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EVALUATION OF EFFICIENT METHOD FOR ACCLIMATIZATION OF AN IMPORTANT ORNAMENTAL RHIZOMATIC PLANT, *Calathea crotalifera*

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ABSTRACT

Calathea crotalifera is one of the important exotic ornamental plants belonging to Marantaceae family. This rhizomatic species can produce a very attractive inflorescence and have been widely used in the horticulture field including landscape and cut flower industry. It usually propagated through cutting rhizome and showed slow growth rate. This study was conducted to evaluate the effects of different propagation substrate (mix soil medium and organic medium) and shade levels (10%, 60% and 90%) on growth, morphogenesis, and physiological development of micropropagated *C. crotalifera* plantlets after two months acclimatized in the natural environment. Transplantation survival and growth rate of plantlets is higher (90%) in an organic medium compared to mix soil medium (75%). It is observed that plantlets grown under 60% shade level produced healthy seedlings with higher plants height, larger specific leaf area, leaf diameter, leaf length, leaf thickness and dry mass. The concentration of chlorophyll a and b, and carotenoid is also higher in this treatment. There are no significant effects on the new shoot production among the treatments. Micromorphological of the leaf surface were evaluated using light and scanning electron microscope. The stomatal frequency per unit leaf area in the adaxial leaf surface decreased significantly ($p \leq 0.05$) with the reduction of light intensity. However, this stomatal frequency was found higher on the abaxial leaf surface from 60% shade level treatment. Moreover, only leaves from 10% shade level treatments show the formation of trichomes on both abaxial and adaxial leaf surface, and exhibited a visual symptom of severe leaf tip burn. The findings revealed that different light intensities strongly affected the morphology and growth index of *C. crotalifera* during *ex vitro* development. The usage of organic medium under 60% shade level demonstrated the most efficient method for acclimatization of micropropagated *C. crotalifera* plantlets.

Key words: *Calathea crotalifera*, acclimatization, light intensity, growth, leaf morphology, leaf physiology

INTRODUCTION

Micropropagation has been extensively used for rapid multiplication and mass propagation production in ornamental plant species consist of tropical and sub-tropical flowers (Rout *et al.*, 2006). The *in vitro* multiplication technique is important to improve horticultural crop production in providing sufficient of good quality planting material of ornamental plants for commercial production including domestic gardens and landscaping. Acclimatization is an important step in micropropagation study to produce a well developed *ex vitro* plantlets that can successfully survive in the field environment (Pospóšilová *et al.*, 1999). However, the process of transplanting of *in vitro* plantlets and establishment of well grown *ex vitro* plant is the crucial stage in most ornamental species. The very special controlled conditions during *in vitro* phase result in the formation of plantlets with abnormal morphology, anatomy and physiology which could reduce their survival capability in the field (Kadleèek *et al.*, 2001; Hazarika, 2006; Bairu, 2011). Pospóšilová *et al.* (1999) reported that these abnormalities can be repaired through gradual changes of environmental conditions including light intensity, relative humidity and temperature to avoid desiccation losses and photoinhibition during the *ex vitro* transfer. In this phase, the plantlets have to correct the abnormalities with some changes especially in development of leaf morphology and anatomy, water relations and photosynthetic parameters (Pospóšilová *et al*., 1999; Faisal & Anis, 2009). The selection of a suitable substrate is also important for acclimatization as well as in the field propagation

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(Moreira *et al.*, 2006; Farzinebrahimi *et al.*, 2013). Furthermore, the using of ABA as antitranspirants to reduce transpiration and the elevating of CO_2 concentration to increase photosynthetic rate could enhanced the acclimatization process (Sutter & Hutzel, 1988; Pospóšilová *et al*., 1999).

