Nodule Cultures of *Paulownia* x *taiwaniana**

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【 Summary 】

Nodule cultures with potential applications in regeneration strategies, automated micropropagation, and *in vitro* phytochemical production in many plants have been reviewed. The 1st nodule cultures of *Paulownia* x *taiwaniana* were established and maintained for more than 1 y by regular subculturing. Vigorous callus clumps were induced from leaf explants cultured on 1/2MS medium containing 3 mg/L IBA and 3 mg/L BA. Nodule cultures were produced after leaf-derived calli were transferred into MS liquid medium containing 3 mg/L each of IBA and BA, and placed on a reciprocal shaker at 130 rpm for 6 wk. Cell growth cycles of the 3 mo-old and 1-y-old subcultures were determined by using settled cell volume and electrical conductivity. Results obtained from these 2 measurements were consistent with each other. Conductivity appeared to be rapid, simple, and reproducible. Histological changes of cell differentiation and vascular organization were also investigated at different growth phases of cultures. Nodules maintained organogenic capability in the 1st few subcultures, but lost it in successive cultures.

Key words : nodule cultures, *Paulownia* x *taiwaniana*.

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台灣泡桐的瘤狀細胞團培養

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摘 要

瘤狀細胞團培養在植物組織培養的再生系統、自動化的微體繁殖與植物化學物的生產上，已被視為具有相當潛力的應用價值。本研究首次建立台灣泡桐的瘤狀細胞團培養並可繼代培養一年以上。首先將葉片培植體培養在 MS 培養基添加 3 mg/L IBA 與 3 mg/L BA 以誘導生長旺盛的癒合組織細胞團，再移植於 1/2 MS 液體培養基添加各 3 mg/L 之 NAA 與 BA，經在 130 rpm 轉速下的迴轉震盪器培養 6 週後，瘤狀細胞團隨即被誘導出來。瘤狀細胞團經繼代培養 3 個月及 1 年後，其生長趨勢可用細胞沈澱法及電導度法加以測定。這兩種測定法所得的結果相當一致，但電導度法較為快速、簡單且重複性高。觀察瘤狀細胞團在各不同生長時期組織切片，可明瞭瘤狀細胞之分化及維管束組織形成的過程。在前數個繼代培養的瘤狀細胞團具有再生芽體的能力，但隨著繼代次數的增加其再生能力即逐漸喪失。

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關鍵詞：瘤狀細胞團培養、台灣泡桐。

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INTRODUCTION

Nodule culture, which offers another pathway to highly efficient shoot regeneration, is as distinct an *in vitro* system as suspension, callus, and shoot tip cultures. It has been proposed as an alternative system which is parallel to somatic embryogenesis, and it has have applications in regeneration strategies, automated micropropagation and *in vitro* phytochemical production in other plant species (McCown *et al.*, 1988). Moreover, a low frequency of somaclonal variation was observed in nodule cultures of *Hemerocalis* spp. (Kirkorian *et al.*, 1981). Nodules after successive subculturing still maintained regenerability in cultures of *Pinus radiata, Populus* hybrids, *Liquidambar styraciflua* (sweetgum), and *Eucalyptus* hybrids (Aitken-Christie *et al.*, 1988; Chen, 1991; Chung and Chung, 1991; McCown *et al.*, 1988; Warrag *et al.*, 1991). In addition to its value as a vegetative propagation method, nodule culture provides an effective culture system for the strict selection of transformed cell lines and the production of transgenic sweetgum plants using *Agrobacterium tumefaciens* (Chen and Stomp, 1992). *Paulownia* is a deciduous tree species of economic importance in Taiwan. *In vitro* cultures of this species were extensively studied (Yang *et al.*, 1996), however, nodule cultures was not performed. We now report on the establishment, maintenance, and organogenesis of nodule cultures of *P.* x *taiwaniana.*

