formation occurred at the basal end of the shoots. Well rooted

plantlets were transferred to pots containing soil:manure:sand in the ratio of 1:1:1 and kept in the greenhouse for acclimatization; about 90 % of the plants survived in the greenhouse (Fig. 1*E*).

Table 2. Shoot bud regeneration from friable calli (300 mg ± 50 mg fresh mass) of *Bambusa vulgaris* after 4-week of culture on different media in the light or in the dark. Data represent average number of shoot buds per callus explant. Shoots shorter than 0.25 cm were not counted. 20 replicates per treatment; repeated three times.

| Growth regulators [μM] | | | | Regenerated culture [%] | | Number of shoot buds [explant$^{-1}$] | |
|---|---|---|---|---|---|---|---|
| BA | Kn | IBA | NAA | light | dark | light | dark |
| 8.88 | 0 | 2.46 | 0 | 52.6±0.2 e | 31.4±0.1 f | 4.36±0.5 d | 1.18±0.3 bc |
| 13.33 | 0 | 2.46 | 0 | 78.4±0.7 f | 42.8±0.3 g | 9.72±0.7 e | 3.21±0.2 e |
| 17.77 | 0 | 2.46 | 0 | 37.2±0.4 cd | 22.6±0.7 d | 3.26±0.2 c | 2.50±0.2 d |
| 13.33 | 0 | 0 | 2.68 | 35.8±0.2 c | 18.4±0.9 c | 3.73±0.2 c | 1.36±0.4 c |
| 8.88 | 0 | 0 | 5.37 | 24.6±0.2 b | 10.2±0.4 b | 1.56±0.4 a | 0.94±0.3 b |
| 13.33 | 0 | 4.92 | 0 | 49.2±0.8 e | 26.6±0.5 e | 4.22±0.4 d | 2.16±0.4 d |
| 8.88 | 0 | 2.46 | 1.34 | 20.8±0.4 ab | 8.4±0.4 ab | 2.77±0.5 b | 0.84±0.3 ab |
| 0 | 13.92 | 2.46 | 1.34 | 18.4±0.4 a | 7.8±0.7 a | 1.36±0.6 a | 0.56±0.2 a |

* Means within a column followed by the same letter are not significantly different, at $P < 0.01$.

Table 3. Effect of various strength of MS liquid media with auxins on rooting (% of shoots rooted; days to rooting in parentheses) of *Bambusa vulgaris* (20 replicates per treatment; repeated three times). a - basal callusing at the cut end; nr - no rooting.

| Concentration [μM] | | MS | 1/2 MS | 1/4 MS |
|---|---|---|---|---|
| NAA | 0.53 | 26.4±0.2 (18) | 46.2±0.3 (14) | 28.4±0.4 (20) |
| | 1.06 | 18.2±0.6 (20) | 24.4±0.3 (18) | 16.7±0.1 (24) |
| | 1.59 | nr | nr | nr |
| | 2.68 | nr | nr | nr |
| IBA | 0.49 | 66.4±0.5 (13) | 82.4±0.4 (10) | 44.8±0.2 (14) |
| | 0.98 | 38.2±0.2 (14) | 60.8±0.5 (12) | 26.8±0.7 (16) |
| | 1.47 | 16.4±0.14 (20a) | 14.2±0.2 (18a) | nr |
| | 2.46 | nr | nr | nr |

## Discussion

The present investigations have shown that it is possible to induce plant regeneration from callus derived from mature excised shoots of *B. vulgaris*. The use of different combinations of auxin and cytokinin for the production of organogenic calli and subsequent plant regeneration of some cereals and grasses is well documented (Rout and Das 1994, Rueb *et al.* 1994). The percentage of cultures showing callus initiation

