Plant Cell Reports (1999) 18: 764–768

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High-frequency in vitro flowering of *Murraya paniculata* (L.) Jack

Received: 10 December 1997 / Revision received: 5 November 1998 / Accepted: 2 December 1998

Abstract Shoots of orange jessamine (*Murraya paniculata*) a member of the Rutaceae family flowered in vitro on half-strength MT basal medium containing 5% sucrose. The highest percentage (95%) of flowering was obtained on medium supplemented with 0.1 mg l^{-1} N^6-benzyladenine and pH 5.7. A "floral gradient" was detected among the stem internodes and root segments derived from seedlings, with shoot and flower formation significantly influenced by position on the shoot internodes and root segments. Flower buds originating from shoots derived from seeds but not other tissues developed into normal flowers and produced zygotic embryos.

Key words Floral gradient · N^6-Benzyladenine · Seedling · *Murraya paniculata* · pH

Abbreviations *BA* N^6-Benzyladenine · *MT medium* Murashige and Tucker medium

Introduction

The ability of explants to form flowers in vitro depends on numerous factors, internal and external, chemical and physical, and virtually all of these factors interact in various complex and unpredictable ways (Tran Thanh Van 1973; Tran Thanh Van et al, 1974; Scorza and Janick 1980; Croes et al. 1985; Lang 1987; Compton and Vielleux 1992). Flowering is considered to be a complex process regulated by a combination of environmental and genetic factors (Tisserat and Galletta 1995). Especially important factors are carbohydrates, growth regulators, light, and pH of the culture medium (Heylen and Vendrig 1988).

Reasons for studying flower formation in vitro can be summarized as (1) to provide a model system for studying flower induction and development, (2) to provide a means for conducting microbreeding, and (3) to provide a source of biochemicals and pharmaceuticals (Tisserat and Galletta 1990).

Flowering can regularly occur from several explants taken from flowering plants of some woody plant species (Scorza 1982). Reduction of flowering ability was observed with increased distance from the apex (Scorza and Janick 1980; McDaniel et al. 1989; Jumin and Nito 1996a, b). The cause of this floral gradient is unknown. Jumin and Nito (1995, 1996b) have successfully induced flowering in vitro from plantlets derived from protoplasts and from branch internodes of *Murraya paniculata*, but they have not yet successfully induced flowering in vitro from epicotyl segments of seedlings. Explants from relatively young vegetative (juvenile) plants regenerate only vegetative buds, even under favorable conditions for flower bud formation (Lang 1987). This paper reports the success obtained in inducing flowering in vitro at high frequency from seeds, stem internodes, and root segments derived from seedlings of *M. paniculata.*

Materials and methods

Seeds of *M. paniculata* used in this study were obtained from mature plants at the Germplasm Collection, Faculty of Agriculture, Saga University, Japan. Seeds were sterilized in sodium hypochloride (1% available chlorine) for 20 min, then rinsed in 70% (vol/vol) ethanol for 1 min followed by three washes in sterile distilled water. The seed coat was removed and a single seed was cultured on half-strength MT (Murashige and Tucker 1969) basal medium containing 5% sucrose and supplemented with either 0.0, 0.001, 0.01, 0.1, or 1.0 mg l^{-1} N^6-benzyladenine (BA). Medium (20 ml) was dispensed into individual 25×150 mm glass culture tubes. The pH was adjusted to 5.7 before autoclaving and 3.0 g l^{-1} Gelrite (Kelco, San Diego, Calif.) was added and finally autoclaved at 121°C for 10 min. The cultures were maintained at 25°C under a 16-h photoperiod using cool, white fluorescent light (52.9 μmol m^{-2} s^{-1}). The effect of

Communicated by J. Widholm

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Fig. 1 In vitro flowering of a *Murraya paniculata* shoot derived from seed on half-strength MT basal medium containing 5% sucrose supplemented with 0.01 mg l^{-1} BA after 60 days of culture (tube diameter is 25 mm)

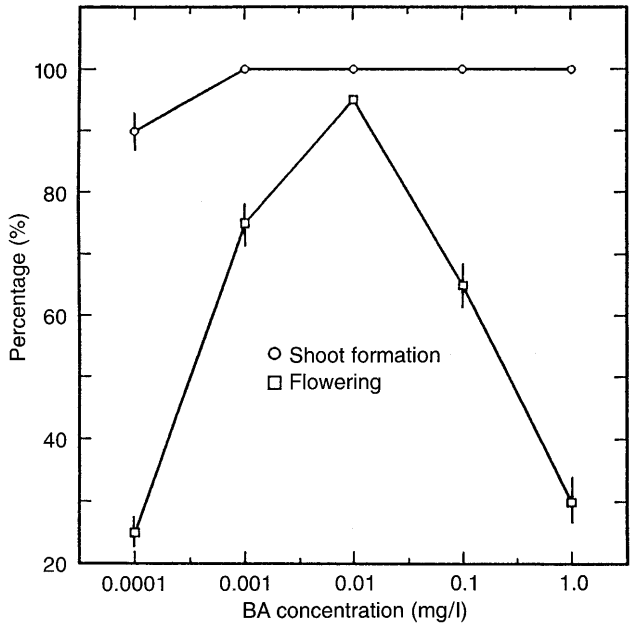

Fig. 2 Effect of BA on in vitro shoot formation and flowering of *M. paniculata* after 90 days of culture (means ± SE of three independent replications)

