Indian J. Plant Physiol., Vol. 17, No. 1, (N.S.) pp. 37-43 (Jan.-Mar., 2012)

FACTORS AFFECTING THE FREQUENCY OF *IN VITRO* FLOWERING OF *BAMBUSA BALCOOA* ROXB.

KALPATARU DUTTA MUDOI* AND MINA BORTHAKUR

Medicinal, Aromatic and Economic Plants Division, CSIR-North East Institute of Science and Technology, Jorhat 785 006, Assam

Received on 21st February, 2011, Revised and accepted on 27th January, 2012

SUMMARY

Factors affecting the frequency of *in vitro* flowering of *Bambusa balcooa* were investigated when multiple shoots (clumps of 1, 3 and 5) were cultured in Murashige and Skoog medium (1962) supplemented with 6-benzylaminopurine (BAP 1.0 mg l^{-1}). With the increase of inoculum density (1–5 clumps) per culture vessel (250 and 500 ml) as well as delaying the sub-culturing period, shoot hyperhydricity, moisture loss and flower formation were observed, with flowers having anthers with pollen grains. The young spikelets were green in colour, which later on turned pink purple. The flowers had viable pollens but did not produce any seeds. Rooting was also induced from flowering shoots, which were transferred successfully into soil.

***Key words*: *Bambusa balcooa*, cytokinin, hyperhydric, *in vitro* flowering**

INTRODUCTION

Bambusa balcooa Roxb., an indigenous widespread bamboo of North – East India is considered the best, as it is the tallest, strongest and highly durable one, having properties mostly for structural use and pulping. Seed production in *B. balcooa* is generally not recorded after gregarious flowering. Moreover, its flowering cycle is reported as 40–100 years and occur only once during its life time (Gogoi 2004).

Flowering in bamboo is a botanical enigma and there is no scientific method yet developed for predicting flowering. Until now, the exact physiological mechanism or ecological factors responsible for bamboo flowering are not known precisely. Generally, in most cases bamboo flowering is recorded at long infrequent intervals, which occurs 2–3 times in a century. Experts have thrown few hypotheses like: (a) parental competition (b) consumer satiation, this suggests that bamboo produce large quantity of seeds and storage of food reserve takes long time (c) climatic periodicity, it said that flowering of bamboo is associated with climatic factors like drought. This assumption is similar to the climatic periodicity hypothesis where bamboo flowering was mentioned to be associated with climatic factors like drought. Drought may trigger mast flowering (Seifriz 1950). Since in most cases bamboo plants die after flowering, it is hypothesised that there may be genes that are involved in programmed flowering followed by cell death. Moreover, this situation may create a competition among the shoot clumps for survival, for which flowering may be formed for generation of offspring.

The first report on *in vitro* flowering of bamboo from axillary shoot cultures of *Dendrocalamus strictus*, *D. brandisii* and *B. arundinacea* (*B. bambos*), which developed viable spikelets and produced fertile seeds caused great excitement (Nadguada *et al.* 1990). Since then *in vitro* flowering has been reported in many

*Corresponding author: kalpataru_dm@yahoo.co.in

varieties of bamboos, generated through seedlings and mature bamboo sources viz. *D. hamiltonii* (Chambers *et al.* 1991), juvenile axillary shoot of *D. giganteus* from shoots of somatic embryonic sources of *D. giganteus, D. strictus and B. vulgaris* (Rajapakse 1992, Rout and Das, 1994, Gielis and Debergh 1998, Chang 1998, Arya and Sharma 1998, Gielis 1999). *In vitro* flowering in axillary shoot cultures was initiated from field culm source in *D. giganteus* and *B. edulis*, respectively (Ramanayake *et al.* 2001, Lin *et al.* 2003a, 2003b, Lin *et al.* 2004). Though *in vitro* flowering from numbers of bamboo species was reported but practical and commercially exploitable results have not been reported yet. Factors that trigger flowering in bamboo are still not clear. As it is not possible to observe the cyclic nature of flowering due to their long life spans, it can be taken as a new area for study of the breeding of bamboo as *in vitro* flowering can open up the possibility of controlled flowering (Rao and Zamora 1995). But many hurdles still need to be taken before the methods really become applicable at agricultural scale.