MATERIALS AND METHODS

Establishment of aseptic seedlings and callus cultures

Seeds of *P.* x *taiwaniana* were surface-sterilized by soaking in 70% alcohol for 1 min, followed by immersing in 1% sodium hypochlorite supplemented with a drop of Tween 20 per 100 mL solution for 15 min. After 3 rinses with sterile water, seeds were plated on MS medium (Murashige and Skoog, 1962) containing 3% sucrose and 0.8% Difco agar (Difco Lab.). Seedlings were 5 to 8 mm tall 4 to 5 wk after germination. To maintain a regular supply of explants and growth of healthy plantlets, shoot tips of seedlings were excised at approximately 3 mm long and subcultured onto fresh medium once every 5 weeks. A factorial experiment was conducted to determine the effects of cytokinin and auxin concentrations on callus formation. Leaf explants (approximately 5 mm square and including central ribs) were placed on MS medium containing various phytohormones. Combination of kinetin or BA and 2,4-D or IAA or NAA or IBA, each at concentrations of 1, 2 and 3 mg/L, were used. Five 6-cm plates containing 10 mL medium, each with 4 leaf explants, were prepared for each of the hormonal treatments. Plates were incubated at 25 ℃ in darkness. Calli produced from different media were subcultured onto fresh media of the same respective composition after 3 wk. After another 3 wk in subculture, callus size and morphology were determined.

Establishment of suspension cultures

Calli (ca. 3 g) obtained in the subcultures on MS medium containing 3 mg/L BA and 3 mg/L IBA were chosen for suspension culture and transferred

into 125-mL Erlenmeyer flasks containing 20 mL MS or half strength of macrosalts of MS medium (abreviated as 1/2 MS), liquid medium containing combined supplements of IBA, NAA, or 2,4-D (each at 1 and 3 mg/L), and BA (at 1 and 3 mg/L) or kinetin (at 1 mg/L). Three flasks were used per treatment. Cultures were placed on a reciprocal shaker at 130 rpm and incubated at 25 ℃. After 3 wk in culture, cell aggregates were sieved by a metal filter with 250- μm pores and transferred to 20 mL fresh liquid medium in a flask. The growth curves and types of cell aggregates were examined at the end of the 1st subculture.

Nodule cultures established in 1/2 MS medium containing 3 mg/L BA and 3 mg/L IBA were maintained in the same medium by regularly subculturing once every 3 wk. To determine the growth cycle, nodules in the stationary phase were sieved using 2 stories of metal filters with 250- and 80- μm pores to obtain 2 size classes: greater than 0.25 cm but less than 0.8 cm, and less than 0.25 cm in diameter. Three-mo- and 1-y-old cultures were sampled to determine cell densities by using settled cell volumes and electrical conductivities. Suspension cultures in the nephalometer flasks with side arms were moved into their 20 mL graduated arm tubes or was 3 mL of sample transferred from 500 mL flasks into graduated centrifuge tubes after allowing cultures to settle for 30 min. Electrical conductivities of the same samples from the 500 mL flasks were then determined using a conductivity meter (SC-17A, Suntex Instruments Co., Ltd.). Treatments were repeated 2 or 3 times. Means of 2 or 3 replicates and standard diviations were calculated.

Plating cultures of nodules

To induce shoot formation, nodules sampled from 1-mo- and 9-mo-old subcultures in the linear growth phase were plated on 1/2 MS medium containing different combinations of IBA (0, 0.1, and 1 mg/L) and BA (1.5, 10, 15, and 20 mg/L), sucrose (3%, 6%, and 9%) and 0.7% Difco agar or agarose (Sigma Co.) or 0.2% gellen gum (Merck Co.). Treatments were repeated 3 times. After 3 wk in culture, organogenesis from nodular tissues was determined.

In these series of experiments, the pH value of the media was adjusted to 5.7 by using KOH before autoclaving at 121 ℃ for 15 min. Cultures, with the exception of callus cultures, were incubated in a culture room at 25 ℃ under fluorescent light of 20-30 $\mu Em^{-2}s^{-1}$ with a 16 h photoperiod.

