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The importance of the sterilization procedure for producing vigorous cherry plants (*Prunus* sp.) *in vitro*

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ABSTRACT

The micropropagation of woody species is a good method for producing juvenile plant material in the short time. The sterilization procedure can have a slightly negative influence on the inoculation of winter buds, the induction and continuous growth of cherry microcuttings. The surface sterilization with the dicloroisocyanuric acid Na_2 salt (DICA) was carried out in two procedures. In the procedure 1, 70% ethanol for 30 sec and 20 g/l of DICA for 20 min were used, while in the procedure 2 only 16.6 g/l of DICA for 15 min was used. Compared with the procedure 1, the procedure 2 showed significantly ($P \leq 0.05$) better induction results and the production of the most vigorous plants (better growth, better root formation).

Key words: *Prunus* species, cherry, sterilization procedure, dicloroisocianuric acid Na_2 salt, induction, rooting

IZVLEČEK

POMEMBNOST POSTOPKA STERILIZACIJE ZA PROIZVODNJO VITALNIH RASTLIN ČEŠENJ (*Prunus* sp.) *in vitro*

Mikropropagacija je uspešna metoda pri lesnatih rastlinah za proizvodnjo juvenilnega materiala v kratkem času. Sterilizacija rastlinskega materiala lahko negativno vpliva na indukcijo in diferenciacijo kulture ter nadaljno rast tudi pri češnjah. Površinska sterilizacija zimskih brstov češenj je bila opravljena z dikloroizocianurno kislino Na_2 soli (DICA) z dvema postopkoma. Pri prvem postopku smo brste sterilizirali 30 sec s 70% etanolom in nato 20 min z 20 g/l DICA. Pri drugem postopku smo uporabili samo 16,6 g/l DICA 15 min. Z drugim postopkom smo v primerjavi s prvim dobili statistično značilno ($p \leq 0,05$) boljše rezultate indukcije brstov in v nadaljnih subkultivacijah vitalnejše poganjke glede rasti in razvoja korenin.

Ključne besede: vrsta *Prunus*, češnja, postopek sterilizacije, dikloroizocianurna kislina Na_2 soli, indukcija, koreninjenje

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1 INTRODUCTION

The knowledge of micropropagation of many woody species is well extended. This holds true for the *Prunus* species (Snir, 1982; Yang, 1994; Preil, 1997) as well. Many cherry rootstocks (Gisela 5, Weiroot, Maxma) are even micropropagated commercially, although several reports consider the micropropagation to be a technique not useful for producing rootstock commercially (Grant and Hammatt, 1999; Osterc and Spethmann, 2000). However, the micropropagation practice points to some steps that are not successful enough in *Prunus*, so they should to be clarified.

YANG (1994) reported difficulties in the rooting process in *Prunus* in his experiment, so the propagation process should have been optimised. He could achieved the best rooting with the use of NAA (α–naphthaleneacetic acid). The use of NAA was already suggested by Snir (1982) who forced better rooting with the basal shoot wounding. Very good rooting results were achieved with the use of IBA (indole-3-butyric acid) in the experiment on the micropropagation of wild cherry *Prunus avium* L. (Meier-Dinkel, 1986).

The problems can be associated also with the sterilization procedure of the plant material in the inoculation phase. The standard sterilization procedure with the use of 3% NaOCl gave very satisfactory results, also in the case of the propagation of *Prunus in vitro* (Meier–Dinkel, 1986; Preil, 1997). Meier-Dinkel (1986) reported about the only 2% contamination with bacteria, but later 55% explants did not survive the multiplication. Anyway, the usefulness of this standard sterilization method, often used together with 70% ethanol, was already shown and discussed in experiments with *Lilium*-bulbs and *Acer* buds (Langens-Gerrits, 1998). Although the hot water treatment (HWT) was used together with the standard sterilisation method in these experiments the contamination rates reached values over 25% at *Acer*-winter buds.

In our article the use of sterilization with DICA is discussed and compared with standard sterilization methods.

