J. AMER. SOC. HORT. SCI. 120(3):435-440. 1995.

Effects of In Vitro-formed Roots and Acclimatization on Water Status and Gas Exchange of Tissue-cultured Apple Shoots

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Additional index words. conductance, photosynthesis, relative water content

Abstract. **Little is known about the physiological changes that occur during acclimatization and how these changes influence plant survival and growth in the new environment. In particular, it is unclear to what extent in vitro-formed roots are functional in water uptake, particularly when the plantlet is exposed to conditions of increasing evaporative demand. Tissue-cultured shoots and plantlets (shoots with roots) were acclimatized by exposing them to a linear reduction in relative humidity (RH) from 99 % to 75%over 4 days. When conductance was measured at 95% RH (21 C), in vitro shoots and plantlets showed a very high initial conductance, followed by a gradual decline, reaching steady state in 12 hours. Acclimatized shoots and plantlets had a 50% lower initial conductance compared to nonacclimatized ones, and reached steady state in 4 hours. The reduction in conductance as a result of acclimatization most likely contributes to a reduced transpiration under conditions of increased evaporative demand. Roots formed in vitro were associated with a higher plant water status, suggesting that these roots were functional in water uptake. Relative water content of the shoot was positively correlated with leaf conductance and net photosynthesis. We suggest that tissue-cultured plantlets behave as hydraulically integrated units, in which there must be a coordination between control of water loss by the shoot and uptake of water by the root to maintain a favorable plant water balance. Our results also indicate that methods that use excised shoots or leaves to determine transpiration gravimetrically may not accurately represent the stomatal water loss characteristics of tissue-cultured plants.**

In vitro shoots and plantlets are grown at low evaporative demand conditions of high relative humidity (RH) and low light *(30* to *75* µmol photons/m^2 per see). Under these conditions, there may be a low rate of transpiration even though stomata are widely open (Shackel et al., 1990). When plantlets are exposed to lower humidities, as occurs during transplanting, they may show high transpiration rates because of their high initial leaf conductance. In many woody species, transpiration after transplanting is often excessive, leading to severe plant water deficits. These water deficits are one of the main causes of plant mortality after transfer from culture to the greenhouse (Preece and Sutter, 1991). To ameliorate the impact of plant dehydration during transplanting, tissue-cultured plants are acclimatized for several weeks by gradually decreasing RH and increasing luminosity. During acclimatization, plants may undergo physiological and morphological changes in response to such alterations in the environment. Despite the intense research in micropropagation, however, little is known about the physiological changes that occur during acclimatization and how these changes influence plant survival and growth in the new environment.

Water deficits in plants will increase whenever transpiration exceeds water uptake. Transpiration is under the control primarily of stomatal conductance which, in addition to environmental factors, depends on the degree of stomatal opening (Kramer, 1983). In tissue-cultured plants, high transpiration rates have been attributed to poor stomatal function (Brainerd and Fuchigami, 1982), reduced leaf epicuticular wax (Sutter and Langhans, 1982) and high stomatal density (Desjardins et al., 1988). Gas exchange measurements of leaf conductance, however, have indicated that stomata from in vitro shoots are functional and close under evaporative demand (Díaz–Pérez, 1994; Shackel et al., 1990). This ability of stomata to close at a low RH might reduce transpiration and be of important horticultural value for the survival of plantlets after transfer from culture to the greenhouse.

Plant water balance depends not only on stomatal regulation of water loss but also on the ability of the plant to take up water. Water uptake can be limited by the extent of the roots, root and soil hydraulic properties, soil (substrate or medium) water potential, and other factors such as salinity, low temperature, and poor aeration in the soil (Kramer, 1983). Compared to roots formed in soil, in vitro roots have been considered nonfunctional by several authors (Debergh and Maene, 1981) because they are hypertrophic (McClelland, 1990), lack root hairs (Ziv, 1986), and have poor vascular connections with the shoot resulting in restricted water transfer from roots to shoots (Grout and Aston, 1977). In vitro roots are also considered unnecessary in some plants during transfer from in vitro to greenhouse conditions because these roots die after transplanting and delay plant growth (Debergh and Maene, 198 1). Another disadvantage of in vitro rooting is that their formation requires an additional step in the production of plantlets, resulting in increased costs (Debergh and Maene, 198 1).

