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Thidiazuron Required for Efficient Somatic Embryogenesis from Suspension-Cultured Cells of *Pimpinella brachycarpa*

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To maintain embryogenic cell lines of *Pimpinella brachycarpa*, we suspension-cultured friable and rapidly growing yellowish calli in an MS liquid medium containing 0.2 μM 2,4-D and 0.5 μM BAP. Efficient somatic embryogenesis was achieved when selected cells were then transferred to an MS medium (0.2% gelrite) that contained 0.2 μM 2,4-D, 0.5 μM BAP, and 10.0 μM TDZ (thidiazuron). These cells were cultured at 27°C under continuous illumination (21.5 μE m^{-2} s^{-1}). Embryogenic calli expanded about four-fold, and developed into pale yellow calli. Somatic embryogenesis was initiated only from glossy and nodular-type calli. After two more weeks of culture, globular embryos appeared on the surface of calli grown in the MS medium that contained 10.0 μM TDZ only, or in combination with 0.5 μM NAA. Experimenting with 2,4-D, an auxin, to promote embryogenic calli resulted in excessive browning and death. We overcame this problem by growing glossy embryogenic and nodular calli on media that contained 10.0 μM TDZ. Calli that were not treated with TDZ turned dark brown and were not viable. Up to 74% of the calli showed somatic embryos when the medium was supplemented with 10.0 μM TDZ and 0.5 μM NAA. Embryos from these TDZ-induced, somatic embryogenic calli grew efficiently, forming multiple shoots and developing into normal plants. Therefore, efficient differentiation of suspension-cultured cell clusters into embryogenic calli, along with treatment of subsequent somatic embryos by TDZ, suggests that TDZ probably helps in establishing the optimum cytokinin-auxin ratio required for induction and expression of somatic embryogenesis.

Keywords: differentiation, embryogenic calli, globular embryo, multiple shoots, plantlet, thidiazuron

Pimpinella brachycarpa, a perennial wild plant belonging to the *Umbelliferae* family, is one of the most expensive and increasingly popular wild edible greens produced in Asian regions, including Korea (Moon et al., 1994). However, because this species normally grows at high elevations, propagation is very difficult. Although plants produce underground rhizomes that branch sympodially, the success rate for vegetative micropropagation of rhizome cuttings is very low, and an efficient multiplication method had not been available. However, recent progress in plant biotechnology makes it possible to develop efficient in-vitro regeneration protocols, thereby adding genetically well-defined traits to the gene pools for useful crops.

Such reproducible protocols require that researchers identify the optimal conditions for plant regeneration, mass propagation, and efficient gene transfer. Few reports have been made about in vitro regeneration of *P. brachycarpa* (Moon et al., 1994), and the efficiencies using these explants have been very low. Feirer and Simon (1991) investigated the biochemical changes that occur during embryogenesis. At the molecular level, the ability to regenerate tissue from calli can enhance the shoot-development potential in plants with normally low regeneration activity (Franz et al., 1989; Aleith and Richter, 1990). Increased gene activity in cultured somatic cells can induce division in resting cells, resulting in a morphological change via physiological and biochemical modifications. The external and internal factors involved in the regulation mechanism for in vitro organogenesis have been identified only through empirical study (Tremblay and Tremblay, 1991; Thorpe, 1993). These approaches have not been adequate.

The use of TDZ prompts multiple shoot formation in a broad range of species (Malik and Saxena, 1992; Murty et al., 1995). In fact, Saxena et al. (1992) have proposed that TDZ can be substituted for the auxins normally required for inducing somatic embryogenesis from intact seedlings and tissue cultures of *Pelargonium*. Based on those preliminary studies, we have now attempted to establish an efficient propagation method for *P. brachycarpa*. Here we report the use of in vitro differentiation to identify the optimum protocol for suspension-cultured cells derived from somatic embryogenic calli, as induced by TDZ.

