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IN VITRO PRESERVATION OF AUTOCHTHONOUS PLUM GENOTYPES

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Abstract

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The paper presents results of the application of 'Cold storage' (CS), an *in vitro* technique for preservation of cultures at +5°C in total darkness. This simple *in vitro* preservation method is effectively employed in large plant gene bank repositories for the purpose of preservation of valuable plant germplasm. In order to protect from extinction autochthonous plums Crvena Ranka (*Prunus insititia* L.), Sitnica *(Prunus domestica* L.) and Cherry plum (*Prunus cerasifera* Ehrh.) a protocol for their *in vitro* preservation has been developed. Upon the establishment of aseptic culture, the studied genotypes were propagated *in vitro* on Murashige and Skoog (1962) medium of different hormonal composition, depending on genotype. During CS, *in vitro* shoots were maintained at +5°C in cold chamber for 3, 6 and 9 months in total darkness. Seven days after respective period, viability of shoots for further propagation was determined as well as multiplication parameters and length of axial and lateral shoots. Three months after maintaining shoots in CS, shoots of Crvena Ranka and Sitnica showed high shoot viability (in excess of 50%), while in Cherry plum viable shoots were etiolated showing symptoms of leaf necrosis (in more than 20%). However, after 6-month preservation under cold conditions, only shoots of Crvena Ranka survived showing necrosis on shoot tips, but they had viable, chlorotic lateral shoots (in excess of 45% of shoots). All three genotypes showed severe signs of necrosis after 9 months of cold storage.

Key words: Autochthonous plums, in vitro culture, 'cold storage', multiplication

Introduction

Plant gene banks under *in situ*, *ex situ* and *in vitro* conditions have been established in most countries of the world. Genetic resources, i.e. gene banks maintained under *in situ* conditions facilitate the work of breeders, however, these require large acreages and rather high costs. In addition, plants are directly exposed to diseases and pests and other external abiotic stress factors.

The establishment of modern germplasm collection most necessarily presupposes the *in vitro* techniques of preservation of plants which tend to be taken as major alternative to the traditional germplasm preservation under field conditions.

It is important to preserve plant tissue at low temperatures for relatively long period of time in order to support mass propagation and active plant gene bank (Klavina et al., 2003). However, it is also of major importance to introduce techniques

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for short- and medium-term cold storage, which enables the development of so-called 'stock plant material' (which may be propagated when required) or germplasm in a smaller space at lower costs and without risks induced by field conditions.

Slow growth storage (also called 'minimal growth storage', 'cold' or 'low temperature storage' and/or 'conservation at above freezing temperature') has repeatedly proved to be a convenient option for medium-term conservation of woody plants (Lambardi and De Carlo, 2003). The technique makes a significant extension to the interval between subcultures possible, thus reducing the costs of stock culture maintenance, as well as risk of contamination during sub culturing. Some experience has shown that the storage period can be prolonged to 2 or even more years (Grout, 1995).

In the early 90's, 'Cold storage' technique was successfully applied in many fruit species, i.e. strawberries (Wilkins et al., 1988; Reed, 1992), *P. avium* (Ružić and Cerović, 1999), *P. domestica* (Ružić and Cerović, 1990), *P. cerasus* (Borkowska, 1986; 1990), *P. persica* (Leva et al., 1992), but over the recent time also in cherry (Petrevica and Bite, 2003) and raspberry (Reed et al., 2008; Ružić et al., 2009).

The aim of this paper was to establish *in vitro* germplasm collection intended for storing germplasm for medium-term conservation, germplasm exchange and potential rapid propagation when circumstances call for it.

Material and Methods

Plant material and media. Plum genotypes Crvena Ranka (*Prunus insititia* L.), Sitnica *(Prunus domestica* L.) and Cherry plum/ Myrobalan (*Prunus cerasifera* Ehrh.) were used as a plant material in these studies.

Aseptic cultures and initiation of shoots were established on medium Murashige and Skoog (MS) (1962) supplemented with 6-Benzyladenine (BA) 2.0; Indole-3-Butyric Acid (IBA) 0.5 and Gibberellic Acid (GA_3) 0.1 mg l^{-1}.

