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Research paper

Establishing an *in vitro* Propagation System for *Prunus campanulata* Maxim. via Plant Fasciation Syndrome

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ABSTRACT

In plant tissue culture, shoot micropropagation is often accomplished through the application of plant growth regulators under *in vitro* conditions. However, an imbalance of endogenous phytohormones may occasionally appear in this process, resulting in physiological or morphological abnormalities. Plant fasciation is one of the abnormalities and is typically considered a negative outcome in *in vitro* culture and therefore often discarded. Fasciated shoot tips are distinguished by the production of multiple, actively proliferating apical meristems. Our laboratory recently developed a large-scale plant propagation system to make use of fasciated tissue. In the present study, this propagation system was applied to *Prunus campanulata* Maxim., a woody ornamental species. An *in vitro* single bud was first cultured for 60 d on the Woody Plant Medium (WPM) supplemented with 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) to induce fasciated tissue with multiple shoot meristems. The sustained shoot fasciation and promoted lateral shoot tip proliferation were achieved through the use of various combinations of BAP and IBA concentrations. By intensively cutting the fasciated tissue, numerous normal morphological shoots were recovered from the cutting debris. From an initial 0.5 cm-wide fasciated tissue fragment, a maximum of 192.4 normal shoots were obtained. Following IBA treatment, 80% of these new shoots rooted, and subsequent acclimatization in a greenhouse achieved a 95% survival rate, yielding morphologically normal plants.

Keywords: apical meristem, fasciation, micropropagation, ornamental cherry, shoot proliferation
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INTRODUCTION

Plant fasciation refers to a morphological abnormality characterized by altered organ development that can occur in various plant organs, including roots, stems, leaves, flowers, inflorescences, cotyledons (White 1945), fruits (Darrow and Borthwick 1954), and rhizomes (Fukai et al. 2000). Binggeli (1990) noted that fasciation occurs more frequently in species with indeterminate growth, citing *Acer pseudoplatanus* L. as an example, and commonly appears in its twigs, petioles, and inflorescences. Choob and Sinyushin (2012) further described that when the shoot apical meristem (SAM) undergoes abnormal expansion due to fasciation, the resulting stem becomes flatter and ribbed compared to its normal counterparts, and the proliferation also occurs laterally. In *Prunus avium* L., leaves and buds on fasciated stem segments tend to cluster near the shoot apex (Kitin et al. 2005). Furthermore, the phyllotaxy of fasciated plants differs from that of normally developing individuals (Tang and Knap 1998, Bykova et al. 2016). Similarly, in *Pisum sativum* L., fasciation is associated with not only altered phyllotaxy but also an increased number of leaves (Sinjushin and Gostimskii 2008).

The onset of fasciation is influenced by physiological mechanisms (Iliev and Kitin 2011, Altae and Dalas 2021) or genetic regulation (Williams and Fletcher 2005, Elhiti and Stasolla 2011, Iliev and Kitin 2011). Environmental zinc deficiency (Rance et al. 1982) and infection by *Rhodococcus fascians* (Tilford 1936) Goodfellow 1984 (Crespi et al. 1992) are known to induce fasciation. These physiologically induced cases are generally attributed to an altered endogenous balance between auxin and cytokinin (Murai et al. 1980, Cakmak et al. 1989, Eason et al. 1996). Regarding genetic regulation, SAM development is coordinately regulated by genes including *CLAVATA* (*CLV*), *WUSCHEL* (*WUS*), *MGOUN* (*MGO*), and *FASCIATA* (*FA*) (Kaya et al. 2001, Graf et al. 2010, Galli and Gallavotti 2016). The key genes *CLV* and *WUS* function through a negative feedback loop to stabilize cell populations within the SAM, thereby ensuring the cells' proper development. Suppression or malfunction of *CLV* signaling disrupts its regulation of *WUS*, leading to *WUS* overexpression. This results in excessive stem cell accumulation within the SAM, accompanied by the stem cells' uncontrolled differentiation into organ primordia, ultimately causing enlargement and overproliferation of the SAM (Schoof et al. 2000). In fasciated stems, this alteration manifests as stem flattening, increased girth, lateral proliferation of the SAM, and the production of numerous diminutive leaves. During signal transduction in plant cells, cytokinin promotes *WUS* expression, either directly or through the suppression of *CLV*, thereby increasing the number of stem cells in the SAM. When excessive cytokinin levels drive uncontrolled stem cell proliferation, fasciation is triggered (Gordon et al. 2009). Therefore, increased cytokinin levels and dysregulation of SAM-related genes are interconnected in the induction of fasciation.

Whether triggered by physiological imbalances or genetic dysregulation, fasciation can ultimately be attributed to the imbalance of the endogenous auxin and cytokinin. In *in vitro* culture procedures, while exogenous cytokinin supplementation is a common practice for mass propagation, it often initiates disruption of the hormonal balance and consequently induces fasciation (Amutha et al. 2006, Iliev et al. 2010, Arellano-Perusquia et al. 2013, Dancheva and Iliev 2015). Cytokinin application is

thus recognized as a pivotal factor in this phenomenon (Isah 2015). Historically, fasciated propagules arising during the *in vitro* cultures have been categorized as aberrant mutations and discarded due to their perceived lack of utilitarian value. In our laboratory, detailed observation of fasciated shoot apices revealed extensive lateral expansion of the SAM, with numerous shoot meristems arranged along the apex. Given that the induction of meristems and the promotion of their growth constitute a critical step in micropropagation, adventitious bud regeneration, and callus organogenesis, this observation led to the hypothesis that if fasciated apices inherently possess multiple preformed meristems, they could be directly utilized as propagation materials, thereby eliminating the need for *de novo* induction.

In studies on carnivorous plants, fasciated organs have been employed for vegetative propagation aimed at preserving the fasciated phenotype for ornamental purposes rather than for increasing the propagule yield, resulting in low propagation efficiency (Wistuba 1983, Hanrahan 1985). In the field of *in vitro* culture, several studies have documented the occurrence of fasciated shoots with detailed data. For instance, Iliev et al. (2003) induced adventitious shoots from the leaf callus of *Betula pendula* Roth. using zeatin [6-(4-hydroxy-3-methylbut-2-enylamino) purine] and reported that less than 5% of the regenerated propagules exhibited fasciation. Similarly, in *P. avium*, the culture of single shoots on medium containing indole-3-butyric acid (IBA) and gibberellic acid (GA_3), followed by treatment with various concentrations of 6-benzylaminopurine (BAP), also yielded a small number of fasciated shoots (Kitin et al. 2005). Papafotiou et al. (2001) also reported the induction of fasciated shoots in *Mammillaria elongata* DC. through the synergistic application of BAP and 1-naphthaleneacetic acid (NAA). These collective findings indicated that although the emergence of fasciated propagules during *in vitro* culture was not uncommon, their frequency was often too low for large-scale propagation, or they were not the primary objective of the original studies. Furthermore, histological analyses of fasciated stems via transverse sections have revealed abnormal enlargement and deformation of cortical parenchyma and pith cells, with some instances of cellular rupture, suggesting aberrant growth patterns (Kitin et al. 2005). As a result, fasciation has received limited attention and has not been further investigated in *in vitro* culture research.

Anderson et al. (1982) identified a multi-apexing phenomenon in the shoot tips of strawberry (*Fragaria* × *ananassa* Duch.), which they characterized as an aberrant manifestation of fasciation. Although they described a process to restore normal growth by isolating these apices and noted that elevated BAP concentrations promoted this condition, they merely suggested its potential as an efficient method for shoot multiplication. Liao and Ji (2014) first verified in *Drosera burmannii* Vahl that the multiple meristems present in fasciated SAMs could proliferate and differentiate under *in vitro* culture conditions, regenerating into numerous morphologically normal plantlets. Liang et al. (2019) took a similar approach with *Vitex rotundifolia* L., using flattened fasciated stem segments as explants to induce the elongation of axillary buds, thereby successfully yielding normal plants. These 2 reports represent the initial attempts to exploit fasciated plant organs in *in vitro* culture and successfully collected a "substantial" multiplication of propagules.

The present study extends this propaga-

tion strategy to a third species, *Prunus campanulata* Maxim. As a notable ornamental plant in Taiwan, *P. campanulata* is widely cultivated in forest recreation areas, private landscapes, and community gardens across the northern regions, resulting in significant market demand for mass-produced plantlets. The genus *Prunus* L. comprises numerous deciduous and evergreen trees and shrubs, including economically important fruit and nut crops worldwide (Chin et al. 2014). Subsequent attempts to extend this propagation method to other ornamental or economically valuable species within the genus hold considerable commercial potential.

MATERIALS AND METHODS

Establishment of aseptic shoots

Basal sprouts were collected from the trunk base of *P. campanulata*. After removing the leaves, the shoots were rinsed under sufficient running tap water and trimmed into segments approximately 5 cm in length. The segments were then immersed in 0.2% (v/v) Anti-A solution (China Chemical & Pharmaceutical Co., Ltd., Hsinchu, Taiwan) for 30 min, followed by washing in 200 mL of tap water supplemented with 1–2 drops of household detergent for 2–3 min. Subsequently, surface sterilization was performed within a laminar airflow cabinet using 20% (v/v) commercial bleach (Clorox®, USA) for 2–3 min, followed by immersion in 75% (v/v) ethanol for 30 s. Finally, explants were rinsed 4 times with autoclaved double-distilled water (ddH_2O), each rinse lasting 3 min. The two ends of the explants, which underwent sterilization treatment, were thinly shaved under aseptic conditions. They were cut into 2–3 cm nodal segments, each containing at least 1 axillary bud, which were used as explants. These explants were then inoculated onto half-strength Murashige and Skoog (½ MS) medium (Murashige and Skoog 1962) supplemented with 8.88 μM BAP (B-3408, Sigma, St. Louis, MO, USA) and 0.5 μM IBA (I-5386, Sigma) to induce axillary bud formation. Subculture was performed every 15 d onto freshly prepared medium of the same composition. After 3 subcultures, the resulting aseptic shoots were excised and transferred to proliferation medium for proliferation and elongation. The proliferation medium consisted of the WPM (Lloyd and McCown 1980) basal medium supplemented with 3% (w/v) sucrose and 0.75% (w/v) Difco Bacto agar (pH 5.7), with an additional 3.0 μM BAP and 0.05 μM IBA. Subcultures were conducted every 30 d. Morphologically normal shoots (NS) harvested from these cultures could serve as the starting material for subsequent fasciation induction experiments.

Effects of BAP and IBA combinations on the induction of fasciated tissue

Aseptic NSs harvested from the previous subcultures were inoculated onto the WPM induction medium supplemented with various combinations of BAP (4.44, 8.88, and 12.32 μM) and IBA (0, 0.1, and 0.5 μM) to trigger the formation of fasciated tissues. Subculture was performed every 15 d for a total of 3 cycles, with growth responses monitored at 20, 40, and 60 d after initial culture. Following the 60-d culture period, the total number of fasciated shoots and the fasciation incidence were recorded. In this stage, each treatment combination comprised 10 explants, with a single shoot inoculated per test tube. The experiment was performed in duplicate.

Histological observation of fasciated tissue and normal shoots

For histological analysis, fragments approximately 2.0 mm in width were excised from fasciated tissues, while shoot apices from NSs served as controls. These samples were fixed in formalin acetic alcohol (FAA) solution (40% formaldehyde: 100% glacial acetic acid: 95% ethanol: ddH_2O = 1:1:10:8; Jung et al. 2005) for 7 d.

Following fixation, tissues were dehydrated through a graded ethanol and tert-butanol (TBA) series (10, 20, 35, 55, 75, and 100% TBA), with vacuum infiltration to ensure thorough reagent penetration. Infiltration was completed in an oven (60–65℃) using melted paraffin (Paraplast, Leica Biosystems, USA). Following embedding, 10-μm-thick sections were obtained using a rotary microtome. The sections were subsequently stained with 1% safranin and 0.5% fast green, mounted, and examined via a light microscope to evaluate morphological differences.

Effects of BAP and IBA combinations on the maintenance of fasciated tissue

After the induction of fasciation, the laterally proliferating shoot apex (indicated by the box in Figure 1A) was excised and divided into multiple small fragments, each approximately 0.5 cm in width (Figure 1B). These fragments were subsequently cultured on the WPM basal medium supplemented with various concentrations of BAP (1.11, 2.22, or 4.44 μM) combined with IBA (0.5 or 1.0 μM). Each treatment group consisted of 6 tissue fragments, with 3 fragments cultured per Petri dish. The cultures were maintained for 84 d with subcultures at days 21, 42, and 63. As the tissue fragments maintained their fasciated characteristics and continued to expand laterally, the width increment of each fragment was measured at each subculture to calculate the mean growth. For subsequent subcultures, a 0.5-cm portion was randomly excised from each expanding fragment to serve as the new explant, thereby maintaining a consistent sample size of 6 fragments per treatment. The final width increment was recorded at day 84. All measurements were performed using a mechanical vernier caliper to determine the width increment every 21 d. The experiment was performed in duplicate.

Effects of BAP and IBA combinations on the formation of recovered normal shoots from fasciated tissue

Fasciated tissue fragments approximately 1.0 cm in width were minced into small fragments using a scalpel in an empty Petri dish. The minced tissue was then inoculated onto the shoot proliferation medium (WPM supplemented with 3.0 μM BAP and 0.05 μM IBA). In addition, 2 different plant growth regulator (PGR) combinations were tested for comparison: 2.22 μM BAP + 1.0 μM IBA and 4.44 μM BAP + 0.5 μM IBA. One milliliter of sterile ddH_2O was added onto the medium surface and mixed with the minced tissue, which was evenly spread using an L-shaped cell spreader (Figure 1C). Each treatment used 15 fasciated fragments (1.0 cm each), with each fragment cultured in 1 Petri dish (Figure 1D). Cultures were maintained for 42 d with a subculture at day 21. During this period, shoots differentiated and emerged from the minced tissue. Recovered normal shoots (RnS) longer than 1.0 cm were excised and transferred individually to the proliferation medium. Undersized or persistently fasciated shoots were excluded from the final count. At the end of the culture period, the number of RnS obtained per 1.0-cm fragment was recorded. The experiment was performed in duplicate.

