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In vitro flowering of orange jessamine (*Murraya paniculata* (L.) Jack)

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Abstract. Branch internodes of *Murraya paniculata* flowered in vitro on half-strength MT medium containing 5% sucrose supplemented with cytokinins. The cytokinins only induced vegetative shoots from the epicotyl of seedlings. The highest percentage of flowering (76.7% of the explants) was achieved from branch internodes of flowering plants close to the apex, on half-strength MT medium containing 5% sucrose and 0.001 mg l^{-1} benzyladenine (BA) in light. Flowering required two days' exposure to BA, but shoot formation could be initiated even without exposure to BA.
Key words. Cytokinin; flowering; internode; *Murraya paniculata*; shoot; sucrose.

Murraya paniculata (L.) Jack is one of the most important genetic resources for *Citrus* improvement, because it has an excellent lime tolerance and resistance to citrus nematodes[1,2]. *M. paniculata* is valued as an ornamental due to its large white flowers and attractive red fruits[3]. Tissue culture techniques have been widely used for micropropagation in the family of Rutaceae. During our studies related to the regeneration of protoplasts via somatic embryogenesis, we observed that flowering occurred on plantlets in half-strength Murashige and Tucker[4] (MT) medium containing 5% sucrose in the light[5]. Prolific flowering in vitro has not yet been reported in citrus-related genera, but has been shown to occur in a few other species[6]. Flowering would regularly form from several explants in some species of woody plants if taken from flowering plants[6]. Flowering has been promoted by cytokinins[7–9], sugars[7], and RNA base analoques[10], but inhibited by gibberellin[5,7] and auxin at high concentrations[10,11]. An in vitro flowering system would be a promising technique for investigating the factors controlling flowering, which are not well characterized in *M. paniculata*.

Reduced flowering with increasing distance from the apex was observed[8]. The cause of this floral gradient is unknown. This work deals with *M. paniculata* plants: a) to investigate the influence of explant position on in vitro flowering; b) to evaluate the effects of cytokinins and sucrose on in vitro flowering.

Materials and methods

Plant materials used for this study were obtained from mature flowering plants at the Germplasm Collection (Faculty of Agriculture, Saga University, Japan) and in vitro seedlings of *M. paniculata* (L.) Jack.

Seeds were sterilized in sodium hypochlorite (1% available chloride) for 20 min, then rinsed in 70% (v/v) ethanol for 1 min, followed by three washes in sterilized distilled water. The seeds were cultured on half-strength MT medium containing 5% sucrose without plant growth regulators. After 2 months, the epicotyls were cut into 5 mm segments. The branch internodes (first to fifth, starting from the apex) of mature plants were disinfected as described above and cut into 5 mm segments.

Benzyladenine (BA), kinetin (KIN), adenine (ADN) and 6-$\gamma\gamma$-dimethylallylaminopurine (DAP) were tested at concentrations of 0 to 1.0 mg l^{-1} for their effects on shoot and flower formation from epicotyls and branch internodes. Cytokinin concentrations were chosen based upon preliminary dose response trials. Twenty ml of medium consisting of half-strength MT medium containing 5% sucrose (basal medium) was dispensed into

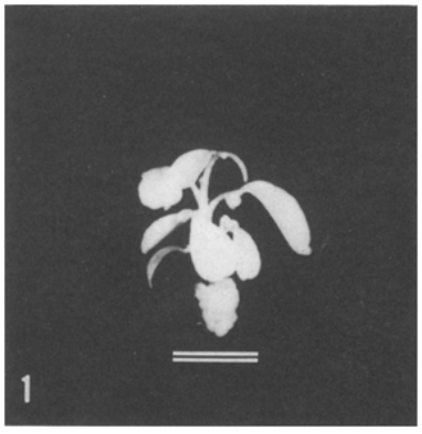

Figure 1. Regeneration of a shoot on an epicotyl segment of *M. paniculata* on half-strength MT medium containing 0.001 mg l^{-1} BA after 90 days in culture (bar = 1 cm).

