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Effect of propagation and rooting conditions on acclimatization of micropropagated plants

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SUMMARY

Plantlets of *Calathea ornata* rooted at frequencies varying between 75 and 100% irrespective of the presence of 6-benzylaminopurine (BAP) or indolebutyric acid (IBA). After transfer to soil all plants grew rapidly with the exception of BAP-rooted plants, probably because these plants lacked lateral roots. Plantlets of *Malus* showed slightly improved rooting and considerably improved survival at increasing sucrose concentration from 20 to 30 g l^{-1} during multiplication. Their survival and performance after planting in soil depended upon the number of roots formed in rooting medium. Elevated CO_2-levels (800 ml m^{-3}) during acclimatization increased survival rate and plant height of rooted and non-rooted plantlets.

Key-words: acclimatization, *Calathea ornata*, *Malus*, micropropagation, rooting.

INTRODUCTION

Micropropagated plants often do not survive acclimatization, or they resume growth only a long time after planting in soil due to suboptimal conditions during the preceding multiplication, rooting and acclimatization stages. Acclimatization can be improved by using fog equipment or by increasing CO_2 levels (Debergh 1991). In addition, conditions during propagation and rooting phases may also enhance the performance after planting. Reduction of humidity in containers by using closures that allow water vapour to escape (Debergh 1991, and references therein) or by bottom cooling (Maene & Debergh 1987) may result in plants with improved water retention capacity. Kozai (1990) favours autotrophic growth *in vitro*, with CO_2 as the sole carbon source at high light intensities.

In this paper, we examine how survival and performance after planting in soil are affected by conditions during multiplication, rooting and the number of regenerated main and lateral roots. The results have been obtained with the herbaceous potplant *Calathea ornata* and the woody species *Malus*.

MATERIALS AND METHODS

Plant materials and culture conditions

Plantlets of *Calathea ornata* had been cultured for more than 2 years by subculturing shoots every 4 weeks on half strength LS-medium (Linsmaier & Skoog 1965) containing 1 mg l^{-1} 6-benzylaminopurine (BAP), and solidified with 7 g l^{-1} agar (BBL, granulated).

Plantlets were grown at 25°C and 12 h light day^{-1}. Light was provided by Philips TLD-33 cool-white fluorescent tubes with a photosynthetic photon flux density of 70 μmol s^{-1} m^{-2}. The mean multiplication factor was calculated as the number of shoots per initial shoot after 4 weeks on multiplication medium. Rooting *in vitro* was carried out on the same medium with hormones added as indicated. Four weeks after transfer to rooting medium, the rooting percentage, the average number of main roots, the average number of lateral roots and the lengths of the roots were determined. In each treatment 27 micro-cuttings were involved.

Plantlets of *Malus* 'Elstar', *Malus* 'M9-COST' and *Malus* 'M26' had been cultured *in vitro* for over 3 years by subculturing every 4 weeks on multiplication medium containing either MS-macroelements (Murashige & Skoog 1962), or Lepoivre (LEP)-macroelements (Quoirin 1977). Furthermore, the medium contained MS-microelements, nicotinic acid (0·5 mg l^{-1}), thiamine HCl (1·0 mg l^{-1}), pyridoxin (0·5 mg l^{-1}), myo-inositol (100 mg l^{-1}), sucrose (30 g l^{-1}), agar (6 g l^{-1}), BAP (1·0 mg l^{-1}) and indolebutyric acid (IBA; 0·1 mg l^{-1}). In one experiment, sucrose concentration was varied at 20, 25 or 30 g l^{-1}.

Rooting medium was composed of LEP macro- and micronutrients at half concentration. Concentrations of vitamins, sugar and agar were equal to those in multiplication medium. BAP was replaced by 0·2 mg l^{-1} IBA. This medium was used with or without proline (100 mg l^{-1}) + riboflavin (1 mg l^{-1}). After transfer to rooting medium, *Malus* shoots were first cultured at 20°C in the dark for 7 days and then transferred to the light (16 h day^{-1}, Philips TLD-33 cool-white fluorescent tubes at an intensity of 70 μmol s^{-1} m^{-2}) at the same temperature. In medium with riboflavin, transfer to the light resulted in a very rapid breakdown of IBA (Gorst *et al.* 1983). In medium without riboflavin, transfer to light also resulted in breakdown of IBA, but at a low rate (W. Van der Krieken, pers. comm.). Thus, the shoots cultured on medium with riboflavin were exposed to IBA for only 7 days, whereas the shoots cultured on medium without riboflavin were exposed to IBA for 28 days. After 28 days, the percentage of plantlets rooted *in vitro* was determined for each medium. The experiment involved 12 treatments for which there were 1440 plantlets per cultivar with six replicates in time. Results were analysed by ANOVA using GENSTAT statistical package preceded by an arcsin or log transformation, if necessary.