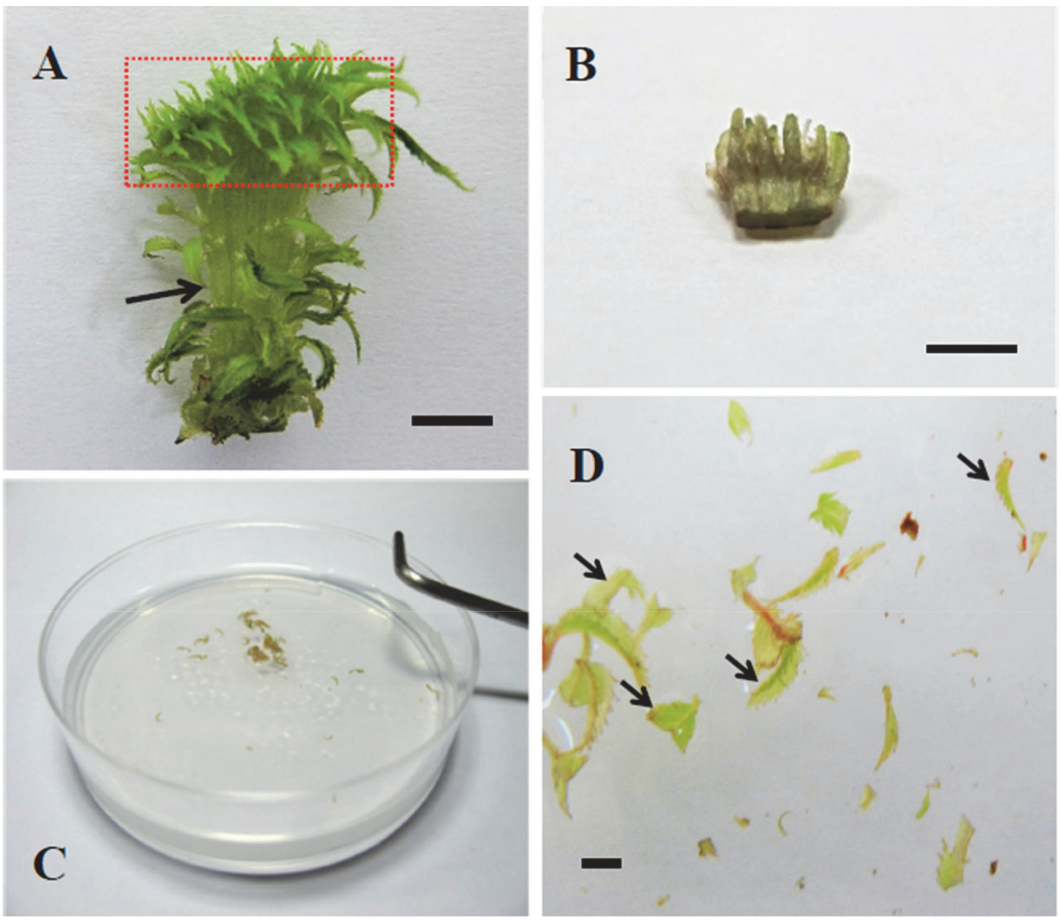

Figure 1. Key culture steps for maintaining shoot fasciation and recovering morphologically normal shoots in *Prunus campanulata* Maxim. (A) A typical fasciated shoot obtained from axillary micropropagation, showing a flattened stem (arrow) and lateral proliferation in the shoot tip (red box); (B) a 0.5 cm-wide fragment excised from the laterally proliferated shoot tip (red box in A), used as the initial explant for fasciation maintenance; (C) cutting fragments from a 1.0 cm-wide fasciated tissue, mixed with ddH_2O and spread onto the shoot proliferation medium using an L-shaped cell spreader; (D) close-up view of the cutting fragments cultured in C, revealing fine dispersed proliferating meristematic cells and larger pieces of broken leaves (arrows). Scale bars in (A) (B) = 5.0 mm; (D) = 2.5 mm.

Methodology for estimating the total yield of recovered normal shoots from fasciated tissues

To estimate the total number of shoots obtainable through the developed propagation system, calculations were based on an initial fasciated fragment 0.5 cm in width. In the "Maintenance of Fasciated Tissue" experiment, the tissues underwent continuous lateral proliferation. At the conclusion of each subculture cycle, the expanded tissues were partitioned into multiple 0.5-cm fragments to initiate the subsequent cycle.

This process was computationally repeated until the end of the experiment to determine the cumulative "total width (cm)" of the proliferated material. Concurrently, the "Formation of Recovered Normal Shoots" experiment established "the number of RnSs produced per 1.0-cm fasciated segment." The total number of shoots was estimated by multiplying the cumulative "total width (cm)" by "the number of shoots produced per cm." However, because full-scale physical propagation of all proliferated material was impractical due to the exponential increase in tissue quantity, parameters used in the estimation were derived from randomized sampling and statistical analysis. Detailed calculations, including the substitution of statistically derived values, are presented in the Results section.

Effects of IBA concentration and induction duration on shoot rooting

The NSs obtained from the proliferation cultures served as the experimental material to determine optimal rooting conditions. These shoots were inoculated onto the WPM basal medium supplemented with different concentrations of IBA (39.36, 49.2, 59.04, and 73.8 μM) and subjected to different induction durations (24, 48, and 72 h). Following induction, the shoots were transferred to the IBA-free WPM medium supplemented with 8.88 μM $AgNO_3$ to facilitate adventitious root elongation. Rooting performance was assessed after 14 d. A shoot was considered successfully rooted when at least 1 adventitious root ≥ 0.5 cm in length emerged from the basal region. The number of rooted shoots and the length of the longest root were recorded. Each treatment consisted of 10 shoots, with 1 shoot cultured per test tube. The experiment was performed in duplicate.

Once the optimal conditions were established, the rooting performance of RnSs was compared with that of subcultured NSs to determine whether shoot origin significantly influenced rooting capability. Forty shoots from each source (RnS and NS) were subjected to the selected optimal rooting treatment. Each shoot was cultured individually in a test tube, and the experiment was performed in duplicate. Root number, mean root length, longest root length, and rooting percentage were recorded to assess potential differences between shoot origins.

Establishment of acclimatization protocol for plantlets

Vigorous rooted plantlets (derived from RnS and NS) were selected for acclimatization. Residual medium at the base was rinsed off with distilled water, and foliage on the lower 50% of the plantlet height was excised. The plantlets were transplanted into perforated rectangular plastic baskets (47 × 37.7 × 13 cm) filled with a mixed substrate (chopped sphagnum moss: akadama soil: vermiculite: perlite = 1:2:2:0.5, v/v). A planting density of 5 × 4 plantlets per basket was maintained. The perforated basket was placed within a larger non-perforated outer container (59 × 47.5 × 16 cm) elevated by 4 Petri dishes (2 cm in height) positioned at the bottom. The outer container was covered with a transparent acrylic plate to maintain high relative humidity, and reverse osmosis (RO) water was applied to keep the substrate moist. During the acclimatization process, the coverage of the acrylic plate was gradually reduced to increase air exchange incrementally. After 4 weeks, the plantlets were transferred to a semi-shaded greenhouse. The acrylic cover was progressively removed to lower the relative humidity and allow adaptation to *ex vitro* conditions. The survival rate was recorded, and the experiment was performed in triplicate.

Environmental conditions across tissue culture stages

For the fasciation induction and rooting experiments, explants were cultured in sterile glass test tubes (20 mm diameter × 115 mm length). For fasciation maintenance and shoot formation experiments, sterile Petri dishes (90 mm diameter × 20 mm height) were used. RnSs were subsequently subcultured in Orchid Cultivation Boxes (90-mm diameter × 104 mm height; Type CK-B, Ching Ke Enterprise Co., Ltd., Taiwan). The culture room was maintained at 24 ± 1℃ under cool white fluorescent lights providing a photon flux density (PPFD) of 30–35 μmol m^{-2} s^{-1} with a 16 h light / 8 h dark photoperiod. During the acclimatization stage, a combination of cool white fluorescent and incandescent lights was employed, with the PPFD increased to 40–50 μmol m^{-2} s^{-1} under the same photoperiod.

Statistical analysis

The fasciation induction experiment (3 × 3) and the NS rooting experiment (3 × 4) were conducted as completely randomized factorial experiments, each performed in duplicate. The data were analyzed using two-way analysis of variance (ANOVA). The fasciation maintenance experiment (3 × 2) was also arranged as a completely randomized factorial experiment, with data analyzed at the end of each subculture cycle to evaluate the effects of plant growth regulator (PGR) combinations on the maintenance of fasciation and to identify the optimal treatment for maximizing lateral width increment. The shoot formation experiment utilized a completely randomized design (CRD), with 3 treatments (15 explants per treatment), each performed in duplicate. Data were analyzed using one-way ANOVA.

All statistical analyses were performed using IBM SPSS Statistics 22.0. When significant differences were detected ($p < 0.05$), treatment means were compared using Fisher's least significant difference (LSD) test. For comparisons between RnSs and NSs in rooting and acclimatization experiments, independent sample *t*-tests were performed to compare the root number, mean root length, longest root length, and survival rate.

RESULTS

Effects of BAP and IBA combinations on fasciation induction

Approximately 20 d after induction culture, the basal region of the shoot explants began to swell, accompanied by the emergence of new shoots. After 40 d of culture, most of the single shoots had developed into clusters of multiple shoots due to the proliferation of axillary buds, and some large shoots exceeding 1.5 cm in length were observed (Figure 2A). After 60 d of culture, fasciation began to emerge at the apices of several large shoots. The shoot apical meristem exhibited lateral expansion rather than vertical elongation, with numerous small leaves arranged along both sides of the laterally expanded apex (Figure 2B).

At the end of the induction stage, both the average number of shoots exhibiting fasciation per explant and the fasciation forming percentage decreased as the BAP concentration increased. Among the treatments, 4.44 μM BAP resulted in a fasciation rate of 7.7%, which was significantly higher than that of the other treatments (Table 1). Furthermore, IBA concentration did not significantly affect fasciation induction, and no significant interaction was observed between BAP and IBA (Table 1).

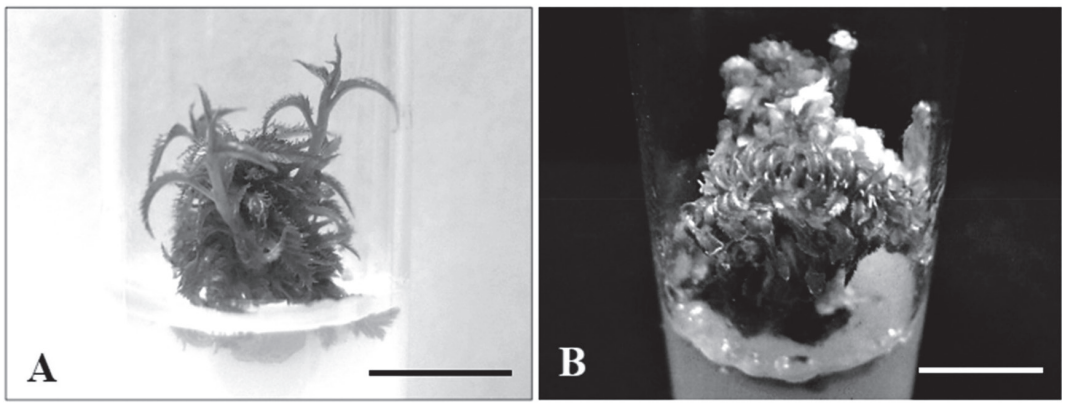

Figure 2. Induction of shoot fasciation in *Prunus campanulata* Maxim. (A) Elongated large shoots derived from axillary micropropagated microshoots; (B) fasciated shoot showing lateral proliferation at the shoot tip with numerous reduced leaves. Scale bars = 1.0 cm.

Table 1. Effect of BAP and IBA concentrations on the induction of shoot fasciation in *Prunus campanulata* Maxim.

Source factor	Levels (μM)	Fasciated shoots [1] (no. explant^{-1})	Ratio of fasciated shoot formation [2] (%)
BAP	4.44	1.6 ± 0.2 [a]	7.7 ± 0.9 [a]
	8.88	0.7 ± 0.1 [b]	3.4 ± 0.6 [b]
	13.32	0.3 ± 0.1 [c]	1.1 ± 0.3 [c]
IBA	0.0	0.8 ± 0.2 [a]	4.7 ± 0.9 [a]
	0.1	0.7 ± 0.1 [a]	3.5 ± 0.7 [a]
	0.5	1.0 ± 0.1 [a]	4.0 ± 0.7 [a]

Source factor	Significance [3]	
	Fasciated shoots	Ratio of fasciated shoot formation
BAP (B)	*	*
IBA (I)	n.s.	n.s.
B×I	n.s.	n.s.

[1,2] Formation of fasciated shoots and its ratio (mean ± SE, n = 10) corresponding to levels of each source factor, where mean values followed by different superscript letters in the same column are significantly different according to Fisher's least significant difference (LSD) test at $p < 0.05$.

[2] Percentage data were all arcsine transformed prior to analysis.

[3] Source factor and interactive effects on fasciated shoot formation, where * represents a significant difference detected by Fisher's LSD test at $p < 0.05$; n.s. indicates no significant difference ($p > 0.05$).

BAP: 6-benzylaminopurine; IBA: indole-3-butyric acid.

Histological characterization of fasciated tissues and normal shoots

Longitudinal sections of the shoot apices revealed a distinct single meristem on the NSs (Figure 3A, arrow), which exhibited a characteristic dome-shaped structure enclosed by developing leaf primordia. By contrast, sections of fasciated tissues exhibited pronounced lateral expansion and broadening

of the shoot apex (Figure 3B). Although the histological section was obtained from only a small portion of the fasciated shoot (approximately 2.0 mm in width), this width was already 10 times greater than the diameter of the NS apex. Considering that a fully developed fasciated shoot can reach a width exceeding 1.0 cm, the degree of lateral expansion compared to NSs is substantially more significant. Numerous protruding meristems were observed on the surface of these expanded regions, composed of small, densely packed cells (Figure 3C, arrow). The cellular organization of these meristems was consistent with that of the NS apical meristem but lacked surrounding leaf primordia and frequently occurred in parallel arrangements. These observations indicated that fasciated

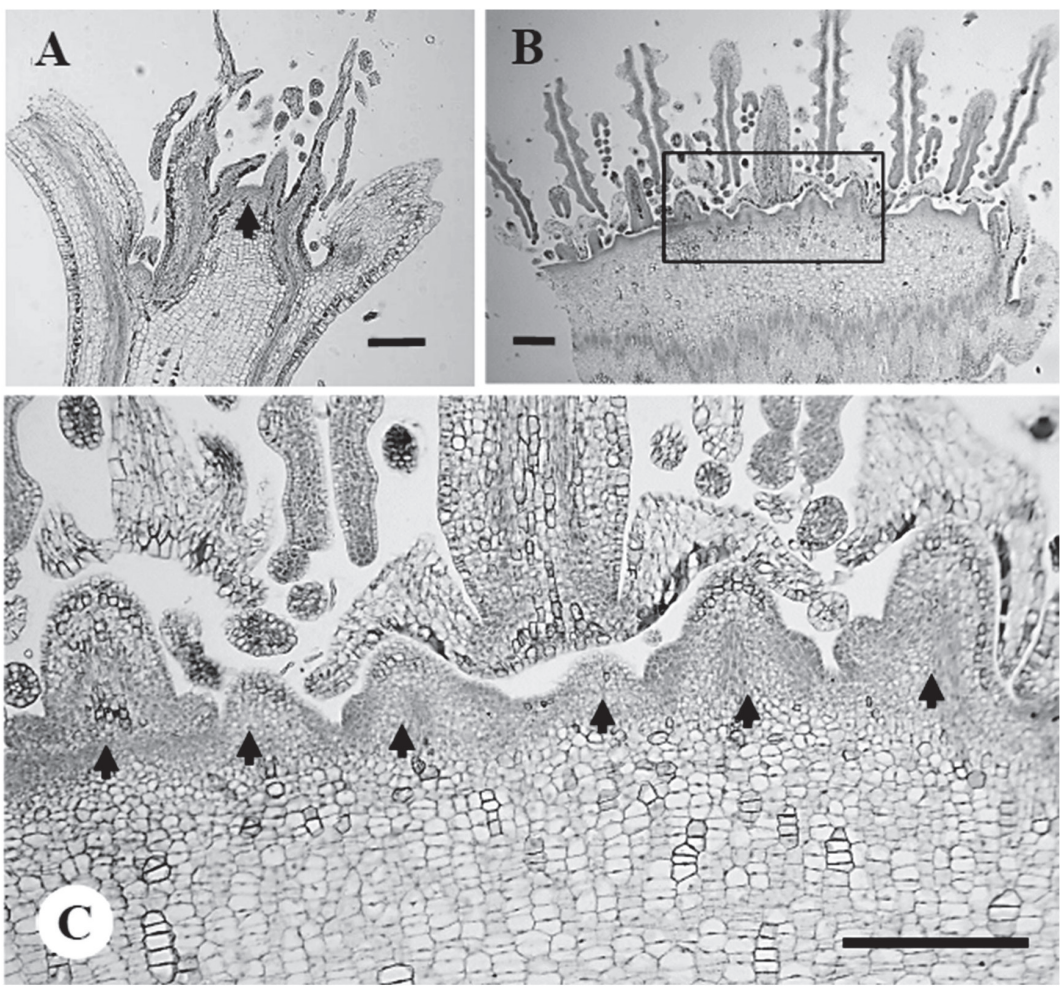

Figure 3. Comparative histological analysis of normal versus fasciated shoots in *Prunus campanulata* Maxim. (A) Longitudinal section of a normal shoot apical meristem showing a single distinct meristem (arrow) enclosed by developing young leaves; (B) longitudinal section of a fasciated shoot tip, revealing laterally proliferating meristems (boxed); (C) magnified view of the region highlighted in B, emphasizing multiple meristems (arrows) that have developed on the flattened upper surface of the shoot tip. Scale bars = 0.2 mm.

shoot apices are characterized by "multiple meristems," which serve as the source for subsequent shoot regeneration, and a higher number of these meristems significantly enhances propagation efficiency.

