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Adaptive features of *in vitro*-derived plantlets of MD2 pineapple during acclimatization process

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ABSTRACT

Tissue culture-derived planting materials require optimal acclimatization period to ensure high survival rates in the nursery and to save labor and production cost. However, there is little information on how the morphology and physiology of *in vitro*-derived plantlets can be affected by culture conditions and acclimatization. We compared different types of media for shoot multiplication using thirty pineapple suckers as explants. Morphological and physiological changes of thirty field-grown plants and *in vitro*-derived plantlets during *ex vitro* acclimatization at 0, 14, 28, 42, 56 and 84 days were evaluated. Experiments were conducted in triplicate. Observations were based on the leaf morphology (number, width, ratio value of dry weight to fresh weight and succulence index of the leaves) and physiological characteristics (photosynthetic rate, stomatal conductance, transpiration rate, stomata opening, trichomes and histological examination). We found that the *in vitro* plantlets undergoing acclimatization showed C3 characteristics and adapted to CAM characteristics after 42 days of acclimatization. The *in vitro* plantlets were compared with field-grown plants and observations were made based on the morphological and physiological characteristics of the leaves. In conclusion, our findings indicated that *in vitro*-derived pineapple plantlets started to develop and achieve similar characteristics with the field-grown CAM plants after 42 days of acclimatization, suggesting that this could be the suitable period prior to transfer into the fields.

Key words: *Ananas comosus*, plant tissue culture, acclimatization, CAM, suckers.

INTRODUCTION

Pineapple has been listed as a major tropical fruit since 2010 (Ahmadian *et al*., 1). Traditionally, MD2 pineapple is propagated using field-collected suckers. This method is slow as only about 6 suckers per plant are produced per year. Moreover, it can easily transmit numerous diseases, such as fusariosis, bud rot in suckers and black rot, from old to new pineapple plantations. Although chemical fungicides can solve this problem, they provide negative impact to the environment and may cause yield losses up to 100% (Sales *et al*., 15). Plant tissue culture is an important technique used in the multiplication and conservation of threatened species. This technique can mass produce disease-free and uniform plantlets in a short period of time (Mengesha *et al*., 12).

The ability of *in vitro*-derived plantlets to successfully undergo acclimatization is a main concern since it determines the high survival and vigorous growth rates of the plants when exposed to natural environment. The plantlets are usually maintained in a shaded nursery before exposing the plantlets to the natural environment to enhance the survival rate. Sudden exposure to drastic environmental changes is detrimental to *in vitro*-derived plantlets. This procedure, however, is extremely expensive since extra labour cost and facilities are required for the process (Aragon *et al*., 2). Previous report has shown low survival rate of *in vitro*-derived MD2 pineapple plantlets and a lengthy period in the acclimatization process (Mengesha *et al*., 12).

To improve the low survival rate of *in vitro*-derived plantlets during the acclimatization process, it is very important to study the morphology and physiology of *in vitro*-derived MD2 pineapple plantlets in comparison to the field grown plants. The characteristics of the MD2 pineapple plants, such as carbon dioxide fixation, could also be determined from the physiological studies. The generated data could be used to determine the optimal acclimatization period before field transfer.

MATERIALS AND METHODS

Pineapple plantlets var. MD2 were micropropagated according to Hamid *et al*. (5) using suckers as explants. For phase I (*in vitro*

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initiation), the explants were cultured on medium containing Murashige and Skoog (MS) basal media supplemented with 3 mg/L 6-benzylaminopurine (BAP), 1 mg/L naphthaleneacetic acid (NAA), 30 g/L sucrose and 2 g/L gelrite. The culture medium for phase II (multiplication) contained MS medium supplemented with 2 mg/L BAP, 1 mg/L NAA and 30 g/L sucrose. For phase III (rooting), the culture medium used to induce roots was MS salts supplemented with 2 g/L gelrite and 6 mg/L activated charcoal. The media were adjusted to pH 5.7 ± 0.2 and autoclaved at 121°C for 20 min. All cultures were incubated at 25 ± 2 °C under a photoperiod of 16 hours daylight with a light intensity of 30 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. The experiments were conducted with a total of ten explants per treatment and were repeated thrice. The number of shoots per explant was recorded after one month of culture. About 3-week old disease-free field-grown pineapple plants purchased from KOSAS company, Selangor, Malaysia, were maintained in a greenhouse until use.

