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Low temperature storage of *Fragaria* sp. and *Pyrus* sp. genetic resources *in vitro*

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Abstract

Climatic conditions, plant diseases and pests constitute a serious hazard to genetic resources maintained in field collections. *In vitro* culture techniques, including storage under growth limiting conditions and cryopreservation, provide storage alternatives for protecting valuable germplasm.

The objectives of present study were to develop a procedure for *in vitro* low temperature storage of strawberry and pear and to investigate the effect of different culture media and incubation duration at low temperature on the state of plants of various genotypes. *In vitro* stored twenty strawberry and nine pear accessions were used for the study at the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry (LRCAF) during 2009–2011.

After a 15-month *in vitro* storage at 4ºC temperature, 40% of strawberry plants were in good condition (average rating point exceeded 2), 60% of plants in poor and bad condition (average rating point below 2). Character of change of plant condition during storage was genotype dependent but was not associated with new shoot and leaf formation. The condition of *Fragaria* × *ananassa* 'Catskill', 'Nida', 'Melody', 'Venta' and *F. virginiana* after storage was the best, while that of *F.* × *ananassa* 'Suvetar', 'Jasna', 'Elsanta', 'Saulenė' and *F. virginiana glauca*, *F. vesca* was the worst.

Pear microshoots were stored successfully at 4ºC temperature for 6 months *in vitro* on MS (Murashige and Skoog) growing medium with 3% sucrose with or without benzylaminopurin (BAP) addition depending on the genotype. Addition of 2% mannitol to the storage medium did not improve microshoots condition during the low temperature storage.

Key words: genetic resources, low temperature, pear, strawberry.

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) and European pear (*Pyrus communis* L.) are worldwide distributed fruit species belonging to the family *Rosaceae*. Pear is one of the most important deciduous fruit trees all over the world and rank second to the apple in production. Strawberry has many traits that make it an attractive model plant for studying genetic resources conservation. Plants have shorter life cycle than other members of the *Rosaceae* family and they are easy to propagate and cultivate *in vitro*. Pear could also be used as a model fruit species for plant growing *in vitro*.

The Institute of Horticulture, LRCAF, in collaboration with the Plant Gene Bank, is involved in the collection, research and conservation of the national genetic resources of horticultural plants. Over 300 accessions of *Pyrus* sp. and over 100 of *Fragaria* sp. are maintained in the collections of Institute of Horticulture, LRCAF, including unique accessions of wild pear. Many of the accessions are unique and exist only in the collection. Climatic conditions, plant diseases and pests constitute a serious hazard to the genetic resources preserved in field collections. Conservation of plant genetic resources via tissue culture could prevent those risks. During the past few decades, different *in vitro* conservation methods have been developed as substitutes for field gene banks and have been widely employed depending on the storage duration required (Ashmore, 1997; Rao, 2004). There are two types of *in vitro* preservation methods used in tissue culture: growth retardation method and cryopreservation or ultra low temperature preservation (Scowcroft, 1984; Sedlak et al., 2001). For short and mid-term 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 (Boong et al., 1996; Reed, 2002; Hassan, Bekheet, 2008; Engelmann, 2011). These alternate preservation techniques are less costly and safe to conserve germplasm (Epperson et al., 1997; Reed, 2002).

A number of individual genotypes were characterised by the majority of the investigations, but large collections often contain accessions or accession groups with specific requirements for storage conditions. Differences in storage requirements among accessions of coffee were evident in a group of 32 diversity groups conserved under slow growth for 3 years. In some groups, all non adapted genotypes were lost during the first subculture, while in others few or none were lost during the entire experiment. Accession in some groups required additional study to provide adequate slow growth conditions (Reed et al., 2004).

Plant viability during storage in low temperature may be associated with cold or winterhardiness in field conditions. Strawberry, pear and other orchard plant ability to withstand temperature changes and other stresses in field conditions during winter depends on the genotype (Rugienius et al., 2009; Kviklys, 2011). Genotypic differences in cold hardiness should be taken into account before planning storage procedures.

Large collections often require quite frequent and periodic assessments of each accession's state and well-timed decision of transplanting time. Response to transplanting depends not only on plant state but also on the genotype. As a result, the new mid-long term storage strategy *in vitro*, where storage duration and conditions should be adjusted for certain genotype groups could be more practical and safe for the preservation of large collections or gene pool specimens in core collections where even negligible losses are not acceptable.

