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ANATOMICAL CHANGES DURING ACCLIMATIZATION IN SOMATIC EMBRYOGENESIS-DERIVED PLANTS OF *HEVEA BRASILIENSIS*

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In *Hevea brasiliensis*, though high frequency plant regeneration protocols *via* somatic embryogenesis have been developed, establishment of these plants in the field has been difficult. A comparison of the anatomical characters of healthy and weak plants derived through somatic embryogenesis, with bud-grafted field-grown plants was carried out in detail. Scanning electron microscopy studies on leaves of healthy plants confirmed the presence of epicuticular wax and its continued increase during hardening. In acclimatized plants, the pattern was identical to that of field-grown control plants. Leaves of *in vitro* weak plants showed less epicuticular wax. Before hardening, stomatal frequency of the *in vitro* healthy plants was higher than that of the weak ones, but the size of the stomatal aperture did not vary significantly. After acclimatization, stomatal frequency of healthy plants was comparable to, or slightly higher than, that of the control plants. Vascular continuity and distribution of latex vessels in the *in vitro* plants are also discussed.

Keywords: Acclimatization, Anatomical changes, *Hevea brasiliensis*, *In vitro* culture, Somatic embryogenesis.

Acclimatization is a process by which tissue culture plants adapt to the uncontrolled external environment, during which normal photosynthetic activity and water relations have to be developed (Desjardins *et al.*, 1995). This process can generate stress (Van *et al.*, 1998). Although the cultural conditions of tissue culture promote rapid growth and development of tissues, the formation of abnormal characteristics like altered leaf morphology, altered mesophyll structure, poor photosynthesis, non-functional stomata and marked decrease in cuticular wax are very common during *in vitro* culture (Ziv, 1986). Upon transplantation, tissue culture plantlets with abnormal leaf development show low survival, mainly due to water loss and desiccation (Ziv *et al.*, 1987). Several reports have indicated that epicuticular wax and stomata in the leaves of tissue culture plants are inadequate or inoperative and are considered to be a major factor responsible for excessive water loss resulting in low survival rate of such plants during hardening (Brainerd and Fuchigami, 1982; Sutter and Langhans, 1982; Sutter, 1988).

Besides micropropagation, the potential use of somatic embryogenesis in molecular genetic manipulation through transgenic

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approaches has been widely recognized for several crops including *Hevea brasiliensis* (Jayashree *et al.*, 2003; Sobha *et al.*, 2003). However, the valuable regenerants may fail to survive upon transplantation. Although a large number of somatic embryogenesis-derived plantlets were regenerated from different explants of *H. brasiliensis* (Jayasree *et al.*, 1999; Sushamakumari *et al.*, 2000; Kala *et al.*, 2005), the establishment of plants in the field was limited due to poor survival of plantlets during acclimatization. The importance of anatomical variations associated with somatic plants is thus high and their role in acclimatization needs to be elucidated. The present study aims at a detailed anatomical investigation on changes occurring in leaves of *in vitro*-derived somatic plants during pre- and post-hardening.

Callus was induced from immature anthers (clone RRII 105) on modified MS medium supplemented with 2.0 mg/L 2, 4–dichlorophenoxyacetic acid and 0.5 mg/L kinetin (Jayasree *et al.*, 1999). The induced calli were then transferred to embryo induction medium containing 0.2 mg/L naphthaleneacetic acid, 0.7 mg/L kinetin and 2.0 mg/L gibberillic acid (GA_3) (Jayasree and Thulaseedharan, 2001). After four months of culture, differentiated embryos were transferred to fresh medium for maturation. Two months later, individual cotyledon stage mature embryos were transferred to plant regeneration medium which consisted of the growth regulators 0.5–1.0 mg/L benzyl adenine combined with 2.0 mg/L GA_3 (Jayasree *et al.*, 2001). Fully - developed plantlets were subjected to hardening.