Recent evidence shows that there is a complete vascular connection between the shoot and the roots in microcultured apple plantlets (Hicks, 1986; Sutter and Luza, 1993) and Asian Jasmine (Apter et al., 1993a) and physiological functionality (Apter et al., 1993b). In vitro roots are also associated with increased survival after transfer as shown in Douglas fir (Mohammed and Vidaver, 1990). Another benefit of in vitro roots is their influence on root development after transplanting, as suggested by the positive correlation between the number of in vitro roots at the time of transplanting and the number of roots formed after transplanting (Stimart and Harbage, 1993). Direct evidence for a positive influence of in vitro roots on plant water status would be a relation between plant water content or water potential to some measure of root size relative to shoot size. Mohammed and Vidaver (1991) reported that roots were functional in water uptake. Their measure

Received for publication 5 July 1994. Accepted for publication 17 Oct. 1994. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.
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of plant water content, however, did not account for transpirational water that was replaced by water taken up from the plant's container.

In this paper, we report the effect of in vitro-formed roots and acclimatization on the water status and gas-exchange of cultured plants. Plant water status is measured as shoot relative water content (RWC), which has been shown to be related to plant function measured as leaf conductance (Díaz–Pérez, 1994). We also compare a porometer-based gas exchange system against the gravimetric method (Kramer, 1983) as a means to measure transpiration. The gravimetric method has been widely used to measure transpiration in tissue-cultured plants (e.g., Conner and Conner, 1984), even though its results might be questioned because this method is commonly based on the use of excised organs, and these organs are often allowed to desiccate severely.

Materials and Methods

Plant material. Apple shoots *(Malus pumila* 'Greensleeves') were multiplied in vitro in Magenta boxes containing Murashige and Skoog (MS) basal salts and vitamins (Murashige and Skoog, 1962) to which 4.4 μM benzyladenine (BA) and 0.5 μM indolebutyric acid (IBA) were added. Shoots were transferred every 4 weeks, and were incubated under standard culture conditions of 40-50 μmol photons/m^2 per sec and a 16-h photoperiod. Individual shoots ≈2 cm long were rooted by incubating them in the dark for 4 days on MS medium supplemented with 15 μM IBA. Shoots were then transferred to the light in 20 ml-blood dilution vials (American Scientific Products, McGraw Park, Ill.) containing 10 ml of half-strength MS basal salts and vitamins. All media were solidified with 0.6% Sigma agar. In this study, a shoot that was rooted as described is called a plantlet. Unrooted shoots used in experiments were transferred from Magenta boxes directly to 20-ml blood dilution vials containing 10 ml of half-strength MS basal salts and vitamins without being exposed to IBA.

Acclimatization. In this paper, acclimatization specifically refers to the application of a gradual increase in vapor pressure difference (VPD) (reduction in RH) to shoots and plantlets over 4 days, as described below. A custom acclimatization chamber was constructed from a transparent plastic box and located in a controlled-temperature room (21 ± 1C, continuous fluorescent light of 3 μmol photons/m^2 per see). The chamber contained a temperature-compensated humidity sensor (IH–3602 Monolithic IC; HY–CAL Engineering, El Monte, Calif.). Chamber humidity was measured and controlled by a data logger (CR 10, Campbell Scientific). The humidity in the chamber was determined by a mix of air from two vessels, one containing water and the other a saturated solution of $CaCl_2$. Air was pumped through these vessels before entering the chamber. A submersible heater in the water vessel was controlled by the data logger and, whenever the VPD inside the chamber was higher that the setpoint, heat was applied to the vessel that contained water. As water was heated, the humidity content of the air pumped into the chamber increased causing a reduction in the chamber VPD. When the VPD was at the setpoint or below, heating was discontinued and the chamber was allowed to passively increase its VPD. The data logger was programmed to increase VPD from 0.026 kPa (99% RH at 21C) to 0.621 kpa (75% RH at 21C) at a rate of 1.03E–4 kPa/min (0.0042%