The objective of this study was to assess storage duration under minimized growth – low temperature conditions for different accession groups of pear and strawberry, taking into account minimal or critical rating point of microshoots state crucial for successful transplanting and recovery after storage.

Materials and methods

Low temperature storage experiments were performed at the Plant Biotechnology Laboratory, Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry in 2009–2011.

Plant material. Each group of stored *Fragaria* and *Pyrus* accessions contained a representative sample of germplasm. Strawberry *F.* × *ananassa* Duch. 'Venta', 'Melody', 'Elsanta', 'Holiday', 'Dangė', 'Nora', 'Nida', 'Jaunė', 'Saulenė', 'Catskill', 'Juni Morgon', 'Suvetar', 'Valotar', 'Vaiva', 'Jasna', KL P8 and *F. moschata* Ouch., *F. vesca* L., *F. virginiana* Duch., *F. virginiana glauca* and pear 'Oranzhevaya', 'Hasselpeare', 'Princesse Dagmara', 'Karalienė Jadvyga', 'Senryo', 'Muskatelka Seda', 'Koncentrat', No. 0408 and *P. pyraster* accessions were used in the study. Twenty strawberry and sixty pear microshoots per cultivar were used in the experiment.

Growth conditions. Strawberry and pear were micropropogated in 130 ml glass jars with 30 ml MS (Murashige, Skoog, 1962) growing medium containing 0.75 mg l^{-1} benzylaminopurin (BAP), 3.0% sucrose and 0.8% agar at pH 5.8, maintained in a growth room at 22 ± 3ºC under a 16 h photoperiod regime. Before placing in the long-term storage, the microshoots were in the best condition.

Storage conditions. After 4–6 weeks microshoots were subcultured to 25 ml glass test tubes with 5 ml medium. Different growing media were used for each crop. Agarized Knop's (Reski, Abel, 1985) medium was used for strawberry. For pear we used three variants of media composition: A – agarized MS medium with 3% sucrose without growth regulators, B – MS medium with 3% sucrose and BAP 0.75 mg l^{-1}, C – MS medium with 3% sucrose and 2% mannitol without growth regulators. Plantlets were cold acclimated for 1 week at 8-h light 22ºC/16-h dark, 4ºC before being placed in storage at 4ºC in the dark. To decrease dehydration and contamination, the glass test tube was sealed with parafilm and stored. Cold storage was performed at 4°C temperature in the dark up to 15 months for strawberry and up to 6 months for pears. Contaminated cultures were discarded. After a specified period of storage, microshoots were transferred on a fresh MS micropropagation medium and grown in the same conditions as before the experiment.

Evaluation. The condition *in vitro* plantlets during cold storage was evaluated every three months for strawberry, every second month for pears and also three weeks after storage.

Mircroshoots were rated on the scale from 0 to 5, based on plant appearance: 0 – dead; 1 – 90–99% microshoots area brown, 1–9% area yellow; 2 – 10–30% yellow, 70–90% brown; 3 – microshoots with etiolation, yellow green 31–70%, brown 69–30%; 4 – microshoots with little etiolation, green 71–95%, brown 5–29%; 5 – microshoots with bright green leaves and stems, brown area less than 5%.

Mean rating values and standard deviations for each cultivar were determined. The data were processed using the software package *SELEKCIJA* (Tarakanovas, Raudonius, 2003).

Results and discussion

Storage of strawberry. The *in vitro* plant storage system was developed taking into account results obtained by different authors (Boong et al., 1996; Reed, 2002; Hassan, Bekheet, 2008). It included cold acclimation in variable temperatures and 16 hour photoperiod, storage at 4ºC in the dark up to 15 months.

Our results show that the survival rate and condition of microshoots varied in a wide range depending on the genotype. At the end of the storage period (after 15 months) the survival rate varied between 11% ('Suvetar') and 100% ('Nida'). This parameter was quite stable during the storage and exceeded 80% for more than half of the accessions. Lower than 50% survival rate was observed only for 'Suvetar' and 'Jasna' (Table 1).