In this paper, we have reported *in vitro* flowering of *B. balcooa* (which is not yet reported earlier in this species) and studied different factors affecting the frequency of flowering.

MATERIALS AND METHODS

In vitro mass multiplication protocol for *B. balcooa* was established from field-grown mature culms source (Dutta Mudoi and Borthakur 2009). For axillary bud break, preliminary experiments were conducted by culturing node explants in MS medium (Murashige and Skoog 1962) containing various cytokinins. After selecting the best treatment (BAP 1.0 mg l^{-1}), shoot clumps were cut into groups of 2–3 shoot clusters and were sub-cultured into the same optimal medium regularly at an interval of 3–4 weeks. The process was repeated to get the desired number of shoots.

Growth medium and culture conditions: In vitro flowering was recorded during sub-culturing of *in vitro* shoots in BAP (1.0 mg l^{-1}) supplemented with MS medium containing 30 g l^{-1} sucrose and 8.0 g l^{-1} agar. All cultures were maintained under 16 h photoperiod with a light intensity of 10 ì mol m^{-2} s^{-1} (cool white fluorescent light) and a temperature of 25±2°C. In this condition, we have recorded a typical morphology of shoots before flowering. Majority of proliferated shoots and leaves became small and stunted which has been referred as hyperhydricity.

Histological study: For histological study, flowers were collected from *in vitro* shoot culture of *B. balcooa* and the anthers were separated from flowers under stereo microscope. Pollen viability was also performed and assessed by tetrazolium test (Hauser and Morrison 1964). Mature pollen grains were freshly collected from the anthers and dusted on a drop of 0.5% TTC (2, 3, 5 triphenyltetraxolium chloride) in sucrose solution and incubated in a humidity chamber at room temperature in dark for 30 min. Then they were observed under the microscope and pollen grains which stained red were scored as viable.

Data analysis: All the experiments were repeated at least thrice. Three-way ANOVA (analysis of variance) of SYSTAT 12 version has been used for the evaluation of the effect of number of clumps, vessel size and days of incubation on shoot multiplication, hyperhydricity, *in vitro* flowering, and moisture loss in *B. balcooa*.

RESULTS

It was observed that, the rate of shoot multiplication of *B. balcooa* was influenced by the vessel size, days of incubation and standard inoculum size. The *F* test in the analysis of variance showed that the number of clumps inoculated, days of incubation and vessel size had highly significant effect on the growth of clumps. Similarly, significant interactions among clump number with days of incubation and day of incubation with vessel size were observed. However, no significant interaction was observed in case of clump number with vessel size and among clump number, days of incubation and vessel size (Fig. 1A).

Under this condition if culturing period became longer (more than 30 days), situation showed hyperhydricity. In this case, we assumed that by delaying subculture, a stress condition was generally created by decreasing the nutritional status along with moisture content of the culture vessels. That stress may ultimately lead to