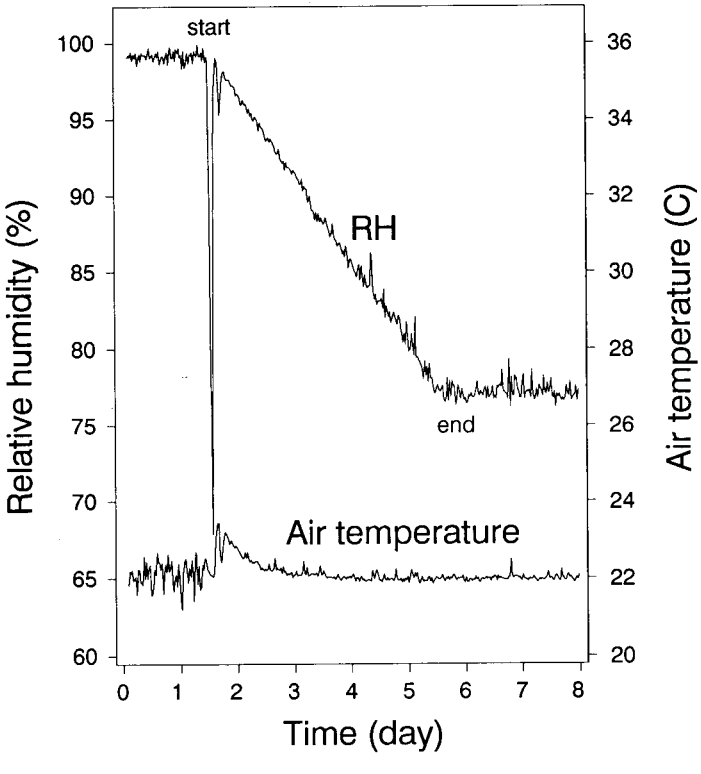


Fig. 1. Changes in relative humidity (RH) and air temperature in the acclimatization chamber during the acclimatization of shoots and plantlets. During acclimatization, RH decreased from 99% (22C) to 77% (22C) at a constant rate over 4 days. Start and end indicate the beginning and the end of the acclimatization period. The sharp drop in RH at the start of acclimatization was associated with opening the chamber.

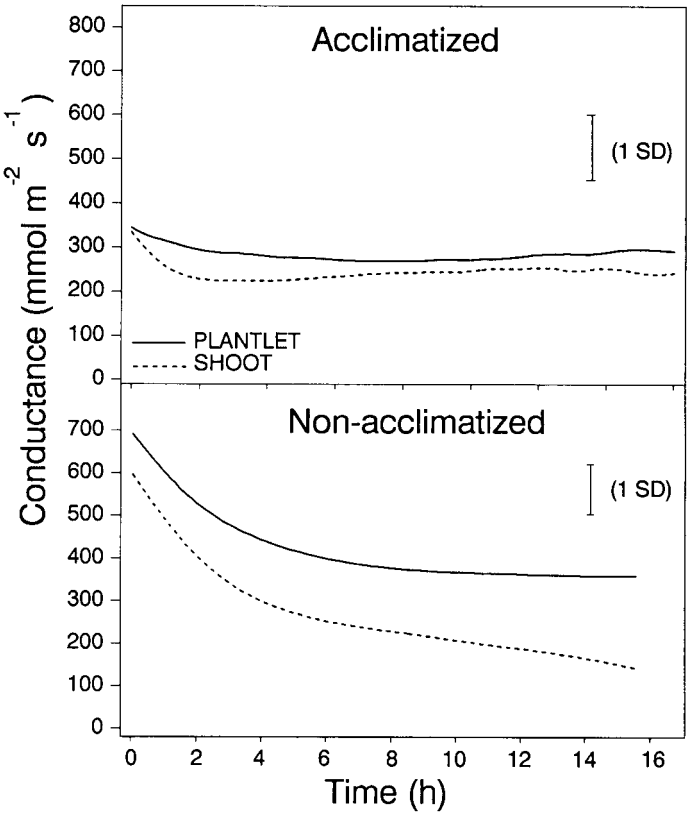


Fig. 2. Effect of acclimatization on leaf conductance over time for cultured shoots and plantlets. Measurements were made at 95% relative humidity, 21C, 350 ppm CO_2, and a photosynthetic photon flux density of 3 μmol photons/m^2 per sec. In each plant, 10-min averages were calculated from conductance values measured every 30 sec over 16 h (as in Fig. 3). Curves are smoothed cubic splines fit through the mean 10-min values of conductance for eight shoots or platlets. Error bars represent a pooled SD and indicate the average variability in the conductance over time among individual shoots or plantlets.

