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Application of antioxidants in rooting of *Prunus avium* L. microshoots

Virge Vasar*

Plant Biotechnological Research Centre EVIKA, Estonian Agricultural University, Teaduse 6a, Saku 75501, Harjumaa, Estonia
*Corresponding author, E-mail: virge.evika@mail.ee

Abstract

The influence of antioxidants on *in vitro* rooting, acclimatization and *ex vitro* growth of sweet cherry (*Prunus avium* L.) microshoots was studied. Trials were conducted with Estonian sweet cherry cultivar 'Kristiina'. Four antioxidants were tested: glutathione (reduced form), dithiothreitol, propyl gallate and ascorbic acid. The *in vitro* rooting and subsequent growth of plants in greenhouse was promoted by antioxidants, but the influence on acclimatization was not always significant. The *in vitro* rooting of microshoots was most improved by 0.25 mM dithiothreitol: 27 % more shoots formed roots compared to untreated shoots. Dithiothreitol 0.25 mM improved the acclimatization of the plants in the greenhouse. The shoot and root growth of cv. 'Kristiina' was most vigorous also on plants that were rooted *in vitro* in the presence of 0.25 mM dithiothreitol.

Key words: acclimatization, antioxidants, micropropagation, *Prunus avium*, rooting, sweet cherry.

Introduction

Micropropagated plants are grown *in vitro* under low levels of light, aseptic conditions, on a medium containing sugar and nutrients to allow heterotrophic growth and at a high relative humidity. Therefore the leaves that develop *in vitro* generally lack well developed epicuticular waxes (Sutter, Langhans 1982), they have malfunctioning stomata (Brainerd, Fuchigami 1981; Marin et al. 1988), and a poorly structured internal anatomy (Marin et al. 1988; Noé, Bonini 1996), and they may not be photosynthetically efficient (Donnelli, Vidaver 1984; Grout 1988). This increases the susceptibility of plantlets to stress under the unstable climatic conditions in greenhouse. Some scientists suggest that rooting of micropropagated plants can be improved by treatment with antioxidants (Stonier 1971). Antioxidants can potentially protect the natural plant rooting hormones from oxidation, enhancing rooting and increasing the tolerance of plants to greenhouse conditions (Lis-Balchin 1989). The aim of the current research was to study the influence of antioxidants on *in vitro* rooting, acclimatization and *ex vitro* growth of sweet cherry microshoots.

Materials and methods

Trials were conducted with sweet cherry cultivar of Estonian origin – 'Kristiina'. In all trials one- or two-years-old tissue culture material was used. Shoots 2.0 to 3.0 cm in

length were rooted on modified Murashige and Skoog (1962; MS) medium containing ½ MS macroelements, full concentration of MS microelements and vitamins, 30 g l^{-1} sucrose, and 2 mg l^{-1} IBA. The pH of media was adjusted to 5.7 - 5.8.

The solutions of antioxidants were sterile-filtrated and added after autoclaving of media. Four antioxidants – glutathione (reduced form, GSH), dithiothreitol (DTT), propyl gallate (PrGl) and ascorbic acid (AscA) were used. Antioxidant concentrations tested were 0, 0.0025 mM, 0.025 mM, and 0.25 mM.

Temperature in the growth room was 22 to 24 °C, photoperiod 16 h and light intensity 35 to 40 μmol m^{-2} s^{-1}. After two weeks of *in vitro* rooting the microshoots were planted in peat substrate in seed trays (VP-96 type) and transferred to greenhouse for acclimatization and *ex vitro* growth. The growth conditions in the greenhouse were set at temperature 20 to 25 °C during the day and 16 to 18 °C at night. After four week acclimatization the survived plants were counted, removed from the substrate and dry massess of shoots and roots were measured.

All trials were conducted in one replication but were repeated three times. For each experiment 100 plants per treatment were planted *in vitro* onto rooting media, and 96 plants were planted *ex vitro* into trays. The results were analyzed statistically using computer software SAS for Windows 8.2 (http://www.sas.com) by single-factor Student's *t*-test. The 95 % least significant difference $LSD_{0.05}$ was determined.

Results

The *in vitro* rooting of cv. 'Kristiina' microshoots varied between treatments, ranging from 49 to 89 % of all planted shoots (Table 1). The highest increase in the number of rooted shoots was achieved by adding DTT to the rooting medium. In the presence of 0.025 mM and 0.25 mM DTT, 27 % more shoots formed roots compared to untreated shoots. Antioxidant treatment affected also the number of roots formed on the shoot. DTT, PrgGl and AscA increased the number of roots per shoot significantly at a 0.25 mM concentration.

