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Relations between Intracellular pH, Water Relations and Morphogenesis in Rose Plants *in vitro*

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Abstract. Variations in water content, relative water content (RWC), pressure potential and intracellular pH (pH_i) had been studied as factors of bud growth in rose plantlets grown *in vitro* for micropropagation. During the phase of healing, pressure potential and pH_i were high enough to allow bud growth. During the following growth phase only buds of the lower part of the stem were able to grow. The growth ability of these buds and the growth inhibition of the others, associated with ΔpH_i, were retained during the last three days of culture, when dry matter content and RWC were the lowest. The results also showed a tight link between pH_i and pressure potential. This may enable to distinguish different stages of plantlet development *in vitro*.

Physiology of plantlets grown *in vitro* has rarely been studied. There are numerous problems in micropropagation, particularly connected with growth and stem development as affected by the components of nutrient medium.

In plants grown *in vitro*, like in field-grown plants, the development is closely linked up with the water relations. Growth is inhibited below a certain value of relative water content (RWC) (*e.g.* SINCLAIR and LUDLOW 1985). This was also confirmed in buds of *Bidens pilosus* L. (COUDRET *et al.* 1987).

Growth requires the cell pressure potential of the tissue in the zone of elongation to be lower than in the nearest adjoining zone (BOYER 1985). A consecutive decrease in pressure and water potential of cell implies formation of water potential gradient necessary for water movement into growing cells (LOCKART 1965). At the same time, the rate of cell extension is thought to be a function of the difference between the actual pressure potential and its threshold value required for growth (COSGROVE and CLELAND 1983).

The morphogenetic potential of the buds is also to a large extent controlled by the supply of metabolites contained from the subjacent parts; the quantity of these metabolites is dependent on intracellular pH of these parts and of the buds (*e.g.* GENDRAUD 1981, GENDRAUD and LAFLEURIEL 1983, TORT and GENDRAUD 1985).

In this study we tried to set up the link between water status, intracellular pH and morphogenesis. In plants cultivated *in vitro* the parameters of water relations are

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really not known; the one established fact is that stomata lose their reactivity and are always open (*e.g.* BRAINERD and FUCHIGAMI 1982, ZIV 1987).

MATERIAL AND METHODS

Plant Material

A rose tree (*Rosa* Madame Delbard (R) Deladel, hybrid of Thee) was micropropagated *in vitro* in the modified medium of Murashige and Skoog. Cultures were grown under 16-h photoperiod, irradiance 50 μmol m^{-2} s^{-1} and day/night temperature of 25/20 ± 1°C.

Measured Parameters

Water content was determined by drying the material at 80 °C and expressed as percentage of dry matter.

Relative water content (RWC). Actual water content was expressed in percentage of water content at full saturation (SINCLAIR and LUDLOW 1985).

Osmotic potential (π). Cell sap was expressed from tissue after exposure in boiling water. Osmotic potential was measured with a freezing-point osmometer (COUDRET 1979).

Water potential (ψ) was measured by Maximov's method. The concentration of sucrose solutions in which plants were incubated was determined with a refractometer. In the material used, comparisons with psychrometric method (WESCOR) gave the same results for both methods.

Pressure potential, turgor, (P) was calculated from the difference between osmotic and water potentials (π and ψ) as $P = \pi - \psi$.

Measurements were always made at the same time in order to eliminate variations in parameters measured due to photo- and thermoperiods. Measured plants were 1, 4, 8, 15, 18 and 21 d old. Each value is a mean of five repetitions.

Intracellular pH (pH_i) of plants was measured using dimethyl oxazolidine [^{14}C] 2–4 dione (DMO) as a probe (KURKJIAN and GUERN 1978, GENDRAUD 1981). The principle of the measurement is based on the penetration of the undissociated molecules which diffuse through the cell membranes. The molecules are dissociated inside and outside the cells according to the intracellular and extracellular pH and a diffusion equilibrium was established between the undissociated molecules in the cells and the incubation medium:

$$\frac{C_i}{C_e} = \frac{1 + 10^{(pKa - pH_i)}}{1 + 10^{(pKa - pH_e)}}$$

where C_i, C_e, pH_i and pH_e are the intracellular and extracellular concentrations of DMO, and intracellular and extracellular pH, respectively. The pKa of DMO was