Effects of BAP and IBA combinations on the maintenance of fasciated tissues

Throughout the 84-d culture period, fasciated tissue fragments in all treatment combinations exhibited a continuous increase in width, indicating that the meristems of the fasciated tissues remained in an actively proliferative state. Statistical analysis revealed that both IBA concentrations had no significant effect on the width increase of the tissue fragments. By contrast, BAP treatments resulted in significant differences in the average width of fasciated tissues during the first 3 subcultures (S1–S3), although no significant differences were observed in the 4th subculture (S4). Among the treatments, 2.22 μM BAP was the most effective in promoting width expansion, yielding the greatest average width increase by the end of each subculture (Table 2) while maintaining a stable fasciated phenotype (Figures 4A, 4B, and 4C). Conversely, the 1.11 μM BAP treatment was less effective, and during the subculture, some tissue fragments reverted to a normal single-shoot morphology, failing to maintain the fasciated phenotype. After 42 d of culture, some explants treated with 4.44 μM BAP exhibited browning, accompanied by the swelling of basal callus and poor growth. Regardless of the concentration applied, all proliferations of the fasciated tissue fragments began to decline after 64 d of culture (S4 stage), and the rate of width increase gradually decreased (Figure 4D; Table 2).

Table 2. Effect of BAP and IBA concentrations on the lateral width proliferation of cultured fasciated *Prunus campanulata* Maxim. shoot fragments.

Source factor	Levels (μM)	Width increase per fasciated fragment (cm) [1]			
		S1 [2]	S2 [2]	S3 [2]	S4 [2]
BAP	1.11	0.46 ± 0.03 [b]	0.82 ± 0.04 [b]	0.62 ± 0.04 [c]	0.28 ± 0.06 [a]
	2.22	0.82 ± 0.03 [a]	1.23 ± 0.05 [a]	1.01 ± 0.05 [a]	0.35 ± 0.05 [a]
	4.44	0.74 ± 0.05 [a]	0.86 ± 0.04 [b]	0.73 ± 0.03 [b]	0.33 ± 0.06 [a]
IBA	0.5	0.67 ± 0.03 [a]	0.96 ± 0.04 [a]	0.77 ± 0.04 [a]	0.31 ± 0.04 [a]
	1.0	0.72 ± 0.04 [a]	0.98 ± 0.05 [a]	0.82 ± 0.05 [a]	0.34 ± 0.05 [a]

Source factor	Significance [3] Width increase per fasciated fragment			
	S1	S2	S3	S4
BAP (B)	*	*	*	n.s.
IBA (I)	n.s.	n.s.	n.s.	n.s.
B×I	n.s.	n.s.	n.s.	n.s.

[1] Mean width increase in cultured fasciated fragments (mean ± SE, $n = 6$) corresponding to levels of each source factor, where mean values followed by different superscript letters in the same column are significantly different according to Fisher's least significant difference (LSD) test at $p < 0.05$.

[2] S1: subculture days 1–21; S2: subculture days 22–42; S3: subculture days 43–63; S4: subculture days 64–84.

[3] Source factor and interactive effects on width increase in fasciated fragments, where * represents a significant difference detected by Fisher's LSD test at $p < 0.05$; n.s. indicates no significant difference ($p > 0.05$).

BAP: 6-benzylaminopurine; IBA: indole-3-butyric acid.

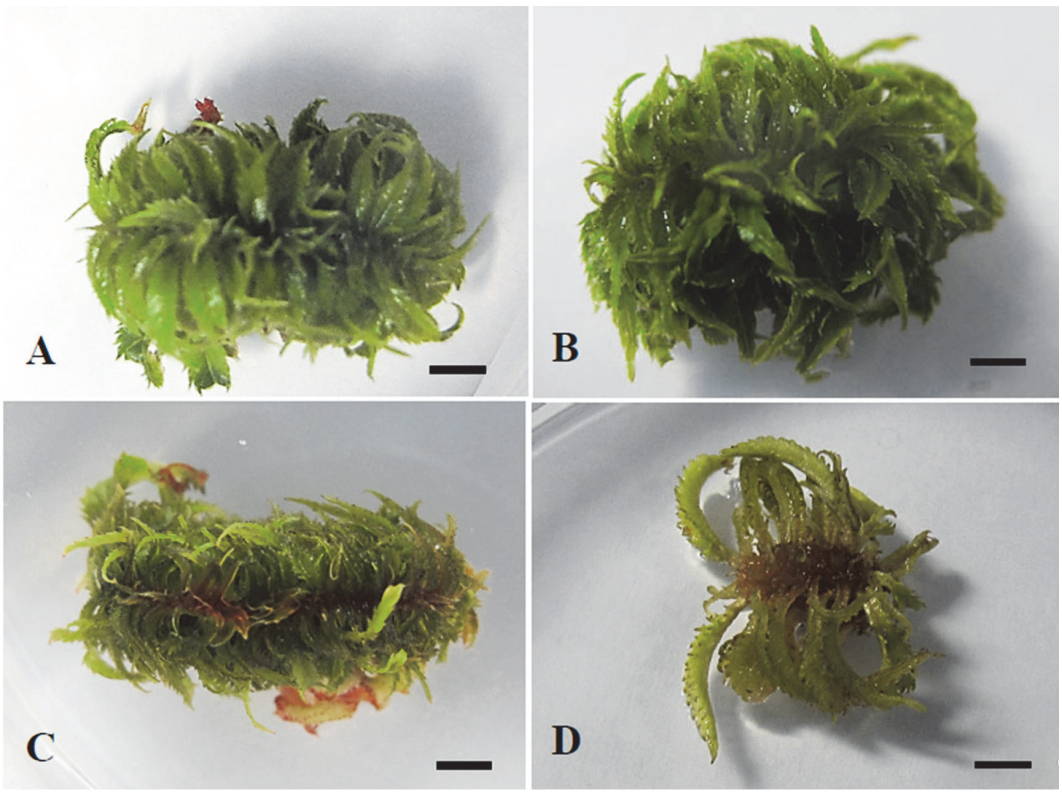

Figure 4. Representative fasciated shoot explants during the "maintenance of fasciation" culture stage. These panels illustrate the laterally proliferated shoot explants at the conclusion of each subculture. The degree of proliferation, as indicated by the increase in explant width, remained consistent across most subcultures, with a notable deduction observed in the 4th subculture. (A) End of the 1st subculture; (B) end of the 2nd subculture; (C) end of the 3rd subculture; (D) end of the 4th subculture. Scale bars = 2.5 mm.

No significant interaction effects between BAP and IBA were detected (Table 2). However, the combination of 2.22 μM BAP and 1.0 μM IBA consistently performed best during S1–S3 (data not shown), with width increments of 0.80 cm (S1), 1.23 cm (S2), and 0.87 cm (S3). These values were subsequently used as parameters for estimating total shoot production.

Effects of BAP and IBA combinations on the regeneration of normal shoots from fasciated tissues

Following intensive fragmentation of fasciated tissue segments and subsequent culture on the WPM proliferation medium, shoots began to differentiate from the minced fragments after 14 d, appearing randomly distributed across the Petri dishes (Figure 5A). By day 21, several RnSs were successfully produced, although some tissues remained in a proliferative fasciated state (Figure 5B). Throughout the 42 d, emerging RnSs were excised and transferred to separate containers (Figure 5C). These shoots exhibited vigorous growth and proliferated into multiple shoots, with no signs of reversion to fasciation (Figure 5D). Once the shoots reached a suitable

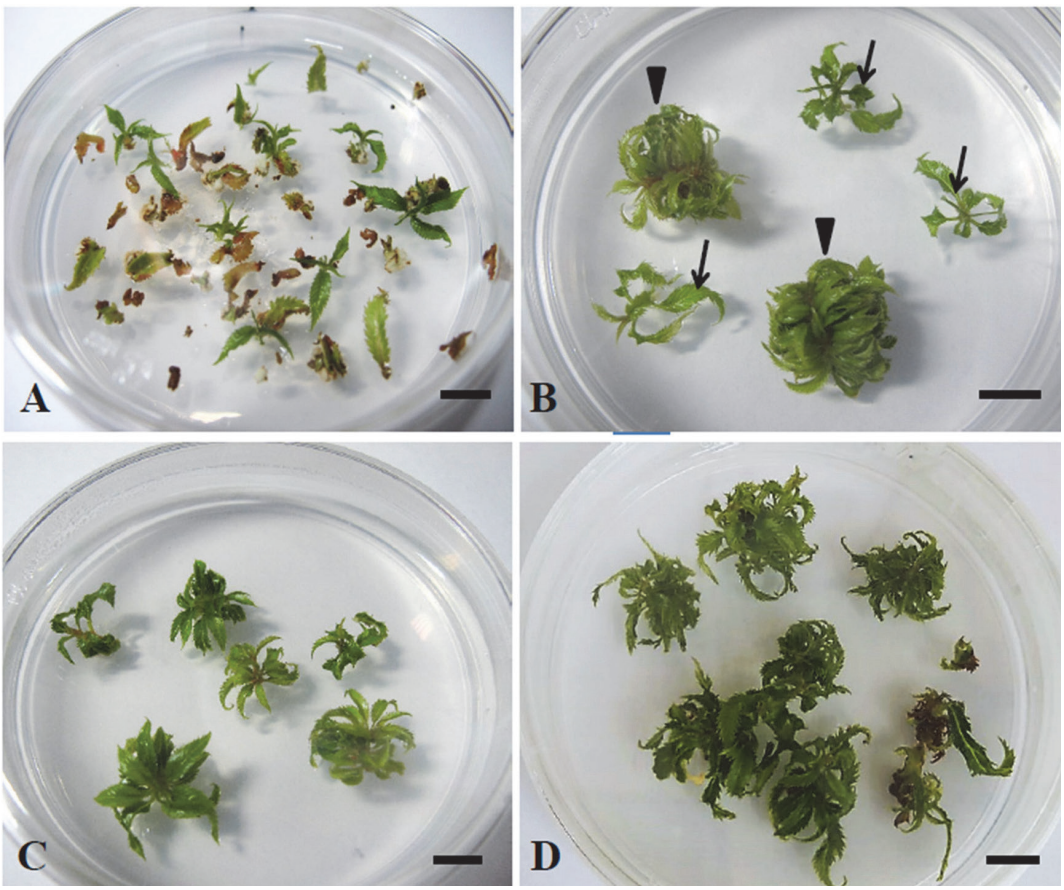

Figure 5. Shoots grown from fasciated *Prunus campanulata* Maxim. explants. (A) New shoots grown from cutting fragments after 14 d in culture; (B) both normal (arrows) and fasciated (arrowheads) shoots developed on the culture medium by day 21 of incubation; (C) individually transplanted normal shoots cultured for further growth; (D) multiple morphologically normal microshoots formed from a single shoot previously cultured as shown in C. Scale bars = 1.0 cm.

height (> 1.5 cm), they could be used as explants for subsequent rooting experiments.

Statistical analysis indicated that shoot regeneration occurred in all treatment combinations following intensive fragmentation of 1.0-cm fasciated tissue segments. However, the combination of 3.0 μM BAP and 0.05 μM IBA resulted in superior shoot regeneration (Table 3). Significant differences were already observed after 21 d of culture, yielding an average of 11.4 large RnSs. After 42 d of culture, this treatment further increased the yield to 15.6 large shoots, the highest among all treatments, with no morphological abnormalities. The other 2 treatment combinations produced 12.9 and 12.7 large shoots, respectively. Consequently, the combination of 3.0 μM BAP and 0.05 μM IBA was adopted as the optimal culture condition for this stage.

Estimation of the total yield of normal shoots induced from fasciated tissues

At the end of the first subculture (S1) in

Table 3. Effectiveness of 3 plant growth regulator (PGR) regimes in promoting the formation of morphologically normal shoots from fine-cut debris of fasciated *Prunus campanulata* Maxim. shoot fragments.

RGR combination		Number of shoots per fasciated fragment [1]	
BAP (μM)	IBA (μM)	Incubation of 21 d	Incubation of 42 d
2.22	1.0	9.9 ± 0.4 [b]	12.9 ± 0.6 [b]
3.0	0.05	11.4 ± 0.3 [a]	15.6 ± 0.4 [a]
4.44	0.5	9.0 ± 0.4 [b]	12.7 ± 0.3 [b]

[1] Mean value of normal shoot formation (mean ± SE, $n = 15$) followed by different superscript letters in the same column are significantly different according to Fisher's least significant difference (LSD) test at $p < 0.05$.
BAP: 6-benzylaminopurine; IBA: indole-3-butyric acid.

the fasciated tissue maintenance experiment, a 0.5-cm fasciated tissue fragment exhibited a width increment of 0.8 cm, resulting in a final fasciated fragment width (FFFW) of 1.30 cm (0.5 + 0.8 cm; Table 4). This length could be partitioned into 2.6 fragments, each 0.5 cm in width (Table 4). Following the second subculture (S2), a 0.5-cm fragment showed a width increment of 1.23 cm, yielding a FFFW of 1.73 cm (0.5 + 1.23 cm). When all 2.6 fragments produced from the previous subculture were further cultured, the total fasciated fragment width (TFFW) reached 4.50 cm (1.73 × 2.6 cm; Table 4), which could then be fragmented into 9.0 segments of 0.5 cm in width (Table 4). Applying the same calculation to the end of the third subculture (S3), each 0.5-cm segment increased by 0.87 cm, resulting in an FFFW of 1.37 cm and a TFFW of 12.33 cm (1.37 × 9.0; Table 4).