It was shown that after the 15 months' storage 40% of strawberry plants *in vitro* were in good condition (average rating point exceeded 2), 60% of plants in poor and bad condition (average rating point below 2). Microshoots condition of *Fragaria* × *ananassa* 'Catskill', 'Nida', 'Melody', 'Venta' and *F. virginiana* was the best, while that of *F.* × *ananassa* 'Suvetar', 'Jasna', 'Elsanta', 'Saulenė' and *F. virginiana glauca*, *F. vesca* was the worst (Table 1).

Table 1. Survival and state mean ratings of strawberry stored for 3, 9, 12 or 15 months at 4°C temperature in Knop's medium

Cultivar	Storage duration, months								Survived after transplanting %
	3		9		12		15		
	survived %	state, points	survived %	state, points	survived %	state, points	survived %	state, points	
'Nida'	100	4.50 ± 0.25	100	4.10 ± 0.24	100	2.40 ± 0.28	100	2.33 ± 0.18	100
'Jaunė'	100	4.10 ± 0.28	100	3.55 ± 0.27	100	3.33 ± 0.24	95	2.20 ± 0.22	95
'Juni Morgon'	100	3.90 ± 0.28	90	2.35 ± 0.35	85	1.95 ± 0.39	84	1.78 ± 0.13	84
'Melody'	95	3.75 ± 0.36	95	2.45 ± 0.25	95	2.40 ± 0.28	94	2.35 ± 0.20	94
'Vaiva'	95	3.20 ± 0.39	94	2.94 ± 0.31	89	2.55 ± 0.44	89	1.92 ± 0.36	89
'Saulenė'	95	2.28 ± 0.29	95	1.85 ± 0.28	90	1.75 ± 0.25	85	1.11 ± 0.26	0
'Nora'	95	2.40 ± 0.33	95	2.30 ± 0.31	95	1.65 ± 0.17	65	1.54 ± 0.18	65
'Valotar'	95	1.95 ± 0.41	95	1.94 ± 0.24	80	1.65 ± 0.28	65	1.35 ± 0.33	65
KL P8	94	3.38 ± 0.44	88	2.55 ± 0.41	72	2.17 ± 0.47	72	2.06 ± 0.41	72
'Dangė'	90	1.90 ± 0.36	90	1.25 ± 0.25	90	1.20 ± 0.14	90	1.22 ± 0.15	90
'Holiday'	90	2.20 ± 0.30	80	2.10 ± 0.35	80	2.10 ± 0.35	80	1.95 ± 0.32	80
'Venta'	85	2.50 ± 0.27	85	2.40 ± 0.26	85	2.40 ± 0.30	85	2.28 ± 0.36	85
'Catskill'	85	3.20 ± 0.43	85	2.95 ± 0.41	85	2.40 ± 0.41	85	2.33 ± 0.39	85
'Elsanta'	75	1.25 ± 0.20	73	1.21 ± 0.29	73	1.16 ± 0.28	70	1.05 ± 0.27	0
'Jasna'	70	1.35 ± 0.31	65	1.26 ± 0.31	40	0.50 ± 0.15	40	0.50 ± 0.15	40
'Suvetar'	50	1.72 ± 0.43	33	0.38 ± 0.14	22	0.22 ± 0.10	11	0.15 ± 0.11	0
Fragaria virginiana	95	4.40 ± 0.32	95	4.10 ± 0.37	95	3.30 ± 0.40	90	2.72 ± 0.36	90
F. moshata	95	2.90 ± 0.35	95	2.15 ± 0.27	90	1.89 ± 0.21	75	1.85 ± 0.34	75
F. vesca	90	1.50 ± 0.22	85	1.33 ± 0.21	75	1.10 ± 0.19	75	1.00 ± 0.16	75
F. virginiana glauca	85	1.61 ± 0.26	80	1.55 ± 0.29	78	1.40 ± 0.24	73	0.95 ± 0.17	73
$LSD_{0.5}$	4.1		4.4		4.9		5.4		5.5

