

1 **Comparative Growth Analysis and Acclimatization of Tissue Culture Derived**
2 **Cocoyam [*Xanthosoma sagittifolium* (L) Schott] plantlets**

3 **Anne E. Sama¹, Mohamed A. Shahba^{2*}, Harrison G. Hughes¹ and**
4 **Mohamed S. Abbas²**

5 ¹**Department of Horticulture & Landscape Architecture, Fort Collins,**
6 **Colorado, 80523-1173, USA.**

7 ²**Department of Natural Resources, Institute of African Research and Studies**
8 **Cairo University, Giza, Egypt 12613.**

9 ***Corresponding author e-mail: shahbam@lamar.colostate.edu**

18 _____
19 **Key words:** tissue culture, cocoyam, epidermal cells, epicuticular wax, stomatal
20 frequency, stomatal index, acclimatization.

21 **Abbreviations:** MS, Murashige and Skoog (1962) medium; TDZ, Thidiazuron; BAP,
22 Benzylaminopurine; BM, basal medium; NAA, 1- naphthaleneacetic acid; AS, Adenine
23 sulphate; EC, Epidermal cells; EW, epicuticular wax; SF, stomatal frequency; SI,
24 stomatal index.

ABSTRACT

The current study was carried out to compare the external leaf structure of tissue culture-derived and conventionally-propagated Cocoyam [*Xanthosoma sagittifolium* (L) Schott] plantlets and to develop an efficient acclimatization protocol for these plantlets. Acclimatization studies were carried out during winter and summer to ascertain seasonal influence relative to plant survival upon transfer from in vitro to natural conditions. Results indicated that, cocoyam leaves have few stomates on both abaxial and adaxial surfaces with fewer on the adaxial surface. High levels of epicuticular wax (EW) found in vitro may have contributed to reduced transpiration rates. The reduced amounts of EW on acclimatized plants could be attributed to the rapid cell enlargement in expanding leaves, more rapid than the rate of wax formation. Acclimatization using humidity tent decreased leaf wilting and damage compared with the control treatment or with the mist treatment. Mist-acclimatized plantlets produced about 50% fewer leaves than those acclimatized in a humidity tent. Similar results were obtained during winter acclimatization with a lower rate of leaf formation compared to summer acclimatization. A relatively high humidity (60-80%) for approximately two weeks reduced leaf injury from wilting and desiccation.

INTRODUCTION

Cocoyam [*Xanthosoma sagittifolium* (L) Schott] is a monocotyledonous crop that belongs to the *Araceae* family. The stem has a starch rich underground structure, the corm, from which offshoots called cormels develop. Flowering is rare, but when it occurs, the inflorescence consists of a cylindrical spadix of flowers enclosed in a 12-15 cm spathe (Purseglove, 1992). It is a staple food in the tropics and subtropics and one of the six most important root and tuber crops world-wide (Onwueme and Charles, 1994). The corm, cormels, and leaves of cocoyam are an important source of carbohydrates for human nutrition, animal feed (Ndoumou et al., 1995; Nyochembeng & Garton, 1998; Sefa-Dedeh and Agyir-Sackey, 2004) and of cash income for farmers (Tambong et al., 1997). Africa produces about 75% of the world production which is about 0.45 million tons (FAO, 2006). Cocoyam breeding and production is labor intensive and requires large amounts of water (Onwueme, 1978). It is highly susceptible to diseases such as cocoyam root rot disease caused by *Pythium myriotylum* (Pacumbaba et al., 1992) and Dasheen Mosaic virus found in the leaves, corm and cormels (Chen and Adams, 2001).

Micropropagation is an efficient method to mass propagate good quality materials that may substantially improve production. It involves the use of defined growth media supplemented with appropriate growth regulators that enable morphogenesis to occur from naturally growing plant parts (Debergh and Read, 1991). Previous studies have shown that shoot multiplication, somatic embryogenesis and tuberization can be induced in shoot tips of cocoyam cultured in vitro on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with various combinations of indol butyric acid (IBA), 1-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), Benzylaminopurine (BAP) and kinetin (Omokolo et al., 1995).

The biochemical aspects of induction of in vitro organogenesis have been investigated in a number of plants including carrot (Choi and Sung, 1984), pea (Stirn and Jacobsen, 1987), summer squash (Ananthakrishnan et al., 2003; Pal et al., 2007), winter squash (Lee et al., 2003), soybean (Joyner et al., 2010), taro (Verma and Cho, 2010), watermelon (Krug et al., 2005), groundnut (Alam and Khaleque, 2010), asparagus (Sarabi and Almasi, 2010), black pepper (Sujatha et al., 2010), canola (Kamal et al., 2007), cotton (Ozyigit, 2008), date palm (Khierallah and Bader, 2007), lentil (Khawar et al.,

2004), common bean (Andrés et al., 2010), sunflower (Mayor et al., 2006), rice (An et al., 2004) and banana (Banerjee and de Langle, 1985). However, the benefit of any micropropagation system can only be realized by the successful transfer of plantlets from tissue-culture vessels to the field conditions (Hazarika, 2003). Most species grown *in vitro* require an acclimatization process in order to ensure that a sufficient number of plants survive and grow vigorously when transferred to soil.

In spite of its importance in many countries, cocoyam has received very little research attention (Watanabe, 2002). The yield potential of cocoyam is seldom realized, mainly because of a lack of knowledge concerning diseases, proper management practices, and physiological determinants that may limit plant growth and development (Goenaga and Chardon, 1995). The objectives of this investigation were to determine an effective acclimatization protocol for micropropagated cocoyam plantlets through a comparison of the external leaf structure of tissue culture-derived plantlets and conventionally-propagated plants in terms of epidermal cells, stomatal frequency and stomatal index, and to determine an effective acclimatization protocol for cocoyam plantlets.

MATERIALS AND METHODS

Source of explants:

Cocoyam ‘South Dade’ white plants were obtained from the Tropical Fruit Company, Homestead, Florida as sprouted corm sections. Sections were potted in polyethylene pots ($\cong 100\text{ cm}^2$) in a mix of peat, perlite and vermiculite (1:1:0.5 by volume). These plants were maintained in a greenhouse under natural photoperiod. Temperature was maintained at $23 \pm 2\text{ }^\circ\text{C}$. Plants were watered as needed with tap water and fertilized with liquid fertilizer containing N:P:K at 20:10:20 by weight twice a week. Eight weeks after planting, sprouts were collected, trimmed to about 5 cm and washed under running tap water for 30-60 minutes. Shoot-tips of 3-5 mm were excised and the apical meristem with 4-6 leaf primordia and approximately 0.5 mm of corm tissue at the base were disinfected in a laminar flow hood using 1% (v/v) sodium hypochlorite solution for 10 minutes before transferred onto the culture medium.

Basal medium (BM):

A modified Gamborg's B5 mineral salts (Gamborg et al., 1968) supplemented with 0.05 μM 1-naphthaleneacetic acid (NAA) was used throughout the study. The modified component of B5 micro-salts was $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ at 10 mg L^{-1} . Organics consisted of myo-inositol (100 mg L^{-1}), thiamine HCl (10 mg L^{-1}), nicotinic acid (1 mg L^{-1}) and pyridoxine HCl (10 mg L^{-1}). Sucrose was provided at 30 g L^{-1} . Whenever a semi-solid medium was desirable, agar (Sigma agar, type A) was added at a concentration of 0.4%. The pH of the medium was adjusted to 5.7 ± 0.02 . A thidiazuron (TDZ) solution containing 0.01% dimethyl sulfoxide (DMSO) was used. Erlenmeyer flasks (125 ml) and test tubes (25 x 150 mm) were used for growing cultures. Aliquots of 25 ml and 15 ml were dispensed into the flasks and test tubes, respectively. Flasks were stoppered with non-absorbent cotton plugs, and then covered with aluminium foil. Test tubes were covered with polypropylene closures, Kaput caps (Bellco Glass, Inc., N. J). The media-containing vessels were then autoclaved for 18 minutes at 121 $^{\circ}\text{C}$.

