Tissue Culture Note

In vitro node culture of seedlings in bamboo plant, *Phyllostachys meyeri* McClure

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Abstract *Phyllostachys meyeri* cultivated in the Fuji Bamboo Garden, Japan showed monocarpic mass flowering from 2004 through 2005. We first examined *in vitro* germination of the caryopses and found that sterilized water supplemented with 0.1% Plant Preservative Mixture™ was effective for their growth with an efficient germination rate (75%). Seedlings were transferred to a liquid modified half strength Murashige and Skoog (m1/2MS) medium for further growth and were acclimatized in soil mixture under *ex vitro* condition. Nodal segments which were collected from *ca.* 1.5 year-old seedlings and 1 year-old tissue cultured clone plants were re-sterilized and then cultured in a liquid m1/2MS medium. Shoot elongation with rooting could be seen within 8 weeks and they were successfully multiplied under *ex vitro* condition.

Key words: Bamboo, caryopsis, node, *Phyllostachys meyeri*.

Bamboo plants can be classified into two types by their growth feature (Uchimura 1990). One is monopodial and single culm-forming type which grows mainly in temperate and subtropical regions and the other is sympodial and clump-forming type which is distributed in tropical regions. Genus *Phyllostachys*, one of major bamboos found in Japan belongs to the former. Since bamboo plants have unique growth characteristics such as fast growing, long life span, and monocarpic mass flowering, it is quite important to investigate physiological and molecular biological events in their growth for better understanding of this unique plant species. To date, plant tissue culture is one of the essential techniques to micropropagate a target plant in a short period of time and it is also a promising tool for elucidating growth characteristics and developmental potential of the plant. There are many reports dealing with tissue culture of bamboos (e.g. reviewed by Chang and Ho 1997; Ramanayake 2006). The sympodial bamboo plants both of genus *Bambusa* and genus *Dendrocalamus* are well documented. On the other hand, little information is available for the genus *Phyllostachys* (Hassan and Debergh 1987; Huang et al. 1989; Huang et al. 2002).

P. meyeri cultivated in the Fuji Bamboo Garden, Japan showed monocarpic mass flowering from 2004 through 2005 (Hisamoto et al. 2005). Since several mature caryopses were collected from this flowering *Phyllostachys* bamboo, we report here a tissue culture protocol for *P. meyeri* with particular attention to *in vitro* germination of the caryopses and plant regeneration from nodal segments of the germinated and tissue cultured seedlings.

Mature infructescences of *P. meyeri* were collected in mid-September and mid-December 2005 at the Fuji Bamboo Garden, Japan. The mass flowering process and morphology of the flower were detailed by Hisamoto et al. (2005). Thirty seven mature caryopses were obtained from *ca.* 2000 spikelets. A sterilization protocol was finalized as follows. The caryopses were washed by soak into tap water with several drops of a detergent for 10 min. Next, they were surface sterilized with 70% ethyl alcohol for 10 min followed by 2% NaClO with several drops of Tween 20 for 20 min and 3% H_2O_2 for 20 min. After the sterilization, they were rinsed 3 times with sterile distilled water containing 0.1 % (v/v) of Plant Preservative Mixture™ (PPM, Nakarai, Japan), dried on sterile paper, and used for a germination test *in vitro*. The caryopses were cultured in either 2 ml of liquid MS (Murashige and Skoog 1962), modified half strength MS (m1/2MS) medium of which concentrations of inorganic elements were reduced to half of the original, or sterile distilled water, with/without 0.1 % (v/v) of PPM in a well of 6 well micro-plate. Seedlings obtained were

Abbreviations: MS medium, Murashige and Skoog medium; PPM, Plant Preservative Mixture™.
This article can be found at http://www.jspcmb.jp/

transferred to a liquid phytohormone-free m1/2MS medium and were then acclimatized in a soil mixture.

Nodal portions, *ca.* 20 mm in length with a lateral bud, were collected from 3 independent seedlings of *ca.* 1.5 year-old maintained in a greenhouse. They were surface sterilized with the same protocol as described above, dried on sterile paper, and used for shoot multiplication test. The nodal explants were cultured in 2 ml solid or liquid MS or phytohormone-free m1/2MS medium in a well of 6 well micro-plate for 4 weeks and then transferred to 10 ml of the same liquid media in 100 ml conical flasks for another 4 weeks. Thirty $g\,l^{-1}$ sucrose was added in all media used and the pH was adjusted to 5.7 before autoclaving. Three $g\,l^{-1}$ gellan gum was added in a medium when it is necessary. The media were autoclaved for 20 min at 120°C. All the cultures were incubated at 25°C, with a 16-h photoperiod under a fluorescent illumination ($65\,\mu mol\,m^{-2}\,s^{-1}$). The tissue cultured clone plants obtained were reused as donor plants for node culture.