Histological study

To examine the histological changes of nodular development, nodules in different growth phases (lag, exponential, linear and stationary phase) were sampled. All samples were fixed in formalin-acetic acid-alcohol (FAA), dehydrated in a tertiary butyl alcohol series (Johanson, 1940), and embedded in paraffin wax. Sections were 10 μm thick and stained with safranin-fast green (Sass, 1958).

RESULTS AND DISCUSSION

Callus cultures

The structures and types of leaf-derived calli cultured on MS medium containing various combinations of kinetin or BA, and 2, 4-D or IAA or NAA or IBA were phytohormone-dependent. Calli induced from medium containing 2,4-D and cytokinins appeared friable were whitish grey and readily turned brown after subculturing. Compact creamy yellow calli proliferated rapidly on NAA or IBA and BA media. This type of callus could be maintained by regular subculturing and further produced nodular tissues. Adventitious buds and roots occurred frequently from callus cultures induced on medium containing NAA and BA. This response is consistent with the results reported by Ho (1994). Callus proliferation was optimal on medium containing 3 mg/L BA and 3 mg/L IBA.

Suspension cultures and nodule formation

Calli obtained from 3 mg/L BA and 3 mg/L IBA medium produced loose, small cell aggregates, and occasionally initiated roots after being transferred to liquid MS medium containing 1 mg/L 2, 4-D. However, these cell aggregates readily turned brown in further subcultures. Calli cultured in NAA and BA medium produced larger cell aggregates and roots, while IBA and BA induced calli to produce nodular tissues, especially in 1/2 MS medium containing 3 mg/L IBA and 3 mg/L BA. Nodules increased in size and multiplied in number more rapidly in 1/2 MS medium than in MS medium. Nodular cultures were subcultured once every 3 wk. Different sizes of nodules exhibited different growth cycles (Fig. 1) and internal structures. Cultures initiated with inoculum of small nodules less than 0.25 cm in diameter, had a longer lag phase lasting for 5 days, followed by a 4 d exponential phase, 4 d of linear growth, and finally a stationary phase. Settled cell volumes doubled in 12 d during the linear growth phase. In contrast, no obvious lag phase was observed when medium-sized nodules, greater than 0.25 cm in diameter but less than 0.8 cm, were inoculated. These cultures showed an exponential phase lasting 5 d, followed by a linear growth phase lasting 13 days before entering a progressively decelerating phase and stationary phase. Settled cell volumes doubled in 8 d. King and Street (1973) pointed out that the occurrence and duration of each phase depends very much upon the cell type, frequency of subculture, initial density, and culture medium. Cell types of the inoculum became critical since other factors were fixed. Restated, dramatic increases of cell volumes, longer periods of growth cycles and more heterogeneous sizes of cell aggregates resulted from using the larger sizes of nodules for the inoculum.

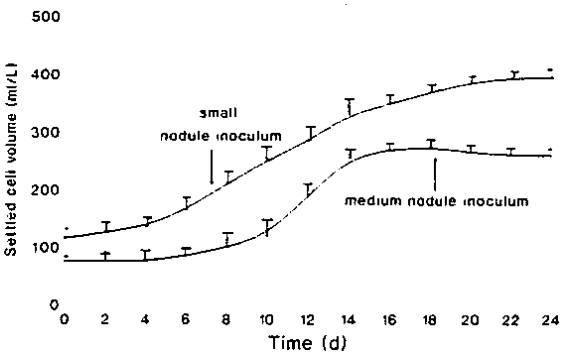

Fig. 1. Time course for changes in settled cell volume during the growth of nodule cultures inoculated with small (less than 0.25 cm) and medium (greater than 0.25 cm but less than 0.8 cm) sizes of nodule inoculum sieving from 3-month-old subcultures. Each point represents the mean of 3 replicates ± standard devision.

