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Comparative Growth Analysis and Acclimatization of Tissue Culture Derived Cocoyam (*Xanthosoma sagittifolium* L. Schott) Plantlets

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AES and HGH designed the study and collected the data. Authors MAS and MSA managed the statistical analysis of the data and wrote the first draft. Author MSA managed the literature searches. Author MAS managed the final report writing while Author HGH managed the final editing. All authors read and approved the final manuscript.

Original Research Article

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

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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.

Keywords: Tissue culture; cocoyam; epidermal cells; epicuticular wax; stomatal frequency; stomatal index; acclimatization.

ABBREVIATIONS

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

1. 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 [1]. It is a staple food in the tropics and subtropics and one of the six most important root and tuber crops world-wide [2]. The corm, cormels, and leaves of cocoyam are an important source of carbohydrates for human nutrition, animal feed [3-5] and of cash income for farmers [6]. Africa produces about 75% of the world production which is about 0.45 million tons [7]. Cocoyam breeding and production is labor intensive and requires large amounts of water [8]. It is highly susceptible to diseases such as cocoyam root rot disease caused by *Pythium myriotylum* [9] and Dasheen Mosaic virus found in the leaves, corm and cormels [10].

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 [11]. 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 [12] 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 [13].

The biochemical aspects of induction of in vitro organogenesis have been investigated in a number of plants including carrot [14], pea [15], summer squash [16,17], winter squash [18], soybean[19], taro [20], watermelon [21], groundnut [22], asparagus [23], black pepper [24],canola[25], cotton [26], date palm [27], lentil [28], common bean [29], sunflower [30], rice [31] and banana [32]. 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 [33]. 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 [34]. 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 [35]. 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.

2. MATERIALS AND METHODS

2.1 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 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± 2°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.

2.2 Basal Medium (BM)

A modified Gamborg's B5 mineral salts [36] supplemented with 0.05 µM 1-naphthaleneacetic acid (NAA) was used throughout the study. The modified component of B5 micro-salts was MnSO$_4$.4H$_2$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°C.

2.3 Acclimatizationof 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°C and 63% respectively. High and low temperatures averaging 26 and 16°C respectively with corresponding relative humidities of 54% and 94%. During winter, the average greenhouse relative humidity was 40 ± 5%, and the temperature was 22 ± 3°C, and light intensities of approximately 400 µmol m^{-2} 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.

2.4 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 [37].

3. RESULTS

3.1 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.

3.2 Stomata in Tissue Culture-Derived and Conventionally-Propagated Plants

3.2.1 Stomatal frequency (SF)

Cocoyam leaves from all sources were amphistomatic. Almost twice as many stomates were found on the abaxialsurface (Fig. 2A). Analysis of variance indicated that SF (the number of stomates per mm^2) was significantly greater in the non-micropropagatedcontrol 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.

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

Table 1. Analysis of variance with mean squares and treatment significance of the effect of different cocoyam plant source and leaf surface on stomatal frequency and index and the effect of plant source on epicuticular wax content on cocoyam leaves and the effect of acclimatization procedures under different durations during summer on leaf damage, leaf 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

Significant at $P \leq 0.05$

3.2.2 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 x 100 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.

3.3 EpicuticularWax 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 plantlets cultured in vitro were found to have greater deposits of EW. Gravimetric determination showed that in vitro leaves had an average of 88.6 µg/cm2, as compared to 50.1 µg/cm2 for plantlets transferred and grown in the greenhouse (Fig. 4).

3.4 Ttissue Culture-Derived Plantlets Behavior and Adaptation to Different Environmental Factors

3.4.1 Summer acclimatization

3.4.1.1 Effects on leaves

Analysis of variance indicated a significant difference among acclimatization procedures on leaf damage and leaf wilting (Table 1). Cocoyam plantlets showed no significant visual desiccation

one hour after transfer to the open bench. After 24 hours, differences were observed in leaf wilting among different treatments. Plantlets acclimatized by uncapping the culture tube showed 14.9% leaf injury after 24 hours of acclimatization compared to only 0.6% in plantlets acclimatized under mist (Fig. 5A). On the other hand, wilting assessment indicated that plantlets acclimated under mist suffered less wilting after 24 hours (Fig. 5B). One week after acclimatization, more wilting was observed as the percentage of damaged leaves increased significantly for open tube acclimatization (43.7 %) as compared to that from the humidity tent which had a damage percentage of 15.9%. Wilting assessment of 2.8 and 3.5 was associated with the previous leaf damage percentages respectively (Fig. 5A and B).

