Foliar Morphology and Anatomy of *Musa* cv. Grande Naine (AAA) Plants Grown *in vitro* and during Hardening as Compared to Field-Grown Plants

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ABSTRACT

The leaves of banana plants (*Musa* cv. Grande Naine, AAA) were studied by comparing leaves of plantlets grown *in vitro* and during the acclimatization phase with those of adult plants grown in the field. Marked morphological and anatomical differences were observed.
The leaves of *in vitro* plants had thin narrow blades and there was little tissue differentiation. During the hardening phase, changes occurred as each new leaf developed: the cuticle increased in thickness and the different leaf layers began differentiating. Field-grown plants had thicker and typically ovate-oblong leaves. The leaf tissues had differentiated thick cuticles, abundant waxy excretions, many chloroplasts in the palisade layer, and a functional pulvinus.

Morphologie et anatomie de feuilles de plants de *Musa* cv. Grande naine (AAA) multipliés *in vitro*, en condition d'acclimatation et au champ.

RÉSUMÉ

Des feuilles de *Musa* cv. Grande naine (AAA) prélevées sur des plants multipliés *in vitro*, sur des vitroplants en phase d'acclimatation et enfin sur des bananiers au champ ont été analysées. Des différences très marquées ont été mises en évidence aussi bien sur le plan de la morphologie que de l'anatomie.
La feuille *in vitro* présente un limbe mince et des tissus encore peu différenciés.
En phase d'acclimatation, des changements interviennent lors de la formation des nouvelles feuilles : la cuticule s'épaissit et les différentes couches de la feuille commencent à se différencier. Les plants au champ ont des feuilles plus épaisses et prennent une forme typiquement ovale oblongue.
La différenciation des tissus est réalisée : cuticule épaisse et excrétion abondante de cire, chloroplastes nombreux dans le parenchyme et pulvinus fonctionnel.

Morfología y anatomía foliar de plantas de *Musa* cv. Gran enano (AAA) *in vitro*, durante la aclimatación y en el campo.

RESUMEN

Se estudió, en forma comparativa, la morfología y la anatomía foliar de plantas de *Musa* cv. Gran enano (AAA), micropropagadas, en aclimatación y adultas en el campo. Se encontraron marcadas diferencias, en aspectos morfológicos y anatómicos. Las hojas de plantas micropropagadas presentan una cutícula muy delgada y una capa fina de cera epicuticular, otros tezidos son aún poco diferenciados.
Durante la fase de aclimatación se observaron varios cambios, usualmente ocurriendo conforme se desarrolla una nueva hoja. La cutícula es más ancha, con mayor cantidad de cera epicuticular, y se observa una gradual diferenciación del resto de tezidos.
Las hojas adultas son ovalo-oblongas. La diferenciación de tezidos es completa : la cutícula es más gruesa con abondante excreción de cera epicuticular, parénquima de empalizada con abundantes cloroplastos y pulvinulo funcional.

Fruits, vol. 49, n°1, p. 37-46

KEYWORDS
Musa, micropropagation, plant anatomy, leaves, *in vitro* culture, adaptation.

MOTS CLÉS
Musa, micropropagation, anatomie végétale, feuille, culture *in vitro*, adaptation.

PALABRAS CLAVE
Musa, micropropagación, anatomía de la planta, hojas, cultivo *in vitro*, adaptación.

introduction

One common objective of micropropagation is to obtain a very large number of identical (clonal) plants from a selected mother plant. Even when no genetic or epigenetic changes occur (Larkin and Scowcroft, 1981; Scowcroft, 1984; Israeli *et al.*, 1991), *in vitro* conditions can affect the morphology and anatomy of the plantlets (Grout and Aston, 1978; Donnelly *et al.*, 1985; Donnelly and Skelton, 1987; Donnelly *et al.*, 1987), with at least part of the variations still noticeable for some time during the acclimatization phase. However, usually all of these changes disappear completely after the plants are transplanted in the field to complete their development.