Uniform microprapagated plantlets of 5.0 cm height with 5-6 true leaves were selected. The rooted plantlets were then rinsed with distilled water and dipped in 1 % (v/v) Imas-Thiram 80 (Imaspro Resources Sdn. Bhd., Malaysia), rinsed with water again and transferred to peat soil in a 7 × 13 cm polyethylene bag. Plantlets were grown in a greenhouse at the University of Malaya, Malaysia (60 % relative humidity, 24 ± 2ºC with photosynthetic photon flux of 400-500 µmol m^{-2} s^{-1}), for 3 months. Mixed fertilizer containing 15 N: 15 P: 15 K (Tesco, Malaysia) was applied for each plantlet at about 2 cm in distance. Plantlets were irrigated for 5 min with an approximately 250 mL per session at 0900 morning each day using an automatic dripper irrigation system that embedded in the soil.

Photosynthetic rate (P_N), stromata conductance (g_s), intercellular CO_2 content (C_i), and transpiration rate (E) of thirty *in vitro*-derived pineapple plantlets were recorded using a portable photosynthesis system (LI6400, Li-COR Biosciences, Nebraska, USA) according to Villalobo *et al*. (19) after 0, 14, 28, 42, 56 and 84 days of acclimatization. Light was fixed at 400 µmol/mol. Field-grown plants at the fruit-setting stage (308 day-old) were used as control.

Histological examination was carried out on the leaves harvested at 0, 14 and 42 days of acclimatization according to Jalil *et al*. (7). Field-grown plants (308 days) were used as control. Slides were mounted with mounting medium for preservation and allowed to dry thoroughly before examining the cross section of the leaves under an inverted microscope (Model IX73PS2F, Olympus, Japan) and photographed using an image capturing system (DP-71, Olympus, USA).

Chlorophyll was extracted from five randomly selected young and mature leaves after 0, 14, 28, 42, 56 and 84 days of acclimatization. Chlorophyll content was measured at 645 and 663 nm according to Aragon *et al*. (2), and calculated using the equations described by Porra *et al*. (13) in mg Chl cm^{-2} leaf area. This method was suggested by Richardson and Berlyn (14). The values of chlorophyll content, fresh weight (FW) and dry weight (DW) were used to find out the succulence index with the formula: SI = (F-D)/C, where F, fresh weight; D, dry weight; C, total content of chlorophyll (Aragon *et al*., 2).

Samples were excised into approximately 1 cm each side in high vacuum to characterize the morphological structure of both abaxial and adaxial sites of the leaves. The diameter of the stomata was observed under a Field Emission Scanning Electron Microscope (model FEG Quanta 450, EDX-OXFORD, UK). Plant growth parameters, namely number of leaves (N), width of leaf (W), and ratio of fresh weight over dry weight (DW/FW) were recorded after 0, 14, 28, 42, 56 and 84 days of acclimatization. The dry weight was recorded after drying the leaves in an oven at 70 °C until constant weight was attained. All observations were conducted with a total of three leaf samples for each physiological and morphological experiments. Each sample repeated for three times. For morphological traits, new leaves were counted after growing at 45º slant. Data were statistically analyzed by one-way ANOVA followed by Tukey's multiple range tests at $p< 0.05$ level using SPSS version 16.0.1 (SPSS Inc., Chicago).

RESULTS AND DISCUSSION

The generated plantlets were acclimatized in the greenhouse before being transferred to the field to ensure high survival rate. All *in vitro* plantlets were survived, grew uniformly, and phenotypically normal as compared to field-grown plants throughout the acclimatization period until 84 days (Fig. 1). In other study, it has been shown that the acclimatization of pineapple var. Smooth Cayenne was not 100% successful and required a lengthy period (Mengesha *et al*., 12). High mortality is usually observed upon the transfer of *in vitro*-derived plantlets to *ex vitro* conditions as the cultured plants have non-functional stomata, poor photosynthetic efficiency, weak root systems and poorly developed cuticles (Tan *et al*.,18). Hence, investigation on the plant physiology and morphology as adaptive features of the *in vitro* plantlets during the acclimatization process may help to address the low survival rate at acclimatization.