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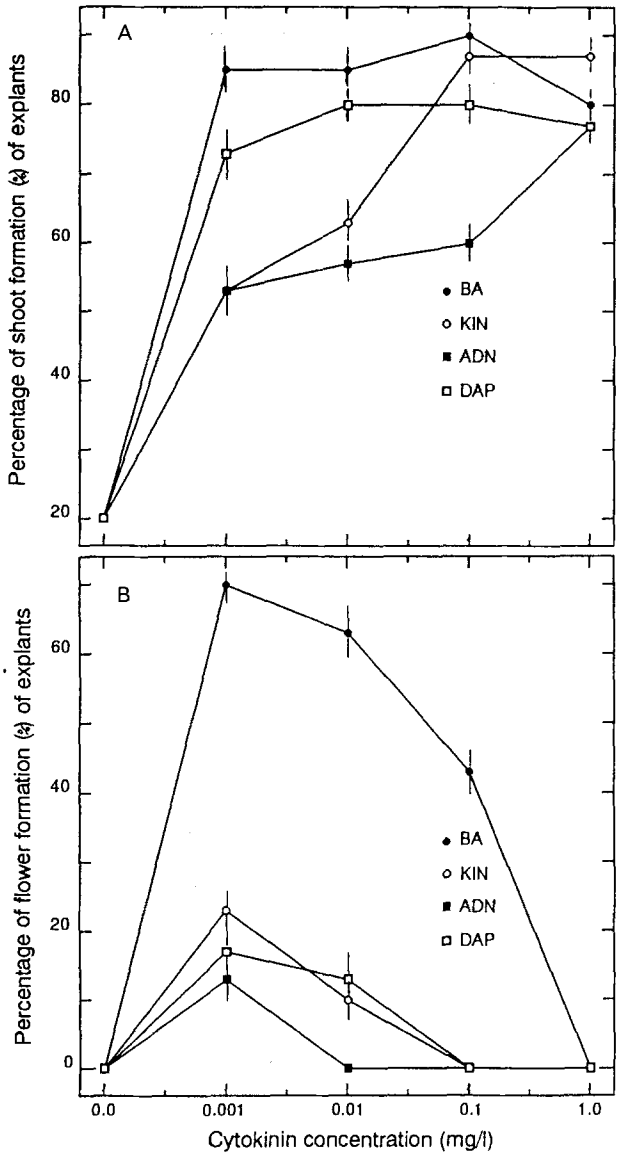

Figure 2. Effects of cytokinins on in vitro shoot *A)* and flower *B)* formation in *M. paniculata* branch internode culture for 90 days. Data collected after 70 to 90 days of culture.

individual 25 × 150 mm glass culture tubes. The pH was adjusted to 5.7, 0.3% Gelrite (Kelco, Division of Merk & Co. Inc., San Diego, California) was added, and the solution 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 photoperiod using cool white fluorescent light (33.3–35.3 μmol m^{-2} s^{-1}). Shoots and flowers were examined and counted at 2-week intervals for each culture.

The experiments were conducted with at least ten cultures per treatment and repeated at least three times.

Results

Flower induction. BA induced flowering from branch internodes of flowering plant explants, but induced only vegetative shoots from epicotyl segments (fig. 1). BA at 0.001 mg l^{-1} was most effective, with 85% of cultures producing shoots and 70% initiating flowers (fig. 2), and this concentration used in all subsequent experiments. Juvenility is characterized by sagittate leaf shape, erect growth, and the absence of tendrils and flowers. A single shoot directly emerged from cortical tissue at the cut top of the explants after 50 to 60 days of culture. A single flower was formed from one developed shoot. In vitro flowering occurred on the leaf axil of developed shoots. Most flowers were small and lacked stamens but a few were large and complete. Flowers with anthers produced pollen in vitro (fig. 3).