Calathea sp. (Marantaceae) or also known as prayer plant has been cultivated widely for commercial purposes especially in landscaping and indoors due to its strongly patterned foliage and colours. *Calathea crotalifera* can produce a very attractive inflorescence with exotic features and have large foliage with a distinctive tropical looks. The unusual conspicuous inflorescence resembles rattlesnake's tail has a long shelf life that making this species suitable for cut flower industry (Bayogan *et al*., 2008) besides as screening plant. It can be propagated by division of old plants, but recovery and the appearance of new shoots are very slow in some species. Hence, several studies on micro-propagation of Marantaceae species have been done to increase its production scale for horticultural industry (Podwyszyñska, 1997; Yang & Yeh, 2008; Rozali *et al.*, 2014). Acclimatization studies on *Calathea* plantlets have been reported recently on *Calathea orbifolia* L. (Yang & Yeh, 2008) and *Calathea louisae* G. 'Maui Queen' (Piqueras *et al.*, 1998). These both species have been used widely as screening and indoor plants due to its small size with attractive foliar colours and variegation patterns.

Piqueras *et al*. (1998) studied the regulation of carbohydrate metabolism and compartmentation during the acclimatization of micropropagated *Calathea* plantlets. The study revealed the changes of plantlets from heterotrophic to autotrophic metabolism, and shows the accumulation of sucrose and starch in leaves due to increasing of light intensity during the acclimatization. Yang & Yeh (2008) reported that the different of *in vitro* condition i.e. semi-solid medium and temporary immersion system could affect the *ex vitro* photosynthetic behaviours, and growth of tissue cultured *Calathea* plantlets. However, the effective transfer medium and shade levels for acclimatization process were not reported yet. Hence, the aim of the present study was to establish an efficient method for acclimatization of *C. crotalifera* and to evaluate the morphological and physiological behaviour of such plantlets on different propagation substrate and under different shade levels in the acclimatization process.

MATERIALS AND METHODS

Plant material

Calathea crotalifera of yellow bract cultivars was used in this study. The axillary shoot buds from the soilless medium were used as explants (Rozali *et al.*, 2014). The explants were surface sterilized prior cultured on the MS basal medium containing 30 g L^{-1} sucrose, 6.0 g L^{-1} plant agar, 1.0 g L^{-1} glutamine, 1.0 g L^{-1} activated charcoal and, supplemented with 3.5 mg L^{-1} BAP and 1.0 mg L^{-1} NAA with pH adjusted at 5.80 ± 0.01. The induced multiple shoots were subcultured in eight to twelve weeks interval. The four months plantlets were rooted in a solid culture medium containing full strength MS salt concentration, 30 g L^{-1} sucrose, 6.0 g L^{-1} agar and augmented with 1.0 mg L^{-1} NAA at pH 5.80 ± 0.01. All cultures were maintained in a growth room with 16/8 h photoperiod under cool-white fluorescent at 25 ± 2ºC.

Plant transfer and acclimatization

Six months of high quality rooted plantlets with about 7.0 cm height were selected from the cultures. Plantlets were removed from the culture vessels and washed gently under running tap water to remove the adhering medium. The plantlets were transferred to the plastic rectangular pot containing three different propagation substrates (Table 1). The planting pots were covered with transparent polythene bags to maintain a relatively high humidity and prevent desiccation. The plants were initially maintained under greenhouse conditions at 25 ± 2ºC with 50% direct sunlight for one month. Each pot was watered with tap water once a day and the transparent plastic was gradually removed before transfer to the natural environment in a shade-house. The survival rate of plantlets were recorded in this first phase. The effect of light intensity was evaluated during the acclimatization period in the natural environment. Shade levels were demonstrated with the use of shade cloth with three different levels (10%, 60% and 90%) of sunlight.

Table 1. Medium substrate for acclimatization of *Calathea crotalifera*

Medium substrate	Ratio	Code
Black soil + red soil + coconut husk + vermiculite	3 : 3 : 1 : 1	M1
Black soil + red soil + coconut husk + perlite	3 : 3 : 1 : 1	M2
Peat moss + black soil	3:1	M3

