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Water Loss and Morphological Modifications in Leaves during Acclimatization of Cork Oak Micropropagated Plantlets

Anabela Romano
Faculdade de Engenharia de Recursos Naturais
Universidade do Algarve
Campus de Gambelas
8005-139 Faro, Portugal
aromano@ualg.pt

M.A. Martins-Loução
Faculdade de Ciências
Universidade de Lisboa
Campo Grande, Bloco C2, Piso 4
1749-016 Lisboa, Portugal

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Abstract

This study characterises some aspects of foliar anatomy of cork oak micropropagated plantlets, and rates of water loss by different types of leaves during the acclimatization period. Water loss from leaves of in vitro cultured plantlets was much higher than that of acclimatized plants or seedlings. Leaves from in vitro plantlets lost 53% of their water within the first 30 min under water stress conditions, while leaves from acclimatized plants lost 14%. Leaves of in vitro grown plants presented open stomata and collapsed guard cells, while acclimatized leaves presented closed stomata. Shade-leaf structure was observed in transverse sections of in vitro leaves, with large intercellular air spaces and a low mesophyll cell density, but with a differentiated palisade cell layer. Leaves from acclimatized plants showed a sun-leaf structure with small intercellular air spaces, high cell density and two or three palisade cell layers. During acclimatization leaf thickness increased, as well as cell compactness and differentiation. Stomatal density, stomatal aperture and guard cell protuberance decreased during the acclimatization period, while trichome density increased.

INTRODUCTION

The transfer of plantlets from in vitro conditions to the glasshouse is still problematic for many species, especially woody plants. The main cause of plant mortality during acclimatization is water stress. This results from excessive transpiration from leaves, stomata and cuticle, or from inadequate uptake of water from roots. Various authors have related water loss to abnormal stomata (Donnelly et al., 1985; Blanke and Belcher, 1989; Santamaria et al., 1993). Others related this water loss to reduced thickness and/or anomalous structure of the cuticle (Dhawan and Bhojwani, 1987; Capellades et al., 1990; Diettrich et al., 1992). Anomalous structure of the leaves, altered stomata density and reduced trichome density, are other factors causing water stress during acclimatization (Donnelly et al., 1985; Lee et al., 1988; Capellades et al., 1990). The goal of this work was to study leaf anatomy and water loss during in vitro acclimatization of cork oak micropropagated plantlets.

MATERIALS AND METHODS

Plant Material and Growing Conditions

The procedures and conditions for establishment, proliferation and rooting of shoot cultures, starting from buds of cork oak adult trees, have been described previously (Romano et al., 1992). For rooting, the basal ends of individual shoots 3 cm in length were dipped for 2 min in 2.5 M indole-3-butyric (IBA) acid followed by culture on Gressoff and Doy (1972) growth regulator-free medium. Four weeks after root induction, plantlets were washed to remove agar and planted into a mixture of peat and perlite (3:1). Plantlets were placed in an acclimatization chamber (Aralab 500 E) set at 90-95% relative humidity, a temperature of 25±2ºC, and a 16h photoperiod (100 µmol $m^{-2}s^{-1}$) for four weeks and then placed under glasshouse conditions.

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Comparative Water Loss Analysis

Water loss was studied in six different types of leaves: i) leaves from in vitro grown plantlets at the end of the rooting phase; ii) persistent leaves from 1-month-old acclimatized plants; iii) new leaves from 1-month-old acclimatized plants; iv) new leaves from 6-month-old acclimatized plants; v) leaves from 3-year-old micropropagated plants, and vi) leaves from 3-year-old seedlings, the latter two growing in the field. The leaves were excised and immersed in distilled water (to ensure full leaf turgidity) and kept at 25°C in diffuse light. After 3h under these conditions their surfaces were gently blotted, and they were placed in shallow aluminium cups with the abaxial surface facing up. Water loss from each leaf was estimated by loss of weight. Fresh weight (FW) was taken every 10 min, for the first hour and every 20 min thereafter, for a total period of 160 min. The temperature (21±2°C) and relative humidity (50%) did not vary appreciably throughout the experiment. After these measurements, the dry weight (DW) of the leaves was determined after oven drying at 80°C for 24h. Relative water content (RWC) was estimated at time (t) as follows: RCW (%) = $[(FW_t - DW) / (FW_s - DW)] \times 100$, where FW_t is the fresh weight at time t, FW_s is the initial fresh weight of saturated leaf, and DW is the dry weight. Each sample consisted of a fully developed leaf. For *in vitro* plantlets groups of four leaves were used to reduce errors of small sample size. The experiment was repeated once and each set consisted of five replicates.

Anatomical and Histochemical Studies

Leaf pieces were fixed in a mixture of 3:1:1 ethanol:acetic acid:formalin for 48h, dehydrated in a graded ethanol series and embedded in glycol methacrylate. Sections (6 µm) were prepared and stained in toluidine blue (0.05%, w/v). For scanning electron microscopy (SEM), leaf pieces were fixed and dehydrated as before and critical point dried in a Denton DCP-1 Critical Point Dryer employing liquid CO_2. Specimens were mounted on aluminium stubs, coated with gold-palladium (in a Polaron E 5300 Freeze Drier coupled with a Polaron E 5350) and observed with a JEOL JSM-T100 SEM.