The factors that induce and influence these changes have not yet been fully defined but high relative humidity and low light levels are probably of prime importance. Few studies have been carried out in other plants to analyse the possible positive or negative effects which could influence the acclimatization process (Wardle *et al.*, 1983; Wetzstein and Sommer, 1983). Grout and Aston (1977 a and b) conducted investigations with *Brassica oleracea* var. *botrytis in vitro* and found that while the plantlets remained under aseptic conditions, there were clear morphological and anatomical differences as compared to conventionally propagated plants. The *in vitro* plantlets had very little epicuticular wax on their leaf surfaces and vascular connections between roots and aerial parts were inefficient. These *in vitro* plantlets were thus quite vulnerable to desiccation after removal from their culture vessels for acclimatization.

Wetzstein and Sommer (1982) considered that *in vitro* plantlets living in heterotrophic conditions do not need to photosynthesize, but the evidence indicates that photosynthesis occurs at a low rate in such plants. The lack of complete differentiation of the mesophyll in leaves of *in vitro* plantlets suggests a reduced photosynthetic potential. Several factors clearly influence *in vitro* development and the acclimatization phase and additional research is needed to better understand these processes (Brainerd and Fuchigami, 1981 and 1982; Dunstan and Turner, 1984; Short *et al.*, 1984).

In *Musa*, although *in vitro* mutiplication has become an important tool for commercial propagation of this tropical crop plant, there is no information available on morphological and anatomical changes occurring during the process and on factors that affect it.

The present study compares the initial manifestations and subsequent gradual form and structure changes in the leaves during the *in vitro* and acclimatization phases with those of adult leaves from field-grown plants.

material and methods

The present investigation was carried out in the Histology Laboratory of the CATIE Biotechnology Unit, Turrialba, Costa Rica. The plant material consisted of *Musa* cv. Grande Naine (AAA) plants grown *in vitro*, in the acclimatization phase and under field conditions.

in vitro stage

The following procedure was used to obtain *in vitro* plantlets. Suckers of field-grown plants were removed. These were reduced to about 5 cm in height and diameter. They were immersed in a commercial bleach during 20 min and rinsed several times with sterile water. Under aseptic conditions, they were cut down to about 2 cm in size. A second disinfection was carried out with the same bleach, but diluted (10% v/v) by the addition of Tween 20 as detergent. After several rinses, the shoot tips were again reduced to about 5 mm in length and diameter. Before inoculation, explants were submersed for 10 min in

an ascorbic acid solution (100 mg/l) to reduce oxidation (SANDOVAL, 1985; SANDOVAL *et al.*, 1991).

Explants were cultured on inorganic medium MURASHIGE and SKOOG (1962), supplemented with 30 g/l sucrose, 100 mg/l myo-inositol, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine-HCl, 0.1 mg/l thiamine-HCl, 2 mg/l glycine, 1 mg/l 6-benzylaminopurine and 7 g/l agar. The pH was adjusted to 5.7 before 10 ml aliquots were placed into test tubes. Specimens were sterilized at 121°C for 15 min. Incubation was carried out at 27°C ± 2°C, 70% relative humidity with a 16L/8D photoperiod (80 mmol/m^2/s).

The initial phase lasted 30 days. Thereafter the explants were transferred to the same medium but with increased cytokinin content (4 mg/l) where they remained for 60 days. They were then separated into individual buds, which were transferred to the same medium but without hormones to stimulate shoot elongation and root formation. After developing into complete plantlets, occupying most of the space in the tubes, they were ready for the analysis. All anatomical observations were carried out on cross-sections for the central-upper portion of the leaves.