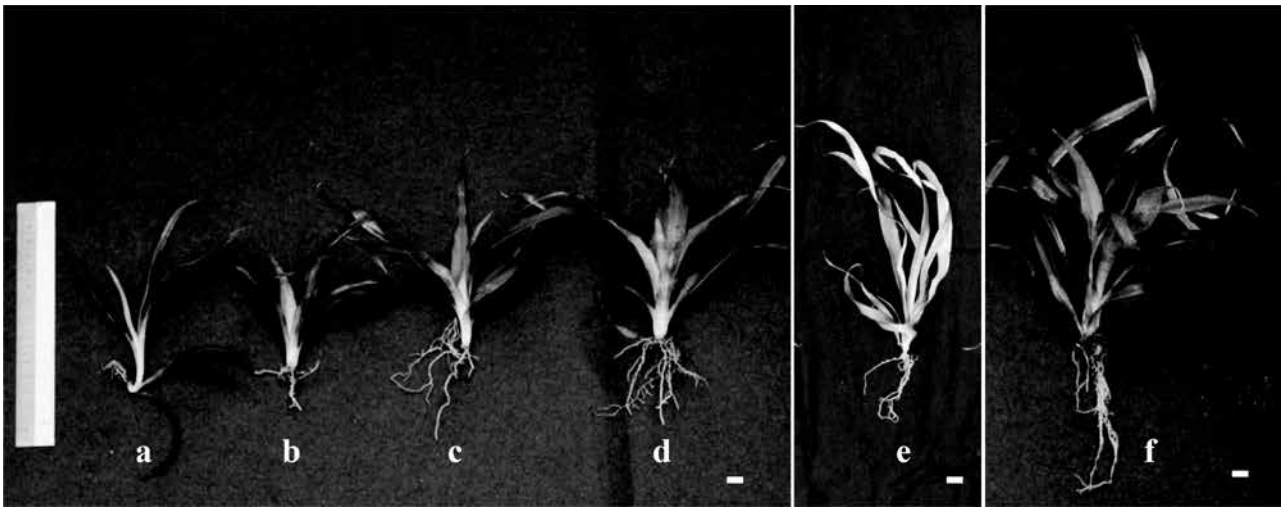

Fig. 1. Stages of acclimatization for *in vitro*-derived pineapple plant (a) 0, (b) 14, (c) 28, (d) 42, (e) 56 and (f) 84 days of acclimatization (Bar = 1 cm)

Leaf gas exchange was recorded throughout the acclimatization period of *in vitro*-derived and field-grown plants. In this study, a correlation between photosynthetic rates, intercellular CO_2, transpiration rates and stomatal conductance was established (Fig. 2).The results showed that the P_N for plants at 0 day in the day (Fig. 2a) and night times (Fig. 2b) were negative, reflecting a negative balance between photosynthesis and respiration (photorespiration). The negative value of photosynthesis rate synchronizes with stomatal conductance and intercellular CO_2 due to the stomatal closure, a characteristic of Crassulacean acid metabolism (CAM) plant. For CAM plant, the stomata in the leaves during day stay shut to decrease evapotraspiration, but the stomata will be open at night to accumulate and allocate carbon dioxide (CO_2) to be absorbed into the mesophyll cells (Males and Griffiths, 10). This is in contrast with C3 plant characteristic where the plant opens its stomata at daytime and close at night (Stutz and Hanson, 17). Moreover, this observation might also be due to sufficient amount of nutrient supply from the previous supplemented media and incomplete autotrophy (Lawson, 9). Hence, the stomatal regulation through opening was not necessary for the plant to fix the carbon dioxide from the atmosphere for photosynthesis. The photosynthetic rate at day time for *in vitro*-derived and field-grown plants started to increase after 14 days of acclimatization and began to decrease after 42 days during the day (Fig. 2a).

