Collezioni *in vitro* di alcune specie autoctone del genere *Prunus* in Albania

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In vitro gene bank collections of autochthonous *Prunus* sp. germplasm from Albania

Abstract. Maintenance and preservation of diversity in autochthons *Prunus* sp. (*Prunus avium* L.*, P. webbii* Spach Vierh.*, P. mahaleb* L) plants are very important to human being for fulfilling their nutritional developments. By the economical point of view, this resource of germplasm needs the discovery, the evaluation, the conservation and the utilization of those forms with higher levels of production and quality and with a high value of dietary and therapeutic components. The increasing presence of wild food products can also be seen by farmhouses or local rural restaurants as a part of the local traditional heritage offered by them. The *in vitro* technique is an effective method for *ex situ* conservation of plant genetic diversity, allowing rapid multiplication from very little plant material and with little impact on wild populations. Establishment of *in vitro* collections by the methods of minimal growth, based in reduction of cell metabolism, but preserving the regeneration possibility, have showed positive results. The major part of this research is result of the experience obtained in Experimental Center of Fruit Trees in Rome, Italy. For micropropagation of *Prunus* sp. germplasm were examined different protocols. For *in vitro* conservation, three different methods of short and medium-term conservation were examined using *in vitro* grown plant cultures: 1- reduction of sucrose and MS salts concentrations; 2- Combination of low temperature and reduction of light regime; 3- Absence of phytohormones or growth regulators in the nutrient medium. The survival and regeneration rate for different periods were evaluated. The examined methods differed significantly in the survival rate of the explants. The effect of low temperature (4°C) combined with reduced light regime is the most effective method of medium term preservation. The optimal time of conservation, without subculture, at 4°C was up to 14 months. Whereas reducing sucrose and MS salt (½MS) concentrations resulted optimal for 5 months preservation.

Key words: micropropagation, *in vitro* conservation, phytohormones.

Introduction

Although the traditional use of wild edibles is largely decreasing due to socioeconomic and ecological changes, wild plants are becoming a part of the new thinking about food: they are very important as health food, and in food security and slow food movements (Luczaj *et al*., 2012). Some of the most important species of *Prunus* genus which originate from autochthons local varieties are *Prunus avium* L*, P. webbii* Spach Vierh and *P. mahaleb* L. By the economical point of view, this resource of germplasm needs the discovery, the evaluation, the conservation and the utilization of those forms with higher levels of production and quality and with a high value of dietary and therapeutic components. An important theme is the need for new theoretical and practical approaches that link the revaluation of plant-based cultural heritage with the conservation and use of biocultural diversity (Pardo de Santayana *et al*., 2010).

Nowadays, different plant conservation methods are developed, especially as *in situ* and *ex situ* ones (Engelmann & Engels, 2002; Sarasan *et al.*, 2006; Sota *et al.,* 2011), using two types of *in vitro* preservation methods: (i) slow or minimal growth method and (ii) cryopreservation. The aim of medium-term storage is to increase the interval period between subcultures by reducing growth through the modified environmental conditions, modified medium, growth retardants, osmotic regulators and/or reduction of oxygen concentration (Kameswara, 2004).

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The aim of this study is to determine the optimal method for the micropropagation of autochthons local varieties of *Prunus* sp. and to establish *in vitro* genetic collections by medium growth storage and cryopreservation (storage at the temperature -196°C of liquid nitrogen).

Materials and Methods

Plant materials

Cultures of different populations of wild cherry (*Prunus avium* L.) vulnerable VU (A_1b), wild almond (*P. webbii* Spach Vierh.) Endangered E., and Mahaleb or rock cherry (*P. mahaleb* L.), grown in Central Albania, were established from apical and lateral buds removed from adult field-grown trees. The preferred explants are shoot tips due to their genetic stability. The buds were collected between January and March, when buds were starting to swell from shoots in dormancy. Zygotic embryos were used as primary explants for *Prunus webbii* Spach Vierh.