Based on the final TFFW of 12.33 cm and the fact that each 1.0-cm fasciated segment can produce 15.6 RnSs using the shoot regeneration protocol described in this study, the total yield was calculated to be approximately 192.4 RnSs (12.33 × 15.6; Table 4).

Effects of IBA concentration and induction duration on shoot rooting

When the NSs were used for rooting experiments, basal swelling was observed in some shoots 5–6 d after transfer to the IBA-free medium. By day 8, adventitious roots were clearly visible at the swollen basal region. By day 10, most roots had elongated

Table 4. Impact of successive subcultures on both the width increase of laterally proliferating fasciated fragments (maintenance stage) and the subsequent recovery of morphologically normal shoots.

Subcultures (d)	IFFW (cm)	Fragment width increase [1] (cm explant^{-1})	FFFW (cm explant^{-1})	TFFW (cm)	No. of 0.5 cm-wide fragments	No. of normal shoots recovered from TFFW
S1 (1–21)	0.50	0.80	0.5 + 0.80 = 1.30	1.30	1.30/0.5 = 2.60	1.30 × 15.6 = 20.3 [2]
S2 (22–42)	0.50	1.23	0.5 + 1.23 = 1.73	1.73 × 2.60 = 4.50	4.50/0.5 = 9.00	4.50 × 15.6 = 70.2 [2]
S3 (43–63)	0.50	0.87	0.5 + 0.87 = 1.37	1.37 × 9.00 = 12.33	–	12.33 × 15.6 = 192.4 [2]

[1] Derived width increase values (see text).
[2] Normal shoot recovery parameter (15.6) (see text for details).
IFFW: initial fasciated fragment width; FFFW: final fasciated fragment width; TFFW: total fasciated fragment width.

and increased in number (Figure 6).

Statistical analysis after 14 d of culture indicated that different induction durations (24, 48, and 72 h) did not significantly affect the root number or root length of NSs (Table 5). However, treatment with 49.2 μM IBA produced the highest number of adventitious roots (5.6 roots per shoot). Although the number of roots gradually decreased as the IBA concentration increased (59.04 and 73.8 μM), no significant differences were detected among these 3 treatments. Similarly, the longest root length showed no significant differences among the 4 IBA concentrations (Table 5). Furthermore, the root number was significantly influenced by the interaction between induction duration and IBA concentration. Detailed analysis indicated that 49.2 μM IBA for 48 or 72 h, and 59.04 μM IBA for 48 h, produced higher numbers of adventitious roots (Table 6). To simplify the protocol while minimizing IBA usage and induction time, 49.2 μM IBA with a 48 h induction period was selected as the optimal rooting treatment.

A comparison of rooting responses between RnS and NS was also conducted using the optimal treatment (49.2 μM IBA for 48 h). After 10 d of culture, both root number and root length increased. The results showed no significant differences between RnSs and NSs in terms of average root number, mean root length, longest root length, or rooting percentage (Table 7). This indicated that the rooting responses of RnSs and NSs were comparable under the optimal induction conditions, regardless of the explant source.

Establishment of acclimatization protocol for plantlets

During the initial phase of acclimatization (the first 14 d), some *P. campanulata* plantlets showed yellowing and abscission of leaves, which was typically accompanied by the emergence of new shoots. After 28 d of acclimatization in the growth chamber, the newly emerged leaves were fully expanded, and additional shoots continued to develop. At this stage, the plantlets were transferred to a shaded greenhouse and transplanted into

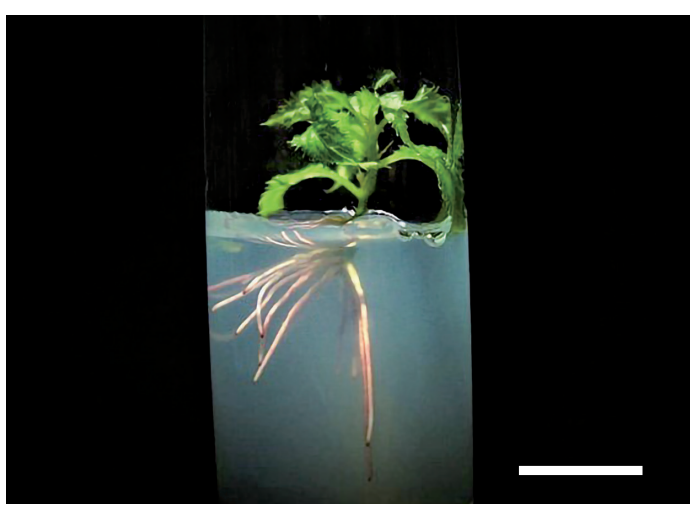

Figure 6. Successful rooting of *Prunus campanulata* Maxim. micropropagated shoots. This image illustrates the optimized rooting conditions enabling subsequent experimental examinations. Scale bar = 1.0 cm.

Table 5. Influence of IBA concentration and induction duration on adventitious rooting in *Prunus campanulata* Maxim. *in vitro*-grown normal shoots.

Source factor	Levels	Root production per shoot [1]	Maximum root length (cm) [2]
IBA (μM)	39.36	3.0 ± 0.6 [b]	1.69 ± 0.21 [a]
	49.20	5.6 ± 0.8 [a]	1.73 ± 0.24 [a]
	59.04	4.4 ± 0.7 [ab]	1.97 ± 0.28 [a]
	73.80	4.0 ± 0.7 [ab]	1.50 ± 0.19 [a]
Induction duration (h)	24	3.7 ± 0.7 [a]	1.42 ± 0.18 [a]
	48	5.0 ± 0.9 [a]	1.80 ± 0.20 [a]
	72	4.2 ± 0.7 [a]	1.89 ± 0.21 [a]

	Significance [3]	
	Root production	Maximum root length
IBA (I)	*	n.s.
Induction duration (T)	n.s.	n.s.
I × T	*	n.s.

[1,2] Root production and maximum root length (mean ± SE, n = 10) corresponding to levels of each source factor, where mean values followed by different superscript letters in the same column are significantly different according to Fisher's least significant difference (LSD) test at $p < 0.05$.

[3] Source factor and interactive effects on root production and maximum root length, where * represents a significant difference detected by Fisher's LSD test at $p < 0.05$; n.s. indicates no significant difference ($p > 0.05$).

IBA: indole-3-butyric acid.

Table 6. Interactive effects of IBA concentration and induction duration, as derived from Table 5, on the adventitious root production of normal *Prunus campanulata* Maxim. shoots.

IBA (μM)	Root production (no.) per shoot [1]		
	Induction of 24 h	Induction of 48 h	Induction of 72 h
39.36	2.2 ± 0.9 [c]	3.0 ± 1.6 [bc]	3.6 ± 1.6 [b]
49.20	4.0 ± 1.5 [b]	7.1 ± 1.5 [a]	5.7 ± 1.4 [a]
59.04	3.0 ± 1.2 [bc]	5.4 ± 1.3 [a]	3.6 ± 1.0 [b]
73.80	4.5 ± 1.4 [ab]	4.3 ± 1.0 [ab]	2.3 ± 1.0 [c]

[1] Mean value of root production (mean ± SE, n = 10) followed by different superscript letters are significantly different according to Fisher's least significant difference (LSD) test at $p < 0.05$.

IBA: indole-3-butyric acid.

Table 7. Comparison of rooting (n = 40) and acclimatization (n = 20) performance in *Prunus campanulata* Maxim. plantlets originated from different shoot explants.

Explant type [1]	Root production (no.) per shoot	Root length (cm)	Maximum root length (cm)	Rooting rate (%)	Acclimatization survival (%)
NS	7.1 ± 0.4	1.50 ± 0.08	1.82 ± 0.09	77.5 ± 5.1	91.7 ± 1.7
RnS	7.2 ± 0.6	1.48 ± 0.07	1.90 ± 0.09	80.0 ± 7.4	95.0 ± 2.9
t-value [2]	-0.392	0.203	-0.578	-0.257	-1.002

[1] NS explants were originated from initially micropropagated normal shoots, whereas RnS explants were normal shoots recovered from fasciated tissue.

[2] All parameters analyzed by an independent sample t-test showed no significant difference between the 2 explant types (t-values < table value $t_{(0.05/2,\ n-2)}$).

nursery pots. By maintaining substrate moisture and nutrient supply, the acclimatization phase was successfully concluded.

The survival rate of rooted RnSs reached 95% (Table 7), showing no significant difference compared to NSs and exhibiting vigorous growth. Furthermore, plantlets produced via this system maintained normal leaf morphology and phyllotaxy during subsequent growth in the greenhouse (Figure 7).

DISCUSSION

Although the same methodological approach was applied, the propagation results obtained for *P. campanulata* exhibited a substantial difference in efficiency compared to those reported for *D. spatulata* (Liao and Ji 2014). During the stage of producing RnSs from fasciated tissues, fragmented 1.0-cm fasciated fragments produced 20.1 RnSs in *D. spatulata*, whereas 15.6 RnSs were obtained in *P. campanulata*. When considering only this stage, the yields of these 2 species are relatively comparable. While fasciated shoots frequently arise during tissue culture, the key factor enabling large-scale propagation lies in the ability of these abnormal propagules to proliferate continuously while stably maintaining the fasciated phenotype. In *D. spatulata*, fasciated shoots underwent continuous lateral expansion on the PGR-free medium for nearly 1 year, and the rate of width expansion did not decline with increasing numbers of subcultures, which represents a key factor enabling its

Figure 7. *Prunus campanulata* Maxim. plantlets recovered from fasciated shoot tissue. These plantlets, grown in a greenhouse, exhibited normal phyllotaxy and leaf size. Scale bar = 15 cm.

high propagation efficiency. By contrast, fasciated fragments of *P. campanulata* failed to survive on the PGR-free medium, making their maintenance and proliferation strictly dependent on exogenous PGRs. Furthermore, during repeated partitioning and subculturing, most *P. campanulata* fragments exhibited physiological decline after 63 d, with a reduced rate of width expansion (Table 2). This represents the most significant difference compared to *D. spatulata* and, therefore, likely accounts for the lower propagation efficiency observed in *P. campanulata* relative to *D. spatulata* when applying the same method. Additionally, both species occasionally showed a small proportion of shoots reverting to normal morphology at a low frequency during the proliferation. A similar phenomenon was termed defasciation during the culture of fasciated *P. sativum* (Sinjushin and Gostimsky 2006). However, we speculated that their underlying mechanisms may differ. In the propagation system developed in this study, the earlier reversion of fasciated shoots to normal morphology implied a reduction in the volume of fasciated tissue available for further proliferation, thereby decreasing the final yield of tissue-culture plantlets. Nevertheless, the frequency of such events was not high enough to cause substantial loss. However, current evidence remains insufficient to explain the mechanism underlying this spontaneous reversion to normal morphology.

In the experiment for RnS production, *P. campanulata* fasciated tissues were intensively fragmented and cultured on the WPM proliferation medium, from which RnSs differentiated from the minced tissue fragments. This procedure represents a form of mechanical (physical) disruption, and when the fragmentation intensity is sufficient to detach and separate the connections among the meristems on the fragments effectively, a larger number of normal plantlets can be obtained. However, evidence remains insufficient to explain how such mechanical disruption triggers the reversion of abnormally structured fasciated tissues to normal morphology. Furthermore, whether alternative fragmentation approaches could enhance RnS production requires further experimental verification.

The propagation method established in this study was based on exploiting the nature of multiple meristems in fasciated shoot apices and integrating this with a culture process that enabled their sustained proliferation. By contrast, Liang et al. (2019) used flattened fasciated stem segments of *V. rotundifolia* as explants to induce the elongation of axillary buds on the segments, a procedure that lacked a continuous proliferation phase. The authors reported that the normal stems of *V. rotundifolia* were approximately 2–3 mm wide, whereas fasciated stems could expand to 5–25 mm in width. Therefore, using the widened and flattened fasciated stems as explants provided a substantially increased surface area for axillary bud formation. Their results showed that an average of 78.0 buds per explant could be induced, effectively improving propagation efficiency. However, as this approach represented a single-cycle operation, introducing a culture phase designed to increase the quantity or volume of fasciated tissues before bud induction could further enhance propagation capacity.

Currently, in conventional tissue culture propagation studies of the *Prunus* species, micropropagation is typically achieved by inducing the growth of apical or axillary buds located on stem nodes. Before rooting induction, shoots must reach a certain developmental standard. This process

is generally divided into several stages, including the establishment of aseptic shoots, shoot proliferation using single-node explants, and shoot elongation (the latter 2 stages may sometimes be combined into a single step, resembling the system proposed in this study). To compare the proliferation efficiency of this method with that reported for other *Prunus* species, studies providing explicit culture data (including culture duration and shoot yield) were reviewed. Research on 2 dwarf cherry rootstocks, 'Maxma-14' (*P. avium* L.) (Muna et al. 1999) and 'PHL-A' (*P. avium* × *P. cerasus*) (Mahdavian et al. 2011), as well as *P. sargentii* Rehder (Sasaki 2003) and *P. campanulata* grown in Brazil (Sanson et al. 2024), has indicated that approximately 84–119 d are required after the establishment of sterile shoots to complete the proliferation and elongation stages. However, the number of shoots produced that meet the required standard ranges only from 3 to 9.57.

Relatively faster shoot proliferation and growth rate have been reported in the *P. mume* L. cultivar 'Xuemei' and in *P. armeniaca* L. The former produced 5.43 well-developed shoots within 63 d (Ning et al. 2007), whereas the latter yielded 3.36 shoots after 28 d of culture (Yildirim et al. 2011).

In the present study, 60 d were required after the establishment of sterile cultures to induce the formation of fasciated tissues. Subsequently, these tissue fragments underwent a maintenance phase. Following the first subculture (21 d), an additional 42 d were required to produce RnSs from the fasciated tissues, resulting in a total yield of 20.3 shoots within 123 d (Table 4). When a second maintenance subculture was performed, the total shoot yield increased to 70.2 (144 d in total). Remarkably, after the third cycle of maintenance, the total shoot yield reached 192.4 within 165 d (Table 4). Compared with conventional micropropagation methods, although this system for *P. campanulata* required a longer proliferation phase, the extended culture period resulted in remarkably high proliferation efficiency. Furthermore, these shoots exhibited an 80% rooting rate and a 95% acclimatization survival rate (Table 7). Consequently, this system represented a highly promising and feasible strategy for large-scale propagation.

CONCLUSIONS

This study used the WPM medium supplemented with 4.44 μM BAP and 0.5 μM IBA to induce fasciation in *P. campanulata* shoots. Fasciated tissue fragments containing shoot meristems were continuously subcultured, allowing the fasciation phenotype to be maintained while the tissue width progressively expanded during a 63-d culture period. Following intensive fragmentation, morphologically normal shoots were regenerated. The complete culture process enabled an initial 0.5-cm fasciated fragment to proliferate and produce 192.4 morphologically normal single shoots. Plantlets obtained through rooting and acclimatization developed into normal plants with no observable morphological variation after 2 years of cultivation. Therefore, establishing a propagation system for *P. campanulata* based on fasciation represents a promising propagation strategy. If this method can be applied to other ornamental cherries or economically important fruit trees within the genus, it may offer considerable commercial potential.