Differentiation among strawberry genotypes in plant state was ascertained after 3 months from the beginning of storage. Difference in microshoots state rating among some genotypes reached 3 rating points. Character of further change of microshoot state during storage was also genotype dependent. Taking into account those results, at least three groups of strawberry accessions could be formed. The first group – *F.* × *ananassa* 'Suvetar', 'Saulenė', 'Elsanta' and 'Jasna'. Microshoot viability in this group was poorest and they should be transplanted after 3–9 months from the beginning of storage. Microshoots of the second group – *F.* × *ananassa* 'Nora', 'Valotar', KL P8, 'Dangė', *F. virginiana glauca* and *F. vesca* survived for 15 months of storage. However, microshoots condition was rather poor (rating close to 1 point). We recommend transplanting them after 12 months. The third group – all remaining genotypes (*F.* × *ananassa* 'Nida', 'Jaunė', 'Catskill', 'Juni Morgon', 'Melody', 'Venta', 'Vaiva', 'Holiday', *F. virginiana* and *F. moschata*) can be stored for up to 15 months without any risk of losing them.

In practice, plants are removed from storage for repropagation when the mean rating point of plant state is 2 (Reed, 1992; Kovalchuk et al., 2009). A comparison of the plant state rating with the percentage of alive plants after 3, 9, 12 and 15-month periods validates this practice, as it was estimated in our experiment, 50% or more 2 rating points rated plants survived and none of the genotypes was lost. Evaluation of plant state is especially important for new accessions, whose behaviour during storage has not been examined before.

Our results show that rating point close to 1 is critical for survival of most of the genotypes. Three of them were lost after transplanting to fresh growth medium – *F.* × *ananassa* 'Saulenė', 'Suvetar' and 'Elsanta'. Another three genotypes – *F.* × *ananassa* cultivar 'Jasna', *F. virginiana glauca* and *F. vesca* survived although state rating was over 1. Therefore, to minimize the risk of losing those genotypes, much earlier transplanting of microshoots is necessary.

Strawberry state declined after 12–15 months' storage at 4ºC temperature, but after transplanting to new medium and transfer to growth room at 22ºC temperature strawberry microshoots of most of the genotypes restored their condition.

The data presented in Figure show that microshoots of *F.* × *ananassa* and four other *F.* species formed new leaves during storage. For all those genotypes, except for *F.* × *ananassa* 'Venta' and 'Jasna', formation of new leaves was observed after 12 months' storage. Strawberry continued new leaf formation until the end of the experiment. Moreover, leaf formation on of *F.* × *ananassa* 'Catskill', 'Melody' and *F. vesca* increased considerably during the last three months of storage. We do not have a clear explanation for this phenomenon. It could be associated also with high cold hardiness of those genotypes, observed in field conditions and *in vitro* treatments (Rugienius et al., 2009).

The data (Fig.) shows that leaf formation rate was genotype dependent – after 12 months, the highest leaf formation rate was observed for *F.* × *ananassa* 'Nida' (1.7 leaves per microshoot, on average) and the

lowest – 'Dangė', 'Valotar' and *F. virginiana glauca* (0.6 leaves per microshoot, on average). At the end of storage, the highest leaf formation rate was observed for 'Catskill', 'Nida' and *F. vesca* (1.6–1.7 leaves per microshoot on average) and the lowest for 'Venta' (0.05 leaves per microshoot, on the average). New leaves were observed on strawberry, which were in good condition. It can be assumed that the formation of new leaves had no negative effect on the state. Similar results were obtained by Kovalchuk et al. (2009), who indicated that emergence of new leaves is associated with good condition of the plants *in vitro*.

