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In vitro conservation of cherry rootstock Gisela 5

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The paper presents results of the application of 'Cold Storage' (CS), a very simple *in vitro* technique preservation of cultures at +5°C in total darkness. A protocol has been developed for *in vitro* preservation of cherry rootstock Gisela 5 based on this method. Upon the establishment of aseptic culture, the studied genotype was propagated *in vitro* on MS medium supplemented with BA at a concentration of 3.37 mg/L. 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 their respective period of time, the shoots were examined for viability for further propagation, together with their multiplication index and length of axial and lateral shoots. Three months after CS, shoots showed high shoot viability (55%), which however declined considerably after 6 and 9 months (15% and 0%, resp.). After 9 months of preservation under cold conditions, shoots showed severe signs of necrosis (55%). The transfer of cultures from the cold chamber to standard growth conditions led to prompt development and greening of leaves which regained morphology and capacity for multiplication and rooting.

Keywords: *In vitro* conservation, Cherry, Rootstock, Cold storage, Propagation, Germplasm preservation

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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, which require large acreages and rather high costs and in addition, plants are directly exposed to diseases and pests and other external abiotic stress factors. It was also confirmed that personnel, energy and materials costs could be reduced with this type of technique[1]. Over the last 30 yrs, there has been a significant increase in the number of plant collections and in accessions in *ex situ* storage centers throughout the world[2].

Fruit species are usually conserved in field gene banks, unfortunately, field tree collections are vulnerable to environmental catastrophes such as high wind, rain, drought, freezing, pests and diseases outbreaks[1]. Large collection of pear (*Pyrus communis* L.) and apple [*Malus domestica* Borkh.(*M. pumila* Mill.)] genetic resources have been preserved through *in vitro* cultures as alternative techniques to the field gene banks[3].

The United States Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository at Corvallis (Oregon, USA) preserves genetic resources for *Rubus*. The *in vitro* collection includes about 200 accessions[4].

For short and midterm storage, the aim is to reduce growth and to increase the intervals between subcultures. Growth reduction is generally achieved by modifying the environmental conditions and/or the culture medium. The most widely applied technique is temperature reduction, combined with a reduction in the concentration of nutritive elements or decrease in light intensity or storage in the dark[5,6]. The plant metabolism can be limited, which is important for cold storige, in several ways: (1) by reducing temperature and light intensity provided to the cultures (cold storage), (2) by addition of osmotic compounds (such as mannitol or sucrose) or (3) by using growth retardants in storage medium[7].

In the early 90's, the 'Cold Storage' technique was successfully applied in many fruit species, i.e. apple[8], strawberries[9,10], *P. domestica*[11], *P. cerasus*[12,13], *P. persica*[14], *P. avium*[15], and more recently in cherry[16], as well as raspberry[4,17], plum[18], pear[1], and some woody species[19]. Rootstock Gisela 5, the interspecies hybrid of *Prunus cerasus* × *Prunus canescens* (No. 148/2), is one of the most successful dwarf cherry rootstock[20]. This leading rootstock induces wide branch angles, good lateral branching

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and early and heavy production[21], and reduces vigour by 50% or more[22]. The objective of this paper is to develop an efficient protocol for long time cold storage of cherry rootstock Gisela 5.

Material and methods

Plant material and culture media

Aseptic cultures and initiation of Gisela 5 shoots were established on medium Murashige and Skoog (MS)[23] supplemented with in mg/L: 6-Benzyladenine (BA) 2.0; Indole-3-Butyric Acid (IBA) 0.5 and Gibberellic Acid (GA_3) 0.1. Upon the establishment of the aseptic culture, the shoots were multiplied on the MS medium supplemented with BA 3.37 mg/L. 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 gm/L and sucrose 20 gm/L.

Cold storage (CS) and multiplication

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 the respective period of time (after CS, the cultures were subsequently transferred to a growth chamber for 7 days), viability of shoots for further propagation was determined as well as fresh (FW) and dry weight (DW) of shoots together with multiplication parameters such as: index of multiplication, length of axial and lateral shoots. Upon removal from the medium the shoots were washed in distilled water and dried with filter paper before their FW was determined. For the DW, shoots were dried in an oven at 65–70°C for 48 hrs.

Following the preparation the cultures were grown under standard growth conditions, i.e. room temperature 23±1°C, 16/8 hrs photoperiod - light/dark and light intensity 8.83 W/m^2 provided by cool white fluorescent tubes 40 W, 6,500°K in strength, and after all three successive subcultures the same multiplication parameters were monitored.