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研究報告

藉由植物綴化現象建立山櫻花(*Prunus campanulata* Maxim.)組織培養繁殖系統

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摘 要

植物組織培養常藉植物生長調節劑誘導培植體(explant)再生芽體，過程中若使培植體內源激素失衡，則有可能導致芽體出現生理或形態上的變化，而綴化(fasciation)現象便屬其一，此現象經常被視為是負面的培養結果，因此常被棄置而少有進一步的利用。植物頂芽綴化後會形成多數頂端生長點(apical meristem)且具增殖特性，本實驗室曾據此建立了一個大量繁殖植物的系統，本研究則將此系統推展應用於山櫻花(*Prunus campanulata* Maxim.)的繁殖。將單一芽體培養於添加6-苯氨基嘌呤(6-benzylaminopurine, BAP)與吲哚-3-丁酸(indole-3-butyric acid, IBA)的WPM (Woody Plant Medium)培養基中，60天後能誘導出具有多數頂端生長點之綴化組織，再以BAP與IBA之不同濃度組合繼代維持綴化組織橫向延展增殖，以密集切割的操作可終止綴化現象並使其形成恢復正常形態之芽體。推估整個培養過程可使初始寬幅0.5 cm之綴化組織形成192.4個形態正常之新芽。這些新芽以IBA誘導後發根率可達80%，出瓶馴化成活率高達95%，由此方法育成之苗木於溫室中生長，外觀形態與正常植株並無明顯差異。

關鍵詞：頂端生長點、綴化、微體繁殖、觀賞櫻花、芽體增殖。

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緒言

植物的綴化(fasciation)現象意指植物器官在形態上的一種異常變化，可能出現在根、莖、葉、花、花序、子葉(White 1945)、果實(Darrow and Borthwick 1954)與根莖(Fukai et al. 2000)等處；Binggeli (1990)指出，綴化現象較易發生在具有無限生長(indeterminate growth)特性的植物，並以岩槭(*Acer pseudoplatanus* L.)為例，說明綴化現象常出現在其嫩枝(twig)、葉柄及花序。Choob and Sinyushin (2012)則描述當莖部的莖頂分生組織(shoot apical meristem, SAM)受綴化影響而異常地延展時，此植物莖部發育就會較正常者寬且帶有稜，而且還會扁平(橫向)擴展。歐洲甜櫻桃(*Prunus avium* L.)綴化莖段上的葉片及芽常集中生長在靠莖頂的位置(Kitin et al. 2005)，此外綴化植物葉序(phyllotaxy)的排列方式也與正常發育者不同(Tang and Knap 1998, Bykova et al. 2016)。而在豌豆(*Pisum sativum* L.)則除了葉序改變，葉片數也會增加(Sinjushin and Gostimskii 2008)。

綴化現象是否發生可受生理機制影響(Iliev and Kitin 2011, Altae and Dalas 2021)或受基因調控(Williams and Fletcher 2005, Elhiti and Stasolla 2011, Iliev and Kitin 2011)。已知環境中缺乏鋅(zinc)元素(Rance et al. 1982)或是植物受香豌豆束莖病菌[*Rhodococcus fascians* (Tilford 1936) Goodfellow 1984]感染(Crespi et al. 1992)都會造成綴化現象，綜觀這些生理因素所引發的綴化現象，都歸因於植物體內生長素(auxin)與細胞分裂素(cytokinin)的平衡發生改變(Murai et al. 1980, Cakmak et al. 1989, Eason et al. 1996)。在基因調控部分，已知SAM的發育受*CLAVATA* (*CLV*)、*WUSCHEL* (*WUS*)、*MGOUN* (*MGO*)及*FASCIATA* (*FA*)等基因的協調控制(Kaya et al. 2001, Graf et al. 2010, Galli and Gallavotti 2016)。關鍵基因*CLV*與*WUS*以負向調節(negative feedback loop)的形式來穩定SAM中細胞的數量並使其正常發育。當*CLV*訊號被抑制或是出現異常時，就失去對*WUS*的調節作用，讓*WUS*大量表達之後，導致SAM中不斷填補累積幹細胞，並使其分化成器官原基的速率也跟著失控，遂出現SAM增大且過度發展的現象(Schoof et al. 2000)。綴化植物的莖部經此變化後，即出現扁平化且增加體積、SAM開始橫向延展以及產生大量縮小的葉片。Cytokinin在進入植物細胞行訊號傳遞的過程中，會透過促進*WUS*或抑制*CLV*等基因的作用來增加*WUS*的表達量，SAM中幹細胞數量會因此增加，當cytokinin濃度失當造成幹細胞增量到失控時就引發綴化(Gordon et al. 2009)。因此增加cytokinin與SAM中相關調控基因的失常導致綴化發生，彼此是互有關聯的。

不論是受生理機制或基因調控異常所導致的綴化，最後都可歸因於植物內生auxin及cytokinin之失衡。而植物組織培養操作為了繁殖大量個體，培養基中添加外源cytokinin是常用的方法，但這往往也是破壞其平衡狀態之開始，因此在組織培養試驗中常會因此誘導綴化現象發生(Amutha et al. 2006, Iliev et al. 2010, Arellano-Perusquia et al. 2013, Dancheva and Iliev 2015)；其中cytokinin的施用應是關鍵因子(Isah 2015)。過往綴化現象出現在組織培養的繁殖體時，常被視為異常之突變，歸類為無利用價值而予以捨棄。本研究室在檢視綴化莖頂時，觀察到橫向延展的SAM，其頂端排列眾多芽生長點，而誘導發生芽生長點是組織培養微體繁殖(micropropagation)、不定芽繁殖(adventitious bud propagation)及癒傷組織器官發生(callus organogenesis)等幾種技術的關鍵步驟，因此產生構想；如果綴化的頂芽都已經形成了多數的芽生長點，為何不利用做為植物繁殖材料，也可省去再誘導的操作。

在食蟲植物(carnivorous plant)之研究，曾有人描述可取其綴化的器官進行無性繁殖，惟其目標是為了使繁殖後代保有綴化特性以供觀賞，並不求數量之突破，因此繁殖效率不高(Wistuba 1983, Hanrahan 1985)。在植物組織培養的研究領域中，亦有學者繁殖出綴化植物並留下詳細數據，例如Iliev et al. (2003)以垂枝樺(*Betula pendula* Roth.)為研究材料，以玉米素[6-(4-hydroxy-3-methylbut-2-enylamino) purine, zeatin]誘導葉的癒傷組織形成不定芽，有低於5%的比例會出現綴化芽體。而歐洲甜櫻桃之單芽培養在含吲哚-3-丁

酸(indole-3-butyric acid, IBA)及激勃素(gibberellic acid, GA_3)的培養基中，再以不同濃度6-苯氨基嘌呤(6-benzylaminopurine, BAP)處理，亦能獲得少量綴化芽體(Kitin et al. 2005)。Papafotiou et al. (2001)在繁殖金星仙人球(*Mammillaria elongata* DC.)時，以BAP組合1-萘乙酸(1-naphthaleneacetic acid, NAA)的培養條件誘導產生綴化芽體。這些文獻紀錄顯示在組織培養過程中綴化芽體的產生並不少見，但不是數量稀少未能達到大量繁殖的層次，就是這些綴化的產出物也並非原本研究設定要收獲之標的。另有報告指出當綴化芽體被誘導形成之後，從莖部橫切面觀察皮層薄壁組織(cortical parenchyma)及髓組織(pith)細胞會異常擴大，有些甚至變形或破碎，並非正常生長(Kitin et al. 2005)。因此綴化現象在組織培養中並未獲重視，也無進一步的研究。

Anderson et al. (1982)曾經在草莓(*Fragaria* × *ananassa* Duch.)莖梢(shoot tip)部位觀察到多莖頂(multi-apexing)現象，認為這是綴化的不正常形態，描述將其分離後可使其恢復正常的過程，也報導了增加BAP濃度容易使這種現象發生的結果，但是僅於文中提出建議，認為這可以是一種高效率的芽體增殖方法。Liao and Ji (2014)以寬葉毛氈苔(*Drosera burmannii* Vahl.)為例首先驗證了利用綴化的SAM頂端有多數生長點的特性，可以於組織培養操作下，讓生長點增殖及分化並復原成多數形態正常的小植株。Liang et al. (2019)則進行類似的操作，以蔓荊(*Vitex rotundifolia* L.)發生綴化的扁平莖段為培植體，誘導該莖段上的側芽抽長生長而獲形態正常植株。這兩篇報導是組織培養開始以綴化植物器官為材料，並實質上有「大量」增殖繁殖體的開始。本研究則是以山櫻花(*Prunus campanulata* Maxim.)為例，再推展將此繁殖法應用到第三個物種。山櫻花為臺灣眾多森林遊樂區及民營景觀熱點之重要觀賞植物，也是北部地區民眾居家庭院或社區中可種植的櫻花種類，大量繁殖有其市場需求及價值。梅屬(*Prunus* L.)植物之類群包含多種落葉或常綠之喬木與灌木，某些物種是世界上重要的經濟水果及堅果作物(Chin et al. 2014)。後續擬嘗試將本繁殖方法推廣應用到同屬其他觀賞或經濟樹種，應頗具潛在商業利益。

材料與方法

無菌芽體之建立

採集山櫻花樹幹基部萌糵枝條，去除葉片後以大量流動自來水沖洗並修剪至每段長約5 cm，接續浸泡於0.2% (v/v)之安期殺菌液(中國化學製藥公司，新竹)30 min，再以200 mL自來水加入1-2滴家用洗滌劑清洗2-3 min，再以20% (v/v)商用漂白水(commercial bleach, Clorox®, USA)於無菌操作台洗滌2-3 min，再使用75% (v/v)乙醇(ethanol)消毒30 s，最後以高溫高壓滅菌後之二次蒸餾水(ddH_2O)清洗4次，每次3 min。在無菌操作下切除枝條兩端點之傷口後再切分成2-3 cm之莖段，每段至少包含一個帶芽點的節作為培植體，接種入含有8.88 μM BAP (B-3408, Sigma, St. Louis, MO, USA)及0.5 μM IBA (I-5386, Sigma)之1/2 MS培養基(Murashige and Skoog 1962)中誘導側芽產生。每15天移至新配製之相同培養基繼代，繼代3次後切取誘導出之無菌芽體，轉移至增殖培養基中增殖與抽長，其為內含3% (w/v)蔗糖及0.75% (w/v) Difco Bacto agar (pH 5.7)，並另外添加3 μM BAP及0.05 μM IBA的WPM基本培養基(Lloyd and McCown 1980)，每30天繼代一次，增殖所得形態正常的芽體(normal shoot, NS)即可作為後續誘導綴化之材料。

BAP與IBA組合處理對誘導綴化組織形成之影響

取前述山櫻花繼代所產出之無菌NS芽體，培養在添加不同濃度BAP (4.44、8.88及12.32 μM)組合IBA (0、0.1及0.5 μM)之WPM誘導培養基中誘導綴化組織形成，每15天繼代一次，共計三次，並在開始培養後的第20、40及60天觀察其生長情形，60天結束後記錄培養基中芽體出現綴化現象的數量與發生率。本階段試驗每個處理組合使用10個培植體，每一芽體置入一試管中，試驗重複2次。

綴化組織與正常芽體之組織切片觀察

切取綴化組織寬幅長約2.0 mm之片段為觀察樣本，另從正常形態芽體莖頂取樣為對照，先以formalin acetic alcohol (FAA)固定液(40% formaldehyde : 100% glacial acetic acid : 95% ethyl alcohol : ddH_2O = 1:1:10:8)(Jung et al. 2005)浸泡7天，以不同比例乙醇與tert-butanol (TBA)配製的混合液進行系列濃度置換脫水，TBA濃度依序為10、20、35、55及75%，直至置換為100%，期間輔以真空幫浦抽氣，於烘箱(60-65℃)中融熔蠟塊(Paraplast, Leica Biosystems, USA)完成滲蠟。包埋樣本後使用切片機切出10 μm厚度之蠟帶，以1% safranin及0.5% fast green進行染色後封片，在光學顯微鏡下觀察樣本之形態差異。

BAP與IBA組合處理對維持綴化組織之影響

在誘導出綴化組織後，將橫向延展之莖頂(Figure 1A方框處)切成多數小片段(寬幅0.5 cm)(Figure 1B)，分別培養於添加1.11、2.22及4.44 μM BAP組合0.5或1.0 μM IBA之WPM基本培養基中，每個處理使用6個組織片段，每3個組織片段置入一培養皿中，進行為期84天的培養，在第21、42及63天時各繼代一次。由於該組織片段保有綴化特性會持續橫向延展，每次於繼代時量取延展中各個綴化組織片段增加之寬幅長度求其均值，並從各組織片段逢機切取0.5 cm組織片段作為下一次繼代培養的培植體，維持每次繼代樣本均為6個組織片段，試驗結束時(第84天)量測一次組織片段增加之寬幅長度。本試驗以機械式游標卡尺進行數據量測，以取得每21天寬幅長度之增加量，試驗重複2次。

BAP與IBA組合處理對綴化組織形成正常芽體之影響

切取寬幅約1.0 cm之綴化組織片段，在空白培養皿中以解剖刀重複將其切割成細碎組織，接種於芽體增殖培養基(即WPM基本培養基添加3 μM BAP組合 0.05 μM IBA)，另將其植物生長調節劑(plant growth regulator; PGR)組合改為2.22 μM BAP組合1.0 μM IBA及4.44 μM BAP組合0.5 μM IBA作為比較。培養基表面注入1.0 mL 滅菌過之ddH_2O與細碎組織混合，利用L型塗抹棒將組織均勻分散(Figure 1C)。各處理皆使用15個寬幅1.0 cm 的組織片段作為培植體，每一組織片段切碎後接種至一個培養皿(Figure 1D)，進行為期42天的培養，在第21天時繼代1次。過程中芽體會自細碎組織中分化發育而出，將形態正常者單獨切離培養於增殖培養基，培養終了時，紀錄每1.0 cm之單位片段經此培養後獲得恢復正常形態芽體(Recovered normal shoot, RnS) (長度 > 1.0 cm)之數量，長度不足或仍呈現綴化之芽體不計，試驗重複2次。

經由綴化組織誘導正常芽體形成數量之估算方法

計算本研究使用的方法產生多少芽體，基本原則是從培養一段寬幅長度為0.5 cm的綴化芽體開始，在「維持綴化組織」的試驗中，使其持續增殖(橫向延展寬幅長度)。每次繼代結束將增殖所得材料，切成更多寬0.5 cm的片段，進入下一次的繼代，重複操作至試驗終了，計算獲得材料增殖的「總長度 (cm)」。另外在「綴化組織形成正常形態芽體」的試驗中，可得知一段寬幅1.0 cm 的綴化片段，能產生形態正常的「芽體數」。「總長度 (cm)」乘以「芽體數」即為芽體總量。惟以上過程因培養物逐次繼代後數量放大，無法將全數材料進行實體操作，因此計算過程所需之參數，為逢機取樣培養後經統計運算所得，本研究在「結果」中會再詳列計算過程並以統計所得數據代入，以估算培養結束可收獲正常形態芽體總量。