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MATERIALS AND METHODS

Plant Material and Experimental Design

We obtained young plants of *P. brachycarpa* from the Alpine Experiment Station, RDA. Petiole explants were grown for three months in a green-house. After harvesting, these explants were surface-sterilized for 15 min with 20% bleach that contained Triton X-100, then washed thoroughly five times with sterilized distilled water. For callus induction, explants were cut into 0.5-cm sections. They were placed on MS basal media treated with 2,4-D alone, or in combination with BAP. The resultant embryogenic calli that were compact, glossy, and nodular were then transferred to an MS (0.2% gelrite) medium supplemented only with 0.1, 0.2, 0.4, or 0.8 μM 2,4-D, or in combination with 0.5 μM BAP. Cultures were held at 27°C in darkness, and somatic embryogenic calli were subcultured on MS solid basal media every 14 d.

One-gram samples from the suspension cultures initiated from embryogenic calli were subcultured in 250-mL Erlenmeyer flasks that contained 50 mL of a liquid MS medium. The flasks were agitated for five months on a reciprocal shaker (100 strokes/min), at one-week intervals. Additional suspension cultures were established at 27°C in the dark in an MS basal medium containing 0.2 μM 2,4-D and 0.5 μM BAP. Large clumps of suspension cultures were removed by filtering them through a stainless, 230-mesh screen.

Somatic Embryogenesis from Suspension-Cultured Cells

To optimize the somatic embryogenesis protocol, we transferred suspension-cultured cells to an MS basal medium that contained 0.2 μM 2,4-D and 0.5 μM BAP only, or in combination with 0.2, 1.0, 5.0, 10.0, or 20.0 μM TDZ. The resulting embryogenic calli were transferred to an MS (0.2% gelrite) medium supplemented with 0.2 μM 2,4-D, 0.5 μM BAP, and 10.0 μM TDZ. Culturing proceeded at 27°C under continuous illumination (21.5 $\mu E\ m^{-2}\ s^{-1}$) for two weeks. The embryogenic calli were subcultured onto MS solid basal media every four weeks.

Differential Developmental Stages of Somatic Embryos and Plant Regeneration

Subcultured, embryogenic calli were cultured on MS (0.2% gelrite) media that contained 10.0 μM TDZ only, or in combination with 0.5 μM NAA, and were held at 27°C under continuous illumination (21.5 $\mu E\ m^{-2}\ s^{-1}$). To promote germination, somatic embryos that appeared on the calli surfaces were separated and placed in an MS liquid medium (no gelrite) that contained 0.6 μM GA_3. The fully germinated plantlets were then placed on an MS solid medium with 0.5 μM NAA. Finally, plants with well-developed roots were transferred to 8-cm pots containing vermiculite, then hardened for one week at 27°C and 85% relative humidity.

RESULTS AND DISCUSSION

Callus Induction and Somatic Embryogenesis from Suspension-Cultured Cells

Petiole explants first were cultured on an MS (0.2% gelrite) medium supplemented with 2,4-D only, or in combination with BAP at 27°C. Calli formed when tissues were held in dark conditions. However, under continuous light, all explants were brown and blighted, showing weak callus formation and unsustained callus induction, regardless of treatment. The optimal concentrations of plant regulators were 0.2 μM 2,4-D and 0.5 μM BAP (data not shown). These calli were subcultured on the MS gelrite medium every 14 d. After four to six weeks, we observed partially nodular embryogenic calli. In similar experiments, nonembryogenic compact and rhizogenic callus types have also been reported (Wang et al., 1984). The embryogenic calli that develop from clusters of small and dense cells are called 'proembryogenic masses' (Binh and Heszky, 1990; Data et al., 1990).

To maintain our embryogenic cell lines, we suspension-cultured the friable, rapidly growing yellowish calli. These cells were separated from the microcalli in a liquid MS medium containing 0.2 μM 2,4-D and 0.5 μM BAP. The small cell clusters became separated after four weeks of culture (Fig. 1, A and B). We also observed dividing clusters derived from those suspension-cultured cells that were maintained in the dark for six weeks (Fig. 1, C and D). To select for embryogenic calli, we transferred the suspension-cultured cells to an MS (0.2% gelrite) medium that had been supplemented with 0.2 μM 2,4-D, 0.5 μM BAP, and various concentrations of TDZ. Culturing proceeded under continuous illumination.