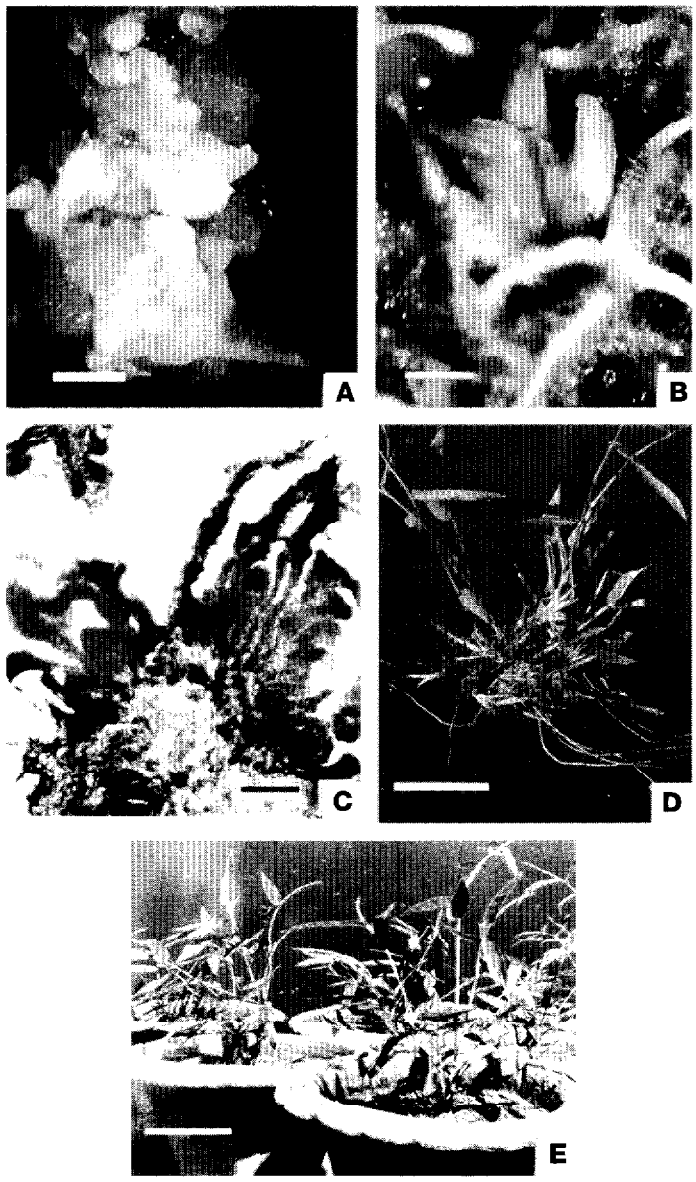


Fig. 1 *A* - Callus proliferation from excised shoots of *Bambusa vulgaris* cultured on MS + 2.22 μM BAP + 9.04 μM 2,4-D + 14.76 μM IBA after 4-week culture (*bar* = 1 mm). *B* - Shoot bud regeneration from friable callus of *B. vulgaris* cultured on MS + 13.33 μM BA + 2.46 μM IBA + 3 % (m/v) saccharose after 4-week culture (*bar* = 2.5 mm). *C* - Section of organogenic calluses with shoot apex surrounded by leaf primordia (*bar* = 0.1 mm). *D* - Rooted plantlets 10 d after transfer to 1/2 MS + 0.49 μM IBA + 2 % (m/v) saccharose containing medium (*bar* = 2.5 mm). *E* - Plantlets established in soil (*bar* = 10 mm).

in darkness was less than that observed with a light as reported earlier in other herbaceous and woody plants (Zimmerman 1985, Zaerr and Mapes 1988).

In *B. vulgaris*, 2,4-D and IBA were found to be suitable for the induction of callus at the initial stage as was reported earlier in switchgrass (Denchev and Conger 1995). IBA and NAA were found to be suitable for the induction of shoot bud regeneration; 2,4-D was not effective in the induction of shoot buds at the concentration tested. NAA and IBA have been widely used for plant regeneration in various cereals and grasses (Naboris *et al.* 1983). With the increase in the concentration of either BAP or IBA, the percentage of the shoot bud regeneration declined in contrast with the earlier reports on herbaceous and woody plant species (Zaerr and Mapes 1988, Sharma *et al.* 1990). Our results demonstrated that, the significant variation were observed among the treatments for both the percent of cultures developing shoot buds and the mean numbers of shoots per culture. The variation of response could be due to the varying concentrations of growth regulators used in the medium and to the culture condition as reported earlier *e.g.* in *Lavandula latifolia* (Calvo and Segura 1989) and in *Trema orientalis* (Samantaray *et al.* 1995). The regenerated shoot buds rapidly multiplied and elongated on similar induction medium; the rate of multiple shoot formation, however, depended on growth regulators as reported earlier in monocots and grasses (Vasil 1982, Wang and Vasil 1982, Finch *et al.* 1992, Samantaray *et al.* 1995). The regeneration ability sharply declined with increase the subculturing of calli. Similar result was reported earlier in bamboo (Rout and Das 1994). The regenerated shoots were rooted in half-strength MS basal salts supplemented with 0.49 μM IBA. The percentage of rooting was maximum at 1/2 MS as compared with full MS or 1/4 strength MS. This may be due to the balancing of the ionic concentration of nutrient salts as reported earlier in other woody plants (Das and Rout 1994, Das *et al.* 1995). This system for efficient regeneration of *B. vulgaris* from callus could be applied for rapid multiplication and mass cloning of the mature culms of other bamboo species and could help in afforestation programmes.

## References

Calvo, M.C., Segura, J.: Plant regeneration from cultured leaves of *Lavandula latifolia* Medicus: influences of growth regulators and illumination condition. - Plant Cell Tissue Organ Cult. **19**: 33-42, 1989.

Das, P., Rout, G.R.: Mass multiplication and flowering of bamboo *in vitro*. - Orissa J. Hort. **19**: 118-121, 1991.

Das, P., Rout, G.R.: Analysis of current methods and approaches on the micropropagation of bamboo. - Proc. nat. Acad. Sci. India **64**: 235-246, 1994.