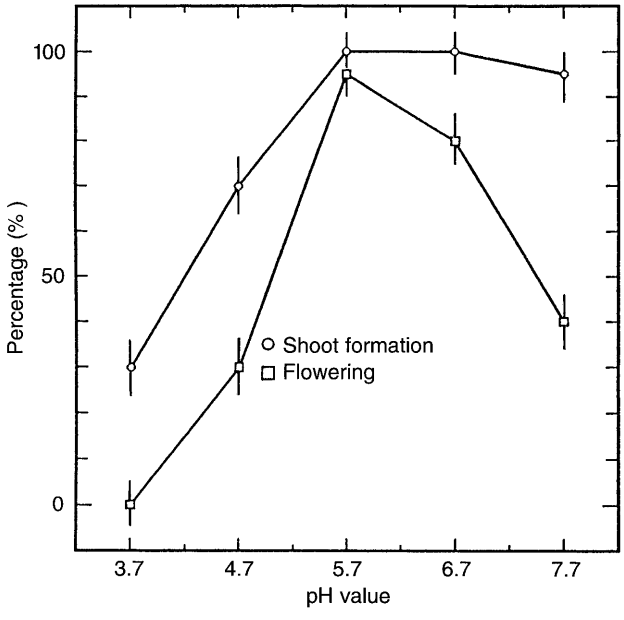

Fig. 3 Effect of medium pH on in vitro shoot formation and flowering of *M. paniculata* after 90 days of culture (means ± SE of three independent replications)

pH on flowering was investigated by culturing seeds in MT basal medium containing 5% sucrose plus 0.01 mg l^{-1} BA at pH 3.7, 4.7, 5.7, 6.7 or 7.7 (as measured before autoclaving). After completion of first flowering, stem internodes (1st to 5th, starting from the apex) and root segments of the seedlings (1st to 5th, starting from the cotyledons) were cut into 5-mm segments. A single explant was cultured on the medium in each culture. Shoots and flowers were examined and counted at 2-week intervals for each culture. The number of flower buds was counted after 60 days, and open flowers were counted after 90 days. Zygotic embryos were observed under an inverted microscope 7–14 days after flower opening if ovaries were >2 mm in diameter. The experiments were conducted with at least ten cultures per treatment with three replications.

Specimens 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-32. The tissue samples were examined under a scanning electron microscope, Topcon ABT-32 at 15 KV.

Results and discussion

Flower induction

BA induced flowers from in vitro shoots of *M. paniculata* at concentrations from 0.001 to 0.1 mg l^{-1} (Fig. 1). BA at 0.01 mg l^{-1} was the most effective with 100% of the seeds forming shoots and 95% initiating flowers (Fig. 2). Srinivasan and Mullin (1978) state that exogenous cytokinin stimulates flowering by activation of endogenous cytokinin in ascending xylem sap. Exogenous cytokinin in apical meristems of *Sinapsis alba* triggered the mitotic cycle that commonly precedes flowering (Bernier et al. 1977). Jumin and Nito (1996a, b) found that cytokinin applied to branch internodes of flowering plants of *Fortunella hindsii* and *M. paniculata* could induce flowering in vitro.

Optimal in vitro flower formation occurred when *M. paniculata* seeds were incubated on medium at pH 5.7–6.7 (Fig. 3). At pH 3.7, only 3% of the seeds produced shoots,