Table 1. Effect of in vitro roots and acclimatization on leaf conductance (g_l), net photosynthesis (P_n), intercellular CO_2 (C_i), and dark respiration (r) of tissue-cultured apple plantlets (+root) and shoots (–root). Each value represents the mean of four observations.

	g_l ($mmol \cdot m^{-2} \cdot s^{-1}$)	P_n ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	c_i (ppm)	r ($\mu mol \cdot m^{-2} \cdot s^{-1}$)
Acclimatized				
Plantlet (+root)	375	+1.99	342	–0.54
Shoot (–root)	289	–0.57	363	–0.71
Nonacclimatized				
Plantlet (+root)	458	+0.73	346	–1.23
Shoot (–root)	153	–0.78	365	–1.13
Significance				
ROOT	**	**	**	NS
ACCLIM	NS	NS	NS	NS
ROOT × ACCLIM	NS	NS	NS	NS

NS,*,**Nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively.

RH/min) over 4 days (Fig. 1), after which the VPD remained at 0.621 kPa. Except with transients associated with opening the chamber, the system was able to maintain the chamber VPD within ±1% of the set VPD.

Gas exchange system. Gas exchange was carried out as described by Shackel et al. (1990) with some modifications to allow for measurements of photosynthetic CO_2 exchange. Leaf conductance (g_l) was determined with a modified steady-state porometer (LI-1600; LI-COR, Lincoln, Neb.) and CO_2 exchange with an infrared gas analyzer (IRGA) (ADC–225 MK3; Analytical Development Co. Limited, Herdfordshire, England). During the CO_2 exchange measurements, the porometer was supplied with air at a constant CO_2 concentration (350 ppm). The air outlet on the sensor head of the porometer was connected to the analysis cell of the IRGA. Before entering the IRGA, the air from the porometer was dried with $CaCl_2$.

Before the gas exchange measurements, a 3–4-mm layer of melted vaseline was applied to the surface of the culture medium to prevent evaporation from the medium during gas exchange determinations. Measurements of gas exchange were made in the controlled-temperature room described above. The porometer was maintained at a steady 95% RH and was connected to a computer (model PC–8201A; NEC, Tokyo) that was programmed to collect data every 30 see, and to calculate and store means for 10-min periods.

During the gas exchange measurements, shoots and plantlets showed a gradual reduction in conductance until they reached a steady state after 12 h under room light (Shackel et al., 1990). Once steady-state conductance was reached at 350 ppm CO_2, a high-light stimulus (350 µmol photons/m^2 per see) was supplied by a tungsten halogen lamp filtered through a 8-cm layer of water to remove infrared radiation. The light applied to the plants [photosynthetic active radiation, (PAR)] was measured with the quantum sensor provided by the porometer. After the application of high light, another steady state was reached 20–40 min later. Under high light conditions, the reported values of leaf conductance and net photosynthesis (P_n) are at steady state (when the values changed< 2%/h). Net photosynthesis was estimated as the CO_2 exchange rate at highlight, while the CO_2 exchange at low light (3 µmol photons/m^2 per see) was an estimate of dark respiration (r). At this low light, errors in the estimate of dark respiration might result from photosynthesis and from a potential suppression of dark respiration by light (Sharp et al., 1984). Based on a value of quantum yield reported by Sharp et al. (1984), our measurements may underestimate dark respiration by 0.28 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Gas exchange rates and intercellular CO_2 (c_i) were calculated according to Field et al. (199 1) from flow rates, transpiration rates and leaf conductance measured with the porometer, and from IRGA determinations of CO_2 concentration in the air before and after leaving the porometer sensor head.

Immediately after gas exchange measurements, the shoot was excised from the roots, weighed to obtain fresh weight, and dehydrated for 10 h at 4C (Díaz–Pérez, 1994) to obtain saturated weight. Leaves were excised from the shoot and total leaf area was determined with a area meter (Delta T; Decagon Instruments, Pullman, Wash.). Roots and shoots were dried at 80C for 48 h to determine dry weights. Relative water content was calculated according to Catsky (1974) as RWC = 100(fresh weight – dry weight)/(saturated weight – dry weight), with saturated weight being the shoot weight immediately after dehydration.

Gravimetric measurements of transpiration. After dehydration, some shoots were removed from the water, blotted dry, placed on a lab bench (40 µmol photons/m^2 per sec of PAR, 24C, and 35% RH) for 6 h, and weighed periodically (shoot fresh weight). Shoot transpiration was calculated as the rate of water loss per unit leaf area. Leaf area and shoot dry weight were measured as described above.