Explant disinfection

The stem sections were washed carefully with water and were shaken for 5 min in ethanol 70 % followed by 20 min treatment with $HgCl_2$ 0.01% and two drops of Tween 20. The embryos were isolated from the seeds of mature wild almond. Double sterilization with $HgCl_2$ 0.01% for 20 min before and after removing seeds tegument followed immediately after.

Tissue culture

Micropropagation. The isolated shoot tips were cultured onto glass tubes containing MS salts and vitamins (Murashige & Skoog, 1962) or in QL medium (Quoirin & Lepoivre, 1977) or in LP medium (Aitken-Christie *et al.*, 1988), and WPM medium (Lloyd & McCown, 1980), combined with different ratios of plant growth regulators (PGRs, auxins, cytokinins and gibberellins) according the fruit tree species. The proliferation nutrient media were supplemented with 3% sucrose and solidified with 0.55% agar. The pH of the media was adjusted 5.7 – 5.8. The incubation conditions were at 25±2 °C in a 16 h light/24 h regime with white fluorescent light of 1500 lux.

In vitro storage:

For medium-term storage were evaluated three methods of minimal growth: elemination of sucrose and reduction of MS salts concentrations (½MS); combination of low temperature and light regime (incubation in 4°C in darkness); absence of phytohormones or growth regulators in the growth media.

The encapsulation - dehydration technique, as a pretreatment method before freezing in liquid nitrogen was used and the capability to encapsulate and dehydrate of beads, without immersion in liquid nitrogen, was tested. For encapsulation, the shoot tips initially were dropped in 3% Na-alginate containing solution and after that in 100mM $CaCl_2$ solution for 30 min. For dehydration, the encapsulated explants were treated with 0.25, 0.5, 0.75, 1.0 and 1.25mM sucrose solution subsequently for 24 h in each of them and finally were dried for 4 h under air flow in aseptic conditions. After this, the beads were inoculated in multiplication medium and incubated at 25±2°C in a 16 h light/24 h regime. In order to determine the optimal amount of sucrose used for dehydration, the regenerations rates were tested.

Statistical analysis

Data collections in experiment were evaluated using the statistical evaluation program JMP 7.0.

Results

Micropropagation and conservation of wild cherry (*Prunus avium* L.)

Some explants and different nutrient media (tab. 1) are used to produce a great number of *in vitro* plants of wild cherry after a cycle of micropropagation (fig. 1). Shoot tips results the most optimal explants for micropropagation of *P. avium* plantlets. The best proliferation and multiplication medium seems to be the MS medium with higher ratio cytokinin/auxin, which plays an important role in the first stages of *de novo* bud formation. During multiplication stage, the high ratio BAP/NAA (70 : 1) (tab. 1), in favor of cytokinin BAP, stimulates the formation of new shoots by inhibiting the apical dominance. The presence of cytokinin BAP has induced the formation of a great number of axillary buds and this phenomenon has increased the number of the plantlets derived from the subcultures. During subcultures was observed not only the production of a considerable number of plantlets, but even increase in length of secondary and tertiary adventitious shoots explants. Three nutrient rooting media containing different concentrations of auxin, α-naphthaleneacetic acid, NAA and macro- and micronutrients, presented in the universal medium MS are compared (tab. 1) and after 4 weeks on rooting medium, rhizogenesis was observed. The explants reacted differently in three types of rooting media. The mineral and NAA concentration of the

Tab. 1 - Uso di diversi metodi di propagazione della coltura in vitro di germoplasma di alcuni *Prunus* sp. autoctoni
Tab. 1 - Use of different methods of in vitro *culture propagation of some autochthonous* Prunus *sp. germplasm.*

