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Callus and cell suspension culture of bamboo plant, *Phyllostachys nigra*

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Abstract A modified 1/2MS medium supplemented with 3 μM 2,4-D was effective for callus induction from bamboo shoots of *Phyllostachys nigra* Munro var. *Henonis*. In the first phase (phase 1), some parts of the explants enlarged and gave rise to whitish-yellow calli after 2-3 weeks of culture. During maintenance subcultures, almost all explants and the initially formed calli turned brown and these calli gradually lost their proliferation capacity (phase 2). Removal of the necrotic potions of explants, and frequent subcultures at phase 2 was essential. Secondary proliferated calli were subsequently produced on the surface of brown tissues (phase 3). These calli could be maintained on both solid and liquid media. The liquid suspension cells had a blue to pale blue autofluorescence in the cell walls. These cells fluoresced strongly when stained with Calcofluor White M2R and Aniline Blue, indicating the presence of callose (β-1,3-glucan) in a cellulosic wall. Endogenous free amino acids analyses indicated that glutamine, γ-aminobutyric acid, and alanine were the major amino acids in callus tissues whereas asparagine and tyrosine were abundant in the regenerated bamboo shoots.

Key words: Bamboo, callus, *Phyllostachys nigra*, suspension culture.

Bamboo plants distribute from tropical to temperate zones of Southeast Asia and they provide useful resources for local economies. The clums of bamboos have a variety of usages. They can be used as materials for house construction, daily sundry goods, agricultural and fisheries tools, and crafting materials. Young shoots are important as food materials as well (Chang 1991; Chang and Ho 1997). As bamboos are fast-growing plants, recently they are considered as a prime renewable resource for biomass production. Furthermore, the importance of bamboo forests as a potential modulator of global environment has been proposed (Bystriakova et al. 2004). Therefore, development of new utilities for bamboo plant is highly recommended. For these reasons, reconstruction methods of bamboo forests for stable supply of materials and analysis of metabolites of bamboo plants for production of new functional chemicals are essential. The common multiplication methods for bamboos are by rhizome planting, culm cutting and seedling cultivations (e.g. Kumar and Pal 2004; Uchimura 1981, 1990). Regarding endogenous metabolites, such as antioxidant, prooxidant, anti-bacterial, or aroma-active compounds, direct chemical analyses have been carried out in different parts of a bamboo plant (Fu et al. 2002; Hu et al. 2000; Nishina et al. 1991).

By adapting the latest plant biotechnologies, new utilities of bamboos could progress beyond the current level. Tissue culture is one essential technique to micropropagate regenerated plant tissues and it is also a pre-requisite for genetic improvement through the use of different transformation strategies. At present, reports dealing with bamboo tissue culture are described mainly for the genus *Bambusa* (Kalia et al. 2004; Lin et al. 2003, 2004; Saxena 1990) and *Dendrocalamus* (Ramanayake et al. 2001; Saxena and Dhawan 1999; Singh et al. 2003; Sood et al. 2002). Little information is available for the genus *Phyllostachys*.

Phyllostachys pubescens Mazel ex Houz de Lehie (mouso-bamboo), *P. bambusoides* Sieb. Et Zucc (madake-bamboo), and *P. nigra* Munro var. *Henonis* (hachiku-bamboo) are the three major bamboo species found in Japan (Takahasi 1991). In order to find new utilities for the Japanese bamboos, tissue culture methods need to be established. The purpose of this study is to establish an efficient cell culture protocol for *P. nigra* and to reveal morphological and physiological characteristics of the cultured cells for further manipulations of the cell culture protocol of this potentially functional bamboo species.

Abbreviations: BA, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; MS medium, Murashige and Skoog medium; Picloram, 4-amino-3,5,6-trichloropyridine-2-carboxylic acid.