Results

Gas exchange

Effect of acclimatization. During the 4 days of acclimatization, no new leaves were formed. Thus all measurements were made on persistent leaves that had been formed during in vitro culture. At 95% RH, plantlets and shoots showed a gradual reduction in g, until they reached steady state or final g_l (Fig. 2). A longer time was required to reach a steady state (>8 h) in nonacclimatized compared to acclimatized shoots or plantlets (2–3 h). Nonacclimatized shoots differed from plantlets and acclimatized shoots in that they failed to reach steady state after 16 h in the gas exchange system (Fig. 2). Additional measurements indicated that nonacclimatized shoots required ≈24 h to reach steady state (data not shown). Acclimatized shoots and plantlets had a 50% lower initial leaf conductance compared to nonacclimatized ones (Fig. 2). Acclimatization had no significant effect on final conductance (Table 1), even though there was a tendency for acclimatized plantlets to have a lower g_l than nonacclimatized plantlets and for acclimatized shoots to have a higher g_l than nonacclimatized shoots. In addition, acclimatization had no significant effect on P_n of plantlets and shoots (Table 1), but there was a tendency for acclimatized

plantlets to have a higher P_n than nonacclimatized plantlets. Respiration tended to be higher in acclimatized corn-pared to nonacclimatized shoots and plantlets, even though the effect was not statistically significant (Table 1).

Effect of in vitro roots. At low light conditions, plantlets (nonacclimatized) had a higher steady-state conductance and were more responsive to light than shoots. After the application of light, plantlets typically responded with an increase in g_l while shoots either maintained the same g_l or decreased it (Fig. 3). The average increase in g_l of plantlets was 12%. Once the light stimulus was removed, conductance returned to a value similar to that before the application of light for plantlets and shoots (Fig. 3). During the gas exchange measurements, plantlets maintained significantly higher steady-state values of g_l, P_n, and c_i than shoots (Fig. 4 and Table 1). There were no statistical differences in dark respiration between the two groups (Table 1). At the completion of the gas exchange measurements RWC was higher in plantlets than in shoots. Conductance and Pn were positively correlated to water status measured as RWC (Fig. 4). The relationships of g_l and P_n with RWC were similar for shoots and plantlets. While all plantlets had positive values of P_n, most shoots had negative values. Below ≈ 70%–75% RWC, the values of P_n were negative, which indicates a negative carbon balance in the shoots (Fig. 4).

The amount of roots relative to shoot size (root: shoot ratio) was positively correlated with plant water status (RWC) and transpiration (Fig. 5). Conductance and root : shoot ratio were also positively correlated with each other (data not shown). Similar relationships were obtained whether root : shoot ratio was expressed as root dry weight per shoot dry weight, or as root dry weight per leaf area.

Gravimetry

Excised shoots initially showed rapidly decreasing transpiration rates, followed by slower rates after 180 min. Within the first 30 rein, nonacclimatized shoots exhibited an apparent increase, followed by a steady decline in transpiration (Fig. 6). Over the duration of the experiment (360 min), RWC and transpiration rate were reduced 10 and 20 times, respectively. Acclimatized shoots had values of transpiration and RWC similar to that of nonacclimatized shoots. There was a trend for acclimatized shoots to have higher values of RWC and lower transpiration rates than nonacclimatized shoots during the first 150 min.

Discussion

Gas exchange

Effect of acclimatization. Many reports suggest that stomata of plants grown in vitro are nonfunctional because they are unable to close under inductive conditions (Brainerd and Fuchigami, 1982). However, recent evidence from microcultured apple shoots indicates that stomata are functional, as shown by reductions of leaf conductance under evaporative demand and by in situ microscopic observations of stomatal closure (Shackel et al., 1990).

In this study, acclimatization resulted in a reduction of leaf conductance as indicated by the lower initial g, of shoots and plantlets (Fig. 2). Such reduction in initial g_l of acclimatized plants is similar to that reported by Sutter et al. (1988). Acclimatized shoots and plantlets also reached steady state faster than nonacclimatized ones. Our data indicate that the faster attainment of steady state in acclimatized compared to nonacclimatized plants is largely a result of a lower initial value of g_l in acclimatizated plants. This lower g, might play an important role in the maintenance of plant water balance just after transplanting from in vitro conditions to the greenhouse. Since shoots have high rates of transpiration immediately after transfer from culture (Pospísilová et al., 1987), dehydration often occurs rapidly as was shown in excised shoots (Fig. 6). Thus, any decrease in transpiration, as would occur as a result of a reduction of g_l, would reduce the extent of water loss and decrease the possibility of dehydration.