Species and status	Explant, nutrient medium	Phytohormones, mgl^{-1}			Authors
		Proliferation	Organ formation, multiplication	Rooting	
Prunus avium L. Vulnerable VU (A1b)	Shoot tips MS	BAP 0,3, IBA 0,1, GA_3 0,3	BAP 0,7, NAA 0,01, GA3 0,1	1. Macro MS/2, micro MS, NAA 0,1	Kongjika *et al*., 2009
	Shoot tips LP	BAP 0,25, IBA 0,6, GA_3 0,3	BAP 0,25, IBA 0,6, GA_3 0,3	2. Macro MS/2, micro MS/2, NAA 0,1	
	leaf pieces MS	NAA 10^{-4} M, BAP 10-5M	NAA 10^{-5} M, BAP 10-4 M	3. Macro MS, micro MS/2, NAA 2	
Prunus webbii (Spach) Vierh. Endangered E	Mature Embryos MS	1.Without phytohormones 2. BAP 1, IBA 0,1	BAP 0,7, NAA 0,01, GA_3 0,1	Macro MS/2, micro MS, NAA 0,1	Sota e Kongjika, 2014
	Cotyledons MS	1. BAP 1, IBA 1 2. BAP 0,5, IBA 1	BAP 0,7, NAA 0,01, GA_3 0,1		
	Shoot tips MS	BAP 0,3, IBA 0,1, GA3 0,3	BAP 0,7, NAA 0,01, GA_3 0,1		
*Prunus mahaleb*L.	Shoot tips	(MS) BAP 0,3, IBA 0,1, GA_3 0,3	(MS) BAP 0,7, NAA 0,01, GA3 0,1	1. Macro MS/2, micro MS, NAA 0,1 2. Macro MS/2, micro MS/2, NAA 0,1 3. Macro MS, micro MS/2, NAA 2	Sota (Mata) e Kongjika, 2011
	Nodal segments, MS, LP	(LP) BAP 0,25, IBA 0,6, GA_3 0,3	(LP) BAP 0,25, IBA 0,6, GA_3 0,3		Sota e Kongjika, 2014

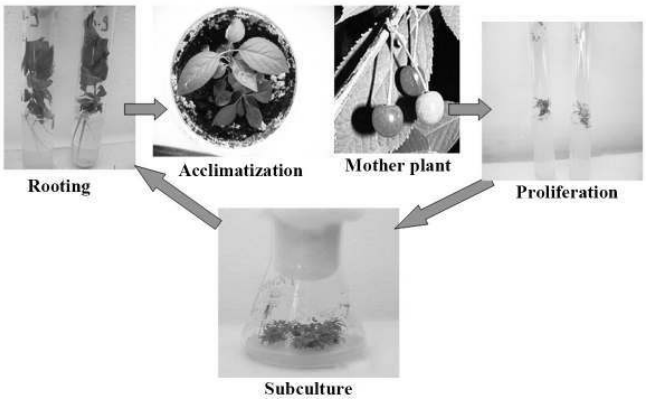

Fig. 1 - Ciclo di micropropagazione di ciliegio selvatico *Prunus avium* L.
Fig. 1 - Micropropagation cycle of wild cherry, Prunus avium *L.*

nutrient media affect rooting percentage and roots characteristics. The most optimal rooting media resulted the one containing ½ MS macronutrients, ½ MS micronutrients, MS vitamins with 0.1 mg l^{-1} NAA. Higher concentrations of NAA tended to induce callus formation on the proximal end of shoots. In this case, the number of roots was high, but those had an abnormal look being two short and thick.

Among some conservation methods, the minimal growth storage appeared to be an ideal method for short to medium-term germplasm preservation (fig. 5). The response of *in vitro* shoots stored for different periods under different storage conditions was assessed on the basis of survival and regeneration rates. The plantlets of *Prunus avium* L. are stored for a long period of 14 months in the reduced regime of temperature and lighting (fig. 2a), while the modified media (reduction of MS salts, sucrose or phytohormones elimination) allowed only up to 4 months (fig. 2b). With the extension of the conservation period, the survival rates may result relatively high, but regeneration rates are reduced drastically for all medium-term conservation methods (fig. 5). The encapsulation - dehydration technique without immersion in liquid nitrogen was used with the aim to determine the capability to encapsulate and dehydrate of beads (fig. 2c). Subsequently dehydration in different concentrations of sucrose solutions resulted optimal up to 0.75mM sucrose solution. In higher concentrations of sucrose, the regeneration rate during cultivation of beads in optimal physical-chemical conditions, was decreased.

Micropropagation and conservation of Mahaleb or rock cherry (*P. mahaleb* L.)