Table 2. Effect of different light intensity on leaf biomass

Shade levels	Number of leaves / plant	Leaf area mass (mm^2)	Leaf dry (mg)	SLA ($m^2\ kg^{-1}$)	LDMC ($mg\ g^{-1}$)	LT (μm)
10%	4.2 ± 0.2a	7.96 ± 1.18a	19.56 ± 2.64a	40.67 ± 2.16ab	260.91 ± 19.45ab	96.20 ± 3.97ab
60%	7.0 ± 0.6b	18.29 ± 2.91b	41.00 ± 8.65b	46.22 ± 2.37b	224.67 ± 3.97a	97.22 ± 4.08b
90%	3.6 ± 0.2a	7.34 ± 0.94a	19.46 ± 3.12a	38.48 ± 1.95a	313.62 ± 25.24b	84.56 ± 3.41a

Values are mean with SE. Means in a column followed by the same letter (a, b) are not significantly different at $p \leq 0.05$ according to DMRT.

Measurement of leaf biomass

The effects of each treatment were evaluated after 60 days of growth in field condition. Biomass analysis was done on ten plantlets that have been randomly selected from the pots. The analysis is based on the number of leaf per plant, plant height, number of new shoots, leaf length, leaf dry biomass, and leaf area as shown in Table 2. Fully expanded leaves were initially weighed to get the fresh weight before drying it in the oven at 60ºC for three days. Leaf area was measured manually using grid paper. Specific leaf area (SLA) was calculated as the ratio of leaf area to leaf dry mass while leaf dry matter content (LDMC) was measured as a ratio of leaf dry biomass to saturated fresh mass. The leaf thickness (LT) were deduced from these two traits, which are much easier to measure as $(SLA \times LDMC)^{-1}$ (Vile *et al*., 2005).

Evaluation of leaf morphology

Fully expanded leaf from five selected plantlets were used for stomata observation. The upper and lower epidermis of the leaves were swabbed with wet cotton to remove any adhered debris or soil. The transparent nail polish was then brushed onto both sides of the leaves. Temporary slide was made by pressing the transparent adhesive tape onto the brushed part of the leaf. Visualization of the slides were conducted using a light microscope (Zeiss Axioscope, Germany) connected to a Sony video camera, supported by VIDAS (Kontron Electronic, Germany). Stomatal frequency was measured as the number of stomata per leaf epidermis field of vision (per mm^2). Micromorphological of stomata and epidermis structure were examined as previously described (Rozali *et al*., 2014), using scanning electron microscope, SEM (Zeiss EVO MA10). The leaf disc with 0.25 cm2 area was fixed in 2% aqueous osmium tetroxide, OsO_4 for overnight at 4ºC. The treated leaf disc were rinsed with distilled water prior to dehydration through ethanol series (10, 20, 30, 40, 50, 60, 70, 80, 95 and 100%) followed by infiltration through ethanol-acetone mixture at 15 minutes each. This dehydration treatment ended with pure acetone for 1 hour before dried using a Critical Point CO_2 Dryer. Dried specimens were then viewed under SEM after being sputter-coated with gold in a sputter coater. The presence of wax on the epidermis layer and morphological of stomata structure were observed and recorded.

Extraction of photosynthetic pigment

The content of photosynthetic pigment was determined in acetone extracts with the ratio of 1.0 g sample to 10 mL 80% acetone. The leaf discs were mashed in a chilled mortar with the addition of calcium carbonate powder. The leaf extracts were centrifuged at 5000 g for five minutes to get the supernatant prior to be analysed spectrophotomatically using UV-VIS Spectrophotometer Model Libra SII at wavelength of 648 nm (Ch b), 666 nm (Ch a) and 480 nm (carotenoid). The concentration of each pigment was determined using Wellburn Equation (Wellburn, 1994).

Statistical analysis

Statistical analyses were performed using software of SPSS Version 17.0. The data were analyzed with a one-way analysis of variance (ANOVA). Differences between means were tested with Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

RESULTS AND DISCUSSION

The survival percentage of plantlets was affected by different medium substrate during the first phase of acclimatization under maintained temperature, low humidity and light intensity. The result revealed that organic medium of M3 has a higher yield (90%) compared to mix soil medium of M1 (75%) and M2 (70%) as shown in Fig. 1. The combination of peat and soil was reported as an effective medium for acclimatization of several micropropagated plantlets (Van Huylenbroeck *et al.*, 1998; Yaacob *et al*., 2013; Sharma *et al.*, 2014). Peat has traditionally been used in most potted, ornamental plants (Tullio *et al.*, 2012) since it possesses good physical and chemical characteristics, stable structure, and favourable application (Kang *et al.*, 2005). Addition of