Acclimatization of cocoyam plantlets:

Acclimatization studies were carried out during winter and summer. Plants used for adaptation were previously proliferated in vitro in 2.0 μM TDZ multiplication medium. Before transplantation, agar was gently washed off the roots with tap water. Plants were transplanted into 10 cm plastic pots containing pre-moistened non-sterile soilless substrate composed of peat, perlite and vermiculite at a ratio of 1:1:0.5 by volume. During transplantation, the number of leaves, roots and plant height were recorded for each plant. Plant height was measured from the basal plate to the lamina tip of the youngest fully expanded leaf. The transplants were then subjected to four different acclimatization treatments for five days as follows: 1) Control, 2) mist, 3) humidity tent, and 4) test tube acclimatization by uncapping. Each treatment had at least 42 plants. Control plants were transferred directly to an open bench in the greenhouse. The initial temperature and relative humidity were 25 $^{\circ}\text{C}$ and 63% respectively. High and low temperatures averaging 26 and 16 $^{\circ}\text{C}$ respectively with corresponding relative humidities of 54% and 94%. During winter, the average greenhouse relative humidity was $40 \pm 5\%$, and the temperature was $22 \pm 3^{\circ}\text{C}$, and light intensities of approximately 400 $\mu\text{mol m}^{-2} \text{S}^{-1}$. The plants subjected to the second treatment were placed under an automatic misting system set for six seconds at eight minutes intervals. To gradually reduce the humidity,

the misting interval was increased to 16 minutes after the first two days for the remaining three days of acclimatization. The third treatment placed plants in a locally constructed plastic humidity chamber with the dimension of 127 X 92 X 62 cm. A humidifier was used to provide an initial relative humidity of 98% with a temperature of 25 °C. Humidity was gradually reduced after the second day by partially opening the flaps of the chamber. On third, fourth and fifth days, the relative humidity was lowered to 96 and 94 and 92% respectively. The fourth treatment was conducted in culture vessels by partial uncapping. The cultures were placed on a bench in the same environment as the control plants. Caps were loosely opened for the first two days, and then totally removed for the remaining three days of acclimatization to expose the plants to the natural environment while in the test tubes. After five days of acclimatization in the test tubes, the plants were taken out, washed and transplanted into the same soilless mix. All plants were transferred to an open bench and grown under standard greenhouse conditions. The number of stomates in tissue culture-derived and conventionally-propagated plants was examined to ascertain stomatal function and influence relative to plant survival upon transfer from in vitro to natural conditions. To count stomates, leaf impressions were made using a thin film of transparent fingernail polish. This was applied to peripheral sections on either side of the midrib and on both abaxial and adaxial surfaces of the lamina. After the dryness of the fingernail polish, the epidermal cell layer was peeled with a transparent adhesive tape. The imprints were then placed on microscope slides for observations. Also, the epicuticular wax content on leaves from tissue culture-derived and conventionally propagated plants was compared.

Data analysis:

Experiments were laid out as a complete block design. All data were subjected to an analysis of variance using unequal replications due to contamination. Treatment means were separated by Tukey's Multiple Range Test at a 5% level of significance (SAS Institute, 2006).

RESULTS

Regenerated plantlets characteristics:

Tissue culture regenerated cocoyam plantlets did not show any obvious deviations, and were morphologically similar to their conventionally propagated counterparts (Fig. 1). The plantlets retained the characteristic sagitate leaves of conventionally-propagated plants without modifications of color or shape.

Stomata in tissue culture-derived and conventionally-propagated plants:

Stomatal frequency (SF):

Cocoyam leaves from all sources were amphistomatic. Almost twice as many stomates were found on the abaxial surface (Fig. 2A). Analysis of variance indicated that SF (the number of stomates per mm^2) was significantly greater in the non-micropropagated control plants than in the acclimatized greenhouse plants (Table 1 and Fig. 3). There was a significant difference in the average SF among control, acclimatized and conventionally-propagated plants (Table 1 and Fig. 2A). The average SF of adaxial and abaxial surfaces were 17.4 and 30.5 respectively. Epidermal cells (EC) of cocoyam leaves from the three sources were polygonal or irregular with undulate anticlinal walls (Fig. 3). However, the anticlinal walls were less distinct in ECs of cultured plantlets. The cells were also varied in size and shape. Stomates of greenhouse plants were elliptical and sunken, while those of in vitro plants were more spherical and raised, but below the level of epidermal cells (Fig. 3). Stomates from all leaf sources were scattered and at unequal distances from one another. However, ECs and stomata along the veins were smaller and aligned in stream-like manner. Abaxial and adaxial stomata were similar to one another, with varying sizes on both sides.

Stomatal index (SI):

Analysis of variance indicated no significant differences in stomatal index calculated as the number of stomata / number of epidermal cells and stomata $\times 100 \text{ mm}^2$ among various cocoyam plantlet sources (Table 1 and Fig. 2B). Values ranged from 8.0 for in vitro propagated plants to 8.6 for control plants on the abaxial surface while it ranged from 4.6 for in vitro plants to 5.3 for control plants on the adaxial surface.

Epicuticular wax content on leaves of culture-derived and conventionally-propagated plants:

Analysis of variance indicated a significant difference among different plant sources in epicuticular wax (EW) formation on cocoyam leaves (Table 1). Cocoyam

1 plantlets cultured in vitro were found to have greater deposits of EW. Gravimetric
2 determination showed that in vitro leaves had an average of 88.6 $\mu\text{g}/\text{cm}^2$, as compared to
3 50.1 $\mu\text{g}/\text{cm}^2$ for plantlets transferred and grown in the greenhouse (Fig. 4).

4 ***Tissue culture-derived plantlets behavior and adaptation to different environmental***
5 ***factors:***

6 **Summer acclimatization:**

7 **Effects on leaves:**

8 Analysis of variance indicated a significant difference among acclimatization
9 procedures on leaf damage and leaf wilting (Table 1). Cocoyam plantlets showed no
10 significant visual desiccation one hour after transfer to the open bench. After 24 hours,
11 differences were observed in leaf wilting among different treatments. Plantlets
12 acclimatized by uncapping the culture tube showed 14.9% leaf injury after 24 hours of
13 acclimatization compared to only 0.6% in plantlets acclimatized under mist (Fig. 5A). On
14 the other hand, wilting assessment indicated that plantlets acclimated under mist suffered
15 less wilting after 24 hours (Fig. 5B). One week after acclimatization, more wilting was
16 observed as the percentage of damaged leaves increased significantly for open tube
17 acclimatization (43.7 %) as compared to that from the humidity tent which had a damage
18 percentage of 15.9%. Wilting assessment of 2.8 and 3.5 was associated with the previous
19 leaf damage percentages respectively (Fig. 5A and B).

20 **Effects on growth habit:**

21 Analysis of variance indicated a significant difference among acclimatization
22 procedures on leaf initiation and leaf shedding (Table 1). After acclimatization, plants
23 continued to grow actively in the greenhouse with normal leaf and whole plant
24 morphology. Significant differences among the different acclimatization treatments were
25 found in the number of new leaves formed in plants (Fig. 5C). Two weeks after
26 acclimatization, an average of 1.2 leaves were produced from plants acclimatized in the
27 humidity tent, as compared to only 0.6 leaves in mist-acclimatized and control plants. An
28 average rate of one new leaf per plant was produced every two weeks. Mist-acclimatized
29 plants produced fewer leaves than the other treatments after four and six weeks and was
30 significantly different from those acclimatized in a humidity tent and uncapped tubes.

The number of leaves shed per plant per treatment was used as indication of the reverse of leaf production. After two weeks of acclimatization, humidity tent plants shed only an average of 0.8 leaves as compared to 1.3 for the control plants (Fig. 5D). A significant difference among treatments was observed after two weeks and disappeared at four and six weeks.