The plantlets bearing two fully-developed leaves with 2-3 cm shoot length and well-developed root system were removed from the culture tube and washed carefully with tap water to remove traces of agar and then transferred to small polybags containing sterile soilrite as planting substrate. After transplantation, high humidity was maintained by covering the planted bag with another polythene bag and keeping it in the glasshouse at 26 ^{0}C. Two weeks after transplantation, the polythene bag covers were perforated, and two weeks later the covers were removed. The plants were maintained in the glasshouse for two more weeks. After full acclimatization, the plants were replanted in large polybags filled with mixture of sand, soil and dried cowdung (1:1:1) and kept in the shade house under natural conditions. During hardening, the plants were irrigated with ½ x MS salts on alternate days for one week, and with tap water thereafter.

To study the anatomical changes during acclimatization, leaves from pre- and post-acclimatized plants were examined. Leaves from 3-year-old bud - grafted plants in the field were used as control. From each category, two plants were taken and mature leaves were collected. For epicuticular wax distribution, leaves from *in vitro* - grown healthy plants (Fig. 1A) and weak plants (Fig. 1B) and from 3-month-old acclimatized healthy plants (Fig. 1C) were collected. Leaf samples were dried using critical point dryer, coated with gold palladium and viewed through a JEOL JSM 35C scanning electron microscope. For stomatal distribution, leaf epidermal peels were taken by boiling the samples in 60 per cent HNO_3 and potassium chlorate (Johansen, 1940), while for anatomical studies, leaf, stem and root were fixed in formaldehyde-acetic acid-alcohol mixture (Johansen, 1940). Fixed samples were dehydrated through a series of tertiary butyl alcohol followed by impregnation of

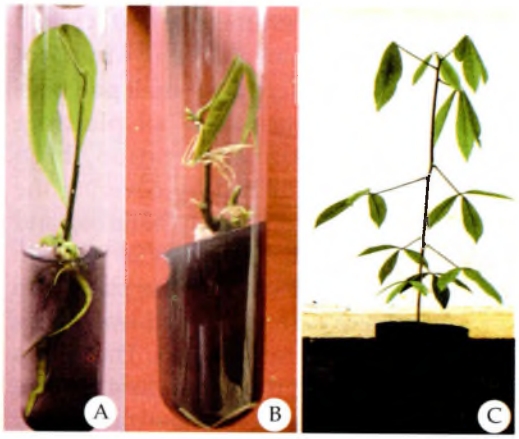

Fig. 1. Somatic embryogenesis - derived plantlets:
A - Healthy *in vitro* plant
B - Weak *in vitro* plant
C - Healthy acclimatized plant

tissue with wax and finally embedded in wax. Serial microtome sections of 8-10 μm thickness were made and stained with toluidine blue O (O' Brien and McCully, 1981).

A large number of plants were produced through somatic embryogenesis which included normal as well as abnormal plantlets. These plants showed morphological variation and were categorized as healthy or weak. Healthy plants had normally spaced internodes, dark green expanded leaves and long roots possessing root hairs (Fig. 1A). The weak plants appeared stunted with close internodes and thin elongated pale green leaves. The root was devoid of hairs (Fig. 1B). In the present study, during hardening, healthy plants showed a survival rate of 50 per cent, while with weak plants only 10 per cent survival was obtained. During *ex vitro* transplantation, the weak plants wilted and dehydrated quickly, resulting in death of plants. One month after acclimatization, all the healthy plants (the 50 per cent that survived hardening) could be transferred to large polybags.

SEM studies revealed that the leaf samples collected from all categories of plants possessed epicuticular wax and invariably there was more wax on the abaxial surface. However, the amount of wax varied with the source of leaf. Before hardening, leaves of weak plants showed low wax deposition (Fig. 2. A, E). Leaves from acclimatized weak plants could not be included for SEM study because the leaves wilted quickly and wrinkled during tissue processing. In the leaves of healthy plants, epicuticular wax started to develop before hardening (Fig. 2. B, F) and was found to thicken during the course of hardening (Fig. 2. C, G). The pattern of wax deposition after hardening was almost identical with that of field-grown control plants (Fig. 2. D, H). Though epicuticular wax was present in all samples, the development was poor in weak plants. This may be one of the reasons for the high mortality rate of weak plants during hardening. Earlier reports in other species have attributed low survival rate of tissue culture plants to high rate of cuticular transpiration (Sutter, 1988) due to poor development of epicuticular wax (Diettrich *et al.*, 1992). Results of the present study revealed that in healthy plants the initial wax deposition was good and continued to increase during the hardening process, which plausibly helped the plants during hardening and resulted in enhanced rate of successful transplantation.

