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Some Morphological and Anatomical Studies of Leaves and Flowers of *Murraya paniculata* (Jack) Linn. *in vivo* and *in vitro*

Rosna Mat Taha and Noorma Wati Haron
Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603, Kuala Lumpur, Malaysia

Abstract: In the present study, various explants of *Murraya paniculata* (Jack) Linn., such as cotyledons, shoots and young stems were cultured on MS medium supplemented with various concentrations of Benzyl Amino Purine (BAP) under 25±1°C with 16 h light and 8 h dark and also 8 h light and 16 h dark to obtain complete plant regeneration. *In vitro* flowering was observed from shoot explants cultured on MS supplemented with 0.5-2.0 mg L^{-1} Naphthalene Acetic Acid (NAA) and also on MS basal medium under similar conditions. The leaves and flowers obtained from both *in vivo* and *in vitro* conditions were examined and compared. Morphological studies such as leaf clearing, epidermal peeling were studied using light and scanning electron microscope. Macromorphological studies of the flowers produced from *in vivo* and *in vitro* conditions were also examined. Morphologically, there were no differences between *in vivo* and *in vitro* flowers except the flowers produced from tissue culture systems were smaller in size with protruding stigmas. Differences were also found in the number of layers of palisade cells and the presence or absence of epicuticle layer of the leaves. Leaves produced from tissue culture system were smaller in size with membranous texture. Stomata were present only on the abaxial surfaces of both *in vivo* and *in vitro* leaves but the stomata were raised above the epidermis in the latter.

Key words: *Murraya paniculata*, tissue culture, *in vitro* flowering, macromorphology, anatomy

INTRODUCTION

Tissue culture is done for various purposes, for instance; for mass propagation, haploid and triploid plant production, somatic hybridisation, genetic improvement, production of secondary metabolites, production of synthetic seeds and also for fundamental understanding of plant basic processes such as division, differentiation and development (Bhojwani and Razdan, 1996). Comparison of processes that occur in cells under *in vitro* environment with that of intact plants can give some understanding of the underlying mechanisms involve. Cells grown *in vitro* are much easier to manipulate compared with the cells in intact plant. Plants derived from tissue culture systems can result in abnormalities or variations either at cellular or morphological level (Larkin and Scowcroft, 1981). Some changes that occur at cellular level is expressed morphologically, whilst some plants such as *Vicia faba* can tolerate the cellular variations and nothing is revealed at morphological level (Taha and Francis, 1990).

In this study, we would like to report some tissue culture studies of *Murraya paniculata* (Jack) Linn. until floral stage in tissue culture system. The leaves and flowers produced *in vitro* were then compared with those produced *in vivo*. The main objective being to investigate whether there are variations between the leaves and flowers obtain under different conditions. For cloning purposes, it is desirable to obtain plants which are the exact copies of the parents, however, variations may be beneficial in certain cases.

MATERIALS AND METHODS

Seeds of *Murraya paniculata* obtained from Rimba Ilmu, University of Malaya, Kuala Lumpur, were washed and sterlised in the usual manner (Taha, 1993) with 70% (v/v) alcohol, 2.5% (w/v) sodium hypochlorite and 0.1% (v/v) Tween-twenty. The seeds were then cultured on MS basal medium (Murashige and Skoog, 1962) to get explant sources. The seedlings used to initiate cultures were 3-4 weeks old. Various explant organs such as leaves, cotyledons, stems and shoots were cut into small pieces (5 mm for shoot and stem and 5×5 mm for leaf and cotyledon). These explants were then cultured on MS media supplemented with different concentrations of NAA and BA. At least 20 replicates were used for each treatment and each treatment was repeated 3 times. The

Corresponding Author: Prof. Dr. Rosna Mat Taha, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia Tel: 603-79674372 Fax: 603-79674173

cultures were maintained under 16 h light and 8 h darkness and also 8 h light and 16 h darkness at 25±1°C. The cultures were examined every alternate day. The leaves and flowers obtained from *in vitro* systems were then compared with those of intact plants.