IBA濃度與處理時間對芽體發根之影響

本研究先將誘導所得之NS芽體作為最佳發根條件的測試材料，培養於添加不同濃度IBA (39.36、49.2、59.04及73.8 μM)之WPM基本培養基中，並以不同誘導時數(24、48及72 h)處理。誘導結束後再移至IBA-free之WPM培養基(含8.88 μM $AgNO_3$)以利不定根伸長，14天後觀察IBA濃

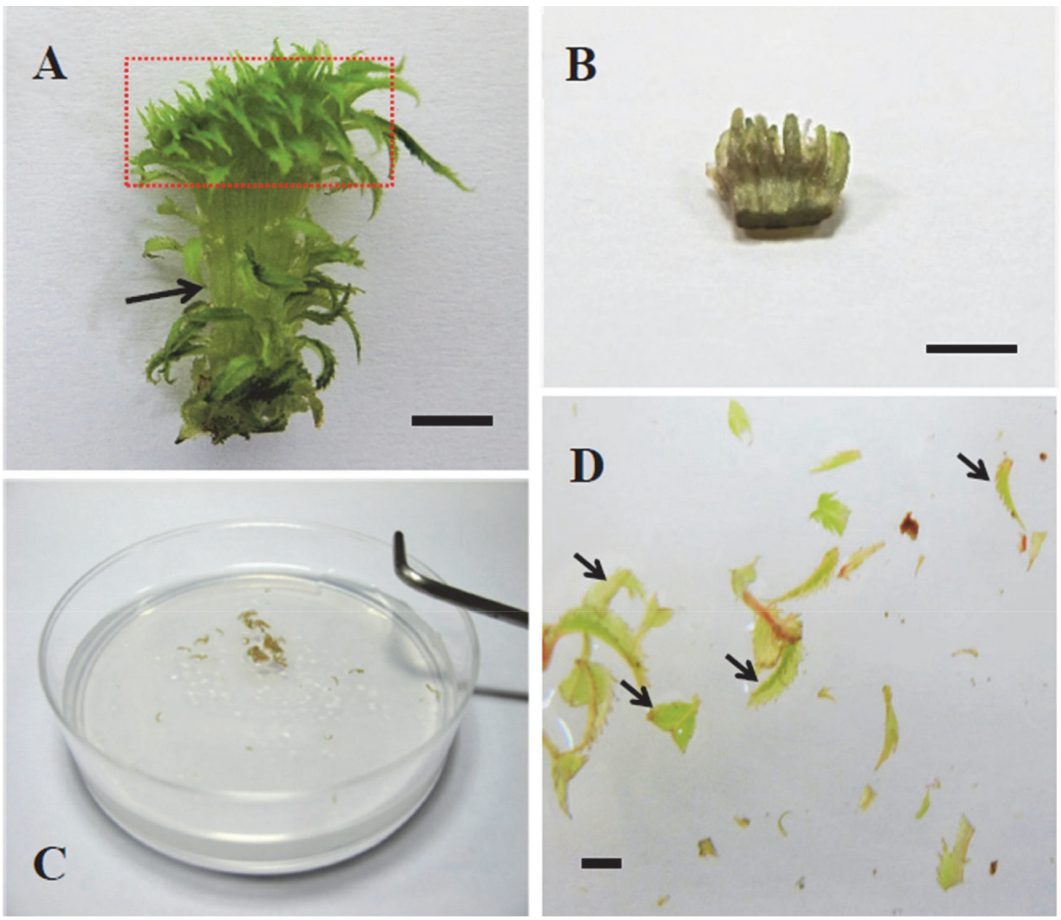

Figure 1. Key culture steps for maintaining shoot fasciation and recovering morphologically normal shoots in *Prunus campanulata* Maxim. (A) A typical fasciated shoot obtained from axillary micropropagation, showing a flattened stem (arrow) and lateral proliferation in the shoot tip (red box); (B) a 0.5 cm-wide fragment excised from the laterally proliferated shoot tip (red box in A), used as the initial explant for fasciation maintenance; (C) cutting fragments from a 1.0 cm-wide fasciated tissue, mixed with ddH_2O and spread onto the shoot proliferation medium using an L-shaped cell spreader; (D) close-up view of the cutting fragments cultured in C, revealing fine dispersed proliferating meristematic cells and larger pieces of broken leaves (arrows). Scale bars in (A) (B) = 5.0 mm; (D) = 2.5 mm.

度與處理時間之組合對芽體發根之影響(芽體基部發出至少一條達0.5 cm長之不定根為成功發根)，記錄發根數與最長根長數據。每組處理培植體各計10個，每個芽體接種入一試管中，試驗重複2次。

取得前述發根試驗之最佳條件後，再使用由綴化片段切碎組織中發育出之正常形態芽體(RnS)與繼代維持的NS芽體進行發根比較，以分析不同來源的芽體，在發根表現上是否存在差異。兩種來源芽體各取40個，以前述最佳發根條件處理，每個芽體接種入一試管中，試驗重複2次，並記錄發根數、平均根長、最長根長以及發根率等數據。

小植株馴化方法之建立

將發根且生長良好的植株(來自RnS及NS)，以清水洗除基部殘餘培養基並去除小植株高度50%以下之葉片進行馴化試驗。試驗容器使用具孔洞的長方型塑膠籃(47 × 37.7 × 13 cm)盛裝混合介質(體積比為碎水苔:赤玉土:蛭石:珍珠石=1:2:2:0.5)，以5 (株) × 4 (株)之密度將小植株種植於內，並將此塑膠籃置於稍大的無孔洞外籃(59 × 47.5 × 16 cm)中，底部使用4個培養皿(高2 cm)墊高，外籃上方以透明壓克力板覆蓋保持籃內相對濕度，施用逆滲透水保持介質濕度，隨馴化期持續將壓克力板的覆蓋面積逐漸縮小，增加空氣交換縫隙。4週後移至半遮蔭之溫室，逐步開啟壓克力板降低相對濕度使植株得以適應室外環境，並記錄小植株成活率，試驗重複3次。

綴化組織培養各階段之環境條件

綴化組織誘導與發根階段之培植體皆培養於20 (直徑) × 115 (長) mm無菌玻璃試管中。而綴化現象維持與芽體形成試驗，使用90 (直徑) × 20 (高) mm無菌培養皿為培養容器，後續恢復正常形態之芽體，則以90 (直徑) × 104 (高) mm蘭花育苗培養盒(Type CK-B, 清科企業，臺灣)繼代培養。生長室環境溫度設定為24 ± 1℃，以冷白螢光燈(cool white fluorescent light)提供30-35 μmol $m^{-2}s^{-1}$之光子通量並維持16 hr light / 8 hr dark光週期。馴化階段時則使用冷白螢光燈與白熾燈泡(incandescent light)兩種光源並調整光子通量為40-50 μmol $m^{-2}s^{-1}$，光週期不變。

試驗數據之統計分析方法

綴化組織誘導試驗(3 × 3)及NS芽體發根試驗(3 × 4)為完全逢機複因子試驗(completely randomized factorial experiment)，皆重複兩次，數據以二因子變異數分析(two-way analysis of variance, two-way ANOVA)進行檢測。綴化現象維持試驗亦採用完全逢機複因子(3 × 2)試驗，每次繼代結束時進行數據分析一次，以逐次檢測PGR組合對培植體維持綴化現象之影響，以取得該次培養使綴化片段寬幅增加量最佳之處理。芽體形成試驗使用完全逢機設計(completely randomized design, CRD)，三種處理皆各使用15個培植體，試驗重複2次，並透過單因子變異數分析(one-way analysis of variance, one-way ANOVA)進行比較。

試驗數據資料皆以IBM SPSS Statistics 22.0軟體進行分析，若處理間差異達顯著水準($p < 0.05$)時，再透過最小顯著差異法(Fisher's least significant difference test, LSD test)比較各處理均數間之差異。惟以RnS及NS芽體進行發根與馴化試驗時，係透過獨立樣本*t*檢定(independent sample *t*-test)依查表所得之*t*值進行分析，比較其根數、平均根長、最長根長及馴化成活率。

結果

BAP與IBA組合處理對誘導綴化組織形成之影響

在誘導培養約20天時，芽培植體基部開始膨大且出現新生的芽體。培養40天後，由於側芽的增殖，這些單芽多數已轉變成叢生之多芽體，且觀察到長度大於1.5 cm之大芽生成(Figure 2A)，直到培養60天時，部分大芽的頂端開始出現綴化現象，莖頂以橫向延展代替垂直抽長，多數小型的葉片排列在橫向延展的莖頂兩側(Figure 2B)。

當誘導階段結束後，單一培植體平均生成綴化組織數與出現綴化現象之百分比皆隨著BAP濃度升高而下降，其中以4.44 μM BAP處理有7.7%的芽體形成綴化變異，顯著高於其它處理(Table 1)；此外，IBA處理在誘導形成綴化現象上並無顯著差異，且與BAP並無交感作用(Table 1)。

綴化組織與正常芽體之組織切片觀察

透過莖梢部分之縱向切片觀察，在形態正常的NS芽體上有明顯的單一生長點(Figure 3A, 箭頭處)，呈現圓穹狀且其周圍有發育初期的幼葉包覆，而在綴化組織則有橫向延展擴大的情形(Figure 3B)。此綴化組織切片樣本僅採集完整綴化芽體的極小部分(寬幅約2.0 mm)，但此樣本寬幅就已約為NS芽體寬幅(直徑)的10倍，而一個完

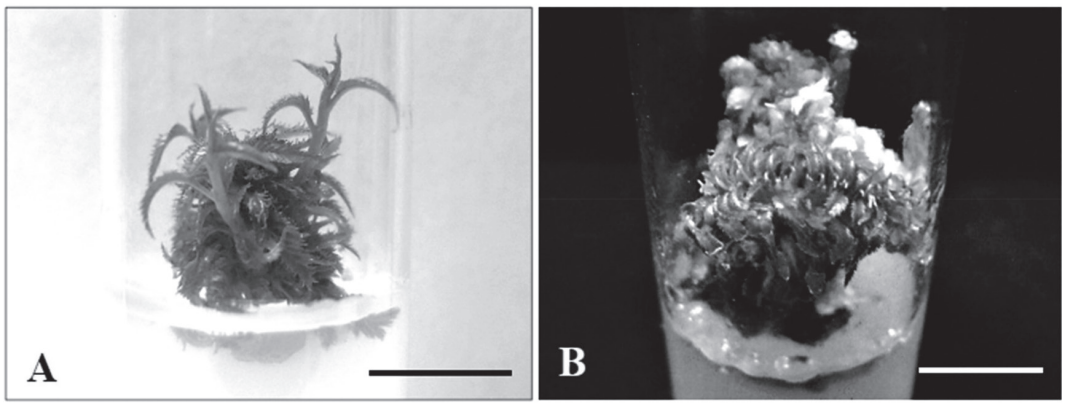

Figure 2. Induction of shoot fasciation in *Prunus campanulata* Maxim. (A) Elongated large shoots derived from axillary micropropagated microshoots; (B) fasciated shoot showing lateral proliferation at the shoot tip with numerous reduced leaves. Scale bars = 1.0 cm.

Table 1. Effect of BAP and IBA concentrations on the induction of shoot fasciation in *Prunus campanulata* Maxim.

Source factor	Levels (μM)	Fasciated shoots [1] (no. explant^{-1})	Ratio of fasciated shoot formation [2] (%)
BAP	4.44	1.6 ± 0.2 [a]	7.7 ± 0.9 [a]
	8.88	0.7 ± 0.1 [b]	3.4 ± 0.6 [b]
	13.32	0.3 ± 0.1 [c]	1.1 ± 0.3 [c]
IBA	0.0	0.8 ± 0.2 [a]	4.7 ± 0.9 [a]
	0.1	0.7 ± 0.1 [a]	3.5 ± 0.7 [a]
	0.5	1.0 ± 0.1 [a]	4.0 ± 0.7 [a]

Source factor	Significance [3]	
	Fasciated shoots	Ratio of fasciated shoot formation
BAP (B)	*	*
IBA (I)	n.s.	n.s.
B×I	n.s.	n.s.

[1,2] Formation of fasciated shoots and its ratio (mean ± SE, n = 10) corresponding to levels of each source factor, where mean values followed by different superscript letters in the same column are significantly different according to Fisher's least significant difference (LSD) test at $p < 0.05$.

[2] Percentage data were all arcsine transformed prior to analysis.

[3] Source factor and interactive effects on fasciated shoot formation, where * represents a significant difference detected by Fisher's LSD test at $p < 0.05$; n.s. indicates no significant difference ($p > 0.05$).

BAP: 6-benzylaminopurine; IBA: indole-3-butyric acid.

整的綴化芽體寬幅可達1.0 cm以上，其橫向擴展程度相較於NS芽體，增加之倍數更為明顯。在擴展區域之表層有多數突起的生長點，由體積小而緊湊聚集的細胞組成(Figure 3C, 箭頭處)，與對照組NS芽體之生長點細胞組成一致，惟其周遭缺乏幼葉包覆，且常見其以多數併列狀態發生，顯示莖頂綴化組織具備「多數生長點」的特性，而這些生長點則是後續培養形成芽體的來源，其數目越多將提升其繁殖效率。

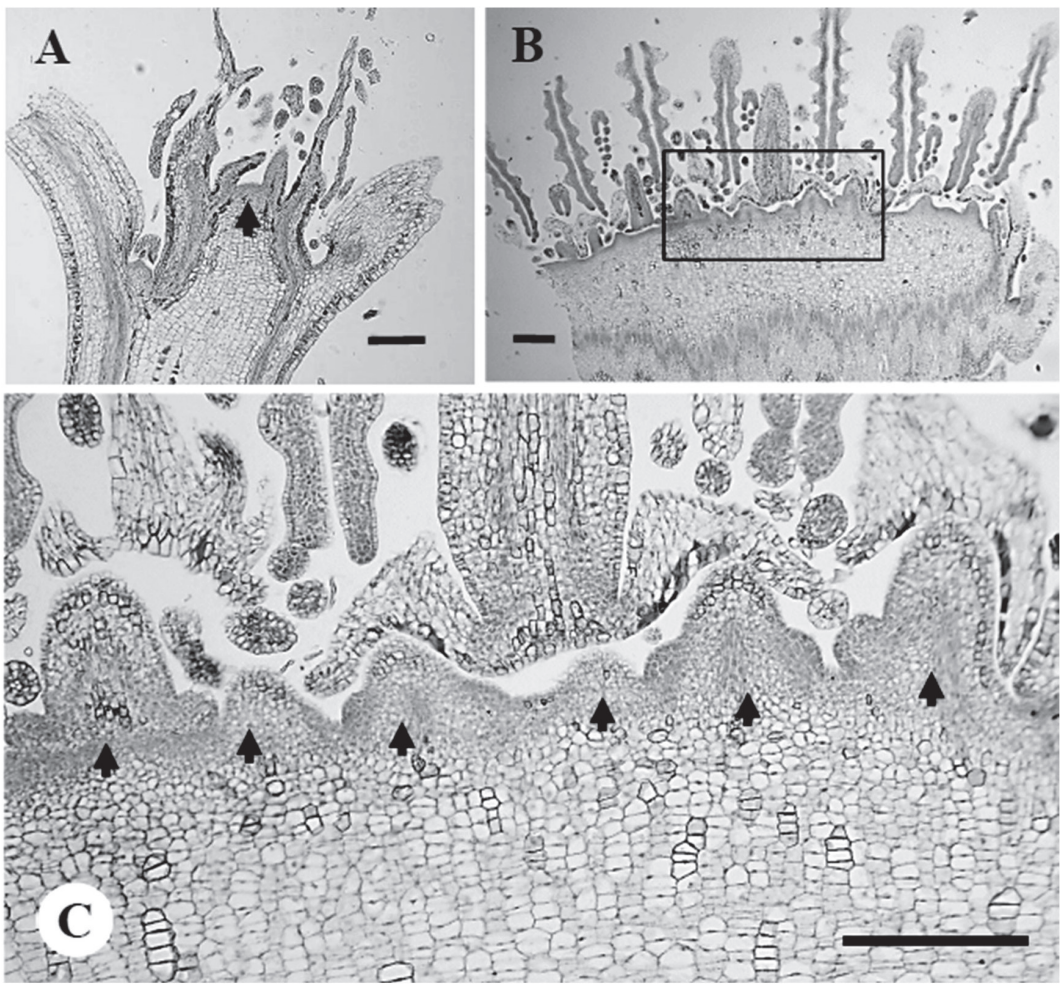

Figure 3. Comparative histological analysis of normal versus fasciated shoots in *Prunus campanulata* Maxim. (A) Longitudinal section of a normal shoot apical meristem showing a single distinct meristem (arrow) enclosed by developing young leaves; (B) longitudinal section of a fasciated shoot tip, revealing laterally proliferating meristems (boxed); (C) magnified view of the region highlighted in B, emphasizing multiple meristems (arrows) that have developed on the flattened upper surface of the shoot tip. Scale bars = 0.2 mm.