2 MATERIALS AND METHODS

For the culture initiation axillary winter buds of *Prunus* species (*Prunus avium*, *Prunus subhirtella* var. *autumnalis* and a hybrid clone Gisela 5, which is used as a rootstock in fruit growing) were used. Stock plants of all species were grown at the same place on the experimental field of Biotechnical Faculty in Ljubljana (Slovenia) under the same conditions. For *Prunus avium* and *Prunus subhirtella* var. *autumnalis* older trees (15 years) were used as stock plants, for Gisela 5 the two-year-old stock plants were used. The surface sterilization was carried out in two ways. The buds of *Prunus avium* und Gisela 5 were rinsed for 30 sec in 70% ethanol, then sterilized in 20 g/l DICA (dicloroisocyanuric acid Na_2 salt, Sigma D-2536) with the addition of a few drops of Tween 20 for 20 min and rinsed three times in the sterilized water. The second sterilization procedure with *Prunus subhirtella* var. *autumnalis* was composed of sterilization with 16.6 g/l DICA with the addition of a few drops of Tween 20 for 15 min and three times rinsed with the sterilized water. After the surface sterilization the bud scales were removed as thoroughly as possible, only in *Prunus subhirtella* var. *autumnalis* at one part of the buds the removal of the bud scales was not so precise.

In the inoculation phase the explants were cultured in 100-mm Petri dishes (8 buds per dish), later in the multiplication phase they were transferred into the glasses. Both, Petri dishes and

glasses were sealed with Parafilm and the explants were exposed to the 16/8 photoperiod at 23 ± 1°C and illumination of 80 μmol $m^{-2}s^{-1}$.

The induction medium (MEIER–DINKEL 1986) was composed of MS macro and micro elements and vitamins, 2 mg/l glycin, 100 mg/l myo-inositol, 30 g/l sucrose and 8 g/l agar (Difco-Bacto). For the induction phase 0.1 mg/l IBA (indole-3-butyric acid), 1.0 mg/l BA (6-benzyladenine) and 0.1 mg/l GA_3 (gibberellic acid) were used. The pH of the medium was adjusted to 5.7 with 1N KOH or HCl before autoclaving at 121 °C for 20 min. In the multiplication and elongation phase the basal medium, vitamins, glycine, myo-inositol, sucrose, agar, pH value, IBA and GA_3 was the same as in the induction medium, only the hormone BA was less concentrated (0.5 mg/l). When the shoots reached the length of about 2 cm, they were transferred into the rooting media. For the rooting process 1/2 concentration of the previously cited MS-medium (for Gisela-cuttings full concentration of MS-medium) was used with 1.0 mg/l IBA as a hormone. Other components were the same as in the induction and multiplication medium. After successfull rooting the shoots with two or more roots with the length of more than 2 cm were planted in the soil and acclimated in the greenhouse.

In the inoculation phase the number of the infected, browned and growing buds were measured. The induction and the infection rates were calculated. Thereafter, the development of growing buds and the time of the transferring into the rooting medium were noted. The rooting results were observed and scored. The one-way ANOVA followed by the Duncan's multiple-range test was conducted to evaluate differences among the treatments.

3 RESULTS AND DISCUSSION

The stock plants of all species grew outside, in the field, and they were usually contaminated before the excision of the axillary buds. It could be expected that the contamination rate was the lowest at the buds of Gisela 5 because the stock plants were micropropagated and they were only two years old. However, after the sterilization procedure 1 (70% ethanol and 20 min with higher concentration 20 g/l DICA) the contamination rate reached 28.6% in Gisela 5 and 8.7% in *Prunus avium* and the rate of browned buds was with 65.2% the highest in the buds of *Prunus avium* and the lowest 28.6% in Gisela 5 (Figure 1). The buds of the ornamental *Prunus* species *Prunus subhirtella* var. *autumnalis* were almost clean after the sterilization procedure 2 without ethanol and shorter time (15 min) with lower concentration (16.6 g/l) of DICA. The contamination rate was only 6.7% at a less precise removal of the bud scales and 15.0% at the precise removal. No browning buds could be found (Figure 2). There were no negative influences noticed in the buds after the sterilization procedure 2 (Figure 2). The results (Figure 2, Table 1) showed that the highest induction of buds (less precise 93.3% or precise 85.0%) without damages was obtained in the sterilization procedure 2. In contrast, during the inoculation phase after procedure 1 statistically significantly less successfully inducted buds (42.9% at Gisela 5 and only 26.1% at *Prunus avium*) were observed compared to the procedure 2 (Table 1).