Materials and methods

Plant materials

Bamboo shoots of *P. nigra* were collected in mid-May 2003 and from early to mid-June 2004 at Takayama, Nara, Japan. Young bamboo shoots 20–30 cm in length were selected as plant materials. Culm-sheaths were removed, and then washed in water with several drops of a detergent for 30 min. After soaking, the shoots were surface sterilized first with 70% ethyl alcohol for 20 min followed by a 2% NaClO with several drops of Tween 20 for 60 min. After sterilization, they were rinsed 3 times with sterile distilled water, dried on sterile paper, and used as explants.

Induction and maintenance of bamboo calli

A modified half strength Murashige and Skoog (m1/2MS) medium (Murashige and Skoog 1962) was used as the basal medium. The concentrations of inorganic elements were reduced to half of the original and 30 g l^{-1} sucrose was added. Plant growth regulators 2,4-D, Picloram, and BA were added to m1/2MS medium at 0, 1, 3, 10, and 30 μM, respectively. The pH of the media was adjusted to 5.7 and 3 g l^{-1} gellan gum was added before autoclaving. The complete media were autoclaved for 20 min at 120°C and a 20 ml aliquot of each medium was poured into a 90×15 mm petri dish. Inner portions of the sterile bamboo shoots (ca. 1–3 cm^2) were excised and 2–4 pieces of the excised tissues were cultured on each dish. Maintenance subculture was carried out on m1/2MS media of the same compositions of the induction media at 3- to 4-week intervals. All the dishes with explants were incubated in the dark at 26°C.

Initiation of a suspension culture

Liquid suspension culture of *P. nigra* was generated as follow. Approximately 500 mg fresh weight of callus tissues derived from bamboo shoots was cultured in 50 ml liquid m1/2MS medium of the same compositions as the induction-maintenance media in a 200-ml conical flask. The flasks were placed on a rotary shaker (SHK-U4, IWAKI, Japan) with a speed of 110 rpm in the dark at 26°C.

Observations

Morphological and histochemical characteristics of calli and suspension cells were observed using a stereo microscope (SZ61, Olympus, Japan) and an inverted fluorescence microscope (IX70, Olympus, Japan). In order to determine histochemical characteristics of the bamboo cells, autoflurorescence was first examined using the fluorescence microscope under U-excitation fluorescence light (U-MWU, excitation filter; 330–385 nm, extinction filter; 420 nm). Then the cells were stained with 0.01% Calcofluor White M2R or 0.05% Aniline Blue in 0.15 M Na_2HPO_4 buffer (pH 8.6) according to Yeung (1984) and observed under B- or GFP-excitation fluorescence lights (U-MNIBA, excitation filter; 470–490 nm, extinction filter; 515–550 nm, U-MGFPHQ, excitation filter; 460–480 nm, extinction filter; 495–540 nm) for the detection of cellulose and callose.

Extraction and analysis of free amino acids

The callus tissues and bamboo shoots (ca. 100–200 mg fresh weight) were collected in a 1.5-ml micro test tube (Eppendorf, Germany), frozen in liquid nitrogen, and stored at −80°C. Soluble free amino acids were extracted from the samples by soaking and homogenizing them twice for 30 min each at room temperature with a total of 2 ml of 1 N HCl. The extracts, which were centrifuged at 15000 *g* for 3 min and filtered using a 0.45 μm mesh filter (Amicon, Ultrafree-MC, Millipore, USA), were used as the free amino acid fractions. The fractions were diluted with 0.02 N HCl at different concentrations of amino acids (ca. 0.5–10 nmol levels) per independent sample. Three independent fractions per sample were measured by using an automatic amino acid analyzer (L-8500, Hitachi, Japan) according to a procedure appended. Free amino acids were identified by co-chromatography with a standard.