Two nutrient media (MS and LP) supplemented with different ratios of three phytohormones (BAP, IBA and GA_3) are used for micropropagation of apical and lateral buds of Mahaleb cherry in proliferation stage, while the same media with the phytohormones (only the auxin IBA is replaced with NAA) are used in multiplication stage. The influence of three rooting media with different concentrations of MS macro-,

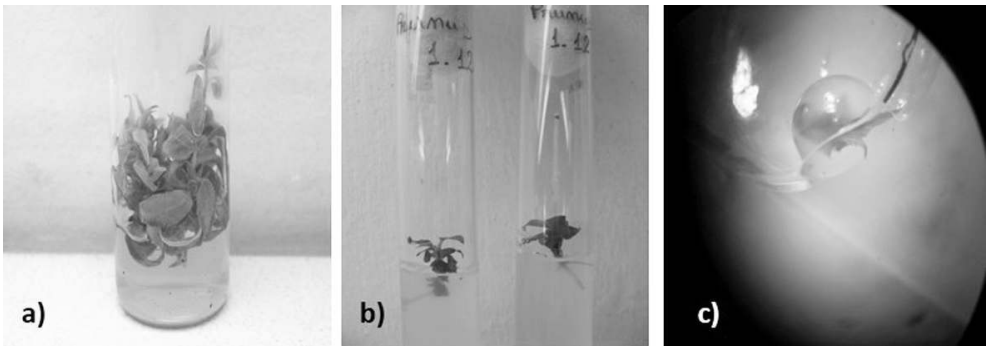

Fig. 2 - Metodi di conservazione delle piantine *in vitro* di *Prunus avium*: **a** - a basse temperature ed oscurità; **b** - in terreno modificato e **c** – gemma incapsulata.
Fig. 2 - Conservation methods of in vitro plantlets of Prunus avium*: **a** - in low temperature and darkness condition; **b** – in modified nutrient medium and **c** – encapsulated shoot.*

micronutrients and auxin NAA is tested for rhizogenesis of new *in vitro* plantlets (tab. 1).

The most optimal nutrient medium is considered MS medium with MS vitamins and combined with 0.3 mg l^{-1} BAP, 0.1 mg l^{-1} IBA and 0.3 mg l^{-1} GA_3, which favors the buds development in the first stage of *in vitro* culture comparing with LP medium (fig. 3a,b). During subcultures was observed not only the production of a considerable number of plantlets, but even increase in length of secondary and tertiary adventitious shoots in the explants multiplicated on MS medium (fig 3c). Best results on rooting percentage were observed in explants cultured on first rooting medium, containing ½ MS macronutrients, MS micronutrients, MS vitamins supplemented with 0.1 mg l^{-1} NAA (fig. 4).

Maintenance of *in vitro* plantlets on 4°C in darkness was shown the most effective method (fig. 5, 6a), because the shoots were stored successfully for longer periods than in the other storage method tested (storage in medium with reduction of salts and sucrose elimination; storage in medium with phytohormones elimination). Mahaleb cherry plantlets reacted positively to encapsulation-dehydration methods, allowing dehydration up to 0.75mM sucrose solution (fig. 6b).

Micropropagation and conservation of wild almond (*P. webbii* Spach Vierh.)

Comparing the two methods of *in vitro* culture (tab. 1), embryo culture seems to be of great impact for propagation of wild almond plants. The germination of zygotic embryos and roots development was observed after three days of cultivation, meantime the leaves developed after 6-7 days. Because of juvenile properties, the embryos possess a great regeneration potential and therefore are considered optimal explants for *in vitro* micropropagation purposes. From the results are observed differences not only related with explants type, but even in their reaction in different induction media. Basal MS medium without PGRs resulted more effective for organogenesis induction and the plant regeneration resulted in a high percentage, when using embryos with part of cotyledons as primary explants. Induction of somatic embryogenesis from cotyledons resulted efficient in nutrient medium MS with cytokinin BAP in 0.7 mg l^{-1} concentration. A great number of new plantlets derived from embryos, cotyledons or shoots culture was obtained during subculture stage. Spontaneous rooting resulted in a high percentage, meanwhile the induced one was in a lower rate (fig. 7).