Rooting

The medium used for rooting was: MS with mineral salts reduced to half, organic complex unchanged supplemented with α-Naphthyl Acetic Acid (NAA) 1 mg/L and GA_3 0.1 mg/L with agar at concentration of 7 gm/L and sucrose 20 gm/L. Duration of subculture was 28 days and after that the following parameters were monitored: percentage of rooting, number and length of roots, as well as height of rooted plants.

As a control, the same age shoots were used, grown in growth room with 20 days subculture/28 days for rooting. 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 $p < 0.05$. The results shown as percentage were transformed by the arcsine transformation.

Results

Although cryopreservation techniques which enable long term storage are nowadays widespread, the CS technique is still being used in the great plant gene bank repositories such as Corvallis, Oregon (USA)[4].

To set up an experiment with CS we started from the very beginning, i. e. from establishment of aseptic culture. This phase was very successful, with more than 80% of explants forming rosettes (Fig. 1).

After 3 and 6 months of cold storage we determined 4 types of shoots of different viability, but after 9 months only 3 types were determined (Table 1; Fig. 2a, b, c, d; Fig. 3a, b, c).

It is obvious that for Gisela 5 genotype the highest viability and regrowth was obtained after 3 months at CS resulting in the highest percentage (55%) of fully viable shoots (Table 1). Some shoots were etiolated after 3 months but the incidence of necrosis was evidenced at the highest level after 6 and 9 months (Table 1; Fig. 2d; Fig. 3c). The transfer of cultures from the cold chamber to standard conditions led to prompt development and greening of leaves which regained morphology and capacity for multiplication. Upon the nine-month maintenance in CS, the multiplication index (4.57) was the highest despite of the axial shoot necrosis owing to viable lateral shoot proliferation (Table 2). FW and DW of callus were highest after 9 months of storage, however FW and DW of both type of shoots, axial and laterals, showed an almost linear decrease (Table 3).

In the first subculture after 3 months of CS shoots already had a normal morphology like control shoots (Fig. 4a, b). After 6 and 9 months of CS shoots also retrieved normal morphology, but with lower multiplication index obtained in 1st subculture after 3 months of CS (Table 4; Fig. 5a, b).

However, multiplication index and length of axial and lateral shoots were significantly lower in comparison to the values of the corresponding