As a preliminary experiment, we tested 3 conditions, i.e. MS, m1/2MS, and sterile distilled water, with a sterilization protocol as follows. Fifteen caryopses soaked into tap water with several drops of a detergent for 10 min were surface sterilized with 70% ethyl alcohol for 5 min followed by a 2% NaClO with several drops of Tween 20 for 20 min. After the sterilization, they were rinsed 3 times with sterile distilled water, dried on sterile paper, and used for germination test *in vitro*. Five caryopses were cultured in each medium condition. This sterilization protocol was not optimal due to a bacterial contamination. However, the treatments did allow for the promotion of germination with the sterile water treatment showing the greatest amount of germination due to the least amount of contamination (data not shown). Based on this observation, the soaking time in 70% ethyl alcohol was changed to 10 min, and 3% H_2O_2 and 0.1% PPM were used as described in materials and methods. In addition, we used sterile distilled water containing 0.1% PPM as a germination medium.

The improved sterilization protocol was quite effective and *in vitro* germination was observed within 2 weeks as shown in Figure 1A. Twelve out of 16 caryopses tested showed high germination ability (75%). After 1 month of culture in sterile distilled water containing 0.1% PPM, propagated seedlings were transferred to a liquid phytohormone-free m1/2MS medium in conical flasks and continued to develop *in vitro* as shown in Figure 1B.

Two to three-month-old seedlings which developed healthy shoots and roots *in vitro* were washed with running tap water and then transferred to a soil mixture in a plant pot. They were cultured for approximately 1 month in a culture room first to naturalize to an air condition, and then transferred to a greenhouse. As shown in Figure 1C and 1D, several culms with newly sprouted leaves elongated and rhizomes also actively formed within 9 months of the acclimatization process.

The effect of medium conditions, both solid and liquid m1/2MS, on node culture of *P. meyeri* was tested. It was observed that liquid m1/2MS medium had a positive effect on elongation of axillary buds (5 out of 9 nodal explants actively elongated) whereas solid m1/2MS medium showed a negative effect on the growth of explants (9 out of 10 nodal explants were turned brown and/or showed stunted shoot growth). Therefore, we investigated the effects of liquid media, both m1/2MS and MS, on shoot elongation and root formation from axillary buds of nodal segments.

Nearly 50% axillary buds of nodal segments sprouted and elongated within 40 days of culture as shown in Figure 1 E. Some of them induced 1-3 roots mainly from the basal node portion of the shoots. It was observed that phytohormone-free m1/2MS medium had a promoting effect on rooting from seedling #1 derived nodal segment after 8 weeks of culture (Table 1, Figure 1E). The average of rooting (29.7%) in m1/2MS medium was higher than in MS medium (21.2%). The regenerated plantlets with well elongated shoots and roots (Figure 1F) were washed with running tap water and then transferred to a soil mixture in a plant pot. These regenerated bamboo plants could be acclimatized to the *ex vitro* condition according to the same protocol.

Then the effect of liquid 1/2MS medium on shoot elongation and root formation was verified by using another 2 independent seedlings and 2 tissue cultured clone plants. *In vitro* rooting was induced from all of the donor plants as shown in Table 1 although the tendency of shoot elongation and/or rooting varied according to the donor plants. Seedling #1 and its tissue cultured clone plants, i.e. #1-1 and #1-2, showed similar shoot growth. Nearly 50% of nodal segments elongated and half of them formed roots (16.7%–29.7%). All of the elongated shoots yielded roots (27.8%) in seedling #2. Seedling #3 showed, however, poor root formation capacity (5.0%). Regenerated plantlets were washed with running tap water and then transferred to a soil mixture in a plant pot. They were all successfully naturalized to the *ex vitro* condition, and then transferred to a greenhouse (Figure 1G) using the same process as the germinated seedlings.

