Stomatal Functioning of *In Vitro* and Greenhouse Apple Leaves in Darkness, Mannitol, ABA, and CO₂

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**Abstract**

Leaves from *in vitro* and greenhouse cultured plants of *Malus domestica* (Borkh.) cv. Mark were subjected to 4 h of darkness; 4 h of 1 M mannitol induced water stress; 1 h of 10⁻⁴ M to 10⁻⁷ M cis-trans abscisic acid (ABA) treatment; 1 h of 0-12% atmospheric CO₂. Stomatal closure was determined by microscopic examination of leaf imprints. In all treatments, less than 5% of the stomata from leaves of *in vitro* cultured plants were closed. The diameter of open stomata on leaves from *in vitro* culture remained at 8 μm. In contrast, an average of 96% of the stomata on leaves of greenhouse grown plants were closed after 4 h in darkness; 56% after 4 h of mannitol induced water stress; 90% after 1 h of 10⁻⁴ M ABA treatment; 61% after 1 h in an atmosphere of 0-12% CO₂. Stomata of *in vitro* apple leaves did not seem to have a closure mechanism, but acquired one during acclimatization to the greenhouse environment. The lack of stomatal closure in *in vitro* plants was the main cause of rapid water loss during transfer to low relative humidity.

**Introduction**

*In vitro* plants must develop ways to prevent water loss to survive after transfer to low humidity (Wardle, Quinlan, and Simpkins, 1979; Brainerd and Fuchigami, 1981). This acclimatization can involve thickening of epicuticular wax (Baker, 1974; Sutter and Langhans, 1979; Fuchigami, Cheng, and Soeldner, 1981) and changes in stomatal functioning (Brainerd and Fuchigami, 1981; Brainerd, Fuchigami, Kwiatkowski, and Clark, 1981). In preliminary studies, we observed that stomata of *in vitro* cultured plum leaves, grown at 26 °C, under 100% relative humidity (r.h.), and 100 μE m⁻² s⁻¹ of a 16 h photoperiod, had no nocturnal closure pattern as did those of greenhouse grown leaves. We also observed that stomata of excised *in vitro* cultured leaves did not close after a 2 h exposure to 45% r.h. at 24 °C during which 95% of the leaf moisture content was lost (Brainerd et al., 1981). The closure mechanism of stomata of *in vitro* cultured leaves may be different from that of acclimatized greenhouse leaves.

Stomata of most mesophytic plants close in response to certain environmental changes. Zelitch (1965) observed that stomata of tobacco leaf disks floating on water, closed completely after 30 min of darkness. Heath and Meidner (1961) observed that 0.4 M mannitol induced water stress caused complete stomatal closure in wheat leaves. In tomato mutants, such as flaccus, which have low endogenous ABA, wilting is prevented by application of exogenous ABA which causes stomatal closure (Imber and Tal, 1970). Stomatal apertures in epidermal strips of *Commelina communis* L. were about 3.0 μm after 2.5 h of 10⁻⁴ M ABA (Wilson, 1981). Raschke (1972) observed the saturation kinetics of

Wardle, Quinlan, and Simpkins (1979) observed that 10⁻⁴ M ABA did not increase stomatal resistance in leaves of in vitro apical meristem cultured Brassica oleracea L. var. Botrytis, but did increase stomatal resistance of seedlings grown in vermiculite.

The objective of this study was to determine if stomata of in vitro and acclimatized greenhouse apple leaves responded similarly to darkness, under mannitol induced water stress, with exogenously applied ABA, and at a CO₂ concentration approximately four times greater than the normal atmospheric level.

MATERIALS AND METHODS

Mark apple plants, obtained from Microplant Nursery in Gervais, Oregon, were used for all experiments. In vitro plants were grown for about 2 months at 25 °C under 16 h photoperiod of approximately 100 μE m⁻² s⁻¹, in 9-0 cm x 8-5 cm jars of root growth promoting medium. Greenhouse acclimatized plants were grown for up to 9 months in peat:perlite (v/v), under natural photoperiod, between 18 °C and 24 °C. The leaves of the 2 and 9-month old plants were at comparable developmental stages. Five culture jars, each containing 10 plants, and five greenhouse plants were selected for each experiment. One leaf was excised from a plant in each jar or greenhouse grown plant so that each treatment had five replicates. Treatment responses of stomata were evaluated by microscopic examination of butylacetate leaf imprints. These imprints were made from silicon-rubber (Dow-Corning compound 3110 RTV) imprints taken from the treated leaf. The number of stomata, with apertures less than 2 μm (Zelitch, 1965), out of 100 stomata counted was expressed as a percentage.