During the night, photosynthetic rate started to increase after 14 days and were similar to the field-grown plants at 56 and 84 days (Fig. 2b), suggesting that the plants might start to adapt to the new environment during the acclimatization period. Villalobo *et al.* (19) explained that this situation happened maybe because the plants undergo CAM. The rate for C_i was similar during the day but decreased at night (Fig. 2c & 2d) throughout the acclimatization period. During the day, a cyclic pattern was observed for the stomatal conductance where the reading was increased for 2 weeks and decreased for the following 6 weeks. At Day 84, the stomatal conductance returned to the same reading as the first cycle and similar to the field-grown plants (Figures 2e & 2f). The changes in g_s were consistent to the transpiration rate readings (Fig. 2g & 2h). During the night, the rates for all leaf gas exchange increased. There was no significant difference between *in vitro*-derived plantlets at 56 days and field-grown plants for photosynthetic and C_i rates. Similar, cyclic pattern was observed for E and g_s at the respective time points tested in this work.

The transmission of CO_2, photosynthesis and transpiration rate was controlled by stomata opening through the guard cells. The opening of stomata during the day time at 14 and 28 days of acclimatization period might be due to the light-independent process in the plant where stomata open during the day for photosynthesis and close during the night. In the light dependent process, CO_2 is fixed by ribulose-1, 5-bisphopsphate carboxylase-oxygenase (RubisCO) during day. Escriba *et al*. (4) suggested that the pattern of CO_2 process described could indicate that the *in vitro*-derived plantlets at 14 and 28 days of acclimatization period under C3 photosynthesis pathway. However, after 42 days of acclimatization, the stomatal conductance was negative, indicating that the stomata were closed during day and used the