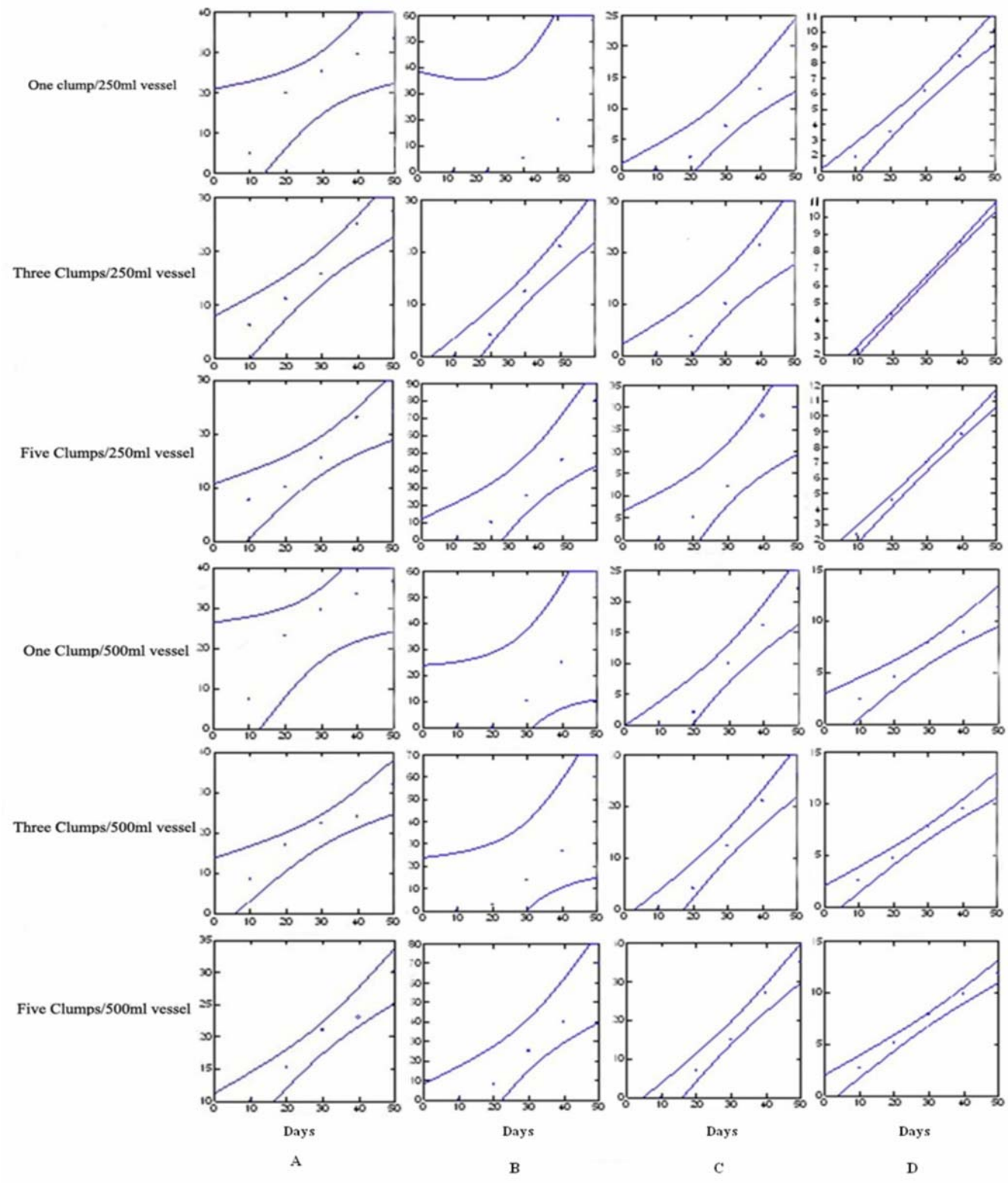

Fig. 1. Effect of clump number, vessel size and days of incubation on A. Shoot multiplication rate, B. Shoot hyperhydricity (%), C. Flower formation (%), D. Moisture loss (g), (Lines-highest and lowest confidence interval at 0.95).

hyperhydricity. The *F* test in the analysis of variance showed that the number of clumps inoculated, days of incubation and vessel size had highly significant effect on the shoot hyperhydricity (%). Similarly, highly significant interactions were observed between clump number with days of incubation, day of incubation with vessel size, clump number with vessel size and among clump number, days of incubation and vessel size (Fig. 1B).

Apart from this, study made by taking different vessel size with varied density of inocula also marked influence towards *in vitro* flowering of *B. balcooa*. The *F* test in the analysis of variance showed that the number of clump inoculated, days of incubation and vessel size had highly significant effect on the flower formation (%). Similarly, a highly significant interaction was observed between clump number with days of incubation, day of incubation with vessel size and among clump number, days of incubation and vessel size. However, interaction was not significant between clump number and vessel size (Fig. 1C).