Upon the establishment of aseptic culture, the shoots of all three cultivars were multiplied on the MS medium supplemented with either BA 1, IBA 0.1 and GA_3 0.1 mg l^{-1} (cvs Crvena Ranka and Sitnica), or BA 0.5, IBA 0.1 GA_3 0.1 mg l^{-1} (Cherry plum).

Prior to autoclaving, the pH value of all the media was adjusted to 5.75 with 0.1 N KOH. The media were sterilized in an autoclave for 20 min at 120°C. All the media contained agar at concentration of 7 g l^{-1} and sucrose 20 g l^{-1}.

Cold storage (CS) – experiment design. During CS, *in vitro* shoots were maintained at +5°C in cold chamber for 3, 6 and 9 months in total darkness. Seven days after respective period of time (after CS, the cultures were subsequently transferred to a growth chamber for 7 days), viability of shoots for further propagation (percentage of fully viable shoots, partially viable shoots and fully necrotic shoots) was determined as well as multiplication parameters.

Twenty days prior and after CS, the cultures were grown under standard growth conditions, i.e. room temperature 23 ± 1°C, 16/8 h photoperiod - light/dark and light intensity 8.83 Wm^{-2} provided by cool white fluorescent tubes 40 W, 6500°K in strength.

As a control we used the same age shoots grown in growth room with 20 days subculture. Fifteen culture vessels x 5 uniform shoots x 2 replications were used for each treatment (150 shoots/treatment).

Data analysis. The data were analysed by ANOVA and F-test, as well as by individual Duncan's Multiple Range Test for $p < 0.05$.

Results and Discussion

Cold storage technique is useful when maintenance of the collection under sterile and controlled conditions is of major priority, and which is suitable when circumstances require rapid propaga-

tion. Developing protocol for the conservation of autochthonous species is a large step on the way to their inclusion into the fruit gene bank. Nowadays cryopreservation techniques which enable long term storage are widespread, however CS technique is still being used in the great plant gene bank repositories e.g. at Corvallis, Oregon (USA) (Reed et al., 2008).

For all 3 genotypes the highest viability and regrowth were obtained after 3 months in CS (Tables 1, 3 and 5). Some shoots were etiolated after 3 months but the incidence of necrosis was evidenced after 6 and 9 months (Figures 1a, b, c; 2a, b, c; 3a, b, c).

Table 1
Viability of cv Crvena Ranka shoots for further propagation after 3, 6 and 9 months of CS

CS period	Shoot types, %			
	Fully necrotic shoots	Necrotic axial shoot with viable lateral once	Viable shoots with etiolated lateral shoots	Fully viable shoots
3 months	6.0	8.0	28.0	58.0
6 months	17.5	37.5	45.0	-
9 months	100.0	-	-	-

Table 2
Multiplication parameters of plum cv Crvena Ranka before CS and after 3 months of CS in 5 successive subcultures

Shoot types	Multiplication index	Length of axial shoot, cm	Length of lateral shoots, cm
Control	1.94 b*	1.19 a	0.68 b
3 months CS + 7 days in GR	2.78 a	1.09 a	1.86 a
1st subculture	1.95 b	1.13 a	0.64 b
2nd subculture	2.43 a	1.09 a	0.68 b
3rd subculture	1.90 b	1.04 a	0.55 b
4th subculture	1.95 b	1.16 a	0.66 b
5th subculture	1.90 b	1.13 a	0.64 b

*Means followed by the same letter within columns are not significantly different at the 5% level of significance using Duncan's Multiple Range Test.

The shoots of all three genotypes after 3 months had viable, etiolated stems, although the incidence of necrosis was sporadically observed on leaves in base section (Figures 1a; 2a; 3a). The transfer of cultures from cold chamber to standard conditions led to prompt development and greening of leaves which regained morphology and capacity for multiplication (Figures 1d; 2d; 3d).

