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In vitro flowering of *Fortunella hindsii* (Champ.)

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Abstract. Branch internodes of mature plants and stem internodes of seedlings of *Fortunella hindsii* flowered *in vitro* on half-strength MT (Murashige and Tucker 1969) basal medium supplemented with benzyladenine, adenine, 6-γ-γ-dimethylallylaminopurine and kinetin. The highest percentage of flowering was achieved with explants originating from branch internodes of flowering plants close to the apex on half-strength MT basal medium containing 5% sucrose and 0.01 mg l^{-1} BA in light. Exposure to darkness for more than 3 weeks followed by re-exposure to light reduced flowering. Flowering required a 4-day exposure to BA, but shoot formation could be initiated even without exposure to BA. First branch internode segments on MT basal medium containing 5% sucrose were prolific in flower (85%) production. The sucrose treatment affected the flower bud size distribution. There were about 13 flower buds per culture in the largest size category (>5 mm).

Key Words: Cytokinin, flowering, internode, *Fortunella hindsii*, shoot, sucrose

Introduction

Fortunella fruit is used for preserve, candy, syrup and fresh consumption because of its special flavor and thick edible rind (Hodgson 1967). *Fortunella* as close relative of *Citrus* is one of the most important genetic resources for *Citrus* improvement (Bitters et al. 1964; Swingle and Recee 1967; Iwamasa et al. 1988; Grosser and Gmitter 1990). During our studies related to the regeneration of protoplasts via somatic embryogenesis of *Murraya paniculata*, we observed that flowering occurred on plantlets in half-strength Murashige and Tucker (1969) medium containing 5% sucrose in the light (Jumin and Nito 1995). Prolific flowering *in vitro* has not yet been reported in *Citrus* related genera, but has been shown to occur in a few other species (Scorza 1982).

A combination of exogenous and endogenous factors influence *de novo* flower morphogenesis (Compton and Veilleux 1992). External factors include plant growth regulators and other promoters, light, and carbon source. Internal factors include the position of the explant on the intact plant at the time of excision, the plant genotype, and the genes expressed during flower morphogenesis.

Increasing of flowering *in vitro* has been promoted by cytokinins (Srinivasan and Mullins 1978; Scorza and Janick 1980; Cousson and Tran Thanh Van 1981; Van den Ende et al. 1984; Nadgauda et al. 1990), RNA base analoqs (Wardell and Skoog 1969b) and auxin (Tran Thanh Van 1973; Cousson and Tran Thanh Van 1981; Van den Ende et al. 1984) but inhibited by gibberellin (Wardell and Skoog 1969a; Gupta and Maheshwari 1970).

Reduction of flowering was observed with increased distance from the apex (Scorza and Janick 1980; McDaniel et al. 1989). The cause of this floral gradient is unknown. The results presented here use *F. hindsii* plants: a) to investigate the influence of position of explants on *in vitro* flowering; b) to evaluate the effects of cytokinins and sucrose on *in vitro* flowering.

Materials and Methods

Plant materials and preparation. Branch internodes were obtained from 5-year old fruit-bearing trees of *F. hindsii* grown at the germplasm collection at Faculty of Agriculture, Saga University, Japan, and from *in vitro* grown seedlings.

In vitro culture. Branch internodes (1st to 5th, starting from the apex) were sterilized in sodium hypochlorite (1% available Cl) for 20 min, rinsed in 70% (v/v) ethanol for 1 min, followed by three washes in sterilized distilled water and were cut into 10 mm segments. Seeds were sterilized as described in the above procedures and cultured on half-strength MT (Murashige and Tucker 1969) basal medium containing 5% sucrose without any plant growth regulators. After 2 months, the first internode between the cotyledons and the first leaves was cut into 10 mm segments. A single explant was planted upright on half-strength MT basal medium containing 5% sucrose and supplemented with either 0.0, 0.001, 0.01, 0.1 or 1.0 mg l^{-1} cytokinin (benzyladenine = BA, kinetin = KIN, adenine = ADN, and 6-γ-γ-dimethylallylaminopurine = DAP). Twenty ml of medium was dispensed into individual 25 x 150 mm glass culture tubes. The pH