Rubber has a dorsiventral leaf and the stomata are confined to the abaxial side of the lamina. The frequency of stomata per unit area in weak plants was low before hardening, though the number increased after

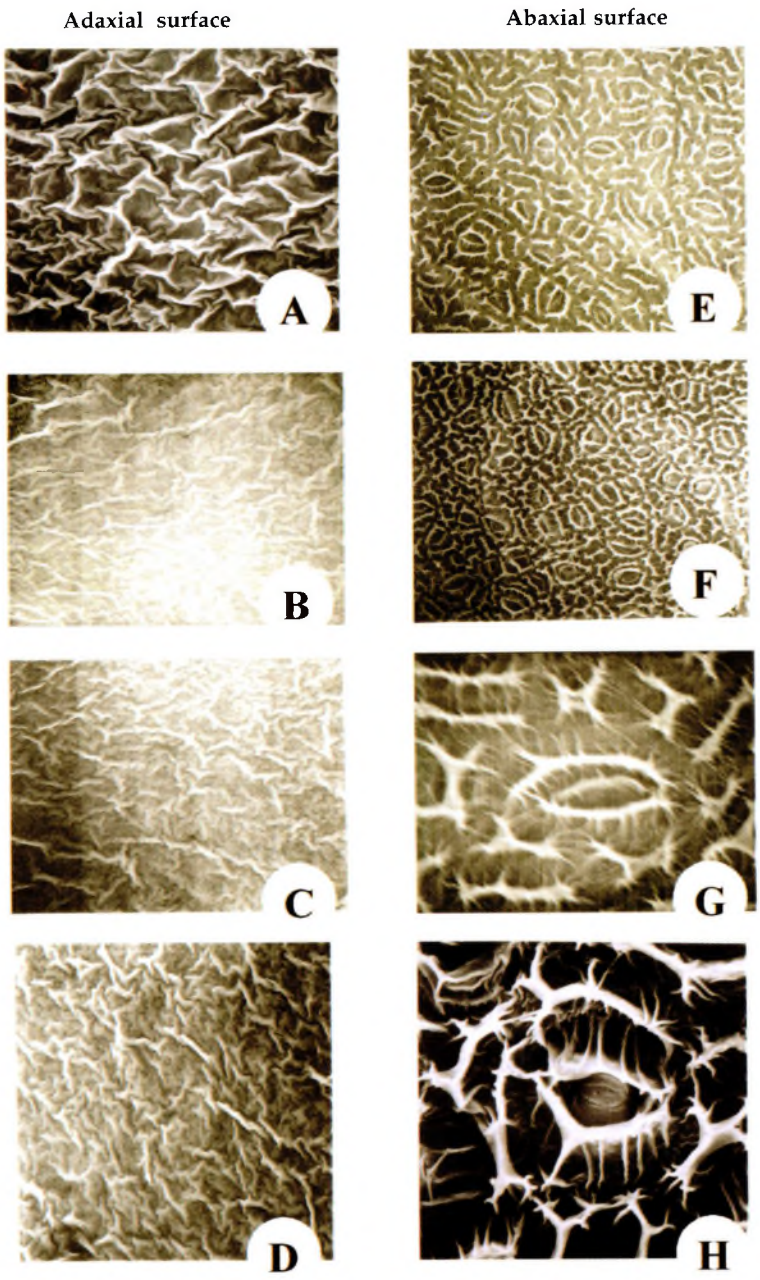

Fig. 2. SEM photographs of epicuticular wax distribution
A-D (adaxial surface of leaves) A- Weak plants (before hardening); B- Healthy plants (before hardening); C- Healthy plants (after hardening); D- Control plants
E-H (abaxial surface of leaves) E- Weak plants (before hardening); F- Healthy plants (before hardening); G- Healthy plants (after hardening); H- Control plants

Table 1. **Stomatal frequency and aperture of somatic plants**

Source of leaves	Stomatal number per mm^2	Stomatal aperture (µm)	
		Length	Width
Weak plant (BH)	129	39.1	18.8
Healthy plant (BH)	213	31.6	8.4
Weak plant (AH)	213	31.3	9.5
Healthy plant (AH)	354	29.5	13.5
Field-grown plant (Control)	326	41	29.3