Upon three-month maintainance in CS, multiplication index of plum Crvena Ranka and Cherry plum showed rise as compared to control (in the second and forth subculture, resp.) (Tables 2 and 6). However, control shoots of genotype Sitnica

Table 3
Viability of plum shoots of Sitnica for further propagation after 3, 6 and 9 months of CS

CS period	Shoot types, %			
	Fully necrotic shoots	Necrotic axial shoot with partially viable chlorotic lateral once	Viable shoots with chlorotic lateral shoots	Fully viable shoots
3 months	10.4	20.8	16.7	52.1
6 months	44.0	56.0	-	-
9 months	82.9	17.1	-	-

a) Viable shoots with etiolated lateral shoots - after 3 months of CS

b) Necrotic shoot tips with etiolated viable lateral shoots – after 6 months of CS

c) Fully necrotic shoots – after 9 months of CS

d) First subculture after 3 months of CS

Fig. 1. Viability of cv Crvena Ranka shoots for further propagation after 3 (a), 6 (b) and 9 (c) months of CS and after CS (d)

had the highest multiplication parameters (Table 4).

The rising tendency of the multiplication index after CS was also observed in other cultures, e.g. sour cherry cultivars (Borkovska, 1990; Petrevica and Bite, 2003), sweet cherry rootstocks Gisela 5 and Tabel Edabriz (Ružić and Cerović, 1999), some peach cultivars (Leva et al., 1992), etc. The occurrence of this in microplants is probably stress-induced owing to the lack of dormancy, which was noticed by Borkowska (1986). However, several raspberry cultivars showed relatively high sensitivity to long-term maintenance at low temperatures, which results in modification of cultural behaviour and significant decline in multiplication rates (Popescu et al., 2004). According to Klavina et al. (2003), raspberry cultures, e.g. grown under cold storage conditions were less resistant to cold than cherries, and these differences were shown by enzyme activities as well. Reed et al. (2008) confirmed that genotype variation is very high for a widely diverse germplasm collection.

Our observation also suggested that the reaction of species and cultivars to *in vitro* cold storing is not identical owing to respective genetic specificities, e.g. plum cv Požegača was stored successful-

a) Fully viable shoots – after 3 months of CS

b) Necrotic axial shoot with partially viable chlorotic lateral once – after 6 months of CS

c) Necrotic axial shoot with viable lateral once – after 9 months of CS

d) Third subculture after 3 months of CS

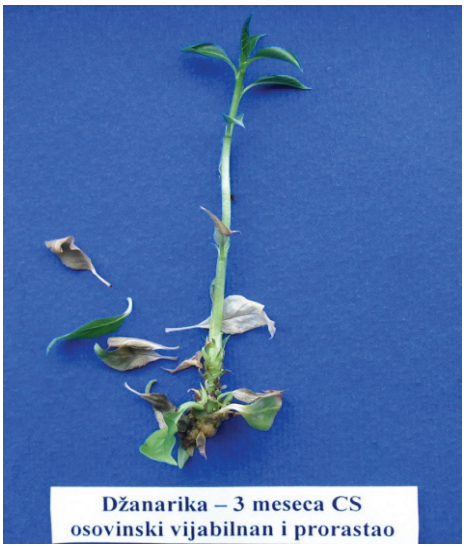
Fig. 2. Viability of cv Sitnica shoots for further propagation after 3 (a), 6 (b) and 9 (c) months of CS and after CS (d)

ly for 10 months in our lab in the same conditions (Ružić and Cerović, 1990).

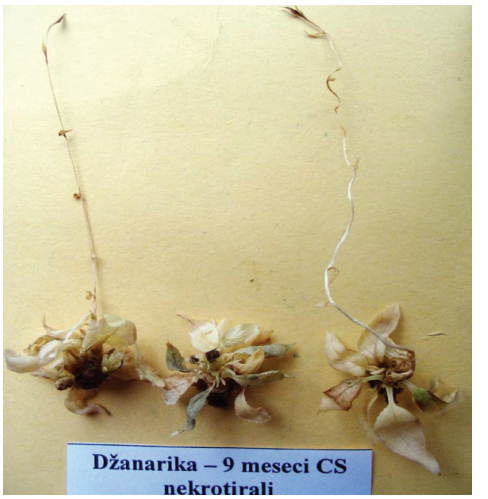
Temperature, light conditions and growth regulators applied also contribute to CS duration. According to Lambardi and De Carlo (2003) almost 60% of species were stored at a temperature between 2°C and 5°C and the maintenace of stored cultures in total darkness is more common. Also, the majority of reserchers stored shoot culture in the same media used during multiplication phase, and only for a limited number of species hormone-free media have been used. Although in our experiments, all genotypes were stored under the most commonly applied conditions (+5°C and total darkness), further optimization of protocol should focus on comparison of the effect of different temperatures and other factors such as hormonal composition of medium on survival and storing capacity of shoots.