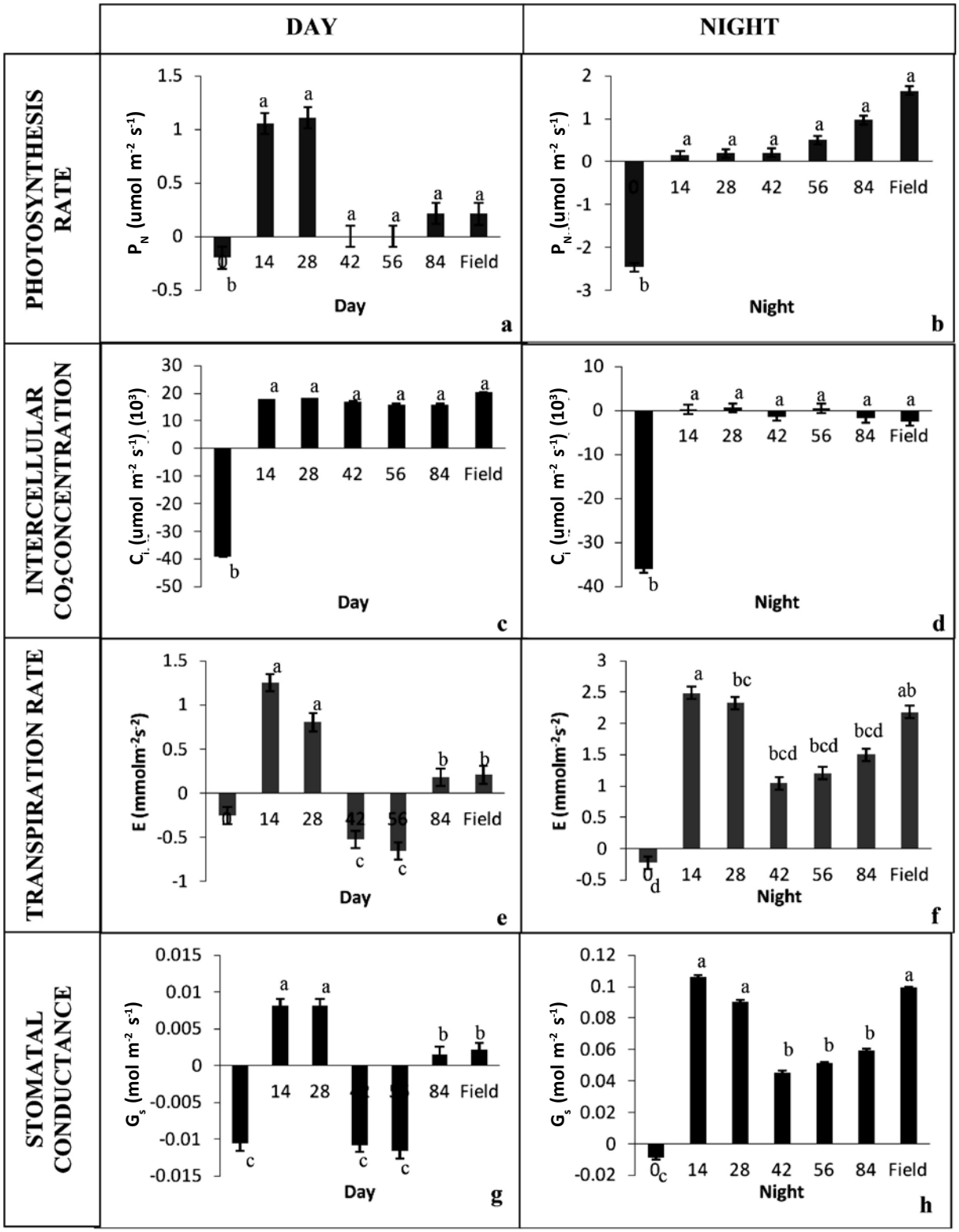

Fig. 2. Specific properties of leaves from micropropagated pineapple plantlets (*Ananas comosus* 'MD2') at different acclimatization periods, compared with field-grown plants (308 days). (a) & (b) photosynthesis rate (P_N); (b) & (d) intercellular CO_2 (C_i); (e) & (f) transpiration rate (E); and (g) & (h) stomatal conductance (g_s). Data represent mean ± SE (n=10), and different letters show significant differences ($p< 0.05$) according to the Tukey Multiple Range Test.

remaining intercellular CO_2 that stored as RubisCO. The process of storing the remaining intercellular CO_2 is called as light independent process, where the CO_2 initiated by phosphoenolpyruvate carboxylase. Then, RubisCO is fixed and used during day in CAM plant (Armero *et al*., 3). This supported the negative readings of the intercellular CO_2 and transpiration rate during day after 42 days of acclimatization. However, photosynthesis was still occurred despite no CO_2 uptake (Fig. 2a). Similar patterns were observed in the field-grown plants where pineapple plants are recognized as CAM plant (Aragon *et al*., 2).

The trichomes and stomata on the upper and lower sides of the leaves of *in vitro*-derived and field-grown plants (control) on 0, 14, and 42 days of acclimatization stages were examined under scanning electron microscope (Fig. 3a-h). Stomata were seen on the abaxial side of the leaves at every stage, whereas the trichomes were negligible on day 0 (Fig. 3a) but gradually developed from day 14 to day 42 (Fig. 3b-d). Stomata covered by large multicellular trichomes on day 42 (Fig. 3c) which were comparable to field-grown plants (Fig. 3d). On the other hand, for the adaxial side, stomata were seen on day 0 (Fig. 3e) and 14 (Fig. 3f). However, large multicellular trichomes were observed on day 42, making the stomata non-visible (Fig. 3g). The edge of trichome protruded upwards due to its abundance in the field-grown plants (Fig. 3h).

During day time, the opening of the stomata at 14 days of acclimatization on the abaxial parts explains the positive readings for photosynthetic rate and transpiration rate (Fig. 3f). Eventually, the readings were shown negative after 42 days of acclimatization. However, the readings were shown to be positive in field-grown plants and *in vitro*-derived plantlets at Day 56. This might be due to the expanding and protruding of multicellular trichomes on the abaxial part of the leaves that covered the stomata completely after 42 days of acclimatization (Fig. 3g) and field-grown plants (Fig. 3h). Our finding was in line with the study carried out by Armero *et al*. (3), whereby they reported that the stomata covered by multicellular trichomes could still open and give positive reading for photosynthetic rate. The multicellular trichomes make the surface of leaves less heated during day. Hence, this explained the positive value for photosynthesis rate during day since stomata still open even it should close to prevent water loss for CAM plant. Furthermore, Kumar and Rao (8) also mentioned that stomatal and trichome structures are one of the factors causing water loss plants. During the night, continuous positive readings were shown in field-grown plants after 56 days of acclimatization.