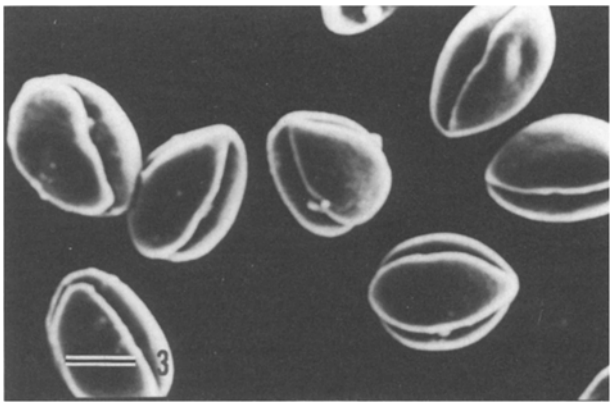

Figure 3. Scanning electron micrograph of pollen produced in vitro 2 days after flower blossom in *M. paniculata* (bar = 15 μm).

Flowering gradient. Preliminary tests indicated that branch internodes of mature plants initiated flowers only if they were obtained from internodes that originated near apices. Explants were taken sequentially starting from the apices (first to fifth internode) to investigate the influence of explant source and position (age) on flowering further. A single 5 mm segment was cut from each internode and one segment was placed on half-strength MT medium supplemented with either 0.0, 0.001, 0.01, 0.1 or 1.0 mg l^{-1} BA. A very low frequency of flowering occurred on the fourth and no flower formation at all on the fifth internodes, indicating the presence of a 'flowering gradient' in both young and old tissues (according to the internode). The combination of young tissue and low (0.001 mg l^{-1}) BA was optimal for inducing flowering (fig. 4). Failure to induce flowering in old tissues suggested either the presence of floral inhibitors in old tissues or of floral promoters in young tissues.

Flowering response to light. Internode 1 explants were cultured on half-strength MT medium containing 0.001 mg l^{-1} BA and grown for up to 5 weeks in darkness before being transferred to light. Few shoots and no flowers formed when explants were in the dark. Exposure to the darkness for more than 2 weeks followed by re-exposure to light reduced flowering (fig. 5).