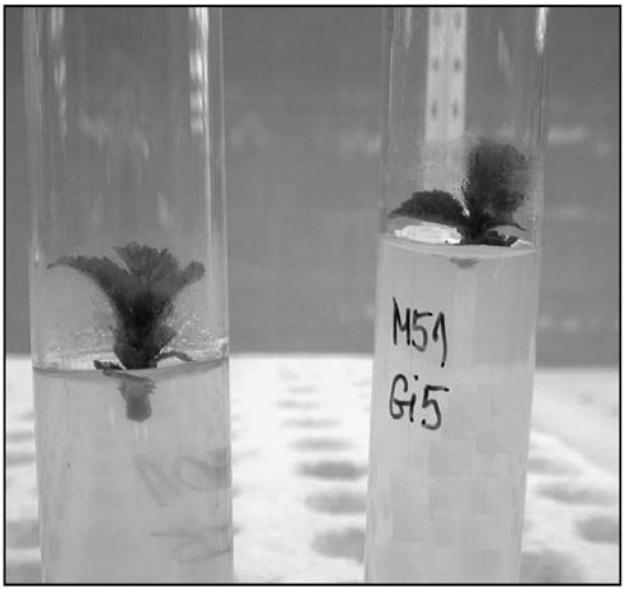

Fig. 1- Rosette initiation

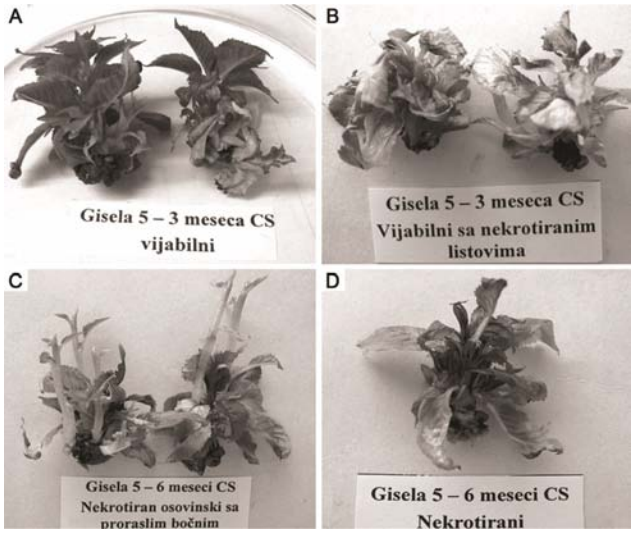

Fig. 2- (a-d) Shoot types of Gisela 5 rootstock after 3 (a and b) and 6 (c and d) months of CS: (a) Fully viable shoots; (b) Viable shoots with partly necrotic leaves; (c) Necrotic axial shoot with viable lateral once; (d) Fully necrotic shoots.

Table 1-Viability of Gisela 5 shoots for further propagation after 3, 6 and 9 months of CS Shoot types (%)

CS period	Fully viable shoots	Viable shoots with partly necrotic leaves	Necrotic axial shoot with viable lateral once	Necrotic axial shoot with etiolated lateral shoots with necrotic tips	Fully necrotic shoots
3 months	55.0^{a*}	12.5^{a}	22.5^{b}	-	10.0^{b}
6 months	15.0^{b}	2.5^{b}	77.5^{a}	-	5.0^{b}
9 months	-	-	27.5^{b}	17.5	55.0^{a}

*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 2-Multiplication parameters of Gisela 5 rootstock after CS (7 days after 3, 6, 9 months of CS)

Storage time	Multiplication index	Length of axial shoot (cm)	Length of lateral shoots (cm)	Average No of buds < 0.5 cm/axial shoots
3 months CS + 7 days in GR	2.12^{c*}	1.48^{a}	1.11^{b}	4.31^{a}
6 months CS + 7 days in GR	2.97^{b}	1.30^{a}	1.78^{a}	3.28^{b}
9 months CS + 7 days in GR	4.57^{a}	-	1.82^{a}	2.07^{c}

*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 3-Fresh weight (FW) and dry weight (DW) of callus and shoots of Gisela 5 rootstock after CS (7 days after 3, 6, 9 months of CS)

Storage time	Shoots FW (mg)			Shoots DW (mg)		
	Callus	Axial shoot	Lateral shoots	Callus	Axial shoot	Lateral shoots
3 months CS + 7 days in GR	301.6^{b*}	298.4^{a}	80.3^{a}	36.7^{b}	46.6^{a}	8.4^{a}
6 months CS + 7 days in GR	295.5^{b}	198.8^{b}	68.8^{b}	39.0^{b}	40.7^{b}	5.9^{b}
9 months CS + 7 days in GR	406.2^{a}	-	67.1^{b}	44.1^{a}	-	6.4^{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 4-Multiplication parameters of Gisela 5 rootstock before CS and in 3 successive subcultures after 3, 6 and 9 months of CS

Storage time/3 subcultures	Multiplication index	Length of axial shoot (cm)	Length of lateral shoots (cm)	Average No of buds <0.5 cm/ axial shoots
Control	2.58[a*]	1.79[a]	0.69[a]	5.88[a]
1st subc. after 3 months CS	1.53[bc]	1.13[cde]	0.61[b]	4.37[b]
2nd subc. after 3 months CS	1.72[b]	1.19[bcd]	0.53[c]	2.23[ef]
3rd subc. after 3 months CS	1.67[b]	1.28[bc]	0.69[a]	3.21[c]
1st subc. after 6 months CS	1.28[cd]	1.32[b]	0.54[c]	2.46[def]
2nd subc. after 6 months CS	1.72[b]	1.11[de]	0.52[c]	2.91[cd]
3rd subc. after 6 months CS	1.47[bc]	1.26[bcd]	0.68[a]	2.66[cde]
1st subc. after 9 months CS	1.00[d]	1.88[a]	-	1.94[f]
2nd subc. after 9 months CS	1.76[b]	1.02[e]	0.56[bc]	2.96[cd]
3rd subc. after 9 months CS	1.25[cd]	0.99[e]	0.57[bc]	3.19[c]

*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-Rooting parameters of Gisela 5 rootstock

Origine of shoots	Rooting %	No of roots	Length of roots (cm)	Length of rooted plants (cm)
Control	70.00[a*]	2.79[a]	3.08[b]	1.31[a]
Shoots after 3 months of CS	36.67[b]	2.28[ab]	4.26[a]	1.37[a]
Shoots after 6 months of CS	16.67[c]	1.80[bc]	3.20[b]	1.37[a]
Shoots after 9 months of CS	20.00[c]	1.25[c]	2.70[b]	1.20[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.