It was noticed that the loss of moisture content was almost more than 5 times within 50 days. Further, it was also observed that the rate of moisture loss was highest in both 250 ml and 500 ml vessels at the same time interval, when maximum shoot clumps (5 nos.) were placed. The *F* test in the analysis of variance showed that the number of clumps inoculated, days of incubation and vessel size had significant effect on moisture loss. However, no significant interaction was observed between clump number with days of incubation, days of incubation with vessel size, clump number with vessel size and clump number, days of incubation and vessel size in relation to moisture loss (Fig. 1D).

We had maintained monoculture of hyperhydric shoots after 3–4 subcultures from *in vitro* multiple shoots of *B. balcooa* (Fig. 2a, b). However, retaining the cultures for a longer period in the same culture vessels/ media became brownish black and ultimately lost their blooming ability (Fig. 2c). Pseudospikelets are characteristics of bamboo species. The glumes of pseudospikelets in bamboos subtend dormant buds that can develop into new spikelets. The proximal parts of the pseudospikelets do not develop into flowers but in tissue culture conditions keep on multiplying indefinitely, allowing establishing of monocultures of pseudospikelets. Similar finding was observed by Gielis *et al.* (2002).

Normal flowers were mainly formed as buds from the nodes of shoots and the sizes of the *in vitro* flowers of *B. balcooa* were 1.0±1.5 cm. The floral structures of bamboo are basically that of the grass family and which are considered primitive among the grasses. The inflorescence itself signifies its primitiveness. When dissected under stereo microscope, it was observed that *in vitro* flowers of *B. balcooa* had contained florets with six stamens, three lodicules, and tricarpellate pistils {Fig. 2d (I–IV)}. The young spikelets were green in colour which eventually turned pink purple.

After flowering, majority of hyperhydric shoots became blackish brown in colour and ultimately died. But normal shoot could be revived from hyperhydricity, by repeated sub-culturing 3–4 times (Fig. 2e).

The flowers had anthers with pollen grains. The stamens which hung pendulously from the spikelets looked normal in shape, but no seed set occurred. Squashing with acetocarmine staining of the unopened (juvenile) flower showed viable pollen grains at early-to-late uninucleate stage {(Fig. 2f (I–II)}. Pollen viability was also assessed by tetrazolium test. However, when *in vitro* pollen germination was attempted in standard Brewbaker and Kwack's medium (1963) with a serial range of sugars, the pollens did not germinate. From this, it could be inferred that there are some physical barriers adversely affecting pollination, which ultimately lead to the failure of seed set. This supported the report of flowering cycle on field grown plants of *B. balcooa* (Gogoi 2004). However, availability of *in vitro* source of viable pollen may lead to further studies towards androgenesis. Moreover, non functional pollen grains or seeds are useful for studying their reproductive biology. As flowering in bamboo is taxonomically important for identification of species but is rare in nature, hence, only vegetative parts are available for identification. In this case flowering associated with seed set could be a perennial source of seed for propagation.

Fig. 2. *In vitro* morphogenesis of *Bambusa balcooa*: a. *In vitro* shoot multiplication, b. monocultures of hyperhydric shoots, c. *in vitro* flower formation, d. an enlarged flower with different floral parts (I–IV), e. development of normal shoots from hyperhydric shoots, f. pollen sacs and uninucleate pollen grains (I–II), g. rooting of flowering shoot, h. flowering branch bearing whorls of spikelets of field grown plant, i. an enlarged view of spikelets of field grown plant.

Similar to our earlier findings, the *in vitro* flowering shoots of *B. balcooa* were also successfully rooted in rooting medium and hardening plantlets showed well established root system (Fig. 2g). The flowering plantlets with vegetative shoots survived when they were transferred to potted soil. The histological studies revealed that *in vitro* flowers of *B. balcooa* were normal like field-grown bamboo flowers but in this case majority of associated florets were empty (Fig. 2h, i).