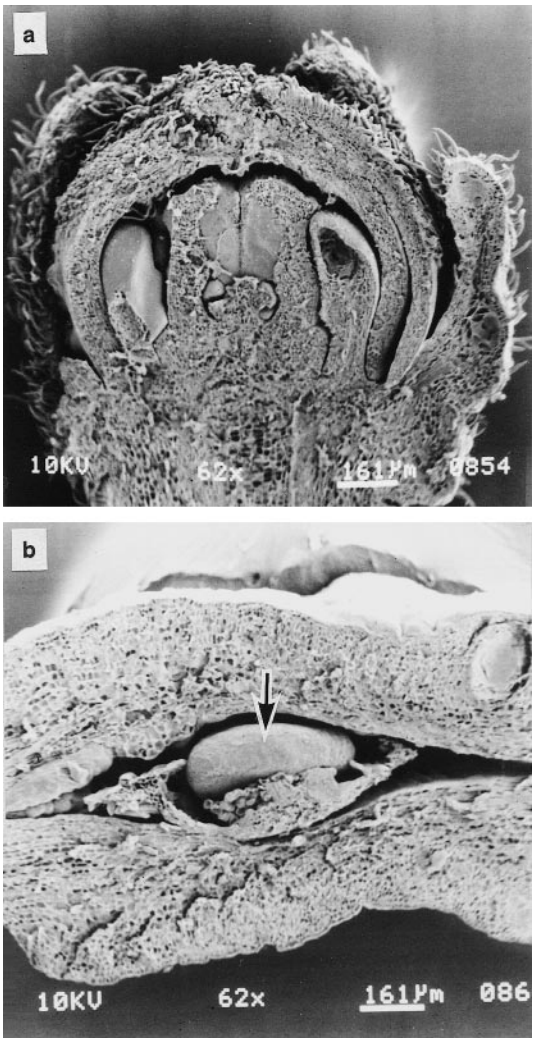

Fig. 4 **a** Scanning electron micrograph of longitudinal section of floral bud (*bar* 161 µm). **b** Scanning electron micrograph of longitudinal section of ovary derived from seed cultures. Zygotic embryo produced 14 days after flower opening (*bar* 161 µm)

but none flowered. At pH 5.7, all seeds grew and 95% flowered. Most flowers were normal and complex, and appeared in the leaf axils. Several flowers were pollinated in vitro and only flowers originating from seeds produced embryos (Fig. 4) but did not set fruit, because the ovary died about 30 days after flower opening. Flowers originating from stem or root segments produced embryos that aborted. The production of zygotic embryos from in vitro flowering is common in citrus relatives (Jumin and Nito 1996a). In *F. hindsii*, floral buds developed into normal flowers and produced zygotic embryos at 0.01 mg l^{-1} BA, 5% sucrose, and pH 5.7 (Jumin and Nito 1996a). Teich and Spiegel-Roy (1972) reported that nucellar embryogenesis was only displayed in polyembryony of citrus. We found that flower production gradually decreased above pH 6.7. The optimal concentration of BA and medium pH obtained from these results were used in all subsequent experiments. With the optimal BA concentration and medium pH, there were a

Table 1 *Murraya paniculata* flower buds (60 days after culture), opened flowers (90 days after culture), and zygotic embryos (7–14 days after flower opening) on half-strength MT basal medium containing 5% sucrose plus 0.01 mg l^{-1} BA (mean±SE)

Explant source	Number per explant		
	Flower buds	Opened flowers	Zygotic embryos
Seed	2.0±0.0	1.7±0.6	0.9±0.6
Stem internode	1.3±0.6	0.8±0.4	0±0.0
Root segment	0.9±0.6	0.7±0.6	0±0.0

mean of 2 buds formed from the inflorescence, 1.3 buds from each shoot that developed from stem internodes, and 0.9 buds from each shoot derived from root segments (Table 1). Cousson and Tran Thanh Van (1981) reported that tobacco thin cell layer stem explants produced either floral buds or floral and vegetative bud at pH 6.5, while only vegetative buds were obtained at pH 5.6.

Floral gradient

A gradient for shoot and flower formation was detected among the stem internode and root segment positions taken from seedlings after the formation of the first flower (about 60 days after germination). The explants were taken from the 0.01 mg l^{-1} BA treatment. Preliminary tests indicated that shoots derived from root segments initiated flowers only if they were obtained from root segments that originated near the cotyledons. Explants (5 mm) were taken sequentially starting from the cotyledons (1st to 5th segments) and from the 0.01 mg l^{-1} BA treatment to investigate the influence of root explant source and position (age) on flowering. About 30% of the shoots from the 1st root segment produced flowers, but shoots initiated from the 4th root segment were all vegetative (Fig. 5). In contrast, shoot formation was not significantly influenced by the position of root segments. Similarly, the shoots that development from stem internodes initiated flowers only if they were taken from stem internodes originating close to the apex. A very high frequency (95%) of flower formation was obtained from shoots that developed from the first stem internodes and flowering gradually decreased with increased distance from the apex (Fig. 6). These results indicate the presence of a “floral gradient” in both young and old tissue (position) of root segments and stem internodes. Branch internodes of *F. hindsii* and *M. paniculata* flowered only if they originated close to the shoot apex (Jumin and Nito 1996a, b). In the present experiment, the ability to form flowers decreased basipetally from the cotyledons in the root. However in stem internodes, the highest ability to induce flowers was obtained from young tissue. Tran Thanh Van (1973) indicated that there is this a pronounced gradient in floral regenerative capacity, decreasing from the apical to the basal regions and expressed in both the number of floral regenerates formed and the degree of commitment to floral regeneration. The existence of a floral