For the dark treatment, plants were placed in a photographic darkroom and after 0, 15, 30, 45, 60, 120, and 240 min, one leaf was removed from each culture jar and greenhouse grown plant, and silicon-rubber leaf imprints were made.

For the mannitol induced water stress treatment, eight leaves were removed from each of five in vitro cultured and greenhouse grown plants and placed abaxial side down in a Petri dish containing distilled H₂O or 1 M mannitol solution, at 22 °C under 100 μE m⁻² s⁻¹. Leaf imprints were taken before and after the treatment. The petioles were not blocked.

For ABA treatment, one leaf was removed from each of five in vitro cultured and greenhouse grown plants and placed abaxial side down in a Petri dish containing distilled H₂O, 10⁻⁴ M ABA, 10⁻³ M ABA, 10⁻² M ABA, 10⁻¹ M ABA or 10⁻⁰ M ABA. Leaf imprints were taken before and after 1 h of floating at 22 °C under 100 μE m⁻² s⁻¹.

For CO₂ treatment, five in vitro cultured plants were removed from separate culture jars by cutting an agar cube surrounding the plant roots. The five plants in the agar cubes were placed in Petri dish covers in a 5 l glass chamber at nearly 100% r.h. Five acclimatized greenhouse plants in 5-0 cm x 5-0 cm pots of peat perlite were also placed in the chamber. One leaf was removed from each plant and imprints were made to determine stomatal aperture prior to the CO₂ treatment. The chamber was closed and a mixture of 0-12% CO₂, 22% O₂, and balance N₂ was pumped through at a rate of 6 l h⁻¹, for 1 h, under 100 μE m⁻² s⁻¹. Leaves were then removed from each plant and stomatal imprints were made.

RESULTS AND DISCUSSION

Stomata in leaves of greenhouse acclimatized apple closed in response to darkness, mannitol induced water stress, exogenous ABA treatment, and high atmospheric CO₂ concentration. In contrast, stomata in leaves of in vitro cultured apple plantlets remained almost completely open in all treatments. In greenhouse grown apple leaves, an average of 96% of the stomata closed after 4 h of darkness (Fig. 1); 56% closed after 4 h in 1 M mannitol (Fig. 2); 90% closed after 1 h of 10⁻⁴ M ABA (Fig. 3); 61% closed after 1 h in 0-12% CO₂ (Fig. 4). Fewer than 5% of stomata of in vitro cultured leaves had apertures less than 2 μm in all treatments. Most stomata had apertures of well above 2 μm and up to 8 μm. Stomatal aperture of in vitro cultured plants was not reduced by a 5 min exposure to 5% CO₂ (data not shown).

Greenhouse apple plants, which were cultured in vitro prior to acclimatization, had
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FIG. 1. Percent stomatal closure in darkness in greenhouse (GH) and aseptically cultured (AC) 'Mac 9' apple leaves. Each point represents the mean of five samples taken at hourly intervals.

FIG. 2. Percent stomatal closure after 4 h in solutions of 1 M mannitol or distilled water in greenhouse (GH) and aseptically cultured (AC) 'Mac 9' apple leaves. Each point represents the mean of five samples.

functional stomata. We previously observed that in *vitro* plants exposed to 4–5 d of low r.h., develop stomata which close when subjected to water stress. Development of a mechanism for stomatal closure in response to environmental stimuli such as darkness, mannitol-induced water stress, ABA and CO₂, occurs during the period of acclimatization.

Although epicuticular wax is less developed on *in vitro* than on greenhouse leaves (Baker, 1974; Sutter and Langhans, 1979; Fuchigami *et al.*, 1981), the major water loss during transfer of *in vitro* plants to a low humidity environment appears to be the lack of stomatal closure in *in vitro* plants.

It appears that the development of functioning mature stomata involves (a) division of the guard mother cell into two closed guard cells (b) initial opening of the pore between the guard cells by ion accumulation and turgor increases (Palevitz and Hepler, 1976), and we now postulate the (c) the development of a closure mechanism. (If this closure mechanism could be developed prior to transfer from the culture jar more plants would survive.)
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FIG. 3. Percent stomatal closure in greenhouse (GH) and aseptically cultured (AC) ‘Mac 9’ apple leaves treated for 1 h with different concentrations of ABA. Values for control were taken from untreated leaves. Each bar graph represents the mean of five samples.

Wardle, Dixon, and Simpkins (1981) measured a lower K:Na concentration in stomatal complexes of in vitro cauliflower plants than in seedlings grown in vermiculite. It is possible that a low K:Na concentration of in vitro apple plants may contribute to their lack of a stomatal closure mechanism and we shall attempt to evaluate K+ involvement in the development of stomatal closure.

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LITERATURE CITED