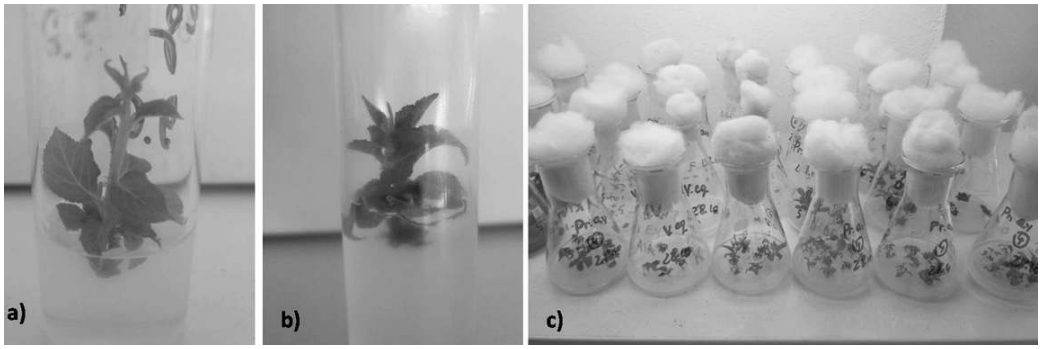

Fig. 3 - Sviluppo delle piantine di ciliegio di Mahaleb: **a** - terreno MS di proliferazione; **b** - terreno LP di proliferazione; **c** – materiale in proliferazione.
*Fig. 3 - Development of plantlets of Mahaleb cherry: **a** - MS proliferation medium; **b** - LP proliferation medium; **c** - plantlets in proliferation stage.*

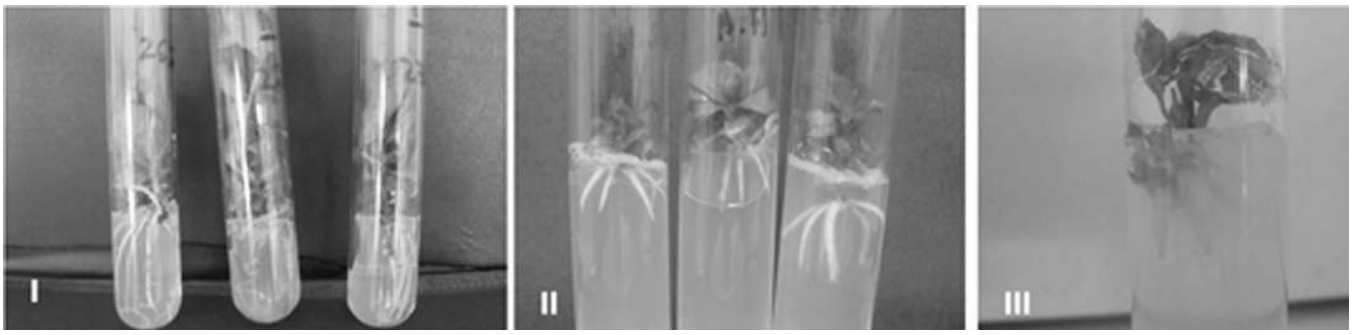

Fig. 4 - Piantine radicate di ciliegio di Mahaleb in tre diversi terreni di radicazione.
Fig. 4 - Rooted plantlets of Mahaleb cherry in three different rooting media.