BH - Before hardening; AH - After hardening

hardening (Table 1). Similarly, stomatal frequency of healthy plants also showed a sharp increase after hardening. However, compared with weak plants, the stomatal frequency of healthy plants before and after hardening was very high. There are contradictory reports on stomatal number of *in vitro* and *in vivo* plants. According to Brainerd *et al.* (1981), in *in vitro* plum plants, the number of stomata per square mm was reduced to half. However, in *in vitro* teak plants, the stomata number was high compared to that of field- grown plants (Bandyopadhyay *et al.*, 2004). In the case of aperture size, though stomatal length was slightly decreased in healthy plants after acclimatization, the width was increased slightly (Table 1). However, in weak plants, before hardening, the stomatal aperture size (length and width) was more. According to Ziv *et al.* (1987) also, the stomatal aperture from weak plant leaves was often twice the size of those from the normal leaves.

The healthy plants had a thicker stem and root (Table 2). Healthy plants before hardening had stiff roots without root hairs, while after hardening the roots elongated with root hairs, as in a normal plant. Anatomically, the proportion of vascular tissue to the total diameter of stem of both healthy and weak plants was the same.

Table 2. **Stem and root measurement of somatic plants**

Stem /Root	Diameter (mm)	Vasculature (mm)	Outer cortex (mm)	Pith (mm)
Stem				
Weak (BH)	0.95	0.12	0.02	0.68
Healthy (BH)	1.86	0.22	0.28	0.72
Weak (AH)	1.98	0.23	0.22	0.85
Healthy (AH)	2.20	0.26	0.25	0.89
Root				
Weak (BH)	0.82	0.16	0.30	0.33
Healthy (BH)	3.06	0.32	0.74	0.30
Weak (AH)	2.50	0.35	0.30	0.90
Healthy (AH)	2.70	0.89	0.29	0.72

BH - Before hardening; AH - After hardening

However, in the case of roots of plants after hardening, the proportion of the vascular tissue in the weak plants was much lower than in the healthy plants. This implies that the casualty observed in the tissue culture plant may partly be due to poor development of vasculature in the root. This needs further confirmation by incorporating more number of samples as the root differentiation and root hair development in tissue culture plants of rubber are crucial. Distribution of latex vessels was observed in the palisade layer of leaves in both healthy and weak plants. Unlike plants generated *via* stem-culturing (Grout and Aston, 1978), in the present study, both healthy as well as weak somatic plants prior to, and post-acclimatization, established a well-developed vascular continuity between the stem and root, similar to seedling plants. This indicated that up to certain stages of development, the weak plants showed growth attributes. Failure to develop further may be due to the poorly developed root devoid of a root tip meristem. According to Apter *et al.* (1999), some micropropagated

plantlets may develop functional roots or may die due to non-functional root system.

Cultural conditions are found to play a crucial role for the development of plant with normal leaf. Environmental conditions including artificial support medium, low-light regimes and high relative humidity imposed during tissue culture induced anatomical, morphological and physiological changes which could induce abnormalities (Kozai, 1991). Ziv (1986) reported that the leaves of plants cultured on low agar or liquid medium lacked surface wax. It was reported that high humidity in the culture vessel (Debergh, 1983), media with high levels of NH_4 (Daguin and Letouze, 1984) and media enriched with cytokinins (Bornman and Vogelmann, 1984) also induced abnormal leaf development. These aspects require more experimental evidence.

During *ex vitro* transplantation, *in vitro*-derived weak plants showed poor survival rate compared to the healthy plants. SEM studies revealed the presence of epicuticular wax in the leaves of healthy plants before hardening, which was found to increase during the course of hardening. In acclimatized plants, the pattern of wax deposition was almost identical with that in field-grown control plants. After acclimatization, stomatal frequency of healthy plants was slightly higher or more or less equal to that of field-grown control plants, though, stomatal aperture size did not increase. Vascular continuity and distribution of latex vessels were well established in *in vitro*-derived plants. Lower epicuticular wax deposition, fewer stomata and poor root vasculature leading to poor development could be the reasons for poor survival of the plant.

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