Costituzione di collezioni *in vitro* di importanti specie arboree "minori" da frutto

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Establishment of *in vitro* collections of under-utilized fruit trees

Abstract. Wild relatives of cultivated fruit trees represent a source of genetic variability and can be very important in breeding programs and cultivation. Plant resources are threatened to extinct because of deforestation, developmental activities and introduction of new varieties. For this reason, it would be of interest to use the micropropagation and *in vitro* conservation as suitable methods for obtaining and preserving a large quantity of genetically homogeneous and healthy plant material and which can be used for planting. The different consumptions of these fruit species, also called 'minor', and even the social-economic impact, justify the need for further evaluation and conservation of these genetic resources. The major part of this research is from the experience obtained in Experimental Center of Fruit Trees in Rome, Italy. Experiments were raised using shoot tips and internodes from *in vivo* collections of *Myrtus communis* L., *Punica granatum* L., *Pyrus pyraster* L., *Pyrus amygdaliformis* Vill. and *Zizyphus jujube* Mill. For all species, different micropropagation protocols were examined. For conservation purposes, effect of reduced sucrose and MS salts concentrations and combination of low temperature and light regime were examined. To test the regeneration of the conserved cultures, they were transferred onto fresh culture medium. These species differed significantly in their survival. However, they were similar in terms of proliferation ability, when they were transferred onto fresh medium. The effect of low temperature (4°C) combined with reduced light intensity is the most effective method of medium term preservation for all the species. The examined micropropagation and *in vitro* conservation protocols can be used effectively for obtaining homogeneous plant material.

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Key words: fruit trees, micropropagation, *in vitro* conservation.

Introduction

The diversity of orographic and climatic conditions makes Albania a rich centre with valuable plant species in the Mediterranean area. Some of the most important species which originate from native local varieties are myrtle *Myrtus communis* L., pomegranate *Punica granatum* L., *Pyrus pyraster* L. (wild pear), *Pyrus amygdaliformis* Vill. and *Zizyphus jujube* Mill. Nowadays, different plant conservation methods are developed, especially as *in situ* and *ex situ* ones (Kongjika *et al.*, 2009; Neveen *et al.*, 2008; Engelmann e Engels, 2002). Slow growth storage by *in vitro* cultures has been reported in many species (Sota, 2012; Maqsood *et al.*, 2010; Kameswara, 2004). The aim of this study was to determine the optimal method for the micropropagation, associated with the investigations of slow growth conditions for the storage of the shoots cultured *in vitro*.

Materials and methods

Cultures of *Myrtus communis* L., *Pyrus pyraster* L., *Pyrus amygdaliformis* Vill. and *Zizyphus jujube* L. were established from apical and lateral buds removed from adult field-grown trees. Zygotic embryos were used as primary explants for *Punica granatum* L. Explants were disinfected with 70% ethanol for 5 min. followed by 20 min. of treatment with $HgCl_2$ 0.01% and two drops of Tween 20. All the procedures are carried out in sterile conditions in the laminar flow. The explants were cultured onto glass tubes containing MS (Murashige e Skoog, 1962), WPM (Lloyd e McCown, 1980) or B5

(Gamborg *et al.*, 1968) salts and vitamins, combined with different concentrations of PGRs (tabb 1, 2 and 3). The incubation conditions were at 25±2° C in a 16 h/8 h light/dark regime with cool, white fluorescent light of 1500 lux. For *in vitro* storage were evaluated the effect of low temperature and light intensity; the effect of reduced sucrose concentrations and MS salts strength; the absence of phytohormones or growth regulators in the growth media (tab. 4).

Results and discussion

In vitro culture

Although the micropropagation of pomegranate by buds considered being better, this method of direct organogenesis, despite many advantages, poses several problems related to explants sterilization and to avoiding the browning caused from polyphenols release in the medium (fig. 1a). Data from previous experiments for direct organogenesis initiated by bud explants show that this development path has no positive results, despite different time of explants isolation, different techniques of sterilization and avoiding polyphenols methods, two nutrient media (MS, WPM) supplemented with different phytohormones have been tested (tab. 1) (Kongjika *et al.*, 2014). Micropropagation by direct organogenesis starting from zygotic embryos inoculated in free-phytohormones MS medium appears as one of the main method of successful proliferation (fig. 1b-e). Mature embryos (fig. 1b) are autotrophic and grown only to speed the germination after the embryo inoculation in less complex medium and free phytohormones. Zygotic embryos are also free of polyphenols. Four weeks after inoculation, the newly plantlets were ready to be subcultured (fig. 1c). This is probably due to the ability of mature zygotic embryo to synthetize phytohormones. In this case, the basal medium MS

Tab. 1 - Uso di coltura *in vitro* di propagazione da germogli di melograno *Punica granatum* L. (organogenesi diretta)
Tab. 1 - Use of in vitro *culture propagation of the pomegranate* Punica granatum *L. buds (direct organogenesis)*