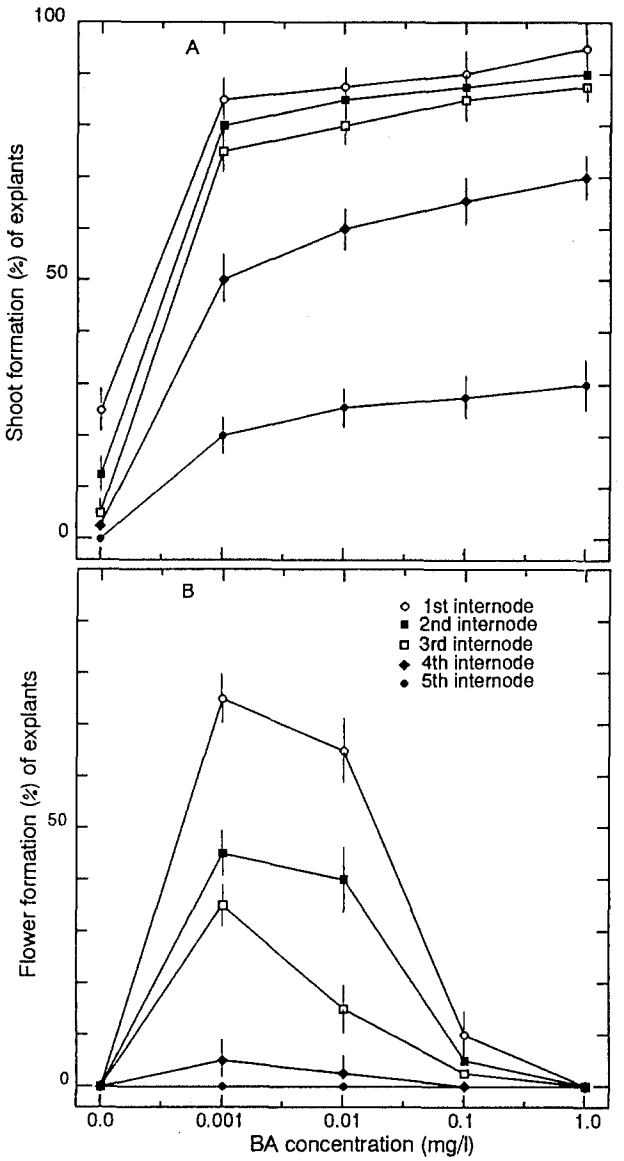

Figure 4. In vitro shoot *A)* and flower *B)* formation as influenced by position of branch internode from *M. paniculata* and BA concentration after 90 days in culture.

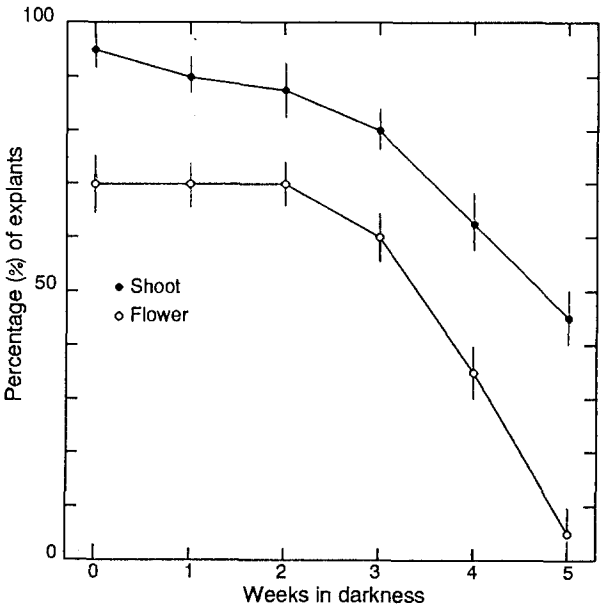

Figure 5. Effects of darkness on shoot and flower formation by branch internode explants of *M. paniculata* 90 days after exposure of light.

Kinetics of BA-induced flowering response. Internode 1 segments were explanted onto half-strength MT medium supplemented with 0.001 mg l^{-1} BA and transferred to BA-free medium at 48 h intervals for up to 8 days. Shoots were produced without BA (fig. 6). Within 14 weeks in the BA-free medium, the number of shoots increased to 30 after 90 days' constant exposure to BA. Flower bud production required 2 days of exposure to 0.001 mg l^{-1} BA, peaked (22 buds) at 8 days' exposure to BA, and was reduced to 19 buds after 90 days' exposure to BA.

Effect of sucrose. The effect of sucrose on flowering was also investigated. First branch internode segments of mature plant cultures on half-strength MT medium containing 0.001 mg l^{-1} BA were kept separately in the dark and under light with a 16 h photoperiod. Organogenesis did not occur in medium without sugar. Shoot and flower formation increased by addition of sucrose up to 5.0%, but was inhibited thereafter (fig. 7).

Discussion

Adventitious shoots produced from branch internodes of flowering plants of *M. paniculata* rapidly produced flowers. Cytokinins appear to be a common requirement for in vitro flowering[8,9,12] and are an absolute requirement in *M. paniculata*. It has been postulated that the flowering stimulus consists of cytokinins that triggered the initial mitotic event preceding flowering[12,13], plus at least one other factor. Our study supports the theory of a multi-factorial control of flowering[14,15]. $T_{(q)}$ stimulus appears to have a short active life time and is dispersed as it is translocated through the plant[14,16]. The floral stimulus was apparently transmitted through the explants to the newly developed shoots. The in vitro flowering habit persisted for several shoot regeneration cycles over a period of at least one year (data not shown). Our finding agrees well with the results reported by Jumin and Nito[5] that flowers of *M. paniculata* could be obtained in vitro when plants derived from protoplasts were cultured on half-strength MT medium in light conditions.