3.4.1.2 Effects on growth habit

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

3.5 Winter Acclimatization

3.5.1 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 fromculture 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.

3.5.2 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).

4. 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 [38,39]. In similar findings, Donnelly and Vidaver, [40] 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 [41] 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 [42]. On the other hand, Brainerd et al. [43] 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 *Linquidambar styraciflua* [44,45], and apple [41].
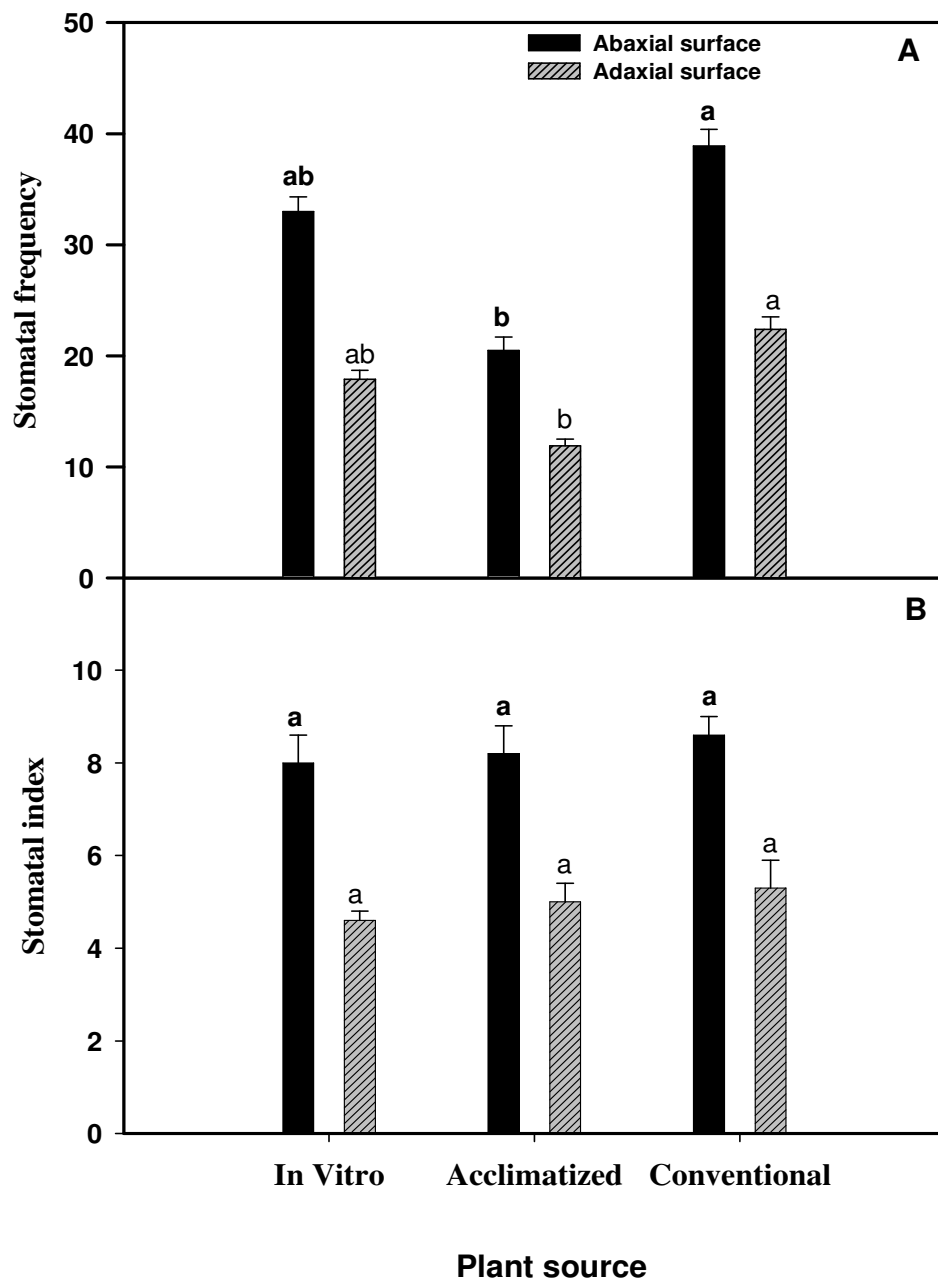
Fig. 2.Stomatal frequencies (A) and indices (B) of the leaves of in vitro, acclimatized and conventionally propagated cocoyam. Columns labeled with the same letter are not significantly different at *P* = 0.05 using Multiple Range Test for plant source comparison at abaxial and adaxial surfaces. Vertical bars at the top represent standard errors
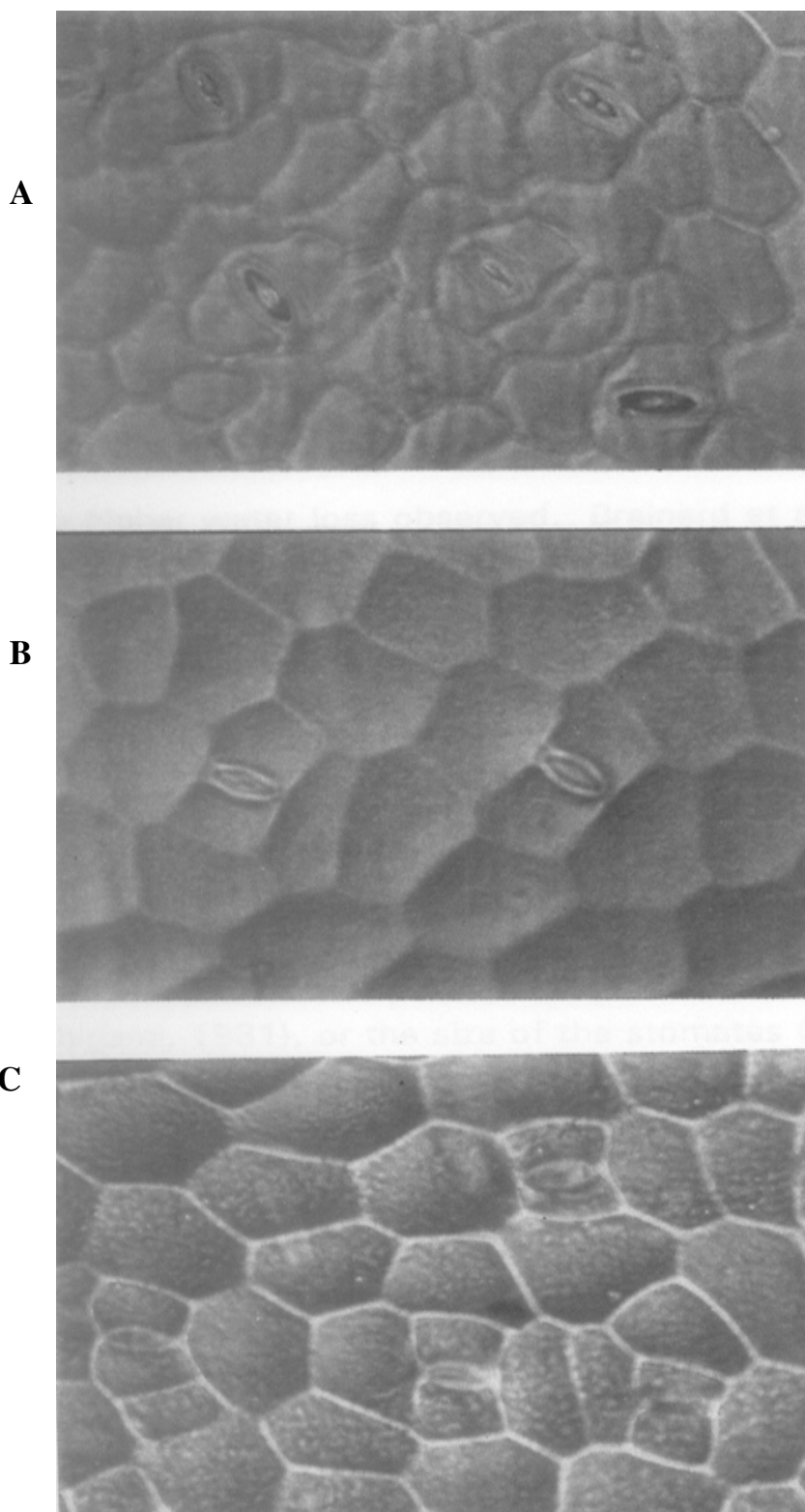
Fig. 3. Stomatal frequency on the abaxial surface of different sources of cocoyam plant as indicated by photomicrographs of the leave imprints (X 600). A. in vitro plants, B. acclimatized plants and C. conventionally-propagated plants
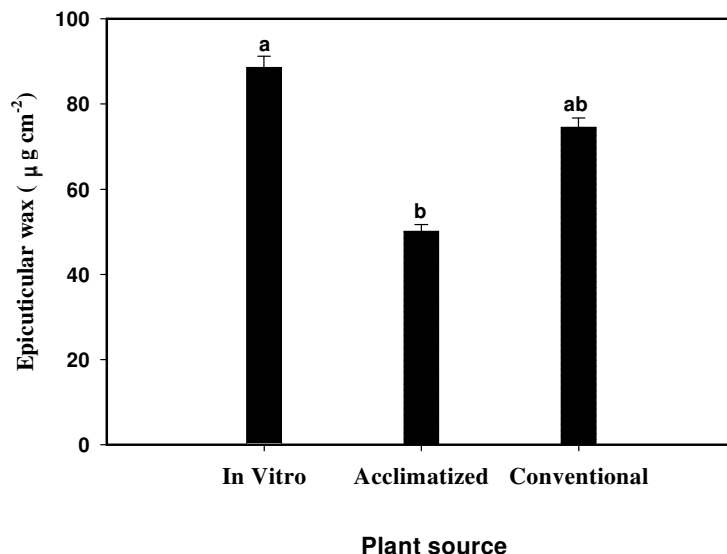