Results

Effect of plant growth regulators on callus induction and its proliferation

As shown in Table 1, the effects of 2,4-D, Picloram, and BA on callus induction were initially tested using the bamboo shoots of *P. nigra* collected in mid-May 2003. The initial sign of callus formation began approximately 2–3 weeks after culture (Figure 1A). In this phase (phase 1), surviving explants (0–85%) enlarged in part and whitish-yellow calli proliferated on the surface of the explants. Abundant callus formation could be found from cut pieces of the shoots in some media containing 3–30 μM of 2,4-D and 3–10 μM of Picloram, whereas BA-containing media had a negative effect on callus induction. The explants with calli were then transferred to m1/2MS media of the same composition as the induction media at 3- to 4-week intervals. During maintenance subcultures (ca. 3–6 months), almost all explants and the calli which were initially formed at phase 1 turned brown in parts (arrowheads in Figure 1B) and the calli gradually lost their proliferation capacity (phase 2). In order to avoid such necrosis, removal of the necrotic potions of the explants and frequent subculture at appropriate 2- to 3-week intervals, were very effective. Secondary calli appeared on the surface of the explants and/or the brown-colored calli in media containing especially 3 and 10 μM 2,4-D (phase 3). Two types of

Table 1. Effect of plant growth regulators on callus induction from bamboo shoots of *P. nigra.*

Plant growth regulator (μM)		Growth response [a]		
		Phase 1	Phase 2	Phase 3
2,4-D	0	No response	Browning	Dead
	1	Callusing slightly	Browning	Secondary callusing
	3	Callusing [b]	Browning [c]	Secondary callusing/root primordia [d,e]
	10	Callusing	Browning	Secondary callusing/root primordia
	30	Callusing	Browning	Dead
Picloram	1	Callusing slightly	Browning	Secondary callusing
	3	Callusing	Browning	Secondary callusing/root primordia
	10	Callusing	Browning	Secondary callusing/root primordia
	30	Callusing slightly	Browning	Dead
BA	1	Callusing slightly	Browning strongly	Dead
	3	Callusing slightly	Browning strongly	Dead
	10	Browning	Browning strongly	Dead
	30	Browning	Browning strongly	Dead

[a] Growth response was summarized based on the features from 15 independent bamboo shoots corrected from 2 different locations. At least 6 pieces of explants were cultured in each condition. During subculture at phase 2, one explant was divided into several chips.
[b] Callusing observed at phase 1 was shown in Figure 1A.
[c] Browning observed at phase 2 was shown in Figure 1B.
[d,e] Secondary callusing/root primordia observed at phase 3 were shown in Figures 1C and D, respectively.

cell tissues, i.e. whitish calli and calli with distinct root primordia could be found at the phase 3 stage (Figure 1C and D, respectively). Since similar results were obtained using bamboo shoots collected in early to mid-June 2004 (duplicated with 6 independent bamboo shoots in total, data not shown), the m1/2MS medium containing 3μM of 2,4-D was the best medium for callus induction from bamboo shoots of *P. nigra*.

Suspension culture and its morphological and histochemical characteristics

The secondary calli induced at phase 3 could be maintained on the solid induction-maintenance media. In order to investigate morphological and histochemical characteristics of these cells, a liquid suspension culture was established. Approximately 500 mg fresh weight of callus tissues was transferred in 50 ml liquid m1/2MS medium containing 3 μM of 2,4-D. Suspension cells were initially generated as shown in Figure 1E within 3–6 weeks after culture. Morphologically, they consisted mainly of cell aggregates of round to oval shaped cells, which were 200–500 μm in diameter. In order to maintain the suspension culture, a portion (2–4 ml) of liquid suspension cells was transferred to fresh m1/2MS liquid medium at 3–4-week-intervals. The suspension cells actively proliferated keeping their morphology. When viewed with a fluorescence microscope, the cell walls fluoresced brightly. A blue to pale blue autofluorescence was present in the walls (Figure 1F). After staining with Calcofluor White M2R or Aniline Blue, a whitish-yellow to green fluorescence was clearly observed in the walls of bamboo cells (Figures 1G, H).

Comparison of endogenous free amino acids between bamboo shoots and calli

As shown in Table 2, 12758.2 μg g fresh weight^{-1} of free amino acids was detected in bamboo shoots. Asparagine and tyrosine were abundant, 35.7% and 21.8% respectively. On the other hand, the quantity of free amino acids in callus tissues was 6743.6 μg g fresh weight^{-1}. Glutamine (41.9%), γ-aminobutyric acid (21.9%), and alanine (14.3%) were the major amino acids in the callus tissues.