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was adjusted to 5.7 before autoclaving and 3.0 g l^{-1} Gelrite (Kelco, Division of Merck & Co. Inc., San Diego, California) was added and finally autoclaved at 121°C for 20 min. A single explant was placed on the medium in each culture and was incubated at 25°C under a 16-h day using cool, white fluorescent lights (35.3 μmol m^{-2} s^{-1}). Shoots and flowers were examined and counted at 2-week intervals for each culture. The number and size distribution (based upon flower bud length) of flower buds were determined after 60 days.

Specimen were prepared for scanning electron microscopy as described by Fowke et al. (1994). Tissue samples were affixed on aluminum stubs with silver paint and coated with gold palladium in a fine coat Ion Sputter Topcon ABT-3. The tissue samples were examined under a scanning electron microscope, Topcon ABT-3, at accelerating voltage of 15 KV.

Results

Formation of floral meristems. BA permitted the formation of floral meristems from branch internodes of flowering plant explants and stem internodes of seedlings. In branch internodes, BA at 0.01 mg l^{-1} was the most effective with 87% of the cultures producing shoots and 80% initiating flowers (Table 1). This treatment was used in all subsequent experiments. A single shoot directly emerged from cortical tissue at the cut surface of stem internodes and branch internodes after 50 to 60 days of culture (Fig. 1). Most flowers appeared from leafy shoots (Fig. 2) but a few developed from leaf axils of large shoots. Most flowers were small and lacked stamens but a few were large and complete. Flowers with anthers produced pollen *in vitro* (Fig. 3). Several flowers were pollinated *in vitro* and produced embryos (Fig. 4) but did not set fruit.

Flowering gradient. A gradient of shoot formation was detected among the branch internode positions. A very high frequency of shoot formation was obtained with the 1st and 2nd branch internodes and a very low frequencies was obtained with the 5th branch internodes. In general, increasing concentrations of BA increased the frequency of shoot formation (Fig. 5). Very low frequencies of flowering occurred on the 4th internode and no flower formation on the 5th internode, indicating the presence of a "flowering gradient" in both young and old tissue (position of internode). Shoots produced from the first internode explants flowered in 80% of the cultures containing 0.01 mg l^{-1} BA, indicating that the combination of young tissues and low (0.01 mg l^{-1}) BA was optimal for initiating flowering.

Flowering response to light. First Internodes were cultured on half-strength MT basal medium plus 0.01 mg l^{-1} BA and grown for 0-5 weeks in darkness before being transferred to light conditions under a 16-h photoperiod. Few shoots and no flowers formed when explants were in the dark. Exposure to darkness for more than 3 weeks followed by re-exposure to light reduced flowering (Table 2).

Kinetics of the BA-induced flowering response. First branch internode segments were explanted on half-strength MT basal medium supplemented with 0.01 or 0.1 mg l^{-1} BA and transferred to BA-free medium at 48 h intervals up to 8-day. Flower and shoot production were then evaluated at 2 week intervals. Shoots were produced without BA (0-day after exposure to BA) and shoot production increased to 95% after 90-day (Table 3). The percentage of explants forming shoots with 0.01 or 0.1 mg l^{-1} BA increased from 20% after a 0-day exposure to 95% after 90-day. Five percent of the explants flowered after a 4-day exposure to 0.1 mg l^{-1} BA and 80% flowered after a 90-day exposure to 0.01 mg l^{-1} BA. Flower production required a 6-day exposure to 0.01 mg l^{-1} BA and peaked at 80% with a 90-day exposure.