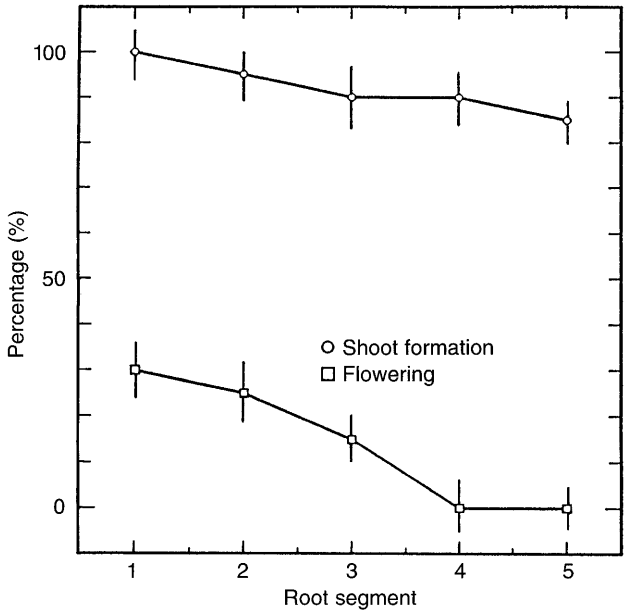

Fig. 5 In vitro shoot and flower formation as influenced by position of root segment of *M. paniculata* after 90 days of culture. The explant was 5 mm long and flower formation was counted after 70–90 days of culture (means ± SE of three independent replications)

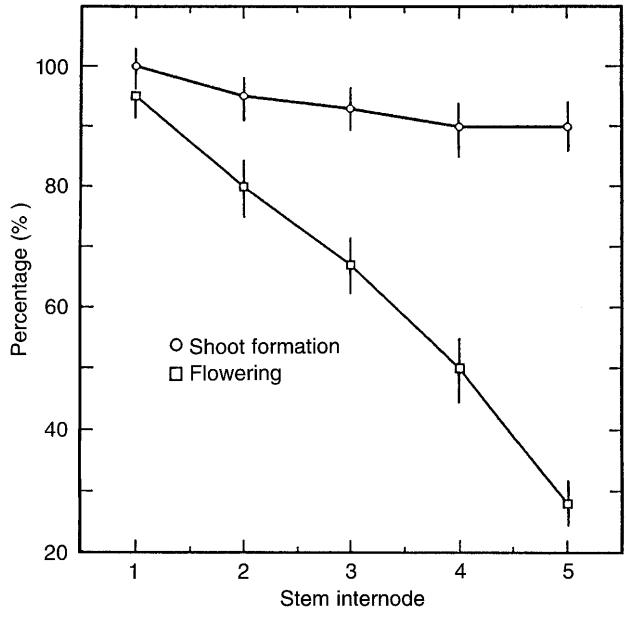

Fig. 6 In vitro shoot and flower formation as influenced by position of stem internode (1st to 5th, starting from the apex) of *M. paniculata* after 90 days of culture. Explants were 5 mm in length and flower formation was counted after 70–90 days of culture (means ± SE of three independent replications)

gradient in vitro has been reported with several species and explant 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 for development, 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, b; Tran Thanh Van 1973; Scorza and Janick 1980; Lang 1987). The position of the explant on the intact plant is extremely important when attempting to obtain de novo flowers in thin-cell-layer explants of a photoperiodically sensitive tobacco cultivar (Bridgen and Veilleux 1985; Rajeevan and Lang 1987; Altamura et al. 1989).

Juvenile plants do not flower due to inability to produce flowering factor(s) or the inability of meristems to respond to a flowering factor (Lang 1965; Hackett 1985). Stem tissue from juvenile tobacco (Wardell and Skoog 1996b) and leaf discs from juvenile *Passiflora suberosa* (Scorza and Janick 1980) do not flower. In the present results, juvenile tissue from 2-month seedlings or stem internodes derived from seedlings could produce flowers at high frequency (95%). Chang and Hsing (1980) reported that the production of embryos from root callus of mature ginseng (*Panax ginseng*), and the embryos formed flowers with fertile pollen within 1 month of subculture. Flowering of explants originating from juvenile plants is rate. This leads to a model system useful for studying microclimates or nutritional effects on plant vegetative and reproductive processes.

Acknowledgements We would like to thank Prof. N. Nito, Faculty of Agriculture, Saga University, Japan for critically reading the manuscript. We would also like to thank Dr. Masood Jelokani-Niaraki for English correction of the manuscript.

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