Litz and Gray (1995) have reported efficient induction of glossy and nodular calli in somatic embryogenesis on MS gelrite media containing 0.2 μM 2,4-D, 0.5 μM BAP, and 10.0 μM TDZ. In the current study,

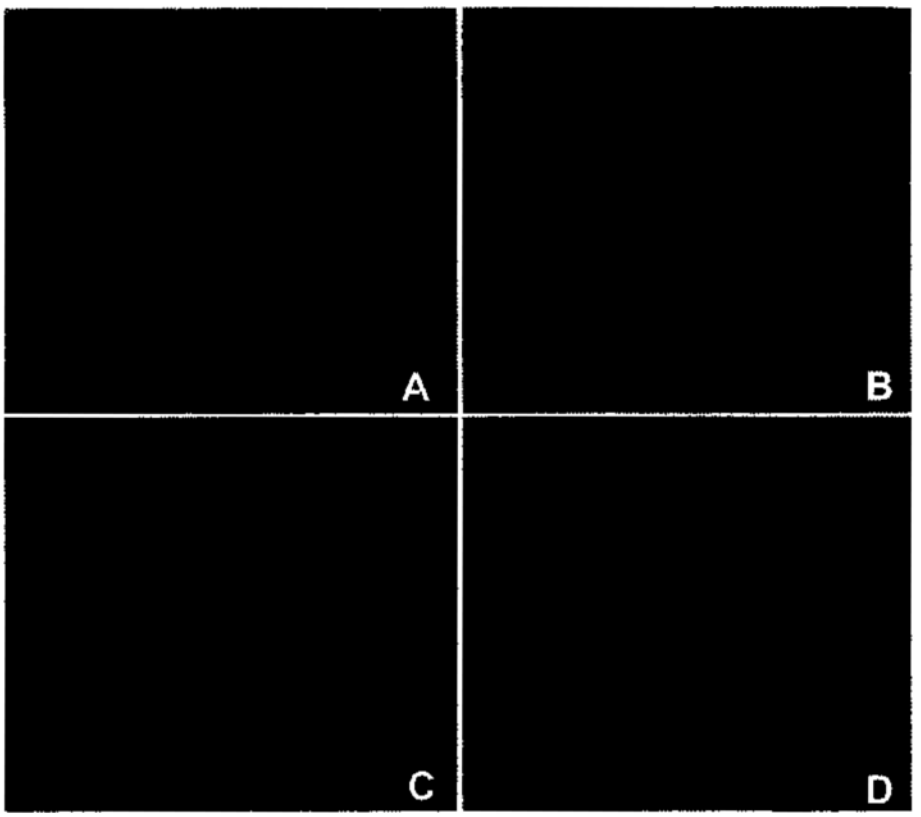

Figure 1. Suspension-cultured cell clusters from *P. brachycarpa* petiole explants. **A.** Cells from the suspension cultures of micro-calli in MS liquid media containing 0.2 μM 2,4-D and 0.5 μM BAP. **B.** Small cell clusters after four weeks of culture. **C.** Single dividing clusters after six weeks of culture. **D.** Suspension-cultured clusters under dark conditions.

the frequency of embryogenic calli was about 86% on this particular medium (Table 1). We also observed 20 to 50 globular embryogenic clusters of small round cells (Fig. 2A). In contrast, the non-embryogenic clusters were elongated and showed excessive browning. Therefore, we determined that induction of glossy and nodular calli, aggregated with small round cells, was the key element for promoting successful somatic embryogenesis. When the somatic embryogenic calli were subcultured to a medium containing 0.2 μM 2,4-D, 0.5 μM BAP, and 10.0 μM TDZ, embryogenesis was initiated after four weeks of culture (Fig. 2B).

Table 1. Effect of plant growth regulators on somatic embryogenesis from suspension-cultured cell clusters of *P. brachycarpa* on MS media (0.2% gelrite) after six weeks of culture.

Plant growth regulator[a] (μM)			Frequency of somatic embryogenic calli
2,4-D	BAP	TDZ	
0.2	0.5	0.0	–
0.2	0.5	0.2	–
0.2	0.5	1.0	+
0.2	0.5	5.0	++
0.2	0.5	10.0	+++ (86%)
0.2	0.5	20.0	++

[a]Plant growth regulators were added to MS basal media.
[b]–, none; +, poor (5 - 30%); ++, moderate (31 - 70%); +++, high (71 - 100%).