Standard procedures were followed for SEM and two to four specimens were examined. A section of 5 mm^2 of the dry leaf lamina (both adaxial and abaxial surfaces) was fixed on to double sided adhesive tape on labelled stubs. The specimens were coated with gold using a Bio-Rad SEM coating system and scanned in Philips SEM 515 Scanning electron microscope. For epidermal studies, samples were taken from midway between the base and apex of lamina. The epidermal characters were investigated from cuticular membrane preparations. Each sample was macerated in 10% nitric acid until the epidermis could be removed. Adaxial and abaxial epidermis were then separated using fine forceps and a dissecting needle. The cleaned epidermis were stained in safranin and mounted in Canada balsam. The method used for anatomical study was after Johansen (1940) with minor modifications. Sectioning of the midrib of the leaf lamina was done using a Reichart Rotary microtome. Photographs were taken using a Photomicroscope. Data obtained are presented in Tables 2 and 3. Light micrographs are shown in Figs. 1, 2, 9 and 10. Scanning electron micrographs of both adaxial and abaxial leaf surfaces are shown in Figs. 3-8. The terminology used is that of Dilcher (1974).

RESULTS

Complete plant regeneration of *Murraya paniculata* was achieved from portions of cotyledons, shoots and young stems cultured on MS supplemented with 1.0 mg L^{-1} BA and also MS basal medium. Rooting of this species could be obtained on either MS basal or MS containing 1.0 mg L^{-1} NAA. *In vitro* flowering was obtained from MS basal and also on MS added with 0.5-2.0 mg L^{-1} NAA. *In vitro* flowering could only occur when cotyledons and shoots were used, but not from plantlets derived from stem and leaf explants. Callus was routinely observed from leaf and stem explants. *In vitro* flowering occurred after 26 days on MS basal medium under 16 h light and 8 h dark but it took longer to flower (35 days) under 8 h light and 16 h darkness on the same medium. The percentage of flowering reduced from 80 to 60% when the cultures were exposed to shorter day length. By adding 0.5-2.0 mg L^{-1} NAA, the *in vitro* flowering was found to occur faster (21 days) (Table 1). GA_3 was not effective for *in vitro* flowering of this species although it may be effective for other species. The leaves and flowers obtained from the regenerants were then subjected to micromorphological and anatomical studies (Tables 2, 3).

Morphologically, there were no differences in the vegetative and reproductive characters between *in vivo* and *in vitro* plant of *Murraya paniculata* except for the sizes and textures of the leaves, the sizes of the flowers and the type of stigmas produced. The size of *in vitro*

Table 1: The percentage flowering of *Murraya paniculata* cultured on MS medium supplemented with different concentrations of NAA and BA (mg L^{-1}) at 25±10°C

	Flowering under different photoperiod (%)		
Medium	16 h light + 8 h dark	8 h light + 16 h dark	Dark
MS basal	80 after 26 days	60 after 35 days	0
MS + 0.5 BA	0	0	0
MS + 1.0 BA	0	0	0
MS +1.5 BA	0	0	0
MS + 2.0 BA	0	0	0
MS + 0.5 NAA	72 after 21 days	45 after 36 days	0
MS+ 1.0 NAA	50 after 21 days	40 after 36 days	0
MS + 1.5 NAA	65 after 21 days	40 after 36 days	0
MS + 2.0 NAA	62 after 21 days	38 after 36 days	0

Table 2: Vegetative and reproductive morphological characters of *Murraya paniculata* plant *in vivo* and *in vitro*

Characters	*In vivo*	*In vitro*
Leaf		
Size	3.0-5.0×1.5-2.0 cm	1.5-2.0×0.5-1.0 cm
Texture	Coriaceous	Membranous
Shape	Oblanceolate	Oblanceolate
Apex	Retus	Retus
Base	Cuneate	Cuneate
Margin	Undulate	Undulate
Vernation	Piunate	Pinnate
Petiole	Winged	Winged
Trichome	Present	Present
Flower		
Colour	Cream	Cream
No. of petals	5	5
No. of sepals	5	5
No. of stamens	10	10
Type of inflorescence	Racemose	Racemose
Position of inflorescence	Terminal	Terminal
Ovary	Superior	Superior

Table 3: Micromorphological and anatomical characters of the leaf lamina of *Murraya paniculata in vivo* and *in vitro*

Characters	*In vivo*	*In vitro*
Stomata		
Position	Level with the epidermis	Raised above the epidermis
Shape	Rounded to elliptical	Rounded to elliptical
Type	Anomocytic	Anomocytic
Structure	Dorsiventral	Dorsiventral
Cuticle	Present	Absent
Epidermal cells		
Size	Adaxial>abaxial	Adaxial>abaxial
Anticlinal wall		
Adaxial	Undulate-straight	Undulate-straight
Abaxial	Undulate-straight	Undulate-straight
Shape		
Adaxial	Isodiametric	Isodiametric
Abaxial	Irregular	Irregular
Palisade cells	Biseriate	Uniseriate
Vascular system	Arc-shaped	Arc-shaped