acclimatization stage

After removing plantlets from the aseptic conditions, most of the spent medium was removed with a jet of water and the plantlets were planted in a sterile loamy soil contained in small black plastic bags (about 1 kg soil per bag). They were immediately placed in the greenhouse for a few days under constant conditions, followed by intermittant misting, for a total of some 35 days. They were then transferred to the open greenhouse space. The greenhouse had a plastic roof allowing about 85% of light to pass. Diurnal temperature was usually 32-35°C and close to 22-25°C at night. Plants were watered daily, and the relative humidity in the greenhouse was maintained at near 80% during the day, reaching the dew point at night. Observations were carried out for about 300 days during acclimatization.

field material

This material was obtained from adult *Musa* plants cv. Grande Naine (AAA) from the CATIE variety collection. Sub-apical portions (about 25 cm from the tip) of completely developed leaves were evaluated for the anatomical study.

light microscopy

Besides the observations on fresh material, the bulk was assessed on permanent slides according to the SASS guidelines (1958); safranine-fast green was used for staining and Permount (® Fisher Scientific Co.) to adhere the cover slips.

scanning microscopy

The material was fixed in FAA (formol - alcohol - acetic acid) for 48 h, passed through a series of ethanol-water mixtures and completely dehydrated with CO_2 in a Hitachi HCP-2 desiccator. After mounting the specimens with silver fixative on aluminum supports, they were shadowcasted with gold (Eiko IB-3). Specimens were examined under a Hitachi HHS-2R scanning microscope. The photos were taken on Kodak Verichrome VP 120 film.

results and discussion

morphology and anatomy of vitroplant leaves

Since *in vitro* cultures are initiated with explants from which most of the leaf primordia had been removed, the new leaves formed in culture first originated from the remaining primordia and thereafter from the apical meristem.

The first leaves which developed after the initial stage of culture were quite atypical. The leaf blade was reduced to a small appendix of the relatively wide sheath (hypopodium), which enveloped the entire bud. With each new leaf formed,

the blade portion increased gradually until well formed, with an extended tip of living cells. Under *in vitro* conditions, the hydathodes (passive) in the leaf tip did not become active due to the high relative humidity in the vessel. There was no true petiole even after many new leaves had been produced. Only a very slight constriction between the lamina and sheath provided a clue to the location where one would form in later stages. There was no pulvinus (tissues composed of large pigmentless parenchymatous cells in the lower epidermis near the midrib allowing folding of half blades along the midrib).

The leaf cross-sections showed the classical features of monocotyledons, with certain differences (Photo 1). The cuticle was very thin. Only small deposits of epicuticular waxes were seen under the scanning electron microscope (Photo 2). Similar results have been reported by other authors for several different species (Grout, 1975; Grout and Aston, 1977a; Sutter and Langhans, 1979 and 1982). The relatively small amount of epicuticular waxes could result from the high relative humidity and low light intensity. Sutter and Langhans (1979) concluded that, besides the presence of growth regulators, high culture temperatures could affect the wax deposits. However, not all species showed marked reduction of the wax layer under *in vitro* conditions, and in these cases survival after transplant was not always correlated with the wax layer. Plantlet death during acclimatization could also be related to inefficient stomate function (Grout, 1975).

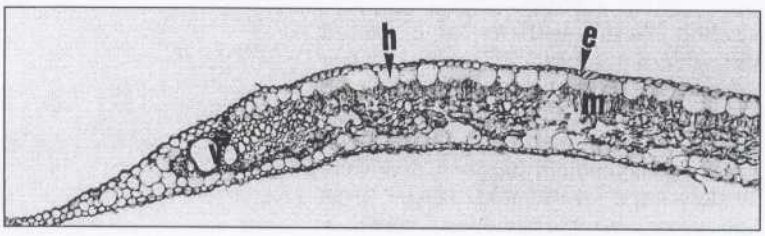


Photo 1
Cross section of a leaf from a micropropagated plant (cv. Grande Naine); e, epidermis; h, hypodermis; m, mesophyll into palisade; v, rudimentary vascular bundle.