BAP與IBA組合處理對維持綴化組織之影響

從綴化組織取來的片段在84天的培養過程中，在所有的處理組合中均可觀察到其寬幅長度持續增加，顯示綴化組織生長點區域保持在增殖狀態。由數據統計分析得知，使用兩種濃度之IBA，其對片段寬幅之增加並無顯著差異。而使用BAP時，除第四次的繼代培養(S4)外，在其他連續幾次的繼代培養(S1-S3)中，BAP處理對綴化組織之平均寬幅具有顯著差異，其中以2.22 μM BAP處理在促進寬幅之增加優於其他處理，不僅在繼代結束時平均寬幅增加量最多(Table 2)，且培植體綴化現象保持不變(Figures 4A, 4B and 4C)。此外，1.11 μM BAP 處理者效果較差，且其於繼代過程中，常有部分組織片段會恢復成正常單株形態，綴化性狀並無法穩定維持。4.44 μM

Table 2. Effect of BAP and IBA concentrations on the lateral width proliferation of cultured fasciated *Prunus campanulata* Maxim. shoot fragments.

Source factor	Levels (μM)	Width increase per fasciated fragment (cm) [1]			
		S1 [2]	S2 [2]	S3 [2]	S4 [2]
BAP	1.11	0.46 ± 0.03 [b]	0.82 ± 0.04 [b]	0.62 ± 0.04 [c]	0.28 ± 0.06 [a]
	2.22	0.82 ± 0.03 [a]	1.23 ± 0.05 [a]	1.01 ± 0.05 [a]	0.35 ± 0.05 [a]
	4.44	0.74 ± 0.05 [a]	0.86 ± 0.04 [b]	0.73 ± 0.03 [b]	0.33 ± 0.06 [a]
IBA	0.5	0.67 ± 0.03 [a]	0.96 ± 0.04 [a]	0.77 ± 0.04 [a]	0.31 ± 0.04 [a]
	1.0	0.72 ± 0.04 [a]	0.98 ± 0.05 [a]	0.82 ± 0.05 [a]	0.34 ± 0.05 [a]

Source factor	Significance [3] Width increase per fasciated fragment			
	S1	S2	S3	S4
BAP (B)	*	*	*	n.s.
IBA (I)	n.s.	n.s.	n.s.	n.s.
B×I	n.s.	n.s.	n.s.	n.s.

[1] Mean width increase in cultured fasciated fragments (mean ± SE, n = 6) corresponding to levels of each source factor, where mean values followed by different superscript letters in the same column are significantly different according to Fisher's least significant difference (LSD) test at $p < 0.05$.
[2] S1: subculture days 1–21; S2: subculture days 22–42; S3: subculture days 43–63; S4: subculture days 64–84.
[3] Source factor and interactive effects on width increase in fasciated fragments, where * represents a significant difference detected by Fisher's LSD test at $p < 0.05$; n.s. indicates no significant difference ($p > 0.05$).
BAP: 6-benzylaminopurine; IBA: indole-3-butyric acid.

BAP 處理在培養42天後，部分培植體出現褐化現象，其基部癒傷組織腫大且生長狀況不良。但不論使用何種濃度，在培養進入第64天(S4期)之後，綴化組織片段的增殖均出現衰退，寬幅長度之增加速度趨緩(Figure 4D)(Table 2)。

分析BAP及IBA組合後產生的培養結果時，並未見到交感效應出現(Table 2)，但以2.22 μM BAP搭配1.0 μM IBA之組合，於各次繼代(S1-S3)中皆為最佳的處理(數據未呈現)，其三次繼代之寬幅增加量依序為0.80 (S1)、1.23 (S2)及0.87 (S3) cm，在之後進行芽體總數估算時即採用此為計算參數。

BAP與IBA組合處理對綴化組織形成正常芽體之影響

將綴化組織片段密集切碎培養於WPM增殖培養基，14天後芽體自細碎之塊狀物上分化形成芽體，且在培養皿中隨機分布(Figure 5A)，培養21天後已有成功恢復正常外觀形態的RnS單芽，但此時仍有持續增殖的綴化組織(Figure 5B)；在42天培養期間凡是有RnS芽體出現，即將其選出於不同容器中另外培養(Figure 5C)，經觀察其生長持續且易增殖形成多芽體，但是都沒有再返回成為綴化芽體的跡象(Figure 5D)。待芽的高生長達合適高度(> 1.5 cm)時，即可作為發根試驗之培植體來源。

經統計分析，每段寬幅1.0 cm的綴化組織片段經過密集切碎後，無論在何種處理組合中均可觀察到芽體生長，但以3.0 μM BAP組合0.05 μM IBA的處理有較佳的芽體生長情形(Table 3)，培養21天時即有顯著差異，可產出RnS大芽11.4個。在42天的培養後，該處理可再增加產量至15.6個大芽，數量最多且外觀均無異常，其餘兩種組合處理，其形成大芽數為12.9與12.7。故本研究在此階段採用3.0 μM BAP與0.05 μM IBA的組合作為培養條件。

估算由綴化組織中可誘導正常形態芽體之總量

當「維持綴化組織」試驗中第一個繼代培養(S1)結束時，一截0.5 cm之綴化組織片段，其寬幅增加量為0.8 cm，因此培養後寬幅長(final fasciated

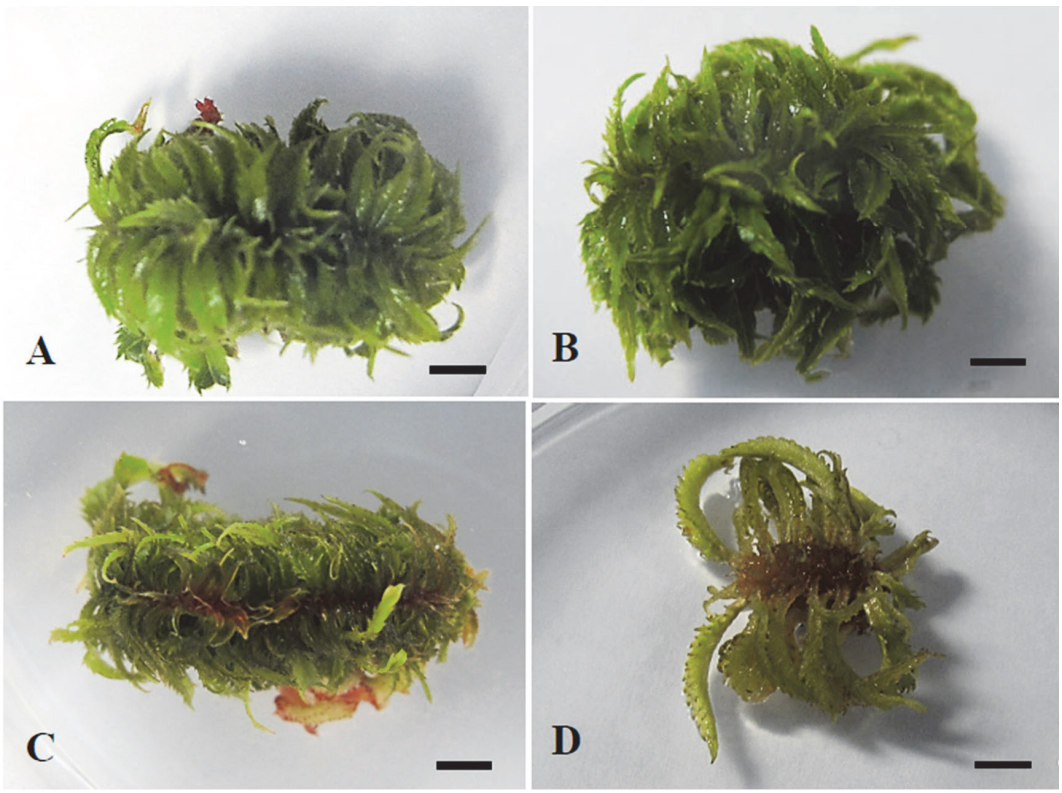

Figure 4. Representative fasciated shoot explants during the "maintenance of fasciation" culture stage. These panels illustrate the laterally proliferated shoot explants at the conclusion of each subculture. The degree of proliferation, as indicated by the increase in explant width, remained consistent across most subcultures, with a notable deduction observed in the 4th subculture. (A) End of the 1st subculture; (B) end of the 2nd subculture; (C) end of the 3rd subculture; (D) end of the 4th subculture. Scale bars = 2.5 mm.

fragment width; FFFW, Table 4)為1.30 (即0.5 + 0.8) cm。該長度可以切分成2.6 (即1.30/0.5)個寬幅0.5 cm的組織片段(Table 4)。在第二次繼代培養(S2)之後，一個寬幅0.5 cm的組織片段增加量為1.23 cm，則培養後寬幅(FFFW)為1.73 (即0.5 + 1.23) cm。計算由前一次繼代操作產出的2.6個片段都進行培養之後，總寬幅(total fasciated fragment width; TFFW, Table 4)可達4.50 (即1.73 × 2.6) cm。該長度又可以切分出9.0 (即4.50/0.5)個寬幅0.5 cm的組織片段(Table 4)。依此計算方式，在第三次繼代培養(S3)結束時，0.5 cm寬幅的組織片段因增加0.87 cm，故寬幅(FFFW)達到1.37 cm，總寬幅(TFFW)長度為12.33 (即1.37 × 9.0) cm (Table 4)。

當取得最後總寬幅12.33 cm的綴化組織片段之後，每1 cm寬幅長的片段，經本研究「綴化組織形成正常芽體」之操作，可產生15.6個正常RnS芽體，因此總計可以產出約192.4 (即12.33 × 15.6)個RnS芽體(Table 4)。

IBA濃度與處理時間對芽體發根之影響

當使用NS芽體進行發根條件之測試時，部分芽體在移至IBA-free培養基中5-6天時，基部出現腫脹情形，培養至第8天時在芽體基部膨大的位置可明顯觀察到不定根形成，至第10天後，多數不定根伸長且數量增加(Figure 6)。

NS芽體經過14天培養後的統計結果顯示，不

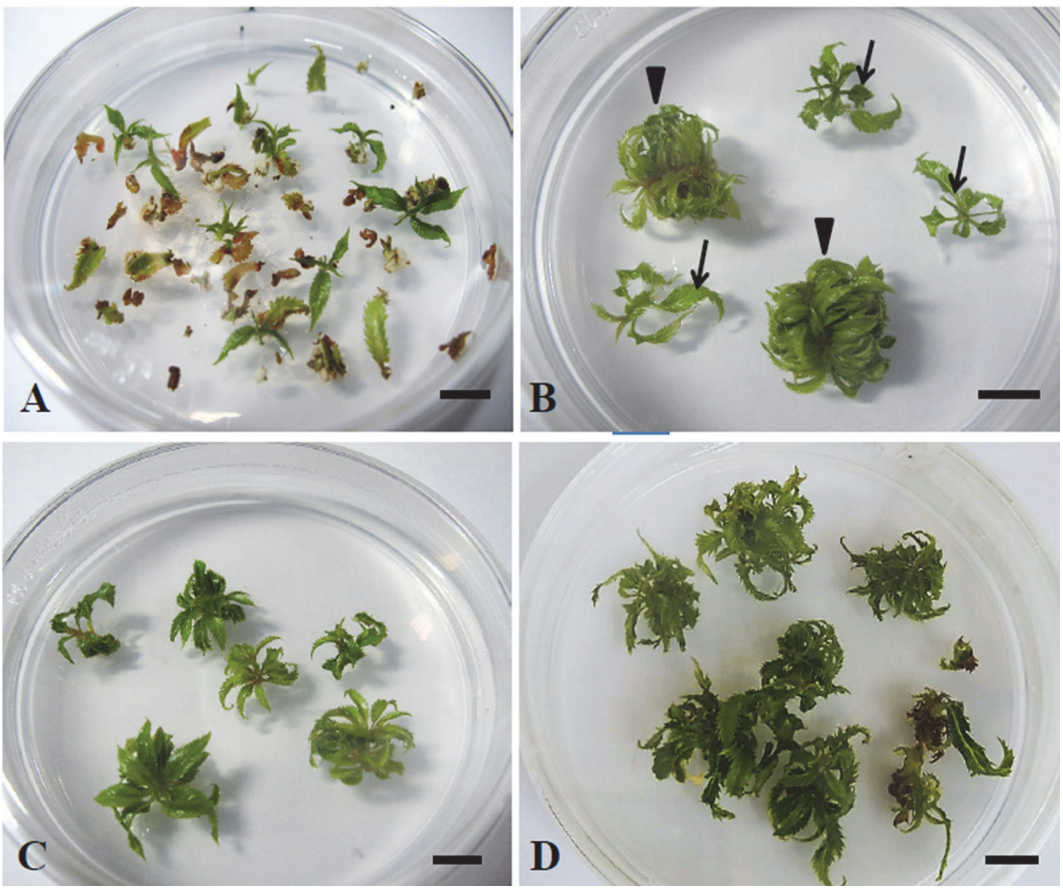

Figure 5. Shoots grown from fasciated *Prunus campanulata* Maxim. explants. (A) New shoots grown from cutting fragments after 14 d in culture; (B) both normal (arrows) and fasciated (arrowheads) shoots developed on the culture medium by day 21 of incubation; (C) individually transplanted normal shoots cultured for further growth; (D) multiple morphologically normal microshoots formed from a single shoot previously cultured as shown in C. Scale bars = 1.0 cm.