taken as 6.3; C_i was measured after 12-h incubation, since this time allowed the diffusion equilibrium to be established. C_e and pH_e were measured directly. C_i could be defined as intracellular DMO quantity (Q_i), relating to intracellular volume (V_i). V_i is the difference between total volume (V_e), determined by mass and density, and intracellular volume (V_e). Apoplastic volumes (V_e) were determined by equilibrating tissues with a radioactive impermeant solute, and measuring the radioactivity associated with the tissues. It was generally near 20 % for our plants irrespective of their age. Q_i is the total radioactivity eluted from extracellular and metabolized DMO. Metabolized DMO was determined after chromatography of an aliquot of the extract on silicagel plates developed in isopropanol-ammonia-water (8/1/1, v/v/v). Measured plants were 1, 4, 8, 15, 18 and 21 d old. Intracellular pH of leaves, stems and buds was determined as shown on Fig. 1. All the data were the means of five repetitions.

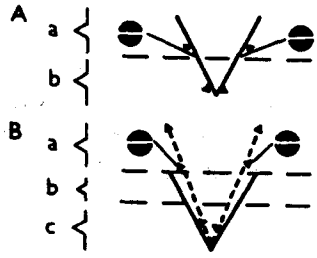

Fig. 1. Scheme of a plantlet grown *in vitro*: a, b, c – upper, lower and middle part of the stem, respectively. – A: plants 1 and 4 d old; B: plants 9, 15, 18 and 21 d old. Full line: old stem; dashed line: young stem; triangle: bud; half discs: leaf.

RESULTS AND DISCUSSION

The course of dry matter accumulation in a plant had three stages (Fig. 2a):

1) 0 to 4 d: during this stage dry mass was stable. Plants, highly trimmed at the time of pricking out were healing.

2) 4 to 17 d: a growth stage during which buds and then stem and leaves were growing.

3) 18 to 21 d: a stage during which growth was stopped before the next pricking out.

Water content (Fig. 2b) decreased during the micropropagation. However, after cutting and pricking out of three weeks old plants an important water uptake was induced that explained the water content increase from 1200 to 1900 % of dry mass during the first two hours after pricking out.

Relative water content. During first days of culture RWC was high; then it diminishes when buds started to grow, and afterwards it remained constant till three weeks (Fig. 2c).

Pressure potential. Pricking out, cutting and water uptake were linked with a very low pressure potential (0.3 MPa) during the first day of micropropagation

(Fig. 2d). From the fourth day, when healing was real, pressure potential was higher and more or less stable (0.6 MPa): the restructured membranes could keep pressure potential high enough for further development of growth potential.

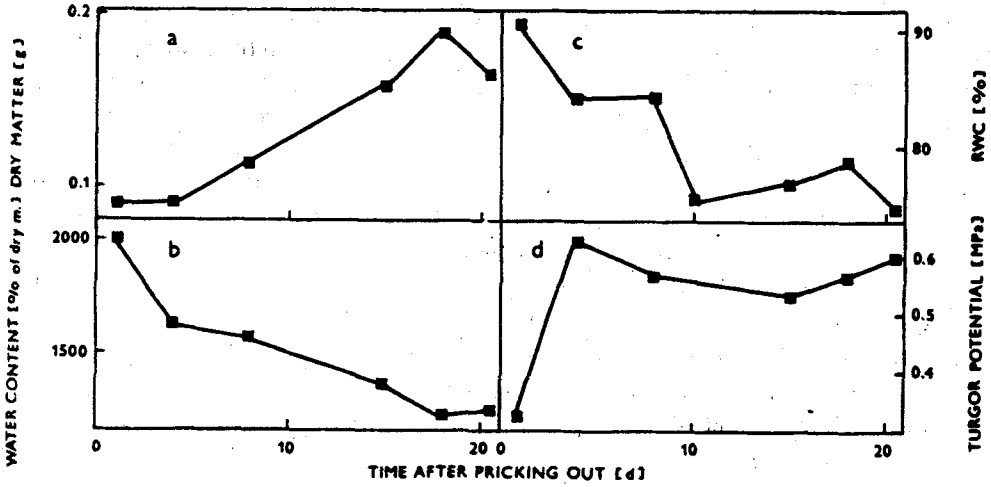

Fig. 2. Dry matter (a), water content (b), relative water content (c) and pressure potential (d) of plants at various periods after pricking out.