Acclimatization

Agar was rinsed from the plantlets with tap water. Plantlets of *Calathea* were transferred to commercial potting soil. *Malus* plantlets were transferred to a 1:1 mixture of peat and perlite. Acclimatization of *Malus* and *Calathea* normally occurred in a greenhouse in transparent plastic tunnels under a Macpenney fog-system at a relative humidity of 96–100%. Greenhouse temperature was kept at a minimum of 18°C. Maximum temperature in summer was 35°C. In winter, additional photosynthetic light was provided by SON-T lamps to a total day-length of 16 h. In one experiment, *Malus* 'M9-COST' plantlets, which were rooted on the medium with proline and riboflavin as mentioned above, were acclimatized in a tunnel under elevated CO_2-atmosphere (concentration was maintained at 800 ml m^{-3}). As a control plantlets were acclimatized in a tunnel without additional CO_2.

For *Calathea*, the leaf area of acclimatized plants was determined 9 weeks after transfer to soil. In *Malus*, the survival and plant height were determined 6 weeks after transfer to soil. To produce the best-fit curve for the results of the CO_2 experiments, GENSTAT non-linear regression analysis was used. One hundred and seventy-nine plantlets were used per treatment.

Table 1. The effect of type and concentration of growth regulator on rooting *in vitro* of microplants of *Calathea ornata*. For each group nine samples of three plantlets were examined

Growth regulator ($mg\,l^{-1}$)	Rooted microcuttings (% ± SE)
BAP (1·0)	85·2 ± 5·8
IBA (0·5)	100
IBA (1·0)	81·5 ± 8·1
IAA (0·5)	74·1 ± 7·4
IAA (1·0)	77·8 ± 7·8
No hormone added	85·2 ± 5·8

RESULTS

Calathea ornata

The mean multiplication factor of *C. ornata* was 2·8. Furthermore, *C. ornata* proved to be an easy-to-root species. Even on propagation medium (that contains 1 $mg\,l^{-1}$ BAP and no auxin), 75–100% of the plants formed one or more roots. A similar percentage of the plantlets rooted within 4 weeks on media where BAP was omitted or replaced by indole-acetic acid (IAA) or IBA (Table 1). The mean number of roots per plantlet was the same on all media. At the start of acclimatization there were no significant differences in size, main-root number (3–4) or main-root lengths (about 10 mm) between plants from the various treatments, but the average number of lateral roots per plantlet was different (Fig. 1a). In plantlets rooted on medium with BAP, lateral roots were absent. Nevertheless, all plants survived the acclimatization. However, in contrast with microplants rooted on the other media, plants rooted on medium with BAP, grew very little after planting. After 9 weeks, the leaf area (which was taken as a parameter for growth) of BAP-plantlets, was substantially lower than that of plantlets from the other treatments (Fig. 1b).

Malus

Rooting was slightly enhanced by the high concentration of sucrose and by using MS-nutrients instead of LEP-nutrients during propagation ($P < 0{\cdot}05$) (Table 2a). Table 2b gives the percentage of *Malus* microcuttings that survived the rooting and acclimatization procedures. Both the sucrose concentration (30 $g\,l^{-1}$ resulted in higher survival than 20 $g\,l^{-1}$) and the mineral composition (MS-nutrients showed higher survival than LEP-nutrients) influenced the survival rate. The duration of the IBA-treatment (7 or 28 days) did not significantly affect the rooting percentage or the survival percentage (data not shown).

The number of roots per microcutting present at the time of transfer to acclimatization conditions was positively correlated to survival and growth (Fig. 2). For high ($\geq 80\%$) survival of the microcuttings under normal acclimatization conditions, a minimum of two roots per plantlet was required (Fig. 2a). When extra CO_2 was added, the survival increased only slightly for rooted plantlets, but almost doubled for non-rooted plantlets or