The epidermis was found to be one-layered and thin-walled. Their cells were sinuous and sometimes irregular in size. They were oriented in the same direction as the leaf axis. The hypodermis was formed by larger cells perpendicular to the orientation of the epidermal cells. There was frequently only one layer and above the vascular bundles the cell size was reduced and the cells were more cubic (Photo 1).

The *in vitro* leaves of Grande Naine are amphistomatic (stomates in upper and lower epidermis of the leaf), with a much higher stomate density on the abaxial side (lower epidermis). The average stomatal size was 38 µm in length and 15 µm in width. The guard cells were surrounded by groups of 4 subsidiary cells. Most stomates showed partially or completely closed ostioles, indicating little or no functioning.

The mesophyll had not yet differentiated into palisade and spongy parenchyma (Photo 1). Both layers formed an almost uniform tissue of chlorenchyma (parenchymatous tissue with chloroplast capable of photosynthesis). Only in the later leaves a so-called one-layered palisade, due to the slightly elongated form of the cells composing it, was observed. There were few chloroplasts in the mesophyll.

The vascular bundles were quite rudimentary with only a few phloem elements and some xylem vessels, indicating the reduced need for transport under aseptic conditions. Usually only a few collenchymatous cells were associated with the bundles and some sclerenchyma fibers were only found in well formed later leaves. The first to appear were located in the mesophyll close to the marginal vascular bundle. Others appeared in the vascular bundles near the outer surface of the sheaths. The very reduced presence of mechanical tissue could be explained by the fact that the leaves inside the vessels were not subjected to mechanical strain.

morphology and anatomy of acclimatized plant leaves

Care must be taken to avoid desiccation of the tender leaves, when transplanting from *in vitro* conditions to soil. However, even when placed immediately under misting conditions the older leaves

produced under aseptic conditions did not live long and were soon replaced by new ones formed at the onset of hardening. Whereas wilting and loss of *in vitro* formed leaves after transfer from the sterile environment may be fatal in species with exposed meristem, in *Musa* the position of the meristem within sheath bases almost always permits full recovery of the plantlets if placed under optimum conditions.

The first leaves formed under the new condition closely resembled the last ones produced under the prior conditions. The petiole was still not very well developed. However due to the large number of stronger leaves, the diameter of the pseudostem increased thus producing a wider sheath which in turn caused a stronger restriction. In the plants studied, formation of a true petiole was only noted for leaf number five. Although the leaf blades were relatively slender at the beginning, they gradually became wider, especially in their middle portion, resulting in a pronounced oval shape. These only gradually acquired the typical ovate-oblong form of leaves of adult plants after transplanting to the field, with formation of many more leaves (Skutch, 1927). Whereas the first leaves formed were still almost perfectly symmetrical, the blade bases were asymmetrical in the later leaves. The development and unfolding of new leaves did not generally differ from the process in adult plants.

The appendix of leaf tips of the younger leaves under acclimatization had a tendency to die but otherwise remained intact, without any typical rupture of the leaf tip which always occurs in adult plants (Skutch, 1927). When the plants were kept under high humidity conditions, the passive hydathodes located in the leaf tip became very active.

Under *in vitro* conditions and during the first part of acclimatization, all leaves were uniformly green. When placed in strong light, the leaves became darker green due to differentiation of the mesophyll and the increased pigment content.

Leaves of plants after less than a month of acclimatization still showed many of the anatomical features of *in vitro* leaves.