Plant meristematic cells generally develop totipotent calli, making possible regeneration via organogenesis and somatic embryogenesis. Based on our morphological observations of callus subcultures and the regeneration frequency of somatic embryos, we conclude that the optimum period for callus induction of *P. brachycarpa* from suspension cultures is 8 to 10 weeks. After 16 weeks of subculture, those calli were no longer embryogenic. Likewise, Nabors et al. (1983) and van Schaik et al. (1996) have demonstrated that embryogenic capacity can be lost during prolonged subculturing periods.

Callus induction was most successful in parenchymatous explants from young plants. These petiole explants frequently formed more or less embryogenic types of calli. Therefore, the particular stage of plant

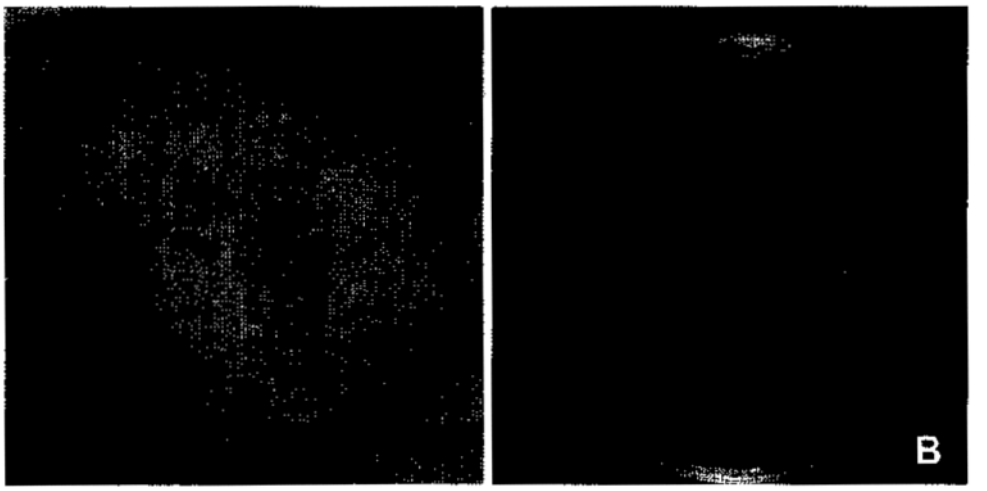

Figure 2. Callus formation and early stage of somatic embryogenesis from *P. brachycarpa* petiole explants. **A.** Proliferation of embryogenic calli derived from suspension-cultured clusters on MS media containing 0.2 μM 2,4-D, 0.5 μM BAP, and 10.0 μM TDZ, and cultured at 27°C under continuous illumination (21.5 E $m^{-2} s^{-1}$) for two weeks. **B.** Early growth of embryogenic calli in subcultures.

development is critical when determining the optimal regeneration protocol from suspension cultures via somatic embryogenesis. Likewise, the addition of TDZ resulted in a high frequency of somatic embryogenic mass cells from the suspension cultures. This should be a very useful tool in establishing a synchronous process for regeneration of *P. brachycarpa*.

Differential Developmental Stages of Somatic Embryos

We subcultured the somatic embryogenic calli that were derived from suspension-cultured cells, using an MS gelrite medium containing 10.0 μM TDZ only, or in combination with 0.5 μM NAA. Culturing proceeded under continuous illumination for four weeks. The tissue mass increased by about four-fold, and pale yellow calli developed on all media tested. However, somatic embryogenesis was initiated only from the glossy and nodular type of callus. After the four-week culturing period, the developed calli were subcultured on the same media type. By two weeks later, somatic embryos had appeared on the surfaces of calli grown either on the MS medium containing 10.0 μM TDZ only, or on the medium supplemented with 0.5 μM NAA.

We also attempted to promote embryogenic calli development on media containing the auxin 2,4-D, but this treatment caused excessive browning and death. To overcome that problem, we then supplemented the MS medium with 10.0 μM TDZ. This addition was successful, in that the calli treated without TDZ turned dark brown and were not viable.