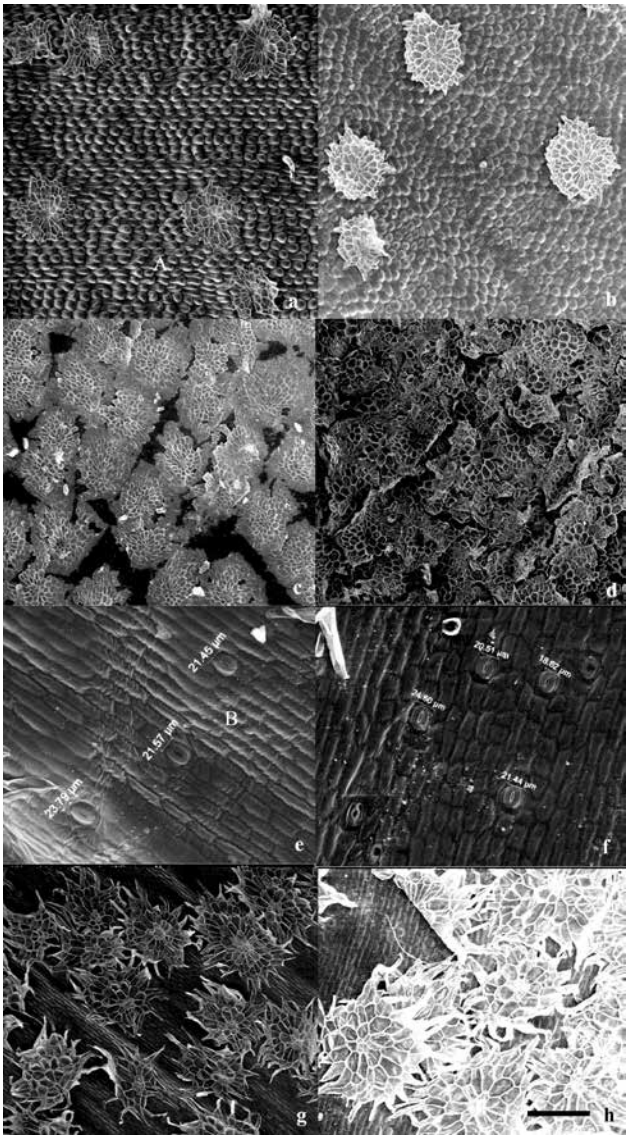

Fig. 3. Upper view of adaxial leaf (upper) and abaxial leaf using field electron microscope (FESEM) after (a) & (e) 0 day, (b) & (f) 14 days, (c) & (g) 42 days and (d) & (h) field-grown (fg) plants of acclimatization. a, b, c, d (adaxial) e, f, g, h (abaxial). A: Trichome; B: Stomata (Bar = 100 µM

This showed that the plants underwent complete transition to CAM pathway where the reading must be positive during night since stomata for CAM plant would only open during night. Similar situation was observed for trichome development on the adaxial part of the leaves.

During the acclimatization period, leaves showed typical plant components, namely upper epidermis (A), mesophyll (B), vascular bundle consisting of xylem (C) and phloem (D), and lower epidermis (G). Stomata (E) and guard cells (F) were found on the