After 1 y in subculture, the growth patterns obtained from 2 sizes of nodule inocula were similar to that of 3-mo-old subcultures (Fig. 2a and b). A long lag phase in smaller nodules and a high growth rate in larger nodules were re-confirmed in measurements to settled cell volumes. Conductivities of cultures changed in an inverse manner of settled cell volumes. Electrical conductivity has been used to determine the cell growth of suspension cultures because of its simplicity, reproducibility, and high correlation with dry-weight changes (Davis *et al.*, 1984; Hahlbrock *et al.*, 1974; Hahlbrock, 1975). In time course, the conductivity for cultures with larger nodule inoculum was 4 d earlier entering the stationary phase than was the settled cell volume. But for small nodule inoculum the trend of entering stationary phase was similar. In addition, the lower precision and higher variability of settled cell volumes when compared to the use of dry-weight measurements has been noted by Davis *et al.* (1984). One of the major reasons for the inaccuracy of settled cell volume is that enlargement of nodule size results in increases of interspaces among

nodules, especially in the late linear growth phase when cells are expanding rapidly.

Small nodules (less than 0.25 cm in diameter) consisted only of parenchyma cells with non-lignified primary walls and had no internal tissue differentiation. During the linear growth phase, nodules increased rapidly in size. Internal differentiation initiated with the organization of "unicenter" nodules was characterized by the formation of xylem elements in the center of nodules. The same internal differentiation was defined in nodular cultures of hybrid poplar and sweetgum (McCown et al. 1988; Chen 1991). For those cultures initiated with medium sized nodule inoculum in which most cell aggregates were unicenter nodules, additional centers of xylem elements differentiated along the subepidermal cells (Fig. 3) creating the "polycenter" or "meganodule" (McCown *et al.* 1988). Some vascular centers were isolated by a few layers of epidermal cells although they were bound in a large mass. These vascular centers could split from the meganodules to become unicenter nodules in the late linear growth phase and stationary phase when cell expansion occurred. Cultures initiated with small sized nodule inoculum appeared to multiply in number more than to increase in size. More uniform cultures, primarily consisting of unicenter nodules, were produced at the very end of the stationary phase. In comparison with these, cultures initiated with medium sized nodule inoculum produced heterogeneous sized nodules.

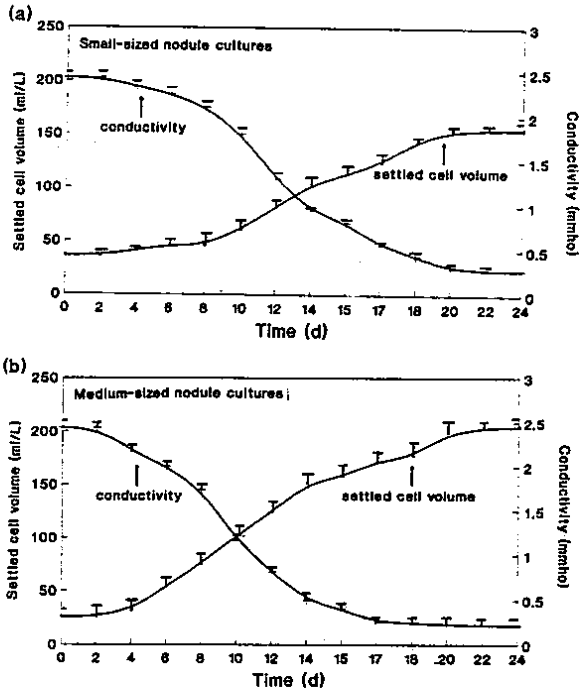

Fig. 2. Time courses for changes in settled cell volume (+) and conductivity (■) during the growth of nodule cultures inoculated with small (a) and medium (b) sizes of nodule inoculum sieved from 1-y-old subcultures. Each point represents the mean of 2 or 3 replicates ± standard deviation.