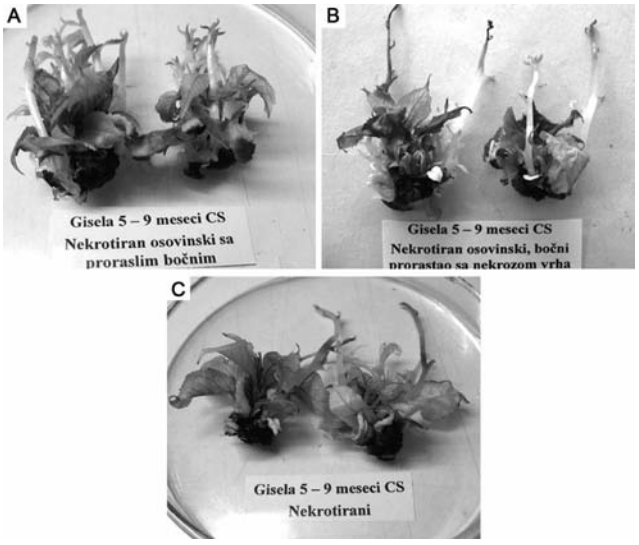

Fig. 3- (a-c) Shoot types of Gisela 5 rootstock after 9 months of CS: (a) Necrotic axial shoot with viable lateral once; (b) Necrotic axial shoot with etiolated lateral shoots with necrotic tips; (c) Fully necrotic shoots.

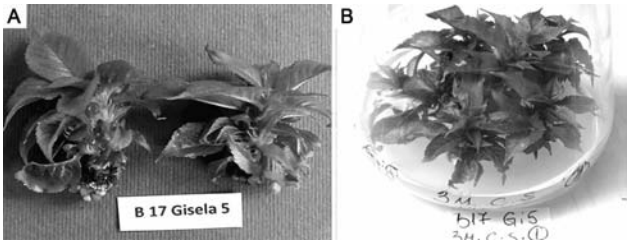

Fig. 4- (a-b) Appearance of Gisela 5 shoots: (a) Control shoots; (b) 1st subculture after 3 months of CS

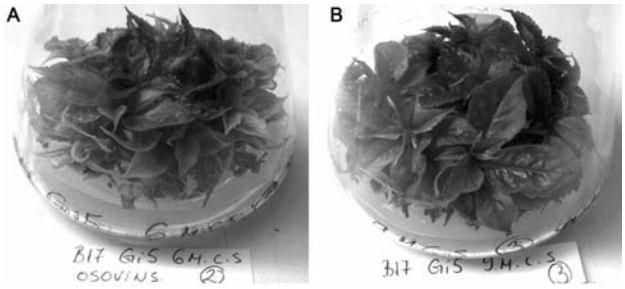

Fig. 5- (a-b) Appearance of Gisela 5 shoots: (a) 2nd subculture after 6 months of CS; (b) 3rd subculture after 9 months of CS

parameters obtained for control shoots. With further subculturing the values of these parameters did not vary significantly, although the tendency of their increase was observed (mainly in 2nd subculture), so that even after three subcultures they have not reached the level of control values.The highest rooting percentage and number of roots were obtained in control, and then after 3 months of CS. However, the highest length of roots and rooted plants were obtained after 3 months of CS (Table 5).The basic

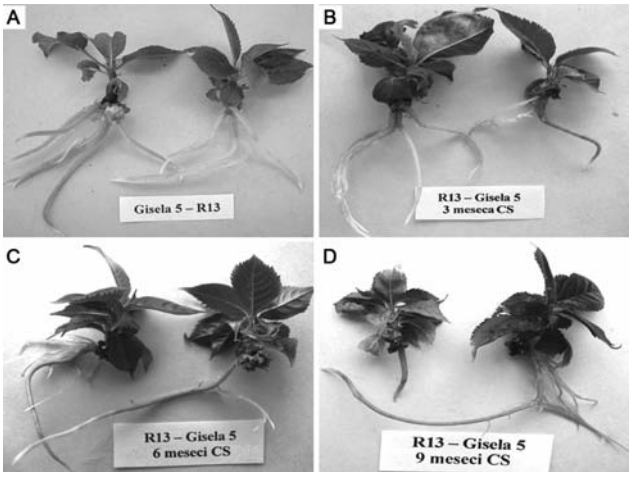

Fig. 6- (a-d) Rooted shoots of Gisela 5 rootstock on medium MS with in mg/L, NAA 1 and GA_3 0.1: (a) Control shoots; (b) After 3 months of CS; (c) After 6 months of CS; (d) After 9 months of CS

characteristics of rooted plants after CS were similar to control: small, firm, nodular callus green in colour; roots were thick, long, radially arranged, no secondary roots, white with pink pigmentation (Fig. 6a, b, c, d).