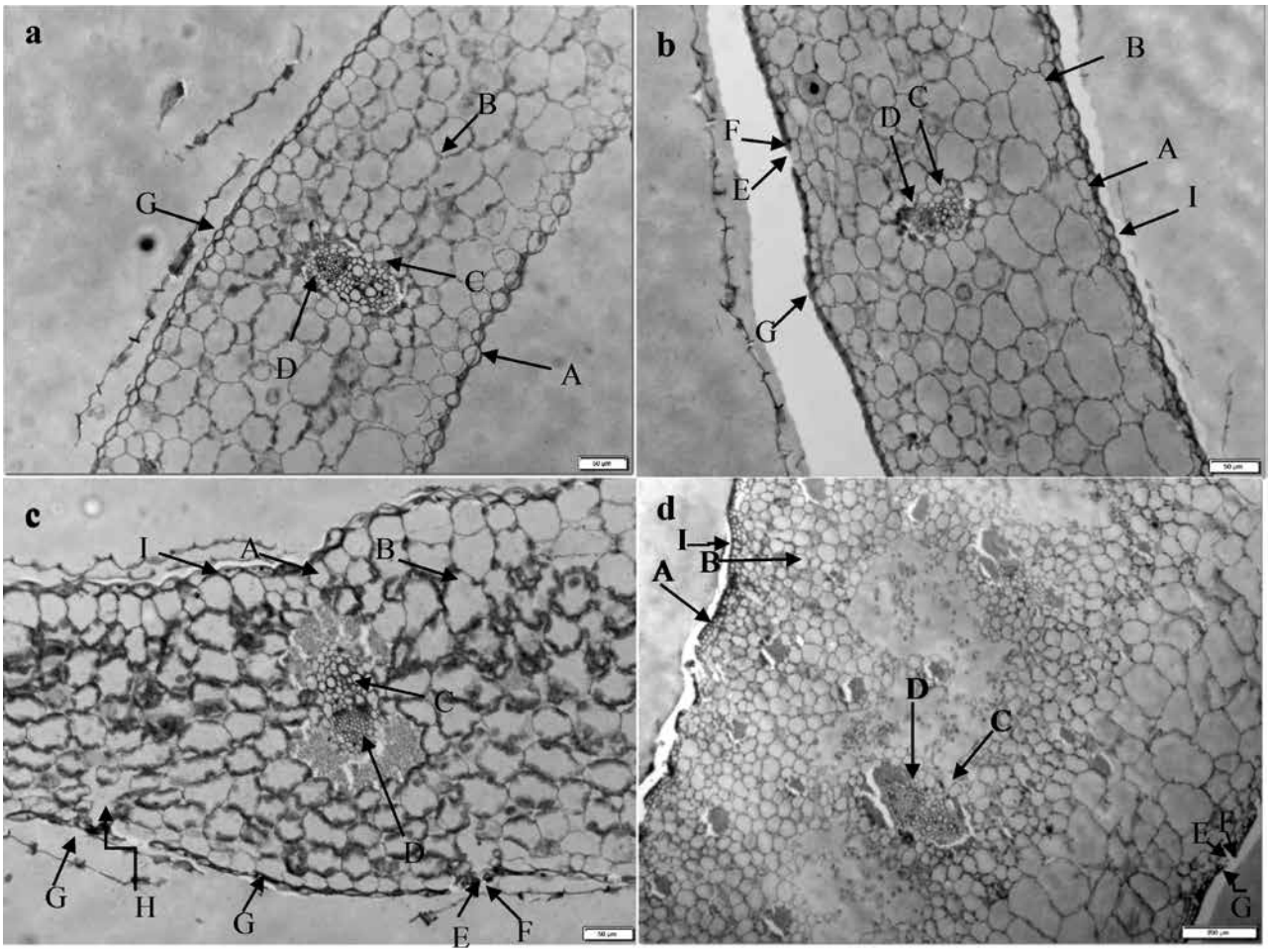

Fig. 4. Cross section of histological process of leaves of *A. comosus* for 0 day (40x) (a), 14 days (40x) (b), 42 days (30x) (c) and field-grown plant (10x) (d). A-Upper epidermis; B- mesophyll; C- xylem; D- phloem (C&D- vascular bundle); E- stomata; F- guard cell; G- lower epidermis; H- air space; I- cuticle.

abaxial section of the leaves at days 0 (Fig. 4A), 14 (Fig. 4b) and 42 (Fig. 4c) as well as field-grown plants (Fig. 4d). The leaf structure at 42 days was similar to the field-grown plants, where palisade mesophyll cells started to develop (Fig. 4c). However, the size of palisade mesophyll and the number of vascular bundles were found in abundance in the field-grown plants.

Histological examination showed the abundance of spongy mesophyll cells compared to palisade mesophyll during 0 and 28 days of acclimatization periods. This explained that C3 photosynthesis pathway mostly occurs in spongy mesophyll cells through stomata opening during day since the shape cells are not organized and compact compared to palisade mesophyll cells (Han *et al*., 6). On the other hand, palisade mesophyll cells started to increase at 42 days which was similar to the field-grown plants. More palisade mesophyll cells were observed after 42 days of acclimatization. CAM photosynthesis pathway happened in palisade mesophyll since the cell are much larger and tightly packed or contain low intercellular airspace, enlarge vacuole to store malic acid that will be used during the day (Han *et al*., 6).