Winter acclimatization:

Effects on leaves:

Plantlet leaves wilted slightly during transplantation, but those acclimatized under mist and humidity tent were able to regain turgidity. Plantlets transferred directly from culture to the open bench were more stressed after 24 hours compared when to other treatments. Plantlets acclimatized under mist and in the humidity tent had wilted leaves only after one week of acclimatization, which were gradually reduced in subsequent weeks. The critical period for leaf injury was the first week after acclimatization. All plants survived in all treatments but less wilting and leave injury were associated with mist or humidity tent as compared with the control.

Effects on growth habit:

The growth and development of plants was not affected by the method of acclimatization during shoot elongation. New leaves were produced by the second week after acclimatization and more grew after four weeks. The rate of leaf formation was low as compared to summer acclimatized plants except for the mist treatment (Fig. 5C).

DISCUSSION

The decrease in stomatal index and increase in epidermal cell size may affect plant growth physiology. Therefore, altered leaf structures might be associated with poor field performance and increased disease susceptibility (Zhao et al., 2003 and 2004). In similar findings, Donnelly and Vidaver, (1984) reported almost twice as many stomates on the abaxial surface in tissue cultured plantlets of *Rubus idaeus*. The reduced SF in leaves of acclimatized plants may have been due to their enlargement. Blanke and Belcher (1989) noticed a drastic decrease in SF of transferred apple plants, which was attributed to leaf expansion. In strawberry plants, the increase in size of persistent leaves was mainly the result of cell enlargement, rather than the increase in cell number (Fabbri et al., 1986). On the other hand, Brainerd et al. (1981) reported significantly reduced cell

length in the upper epidermis of transferred 'Pixy' plum plants as compared to those aseptically and field grown. Comparable findings, where SF was greater with in vitro plantlets than those that had been removed from culture, were observed in *Liquidambar styraciflua* (Wardle et al., 1983; Wetzstein and Sommer, 1983), and apple (Blanke and Belcher, 1989).

The average SF of 17.4 and 30.5 for adaxial and abaxial surfaces of cocoyam, respectively, are relatively low, when compared to 27.5 and 150 for *Rubus idaeus* (Donnelly and Vidaver, 1984), 184.5 (adaxial only) for *Vitis sp.* 'Valiant' (Dami, 1991). This low SF may have contributed to low transpiration rates, which resulted in less wilting and high survival rates of cocoyam plantlets after transplantation. In contrast, Brainerd and Fuchigami (1981) suggested that the high SF of apple micropropagated plantlets was responsible for the higher water loss observed. The rapid water loss could be due to stomatal malfunction (Brainerd and Fuchigami, 1981) or the size of the stomates (Wetzstein and Sommer, 1983). Wetzstein and Sommer (1983) found that stomata were also larger in vitro plantlets of sweet gum, in addition to their greater densities. As indicated by Brainerd and Fuchigami (1981) and Conner and Conner (1984), stomates have a greater part in water loss of plantlets than epicuticular wax. No significant differences in stomatal index among various cocoyam plantlets sources were found. These results corroborate previous findings that were reported for *Solanum laciniatum* (Conner and Conner, 1984), *Rosa multiflora* (Capellades et al., 1990), and *Vitis sp.* 'Valiant' (Dami, 1991) in comparisons made between in vitro and field grown plants. Dami (1991) found significantly greater stomatal densities in leaves of greenhouse-grown plants than in *in vitro* cultured leaves but found none when SI comparisons were made. These results agree with the idea that SI is a better estimate than SF in comparisons involving leaves with stomates of different sizes (Conner and Conner, 1984; Donnelly and Vidaver, 1984; Dami, 1991). However, Zhao et al. (2006) found that micropropagated regenerants had produced a significantly lower stomatal index, but larger epidermal cell size than conventional plants when they investigated the alterations in leaf trichomes, stomatal characteristics and epidermal cellular features in micropropagated rhubarb (*Rheum raphonticum L.*).

Quantitative variation is frequently found among regenerants derived from tissue culture and often indicates alteration of numerous loci (Kaeppler et al., 2000). Quantitative variation has been described for many phenotypes including plant growth habit and agronomic performance (Kaeppler et al., 2000; Anu et al., 2004; Zhao et al., 2005). The causes of somaclonal variations are believed to result from a range of genetic events during plant tissue culture, but it is difficult to interpret somaclonal variation in a genetic mode (Scowcroft, 1985; Larkin et al., 1989; De Klerk et al. 1990). In recent years the genetic analysis of plants regenerated from tissue culture has revealed that extensive genetic changes apparently occur during tissue culture. The majority of morphological variants observed in tissue cultured plants were due to numerical and structural chromosome changes induced during culture (D'Amato, 1985; Duncan, 1997).

Cocoyam plantlets cultured in vitro were found to have greater deposits of EW. Apparently, there was less wax deposits per unit area after transplantation. Sutter (1988) found a similar phenomenon with apple plants, with more EW in vitro and less after acclimatization. It was suggested that the decrease may be related to two possible causes: leaf enlargement that exceeded the synthesis of additional wax to cover the additional surface area; and wax metabolism during acclimatization, since previous studies have shown that wax biosynthesis and degradation is a continual and dynamic process (Cassagne and Lessire, 1974; Sutter, 1988). These results are in contrast to reports where more extensive wax deposits were observed in greenhouse and field plants than observed in vitro. Examples include cauliflower (Grout, 1975), carnation (Sutter and Langhans, 1979), cabbage (Sutter and Langhans, 1982), strawberry (Fabbri et al., 1986), chrysanthemum (Sutter, 1988) and grape (Dami, 1991). Wax deposition after plantlet transplantation occurs with time. Wax formed after 10-14 days in *Brassica oleracea* (Grout and Aston, 1977; Wardle et al., 1979) and 17/18 days in carnation (Sutter and Langhans, 1979). Fabri et al. (1986) observed an increase in EW deposits of transferred strawberry plantlets during the first 20 days, while similar findings were observed in *Solanum laciniatum* acclimatized plants after a month (Conner and Conner, 1984). The results obtained in this study showed a decrease in wax content per unit area in transferred plantlets at 9 and 12 weeks from transplantation. The previous results indicate that wax deposition and breakdown are species-dependent.

1 Sutter and Langhans (1979) and Wetzstein and Sommer (1983) indicated that the
2 environment in which a plant grows determines its morphology and chemical
3 composition. The in vitro conditions in which cocoyam plantlets were grown seemed
4 favorably for EW formation. This high EW content may have contributed to plantlet
5 survival upon transfer ex vitro. On the other hand, Brainerd and Fuchigami (1981) and
6 Conner and Conner (1984) showed that EW was less important than stomates in
7 determining the amount of water loss in plants. The sunken and ellipsoidal stomata of
8 cocoyam leaves in vitro, in addition to their high EW content, may have been invaluable
9 in conferring plantlet survival. The low wax content of acclimatized plants may have
10 been caused principally by the rapid leaf expansion that suppressed wax formation. The
11 environmental conditions were not optimum (Sutter and Langhans, 1979; Wetzstein and
12 Sommer, 1983), but did favor wax formation in vitro. Another possible cause for the
13 high amounts of EW observed in vitro may have been the dissolution of internal lipids
14 from open stomata of in vitro plants to close upon removal from culture (Brainerd and
15 Fuchigami, 1981; Wetzstein and Sommer, 1983; Conner and Conner, 1984). This could
16 be true for cocoyam. It could also relate to the fact that cocoyam typically grows in high
17 humidity, and thus may have wax production even under high humidities.

18 The relatively poor growth performance of plantlets acclimatized by mist system
19 may be attributed to the wet conditions they were subjected to. Griffis et al. (1983)
20 reported that nutrients are leached under a misting system, and that the wetness creates an
21 environment favorable for microorganism growth. Cocoyam, unlike taro, cannot
22 withstand water-logging under natural conditions (Onwueme, 1978; Cavensess et al.,
23 1986; FAO, 1988). Continuous misting for a period of five days, in addition to the high
24 humidity, may have been too wet to ensure normal growth. However, the overall trend
25 was that more leaves were produced than shed. Reduction in growth upon transplanting
26 of tissue culture plantlets has been frequently reported in the literature (Grout and Aston,
27 1977, 1978; Grout and Millam, 1985).