Effect of sucrose. The effect of sucrose on flowering was investigated. First internode segments cultures on half-strength MT basal medium containing 0.01 mg l^{-1} BA were kept separately in the dark and under a 16-h photoperiod. Organogenesis did not occur in medium without sugar or without exposure to light even with sugar. Explants kept under light with 1% sucrose produced 40% shoots and did not flower, and 3% sucrose produced 90% shoots and 65% flowers; whereas those kept under light with 5% sucrose were prolific in both shoot (92.5%) and flower (85%) production. Shoot and flower formation decreased with 7% sucrose (Table 4). Sucrose also affected the flower bud size in the cultures (Table 5). With 5% sucrose, there were about 13 buds per culture in the largest size category (>5 mm).

Table 1. Effects of cytokinins on *in vitro* shoot and flower formation from *F. hindsii* stem internodes of seedlings and branch internodes (random from 1st to 5th) of mature plant explants cultured 90 days.

Cytokinin concentrations (mg l^{-1})		Stem internodes of seedlings		Branch internodes of mature plants	
		Shoot (%)	Flower (%)	Shoot (%)	Flower (%)
Nocytokinin		20±5.8	0	20±5.8	0
BA	0.001	40±5.8	0	30±11	10±0.0
	0.01	90±5.8	27±3.3	87±3.3	80±5.8
	0.1	87±3.3	13±3.3	90±5.8	63±3.3
	1.0	80±5.8	0	80±5.8	27±3.3
KIN	0.001	20±5.8	0	13±3.3	23±3.3
	0.01	46±3.3	0	63±3.3	30±5.8
	0.1	87±3.3	0	87±3.3	17±3.3
	1.0	80±5.8	0	87±3.3	0
ADN	0.001	0	0	13±3.3	0
	0.01	47±3.3	0	36±8.8	13±3.3
	0.1	87±3.3	0	60±5.8	0
	1.0	80±5.8	0	47±3.3	0
DAP	0.001	43±3.3	0	33±3.3	0
	0.01	46±3.3	0	60±5.8	13±5.8
	0.1	73±3.3	0	60±5.8	17±3.3
	1.0	60±5.8	0	10±5.8	0

The data represent the means ± SE of three experiments, each of five replicates.