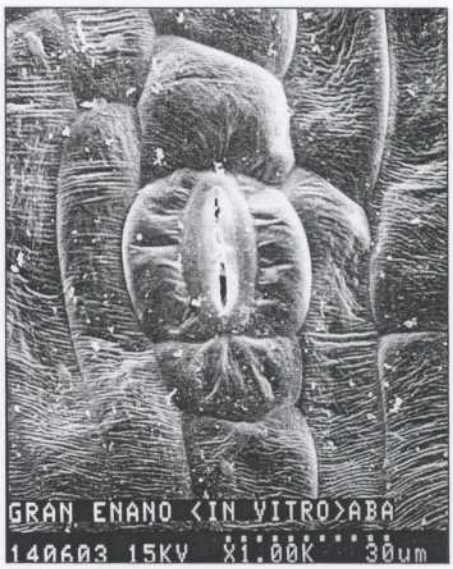
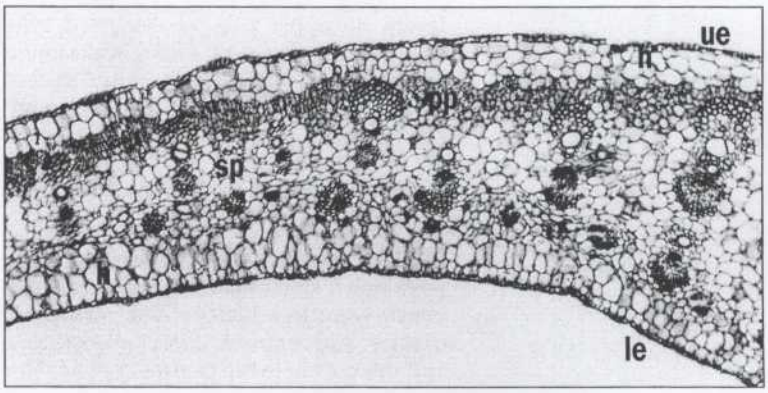


Photo 2
Leaf surface (abaxial side) of the plant under in vitro *conditions. The surface streaked epidermal cells show very little epicuticular wax (cv. Grande naine).*

The cells were more elongated in the palisade parenchyma, thus close to their typical form. There was only one distinctive layer. Contrary to later leaves, the mesophyll did not yet have any large intercellular spaces and extended from one vascular bundle to the next without interruption. The vascular bundles then consisted of more elements, including collenchyma and sparce sclerenchyma fibers (Photo 3). Droplets of water stayed on the surface of the leaves without wetting them, suggesting the presence of a waxy layer. Under the scanning microscope we noted that the waxy excretions had no definite pattern but more or less uniformly covered the leaf surface, being more conspicuous on the lower surface (Photo 4). The waxy layer became gradually thicker with each new leaf formed.

Photo 3
Cross section of a Musa *cv. Grande Naine leaf near the midrib, after three months under acclimatization;*
ue, upper epidermis;
le, lower epidermis;
h, hypodermis;
pp, palisade parenchyma;
sp, spongy parenchyma.



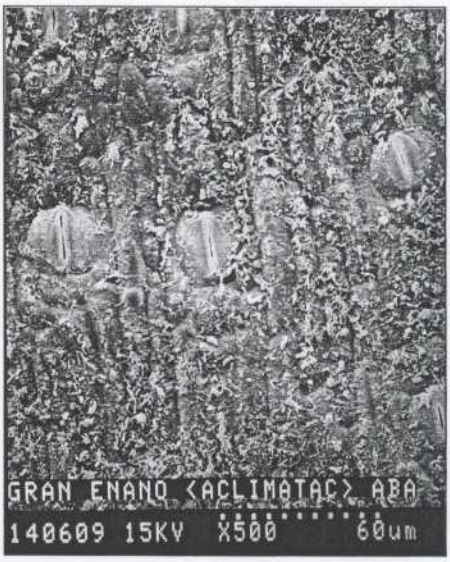


Photo 4
Leaf surface (abaxial side) of the plant during the hardening phase. On the epidermal surface we observe stronger formation of epicuticular wax layer (cv. Grande Naine).

In *Musa*, the relatively small quantity of epicuticular wax found in the first leaves formed under acclimatization conditions, as compared to those grown in the field, suggests the influence of the high humidity and high temperature conditions in the greenhouse. In greenhouse plants, no wax was ever found to cover the stomates, a fact which could negatively affect the gas exchange (Freeman and Turner, 1985). The wax is formed in the periclinal region of the cuticle and its synthesis occurs in the epidermal cells, then passing through the cuticle to the outer surface. However, the exact mechanism is still unknown (Freeman and Turner, 1985).