Since *in vitro* germination of embryos was reported by Alexander and Rao (1968), infructescence organs, such as embryos and caryopses, were often used for explants of micropropagation in bamboo plants. There are many reports on micropropagation of sympodial and clump forming bamboos which are distributed in tropical regions, like genus *Bambusa* and *Dendrocalamus* (see Ramanayake 2006). However, little information is available for the genus *Phyllostachys* because of the difficulty for getting infructescence organs due to their

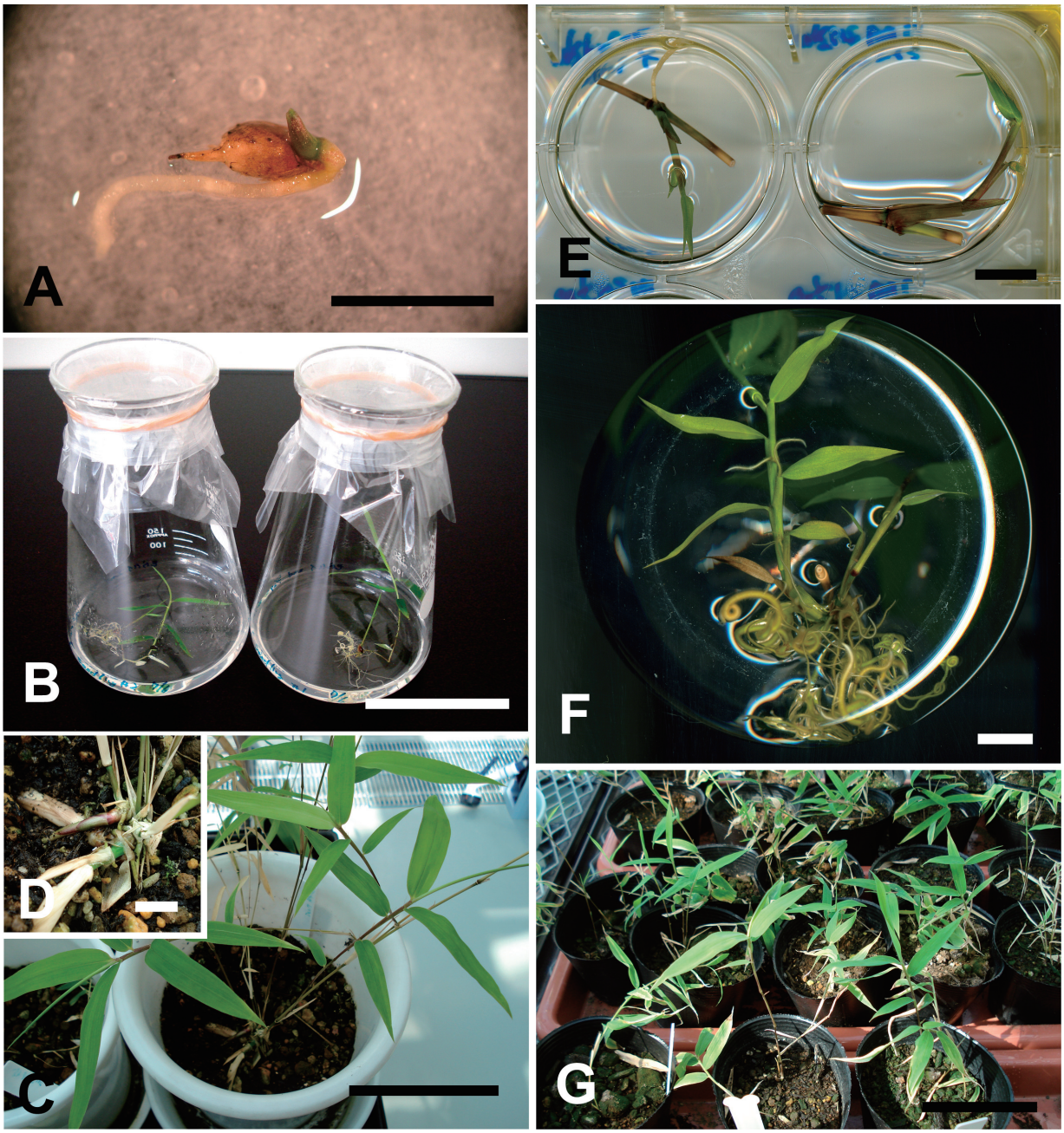

Figure 1. *In vitro* germination and node culture of *Phyllostachys meyeri* McClure. (A) Germination of mature caryopse in 2 weeks. (B) 3-month-old seedlings in a phytohormone-free liquid modified 1/2MS medium. (C) Morphological characteristics of 9 month-old seedling. (D) A magnified photograph of (C) indicating newly formed rhizomes. (E) Nodal segments with sprouted axillary buds and adventitious root after 40 days of culture. (F) Regenerated plantlet with well elongated shoots and roots after 90 days of culture. (G) 4 month-old regenerated plantlets in a soil condition.
Scale bars in (A) (B) (C) (D) (E) (F), and (G) represent 5 mm, 5 cm, 10 cm, 1 cm, 1 cm, 1 cm, and 5 cm, respectively

long life span and rare flowering period. In the present study, a tissue culture protocol in a liquid medium condition for *P. meyeri* was successfully achieved with particular regard to *in vitro* germination of the caryopses and plant regeneration from nodal segments of the germinated seedlings and tissue cultured clone plants. The protocols are simple and effective, though this could be applied only when mature caryopses are available.