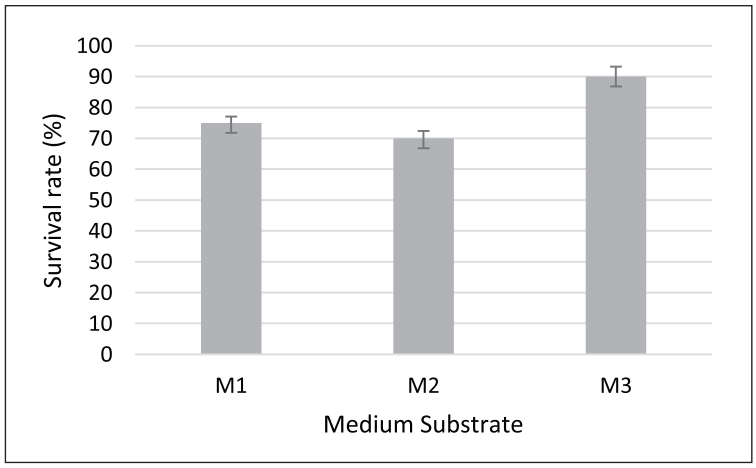

Fig. 1. Effect of different substrate medium on survival rate of acclimatized *Calathea crotalifera* plantlets.

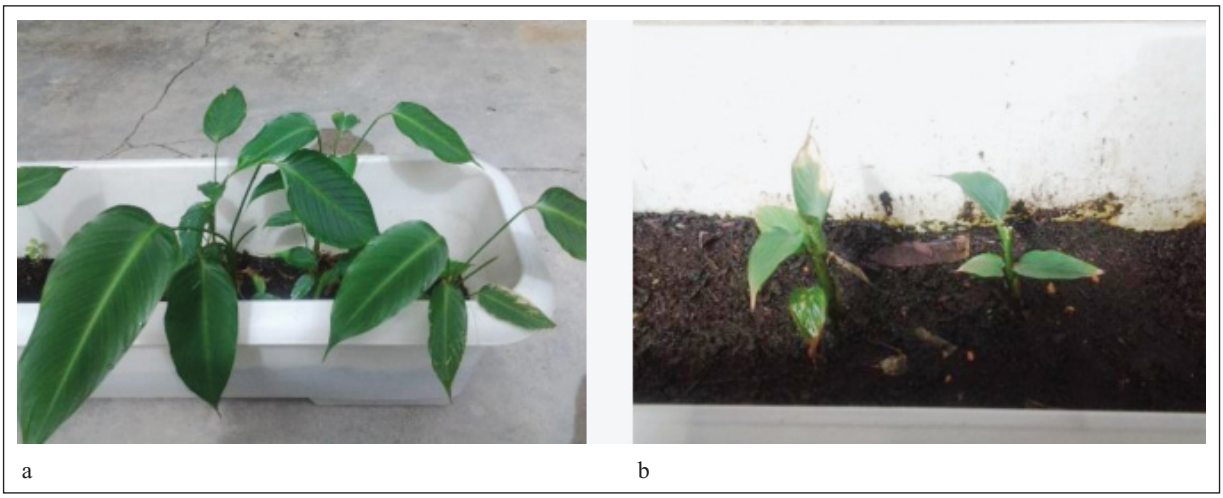

Fig. 2. Morphological development of leaf in acclimatized *Calathea crotalifera* under different light intensity. (a) Dark green leaf with large surface area under 60% shade level; (b) Small leaf with necrotic effect on leaf tip under 10% shade level.

vermiculite and perlite in the mix soil medium did not enhance the medium quality and survival rate in the presence study. Hercílio *et al.* (2005) indicated that the use of vermiculite should not be recommended for the acclimatization of Heliconiaceae. However, the optimum ratio of soil and vermiculite or perlite could enhance the growth rate for several species (Kadleèek *et al*., 2001). After one month, the survived seedlings in these three different mediums show low emission of new roots and the existing roots originated during in vitro rooting were not functional with no apex development. Hence, medium substrate M3 that consist of peat moss and black soil was used for acclimatization in the field environment to evaluate the effect of the various shade levels.