After three months, the leaf cross-sections showed thick sclerenchyma strands around the vascular bundles. The palisade parenchyma consisted of two layers, an upper layer of elongated cells and a lower layer of more isodiametric cells. There were large airy spaces resembling small cavities in the spongy parenchyma. However, these were not yet as pronounced as in adult leaves, so no real compartmentation had occurred by this point (Photo 3).

After 5 months, the hypodermal cells were much larger than the epidermal cells which were less sinuous. The mesophyll cavities had become more pronounced and there were many oxalate crystals. The palisade tissue was sometimes already composed of three layers, two of elongated cells, followed by a layer of more isodiametric cells. Three types of vascular bundles could be distinguished, although not always very clearly: primary, secondary and tertiary. The primary bundles extended clear to the epidermis, whereas the others had a relatively thick layer of chlorenchyma between them and the hypodermis. Sclerenchyma accompanied all vascular bundles, but was especially noticeable in the primary bundles. All bundles contributed to compartmentation of the mesophyll. An incipient pulvinus was detected. At 6 to 7 months of acclimatization, the leaves still had not yet attained the complete structure of adult leaves. The pulvinus was now well formed on both sides of the lamina near the midrib. This tissue consisted of elongated parenchymatous cells, with very thin walls, oriented perpendicular to the epidermis. There was no hypodermis in this part. The pulvinus tissue extended along both sides of the midrib in the form of a continous band. By osmotic regulation, it allowed folding of the lamina halves along the midrib.

morphology and anatomy of adult leaves

The morphology and anatomy of *Musa* leaves have been well investigated. The exhaustive research of Skutch in 1927 can still be considered as the fundamental work on this subject. The typical leaf of an adult plant is ovate-oblong. Between blade and sheath there is a well defined petiole. However, the transition from one part to the other is always somewhat gradual and not sharp.

When comparing the anatomy of leaves from adult plants with those from *in vitro* plants, especially those undergoing acclimatization, it becomes apparent that differences are basically quantitative, with a much higher level of differentiation (Photo 5).

The leaves of adult plants were found to be much larger and thicker. The entire epidermis was covered with a thick layer of cuticle. The amount of epicuticular

wax was increased, being more evident on the abaxial surface (Photo 6). Wax deposits often followed the outline of the epidermal cells, indicating increased excretion through the walls. Wax deposits also sometimes partially covered the stomates. On average, these measured 27 µm in length and 17 µm in width. The well defined substomatal cavities which extended through the hypodermis to the mesophyll were more noticeable. Both epidermal and hypodermal cells often contained large single crystals. The palisade layer usually consisted of 3 layers of elongated cells and 1 layer of more isodiametric cells. The latex vessels which accompanied the vascular bundles were also more differentiated (Photo 5).

There was much more sclerenchyma accompanying all vascular bundles. Contrary to the conditions under acclimatization, the sclerenchyma was significantly pronounced, especially along the midrib. Although the sclerenchymal strands were sometimes in direct contact with the hypodermis, several layers of colenchymatous cells were intercalated between both especially in tertiary bundles. The phloem consisted of small elements separated from the xylem by colenchyma-like cells. The xylem usually consisted of several large tracheas and various tracheids. The entire bundle was usually surrounded by plastid-free cells in the form of a sheath, which was not yet visible in plants under acclimatization conditions. Since all vascular bundles separated the mesophyll into small areas, with a very large cavity, compartmentation of the leaves was complete (Photo 5).

Although present in later leaves under acclimatization, the typical pulvinular band was only observed in leaves of adult plants. It consisted of elongated cells without pigmentation, thin walls, accompanied by more isodiametric cells above and below.