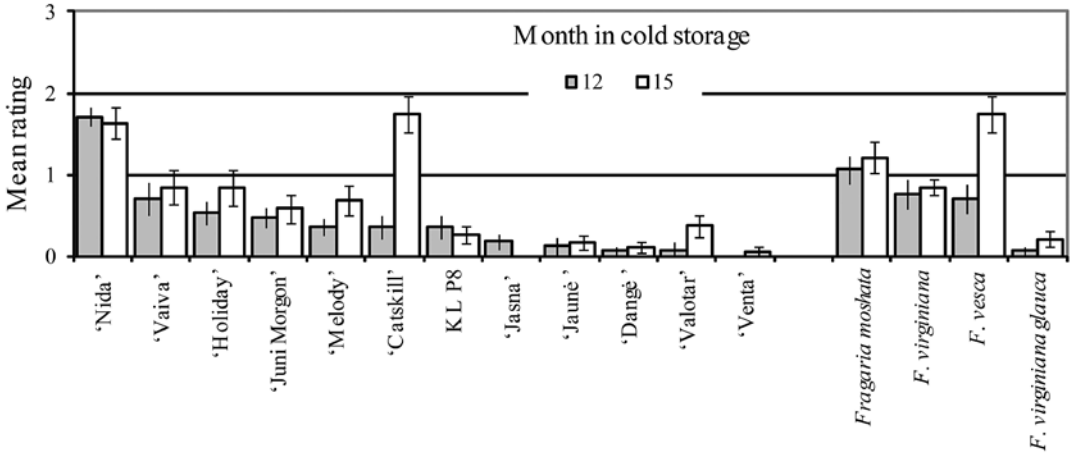

Figure. Formation of new leaves in strawberry during storage at 4°C temperature in Knop's medium

Storage of pear. Genotypes for pear mid-term storage experiments were chosen as representatives of pear genotype segregation groups revealed by polymorphism studies (data not shown) using microsatelite markers. In the current study, we evaluated the viability and state of different pear groups after 6 months' storage in low temperature (4ºC). Storage media were preselected considering our previous experiments, where optimal conditions for cold acclimation of pear groups (A, B, C) were established. We supposed that growth media, optimal for cold acclimation of a particular genotype group, could be suitable for mid-term storage of those genotypes in low temperature as well.

Results show that 75–100% of pear microshoots stored on standard MS growth medium without growth regulators and on the MS medium with BAP 0.75 mg l^{-1} survived during the storage, while only 23–46% of pear microshoots stored on the MS medium with 2% of mannitol survived. The condition of microshoots stored on MS medium with sucrose with or without BAP was rather good – varied between 3.2 and 4.5 rating point (Table 2). This means that both of the medium variants are quite suitable for mid-term storage of the two cultivar groups. In the medium with addition BAP, microplant condition of most of the genotypes was slightly better and more stable during the storage time than in medium without BAP, but for cultivar 'Senryo' the opposite tendencies were observed. Deterioration of plant state after 6 months' storage was most prominent for this cultivar, although the variation in the two groups was not high.

Table 2. Survival and state mean ratings of pear microshoots after 2, 4, 6 months' storage at 4°C temperature in different MS media: A – with 3% sucrose without growth regulators, B – with 3% sucrose and BAP 0.75 mg l^{-1}, C – with 3% sucrose and 2% mannitol without growth regulators

Genotype	Cultivar group (growth dium)	Storage duration, month						Survived after transplanting %
		2		4		6		
		survived %	state, points	survived %	state, points	survived %	state, points	
'Princesse Dagmara'	A	100	4.32 ± 0.1	100	4.02 ± 0.09	100	3.63 ± 0.09	98.31
Pyrus pyraster	A	96	4.58 ± 0.11	94	4.32 ± 0.2	94	4.24 ± 0.1	100
'Senryo'	A	100	4.15 ± 0.12	96	3.72 ± 0.11	84	3.2 ± 0.1	100
'Hasselpeare'	A	95	4.46 ± 0.14	93	4.16 ± 0.15	89	3.93 ± 0.14	100
No. 0408	A	91	3.95 ± 0.2	88	3.76 ± 0.21	86	3.71 ± 0.13	100
'Senryo'	B	97	3.99 ± 0.13	97	3.43 ± 0.13	97	3.21 ± 0.12	100
'Oranzhevaya'	B	100	4.47 ± 0.07	100	4.36 ± 0.07	75	4.36 ± 0.07	100
'Muskatelka Seda'	B	98	4.68 ± 0.06	98	4.54 ± 0.07	98	4.47 ± 0.07	100
'Karalienė Jadvyga'	B	98	4.59 ± 0.07	98	4.46 ± 0.07	98	4.42 ± 0.09	100
No. 0408	C	78	2.39 ± 0.24	59	1.53 ± 0.25	46	1.5 ± 0.36	75
'Koncentrat'	C	51	1.02 ± 0.18	25	0.25 ± 0.6	23	0.24 ± 0.6	20.29
$LSD_{0.5}$		2.7		2.8		3.4		3.2

In the medium with mannitol, survival rate and state of No. 0408 were considerably higher than those of cv. 'Koncentrat' (Table 2). State of plants, stored in medium with 2% mannitol varied between 0.24 and 1.5 points. Survival rate of pear considerably decreased when rating became close to 1 point and this is in agreement with the results obtained with strawberries.