Discussion

Cryopreservation, when available, is the preferred form for long-term (base collection) conservation, with *in vitro* storage as the second choice and field collections as the third[24].

Medium-term *in vitro* cold storage of fruit species was investigated such as for *Rubus* germplasm using various temperatures, photoperiods, and storage containers. Results of these studies suggest that most *Rubus* germplasm can be stored safely at 4°C with 12 hours of light[25]. Temperature, light conditions and growth regulators applied also contribute to CS duration. Almost 60% of species were stored at a temperature between 2°C and 5°C and the maintenance of stored cultures in total darkness is more common[26], so we have chosen to store our genotype under the most commonly applied conditions (+5°C and total darkness).

Different authors have so far obtained different results applying these most widely used CS conditions.

After a 15-month *in vitro* storage at 4°C temperature, 40% of strawberry plants were in good condition, 60% of plants in poor and bad condition, but, pear microshoots were stored successfully for 6 months[6]. Incubating temperature treatments i. e., 5°C and 10°C, proved successful for preservation of apical pear shoots for 3 months, however, the shoots kept at a higher temperature for a longer period survived with poor re-growth. Hence it was concluded that the shoot tips of pear genotypes can be successfully stored *in vitro* for short to medium terms at reduced incubation temperatures[1] which is similar to our obtained results. In our previous experiment with three plums on CS Crvena Ranka (*Prunus insititia* L.), Sitnica *(Prunus domestica* L.) and Cherry plum (*Prunus cerasifera* Ehrh.), the best survival rate for all also was after 3 months of CS[18]. Our observation also suggested that the reaction of species and cultivars to *in vitro* cold storing is not identical owing to respective genetic specificities. For example, the plum cv Požegača was stored successfully for 10 months in our lab at the same conditions[11].

Although the cold storage seems to improve the proliferation capacity of the cultures, when they are transferred to light conditions this capacity proves to be transitory, and after 2–3 further subcultures they acquire the normal values of non-cold-stored cultures[27]. The morphological differences between stored and control cultures have not found for at least 1 year without subculturing wild cherry, oak and chestnut cultures[27].

Very important results obtained with Gisela 5 shoots reveal that after CS normal values and morphological properties appeared already in the 1st subculture. Certain linear increase toward 2nd subculture was recorded in multiplication index. The rising tendency of the multiplication index after CS was also observed in other cultures, and the occurrence of this in microplants is probably stress-induced due to a lack of dormancy[12]. However, some species, such as raspberry cultivars showed relatively high sensitivity to long-term maintenance at low temperatures, which resulted in modification of cultural behaviour and significant decline in multiplication rates[28]. It was also confirmed that genotype variation is very high for a widely diverse germplasm collection[4].

It is very important that shoots/plants of Gisela 5 originating from CS, specially after 3 months of CS were capable for rooting, especially after 3 months of CS, thus indicating the possibility of rounding up the whole process, and the purpose for CS.

Since the *in vitro* storage of the germplasm is a very simple technique, the loss of the material being only accidental, 3-month maintenance of cherry

rootstock Gisela 5 under conditions in this experiment has proved to be beneficial, accompanied by high survival and viability rate. Variability in survival rates of the genotypes might be due to their genetic makeup. Variety dependent variability in survival rate during *in vitro* preservation is confirmed in apple[9].

Finally, it was 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[29].

This study showed that the shoots of cherry rootstock Gisela 5 can be maintained successfully for 3 months by *in vitro* slow growth preservation technique under described simple conditions (at +5°C, in darkness) to obtain a high survival rate and to multiple the shoots/plants when necessary.Three and six months of storage periods were proved better than longer durations.

These results provide a firm base for the development of standard protocol for maintenance in *in vitro* fruit germplasm and its introduction into our country, especially for the formation of the national fruit *in vitro* gene bank which represents the future in the field of plants conservation.

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