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Microculture effects on leaf epidermis and root structure in *Sclerocarya birrea* subsp. *caffra*

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Abstract

Micropropagation of woody plants is beset by a number of challenges such as cell and tissue abnormality, which ultimately affect survival of plantlets *ex vitro*. The aim of the study was to evaluate the effects of *in vitro* microenvironments, in particular photoperiod, on the structure of the leaf epidermis and adventitious roots under photomixotrophic conditions. Control plants were grown under photoautotrophic conditions in a growth chamber. Stomatal density (number of stomata per mm^2) on the abaxial leaf surfaces was highest for the control (221.9±13.99) and the 16-h photoperiod treatment (206.6±15.28). Stomata of plants grown under a 16-h photoperiod had fully expanded kidney-shaped guard cells that closely resembled stomata in the control plants. Formation of epicuticular wax was least for *in vitro* plants grown under a 24-h photoperiod. One of the main structural differences was the relative thickness of the inner and outer periclinal cell walls of the root epidermis between the control plants and *in vitro* plantlets. Control plants developed a thicker epidermis compared to that of *in vitro* plantlets. The root endodermis in the control plants was also comparatively thicker and more regular than that of *in vitro* plantlets. Control plants had a greater proportion of vascular tissue relative to the total area of the root cross-section compared to *in vitro* plants due to the development of xylem and phloem tissues. A micropropagation system that facilitates the modification of the *in vitro* microclimate may reduce the structural abnormalities of conventional microcultures.

Keywords: Histology; Microculture; Micropropagation; Microscopy; Stomata; Ultrastructure

1. Introduction

Plant morphogenesis *in vitro* is a complex developmental process that is controlled and regulated by the intricate interactions of several intrinsic and environmental stimuli. Microculture involves the aseptic manipulation of plant tissues growing in photomixotrophic conditions on a carbohydrate and nutrient-enriched basal medium. The micro-environmental conditions, carbohydrate-enriched basal medium as well as the balance of nutrients within it, constitute some of the major factors influencing growth of plantlets *in vitro*. Optimisation of the microenvironment is a key step in micropropagation and ensures the production of good quality plantlets that have high chances of surviving the *ex vitro* conditions in greenhouses and ultimately the natural environment. In micropropagation, the culture vessel can thus be viewed as a miniature growth chamber with tightly controlled conditions (Kozai et al., 1997). The characteristics of this controlled *in vitro* environment not only include: constant temperature, high relative humidity, low photosynthetic photon flux (PPF), optimised concentrations of sugars, salts and plant growth regulators, and aseptic conditions, but also accumulation of secondary metabolites that may be toxic to the growing plantlets (Kozai et al., 1997).

Temperature is one of the most important factors regulating *in vitro* plant development (Franklin, 2009). In nature the perception of ambient temperature allows for the maintenance of plant homeostasis, thereby buffering against potential disruptive effects on cellular stability (Franklin, 2009). In contrast to the natural environment, under microculture a constant temperature regime is normally maintained and this

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may have effects on the development of plants (Nadgauda et al., 1997). Light, which is the source of energy for photosynthesis, is another critical factor regulating plant growth and development *in vitro*. Though the light requirement for photosynthesis of *in vitro* plants is of less importance compared to *in vivo* plants (Economou and Read, 1987), it still profoundly regulates plant development through the influence of intensity, photoperiod and spectral quality (Da Silva and Debergh, 1997). Low light intensities (15–65 μmol m^{-2} s^{-1}) are normally used in micropropagation systems, as exposure to levels above the photosynthetic photon flux (PPF) saturation point leads to photo-inhibition and photo-oxidative damage of the fragile photosynthetic tissues of *in vitro* plantlets (Kodym and Zapata-Arias, 1998). Low *in vitro* PPF levels are preferred for stimulating the production of relatively higher concentrations of chloroplast pigments, electron carriers, and increased numbers of chloroplasts (Walters et al., 2003). In addition to light intensity, photoperiod is another critical light-dependent factor that regulates plant developmental processes including stem elongation, bud dormancy, leaf growth (Kühn et al., 2009), pigment accumulation, hormone synthesis, gene expression and ion influx changes (Hauser et al., 1998). Plants have evolved a system of sensory photoreceptors that perceive changes in the ambient light environment (Gyula et al., 2003). The perception and regulation of the light signal are controlled by a network of photoreceptors, in particular phytochromes (red/far-red receptors), cryptochromes (blue/UV-A light receptors) and phototropins (phot1 and phot2) (Walker and Bailey, 1968; Rudiger et al., 1983; Mockler et al., 2003). One advantage of plant tissue culture is that the photoperiod can be optimised to suit specific growth conditions. In nature, natural photoperiods are characterised by twilight phases, which may constitute a critical part in the daily cycle of a plant's physiology and development (Kühn et al., 2009). In contrast, artificial photoperiods in micropropagation lack the critical twilight period, as the transition between light and dark phases is abrupt (Kühn et al., 2009). This major difference in photoperiod dynamics between the artificial conditions in growth chambers and the natural environment may have physiological implications on plant development and affect growth during acclimatisation.