Table 3. Effectiveness of 3 plant growth regulator (PGR) regimes in promoting the formation of morphologically normal shoots from fine-cut debris of fasciated *Prunus campanulata* Maxim. shoot fragments.

RGR combination		Number of shoots per fasciated fragment [1]	
BAP (μM)	IBA (μM)	Incubation of 21 d	Incubation of 42 d
2.22	1.0	9.9 ± 0.4 [b]	12.9 ± 0.6 [b]
3.0	0.05	11.4 ± 0.3 [a]	15.6 ± 0.4 [a]
4.44	0.5	9.0 ± 0.4 [b]	12.7 ± 0.3 [b]

[1] Mean value of normal shoot formation (mean ± SE, $n = 15$) followed by different superscript letters in the same column are significantly different according to Fisher's least significant difference (LSD) test at $p < 0.05$.
BAP: 6-benzylaminopurine; IBA: indole-3-butyric acid.

Table 4. Impact of successive subcultures on both the width increase of laterally proliferating fasciated fragments (maintenance stage) and the subsequent recovery of morphologically normal shoots.

Subcultures (d)	IFFW (cm)	Fragment width increase [1] (cm explant^{-1})	FFFW (cm explant^{-1})	TFFW (cm)	No. of 0.5 cm-wide fragments	No. of normal shoots recovered from TFFW
S1 (1–21)	0.50	0.80	0.5 + 0.80 = 1.30	1.30	1.30/0.5 = 2.60	1.30 × 15.6 = 20.3 [2]
S2 (22–42)	0.50	1.23	0.5 + 1.23 = 1.73	1.73 × 2.60 = 4.50	4.50/0.5 = 9.00	4.50 × 15.6 = 70.2 [2]
S3 (43–63)	0.50	0.87	0.5 + 0.87 = 1.37	1.37 × 9.00 = 12.33	–	12.33 × 15.6 = 192.4 [2]

[1] Derived width increase values (see text).
[2] Normal shoot recovery parameter (15.6) (see text for details).
IFFW: initial fasciated fragment width; FFFW: final fasciated fragment width; TFFW: total fasciated fragment width.

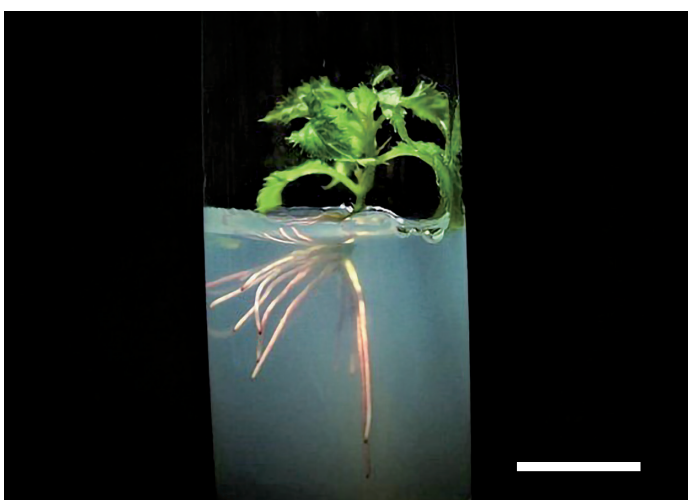

Figure 6. Successful rooting of *Prunus campanulata* Maxim. micropropagated shoots. This image illustrates the optimized rooting conditions enabling subsequent experimental examinations. Scale bar = 1.0 cm.

同的誘導時間(24、48及72 h)對於芽體發根數量與根長的影響沒有顯著差異(Table 5)。但是以49.2 μM IBA處理後芽體生成最多不定根(5.6根)，且隨著濃度的提高(59.04及73.8 μM IBA處理)，不定根數目逐漸減少，但三種處理間並無顯著差異；而最長根長則在四個IBA濃度的處理間並無顯著差異(Table 5)。除此之外芽體發根數目受到誘導時間與IBA濃度交感效應之影響，進一步分析後得知以49.2 μM IBA處理誘導48及72 h或59.04 μM IBA誘導48 h，這三種處理組合使NS芽體形成較多的不定根(Table 6)。為使IBA用量與誘導時間以極簡為佳，選擇較低濃度(49.2 μM)搭配較短誘導時間(48 h)作為本試驗發根的最佳處理。

另外，以RnS及NS的芽體進行發根比較，利用此最佳處理條件(49.2 μM IBA誘導48 h)培養後，在培養第10天後觀察到不定根數量增加且根長增長之現象。試驗結果顯示無論芽體為RnS或是NS，兩者在平均發根數、平均根長及最長根長的表現上皆沒有顯著差異，發根率亦相近(Table 7)，即綴化後再復原之RnS芽體與正常形態NS芽體，對發根試驗最佳的誘導條件反應相同，並未因培植體來源不同而有差異。

Table 5. Influence of IBA concentration and induction duration on adventitious rooting in *Prunus campanulata* Maxim. *in vitro*-grown normal shoots.

Source factor	Levels	Root production per shoot [1]	Maximum root length (cm) [2]
IBA (μM)	39.36	3.0 ± 0.6 [b]	1.69 ± 0.21 [a]
	49.20	5.6 ± 0.8 [a]	1.73 ± 0.24 [a]
	59.04	4.4 ± 0.7 [ab]	1.97 ± 0.28 [a]
	73.80	4.0 ± 0.7 [ab]	1.50 ± 0.19 [a]
Induction duration (h)	24	3.7 ± 0.7 [a]	1.42 ± 0.18 [a]
	48	5.0 ± 0.9 [a]	1.80 ± 0.20 [a]
	72	4.2 ± 0.7 [a]	1.89 ± 0.21 [a]

	Significance [3]	
	Root production	Maximum root length
IBA (I)	*	n.s.
Induction duration (T)	n.s.	n.s.
I × T	*	n.s.

[1,2] Root production and maximum root length (mean ± SE, n = 10) corresponding to levels of each source factor, where mean values followed by different superscript letters in the same column are significantly different according to Fisher's least significant difference (LSD) test at $p < 0.05$.

[3] Source factor and interactive effects on root production and maximum root length, where * represents a significant difference detected by Fisher's LSD test at $p < 0.05$; n.s. indicates no significant difference ($p > 0.05$).

IBA: indole-3-butyric acid.

Table 6. Interactive effects of IBA concentration and induction duration, as derived from Table 5, on the adventitious root production of normal *Prunus campanulata* Maxim. shoots.

IBA (μM)	Root production (no.) per shoot [1]		
	Induction of 24 h	Induction of 48 h	Induction of 72 h
39.36	2.2 ± 0.9 [c]	3.0 ± 1.6 [bc]	3.6 ± 1.6 [b]
49.20	4.0 ± 1.5 [b]	7.1 ± 1.5 [a]	5.7 ± 1.4 [a]
59.04	3.0 ± 1.2 [bc]	5.4 ± 1.3 [a]	3.6 ± 1.0 [b]
73.80	4.5 ± 1.4 [ab]	4.3 ± 1.0 [ab]	2.3 ± 1.0 [c]

[1] Mean value of root production (mean ± SE, n = 10) followed by different superscript letters are significantly different according to Fisher's least significant difference (LSD) test at $p < 0.05$.

IBA: indole-3-butyric acid.

Table 7. Comparison of rooting (n = 40) and acclimatization (n = 20) performance in *Prunus campanulata* Maxim. plantlets originated from different shoot explants.

Explant type [1]	Root production (no.) per shoot	Root length (cm)	Maximum root length (cm)	Rooting rate (%)	Acclimatization survival (%)
NS	7.1 ± 0.4	1.50 ± 0.08	1.82 ± 0.09	77.5 ± 5.1	91.7 ± 1.7
RnS	7.2 ± 0.6	1.48 ± 0.07	1.90 ± 0.09	80.0 ± 7.4	95.0 ± 2.9
t-value [2]	-0.392	0.203	-0.578	-0.257	-1.002

[1] NS explants were originated from initially micropropagated normal shoots, whereas RnS explants were normal shoots recovered from fasciated tissue.

[2] All parameters analyzed by an independent sample t-test showed no significant difference between the 2 explant types (t-values < table value $t_{(0.05/2, n-2)}$).

小植株馴化方法之建立

山櫻花小植株在馴化過程初期(前14天)，有部分個體葉片會枯黃掉落，而老葉的脫落會伴隨新芽的出現，待植株於生長室馴化28天後，此時新芽葉片已充分伸展且有新芽持續萌發，在此階段可將植株移置遮陰溫室並移植於育苗盆中，保持介質濕度與養分供給，即完成植株馴化階段。

發根的RnS小植株馴化成活率達95% (Table 7)，與NS並無差異且生長狀況良好，而由此方法育成之苗木後續於溫室中生長，葉片形態與葉序亦無變異(Figure 7)。

討論

將山櫻花進行研究所獲之繁殖結果與寬葉毛氈苔的例子(Liao and Ji 2014)比較，雖使用相同的方法，但兩者繁殖效率差異甚大；從綴化組織中恢復正常形態芽體的培養階段，寬葉毛氈苔每1.0 cm綴化片段切碎後形成之正常芽體為20.1個，而山櫻花為15.6個，單純比較此階段所獲數值兩者仍屬接近。綴化芽體的發生雖常在組織培養操作中出現，惟要能使該異常繁殖體能持續增殖且穩定維持綴化性狀，才是能夠大量繁殖的關鍵。在寬葉毛氈苔的例子中，綴化芽體能夠在完全無添加PGR的培養基中持續地橫向延展生長接近一年，且不會隨著繼代次數的增加而使寬幅長度延展的速率趨緩，是使其能大量繁殖的重要因素。而山櫻花綴化片段在完全無添加PGR的培養基中無法成活，因此其綴化組織之維持與增殖須建立在添加外源PGR的前提之下，且在切分綴化片段並連續繼代時，多數片段在培養63天後，生長出現衰退且使寬幅長度之延展趨緩(Table 2)，這是與培養寬葉毛氈苔相較最大的不同，推測此為

Figure 7. *Prunus campanulata* Maxim. plantlets recovered from fasciated shoot tissue. These plantlets, grown in a greenhouse, exhibited normal phyllotaxy and leaf size. Scale bar = 15 cm.

山櫻花以相同方法繁殖但效率不及寬葉毛氈苔的原因。此外，上述兩者在增殖過程中偶有部分綴化芽體會恢復正常形態，惟其發生比例並不高，類似情況亦在綴化豌豆的培養過程中被描述為去綴化作用(defasciation) (Sinjushin and Gostimsky 2006)，但推測其機制有所差異。在本研究所發展的繁殖流程中，綴化芽體越早恢復正常，表示可以增殖的綴化組織材料減少，亦即降低最後的組培苗總產量；然而其比例不高而不足以構成芽體嚴重減損，惟目前仍未有足夠的證據解釋此種自發性恢復正常形態的機制。

在恢復正常形態芽體的試驗中，將山櫻花綴化組織片段密集切碎培養於WPM增殖培養基，即從細碎組織中分化形成正常形態單芽，此操作屬機械性(物理性)破壞，若有足夠切割強度，確實切離並分開片段上生長點間的連結，就能有效從中獲得更多的正常植株，惟機械性破壞如何使形態異常之綴化組織恢復正常，又是否有更佳的操作方式可以切碎綴化組織以獲取更多RnS芽體，尚需進一步試驗證實。

本研究所建立之繁殖方法，關鍵在於利用綴化莖頂組織有多數生長點的特性，並能結合維持其再增殖的培養過程。而Liang et al. (2019)則是以蔓荊發生綴化現象之扁平莖段為培植體，誘導該莖段上的側芽抽長後加以利用，過程中缺少一個再增殖的步驟；該文作者指出，正常形態之蔓荊莖部約2-3 mm寬，而發生綴化後莖部寬度可延展至5-25 mm，故以綴化後寬扁的莖部為培植體，在顯著增加的表面積上自然可收集較多側芽，結果顯示單一個培植體平均可誘導出78.0個芽，雖已有效提升繁殖效率，惟這僅是一次性的操作，若能先導入增加綴化組織數量或體積的培養，再誘導其上形成的芽體生長，將會再次提升產能。

目前梅屬植物在傳統的組織培養繁殖研究中，有透過誘使頂芽或莖節上之側芽生長以進行微體繁殖，其在進入誘導發根前，須培養達一定標準之大芽，此過程概略區分為誘導無菌芽體之建立、取單芽進行增殖及芽體抽長等階段(後兩階段或可一次完成與本研究所呈現之方法屬近似之系統)。為瞭解本研究呈現方法之增殖效率與傳統方法應用在其他作物之異同，檢索具有明確提出培養數據(包括時間與芽體收獲量)之相關文獻，結果顯示兩種矮生型櫻桃砧木Maxma-14 (*P. avium* L.) (Muna et al. 1999) 與PHL-A (*P. avium* × *P. cerasus*) (Mahdavian et al. 2011)、大山櫻(*P. sargentii* Rehder) (Sasaki 2003)及在巴西(Brazil)生長的山櫻花(Sanson et al. 2024)等研究指出，建立無菌芽體之後約需要84-119天的時間完成後兩階段的培養，但誘導形成合乎需求之芽體數目僅有3-9.57個。

芽體增殖與生長較快者有梅的栽培種(*P. mume* L. “Xuemei”)與杏(*P. armeniaca* L.)，前者利用63天時間可產出5.43個大芽(Ning et al. 2007)，後者則在28天培養後收獲3.36個大芽(Yildirim et al. 2011)。本研究結果顯示，在無菌芽體建立後需60天的時間誘導芽體形成綴化組織，再取組織片段進行綴化現象維持之培養階段，經第一次繼代後(需21天)要再利用42天從綴化組織中培養形成正常形態之大芽，總計需要123天，如此可獲得20.3個芽體(Table 4)；但是當進行第二次綴化組織維持培養後，則芽體總數可增加到70.2個(總計需時144天)；若完成三次綴化組織維持培養，則芽體總數將達到192.4個 (總計需時165天) (Table 4)。與傳統微體繁殖法相比，山櫻花利用植物綴化現象建立組織培養的繁殖系統，在芽體的增殖階段雖需時甚久，但較多投入時間將可獲得極佳的增殖效率，此外這些芽體具有80%的發根率與95%的馴化存活率(Table 7)，因此是為極具潛力且可行之繁殖方式。

結論

本研究以WPM培養基添加4.44 μM BAP與0.5 μM IBA的組合誘導山櫻花芽體形成綴化組織，取具有芽體生長點之綴化組織片段持續繼代培養，能於63天培養期間維持其綴化性狀並持續擴展其寬幅，經密集切碎操作後可形成具有正常形態之芽體，整個完整的培養過程讓初始0.5 cm寬幅之綴化片段可增殖形成192.4個形態正常之單芽，再經發根及馴化育成之苗木，經2年之培育後長成

外觀形態並無變異的正常植株。故以植物綴化現象建立山櫻花繁殖系統為一具潛力之方法，未來若能將此方法應用其他觀賞櫻花或同屬之經濟果樹，應當具有極大商機。

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