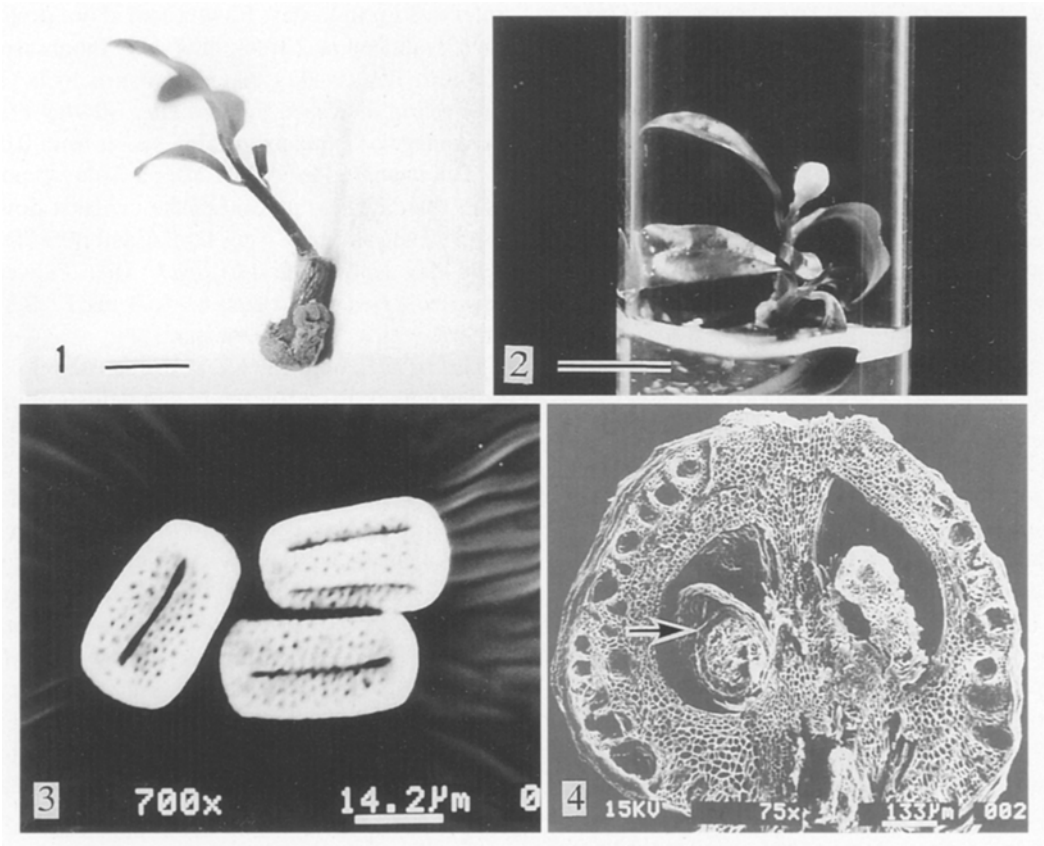

Fig. 1. Regeneration of shoot on a branch internode of *F. hindsii* on half-strength MT basal medium plus 0.01 mg l^{-1} BA after 60 days of culture (bar = 1 cm). **Fig. 2.** Flowering shoot regenerated from a branch internode of *F. hindsii* on half-strength MT basal medium plus 0.01 mg l^{-1} BA after 90 days of culture (bar = 1 cm). **Fig. 3.** Scanning electron micrograph of the pollen produced *in vitro* 2 days after flower blossom formation in *F. hindsii* (bar = 14.2 um). **Fig. 4.** Scanning electron micrographs of longitudinal section of zygotic embryo produced *in vitro* 15 days after flower blossom formation in *F. hindsii* (bar = 133 um).