The effect of different ratios of inoculum density and culture vessel size on shoot growth and multiplication of *B. balcooa* revealed that the one clump (2–3 propagules/ culture vessels) inoculum density resulted in about 10–11-fold increase in total number of shoots per 250 and 500 ml culture vessel. Use of one clump/250 ml culture vessel was optimal for shoot multiplication. But higher inoculum density exhibited only 5–8-fold increase in shoot multiplication. It was observed that when inoculum density increased up to 5 clumps/culture vessel, the rate of shoot multiplication decreased thereby increasing the shoot hyperhydricity, flower formation and moisture loss.

DISCUSSION

In general, development of the morphological symptoms of hyperhydricity depends upon multiple factors expressed over time. It can be assumed that changes in anatomy, morphology and physiology had begun as soon as an explant was placed in culture

medium (Debergh *et al.* 1992). Moreover, the influence of type and concentration of growth regulators used in the medium may be one of the causes. Likewise, shoot cultures of *Sequoia sempervirens* became hyperhydric when kept continuously on a medium containing BAP (Franclet 1991). By repeated sub-culturing, generation of healthy shoot proliferation was recorded from hyperhydric shoot cultures of *S. sempervirens*. The presence of cytokinin and stress attributed *in vitro* flowering was reported by various workers (Kataeva *et al.* 1991, Gielis *et al.* 1997, Nadguada *et al.* 1997, Ramanayake *et al.* 2001). Similarly, supplementation of BAP exogenously may be one of the responsible factors for *in vitro* flowering of *B. arundinacea*, *D. brandisii* and *D. hamiltonii* (Joshi and Nadguada 1997). Hence, it can be said that BAP plays an important role in the induction of flowering when applied exogenously to the shoot cultures. Positive response of BAP towards *in vitro* flowering was reported in case of other plant species like in *Pisum sativum*, *Perilla frutescens* when applied alone or in combinations (Franklin *et al.* 2000, Zhang 2007).

Control and reversion of flowering is very important in bamboo (Gielis *et al.* 2002). Use of optimal shoot clump/culture vessel was the prerequisite factor for controlling *in vitro* flowering and shoot multiplication of *B. balcooa*.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. P.G. Rao, Director, CSIR-North East Institute of Science & Technology, Jorhat, Assam, India for his kind permission to carry out the research work and for providing the facilities during the period.

REFERENCES

Arya, S. and Sharma, S. (1998). Micropropagation technology of *Bambusa bambos* through shoot proliferation. *Indian For.* **124:** 725-731.

Brewbaker, J.L. and Kwack, B.H. (1963). The essential role of calcium ion in pollen germination and pollen tube growth. *Am. J. Bot.* **50:** 747-858.

Chambers, S.M., Heuch, J.H.R. and Pirrie, A. (1991). Micropropagation and *in vitro* flowering of the bamboo (*Dendrocalamus hamiltonii*) Munro. *Plant Cell Tissue Organ Cult.* **27:** 45-48.

Chang, W.C. (1998). Micropropagation of *Bambusa edulis* through nodal explants of field grown culms and flowering of regenerated plantlets. *Plant Cell Rep.* **17:** 617-620.

Debergh, P.T., Aitken-Christie, J., Cohen, D., Grout, B., Von, A.S., Zimmerman, R. and Ziv, M.L. (1992). Reconsideration of the term 'vitrification' as used in micropropagation. *Plant Cell Tissue Organ Cult.* **30:** 135-140.

Dutta Mudoi, K. and Borthakur, M. (2009). *In vitro* micropropagation of *Bambusa balcooa* Roxb. through nodal explants from field-grown culms and scope for upscaling. *Curr. Sci.* **96:** 962-966.

Franclet, A. (1991). Biotechnology in 'rejuvenation': hope for the micropropagation of difficult woody plants. *Acta Hort.* **289:** 273-282.

Franklin, G., Pius, P.K. and Ignacimuthu, S. (2000). Factors affecting *in vitro* flowering and fruiting of green pea (*Pisum sativum* L.). *Euphytica* **115:** 65-74.