Fig. 4. Effect of plant source on epicuticular wax formation on cocoyam leaves. Columns labeled with the same letter are not significantly different at *P* = 0.05 using Multiple Range Test for plant source comparison. Vertical bars at the top represent standard errors

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* [40], 184.5 (adaxial only) for *Vitis sp.* 'Valiant' [46]. 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 [47], 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 [47] or the size of the stomates [45]. Wetzstein and Sommer [45] found that stomata were also larger in vitro plantlets of sweet gum, in addition to their greater densities. As indicated by Brainerd and Fuchigami [47] and [48], 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* [48], *Rosa multiflora* [49], and Vitis sp. 'Valiant' [46] in comparisons made between in vitro and field grown plants. Dami [46] 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 [48, 40, 46]. However, Zhao et al. [38] found that micropropagatedregenerants 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 rhaponticum L.*).

Quantitative variation is frequently found among regenerants derived from tissue culture and often indicates alteration of numerous loci [50]. Quantitative variation has been described for many phenotypes including plant growth habit and agronomic performance [50-52]. 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 [53-55]. 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 [56,57].

Cocoyam plantlets cultured in vitro were found to have greater deposits of EW. Apparently, there was lees wax deposits per unit area after transplantation. Sutter [58] 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 [58,59]. 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 [60], carnation [61], cabbage [61], strawberry [42], chrysanthemum [58], and grape [46]. Wax deposition after planlet transplantation occurs with time. Wax formed after 10-14 days in *Brassica oleracea* [62,63] and 17/18 days in carnation [61]. Fabri et al. [42] 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 [48]. 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.
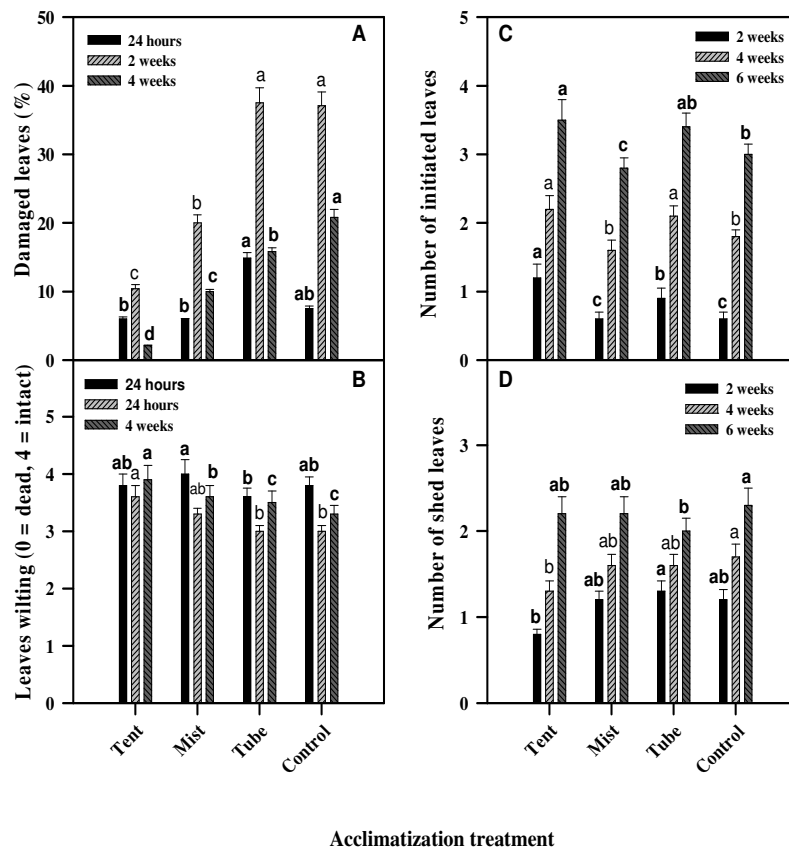