Effect of in vitro roots. During the gas exchange measurements, the presence of in vitro roots resulted in a higher RWC, g_l, and P_n in plantlets compared to that in shoots. This positive impact of roots on g_l and P_n probably occurred as a result of improved water status as indicated by the positive correlation of g_l and P_n with RWC (Fig. 4). The correlation of both g_l and P_n with RWC shows that RWC is useful as an indicator of shoot water status in tissue-cultured plants.

Plantlets had small but positive values of P_n while most shoots had negative values of P_n, showing a major difference in carbon balance between the two groups. The difference in P_n between shoots and plantlets was apparently not a result of differences in dark respiration, because respiration was similar for shoots and plantlets (Table 1).

Photosynthesis can be limited by nonstomatal and stomatal factors (Farquhar and Sharkey, 1982). However, the low photosynthetic values of in vitro plants have been largely attributed to nonstomatal factors such as low chlorophyll concentrations and a reduced Rubisco activity (Grout and Donkin, 1987) or to chloroplast malformations (Lee et al., 1985). Our results support the presence of nonstomatal inhibitions of photosynthesis in tissue-cultured plants, as indicated by the low values of P_n despite the

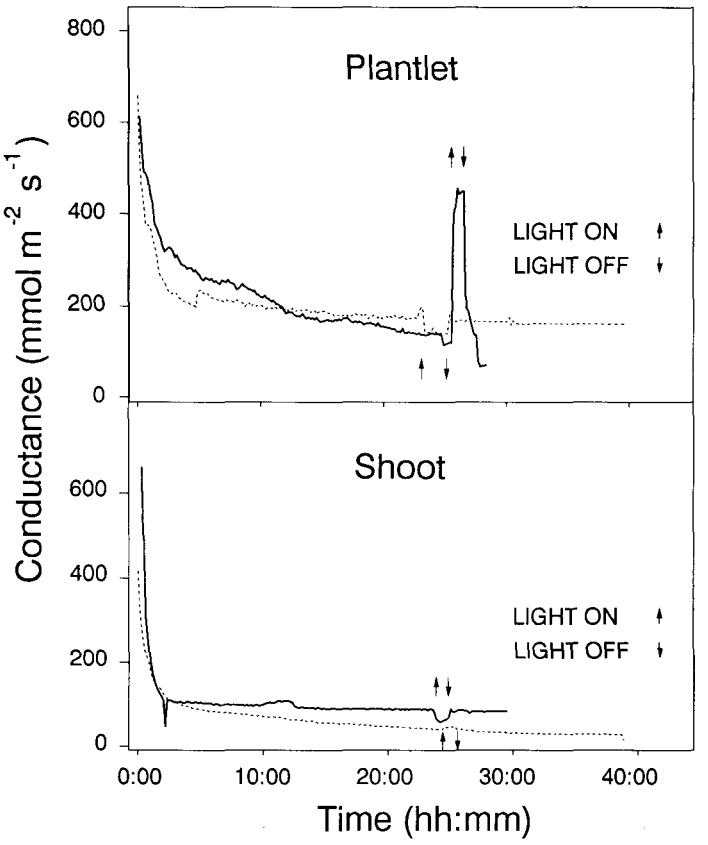


Fig. 3. Leaf conductance over time of nonacclimatized shoots and plantlets. Each curve represents a single shoot or plantlet. A light stimulus (350 µmol photons/m² per sec) was applied once conductance reached steady state. Arrows were used to indicate when lights were turned on and off. The examples shown represent the range in conductance responses to the light stimulus.

relatively high values of C_i and g_l (Table 1). Our results also indicate, however, that reductions of P_n might also be due to stomatal limitations as suggested by the correlation of g_l and P_n with RWC (Fig. 4). These stomatal limitations might be a consequence of a low water status of the plant as has also been found in cultured asparagus plants (Yue et al., 1992). In addition to reductions in g_l as a result of low values of RWC, it is also possible that a low RWC might have a direct effect on the photosynthetic capacity of the plant (Kaiser, 1982). Thus, by having roots, plantlets had an increased water uptake, which resulted in a higher water status, and higher water status probably allowed a higher g_l and P_n.