A summary of the morphological and anatomical characteristics in leaves of *in vitro* plants, in acclimatization conditions and in the field are presented in Table 1.

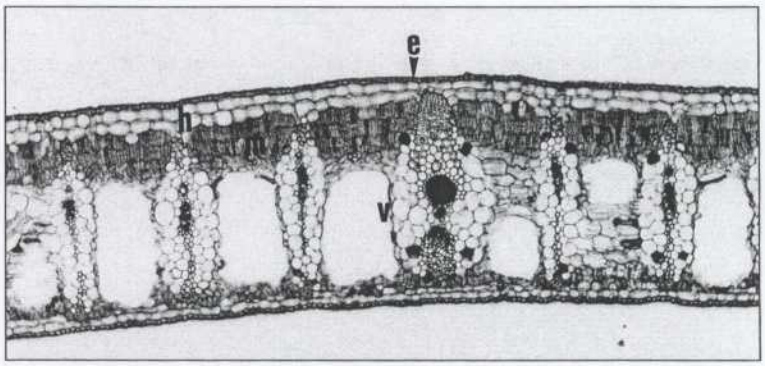


Photo 5
Cross section of the leaf from a field-grown plant (cv. Grande Naine); e, epidermis; h, hypodermis; m, mesophyll into palisade; v, vascular bundle.

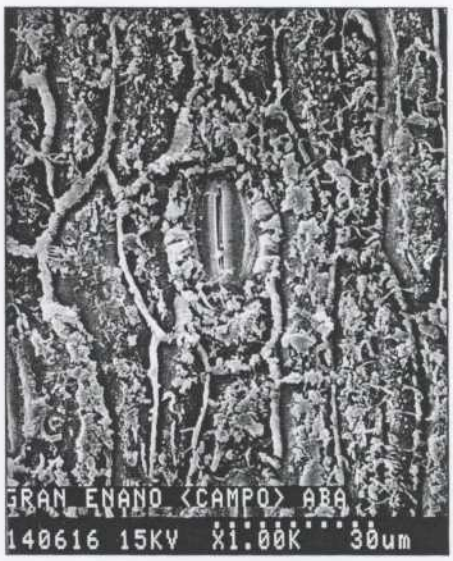


Photo 6
Leaf surface (abaxial side) the field-grown plant with abundant waxy excretion (cv. Grande Naine).

conclusions

The following conclusions were drawn from the results.

in vitro plantlets

The leaf morphology and anatomy was quite different from those of plants undergoing acclimatization and especially field-grown plants.

Leaf formation underwent a similar process to that observed in seedlings: the first leaves had no blade or only one in the form of a small appendage. The complete leaf only gradually emerged but no petiole was formed.

Table 1
Summary of anatomical characteristics in leaves of *in vitro* plants, in acclimatization conditions and in the field.