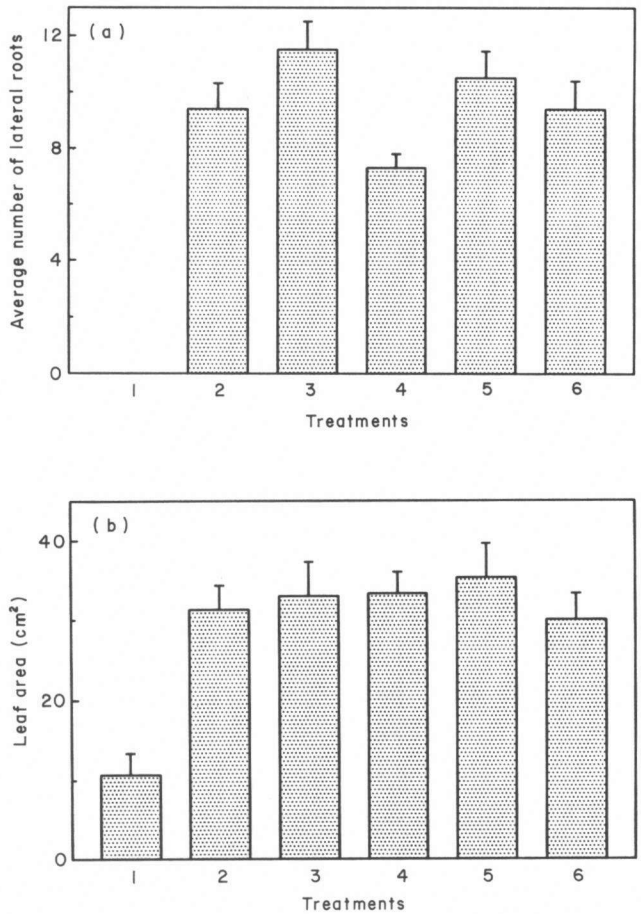

Fig. 1. Effect of type and concentration of growth regulator on average number of lateral roots per *Calathea ornata* plantlet formed on rooting media with different hormones (a) and on leaf area of acclimatized plants of *Calathea ornata* 9 weeks after transfer to soil, rooted on media with different hormones (b). In each treatment 27 plantlets were involved. 1: 1·0 mg l^{-1} BAP; 2: 0·5 mg l^{-1} IBA; 3: 1·0 mg l^{-1} IBA; 4: 0·5 mg l^{-1} IAA; 5: 1·0 mg l^{-1} IAA; 6: no hormone added.

plantlets with only one root. From the *Malus* plantlets that had not formed roots during the rooting period on agar, 20% (without extra CO_2) or 50% (with extra CO_2) still survived acclimatization (Fig. 2a), but their height at the end of the acclimatization period had hardly increased (data not shown). However, growth of the rooted plantlets increased from 50 to 100% under elevated CO_2 (Fig. 2b).

DISCUSSION

The most important parameter for successful micropropagation is, of course, survival and performance after acclimatization. The results presented in this paper show that successful acclimatization of micropropagated plants is influenced not only by the conditions during the rooting and acclimatization phases, but also by conditions during the propagation stage. The positive effect of high sucrose concentration during propagation on the final

Table 2. The effect of sucrose concentration and mineral salts (MS or LEP) in the propagation medium on rooting percentage and on survival of *Malus* 'Elstar' and *Malus* 'M26'. Rooting was scored 28 days after transfer to rooting medium (a). Survival is expressed as the percentage of initially unrooted explants that survive and grow after acclimatization (b). Results were analysed by ANOVA using GENSTAT statistical package, if necessary preceded by an arcsin or log transformation. Rooting differed significantly at $P=0{\cdot}05$ for sucrose concentrations and mineral salts. For survival, sucrose concentrations differed significantly at $P=0{\cdot}001$, mineral salts at $P=0{\cdot}05$

	Mineral salts ($g\,l^{-1}$ sucrose)	'Elstar'		'M26'	
		MS	LEP	MS	LEP
(a) Rooting (%)	20	81·2	82·6	83·2	88·4
	25	90·2	79·6	92·2	83·8
	30	92·8	83·8	92·9	89·2
(b) Survival (%)	20	52·1	66·0	65·6	57·2
	25	69·6	63·6	74·8	60·6
	30	75·2	77·4	83·4	75·3

survival of *Malus* may be directly related to accumulation of carbohydrates (Debergh 1991; Debergh *et al.* 1992). This accumulation could compensate for the reduced photosynthetic activity in the first days of acclimatization during which the photosynthetic system is initiated (see Debergh *et al.* 1992). In strawberry, carbon fixation of leaves produced *in vitro* was low and insufficient to sustain autotrophic growth and did not improve significantly during acclimatization (Grout & Millam 1985). *Ex vitro* plantlets, therefore, depended on stored products in shoots and roots for subsequent development during acclimatization, until new productive leaves were produced. In addition, the increase in osmolality of the medium by increased sucrose concentrations may render the microcuttings better equipped to manage the drought conditions *ex vitro*.

The effect of mineral composition on survival is difficult to explain. The difference between MS-medium and LEP-medium is that the latter contains about one-quarter of the concentration of NH_4NO_3 present in MS-medium and 5 mM $Ca(NO_3)_2$ instead of 3 mM of $CaCl_2$ present in MS-medium. Apparently the decreased ammonium concentration and increased calcium concentration are sufficient to cause the effects observed.