The presence of in vitro roots in plantlets increased water status and improved physiological activity, suggesting that in vitro roots are functional in water uptake. In vitro roots improved water status by increasing the water uptake capacity of the plant. But increases in water status came not only from the very presence of the roots but also from the amount of roots relative to shoot size, as indicated by the positive correlation of RWC and transpiration per unit leaf area with root: shoot ratio (Fig. 5). Such correlations suggest that roots improve water uptake in plantlets because relative increases in root mass are associated with higher transpiration and RWC. Further support for the normal function of in vitro roots is their ability for phosphorus uptake and translocation to shoots, even though uptake was at lower rates compared to roots formed in soil (Apter et al., 1993b).

Gravimetry

When transpiration of excised shoots was measured by gravimetry, differences between acclimatized and nonacclimatized plants were not as apparent as when transpiration was measured by gas exchange. This discrepancy might be due to errors from the use of excised material. One possible source of error is the Ivanov effect (Kramer, 1983), which may have occurred as evidenced by the increase in transpiration after excision of nonacclimatized shoots. Another potential problem is that excised shoots desiccate very rapidly reaching deleterious values of RWC that are lower than physiological values. For example, our shoots declined from 100% to 40% RWC 60 min after excision. At such low RWC values, it is doubtful that any cells are exhibiting normal physiological activities. The use of a more technically and physiologically sound method to measure transpiration such as gas exchange, which is also nondestructive and allows strict environmental control, probably results in more accurate comparisons than the gravimetric method.

In summary, our results indicate that roots formed in vitro are functional in water uptake and are associated with improved plant water status. Shoot RWC may be used as an indicator of water status of tissue cultured plants because it was positively correlated to physiological activity such as g_l and P_n. Acclimatization promoted a reduction of leaf conductance but had no apparent effect on the photosynthetic characteristics of these plants. We suggest that tissue-cultured plantlets behave as hydraulically integrated units, in which there must be a coordination between control of water loss by the shoot and water uptake by the root to maintain a favorable plant water balance. More experiments are required to understand stomatal responses, as affected by root: shoot ratio and water deficits, and how stomatal regulation of transpiration and root activity determine the maintenance of water status of plantlets during the transfer from culture to the greenhouse.

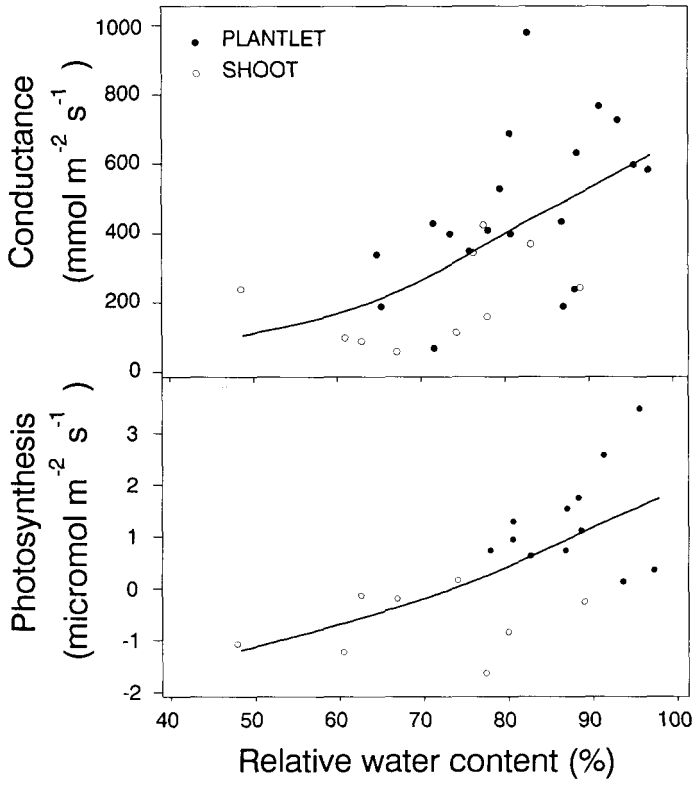
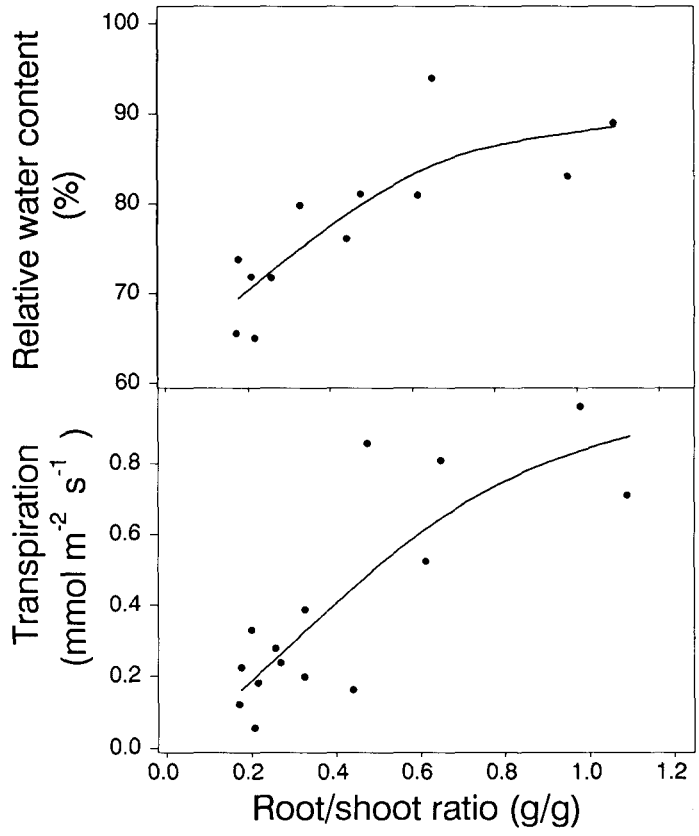