28 The number of leaves shed was comparatively lower than that encountered from
29 non-tissue culture derived plants under field conditions (Spence, 1970). This could be due
30 to the use of growth regulators while in culture. Spence (1970) observed that field grown
31 cocoyam plants were wasteful in the manner in which they produced and maintained their

1 leaves, and suggested the use of growth regulators to alleviate the shedding. The
2 continuous turnover of large leaves reduced photosynthetic productivity of the plants.

3 The ability to successfully transfer cocoyam plantlets from culture at a relatively
4 low cost with minimal loss is important to the micropropagation technique, especially at
5 the commercial scale. In general, many tissue culture regenerated plants are lost during
6 transfer to normal growth conditions. These losses are associated with rapid water loss
7 and desiccation during the acclimatization phase. Mist systems and humidity chambers
8 are most commonly utilized in an attempt to mitigate plant loss (Wardle et al., 1983).
9 Short et al. (1987) evaluated the success of a micropropagation system by the percentage
10 of plants that are successfully transferred from culture to natural soil conditions.

11 In this study, all cocoyam plants transferred from culture to in vivo conditions
12 survived, even without acclimatization. Onokpise et al. (1992, 1993 and 1999) also
13 obtained 100 % survival with different acclimatization studies. Staritsky et al. (1986)
14 reported that rootless cocoyam shoots could be easily rooted and would rapidly develop
15 into plantlets when transferred into soil. Acclimatization procedures may be either
16 unnecessary or just advantageous for a short period, especially in areas such as the humid
17 tropics with relatively high humidities. Otherwise, a humidity tent or cheaper method of
18 maintaining a moderately high humidity is recommended, rather than an expensive
19 misting system, in areas with low relative humidities.

20 The lag in growth in the case of winter acclimatization could be related to the low
21 temperatures, humidity, and lower light intensities in winter conditions within the
22 greenhouse. This probably slowed conversion from heterotrophic to autotrophic nutrition.
23 Tsafack et al. (2009) mentioned that the tuberization rate, the number and weight of
24 microtubers and the leaf weight were affected by day length and temperature. Omokolo et
25 al. (2003) obtained the highest tuberization rate (83%) of the white cocoyam cultivar with
26 an inductive medium containing 6-benzylaminopurine (BAP) under Short day regime.
27 Tsafack et al (2009) confirmed the findings of Gopal et al. (1998) and Tsafack et al.
28 (2004) who reported that tubers could be induced in vitro without the use of plant growth
29 regulators (PGRs). The use of media without PGRs was important to judge the innate
30 capacity of genotypes to produce microtubers and to avoid the possibility of any
31 undesirable carry-over effect of PGRs on morphogenesis and sprouting.

CONCLUSION

Evaluation of stomatal number showed that cocoyam leaves have few stomates on both abaxial and adaxial surfaces with fewer on the adaxial surface. High levels of epicuticular wax found in vitro may have contributed to reduced transpiration rates. The reduced amounts of EW on acclimatized plants could be attributed to the rapid cell enlargement in expanding leaves, more rapid than the rate of wax formation. Erlenmeyer flasks and test tubes did not prove to be the best culture vessels. A wider-mouthed culture vessels should be used so that the mass of proliferated tissue can be removed easily. The culture derived plants should be grown in the field under normal conditions to evaluate trueness-to-type. This study provides additional evidence of somaclonal variation in these regenerants. Further investigations on physiological parameters will be beneficial to understand the effect of altered leaf structure on plant growth and abnormal plants. A relatively high humidity (60-80%) is required for approximately two weeks to prevent leaf injury resulting from wilting and desiccation. Evaluation of stomatal number showed that cocoyam leaves have few stomates on both abaxial and adaxial surfaces.

LITERATURE CITED

- Alam AKMM, M.A. Khaleque. 2010. In vitro response of different explants on callus development and plant regeneration in groundnut (*Arachis hypogaeae* L.). Int. J. Expt. Agric. 1:1-4.
- An, Y.R., X.G. Li, H. Y. Su and X. S. Zhang. 2004. Pistil induction by hormones from callus of *Oryza sativa* in vitro. *Plant Cell Rep.* 23:448–452.
- Ananthakrishnan, G., X. Xia, C. Elman, S. Singer, H.S. Paris, A. Gal-On, and V. Gaba. 2003. Shoot production in squash (*Cucurbita pepo*) by in vitro organogenesis. *Plant Cell Rep.* 21:739-46.
- Andrés M. Gatica Arias, A.M.G., J.M. Valverde, P. R. Fonseca and M.V. Melara. 2010. *In vitro* plant regeneration system for common bean (*Phaseolus vulgaris*): effect of N6-benzylaminopurine and adenine sulphate. *Electronic Journal of Biotechnology* 13:1-8.
- Anu, A., K.N. Babu and K.V. Peter. 2004. Variations among somaclones and its seedling progeny in *Capsicum annum*. *Plant Cell, Tissue Organ Cult.* 76: 261–267.
- Banerjee, N. and E. de Langhe. 1985. A tissue culture technique for rapid clonal propagation and storage under minimal growth conditions of *Musa* (Banana and plantain). *Plant Cell Rep.* 4:351–354.
- Blanke, M. M. and A. R. Belcher. 1989. Stomata of apple leaves cultured *in vitro*. *Plant Cell Tiss. Org. Cult.* 19: 85–89.
- Brainered, K. E. and L. H. Fuchigami. 1981. Acclimatization of aseptically cultured apple plants to low relative humidity. *J. Am. Soc. Hort. Sci.*, 106: 515-518.
- Capellades, M., R. Fontarnau, C. Carulla and P. Debergh. 1990. Environment influences anatomy of stomata and epidermal cells in tissue cultured *Rosa multiflora*. *J. Amer. Soc. Hort. Sci.* 115 (1): 141–145.
- Cassagne, C. and R. Lessire. 1974. Studies on alkane biosynthesis in the epidermis of *Allium porrum* L. leaves. *Arch. Biochem. Biophys.*, 165: 274-280.
- Caveness, F. E., S. K. Hahn, and M. N. Alvarez. 1986. Sweet potato, yam, and cocoyam production. In: J. Cock (ed.), *Global Workshop on Root and Tuber Crops Propagation*. Proceedings of a regional workshop held in California, 13-16 September, 1983. CIAT, Cali, Colombia, pp. 22-31.

1 Chen, J., and M. J. Adams. 2001. Molecular characterization of an isolate of Dasheen
2 mosaic virus from *Zantedeschia aethiopica* in China and comparisons in the
3 genus *Potyvirus*. Archives of Virology 146, 1821-1829.

4 Choi, J. H. and Z. R. Sung. 1984. Two dimensional gel analysis of carrot somatic
5 embryogenesis proteins. Plant Mol. Biol. Rep. 2: 19–25.

6 Conner, L. N. and A. J. Conner. 1984. Comparative water loss from leaves of *Solanum*
7 *laciniatum* plants cultured in vitro and in vivo. Plant Sci. Lett., 36:241-246.

8 D’Amato, F. 1985. Cytogenetics of plant cell and tissue cultures and their regenerates.
9 CRC Crit. Rev. Plant Sci. 3: 73–112.

10 Dami, I. 1991. In vitro acclimatization of tissue cultured grape (*Vitis sp.* ‘Valiant’)
11 plantlets. M.S. Thesis, Colorado State University.

12 De Klerk, G.J., J. Ter Brugge and H. Bouman. 1990. An assay to measure the extent of
13 variation in micropropagated plants of *Begonia hiemalis*. Acta Bot. Neerl. 39:
14 145–151.