Malformations and poor flower quality frequently observed in the flowers produced in vitro may have been at least partially due to competition and/or nutritional deficiencies[17]. Transplanting separated shoots with a floral bud to fresh medium led to an increase in the

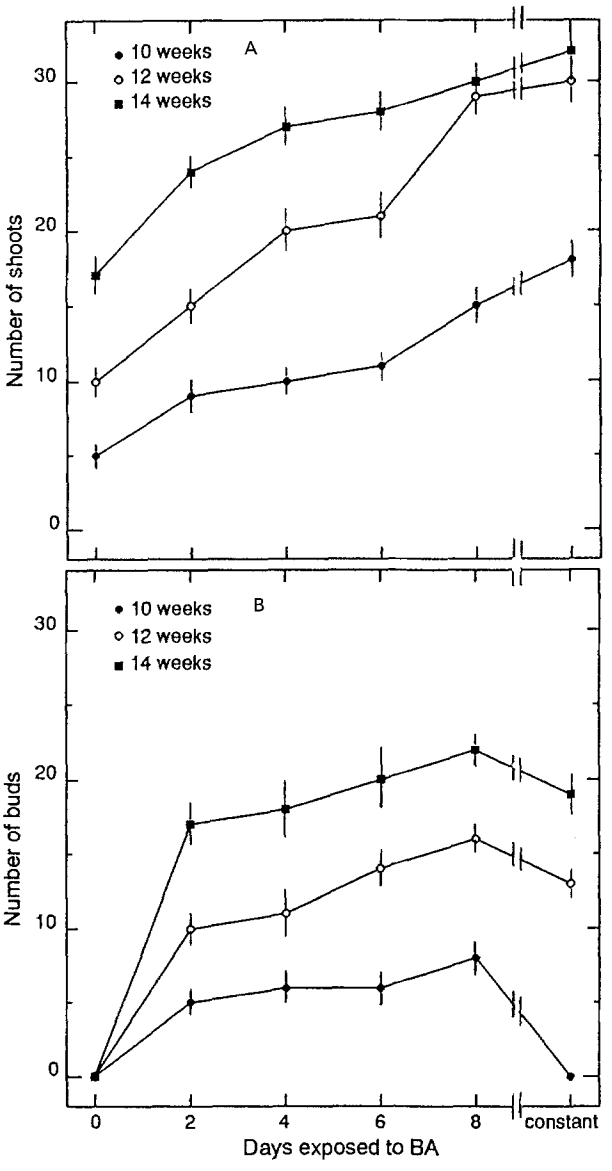

Figure 6. Numbers of shoots *A)* and flower buds *B)* at 2-week intervals following placement of segments of the youngest internode of *M. paniculata* branches on half-strength MT medium after exposure to 0.001 mg l^{-1} BA.