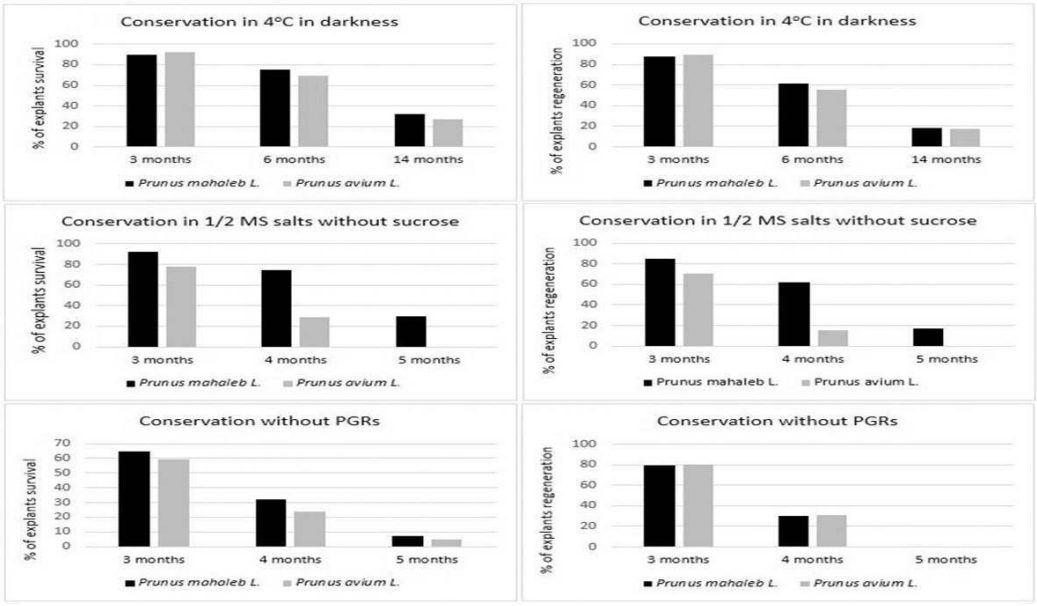

Fig. 5 - Tassi di sopravvivenza e rigenerazione di piantine di *P. avium* e *P. mahaleb* durante la conservazione nelle condizioni testate.
Fig. 5 - Survival and regeneration rates of P. avium *and* P. mahaleb *plantlets during minimal growth conditions.*

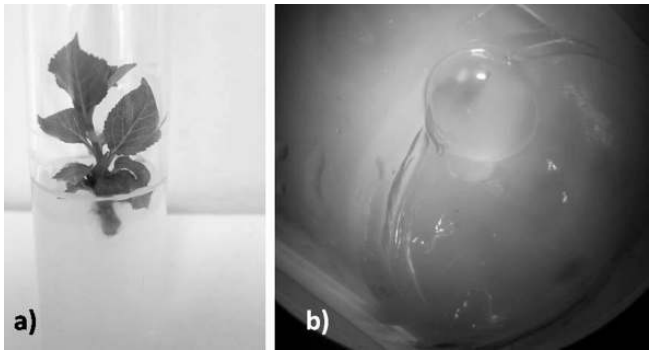

Fig. 6 - *a* - Piantine rigenerate di *Prunus mahaleb* dopo 10 mesi di conservazione a bassa temperatura ed in oscurità;
b - germoglio incapsulato.
Fig. 6 - a - Regenerated plantlets of Prunus mahaleb *after 10 months conservation in low temperature and darkness;*
b - encapsulated bead.

All *in vitro* plantlets produced by different methods beginning from some explants (mature embryos, cotyledons, buds) underwent *in vitro* conservation by minimal growth in low temperature (4ºC) and darkness. The result of this method is the storage of *in vitro* plantlets for 6 months, preserving the possibility of plant regeneration after this period.

Discussions and conclusions

The application of plant biotechnology can make considerable contributions towards the genetic and phytosanitary improvement of fruit trees and other species economically important. Micropropagation of autochthonous species of *Prunus sp.* germplasm is an efficient method for their multiplication. The most optimal nutrient medium results MS medium combined with PGRs such BAP, NAA and GA_3. During subcultures is observed a very high micropropagation coefficient in plantlets cultivated in MS medium supplemented with 0.7 mg l^{-1} BAP, 0.01 mg l^{-1} NAA and 0.1 mg l^{-1} GA_3. Better results obtained in *in vitro* culture of *Prunus sp.* germplasm in MS medium comparing to LP one could be related to different composi-