15 Debergh, P. C. and P. E. Read. 1991. Micropropagation. In: P. C. Debergh and R. H.
16 Zimmerman (eds.), Micropropagation, Technology and Application, pp. 1-13,
17 Kluwer Academic Publishers, Netherlands.

18 Donnelly, D. J. and W. E. Vidaver. 1984. Leaf anatomy of red raspberry transferred from
19 culture to soil. J. Am. Soc. Hort. Sci., 109: 172-176.

20 Duncan, R.R. 1997. Tissue culture-induced variation and crop improvement. Adv. Agron.
21 58: 201–240.

22 Fabbri, A., E. Sutter, and S. J. Dunston. 1986. Anatomical changes in persistent leaves of
23 tissue-cultured strawberry plants after removal from culture. Scientia Hort.,
24 28:331-337.

25 FAO. 1988. Root and tuber crops, Plantains and bananas in developing countries:
26 challenges and opportunities. FAO plant production and protection paper 87,
27 Rome, Italy.

28 FAO. 2006. Food and agriculture organization statistical database: world production of
29 fruits and vegetables. Available at
30 <http://www.ers.usda.gov/publications/vgs/tables/world.pdf>.

- 1 Gamborg, O.L., R.A. Miller, K. Ojima. 1968. Nutrient requirements of suspension
2 cultures of soybean root cells. *Exp. Cell Res.* 50:151-158.
- 3 Goenaga, R. and U. Chardon. 1995. Growth, yield and nutrient uptake of taro grown
4 under upland conditions. *Journal of Plant Nutrition* 18 (5), 1037-1048.
- 5 Gopal, J. , J. L. Minocha, and H. S. Dhaliwal. 1998. Microtuberization in potato
6 (*Solanum tuberosum* L.). *Plant Cell Rep* 17:794–798.
- 7 Griffis, Jr., J. L., G. Hennen, and R. P. Oglesby. 1983. Establishing tissue cultured plants
8 in soil. *Comb. Proc. Intl. Plant Prop. Soc.*, 33:618-622.
- 9 Grout, B. W. W. 1975. Wax development of leaf surfaces of *Brassica oleracea* var.
10 currawong regenerated from meristem culture. *Plant Sci. Lett.*, 5:401-405.
- 11 Grout, B. W. W. and M. J. Aston. 1977. Transplanting of cauliflower plants regenerated
12 from meristem culture. I. Water loss and water transfer related to changes in leaf
13 wax and to xylem regeneration. *Hort. Res.*, 17: 1-7.
- 14 Grout, B. W. W. and M. J. Aston. 1978. Modified leaf anatomy of cauliflower plantlets
15 regenerated from meristem culture. *Ann. Bot.*, 42:993-995.
- 16 Grout, B. W. W. and S. Millam. 1985. Photosynthetic development of micropropagated
17 strawberry plantlets following transplanting, *Ann. Bot.* 55 (1985), pp. 129–131.
- 18 Hazarika, B. N. 2003. Acclimatization of tissue-cultured plants. *Current Science*, 85: 12-
19 25.
- 20 Joyner, E.Y., L.S. Boykin and M.A. Lodhi. 2010. Callus induction and organogenesis in
21 soybean [*Glycine max* (L.) Merr.] cv. Pyramid from mature cotyledons and
22 embryos. *The Open Plant Science Journal* 4: 18-21.
- 23 Kaeppeler, S.M., H.F. Kaeppeler and Y. Rhee. 2000. Epigenetic aspects of somaclonal
24 variation in plants. *PlantMol. Biol.* 43: 179–188.
- 25 Kamal, G. B., K. G. Illich and A. Asadollah. 2007. Effects of genotype, explant type and
26 nutrient medium components on canola (*Brassica napus* L.) shoot *in vitro*
27 organogenesis. *African Journal of Biotechnology* 6:861-867.
- 28 Khawar, K. M., C. Sancak, S. Uranbey and S. Zcan. 2004. Effect of thidiazuron on shoot
29 regeneration from different explants of lentil (*Lens culinaris* Medik.) via
30 organogenesis. *Turk J Bot* 28: 421-426.

- 1 Khierallah, H.S.M. and S.M. Bader. 2007. Micropropagation of date palm (*Phoenix*
2 *dactylifera* L.) var. Maktoom through direct organogenesis. *Acta Hort* 736: 213-
3 224.
- 4 Krug, M. G. Z., L. C. L. Stipp, A. P. M. Rodriguez and B. M. J. Mendes. 2005. In vitro
5 organogenesis in watermelon cotyledons. *Pesq. agropec. bras.*, Brasília, 40:861-
6 865.
- 7 Larkin, P.J., P.M. Banks, R. Bhati, R.I.S. Berttel, P.A. Davies, S.A. Ruan, W.R.
8 Scowcroft, L.H. Spindler and G.J. Tanner. 1989. From somatic variation to
9 variant plant: mechanisms and application. *Genome* 31: 705–711.
- 10 Lee, Y.K., W. Chung and H. Ezura. 2003. Efficient plant regeneration via organogenesis
11 in winter squash (*Cucurbita maxima* Duch.). *Plant Science* 164:413-418.
- 12 Mayor, M.L., G.Nestares, R. Zorzoli, and L.Picardi. 2006. Analysis for combining ability
13 in sunflower organogenesis-related traits. *Australian Journal of Agricultural*
14 *Research*, 57:1123–1129.
- 15 Murashige T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with
16 tobacco tissue culture. *Plant Physiol.* 15: 473–497.
- 17 Ndoumou, D.O., G. N. Tsala, G. Kanmegne, and A. P. Balange, A.P. 1995. In vitro
18 induction of multiple shoots, plant generation and tuberization from shoot tips of
19 cocoyam. *C. R. Acad. Sci. Paris, Sciences de la vie/Life Sciences* 318, 773-778.
- 20 Nyochembeng, L. and S. Garton. 1998. Plant regeneration from cocoyam callus derived
21 from shoot tips and petioles. *Plant Cell, Tissue and Organ Culture* 53, 127-134.
- 22 Nyochembeng, L. and S. Garton. 1998. Plant regeneration from cocoyam callus derived
23 from shoot tips and petioles. *Plant Cell, Tissue and Organ Culture* 53, 127-134.
- 24 Omokolo, N. D., N. G. Tsala, G. Kanmegne, and A. P. Balange. 1995. Production of
25 multiple shoots, callus, plant regeneration and tuberization in *Xanthosoma*
26 *sagittifolium* cultured in vitro. *C R Acad Sci.*, 318:773–778
- 27 Omokolo, N. D., T. Boudjeko, T. J. J. Tsafack. 2003. In vitro tuberization of *Xanthosoma*
28 *sagittifolium* (L.) Schott: effects of phytohormones, sucrose, nitrogen and
29 photoperiod. *Sci Horti* 98:337–345. doi:10.1016/s0304-4238(03)00066-9
- 30 Onokpise, O. U., J. G. Wutoh, X. Ndzana, J. T. Tambong, M. M. Mebeka, A. E. Sama,
31 Nyochembeng L, Agueguia A, Nzietchueng S, Wilson JG, Borns M (1999)

1 Evaluation of macabo cocoyam germplasm in Cameroon. In: Janick J (ed)
2 Perspectives on news crops and news uses. Ashs Press, Alexandra VA USA, pp
3 394–396.

4 Onokpise, O.U., J.T. Tambong, L. Nyochembeng and J.G. Wutoh. 1992.
5 Acclimatization and flower induction of tissue culture derived cocoyam
6 (*Xanthosoma sagittifolium* Schott) plants. *Agronomie*, 12: 193-199.

7 Onokpise, O.U., M.M. Meboka, and A.S. Eyango. 1993. Germplasm collection of
8 macabo cocoyams in Cameroon. *African Tech. Forum* 6:28–31.

9 Onwueme, I. C. 1978. The tropical tuber crops: yams, cassava, sweet potato, cocoyams.
10 John Wileys and sons Ltd, U. K., 234p.