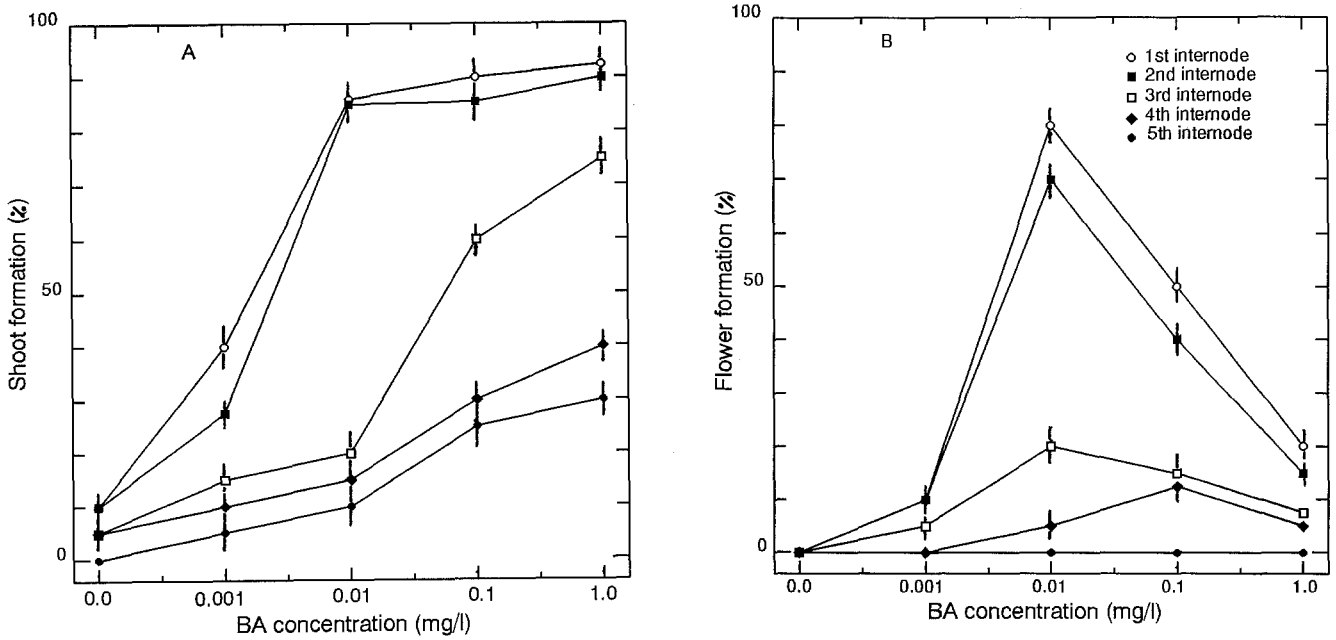

Fig. 5. *In vitro* shoot and flower formation from *F. hindsii* as influence by position of branch internodes and BA concentrations after 90 days of culture (A = shoot formation, B = flower formation).

Discussion

Exogenous cytokinin stimulated flowering by acting on the endogenous cytokinin in the ascending xylem sap in grapevine (Srinivasan and Mullin 1978). Bernier et al. (1977) found that cytokinin applied to apical meristem of *Sinapsis alba* triggered the mitotic cycle that commonly precedes flowering. Formation of flowers in *F. hindsii* required exogenous BA at concentrations from 0.001 to 0.1 mg l^{-1}. It has been postulated that the flowering stimulus consists of cytokinin to trigger the initial mitotic event that precedes flowering (Havelange et al. 1986; Bernier et al. 1977), plus at least one other factor. Our study supports the theory of a multiple-factorial control of flowering (Bernier et al. 1993; Evans 1969).

Branch internodes of *F. hindsii* flowered only if they originated close to the shoot apex, and flowering decreased with increased distance from the apex. The existence of a flowering gradient *in vitro* has been reported with several species and explants sources (Scorza 1982). Scorza and Janick (1980) and McDaniel et al. (1989) suggested that this apex to base floral gradient may be a result of endogenous gradients of growth substances in the stem. The floral gradient may result from changes in the capacity of cells to enter into a reproductive state in the course of ontogeny; changes in the capacity of particular cells to produce or react to a floral stimulus; a gradient of a floral promoter or inhibitor at the time of explant excision, or a combined promoter-inhibitor gradient (Wardell and Skoog 1969a, 1973; Tran Thanh Van 1973: Scorza and Janick 1980; Lang 1989). The ability of tissue to form flowers decreases basipetally from the apical end of a flowering plant (Compton and Veilleux 1992). Tran Thanh Van (1973) demonstrated that thin cell layer explants taken from the base of greenhouse-grown 'Wisconsin 38' plants formed only vegetative shoots, whereas those explants excised from the inflorescence formed flower buds. The position of the explant on the intact plant is extremely important when attempting to obtain *de novo* flowers on thin cell layer explants of photoperiodically sensitive tobacco cultivars (Compton and Veilleux 1992). Rajeevan and Lang (1987) suggest that the reduced flowering observed for short-day cultivars may result from a reduction in the number of cells competent for *de novo* flower morphogenesis. In addition, the increase in flower response among explants derived from tobacco inflorescences has been correlated with an increase in nuclear DNA content in that region (Wardell and Skoog 1973).

Sugars are necessary carbon sources in culture media for reliable induction and development of flowers (Scorza 1982). Addition of 3 to 7% sucrose to the medium may be necessary for induction of floral stimulus. The effect of the interaction of sucrose and light to promote *in vitro* flowering has been reported in a number of species (Scorza 1982). *Passiflora suberosa* stem internodes cultures on a medium containing 3% sucrose did not flower in darkness (Scorza and Janick 1980). *Murraya paniculata* plantlets derived from protoplasts cultured on half-strength MT medium containing 5% sucrose did not flower in darkness, but did in light (Jumin and Nito 1995). As shown in this work, branch internodes of *F. hindsii* also did not flower in darkness. Prolific flowering occurred in the light on half-strength MT basal medium containing 5% sucrose. This indicates, that sucrose is essential for *in vitro* flowering in light conditions.