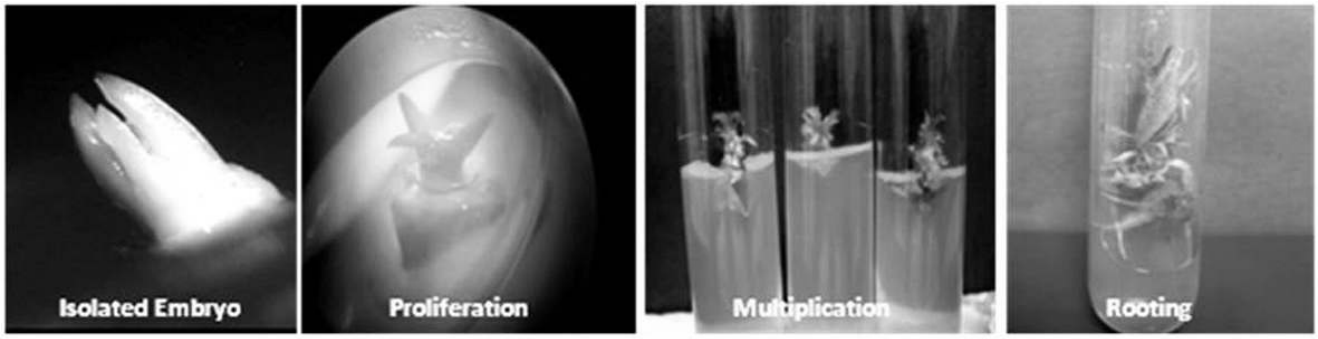

Fig. 7 - Diverse fasi della coltura embrionale di mandorlo di Webb, *Prunus vebbii*
Fig. 7 - Different stages of embryo culture of wild almond, Prunus vebbii.

tion of these media. The major differences in macronutrients among these two basal media are in ammonium and nitrate ion concentrations and total ion concentration. Full-strength MS medium is highest in ammonium and nitrate, while LP is a low ammonium medium. MS medium has even potassium nitrate as a major nitrogen source, while LP medium has calcium nitrate. Beside this, some micronutrients are present only in the MS medium.

For *Prunus webbii* using zygotic embryo as primary explants, embryo culture results an optimal method during proliferation in MS medium without PGRs or phytohormones. PGRs are not necessary, because the embryo has a considerable size and is in an autotrophic phase. As reported by other authors (Raghavan *et al*., 1982), there is no specific need for additional amounts of PGRs in the nutrient media for a large broad of wild plants. Shoots did not show callus genesis, but were developed through direct organogenesis.

Medium term conservation techniques are now routinely used in commercial laboratories, due to their immediate advantages and the low necessary investments. Conservation in 4°C in darkness is an effective method for medium term conservation. Preserving of *Prunus* sp. germplasm for 10 months in such conditions gives high percentage of survival and regeneration parameters. As far as duration of preservation is concerned, 3 and 6 months of storage periods are proved better than longer durations for the two other conservation methods tested. With increase in storage period, survival, as well as regeneration rate, is reduced significantly. In the cultures, which are stored for the period of 10 months, their survival and regenerating percentage remained below 60 percent. The present investigation revealed that the higher survival rates had positive effect on regeneration percentage of the shoots.

In the present study, the most effective method of medium-term conservation results the conservation at low temperature in darkness up to 10 – 14 months according to the species. These results confirmed the findings of other workers who reported the effectiveness of storage at low temperatures (Bell & Reed, 2002; Orlilkowska, 1992; Negri *et al*., 2000).

Riassunto

La conservazione della biodiversità delle piante autoctone del genere *Prunus* (*Prunus avium* L.*, P. webbii* Spach Vierh.*, P. mahaleb* L.) è molto importante per soddisfare le esigenze nutrizionali umane. Per la conservazione a medio termine sono state saggiate tre tecniche: 1) riduzione della concentrazione di saccarosio e dei sali del substrato MS; 2) riduzione della temperatura e della intensità luminosa; 3) eliminazione dei fitormoni o regolatori di crescita nei terreni di coltura. La combinazione della bassa temperatura (4°C) e della riduzione della intensità luminosa è risultata la tecnica migliore di conservazione a medio termine, permettendo la sopravvivenza dei germogli fino a 14 mesi.

Parole chiave: micropropagazione, conservazione *in vitro*, fitormoni

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