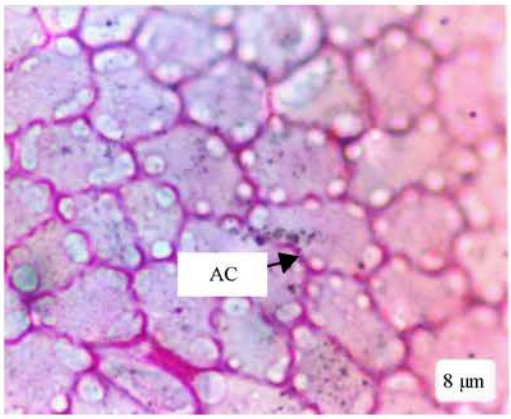

Fig. 1: Light micrograph of adaxial surface of leaf showing the anticlinal wall of the epidermis (AC)

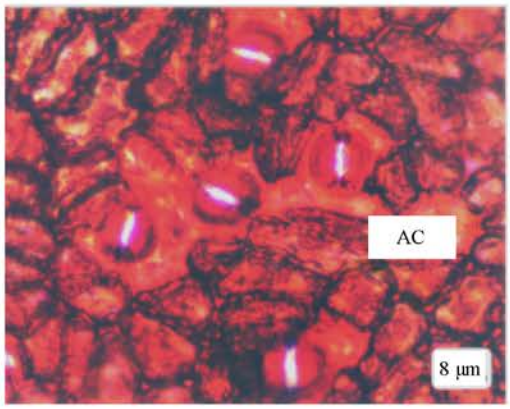

Fig. 2: Light micrograph of abaxial surface of leaf showing anomocytic stomata and anticlinal wall of the epidermis (AC)

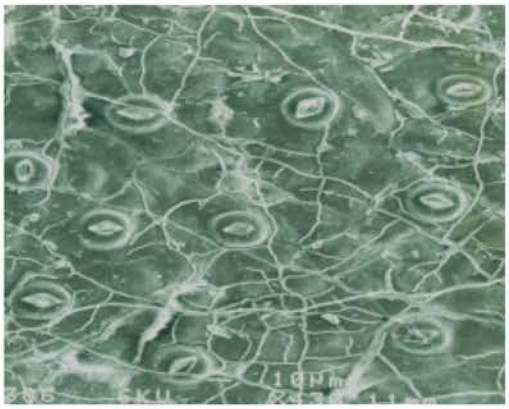

Fig. 3: Scanning electron micrograph of abaxial surface of *in vivo* leaf showing the distribution of stomata

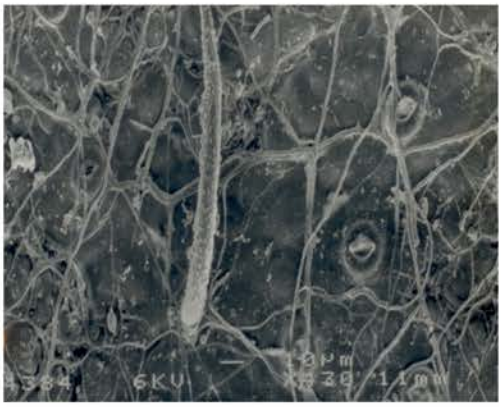

Fig. 4: Scanning electron micrograph of abaxial surface of *in vivo* leaf showing the structure of the trichome

leaves ranges from 1.5-2.0×0.5-1.0 cm whereas *in vivo* leaves were larger in size, ranging from 3.0-5.0×1.5×2.0 cm. The texture of *in vitro* leaves was membranous while *in vivo* leaves were coriaceous. Trichomes were observed on the abaxial surfaces of both types of leaves but *in vitro* leaves produced a smooth type of trichome (Figs. 6, 8). Palisade cells were uniseriate in *in vitro* species while they were biseriate in *in vivo* species (Figs. 9, 10). *In vitro* flowers were smaller in size compared with *in vivo* flowers and the former also produced protruding stigmas. Studies on micromorphological and anatomical characters of the leaf lamina of *in vivo* and *in vitro* species of *Murraya paniculata* also showed little differences between *in vitro* and *in vivo* leaves. Both leaves were dorsiventral and hypostomatic. Stomata on *in vitro* leaves were observed to be raised above the epidermis but in *in vivo* leaves the stomata were found to be leveled with the epidermis. The stomata were dispersed randomly over the whole abaxial surface and they appear rounded to elliptical in outline when viewed with the SEM (Figs. 5, 7). Cuticle was absent in *in vitro* leaves.