A requirement for *in vitro* flowering of *F. hindsii* is the mature state of the explant source. *F. hindsii* undergoes a distinct juvenile stage lasting several months. Juvenility is characterized by sagitate leaf shape, erect growth, and the absence of tendrils and flowers. Juvenile plants do not flower due to an inability to produce a flowering factor or the inability of meristems to respond to a flowering factor (Lang 1965; Hackett 1985). Stem tissue from juvenile tobacco (Wardell and Skoog 1969b) and leaf discs from juvenile *Passiflora suberosa* (Scorza and Janick 1980) do not flower. Similarly, the 5th branch internodes of *F. hindsii* cultures on a 5% sucrose medium produce shoots which did not flower. Thus, juvenility in these 3 species seems to be a plant phenomenon and is not restricted to meristems since excised tissues devoid of buds did not flower.

Our investigations indicate that shoot and flower formation differ in their requirement for BA. Shoot production is evidently dependent upon a high concentration of BA although a low concentration is effective over a long exposure period (90-day). Flower production is greatest at a low concentration of BA or short exposures to a high concentration. At least a 4-day exposure to 0.1 mg l^{-1} BA or a 6-day exposure to 0.01 mg l^{-1} BA are necessary for flowering and vegetative shoot initiation. It is suggested that BA first induces meristem formation and then induces the flowering process. *In vitro* flowering of *F. hindsii* is affected by maturity, cytokinins and sucrose in light. The simplicity, yet plasticity, of the *in vitro* system as presented in this investigation suggests culture of *F. hindsii* as a promising technique for investigating flowering.

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Table 2. Effect of darkness on shoot and flower production from the 1st branch internode segments of *F. hindsii* cultured on half strength MT basal medium plus 0.01 mg l^{-1} BA 90 days after exposure to light under a 16–h photoperiod

Weeks in Darkness	Shoot (%)	Flower (%)
0	82.5a[z]	45.0a
1	80.0ab	45.0a
2	85.0a	45.0a
3	72.5bc	40.0a
4	60.0c	15.0b
5	65.0c	2.5c

[z]Means within a column followed by the same letter are not significantly different at p = 0.05.

Table 3. Effect of *in vitro* BA exposure on shoot and flower formation from the 1st branch internode segments of *F. hindsii* cultured 90 days after exposure to BA.

Days exposure to BA	BA concentrations (mg l^{-1}			
	0.01		0.1	
	Shoot (%)	Flower (%)	Shoot (%)	Flower (%)
0	20.0a[z]	0	20.0a	0
2	20.0a	0	25.5a	0
4	30.0a	0	35.0ab	5.0a
6	30.5a	5.3a	40.5b	23.0b
8	70.5b	42.5b	77.5c	45.0c
Constant	95.0c	80.0c	95.5c	27.5b

[z]Means within a column followed by the same letter are not significantly different at p = 0.05.

Table 4. Effect of sucrose on shoot and flower formation from the 1st branch internode segments of *F. hindsii* cultured on half–strength MT basal medium plus 0.01 mg l^{-1} BA.

Sucrose concentration (%)	Light		Dark	
	Shoot (%)	Flower (%)	Shoot (%)	Flower (%)
0	0.0a[z]	0.0a	0.0a	0.0a
1	40.0b	0.0a	0.0a	0.0a
3	90.0c	65.0b	0.0a	0.0a
5	92.5c	85.0c	0.0a	0.0a
7	42.5b	25.0d	0.0a	0.0a

[z]Means within a column followed by the same letter are not significantly different at p = 0.05.

Table 5. The effect of sucrose concentration on *F. hindsii* flower bud size distribution from the 1st branch internode segments cultured on half–strength MT basal medium plus 0.01 mg l^{-1} BA.

Sucrose concentration (%)	Bud length (mm)		
	>5	3–5	1–3
	Number of buds		
1	0	0	0
3	1±0.6*	0	8±0.6
5	13±0.6	6±0.6	9±0.6
7	4±0.4	3±0.6	5±0.4

*±values indicated standart error of the mean.