11 Onwueme, I.C. and W. B. Charles. 1994. Cultivation of cocoyam. In: Tropical root and
12 tuber crops. Production, perspectives and future prospects. FAO Plant Production
13 and Protection Paper 126, Rome, pp. 139-161.

14 Ozyigit, I.I. 2008. Phenolic changes during *in vitro* organogenesis of cotton (*Gossypium*
15 *hirsutum* L.) shoot tips. *African Journal of Biotechnology* 7:1145-1150.

16 Pacumbaba, R. P., J. G. Wutoh, A. E. Sama AE, J. T. Tambong and L. M.
17 Nyochembeng. 1992. Isolation and pathogenicity of rhizosphere fungi of
18 cocoyam in relation to the cocoyam root rot disease. *J. Phytopath.* 135: 265–273.

19 Pal, S.P., I. Alam, M. Anisuzzaman, K.K. Sarker, S. A. Sharmin, M.F. Alam. 2007.
20 Indirect organogenesis in summer squash (*Cucurbita pepo* L.). *Turk. J. Agric.*
21 *For.* 31:63-70.

22 Purseglove, J. W. 1985. Tropical Crops: Monocotyledons (p 97-117) Longmans, London.

23 Sarabi, B. and K. Almasi. 2010. Indirect organogenesis is useful for propagation of
24 Iranian edible wild asparagus (*Asparagus officinalis* L.). *Asian Journal of*
25 *Agricultural Sciences* 2: 47-50.

26 SAS Institute. 2006. SAS/STAT user's guide. SAS Institute, Cary, N.C.

27 Scowcroft, W.R. 1985. Somaclonal variation: the myth of clonal uniformity. In: Hohn B
28 and E.S. Dennis (eds.) *Plant Gene Research: genetic flux in plants* (pp 215–245).
29 Springer-Verlag, New York.

1 Sefa-Dedeh S. and K. E. Agyir-Sackey. 2004. Chemical composition and effect of
2 processing on oxalate content of cocoyam *Xanthosoma sagittifolium* and
3 *Colocasia esculenta* cormels. Food Chem 85:479–487.

4 Short, K. C., J. Warburton, and A. V. Roberts. 1987. In vitro hardening of cultured
5 cauliflower and chrysanthemum plantlets to humidity. Acta Hort., 212:329-334.

6 Spence, J. A. 1970. Growth and development of tannia (*Xanthosoma* sp.). In: Tropical
7 root and tubers crops tomorrow 2. Proceedings of the 2nd International
8 Symposium on Tropical Root and Tuber Crops, Honolulu, Hawaii, pp. 47-52.

9 Staritsky, G., A. J. Dekkers, N. P. Louwaars and E. A. Zandvoort. 1986. In vitro
10 conservation of aroid germplasm at reduced temperatures and under osmotic
11 stress. In: L. A. Withers and P. G. Alderson (eds.). Plant tissue culture and its
12 agricultural applications, pp. 277-283, Butterworths, London.

13 Stirn S. and H. J. Jacobsen. 1987. Marker proteins for embryogenic differentiation
14 patterns in pea callus. Plant Cell Rep. 6: 50–54.

15 Sujatha, R., L.C. Babu and P.A. Nazeem. 2010. Histology of organogenesis from callus
16 cultures of black pepper (*Piper nigrum* L.). Journal of Tropical Agriculture 41:
17 16-19.

18 Sutter, E. 1988. Stomatal and cuticular water loss from apple, cherry, and sweet gum
19 plants after removal from *in vitro* culture. J. Amer. Soc. Hort. Sci., 113:234-238.

20 Sutter, E. and R. W. Langhans. 1979. Epicuticular wax formation on carnation plantlets
21 regenerated from shoot tip culture. J. Am. Soc. Hort. Sci., 104: 493-496.

22 Sutter, E. and R. W. Langhans. 1982. Formation of epicuticular wax and its effect on
23 water loss in cabbage plants regenerated from shoot-tip culture. Can. J. Bot.,
24 60:2896-2902.

25 Tambong, J.T, X. Ndzana, J. G. Wutoh, J.G. and R. Dadson. 1997. Variability and
26 germplasm loss in the Cameroon national collection of cocoyam (*Xanthosoma*
27 *sagittifolium* Schott (L.)). Plant Genetic Resources Newletters 112, 49-54.

28 Treche, S., Guion, P., 1979. Etude des potentialite's nutritionnelles de quelques
29 tubercules tropicaux au Cameroun. Agron. Trop. 34, 127–146.

30 Tsafack, T. J. J., P. C. Gilbert, A. Hourmant, N. D. Omokolo, M. Branchard. 2009. Effect
31 of photoperiod and thermoperiod on microtuberization and carbohydrate levels in

1 Cocoyam (*Xanthosoma sagittifolium* L. Schott). Plant Cell Tiss Organ Cult. 96:
2 151-159.

3 Tsafack, T. J. J., T. Boudjeko, H. D. Mbouobda, N. D. Omokolo .2004. Effect of nitrogen
4 nutrition on in vitro tuberization of *Xanthosoma sagittifolium* L Schott
5 (Cocoyam). J Cam Acad Sci 4:337–344

6 Verma, V.M. and J.J. Cho. 2010. Plantlet development through somatic
7 embryogenesis and organogenesis in plant cell cultures of *Colocasia*
8 *esculenta* (L.) Schott. AsPac J. Mol. Biol. Biotechnol. 18: 167-170.

9 Wardle, K., A. Quinlan, and I. Simpkins. 1979. Absciscic acid and the regulation of water
10 loss in plantlets of *Brassica oleracea* L. var. botrytis regenerated through apical
11 meristem culture. Ann. Bot. 43:745-752.

12 Wardle, K., E. B. Dobbs, K. C. Short. 1983. In vitro acclimatization of aseptically
13 cultured plantlets to humidity. J. Amer. Soc. Hort. Sci., 108:386-389.

14 Watanabe, K.Z. 2002. Challenges in biotechnology for abiotic stress tolerance on root
15 and tubers. JIRCAS Working Reports, 75-83.

16 Wetzstein, H. Y. and H. E. Sommer. 1983. Scanning electron microscopy of in vitro-
17 cultured *Liquidambar styraciflua* plantlets during acclimatization. J. Amer. Soc.
18 Hort. Sci., 108:475-480.

19 Zhao, Y., B.W.W. Grout and P. Crisp. 2003. Inadvertent selection for unwanted
20 morphological forms during micropropagation adversely affects field
21 performance of European rhubarb (*Rheum rhaponticum* L.). Acta Hort. 616: 301–
22 308.

23 Zhao, Y., B.W.W. Grout and P. Crisp. 2004. Unexpected susceptibility of novel breeding
24 lines of European rhubarb (*Rheum rhaponticum* L.) to leaf and petiole spot
25 disease. Acta Hort. 637: 139–144.

26 Zhao, Y., B.W.W. Grout and P. Crisp. 2005. Variation in morphology and disease
27 susceptibility of micropropagated rhubarb (*Rheum rhaponticum*) PC49, compared
28 to conventional plants. Plant Cell, Tissue Organ Cult. 82: 357–361.

29 Zhao, Y., Y. Zhou and B. W.W. Grout. 2006. Variation in leaf structures of
30 micropropagated rhubarb (*Rheum rhaponticum* L.) PC49, Plant Cell, Tissue and
31 Organ Culture (2006) 85: 115–121.

- 1
- 2

1 Table 1. Analysis of variance with mean squares and treatment significance of the effect
2 of different cocoyam plant source and leaf surface on stomatal frequency and index and
3 the effect of plant source on epicuticular wax content on cocoyam leaves and the effect of
4 acclimatization procedures under different durations during summer on leaf damage, leaf
5 wilting, leaf initiation and leaf shedding of tissue culture-derived cocoyam plants.