Plating cultures of nodules

Root and shoot regeneration from nodules occurred only in the early few subcultures when nodules were plated on solid 1/2 MS medium or cultured in liquid 1/2 MS medium containing 3 mg/L IBA and 3 mg/L BA (Fig. 4a and b). Since the regeneration capability has been proved to be difficult for callus cultures of *P.* x *taiwaniana* (Ho, 1992), nodule cultures appear to maintain longer

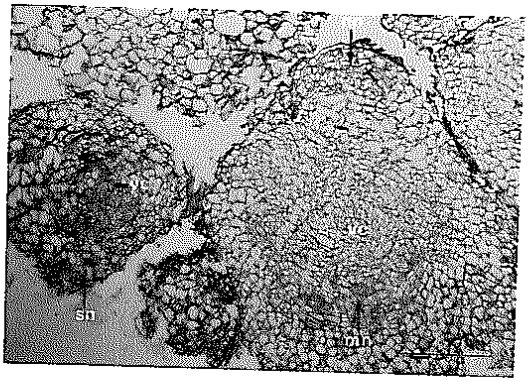

Fig. 3. Partial section of a meganodule with a mature nodule (mn) containing a clear vascular center (vc), a newly-formed nodule (nn) emerging along subepidermal layers, and a separate nodule (sn) in the process of splitting away and becoming independent. Bar = 5 mm.

regenerability. However, this organogenic capability was finally lost after continued subculturing even though various phytohormonal combinations were used to induce nodules in plating cultures or liquid medium to regenerate shoots. Green callus clusters with vigorous growth were commonly observed in 3 wk after nodules were plated on MS medium containing 0.01 or 0.1 mg/L NAA, 5 mg/L BA, 0.2% gellen gum and 3% sucrose (Fig. 4c). Increases of sucrose concentrations in the medium caused *Paulownia* nodule browning, even though the osmolarity of the medium is important for obtaining, retaining, and reviving the high regeneration frequency of callus cultures for many other plant species (Debergh, 1983; Ho, 1994; Pochet *et al.*, 1991; Tsukahara and Hirosawa, 1992).

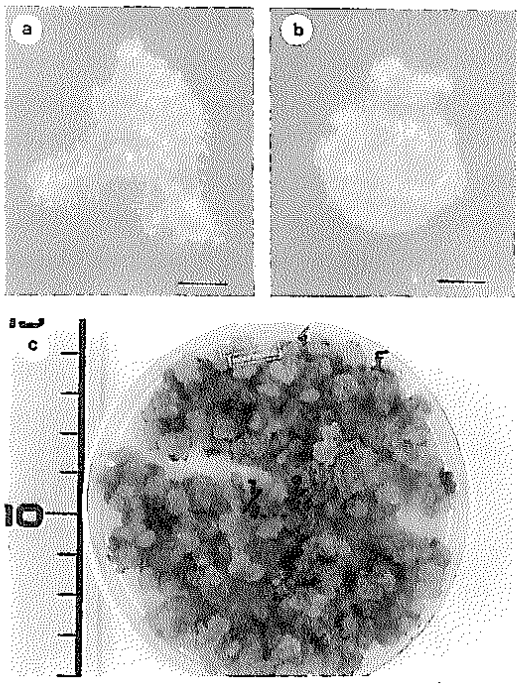

Fig. 4. Plating cultures of nodule tissues. Root (a) and bud (b) formation which occurred in early nodule subcultures; (c) vigrously growing green cell clusters from nodules of 1-y-old subcultures. Bar in Fig. a and b is 5 mm, and in Fig. d is 1 cm.

CONCLUSION

Nodule cultures with the advantages of both shoot cultures and suspension cultures were the focus of this study and other reports described above. Liquid cultures of nodules can produce uniform mass-nodules with the ability of shoot regeneration using the same techniques as with suspension cultures. There are many topics requiring further study: the theoretical basis of the nodular developmental pathway; the regeneration rate of nodules *in vitro*; and the genetic and physiological uniformity of nodule-derived plants as pointed out by McCown *et al.* (1987). The retention of morphogenetic capacity in long-term cultures is also another topic which should be addressed.

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