Both in *Calathea* and *Malus* the presence of a functional root system appears to be more important for performance than for survival. In *Calathea*, microplants without lateral roots survived the acclimatization stage, but their growth was less than that of plants with lateral roots. The slow growth is probably correlated to the complete absence of lateral root formation on BAP-medium, although the mean lengths of the main roots from the different treatments were similar. Cytokinins inhibit the formation of lateral roots (Torrey 1962). It should be noted that the concentration of cytokinin that inhibited formation of lateral roots in *Calathea*, allowed the regeneration of adventitious main roots from the stem. Apparently, regeneration of lateral roots on the main root was more sensitive to BAP than regeneration of adventitious roots on the stem.

In *Malus*, the number of roots per plantlet appeared to be more important for survival and subsequent growth than the presence of lateral roots. Plantlets with less than three roots had a lower survival rate. Nevertheless, 20% of the non-rooted shoots still survived

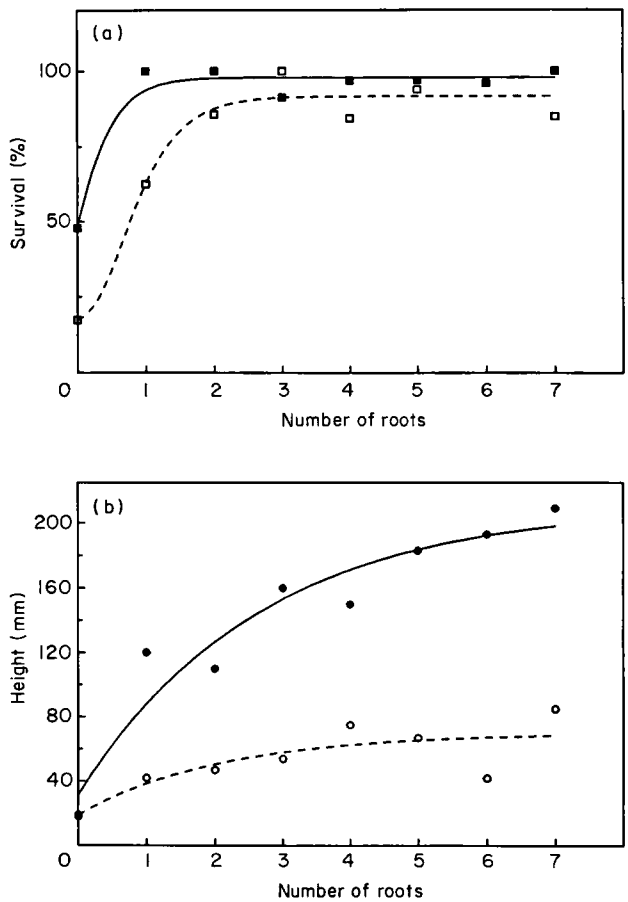

Fig. 2. Effect of a constant CO_2-level of 800 ml m^{-3} during acclimatization (filled symbols) or no additional CO_2 (open symbols) in relation to the number of roots of *Malus* plantlets on the survival rate after acclimatization (a) and the plant height at the end of the acclimatization period (b). Microcuttings were rooted as described in Materials and Methods. After rooting, plantlets were transferred to a peat-perlite (1:1) mixture and acclimatized. In each treatment 186 plantlets were used. The numbers of plantlets analysed were 23, 16, 14, 23, 32, 33, 25 and 20 for 0–7 roots per plantlet, respectively. Curves were fitted by non-linear regression analysis using GENSTAT statistical package.

acclimatization and had formed roots after this period. The reason why these initially non-rooted plantlets formed roots in soil might be caused by the higher availability of oxygen in soil than in agar. Rooting is improved by aerobic conditions (Pierik & Steegmans 1975). A second possible cause could be that during the period *in vitro*, light inhibited the progress of the rooting process.

Elevated CO_2 had a striking effect on both survival and growth of *ex vitro* plantlets. The survival rate of non-rooted plantlets was more than doubled. In strawberry, CO_2-enrichment during acclimatization resulted in significant increases in root dry weight (Desjardins *et al.* 1987). The plantlets apparently divert photosynthates to produce roots first, at the expense of shoot growth. A second explanation could be that, in both *Calathea* and in *Malus*, the onset of growth was retarded because the absence of functional roots rendered the plantlets lacking in the possibility of supplying sufficient H_2O to exploit the

photosynthetic capacity to its full extent. High humidity during the acclimatization period would then merely be necessary for the maintenance of the turgor and not the support of photosynthesis.

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