Source	DF	Mean squares	P-value*
Stomatal frequency:			
Plant source (S)	2	1052.3	0.001
Leaf surface (F)	1	935.0	< 0.0001
S x F	2	2251.0	< 0.0001
Rep	41	10222.0	0.35
Stomatal index:			
Plant source (S)	2	152.3	0.003
Leaf surface (F)	1	235.0	< 0.0001
S x F	2	241.0	< 0.0001
Rep	41	952.0	0.44
Cuticular wax:			
Plant source	2	4321.0	< 0.0001
Rep	41	789.0	0.70
Leaf damage:			
Acclimatization treatment (T)	3	1252.3	0.005
Duration (D)	2	1335.0	< 0.0001
T x D	6	3251.0	< 0.0001
Rep	41	15222.0	0.14
Leaf wilting:			
Acclimatization treatment (T)	3	252.3	0.001
Duration (D)	2	335.0	< 0.0001
T x D	6	351.0	< 0.0001
Rep	41	1522.0	0.54
Leaf initiation:			
Acclimatization treatment (T)	3	211.0	0.01
Duration (D)	2	195.0	< 0.0001
T x D	6	466.0	< 0.0001
Rep	41	952.0	0.24
Leaf shedding:			
Acclimatization treatment (T)	3	122.0	0.015
Duration (D)	2	95.0	< 0.0001
T x D	6	166.0	< 0.0001
Rep	41	789.0	0.90

6 *Significant at $P \leq 0.05$

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28

Figures captions

Fig. 1. A morphological comparison between conventionally-propagated and tissue cultured (3 months in greenhouse) cocoyam plants growing in the greenhouse.

Fig. 2. Stomatal frequencies (A) and indices (B) of the leaves of in vitro, acclimatized and conventionally propagated cocoyam. Columns labeled with the same letter are not significantly different at $P = 0.05$ using Multiple Range Test for plant source comparison at abaxial and adaxial surfaces. Vertical bars at the top represent standard errors.

Fig. 3. Stomatal frequency on the abaxial surface of different sources of cocoyam plant as indicated by photomicrographs of the leave imprints (X 600). A. in vitro plants, B. acclimatized plants and C. conventionally-propagated plants.

Fig. 4. Effect of plant source on epicuticular wax formation on cocoyam leaves. Columns labeled with the same letter are not significantly different at $P = 0.05$ using Multiple Range Test for plant source comparison. Vertical bars at the top represent standard errors.

Fig. 5. Effect of acclimatization procedures during summer on leaf damage (A), leaf wilting (B), leaf initiation (C) and leaf shedding (D) of tissue culture-derived cocoyam plants after different durations. Columns labeled with the same letter are not significantly different at $P = 0.05$ using Multiple Range Test for treatment effect comparison at different durations. Vertical bars at the top represent standard errors.



Fig. 1

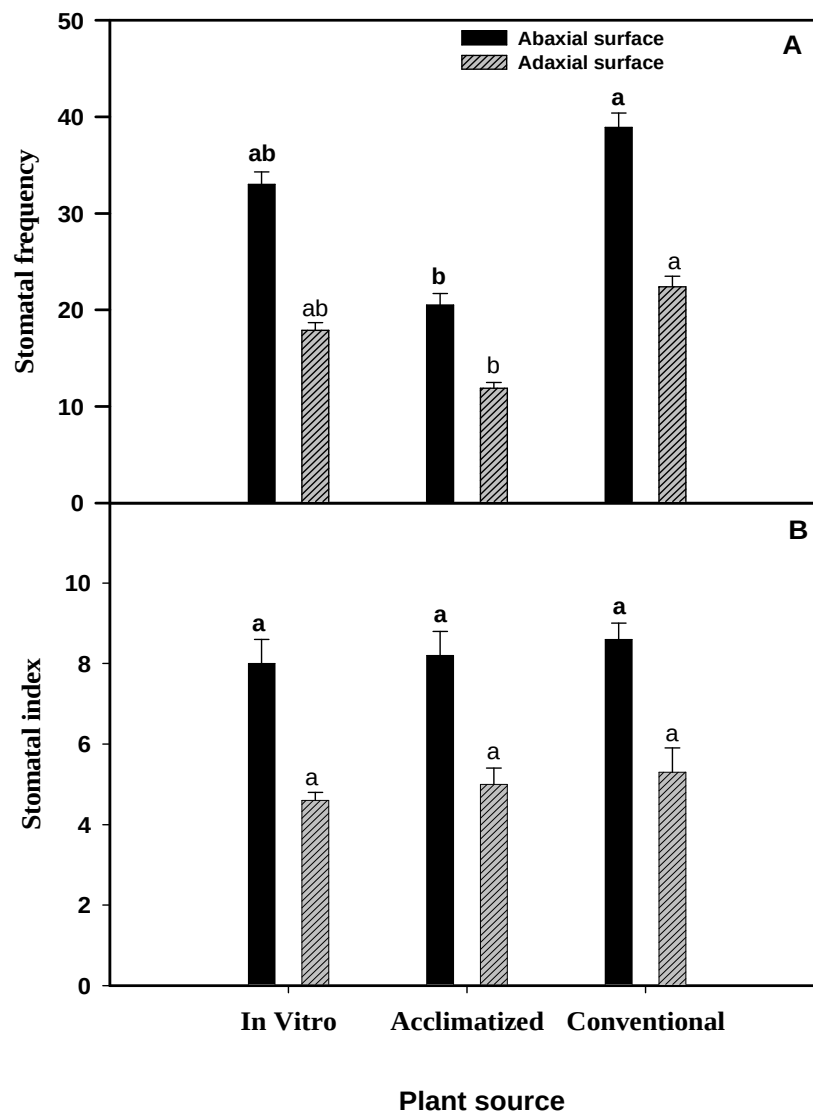
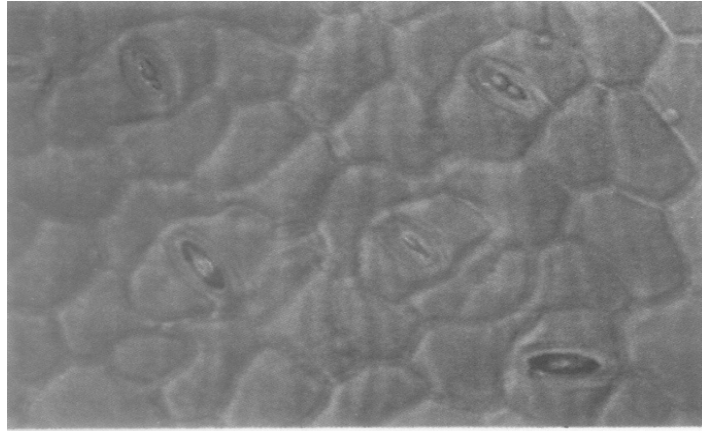