The morphological characteristics and germination capacity of caryopses in *P. meyeri* were reported by Hisamoto et al. (2005). They pointed out that the germination rates varied from 3.6% to 54.9% in *ex vitro* conditions. Growth of *P. pubescens* seedlings under the different environmental conditions was also investigated by Uchimura (1990). He suggested that the germination rates were 7% to 60% depending on culture conditions. Mature caryopses of *P. meyeri* exhibited high germination capacity (75%) although a small number of samples were used for the experiment. The resulting seedlings grew healthily under *in vitro* conditions. In

Table 1. Shoot elongation and root formation from nodal segments of 3 independent seedlings and 2 tissue cultured clones in *Phyllostachys meyeri* McClure.

Donor plants*1	Medium condition	Number of nodal explants	Number of explants with no response	(%)	Number of explants with shoot elongation	(%)	Number of explants with shoot elongation and rooting*2	(%)
Seedling #1	m1/2MS	64	29	(45.3)	16	(25.0)	19	(29.7)
Seedling #1	MS	52	25	(48.1)	16	(30.8)	11	(21.2)
Seedling #2	m1/2MS	18	13	(72.2)	0	(0.0)	5	(27.8)
Seedling #3	m1/2MS	20	12	(60.0)	7	(35.0)	1	(5.0)
TC clone #1-1	m1/2MS	17	9	(52.9)	4	(23.5)	4	(16.7)
TC clone #1-2	m1/2MS	18	12	(66.7)	3	(16.7)	3	(19.3)

The data were obtained after 8 weeks of culture.

*1 Three independent seedlings of *ca.* 1.5 year-old and 2 independent tissue cultured (TC) clonal plants of *ca.* 1 year-old from seedling #1 were used as donor plants for nodal segment culture.

*2 The explants which have elongated shoots with rooting as shown in Figure 1.

some cases, multiple shoots with more than 6 culms were obtained from 1 caryopsis (data not shown). They were successfully naturalized *ex vitro* condition in a greenhouse.

In general, agar- or gellan gum-solidified medium is used for tissue culture of plants. Liquid medium is sometimes preferred for bamboo tissue culture. Sood et al. (2002) tested the effects of agar-solidified medium and static liquid medium on shoot multiplication and root formation in *D. hamiltonii*, and suggested that a liquid culture condition is more suited for tissue culture of this bamboo species. Das and Pal (2005) used liquid MS medium on multiple shoot formation from axillary buds of field-grown culms of *B. balcooa*. Shirin and Rana (2007) also used liquid culture condition for shoot development in *B. glaucescens*. Based on these reports, we checked the effect of medium conditions, both solid and liquid m1/2MS, on node culture of *P. meyeri*, and found that liquid m1/2MS medium was suitable for elongation of axillary buds. Furthermore, shoot elongation and root formation were achieved in liquid m1/2MS and phytohormone-free MS media in this bamboo species. It was observed that m1/2MS medium had a promoting effect on rooting. The average of rooting on m1/2MS medium was 29.7% compared to 21.2% on MS medium (Table 1). We have also previously reported that m1/2MS medium was effective as a basal medium for callus and cell suspension cultures of 3 *Phyllostachys* bamboos, namely *P. pubescens* Mazel ex Houz de Lehie, *P. bambusoides* Sieb. et Zucc., and *P. nigra* Munro var. Henonis (Ogita 2005; Ogita et al. 2008). Shirgurkar et al. (1996) reported that liquid 1/2MS medium was conducive for shoot development and root formation in *D. strictus* Nees. With these results, it is safe to conclude that liquid m1/2MS medium condition is adaptable for tissue culture of bamboos including genus *Phyllostachys*. Seedlings from mature caryopses and tissue cultured clonal plants were multiplied by the node culture in phytohormone-free liquid m1/2MS medium, although the rooting rates were not very high (5.0%–29.7%) and they varied according to the donor plants. Further experiments, e.g. addition of auxins (Shirin and Rana 2007), TDZ (Lin et al. 2004), and other growth promoting substances, to enhance rooting rate in *P. meyeri* are required. To the best of our knowledge, this is the first report on *in vitro* node culture of *P. meyeri*. The results suggest that clonal bamboo plants which are produced by the *in vitro* node culture technique could be used not only for conservation and propagation but also further characterization of physiological and molecular biological events of this bamboo species.

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