The reduction in the contamination rate by using only 16.6 g/l DICA, 15 min was very successful so the method can be regarded as a very positive one when compared with the literature data where the sterilization results showed at *Acer*-winter buds over 25% contamination (Langens-Gerrits *et al*., 1998). This positive effect of the second sterilization procedure could be observed in both cases – when the bud scales were removed precisely or not (Figure 2). On the other hand, the precise removal of the bud scales in Gisela 5 and *Prunus avium* could not stop the contamination. The results

with the ornamental *Prunus* species showed that the lowest concentration of DICA stopped the contamination compared with the higher concentration of DICA and ethanol (Table 1). The worse sterilization results (high percentage of the browning buds) of the procedure 1 were probably caused by the effect of ethanol or by the comparison with the higher concentration of DICA (Figure 1).

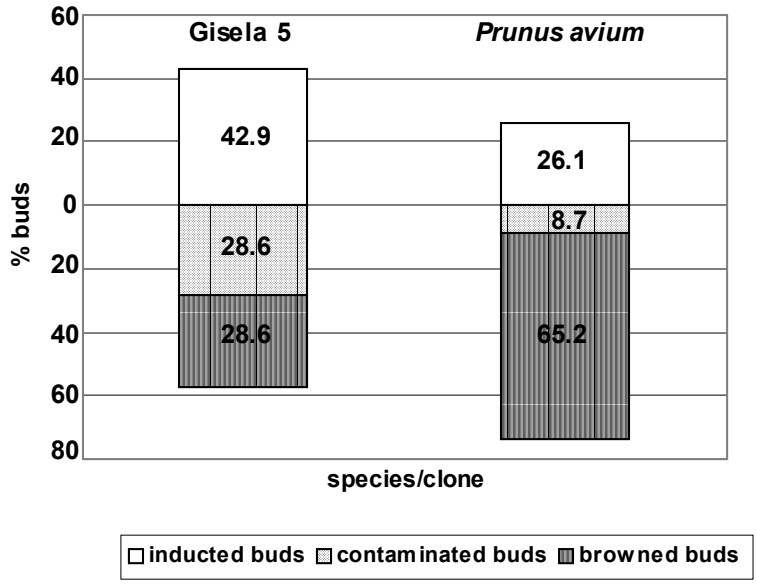

Figure 1. The contamination and the induction results of the axillary winter buds at two different *Prunus* species after sterilization procedure 1.

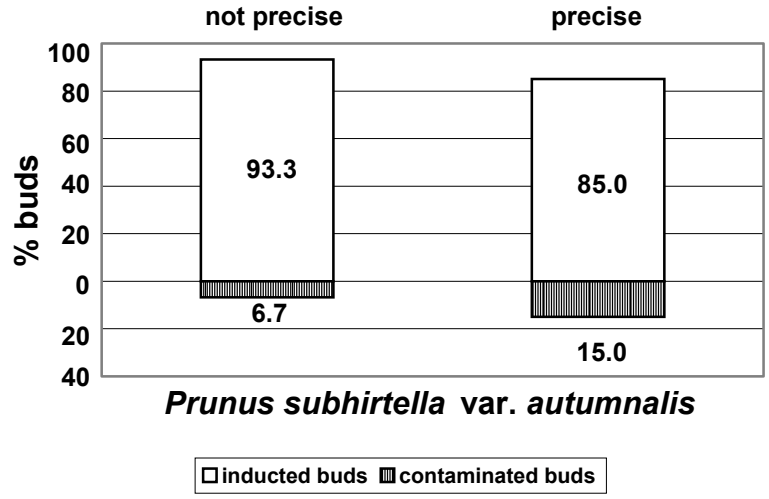

Figure 2. The contamination and the induction results of the axillary winter buds at *Prunus subhirtella* var. *autumnalis* after sterilization procedure 2 with regard to the degree of the removal of the bud scales.

Also the future development of the buds after their induction differed among the species with regard to the sterilization procedure. Even the effect of the sterilization procedure 1 differed significantly between Gisela 5 and *Prunus avium* (Figure 1). 3 to 5 days after the inoculation the buds colour turned from green to light or dark brown.