The leaf blade of plantlets was wilting in the first three to four days. Several leaves were severely affected and showing necrosis at the tip especially for plants under 10% shade level (Fig. 2). However, the wilting percentage was decreased after seven days onwards. The abnormalities caused by *in vitro* conditions could be the reason for these problems (Pospóšilová *et al*., 1999; Estrada-Luna *et al.*, 2001; Hercílio *et al*., 2005). After a few days, the leaf expanded and the stems started to harden. The new leaf and roots emerged after one month acclimatized in the field. The observed gradual development indicated that the plantlets have adapted to the field. The plantlets have transformed from heterotrophic to autotrophic metabolism (Piqueras *et al*., 1998) due to the stabilization and improvement water status and gas exchange (Estrada-Luna *et al*., 2001). The morphology and physiology of the leaf were significantly affected by the different shade level treatments. The leaf biomass i.e. shoot height, leaf number, leaf area and leaf dry mass of *C. crotalifera* plantlets produced under 60% shade level was two

times higher as compared to 10%, followed by 90% shade levels respectively (Fig. 2 and Table 2). These conditions contributed to the increasing of SLA and leaf thickness as compared to *in vitro* leaf (Yang & Yeh, 2008). Previous reports supported that the intermediate shade level at between 50% and 60% was suitable for acclimatization and propagation of several plantlets or plant seedlings (Hercílio *et al*., 2005; Farzinebrahimi *et al*., 2013).

Total chlorophyll (a+b) and carotenoid concentration was also higher in leaves under 60% shade level (30.28 µg/mL; 3.26 µg/mL) followed by 90% (23.03 µg/mL; 2.82 µg/mL) and 10% (22.65 µg/mL; 2.76 µg/mL) respectively (Table 3). The plantlets under low shade levels would produce dark green leaf as shown in Fig. 2. The results observed implied that the higher light intensity could reduce the concentration of photosynthetic pigment in the leaf as reported by Faisal & Anis (2009). The increasing of light intensity above the optimum level during acclimatization phase could decrease the photosynthetic activity of plantlets (Piqueras *et al*., 1998). The increasing of photosynthetic pigments in plantlets was associated with gradual changes of photosynthetic performance that could indicated the adaptability of plantlets in the new condition under intermediate light intensity with high humidity (Piqueras *et al*., 1998; Kadleèek *et al*., 2001; Jeon *et al*., 2006). Besides that, photosynthetic pigments were also found higher in acclimatized plantlets with better supplied of CO_2 and ABA treatments (Pospóšilová *et al*., 1999).

The stomatal morphology in each shade levels showed some variances. The length of stomatal (adaxial and abaxial) in 60% shade level was longer whereas the stomatal width in 10% shade levels has the larger size as compared to other leaves (Table 4). Stomatal size in 90% shade level was poor developed with smallest size and lowest frequency. The leaf is thicker in 60% (97.22 µm) and 10% (96.20 µm) shade level indicated that the photosynthetic tissues consist of palisade parenchyma, spongy parenchyma and chlorenchyma were well developed. Similar results have been reported regarding the leaf thickness in *C. orbifolia* (Yang & Yeh, 2008), *Gardenia jasminoides* (Serret & Trillas, 2000) and young tomato (Fan *et al*., 2013). Adaxial surface of leaf in 10% shade level contains higher stomatal frequency (12.43 stomata/mm^2) while the higher frequency of stomata on abaxial surface was found in 60% shade level (67.00 stomata/mm^2). The stomatal frequency was varied due to the different size of leaf area as a response to the environmental changes (Marín *et al.*, 1988; Drew *et al.*, 1992). *C. crotalifera* has amphistomatic leaves where the stomatal density on the abaxial surface is higher than on adaxial surface, similar to other angiosperm species (Tichá *et al.*, 1999; Khan *et al*., 2014). As shown in Fig. 3d, the shape of *C. crotalifera* stomata is brachyparacytic where two irregular and wavy epidermis cells were flanking the sides of the guard cells, but not completely enclosing them (Khan *et al*., 2014).