Intracellular pH. Micropropagation by clump separation required that only buds from the lower part of the stem grew. Intracellular pH measurements in buds, as a function of the age of plants and of the insertion level on the stem, showed that growth ability appeared between the 18th and the 21st days of growing: then, their pH became highly alkaline (Fig. 3).

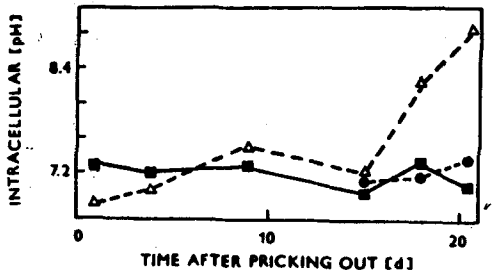

Fig. 3. Intracellular pH of buds of the upper (■), middle (●) and lower (△) part of the stem at various periods after pricking out.

However, there was an alternation between both phenomena: the upper leaves got more alkaline and the lower part of the stem became less alkaline. Immediately after pricking out, the lower part of the stem got more acid; in contrary the leaves kept the same alkaline pH_i for 21 d.

From the first to the fourth day, pH_i of the lower part of the stem increased similarly as pH_i of upper leaves (Fig. 3). Buds of the lower part of the stem grew in

9-d old plantlets, when the stem was alkaline and was also able to take up metabolites from the nutrient medium. From the 9th to 15th day, the pH_i of upper leaves increased, in contrary pH_i of the lower part of the stem decreased; metabolites did not accumulate in the cells of the lower part of the stem, being used for growth.

From the 18th day, growth rate decreased and, in the same time, the gradient of pH_i was inverted: there was an accumulation of metabolites in the lower part of the stem and this was the sign of considerable organogenesis in the buds of the same part.

Changes in the parameters of water relations in plantlets grown *in vitro* indicate three different stages of development: 1) healing stage till the fourth day, including restructuring of membranes necessary for growth; 2) cell growth stage from the 4th to the 17th day; 3) stage during which the plants look like mast organs and are characterized by a weak growth and low water content.

Immediately after pricking out, pH_i of the lower part of the stem became acid: cells below buds were not able to keep metabolites needed for growth and taken up from the medium. In the lower part of the stem the transmembrane H^+ gradient reached its initial level and allowed growth of buds between the fourth and the ninth days.

Till the 17th day, leaves obtain metabolites. At the end of growing, nutrients passed into the lower part of the stem: this allowed buds to grow. The increase in bud pH_i on the lower part of the stem was needed to allow micropropagation by clump separation; in fact, if picking out (cutting the top off, cutting off dead structures of the lower part of the stem, transplantation on new medium) took place the 18th day, *i.e.* before the increase in bud pH_i, all buds may grow.

High RWC is needed for bud growth. Buds do not grow at RWC lower than 70 % (end of subculture) or lower than 75 % (beginning of a subculture). However, when RWC is very high (more than 98 %) a majority of buds grow rapidly, and vitrification can occur (HARBAOUI and DEBERGH 1982). We found similar results with *Biddens pilosus* L. (CHANTELOUBE 1987).

A very tight link could be observed between water status and intracellular pH. 24 h after pricking out, water content was high and pressure potential was very low; this could indicate a disorganization of membranes. H^+ gradient on the plasmalemma decreased and the pH_i became acid. The first four days of growing, healing was simultaneous with an increase in pressure potential and alkalization of the lower part of the plant. This showed changes in membrane structures implied in the increase in pressure potential and accumulation of metabolites (ATPases and functioning of co-transport system).

During the stage of new clump growth, turgor potential became stable, water content decreased and the alkaline pH_i signified that cells of leaves could be able to keep metabolites; the cells were in meresis and auxesis. At the end of growth, a low water content showed that organs were not get young: the metabolites passed again to the lower part of the stem. Growth stopped; an organogenesis stage was responsible that the plants became more mast-like than before and the buds of the lower part of the stem could be able to grow.

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