Fig. 4. Leaf conductance and net photosynthesis as a function of shoot relative water content for shoots and plantlets. Measurements were made at 95% relative humidity, 21C, 350 ppm CO_2, and a photosynthetic photon flux density of 350 µmol photons/m' per sec. Each point represents a measurement on a single shoot or plantlet. The solid lines represent a smoothed cubic spline.



Fig, 5. Shoot relative water content and transpiration as a function of root/shoot ratio under conditions of 95% relative humidity, 21C, 350 ppm CO_2, and a photosynthetic photon flux density of 350 µmol photons/m^2 per sec. Each point represents a measurement on a single plantlet. The solid lines represent a smoothed cubic spline.

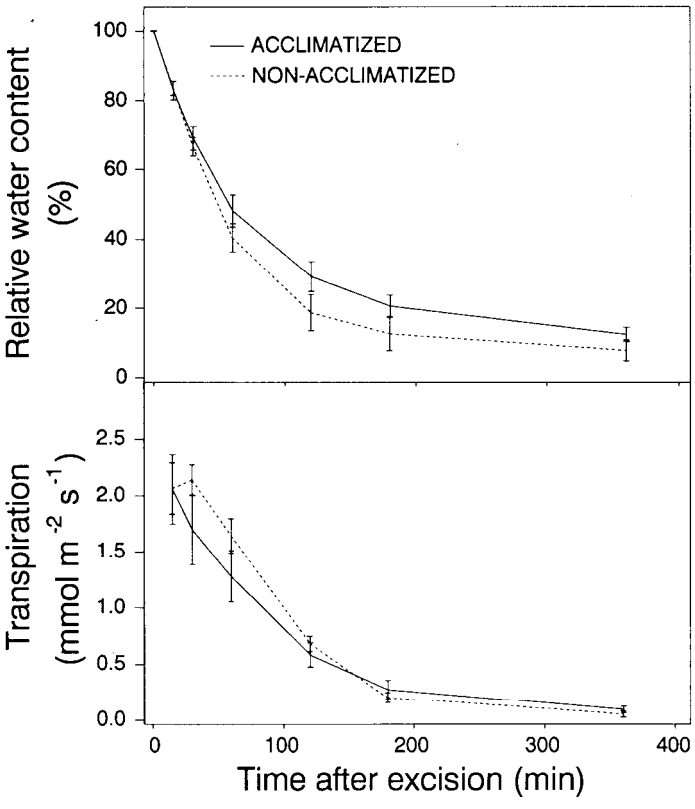


Fig. 6. Shoot relative water content and transpiration of acclimatized and nonacclimatized excised shoots. Each point is a mean of eight observations. Values are shown ± 2 SE,

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