Gielis, J., Geotghebeur, P. and Debergh, P. (1997). Morphological and biochemical aspects of flowering in bamboo- the development of model systems. *In*: G.P. Chapman (ed.), The Bamboos, (Academic Press, London), pp. 179-186.

Gielis, J. and Debergh, P.C. (1998). *In vitro* flowering of bamboos. *The flowering Newsletter*, **26:** November.

Gielis, J. (1999). Micropropagation and *in vitro* flowering of temperate and tropical bamboos. *In*: S.P. Raychaudhuri and K. Maramorosch (eds.), Biotechnology and plant protection in forestry sciences. (Science Publishers, Inc., USA), pp. 13-38.

Gielis, J., Peeters, H., Gillis, J. and Debergh, P.C. (2002). Tissue culture strategies for genetic improvement of bamboo. *In*: Van, H.J. Van, B.E. and Debergh, P. (Eds.) Proceedings of the twentieth International Eucarpia Symposium. *Acta Hort.* **552:** 195-203.

Gogoi, P. (2004). Sachyor uttam banh. *The Dainik Janambhumi* **4:** 31 October.

Hauser, E.J.P. and Morrison, J.H. (1964). The cytochemical

reduction of nitroblue tetrazolium as an index of pollen viability. *Am. J. Bot.* **51:** 748-752.

Kataeva, N.V., Alexandrova, I.G., Butenko, R.G. and Dragavtceva, E.V. (1991). Effect of applied and internal hormones on vitrification and apical necrosis of different plants cultured *in vitro*. *Plant Cell Tissue Organ Cult.* **27:** 149-154.

Joshi, M.S. and Nadguada, R.S. (1997). Cytokinin and *in vitro* induction of flowering in Bamboo: *Bambusa arundinacea* (Retz.) Wild. *Curr. Sci.* **73:** 523-526.

Lin, C.S., Chen, C.T. and Lin, C.C. (2003a). A method for inflorescence proliferation. *Plant Cell Rep.* **21:** 838-843.

Lin, C.S., Lin, C.C. and Chang, W.C. (2003b). *In vitro* flowering of *Bambusa edulis* and subsequent plantlet survival. *Plant Cell Tissue Organ Cult.* **72:** 71-78.

Lin, C.S., Lin, C.C. and Chang, W.C. (2004). Effect of thidiazuron on vegetative tissue-derived somatic embryogenesis and flowering of *Bambusa edulis. Plant Cell Tissue Organ Cult.* **76:** 75-82.

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

Nadguada, R.S., Parasharsmi, V.A. and Mascarenhas, A.F. (1990). Precocious flowering and seeding behaviour in tissue cultured bamboos. *Nature* **344:** 335.

Nadguada, R.S., John, C.K., Joshi, M.S., Parasharami, V.A. and Mascarenhas, A.F. (1997). Application of *in vitro* techniques for bamboo improvement. *In*: G.P. Chapman (ed.), The Bamboos. (Academic Press, London), 163-177.

Rajapakse, M.C. (1992). Studies on the *in vitro* development of *Dendrocalamus giganteus*. M. Phil thesis, Post Graduate Institute of Agriculture. University of Peradeniya, Sri Lanka.

Ramanayake, S.M.S.D., Wanniarachchi, W.A.V.R. and Tennakoon, T.M.A. (2001). Axillary shoot proliferation and *in vitro* flowering in an adult giant bamboo *Dendrocalamus giganteus* Wall. Ex. Munro. *In Vitro Cell Dev. Biol. (Plant)*, **37:** 667-671.

Rao, I.V.R. and Zamora, A.B. (1995). *In vitro* flowering and its implications for bamboo development. *Genetic Enhancement of Bamboo and Rattan* **7:** 61-67.

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

Seifriz, W. (1950). Gregarious flowering of *Chusquea. Nature* **22:** 635–636.

Zhang, T. (2007). Studies on *in vitro* flowering and fruiting of *Perilla frutescens. Agri. Sci. China* **6:** 33-37.