Mannitol was reported to be one of the substances lengthening the storage life of *in vitro* grown tissues (Shibli et al., 2006). However, Hassan et al. (2008) found that addition of mannitol or increased sucrose concentration in the medium did not produce acceptable survival rates of shoot tips of pear. In our study, mannitol decreased viability of plants during storage and at the concentration used it appeared to be an unsuitable source of carbohydrates in the medium for long – mid-term storage of pears *in vitro*.

Our results suggest that optimal conditions for pear cold acclimation are not always the best for long and medium term preservation of plants in low temperature.

The present study is our first time attempt to optimize growth medium composition for low temperature mid-term storage of three groups of genotypes, differentiating in genetic structure, taking into account their requirements for cold acclimation.

In vitro conservation will never replace conventional technologies entirely but will complement them within a strategy that balances the traditional approaches of *in situ*, seed and field genebank conservation with the best of the new approaches of using *in vitro* conservation, pollen storage and even DNA storage (Shibli et al., 2006).

Conclusions

1. Eighty-five percent of the investigated strawberry genotypes sustained 15 months' storage at 4ºC temperature *in vitro*, 40% of strawberry microshoots were in good condition. The survival rate during storage was genotype dependent and was not associated with new shoot and leaf formation.

2. Pears were stored successfully at 4ºC temperature for 6 months *in vitro* on Murashige, Skoog (MS) growth medium with 3% sucrose with or without benzylaminopurin (BAP). Addition of 2% mannitol to the storage medium did not improve microshoots condition during the low temperature storage.

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Braškės (*Fragaria* × *ananassa* Duch.) ir kriaušės (*Pyrus communis* L.) genetinių išteklių saugojimas žemoje temperatūroje *in vitro*

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Santrauka

Klimato sąlygos ir augalų ligos kelia pavojų augynuose saugojamiems genetiniams ištekliams, todėl kuriamos ir taikomos saugojimo *in vitro* technologijos. Alternatyvūs būdai išsaugoti vertingus genetinius išteklius yra augalų augimą slopinančios *in vitro* technologijos ir kriosaugojimas. Tyrimų tikslas – įvertinti būdingų braškės (*Fragaria* × *ananassa* Duch.) bei kriaušės (*Pyrus communis* L.) genotipų ilgalaikio saugojimo žemoje temperatūroje *in vitro* sąlygomis galimybes ir parinkti tinkamus saugojimo metodų parametrus. Tyrimams naudoti *in vitro* kolekcijose saugomų 20 veislių braškių ir 9 veislių kriaušių mikroūgliai. Tyrimai atlikti 2009–2011 m. Lietuvos agrarinių ir miškų mokslų centro Sodininkystės ir daržininkystės institute.

Tyrimų duomenimis, po 15 mėnesių saugojimo 4º C temperatūroje 40 % braškių mikroūglių buvo geros būklės. Augalų būklės kitimas priklausė nuo genotipo, bet nebuvo susijęs su naujų lapelių formavimu ilgalaikio saugojimo metu. Po 15 mėnesių saugojimo žemoje teigiamoje temperatūroje geriausios būklės buvo *Fragaria* × *ananassa* veislių ‘Catskill’, ‘Nida’, ‘Melody’, ‘Venta’ ir *F. virginiana* mikroūgliai, prasčiausios – *F.* × *ananassa* veislių ‘Suvetar’, ‘Jasna’, ‘Elsanta’, ‘Saulenė’, *F. virginiana glauca* ir *F. vesca* mikroūgliai.

Kriaušių mikroūgliai buvo sėkmingai saugojami *in vitro* 4º C temperatūroje 6 mėnesius, pasodinti MS (Murashige ir Skoog) maitinamojoje terpėje su 3 % sacharozės priedu be arba su benzilaminopurinu (BAP), priklausomai nuo genotipų grupės. Manitolio 2 % priedas terpėje saugojimo metu mikroūglių būklės nepagerino.

Reikšminiai žodžiai: braškė, genetiniai ištekliai, kriaušė, žema temperatūra.