As *in vitro* storage of the germplasm could be easily achieved, the loss of the material being only accidental, 3-month maintenance of plum genotypes under cold temperature conditions in this experiment has proved to be beneficial, accompanied by high survival and viability rate.

Finally, genetic stability of cold stored plants



a) Etiolated shoots with leaf necrosis - after 3 months of CS



c) Fully etiolated necrotic shoots - after 9 months of CS

b) Necrotic axial shoot with partially viable chlorotic lateral once – after 6 months of CS

d) Third subculture after 3 months of CS

Fig. 3. Viability of Cherry plum shoots for further propagation after 3 (a), 6 (b) and 9 (c) months of CS and after CS (d)

is very important task, which was confirmed by Hiraoka et al. (2003) who demonstrated that cold storage of *in vitro* shoot cultures can be used as a germplasm preservation system for short or medium duration without deterioration of their biological and biochemical characteristics.

Conclusions

Our results have shown that *in vitro* propagated plums can be stored under common conditions (at +5°C, in darkness) 3 months so as to obtain a high survival rate.

It is possible to improve the shoot multiplication *in vitro* by replacing natural period of dormancy with a short-term maintenance in cold storage.

These results provide firm base for the development of standard protocol for maintenance in *in vitro* fruit germplasm and its introduction into our country, which eventually focuses on revitalisation and further formation of the national fruit gene bank.

Table 4
Multiplication parameters of plum Sitnica before CS and after 3 months of CS in 5 successive subcultures

Shoot types	Multi-plication index	Length of axial shoot, cm	Length of lateral shoots, cm
Control	2.11 a*	1.19 a	0.61 a
3 months CS + 7 days in GR	1.26 bc	0.89 bc	0.55 ab
1st subculture	1.05 c	0.67 d	0.50 b
2nd subculture	1.52 b	0.74 d	0.56 ab
3rd subculture	1.52 b	0.86 c	0.52 b
4th subculture	1.43 bc	0.87 bc	0.56 ab
5th subculture	1.24 bc	0.99 b	0.53 b

*Means followed by the same letter within columns are not significantly different at the 5% level of significance using Duncan's Multiple Range Test.

Table 5
Viability of Cherry plum shoots for further propagation after 3, 6 and 9 months of CS

CS period	Shoot types, %			
	Fully necrotic shoots	Necrotic axial shoot with partially viable lateral once	Etiolated shoots with leaf necrosis	Etiolated axial shoot with shoot tip necrosis
3 months	28.9	26.7	44.4	-
6 months	87.5	2.5	-	10.0
9 months	100.0	-	-	-

Table 6
Multiplication parameters of Cherry plum shoots before CS and after 3 months of CS in 5 successive subcultures

Shoot types	Multi-plication index	Length of axial shoot, cm	Length of lateral shoots, cm
Control	1.22 c*	1.34 abc	0.67 b
3 months CS + 7 days in GR	1.32 c	1.47 ab	1.96 a
1st subculture	1.80 ab	1.52 a	0.78 b
2nd subculture	1.57 bc	1.07 c	0.62 b
3rd subculture	1.48 bc	1.09 c	0.61 b
4th subculture	2.05 a	1.21 bc	0.77 b
5th subculture	1.48 bc	1.10 c	0.61 b

*Means followed by the same letter within columns are not significantly different at the 5% level of significance using Duncan's Multiple Range Test.

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