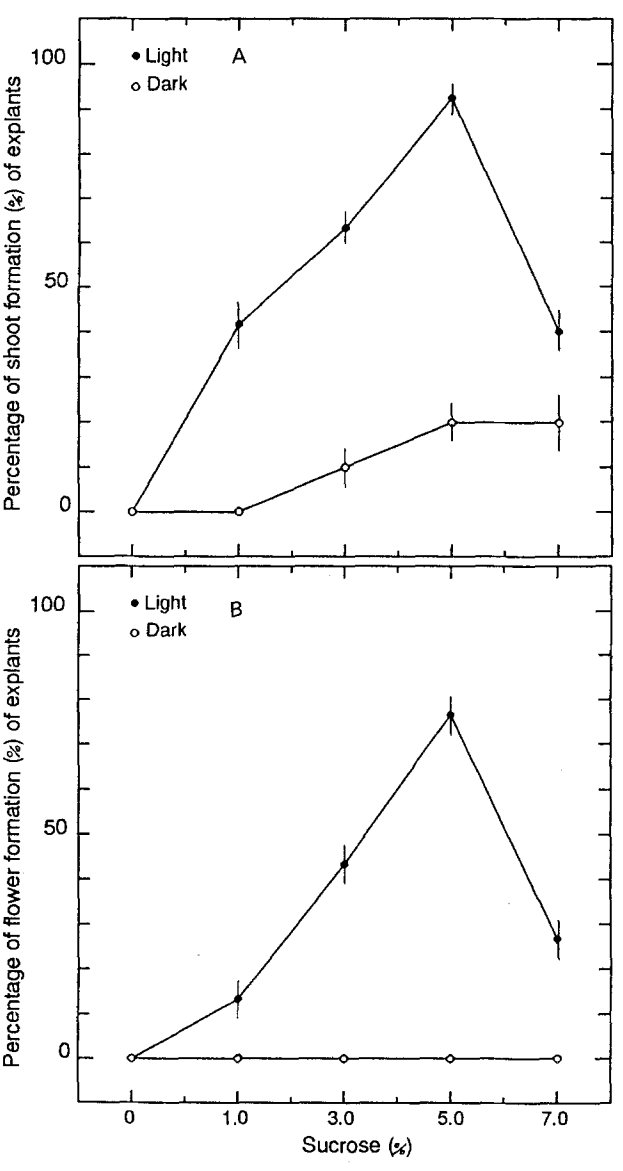

Figure 7. Effects of sucrose and light on shoot *A)* and flower *B)* formation from segments of the youngest internode of *M. paniculata* branches on half-strength MT medium containing 0.001 mg l^{-1} BA after 90 days of culture.

quality of flowers produced in vitro (data not shown). Fresh subculture medium may be necessary to eliminate the nutritional deficiency during flower development. More work is needed to substantiate this possibility.

Precocious flowering is generally an undesirable trait for propagation[18]. Indeed, with *M. paniculata* the persistent flowering nature of shoots derived initally from branch internodes of mature plants resulted in production of poor quality plantlets for mass propagation purpose. Nevertheless this research provides information on the acceleration of generation turnover with immediate application to citrus breeding programs. Further experiments should lead to a better understanding of the physiological and molecular events underlying the shift from the vegetative to the floral state.

Flowering of internode segments of *M. paniculata* decreased with increasing distance from the apex. The existence of a flowering gradient in vitro has been reported with several species and explant sources[6]. Scorza[6] suggested that this apex-to-base floral gradient may be a result of endogenous gradients of growth substances in the stem.

Sugars are necessary carbon sources in culture media for reliable induction and development of flowers[6]. Explants cultured in a medium with 5% sucrose in darkness or without sucrose in the light did not flower. It is indicated that sugars interact with light to show their effect. Addition of sucrose to the medium may be necessary for induction of floral stimulus. Sugar production by photosynthesis helped to induce the production of

the floral stimulus under inductive photoperiod[19,20]. Reid et al[21] mentioned that the assimilate transported to the apex after high light irradiance reflects a need for carbohydrates (mainly sucrose) and other assimilates by the induced apex, but a particular floral stimulus may also be transported in the phloem. Sucrose cannot substitute for insufficient light[14]. The effect of the interaction of sucrose and light to induce in vitro flowering has been reported in a number of species[6]. *Fortunella hindsii* branch internode cultured on a medium containing 5% sucrose did not flower in darkness[22] but did in light. As shown in this work, branch internodes of *M. paniculata* did not flower in darkness either.

A requirement for in vitro flowering of *M. paniculata* is maturity of the explant source. 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[23,24]. Stem tissue from juvenile tobacco[10], leaf discs from juvenile *Passiflora suberosa*[8], and stem internodes of *Fortunella hindsii* seedlings[22] do not flower, and a similar result was observed in the epicotyls of *M. paniculata* seedlings. Thus, juvenility in these four species seems to be a whole-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 on a high concentration of BA although a low concentration is effective over a long exposure period (90 days). Flower production is greatest at low BA concentration. At least 2 days' exposure to 0.001 mg l^{-1} BA is 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 *M. panicutala* in light is affected by maturity, cytokinins and sucrose. The simplicity, yet plasticity, of the in vitro system presented in this investigation suggests *M. paniculata* culture is a promising technique for investigating flowering.

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