DISCUSSION

***In vitro* flowering:** Not all plant species can easily flower *in vitro*. The utilisation of plant hormones is important to trigger *in vitro* flowering, besides carbon source, day length and lighting. So far *in vitro* flowering has been reported in species like tobacco, bamboo, orchids, *Murraya paniculata* etc. In species such as *Lolium temulentum*, a long day plant, the shoots could only flower *in vitro* when they were exposed to long hours of light. When the shoot explants were exposed to short day treatment, the shoots turned to vegetative phase of growth (McDaniel *et al*., 1991). The interaction

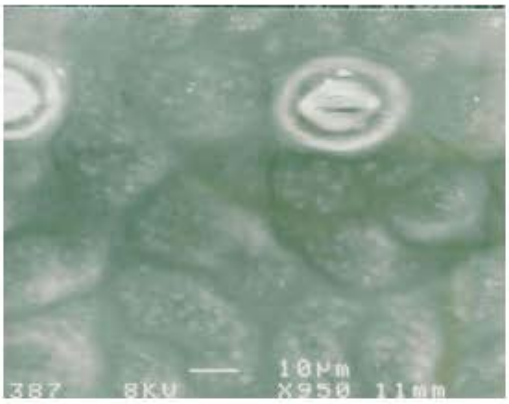

Fig. 5: Scanning electron micrograph of abaxial surface of *in vivo* leaf showing anomocytic stomata

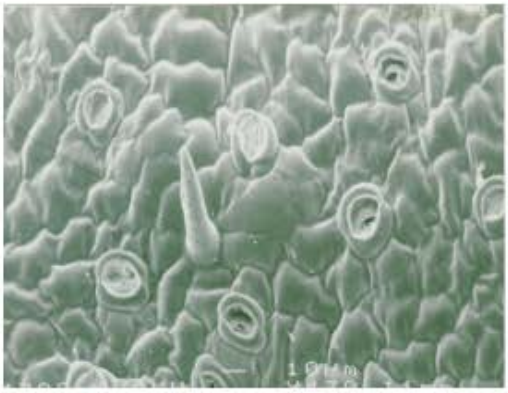

Fig. 6: Scanning electron micrograph of abaxial surface of *in vitro* leaf showing the distribution of the stomata and structure of trichome

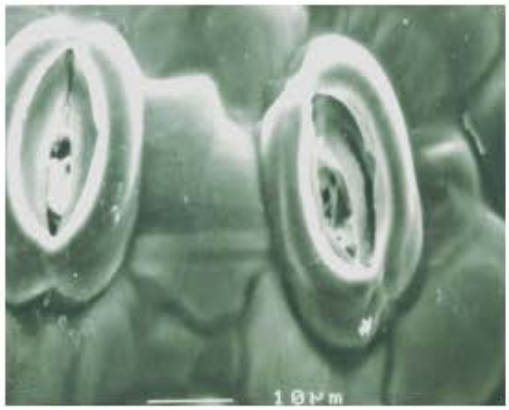

Fig. 7: Scanning electron micrograph of abaxial surface of *in vitro* leaf showing anomocytic stomata

of sucrose and plant hormones, minerals and phenolic in *in vitro* flowering were also discussed in *Torenia* sp. (Tanimoto, 1981; Tanimoto and Harada, 1981).

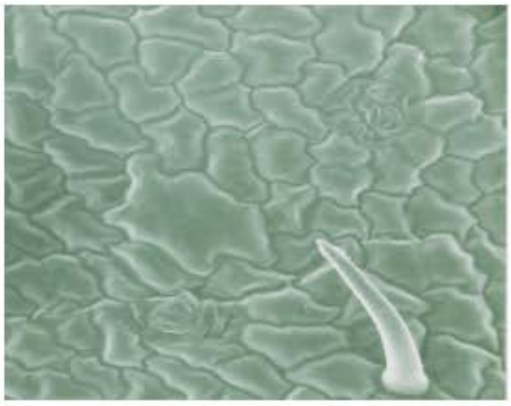

Fig. 8: Scanning electron micrograph of abaxial surface of *in vitro* leaf showing the structure of the trichome

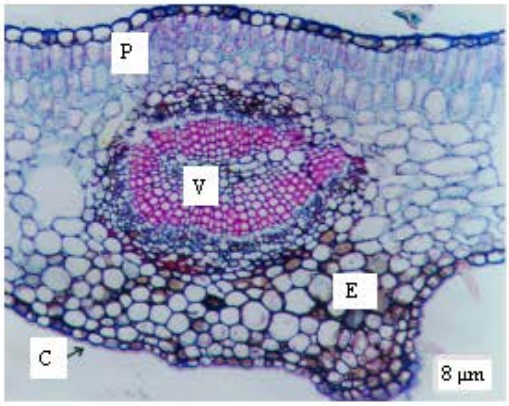

Fig. 9: Light micrograph showing transverse section of *in vivo* lamina with cuticle (C), epidermis (E), palisade cells (P) and vascular bundle (V)