Das, P., Rout, G.R., Samantary, S.: Regeneration of plantlets from callus of *Dalbergia* sp. *in vitro*. - Biologia **51**: 49-54, 1996.

Denchev, P.D., Conger, B.V.: *In vitro* culture of switchgrass: Influence of 2,4-D and picloram in combination with benzyl adenine on callus initiation and regeneration. - Plant Cell Tissue Organ Cult. **40**: 43-48, 1995.

Finch, R.P., Baset, A., Slamet, I.H., Cocking, E.C.: *In vitro* shoot culture of wild oryzae and other grass species. - Plant Cell Tissue Organ Cult. **30**: 31-36, 1992.

Lee, B.C., Kim, J.H., Park, J.I.: Induction of plantlets by bud culture in *Quercus acutissima*. - Res. Rep. Inst. Forest Genetics (Korea) **21**: 104-108, 1985.

Marascuilo, L.A., McSweeney, M.: Nonparametric and Distribution Free Methods for the Social Sciences Books/Cole Publ. Co. 1977.

Murashige,T., Skoog, F.: A revised medium for rapid growth and bioassys with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nabors, M.V., Heyser, J.W., Dykes, T.A., DeMott, K.J.: Long duration, high frequency plant regeneration from cereal tissue culture. - Planta **157**. 385-391, 1983.

Nadgir, A.L., Phadke, C.H., Gupta, P.K., Parsharami, V.A., Nair, S., Mascarenhas, A.: Rapid multiplication of bamboo by tissue culture. - Silvae Genet. **33**: 219-223, 1984.

Nehra, N.S., Stushnoff, C., Kartha, K.K.: Regeneration of plants from immature leaf-derived callus of strawberry (*Fragaria* × *Ananassa*). - Plant Sci. **66**: 119-126, 1990.

Rao, I.V., Rao, I.V.R., Narang, V.: Somatic embryogenesis and regeneration of complete plantlets in the bamboo, *Dendrocalamus strictus*. - Plant Cell Rep. **4**: 191-194, 1985.

Rout, G.R., Das, P.: Somatic embryogenesis and *in vitro* flowering of 3 species of bamboo. - Plant Cell Rep. **13**: 683-686, 1994.

Rueb, S., Leneman, M., Schilperoort, R.A., Hensgens, L.A.M.: Efficient plant regeneration through somatic embryogenesis from callus induced on mature rice embryos (*Oryza sativa* L.). - Plant Cell Tissue Organ Cult. **36**: 259-264, 1994.

Samantaray, S.S., Rout, R.R., Das, P.: *In vitro* plant regeneration from leaf base and mesocotyl cultures of *Echinochloa colona*. - Plant Cell Tissue Organ Cult. **40**: 37-51, 1995.

Samantaray, S.S., Rout, G.R., Das, P.: An *in vitro* study on organogenesis in *Trema orientalis* (Blume) Linn. - Plant Sci. **105**: 87-95, 1995.

Sharma, K.K., Bhojwani, S.S., Thorpe, T.A.: Factors affecting high frequency differentiation of shoots and roots from cotyledons explants of *Brassica juncea* (L.) Czern. - Plant Sci. **66**: 247-253, 1990.

Sexena, S.: *In vitro* propagation of the bamboo (*Bambusa tulda* Roxb.) through shoot proliferation. - Plant Cell Rep. **9**: 431-434, 1990.

Vasil, I.K.: Plant cell culture and somatic cell genetics of cereals and grasses. - In: Vasil, I.K., Scowcroft, W.R., Frew, K.J. (ed.): Plant Improvement and Somatic Cell Genetics. Pp. 179-203. Academic Press, New York 1982.

Wang, D.Y., Vasil, I.K.: Somatic embryogenesis and plant regeneration from inflorescence segments of *Pennisetum purpureum* Schum. (napier or elephant grass.) - Plant Sci. Lett. **25**: 147-154, 1982.

Woods, S.H., Phillips, G.C., Woods, J.E., Collins, G.B.: Somatic embryogenesis and plant regeneration from zygotic embryo explants in Mexican weeping bamboo, *Otatea acuminataazecorum*. - Plant Cell Rep. **11**: 257-261, 1992.

Yeh, M.L., Chang, W.C.: Plant regeneration through somatic embryogenesis in callus culture of green bamboo (*Bambusa oldhamii* Munro). - Theor. appl. Genet. **78**: 161-163, 1986.

Zaerr, J.B., Mapes, M.O.: Action of growth regulators. - In: Bonga, J.M., Durzan, D.J. (ed.): Tissue Culture in Forestry. Pp. 231-255. Martinus Nijhoff Publ., Dordrecht - Boston 1988.

Zimmerman, R.H.: Application of tissue culture propagation to woody plants. - In: Henke, R.H., Hughes, K.W., Constantin, H.J., Hollaender, A. (ed.): Tissue Culture in Forestry and Agriculture. Pp. 165-177. Plenum Press, New York 1985.