Discussion

Callus can be obtained from various bamboo tissues using media containing an auxin such as 2,4-D and Picloram (e.g. See Chang 1991). The author reported here a callus and cell suspension culture method for *P. nigra*. In order to induce callus from bamboo shoot tissues, the addition of 3 μM 2,4-D is essential. On the other hand, BA, a cytokinin, has a negative effect on callus induction and proliferation. The treatment of BA (1–30 μM) caused strong browning of the explants at phase 2, and secondary callus formation was inhibited at phase 3. Browning of excised explant tissues as well as tissue culture media occurs frequently and this is most likely the mean reason for recalcitrancy of tissues *in vitro*. To avoid browning, activated charcoal and/or polyvinylpyrrolidone (PVP) are often added to tissue culture media. Saxena and Dhawan (1999) reported that the addition of 250 mg l^{-1} PVP to a medium was effective in overcoming the browning of the culture medium and promoted the growth of somatic embryos in a bamboo (*Dendrocalamus strictus*) tissue culture. The author had also tested the effect of PVP (0, 500, 1000 mg l^{-1}) on callus induction from bamboo shoots of *P. nigra* and

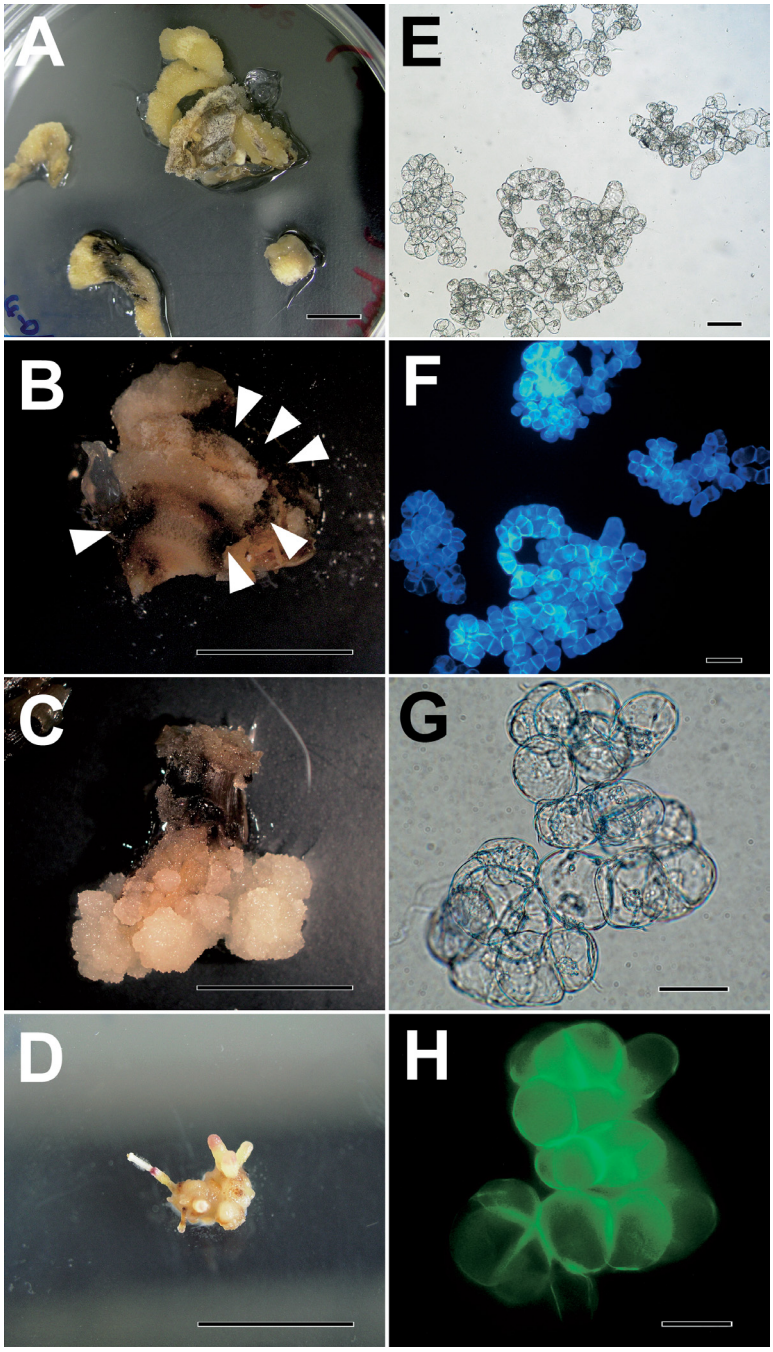

Figure 1. Callus and suspension culture of *P. nigra*. (A) A cut piece of bamboo shoots cultured on m1/2MS medium supplemented with 3 μM of 2,4-D. The explants enlarged in part and yellow-whitish calli proliferated on the surface (phase 1). (B) Browning of the explants (arrowheads) during phase 2. (C) Secondary proliferation of calli on the surface of the explants (phase 3). (D) The appearance of a bunch of root primordia derived from a bamboo shoot. (E) and (G) The appearance of suspension cells. (F) A blue to pale blue autofluorescence suggests abundant deposition of cell wall compounds in the cells. (H) These cells fluoresced greenly under GFP-excitation fluorescence light when stained with Aniline Blue, which is a specific dye for β-1,3-glucans. Scale bars in A–D = 1 cm, in E and F = 100 μm, in G and H = 50 μm, respectively.