Epicuticular wax of leaves were formed on the *ex vitro* leaf and absent on the *in vitro* leaf (Fig. 3a and 3b). The composition of epicuticular wax is the most important changes during the acclimatization process due to the changes of environment that could reduce cuticular transpiration rates of leaf in plantlets and leading to stabilization of water status (Drew *et al*., 1992; Pospóšilová *et al*., 1999; Yang & Yeh, 2008). Formation of trichomes was observed to be presence only on the leaf surface in 10% shade level. The environmental changes especially increasing of light intensity associated with high temperature and low humidity induced the formation of trichomes during acclimatization (Donnelly & Vidaver, 1984; Perez-Estrada *et al*., 2000; Bandyopadhyay *et al.*, 2004). This modified epidermal structure is essential to alter the heat loss and to reduce water transpiration (Brutti *et al.*, 2002; Bandyopadhyay *et al*., 2004). Microscopic studies revealed that the type of trichomes in *C. crotalifera* is stellate-branches (Bandyopadhyay *et al*., 2004).

CONCLUSIONS

The study revealed that the substrate medium and light intensity significantly influenced the morphology and physiology development of tissue cultured plantlets of *C. crotalifera* during the

Table 3. Effect of different light intensity on photosynthetic pigment concentration of *Calathea crotalifera* leaf

Shade levels	Ch a (µg/mL)	Ch b (µg/mL)	Total chloropyll (Ch a + Ch b) (µg/mL)	Carotenoid (µg/mL)
10%	16.87 ± 0.20^a	5.78 ± 0.09^a	22.65^a	2.76 ± 0.06^a
60%	21.19 ± 0.38^b	9.09 ± 0.26^b	30.28^b	3.26 ± 0.05^b
90%	17.04 ± 0.31^a	5.99 ± 0.14^a	23.03^a	2.82 ± 0.07^a

Values are mean with SE. Means in a column followed by the same letter (a, b) are not significantly different at $p \leq 0.05$ according to DMRT.

Table 4. Effect of different light intensity on stomata morphology on lower (abaxial) and upper (adaxial) epidermis of acclimatized *Calathea crotalifera* leaf

Shade levels	Length (μm)		Width (μm)		Stomata frequency (stomata·mm^2)	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
10%	31.54 ± 1.21^b	31.36 ± 0.65^b	17.58 ± 0.54^c	13.68 ± 0.69^b	12.43 ± 0.37^b	45.29 ± 1.25^a
60%	37.43 ± 0.35^c	35.15 ± 0.57^c	13.97 ± 1.02^b	10.64 ± 0.52^a	7.71 ± 0.36^a	67.00 ± 2.64^c
90%	27.65 ± 0.71^a	26.41 ± 0.64^a	10.55 ± 0.33^a	10.31 ± 0.40^a	6.43 ± 0.78^a	53.29 ± 1.25^b

Values are mean with SE. Means in a column followed by the same letter (a, b and c) are not significantly different at $p \leq 0.05$ according to DMRT.