The dark browning buds did not develop later or started developing and then stopped or showed only limited growth. The light browning buds started growing later and more slowly than the undamaged green buds. The buds of *Prunus avium* survived till the end of the induction phase but their vigour was not satisfactory. They did not survive the following subculturing at all (Table 1).

Table 1: The induction results at different *Prunus* species with regard to the sterilization procedure

Plant species	Removal of the bud scales	Sterilization procedure	Time of the inoculation	Inducted buds (%)*	Transfer into the multiplication medium (weeks)	Transfer into the rooting medium (weeks)
Gisela 5	precise	1	20. 03. 2001	42.9 b	5	24
Prunus avium	precise	1	20. 03. 2001	26.1 a	5	no survival
Prunus subhirtella var. *autumnalis*	not precise	2	27. 03. 2001	93.3 c	4	18
Prunus subhirtella var. *autumnalis*	precise	2	27. 03. 2001	85.0 c	4	18

* Percentages with identical letters following the values indicate no significant differences according to the Duncan's multiple-range test ($p<0.05$).

The time needed for forming the plants which could be rooted well was in Gisela 5 six weeks longer than in *Prunus subhirtella* var. *autumnalis* (Table 1). The buds of *Prunus subhirtella* var. *autumnalis* were not damaged during the sterilization procedure 2. All of the inoculated buds were still green (Figure 2) and after the first 5 to 10 days the explants doubled or more than doubled their size. After the next 10 days the difference between *Prunus subhirtella* var. *autumnalis* and Gisela 5 became more distinct. In the multiplication medium inside the multiple shoot structure from 3 to 5 or more individual shoots were obtained. This also means that the time needed for producing ready plants (propagation time) was the longest with the damaged buds. The duration of the entire propagation period was dependent on the sterilization method as well.

The unsatisfactory results in the propagation Gisela 5 and *Prunus avium* were mainly achieved due to the bad inducted and elongated bud material which was transferred into the multiplication medium. The induction of these buds was interrupted due to the damages during the sterilization procedure. The appearance of such damages was already published (Meier–Dinkel, 1986). In his results the 55% explants which did not survive the multiplication could be damaged through the use of ethanol and 3% NaOCl.

The rooting results varied very strongly among several species. The rooting rates and the number of roots per rooted plant were with 100% from 4.4 to 5.2 roots per plant greater with *Prunus subhirtella* var. *autumnalis* than with Gisela 5 where only 75% of explants were rooted with 1.7 roots per plant (Table 2). These worse results with Gisela 5 could be ascribed also to a different sterilization procedure. After two or

more subcultures in the multiplication media, vigorous shoots can be the same (rooting and the root number) in both procedures. We lost only the time for producing vigorous plants.

Table 2: Rooting results at different *Prunus* species with regard to the different sterilization procedure

Plant species	Removal of the bud scales	Sterilization procedure	**Rooting** (%) *	**Number of roots/explant ***
Gisela 5	precise	1	75 a	1.7 a
Prunus subhirtella var. *autumnalis*	not precise	2	100 b	4.4 b
Prunus subhirtella var. *autumnalis*	precise	2	100 b	5.2 b

* Percentages with identical letters following the values indicate no significant differences according to the Duncan's multiple-range test ($p<0.05$).

The plants with two or more roots were planted in the soil and after a month the shoots, especially the roots became larger and multiplicated (Figure 3). The survival of the plants during the acclimatization was nearly 100% and they had a good juvenile condition. These results were better than those reported by Meier-Dinkel (1986).

Figure 3: The well formed rooting system of Gisela 5 after successful acclimatization as the base of production vigorous plants

Many literature data support the idea of performing one, two or maybe three optimal sterilization procedures for most woody plants. The most frequently used procedure is the sterilization with 70% ethanol and 1-3% NaOCl (Meier-Dinkel, 1986; Preil, 1997). Our results show clearly that in some cases during the sterilization period some other chemicals (for example DICA) can give better results for the elimination of the microorganisms and do not damage or just cause minimal damage to the plant material. This means also the shorter time needed for producing plants.

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