found that the addition of PVP did not promote callus formation (data not shown). This observation confirms the report by Huang et al. (2002) that PVP failed to completely suppress the browning of *P. nigra* shoot culture.

When the brown necrotic portions of tissues were removed from the explants and frequent subcultures were performed at phase 2, secondary calli (phase 3) appeared. Hence, it is clear that even though the explants gradually turned brown on the callus induction medium, inhibitory factor(s) could be removed from the culture system by simple manual manipulation as described

Table 2. Endogenous free amino acids in bamboo shoots and callus tissues of *P. nigra.*

Amino acid (μg g fresh weight^{-1})	Bamboo shoots			Callus tissues		
	Average	SD	% total	Average	SD	% total
Aspartic acid	510.7 ±	13.9	(4.00)	58.9 ±	8.0	(0.87)
Threonine	168.5 ±	26.4	(1.32)	185.8 ±	49.6	(2.76)
Serine	531.5 ±	72.8	(4.17)	183.4 ±	14.5	(2.72)
Asparagine	4548.8 ±	973.0	(35.65)	41.3 ±	8.1	(0.61)
Glutamic acid	725.4 ±	73.3	(5.69)	232.9 ±	21.5	(3.45)
Glutamine	477.6 ±	67.3	(3.74)	2828.0 ±	388.7	(41.94)
Glycine	54.6 ±	13.1	(0.43)	62.3 ±	16.6	(0.92)
Proline	80.1 ±	7.8	(0.63)	67.8 ±	14.4	(1.01)
Alanine	216.7 ±	38.3	(1.70)	960.8 ±	129.0	(14.25)
Citrulline	11.0 ±	1.8	(0.09)	20.4 ±	5.8	(0.30)
Valine	338.3 ±	59.0	(2.65)	142.3 ±	18.1	(2.11)
Cystine	41.5 ±	10.7	(0.33)	ND		(–)
Methionine	127.2 ±	25.4	(1.00)	53.9 ±	14.6	(0.80)
Isoleucine	253.8 ±	38.7	(1.99)	52.0 ±	10.7	(0.77)
Leucine	273.4 ±	41.5	(2.14)	72.3 ±	19.2	(1.07)
Tyrosine	2779.5 ±	383.9	(21.79)	39.6 ±	4.8	(0.59)
Phenylalanine	222.2 ±	35.5	(1.74)	23.0 ±	7.4	(0.34)
β-Alanine	45.3 ±	9.2	(0.36)	42.1 ±	3.7	(0.62)
γ-Aminobutyric acid	70.1 ±	26.8	(0.55)	1475.8 ±	264.6	(21.88)
Ornithine	3.7 ±	1.9	(0.03)	8.4 ±	0.6	(0.13)
Lysine	407.0 ±	89.5	(3.19)	49.8 ±	15.0	(0.74)
Histidine	259.4 ±	27.8	(2.03)	19.1 ±	4.1	(0.28)
Arginine	538.4 ±	147.7	(4.22)	32.9 ±	7.8	(0.49)
Ethanolamine	15.3 ±	0.9	(0.12)	25.0 ±	2.9	(0.37)
Ammonium chloride	58.4 ±	11.6	(0.46)	65.5 ±	1.7	(0.97)
Total	12758.2		(100)	6743.6		(100)