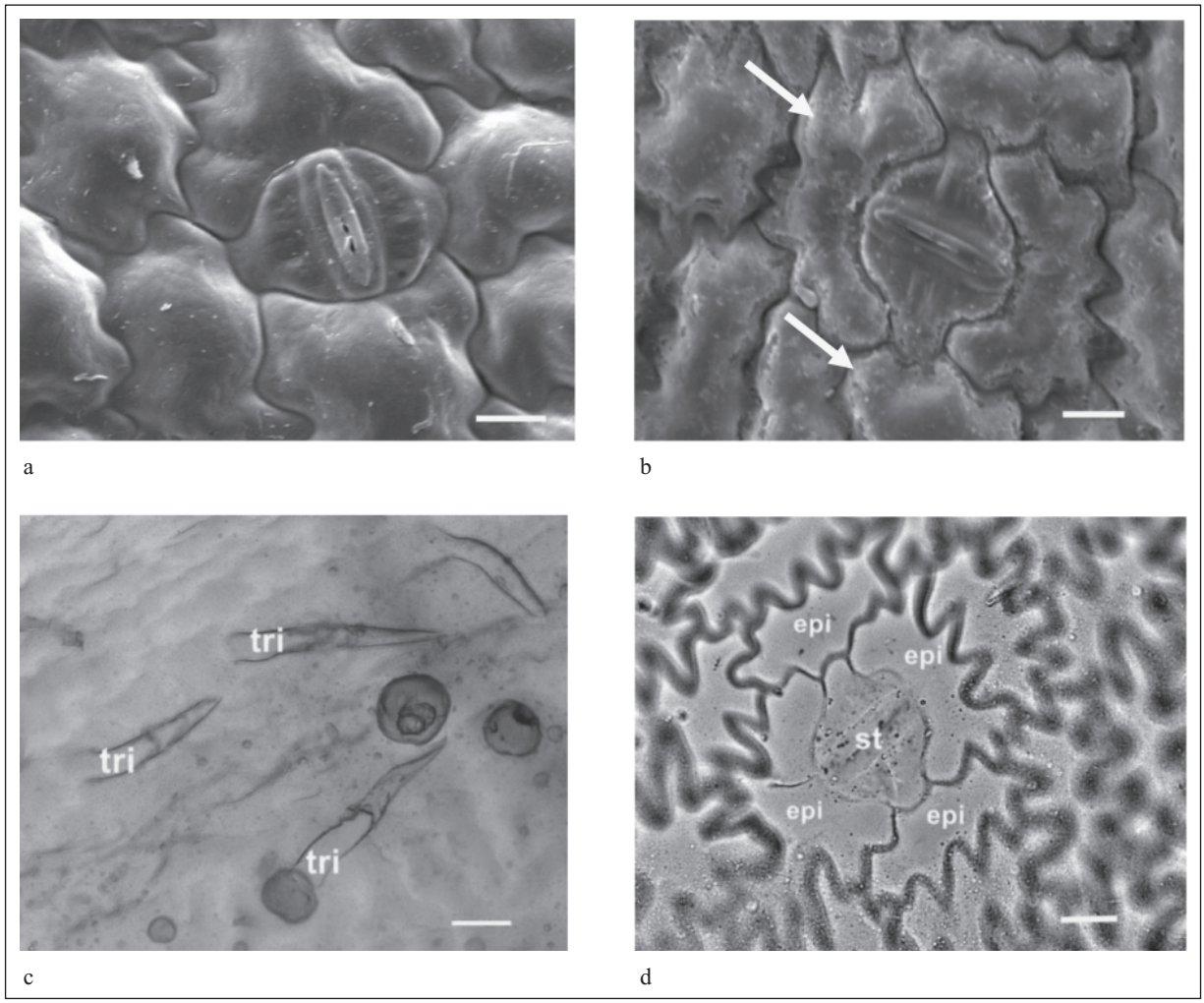

Fig. 3. Micromorphological development of leaf surface in *Calathea crotalifera* plantlet. (a) Abaxial surface of leaf in *in vitro* plantlets with no epicuticular wax (arrow) present viewed under SEM (bar 10 μm); (b) Abaxial surface of leaf in *ex vitro* acclimatized plantlets with epicuticular wax present viewed under SEM (bar 10 μm); (d) Formation of trichomes (tri) on adaxial surface of leaf viewed under light microscope (bar 20 μm); (e) Brachyparacytic structure of stomata (st) with irregular and wavy epidermis (epi) viewed under light microscope (bar 20 μm).

acclimatization process. Organic medium containing peat moss and black soil in the ratio of 3:1 and 50% shade level produced better development of acclimatized plantlets. The changes of the leaf morphology and photosynthetic pigments demonstrated the adaptability of plantlets to survive in the field environment. Therefore, shading at 50% using shade cloth together with the organic medium

of peat moss and black soil could be applied in industrial cultivation of *in vitro* ornamental rhizomatous plant species.

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