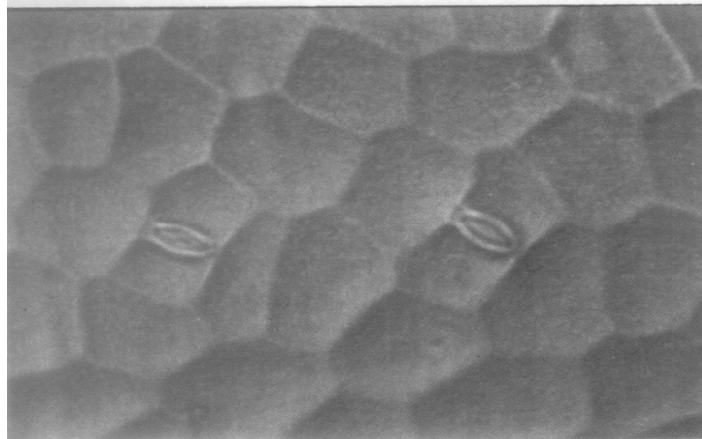
Fig. 2

1
2
3
4
5
6
7
8

A



B



C

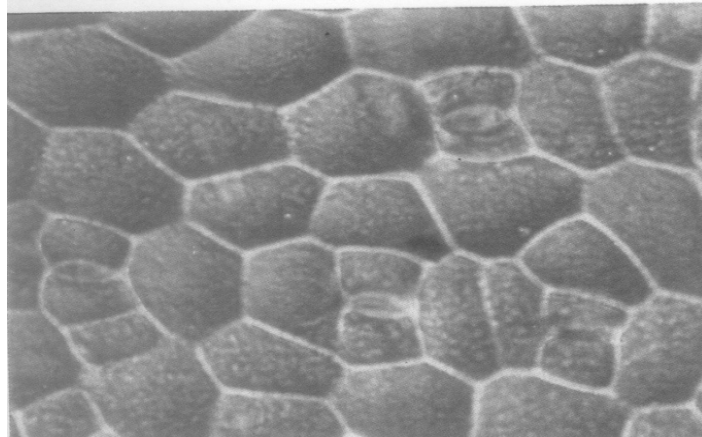


Fig. 3

1
2
3
4
5

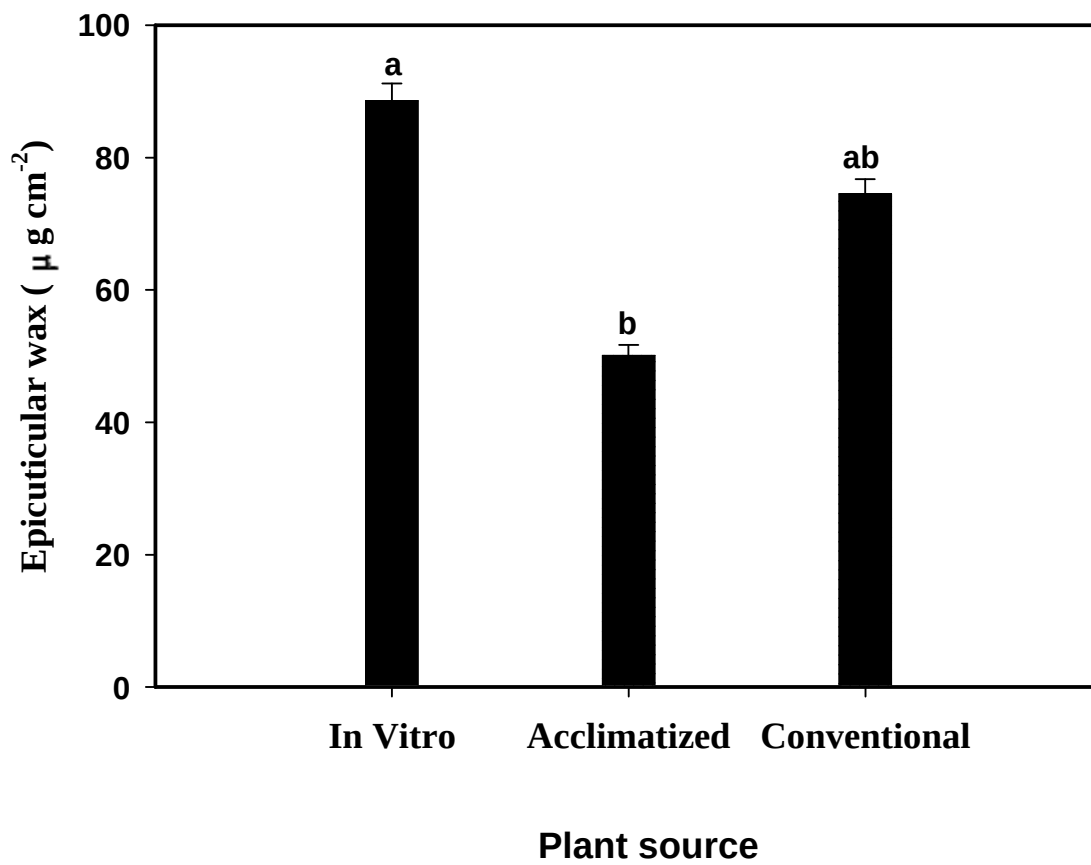
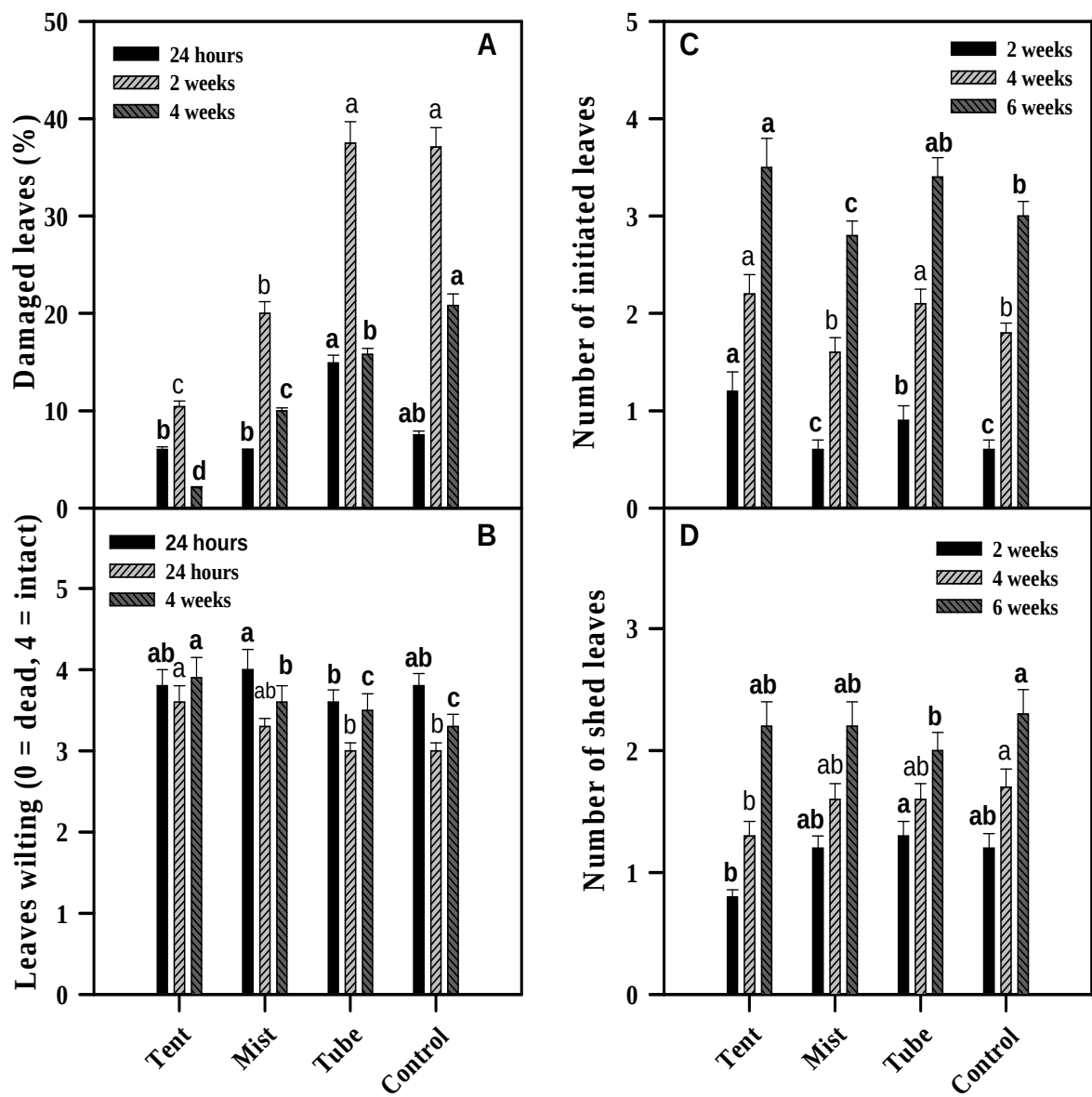


Fig. 4

1
2
3
4
5
6
7
8
9
10
11



Acclimatization treatment

1
2
3

Fig. 5