Three independent fractions per each sample were measured. ND: not detected.

above. Since this protocol was also applicable for callus induction from bamboo shoots of *P. bambusoides* and *P. pubescens* (unpublished result), it is safe to conclude that the callus initiation process is similar for the Japanese *Phyllostachys* bamboos.

The suspension culture system offers many advantages for examining the metabolic role of nutrients and their utilization in plant cells of interest. In order to use the suspension culture system for examinations, a stable morphology and synchronous growth of cells are required. Furthermore, in order to elucidate physiological and molecular biological features of plant cells, a reproducible culture system is required. In this study, the author established an efficient suspension culture protocol and investigated morphological, histochemical and physiological characteristics of the bamboo cells for further manipulations. Suspension cells of *P. nigra* derived from secondary calli proliferated on m1/2MS medium containing 3 μM of 2,4-D had a homogenous feature from a morphological viewpoint. The most important observation of this study is that suspension cells of bamboo had a high proliferation potential with abundant deposition of cell wall compounds. Cell wall compounds of bamboos (*Arundinaria japonica* and *A. anceps*) have been studied (Kenneth et al. 1977). β-glucans having (1,3-) and (1,4-) links have been isolated from the leaves and stems of plants of both species. In this study a histochemical approach was carried out to complement the biochemical study. The cell wall compounds were Calcofluor White M2R positive (data not shown). This indicates the presence of β-1,4-glucans and/or β-1,3-glucans. The cells also fluoresced whitish-yellow to green when stained with Aniline Blue, which is a specific dye for β-1,3-glucans. GFP-excitation fluorescence light (U-MGFPHQ) served for the detection of callose. From these novel findings, cell wall biosynthesis appears to be unique in the bamboo cultured cells.

Amino acids and their derivatives are considered as a good physiological marker for plant cells of interest (Wallsgrove 1995). Comparison of endogenous free amino acids between bamboo shoots and calli gave unique information for further manipulations of the tissue culture protocol. In bamboo shoots of *P. nigra*, tyrosine was the major amino acid as described by Kozukue et al. (1988). On the other hand, tyrosine content in the bamboo calli was remarkably low. Glutamine, γ-aminobutyric acid, and alanine were abundant in the calli instead. Glutamine derived from nitrogen assimilation has been known as a key metabolic regulatory molecule in higher plants (Wallsgrove 1995). Glutamine is utilized in different metabolic pathways in various parts of the plant at different stages of development. In plant tissue culture, glutamine has also

been recognized to be an effective compound which assists in the growth of plant tissues *in vitro*. For example, endogenous and/or exogenous glutamine played an important role in embryogenic tissues proliferation and development in *Cryptomeria japonica* (Ogita et al. 2001). The pool of glutamine in the calli of *P. nigra* could play an important part in the organogenetic process. The content of γ-aminobutyric acid, a metabolite of the catabolism of glutamic acid and arginine (Mazelis 1980), was extremely high compared with that in bamboo shoots. Furthermore, alanine, a major derivative of pyruvate (Buchanan et al. 2000), was abundant. These results also suggest that typical amino acid metabolisms and/or glycolysis would be activated in the bamboo calli.

With these results, the callus and suspension culture protocol presented in this study can provide a tool not only to study the physiological and molecular biological events of bamboo plants, but also to develop new utilities of bamboo cells for industrial applications as reviewed by Hellwig et al. (2004).

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