The compound TDZ is involved in many physiological systems, and has been used to elicit multiple shoot formation in a broad range of species (Malik and Saxena, 1992; Murty et al., 1995). For example, TDZ has induced somatic embryos in intact seedlings of the peanut (*Arachis hypogaea*; Saxena et al., 1992). Likewise, somatic embryogenesis and a high frequency of shoot regeneration were observed in tobacco (*Nicotiana tabacum*), watermelon (*Citrullus* sp.), and white ash (*Fraxinus* sp.) in response to TDZ (Murty et al., 1995). Saxena et al. (1992) proposed that TDZ could substitute for the auxins normally required for inducing somatic embryogenesis in intact seedlings and tissue cultures of peanut and *Pelargonium*. This claim was substantiated by Murty et al. (1995), who found that the level of cytosolic IAA increased in cotyledons and hypocotyls of TDZ-treated peanut seedlings.

In the current study, petiole explants of *P. brachycarpa* gave rise to embryogenic calli, an effect similar to that elicited by 2,4-D and BAP. In addition, the efficient differentiation of suspension-cultured cell clusters into embryogenic calli, as well as the subsequent somatic embryos induced by TDZ treatment, suggests that TDZ probably helps in establishing the optimum cytokinin-auxin ratio required for induction and expression of somatic embryogenesis. Thomas and Katterman (1986) also have reported physiological responses to TDZ (usually mediated by adenine-based cytokinins) that exhibit cytokinin-like activity.

Table 2. Effect of plant growth regulators on somatic embryo production from embryogenic calli on MS media (0.2% gelrite) at 27°C under continuous illumination (21.5 μE m^{-2} s^{-1}) for four weeks.

Plant growth regulator[a] (μM)		Frequency of SE[b]
2,4-D	TDZ	
0	0.5	–
10	0.0	++
10	0.5	+++ (74%)
10	1.0	++

[a]Plant growth regulators were added to MS basal medium. [b]–, none; +, poor (5 - 30%); ++, moderate (31 - 70%); +++, high (71 - 100%).

The frequency of somatic embryo production per embryogenic callus was about 74% on the MS medium containing 10.0 μM TDZ and 0.5 μM NAA (Table 2). Afterward, the embryos were separated from the calli and placed in a liquid MS medium (no gelrite) that contained 0.6 μM GA_3 (Gmitter and Moore, 1986; Chalupa, 1990; Bueno et al., 1992). Fully germinated embryos matured into plantlets within two to three weeks (Fig. 3D). Compared with results from media containing 0.2% gelrite, the liquid MS medium supplemented with the GA_3 was more suitable for promoting rapid primordial shoot development from somatic embryos.

For in vitro regeneration, we placed somatic embryo-derived plantlets in an MS gelrite medium with 0.5 μM NAA (Fig. 4A). The rooted regenerants that resulted then developed into normal plants (Fig. 4B), eventually producing shoots and leaves.

One of the most important attributes of somatic embryogenesis is the ability of plant tissue to proliferate indefinitely. The activity of these embryogenic cells is influenced by several factors, including those involved in the induction of embryogenic calli and embryo formation. This synchronous and continuous proliferation provides potential for research efforts on mass propagation and gene transfer technology (Thorpe, 1993). In fact, this study represents the first time that TDZ-induced somatic embryogenesis has been demonstrated from suspension-cultured cells in *P. brachycarpa*. The somatic embryogenesis protocol from