The subsequent influence of *in vitro* rooting conditions on the survival of microplants in the greenhouse varied between treatments (Fig. 1). Among the tested antioxidants, only DTT and AscA had improved the survival of microplants. The survival in greenhouse was

Table 1. The influence of antioxidants on the *in vitro* rooting of *Prunus avium* L. microshoots of cv. 'Kristiina'. Evaluation after 2 weeks of *in vitro* rooting. Values represent the mean of 100 explants per treatment of three repeated tests. GSH, glutathione (reduced form); DTT, dithiothreitol; PrGl, propyl gallate; AscA, ascorbic acid. *, values significantly different from untreated control

Concentration	Root-forming shoots *in vitro* (%)				No. of roots per shoot			
(mM)	GSH	DTT	PrGl	AscA	GSH	DTT	PrGl	AscA
0	73	49	64	58	5.3	5.1	5.4	5.4
0.0025	64	68*	78*	61	5.3	5.1	6.5	5.9
0.025	79	76*	72	67	6.8*	5.1	5.5	5.8
0.25	65	76*	89*	65	5.9	6.4*	8.7*	7.3*
$LSD_{0.05}$	13	13	12	14	1.4	1.3	1.6	1.6

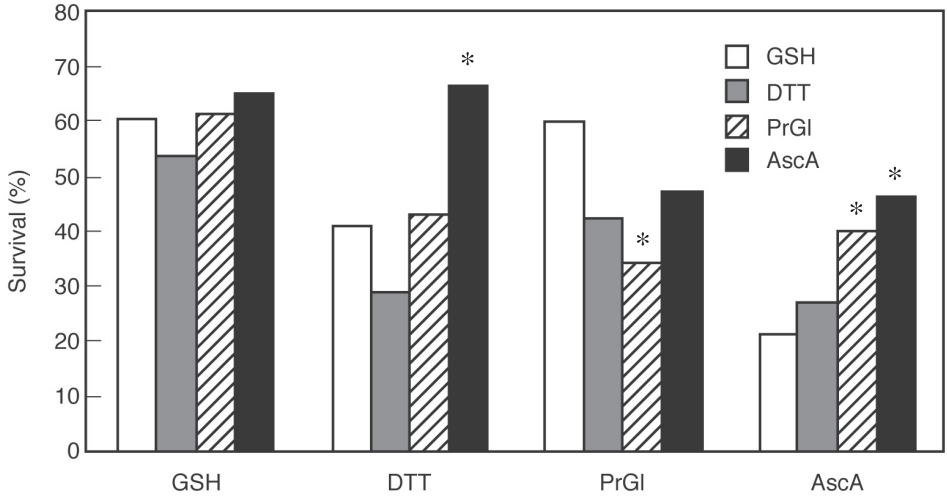

Fig. 1. The influence of antioxidants on survival of *Prunus avium* L. microshoots *ex vitro*. $LSD_{0.05}$ GSH = 14; DTT = 14; PrGl = 15; AscA = 14. Evaluation after 4 weeks of *ex vitro* growth. Values represent the mean of 96 explants per treatment (three repeated tests). GSH, glutathione (reduced form); DTT, dithiothreitol; PrGl, propyl gallate; AscA, ascorbic acid. *, values significantly different from untreated control.

improved 1.6 fold when shoots were rooted on the medium containing 0.25 mM DTT, and 2.1 fold when they were rooted at 0.25 mM AscA as compared to untreated shoots.

There was no clear relationship between survival percentage and dry mass ofshoots and roots of plants acclimatized in the greenhouse. The dry mass of shoots and roots was raised by treatment with DTT at the concentration 0.25 mM (Fig. 2). The other antioxidants demonstrated a slight positive effect on the growth of shoots and/or roots but the difference was insignificant compared to the untreated plants (data not shown).

Discussion

The rooting intensity of sweet cherry *in vitro* varied in relatively large scale between untreated microshoots. The rooting capacity of microshoots depends on many factors – the age of explants (number of subcultures); amount of BA in culture medium and the amount of BA taken up by the tissues during *in vitro* cultivation; the ratio between cytokinins and auxins in culture medium etc. (George 1993). It has been found that the rooting capacity of apple microshoots varied not only within the cultivar but even within the same shoot cluster (Hicks 1987; Welander, Pawlicki 1993). In the present study it was rather difficult to select microshoots by their rooting ability. There were no visual symptoms referring to low rooting capacity. All malformed shoots (e.g. vitrified or presenting other epigenetic changes) were immediately discarded.