Fig. 5. Effect of acclimatization procedures during summer on leaf damage (A), leaf wilting (B), leaf initiation (C) and leaf shedding (D) of tissue culture-derived cocoyam plants after different durations. Columns labeled with the same letter are not significantly different at *P* = 0.05 using Multiple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

Sutter and Langhans [61] and Wezstein and Sommer [45] indicated that the environment in which a plant grows determines its morphology and chemical composition. The in vitro conditions in which cocoyam plantlets were grown seemed favorably for EW formation. This high EW content may have contributed to plantlet survival upon transfer ex vitro. On the other hand, Brainerd and Fuchigami [47] and Conner and Conner [48] showed that EW was less important than stomates in determining the amount of water loss in plants. The sunken and ellipsoidal stomata of cocoyam leaves in vitro, in addition to their high EW content, may have been invaluable in conferring plantlet survival. The low wax content of acclimatized plants may have been caused principally by the rapid leaf expansion that supressed wax formation. The environmental conditions were not optimum [45,61], but did favor wax formation in vitro. Another possible cause for the high amounts of EW observed in vitro may have been the dissolution of internal lipids from open stomata of in vitro plants to close upon removal from culture [45,47,48]. This could be true for cocoyam. It could also relate to the fact that cocoyam typically grows in high humidity, and thus may have wax production even under high humidities.

The relatively poor growth performance of plantlets acclimatized by mist system may be attributed to the wet conditions they were subjected to. Griffis et al. [64] reported that nutrients are leached under a misting system, and that the wetness creates an environment favorable for microorganism growth. Cocoyam, unlike taro, cannot withstand water-logging under natural conditions [8,65,66]. Continuous misting for a period of five days, in addition to the high humidity, may have been too wet to ensure normal growth. However, the overall trend was that more leaves were produced than shed. Reduction in growth upon transplanting of tissue culture plantlets has been frequently reported in the literature [62,67,68].

The number of leaves shed was comparatively lower than that encountered from non-tissue culture derived plants under field conditions [69]. This could be due to the use of growth regulators while in culture. Spence [69] observed that field grown cocoyam plants were wasteful in the manner in which they produced and maintained their leaves, and suggested the use of growth regulators to alleviate the shedding. The continuous turnover of large leaves reduced photosynthetic productivity of the plants.

The ability to successfully transfer cocoyam plantlets from culture at a relatively low cost with minimal loss is important to the micropropagation technique, especially at the commercial scale. In general, many tissue culture regenerated plants are lost during transfer to normal growth conditions. These losses are associated with rapid water loss and desiccation during the acclimatization phase. Mist systems and humidity chambers are most commonly utilized in an attempt to mitigate plant loss [44]. Short et al. [70] evaluated the success of a micropropagation system by the percentage of plants that are successfully transferred from culture to natural soil conditions.

In this study, all cocoyam plants transferred from culture to in vivo conditions survived, even without acclimatization. Onokpise et al. [71-73], also obtained 100 % survival with different acclimatization studies. Staritsky et al. [74] reported that rootless cocoyam shoots could be easily rooted and would rapidly develop into plantlets when transferred into soil. Acclimatization procedures may be either unnecessary or just advantageous for a short period, especially in areas such as the humid tropics with relatively high humidities. Otherwise, a humidity tent or cheaper method of maintaining a moderately high humidity is recommended, rather than an expensive misting system, in areas with low relative humidities.

The lag in growth in the case of winter acclimatization could be related to the low temperatures, humidity, and lower light intensities in winter conditions within the greenhouse. This probably slowed conversion from heterotrophic to autotrophic nutrition. Tsafack et al. [75] mentioned that the tuberization rate, the number and weight of microtubers and the leaf weight were affected by day length and temperature. Omokolo et al. [76] obtained the highest tuberization rate (83%) of the white cocoyam cultivar with an inductive medium containing 6-benzylaminopurine (BAP) under Short day regime. Tsafack et al [75] confirmed the findings of Gopal et al. [77] and Tsafack et al. [78] who reported that tubers could be induced in vitro without the use of plant growth regulators (PGRs). The use of media without PGRs was important to judge the innate capacity of genotypes to produce microtubers and to avoid the possibility of any undesirable carry-over effect of PGRs on morphogenesis and sprouting.

5. 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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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