Species	Explant, nutrient medium	Phytohormones, mg l^{-1} in different stages			
		Proliferation	Organ formation	Subculture	Rooting
Punica granatum L.	Buds, MS, WPM	BAP 1, NAA 0.1	BAP 0.3, IBA 0.1, GA_3 0.3	BAP 0.5, NAA 0.1	MS/2, IBA 1
	Zygotic embryos, MS	PGRs free	BAP 0.5, NAA 0.1	BAP 0.5, NAA 0.1	MS/2, IBA 1

Tab. 2 - Uso di coltura *in vitro* di propagazione da radici provenienti dalla coltura embrionale di melograno *Punica granatum* L. (embriogenesi somatica indiretta)
Tab. 2 - Use of in vitro *culture propagation from the roots derived from embryo culture of pomegranate Punica granatum* L. *(indirect somatic embryogenesis)*

Species	Explant, nutrient medium	Phytohormones, mg l^{-1} in different stages			
		Proliferation	Globular embryos	Heart and torpedo embryos	Mature embryos,new plantlets
Punica granatum L.	Roots from embryo culture, B5-Gamborg	(1). NAA 1 (2) 2,4-D 0.5 (3) BAP 0.5 (4) Kinetin 2	BAP 0.5	BAP 0.5	NAA 0.01,BAP 0.5, Kinetin 2

Tab. 3 - Uso di coltura *in vitro* di propagazione di alcuni minori specie da frutto (organogenesi diretta da germogli)
Tab. 3 - Use of in vitro *culture propagation of some minor fruit trees species under utilization (direct organogenesis from buds)*

Species	Explant, nutrient medium	Phytohormones, mg l^{-1} in different stages		
		Proliferation	Subculture	Rooting
Myrtus communis L.	Buds, MS	(1) BAP 0.65, NAA 0.01, GA_3 0.1 (2) BAP 2, NAA 0.05, GA3 0.1	BAP 0.65, NAA 0.01, GA_3 0.1	(1) MS+IBA 0.1 (2) Macro MS/2, micro MS, NAA 0.1
Zizyphusjujube Mill.	Buds, MS	BAP 1, IBA 0.5	BAP 1, IBA 0.5	IBA 1
Pyrus pyraster L.	Buds, MS	BAP 1, NAA 0.1, GA_3 0.3	BAP 1, NAA 0.1	(1) MS + IBA 0.1, (2) MS + IBA 0.1, Sequestrene 138
Pyrus amygdaliformis Vill.	WPM	BAP 1, NAA 0.1, GA3 0.3	BAP 1, NAA 0.1	MS + IBA 0.1

Tab. 4 - Uso di diversi metodi di conservazione *in vitro* di alcuni minori specie
Tab. 4 - Use of different methods of in vitro conservation of some fruit trees species under utilization

Species	Explant	Type of morphogenesis	Conservation method	Period of conservation
Myrtus communis L.	Buds	Direct organogenesis	Low temperature 4ºC, darkness	10 months
			Modified medium (Reduction of MS salts, sucrose elimination)	5 months
			Modified medium (phytohormones elimination)	4 months
Pyrus pyraster L. *P. amygdaliformis* Vill.	Buds	Direct organogenesis	Low temperature 4ºC, darkness	10 months
Zizyphus jujube L	Buds	Direct organogenesis	Low temperature 4ºC, darkness	14 months
			Modified medium (Reduction of MS salts, sucrose elimination)	5 months
			Modified medium (phytohormones elimination)	5 months

Fig. 1 - Sviluppo delle piantine di melograno mediante organogenesi diretta: (a) segni di brunitura di terreno da polifenoli liberati da germogli; (b) embrione zigotico isolato; (c) piantine durante la fase di proliferazione in terreno MS senza regolatori di crescita; (d) piantine di melograno, derivati da micropropagazione in terreno MS con BAP 0,5 mg l^{-1} e ANA 0,1 mg l^{-1} (e) effetto di auxina AIB 1 mg l^{-1} in rizogenesi.
Fig. 1 - Pomegranate plantlets development by direct organogenesis: (a) browning signs of nutrient medium from polyphenols released from buds; (b) isolated zygotic embryo; (c) plantlets during proliferation stage in free-PGRs MS medium; (d) micropropagated pomegranate plantlets in MS medium with BAP 0.5 mg l^{-1} e NAA 0.1 mg l^{-1} (e) effect of auxin IBA 1 mg l^{-1} in rhizogenesis.

only support the growth and regeneration process. A great number of new plantlets were obtained during the subculture stage in MS medium supplemented with BAP 0.5 mg l^{-1} and NAA 0.1 mg l^{-1} (fig. 1d). Comparing the effect of two auxins present in MS medium, IBA (1 mgl^{-1}) and NAA (0.1 mgl^{-1}) (table 1) during rhizogenesis highlights the key role of auxin IBA as "rooting factor" (fig. 1e).