There was no increase in the number of leaves (N) within the acclimatization period until 84 days, but the width increased significantly after 56 days compared to 42 days (Table 1). The root length was significantly increased after 14 days of acclimatization (Table 1). The ratio of dry weight over fresh weight (DW/FW) and succulence index were significantly higher in plants acclimatized after 42 days compared to 0, 14 and 28 days of acclimatization (Table 1). Succulent characteristic is well known to be related to CAM plants, such as pineapple, that enables the plant to store water longer, making the plants able to survive under drought conditions (Spinelli *et al*., 16).

Table 1. Morphological traits of acclimatized *in vitro* and field-grown plants of *A. comosus* var. MD2 during acclimatization. Data on number of new leaves (N), width of leaves (W), root length (R), ratio of dry weight to fresh weight (DW/FW), and succulence index (S) were recorded.

Day	N	W (cm)	R (cm)	DW/FW (g/g)	S (mg/µg)
0	9.2 ± 0.3^{b}	1.2 ± 0.0^{bc}	2.0 ± 0.3^{d}	0.10 ± 2.3^{b}	40^{b}
14	10.1 ± 5.1^{ab}	1.3 ± 0.5^{bc}	3.0 ± 0.3^{d}	0.13 ± 2.1^{b}	45^{b}
28	11.0 ± 1.2^{ab}	1.4 ± 2.1^{b}	7.4 ± 0.6^{c}	0.15 ± 2.5^{b}	55^{b}
42	11.0 ± 0.3^{ab}	2.0 ± 1.1^{ab}	8.2 ± 0.4^{bc}	0.33 ± 2.9^{a}	80^{a}
56	12.0 ± 2.1^{ab}	2.1 ± 5.6^{a}	8.4 ± 1.2^{bc}	0.33 ± 0.8^{a}	84^{a}
84	12.0 ± 2.5^{ab}	2.2 ± 0.3^{a}	10.6 ± 0.4^{b}	0.37 ± 3.3^{a}	89^{a}
Field (308 days)	16.0 ± 1.5^{a}	2.4 ± 0.0^{a}	16.3 ± 1.4^{a}	0.26 ± 0.0^{a}	98^{a}

New leaves were counted after growing at 45º slant; Mean ± SE (n = 10) within each column with different letters are significantly different (p< 0.05) according to Tukey Multiple Range Test (TMRT); Means of the treatment in the same column followed by different letters are significantly different from each other at the 5 % level; About 3-week old field-grown pineapple plants were used.

In this study, the succulence index data of *in-vitro* pineapple plantlets were significantly lower when comparing the acclimatization period before and after 42 days. The dry mass/fresh mass ratio (DM/FM) is sufficient for the plantlets to complete the transition from C3-dependent photosynthesis to CAM assimilation (Maxwell *et al.*, 11). This might indicate a gradual change of plantlets to CAM trait after 42 days of acclimatization. For all the traits, there were no significant difference observed between plant acclimatized after 42 days and field-grown plants (Table 1).

Originally, pineapple is a CAM plants in which the plants use carbon fixation pathway to adapt to the arid environment. Stomata remain closed during the day because of evapotranspiration and open during night to accumulate CO_2 for photosynthesis (Spinelli *et al.,* 16). This contradicts to C3 plant where the stomata opened during day and closed during night. Aragon *et al*. (2) stated that *in vitro* pineapple plantlets which are C3 plant might change to CAM in response to stress environmental changes, such as humidity, temperature and light intensity, due to plastic morphology. In this study, the patterns of photosynthesis rate, stomatal conductance and transpiration rate during day showed high reading, indicating that stomata were opened before *in vitro* plantlets acclimatized for 42 days. This suggested that CAM plantlets might begin with C3 due to high humidity, controlled temperature and light intensity and eventually switched to CAM plant after 42 days of acclimatization.

In conclusion, the results from physiological and morphological studies indicated that the *in vitro* pineapple plantlets have C3 characteristics but transformed to plants with CAM characteristics after 42 days of acclimatization. It was also shown that these plantlets underwent changes in morphological and physiological traits which attained similar readings to the field-grown plants after 42 days of acclimatization, suggesting that this could be the suitable period for acclimatization before transferring. Further examination on morphological and physiological changes on *in vitro*-derived plants into the fields will be continued.

DECLARATION

The authors declare no conflict of interest.

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