Sclerocarya birrea subsp. *caffra* (marula, Anarcadiaceae), which was the experimental plant, is highly valued in sub-Saharan Africa for its unique medicinal and nutritional properties (Moyo et al., 2009a). However, despite the industrial potential of *S. birrea*, few plant tissue culture and related biotechnology research studies that would help to catapult the plant into plantation horticulture have been carried out (Mollel and Goyvaerts, 2004; Moyo et al., 2009b, 2011). The current research was done following the challenges reported by Moyo et al. (2011) in the acclimatisation of *in vitro* rooted plantlets. The objective of the present study was to evaluate the effects of the *in vitro* microenvironment on the development of plant cells and tissues using histological techniques. This was achieved by comparing the structure of the leaf epidermis and endodermis and adventitious roots, and the root ultrastructure of *in vitro* plantlets and photoautotrophic seedling plants.

2. Materials and methods

2.1. Chemicals

Indole-3-butyric acid (IBA), myo-inositol, vitamins (thiamine HC1, nicotinic acid, pyridoxine HC1) and glycine were obtained from Sigma-Aldrich (St. Louis, USA); bacteriological agar from Oxoid Ltd (Basingstoke, England); polyvinylpyrrolidone and toluidine blue from BDH Chemicals (Poole, England); glutaraldehyde, 2,4,6-tri(methylamino ethyl)phenol (DMP-30), sodium cacodylate, osmium tetroxide, propylene oxide, Epon 812 and Araldite CY212 from Agar Scientific Ltd (Essex, UK); and polyoxyethylene sorbitan monolaurate (Tween 20) from Saarchem, (Krugersdorp, South Africa). The plant growth regulator, *meta*-topolin [6-(3-hydroxybenzylamino) purine (*m*T)] was provided by Dr Karel Doležal, Laboratory of Growth Regulators, Olomouc, Czech Republic. All other chemicals used to prepare growth media and specimens for microscopy were of analytical grade.

2.2. Plant material

Initial shoot explants were obtained from 30–60-day-old *S. birrea* seedlings grown on nutrient-free vermiculite in plant growth chambers (Controlled Environments Ltd, Manitoba, Canada) at 25±2 °C under a 16-h photoperiod at a photosynthetic photon flux of 90–100 μmol m^{-2} s^{-1} provided by cool white fluorescent light (Osram L 58W/640, Germany). Subsequent shoots that were used in the experiments were obtained from the resultant *in vitro* cultured explants. Explants from seedlings raised in plant growth chambers were rinsed thoroughly in running tap water, and surface-decontaminated in 70% alcohol (v/v) for 1 min followed by 2% sodium hypochlorite (v/v) with Tween 20 for 20 min. The explants were thoroughly rinsed in sterile distilled water (3 times) and cultured on MS growth medium supplemented with vitamins and plant growth regulators.

2.3. Growth media and conditions

The basal medium consisting of MS (Murashige and Skoog, 1962) salts supplemented with vitamins (thiamine HCl, nicotinic acid, pyridoxine HC1), glycine, sucrose (30 g l^{-1}), myo-inositol (0.1 g l^{-1}), polyvinylpyrrolidone (3 g l^{-1}) and plant growth regulators (8.0 μM *m*T or 4.0 μM IBA) was used. The medium was adjusted to pH 5.8 using 1.0 M KOH before adding the gelling agent (8 g l^{-1} agar) and autoclaving at 121 °C, 15 kPa for 20 min. The culture tubes contained 10 ml of MS medium for all experiments. Cultures were maintained at a temperature of 25±2 °C under either a 16-h or 24-h photoperiod. Unless specified otherwise, a constant PPF of 40 μmol m^{-2} s^{-1} was provided by cool white fluorescent light as measured at the culture tube level. Photosynthetic photon flux density was measured using a quantum radiation sensor (Model Skp 215, Skye Instruments Ltd, Llandridod Wells, UK).