Our trials were conducted during three years. The initial test material was renewed periodically in order to use shoots of the same age in all the trials. Because of persistent renewal, the microshoots used in different trials originated from different mother plants which may have caused the large variation in rooting capacity. Nevertheless, it was evident that in the case of low rooting capacity of microshoots the formation of roots

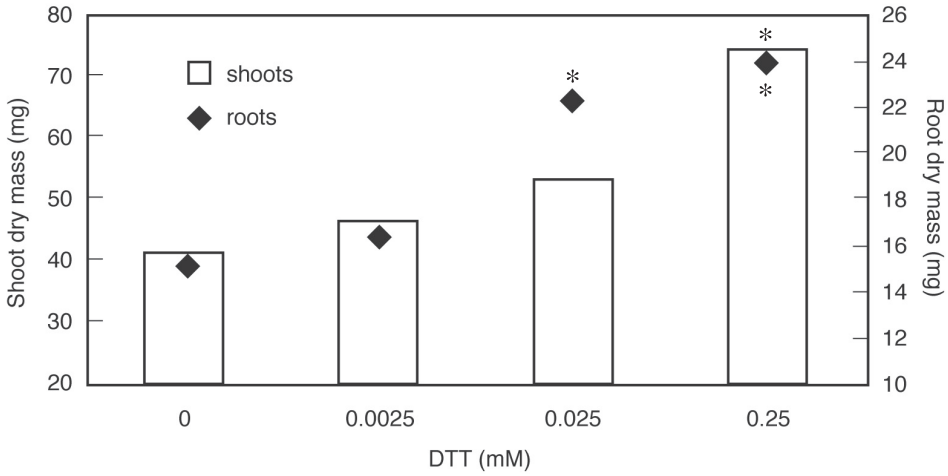

Fig. 2. The influence of dithiothreitol (DTT) on dry mass of shoots and roots of micropropagated *Prunus avium* L. *ex vitr*o. $LSD_{0.05}$ shoots = 15; roots = 5. Evaluation was performed after 4 weeks of *ex vitro* growth. Values represent the mean of 96 explants per treatment (three repeated tests). *, values significantly different from untreated control.

in vitro was enhanced by supplementing the rooting medium with a certain amount of antioxidant. Similar to that, the variation in survival percentage of untreated microplants can be affected by the time of the year the tests were performed.

The effect of antioxidants depends on their concentration in the medium as well as on the plant genotype. Bonner and Axtmann (1937) demonstrated that the rooting of pea microshoots was promoted by 50 mg l^{-1} (0.25 mM) ascorbic acid, while 25 mg l^{-1} (0.126 mM) and 100 mg l^{-1} (50 mM) were detrimental. The best *in vitro* rooting of soybean was achieved with 0.1 mM GSH and DTT, and the best rooting of apple with 0.25 mM DTT or 0.075 mM GSH (Auderset et al. 1996). In our trials rooting was improved by antioxidants at a 0.25 mM concentration. Although the rooting percentage was increased already by 0.0025 mM DTT, a 0.25 mM concentration was needed to increase also the number of roots.

During the acclimatization process plants have to adapt to the new environmental conditions such as lower relative humidity, higher light density, fluctuating temperatures and constant disease stress (Preece, Sutter 1991). A prerequisite for the survival of rooted microcuttings is that the roots support the plant while new leaves and stems are produced during acclimatization (Nemeth 1986). It has been observed that *ex vitro* rooting and simultaneous acclimatization for different crops can save time and other resources (Maene, Debergh 1983; Preece, Sutter 1991). In our trials the survival of shoots that had not formed roots *in vitro* varied within the same range as that of rooted microplants. The positive effect of antioxidants was more pronounced in the trials where the overall survival of microplants was low.

In our trials the survival of shoots that had not formed roots *in vitro* varied at the same range as that of rooted microplants. However, the survival of untreated shoots was correlated to the formation of roots *in vitro* of the same treatment and depended on the physiological conditions of microplants at the moment of planting *ex vitro*. The

rooted microshoots were apparently stronger and therefore better adapted to greenhouse conditions than unrooted shoots. Similarly to *in vitro* rooting results, the positive effect of antioxidants was more expressed in the trials where the overall survival of microplants was low.

There was no relationship between the *in vitro* rooting of microshoots and *ex vitro* growth of plants. This is consistent with the results of micropropagated grapes where the number new roots produced *ex vitro* was independent from roots formed *in vitro* (Thomas, Ravindra 1997).

It has been demonstrated that a mixture of two antioxidants can affect the rooting of cuttings synergistically. There was a considerable enhancement in rooting of *Geraniceae* cuttings after treatment with a mixture of vitamins C and E or propyl gallate and butyl-hydroxyanisole than when these components were used alone (Lis-Balchin 1989). In our trials all antioxidants were used alone, not in mixtures, which can may explain why the effect of some of the compounds remained relatively low. In future studies we are planning to test the efficacy of combinations of different antioxidants on the *in vitro* rooting, subsequent acclimatization and *ex vitro* growth of micropropagated plants.

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