	In vitro	Acclimatization*	Field
Petiole	Does not exist.	After several leaves noticeable as constriction, then formed.	Well formed.
Pseudostem	Does not exist, sheath only together in lower portion.	Slender, soft.	Tall, very strong support, thick.
Leaf anthocyanin	None.	After a few leaf blades with irregular spots all over laming (characteristic), sometimes in leaf margin.	
Leaf blade	On first leaves only as small appendix, gradually elongating, then lanceolate-elliptical.	Lanceolate-elliptical later ovate.	Ovate-oblong.
Symmetry of base of lamina	Symmetrical.	Latest leaves start to show slight asymmetry.	Pronounced asymmetrical (characteristic).
Cuticule	Extremely thin.	Thin, later thicker.	Thick layer.
Epicuticular wax	Scattered extrusions of small particles on lower epidermis.	Gradually extrusions larger, more close on lower epidermis, on upper slight layer.	Heavy extrusions, very close, sometimes covering stomates on lower epidermis, upper with thicker layer.
Epidermis	Relatively large cells, outer wall thin, slightly arched outward, triated.	Smoother, thicker outer wall.	Smooth, very thick outer wall, cells relative small.
Stomates	Mostly on lower epidermis, four subsidiary cells, irregular arrangement, not all functioning.	On both epidermis, four subsidiary cells, more regular arrangement in rows.	Sometimes irregular number of subsidiary cells, strict arrangement in rows.
Substomatal cavity	Not developed.	Gradually developing.	Well developed, reaching through hypodermis.
Hypodermis	Large cells, thin walls, one continuous smooth layer.	One, much later two layers, relatively smooth, larger cells in upper.	Two or more layers, especially over vascular bundles, where irregular, smaller cells, orientation cross-wise to longitudinal epidermal walls.
Pulvinus	None.	First leaves none, later initiation, not very functional yet.	Well developed, fully functional.
Mesophyll	Not differentiated.	Differentiation starts.	Well differentiated.
Palisade	One layer, isodiametric cells, close contact to spongy parenchyma.	Later two layers, elongated cells, still contact with spongy parenchyma.	Three to four layers, first layers elongated cells, separated from spongy parenchyma.
Spongy parenchyma	Up to two irregular layers.	Two, later three layers, irregularly arranged.	Several layers covering walls of alveoli, separate from palisades.
Chloroplasts	Very few, low pigment content.	Gradually more, darker green.	Cells well filled with dark green ones.
Alveoli	None, only small intercellular spaces.	Later leaves with small ones, especially near midrib, then larger ones.	Large, well defined, separating palisade from spongy parenchyma.

Table 1 *(cont'd)*.
Summary of anatomical characteristics in leaves of *in vitro* plants, in acclimatization conditions and in the field.

	In vitro	Acclimatization*	Field
Vascular bundles	Rudimentary, few small vessels.	Gradually developing.	Well developed, large tracheas, primary, secondary and tertiary, resulting in compartmentation of mesophyll.
Sclerenchyma	None or very occasional fibers associated with bundles in larger leaves.	Gradually increasing number of fibers with vascular bundles, also in midrib.	Large compact strands of fibers, especially on upper part of bundles; also strands not associated with bundles.
Average thickness	200 - 250 µm (largest leaves).	250 - 300 µm.	400 - 500 µm.

* Most changes indicated are very gradual with each new leaf formed during this phase.

The thin cuticle, rudimentary layer of epicuticular wax reduced epidermis with very thin walls, lack of a pulvinus and differentiation of the mesophyll into palisade and spongy layer, absence of cavities in the spongy layer and the reduced chlorophyll content can all be attributed to normal plant development under quite inadequate growing conditions, such as reduced light level, spectral quality of the light, reduced gas exchange and vapour saturated atmosphere.

Similarly, the almost complete absence of sclerenchyma was probably due to the fact that these leaves were not subjected to any mechanical stress.

plants under acclimatization and field-grown plants

All morphological and anatomical changes occurred gradually with the development of each new leaf.

The reduced relative humidity in the greenhouse caused an increase in the thickness of the cuticle and extrusion of more epicuticular wax.

When conditions of high relative humidity and soil moisture prevailed, fast guttation took place through passive hydathodes located only near the tip of the leaves.

The leaf form passed from elliptical through oval to the final ovate-oblong form but required many intermediate leaves and a well developed root system.

Differentiation of the mesophyll and the large intercellular spaces in the spongy parenchyma between vascular bundles gradually led to alveoli and thus compartmentation, typical of leaves from adult plants.

Development of the final leaf form and gradual changes – except for the initial stages under *in vitro* conditions – as well as the typical anatomical features, closely resembled the development of plants from seed (types diploids) and not from suckers. ●

acknowledgements

The first author wishes to thank CATIE for making it possible for him to obtain his Master's degree at this institution. The present work was part of the investigations required for graduation. We thank Nelly Vásquez (Magister Scientiae; Biotechnology Unit, CATIE) for her advice and assistance with the scanning electron microscopy.

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