RESULTS AND DISCUSSION

In a cork oak plantlet, three months after transfer to soil, different leaf types are distinguished: i) leaves grown under in vitro conditions, but persisting after acclimatization (persistent leaves); ii) leaves induced during in vitro culture, but developed after transfer to soil, which show intermediate characteristics (intermediate leaves); and iii) leaves differentiated in the glasshouse (new leaves). The rates of water loss observed for the different types of leaves are given in Fig 1. As expected, water loss by leaves from in vitro plantlets was much higher than that of acclimatized plants or seedlings (Fig. 1). Leaves from in vitro plantlets lost 53% of their water within the first 30 min of the dehydration treatment, while leaves from acclimatized plants lost 14% (Fig. 1). New leaves from plantlets after one month of acclimatization lost 14% water after 30 min of holding at 50% relative humidity at room temperature, compared to 29% from old leaves of 1-month-old plantlets. At the end of the dehydration period in vitro leaves presented open stomata and collapsed guard cells, while acclimatized leaves presented closed stomata (Table 1). Stomata of in vitro plantlets had raised, round guard cells compared to normal elliptical, sunken guard cells. Those stomata did not close under water stress conditions, while those of acclimatized plants were closed under these conditions. Stomata of a 1-month-old acclimatized plant had an intermediate response (Table 1). This might explain the poor control of water stress shown by these leaves (Ghashghaie et al., 1992; Santamaria et al., 1993). An increment on stomatal and trichome densities were observed during acclimatization process (Table 1). After 6 months of acclimatization the number of stomata per square millimetre was similar to what has been observed in the field (Molinas, 1991).

A shade-leaf structure (Molinas, 1991) was observed in transverse sections of in vitro leaves, with large intercellular air spaces and a low mesophyll cell density, but with a differentiated palisade cell layer (Table 1). These leaves generally die and only the new

leaves resist acclimatization stress. New leaves from acclimatized plants showed a sun-leaf structure with small intercellular air spaces, high cell density and two or three palisade cell layers (Table 1). The characteristics observed in in vitro leaves, persist in the first leaves developed ex vitro, the transition leaves. Vascular tissues of in vitro leaves were less developed and development increased during the acclimatization period (Table 1).

Morpho-physiological studies confirm the earlier reports that cultured plants have poor control of water loss (Dhawan et al., 1987; Diettrich et al., 1992; Ghashghaie et al., 1992). The results observed in this work suggest that the main factor causing death and slow recovery of micropropagated cork oak plantlets during the period of acclimatization is the poor control of water loss, until new leaves are developed. Protecting *in vitro* cultured plantlets from water stress by maintaining plantlets under high humidity over an interval of four weeks was found to be essential for successful acclimatization of cork oak micropropagated plantlets.

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Tables

Table 1. Features of leaf anatomy of cork oak plants at different acclimatization phases.

	In vitro	Acclimatization	
		1 month (new leaves)	6 months (new leaves)
Epidermis	Thin, lacking cuticle	Thin with a thin cuticle	Thick, with cuticle
Palisade cell layers	1 layer of loosely packed cells	2 layers	3 compact layers
Spongy parenchyma	Spherical cells with large intercellular spaces	Differentiated	Highly differentiated, small intercellular spaces
Vascular tissues	Less developed	Developed	Highly developed
Trichome	Rare	Few	Abundant
Stomatal density	364 ± 12	372 ± 14	456 ± 17
Stomata	Abundant stomata,large and raised guard cells	Guard cells with low protuberance	Few stomata with depressed guard cells
Stomata response to water stress	None	Reduced	High

Figures

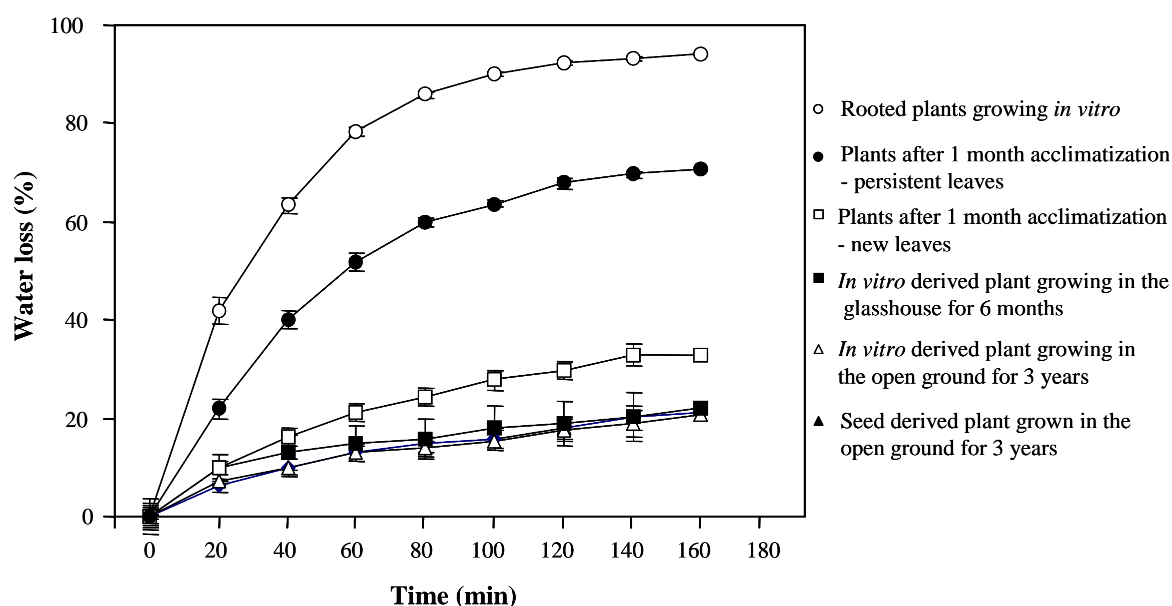

Fig. 1. Changes in relative water content of excised leaves (initially fully turgid) as a function of time. Each point represents the mean of 4 replicates (for details see text).