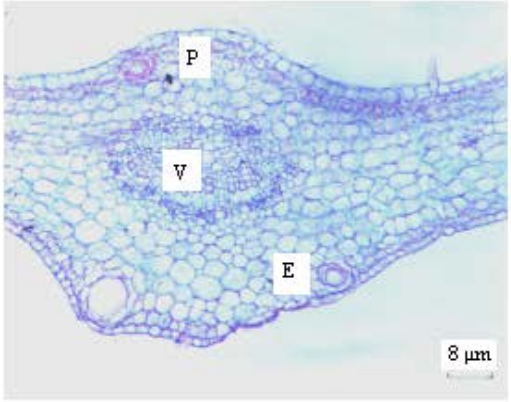

Fig. 10: Light micrograph showing transverse section of *in vitro* lamina with epidermis (E), palisade cells (P) and vascular bundle (V)

Hormones such as BA, NAA and GA_3 (Zeevart, 1967) were often used to induce flowering. For instance, GA_3

could induce *in vitro* flowering in *Petunia hybrida* grown in culture (Abdullah, 1998). However, GA_3 could also inhibit *in vitro* flowering in *Fuchsia hybrida* (Sachs *et al*., 1967). In this study, *M. paniculata* was found to produce flowers under tissue culture system when 0.5-2.0 mg L^{-1} NAA was supplemented to the MS medium. However, flowering could also occur on MS basal. This could be due to endogenous hormones already present in the explants. BA was not effective in promoting *in vitro* flowering of this species, although BA was capable of giving rise to plant regeneration. Exogenous hormones including BA and NAA supplied to the medium and endogenous hormones already present in the explants can stimulate *in vitro* flowering in species such as tobacco (Pierik, 1967), but in this study, BA was totally inhibitory for *in vitro* flowering of *Murraya paniculata*.

Longer period of light (16 h) was favourable compared to shorter day length (8 h light). By subjecting the cultures to only 8 h light, *in vitro* flowering was reduced to almost half. This is in agreement with observations made by Pierik (1967) working on *Lunaria annua*. This could be due to the photosynthetic light affecting the overall growth of the plant; when more light available, the rate of photosynthesis will be higher, thus because flower initiation should be considered as part of growth process, all factors normally affecting growth also affect flower bud initiation.

Morphological and anatomical studies

Cuticle: The presence and thickness of the cuticle is dictated by environmental factor. It is therefore more of ecological rather than of taxonomic importance. Wilkinson (1979) provides a list of environmental factors that affect cuticle thickness and notes that it is more frequently of value as an indicator of climate or habitat. The presence of cuticle in *in vivo* leaves and its absence in *in vitro* leaves has been expected since *in vivo* leaves is exposed to the sun.

Epidermis: The adaxial and abaxial epidermal cells of both *in vitro* and *in vivo* leaves vary from being isodiametric to irregular in shaped. The adaxial epidermal cells are larger than the abaxial epidermal cells. However, this is of no taxonomic significance since this characteristic is universal in the dicotyledons.

Mesophyll: The mesophyll in leaf transection showed a clear differentiation between a well-formed palisade and the adjacent spongy layer. Many studies on the ecological anatomy of tree species (Hanson, 1917; Ryder, 1954) indicate that the degree of mesophyll differentiation is highly dependent on the degree of exposure to the sun. According to them, leaves growing in sunshine have more palisade layers compared to leaves in shade and the palisade cells are long, large and close together. In leaves in heavy shade the palisade cells tend to become short, thin and loosely arranged. This may explain why *in vivo* leaves have biseriate palisade layers.

Vascular bundles: The vascular bundles are consistent in both *in vivo* and *in vitro* leaves as only arc-shaped vascular bundles were observed.

CONCLUSIONS

Longer period of light (16 h) was found to be more effective to trigger *in vitro* flowering of this species compared to short day. BA was inhibitory to *in vitro* flowering of this species.

The present study also suggest that the anatomical differences observed in both *in vivo* and *in vitro* leaves were due to the differences in the environment of the plants. Hence, when the *in vitro* plant is transferred to the soil, having the same environment as *in vivo* plant, no anatomical as well as morphological differences are to be expected.

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