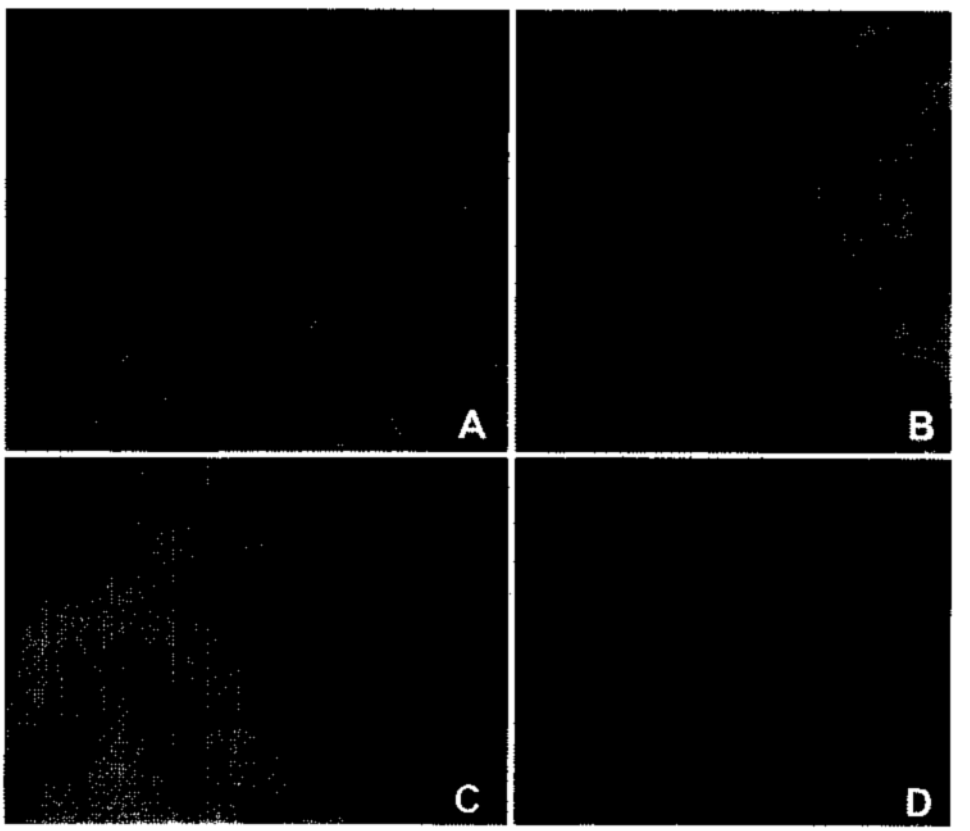

Figure 3. Various developmental stages of somatic embryos derived from embryogenic calli on MS media containing 10.0 μM TDZ and 0.5 μM NAA. **A.** Embryogenic callus after two weeks of culture. **B** and **C.** Globular embryos and heart-shaped embryos after four weeks of culture. **D.** Multi-matured somatic embryos after five weeks of culture.

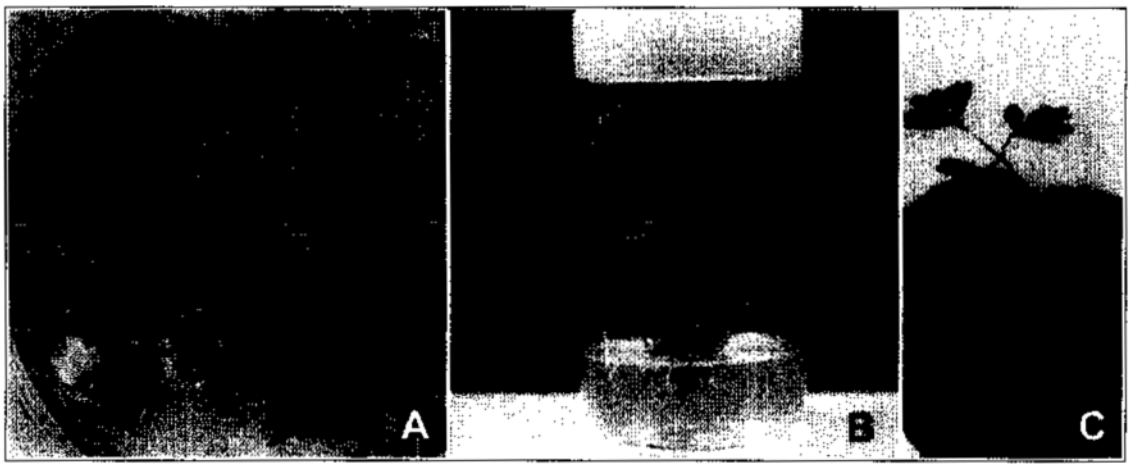

Figure 4. In vitro plant regeneration and regenerants from *P. brachycarpa* petiole explants. **A.** Germinated plantlets with two or three shoots in an MS liquid medium containing 0.6 μM GA_3 after two weeks of culture. **B.** Somatic embryo-derived plantlets in an MS gelrite medium containing 0.5 μM NAA. **C.** Regenerants potted in soil.

suspension-cultured cell clusters, as reported here, may be ideal for obtaining synchronous embryogenic cell masses, while enhancing our understanding of the morphogenic process. We suggest that, because of its high frequency during regeneration, this versatile tool will be critical to the further refinement of an efficient gene transfer system for *P. brachycarpa*.

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