During indirect somatic embryogenesis, B5-Gamborg medium of inoculation and proliferation of pomegranate root derived from embryo culture was enriched separately with different PGRs: (1 mg l^{-1} NAA, 0.5 mg l^{-1} 2,4-D; 0.5 mg l^{-1} BAP, 2 mg l^{-1} Kinetin). From four different experiments, after 2 months, only root explants, under the action of cytokinin BAP 0.5 mg l^{-1}, generated callogenesis (fig. 2a). From these embryogenic calli, initial somatic embryos in globular forms (fig. 2b) were developed within 15 days in subculture B5-Gamborg medium with 0.5 mg l^{-1} BAP as the most effective dose. After the somatic embryos maturation stage of 4 months, the globular embryos were transformed into distinct bipolar structures (fig 2c). Plantlet differentiation from somatic to mature embryos and new plantlets were observed after the transfer on B5-Gamborg medium with 0.01 mg l^{-1} NAA, 0.5 mg l^{-1} BAP, 2 mg l^{-1} kinetin (fig. 2d). The plantlets isolated from callus were transferred into fresh MS medium supplemented with BAP 0.5 mg l^{-1} for further development and for successive subcultures. After the first subculture, the average multiplication coefficient is 3.2. It seems that somatic embryogenesis is considered as an alternative technique for *in vitro* clonal propagation of pomegranate plants.

The most optimal nutrient medium for micropropagation of *Myrtus communis* L. (fig. 3a-d) and *Zizyphis jujuba* Mill. (fig. 4a-c) is considered MS medium (tab. 3) with higher ratio cytokinin/auxin, which plays an important role in the first stages of *de novo* bud formation (proliferation and subculture stage) (fig. 3a, b and 4a). The ratio BAP/NAA is very high for micropropagation of myrtle (from 40:1 to 65:1) (tab. 3) (fig. 3c), whereas for jujube only 2:1 proportion (fig. 4b) in favour of the cytokinin BAP induced the formation of new shoots by inhibiting the apical dominance (Mata e Kongjika, 2010; Sota eKongjika, 2014). Despite the difference in this ratio, in both cases of myrtle and jujube, the multiplication

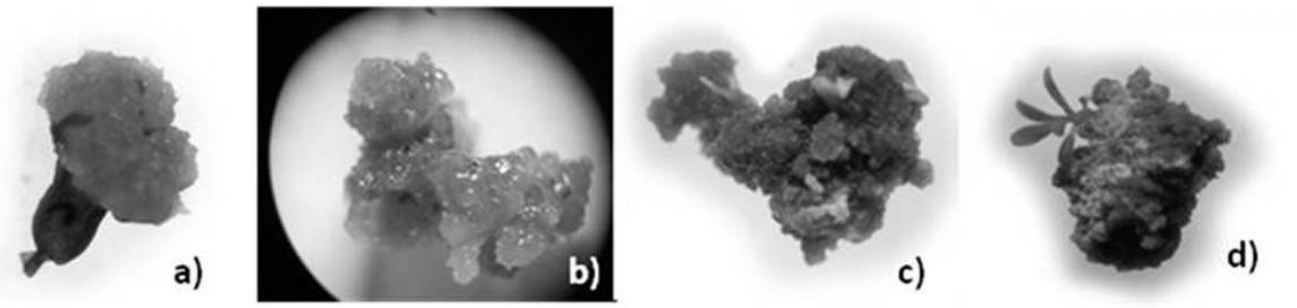

Fig. 2 - Diverse fasi di embriogenesi somatica indiretta di segmenti di radici di melograno sotto l'effetto di BAP: (a) callo embriogenico all'estremità del segmento della radice in terreno B5-Gamborg con BAP 0.5 mg l^{-1}; (b) embrioni somatici globulari alla superficie di differenti segmenti di radici; (c) fase di maturazione di embrioni somatici e (d) differenziazione di piantine dagli embrioni somatici in terreno B5-Gamborg con ANA 0.01 mg l^{-1}, BAP 0.5 mg l^{-1} e kinetina 2 mg l^{-1}.

Fig. 2 - Different stages of indirect somatic embryogenesis of pomegranate root segments under the effect of BAP: (a) embryogenic callus at the end cut of root segment in MS medium with BAP 0.5 mg l^{-1}; (b) initial globular somatic embryos at the surface of different root segments; (c) maturation stage of somatic embryos and (d) plantlet differentiation from somatic embryos in MS medium with phytohormones NAA 0.01 mg l^{-1}, BAP 0.5 mg l^{-1} e kinetin 2 mg l^{-1}.

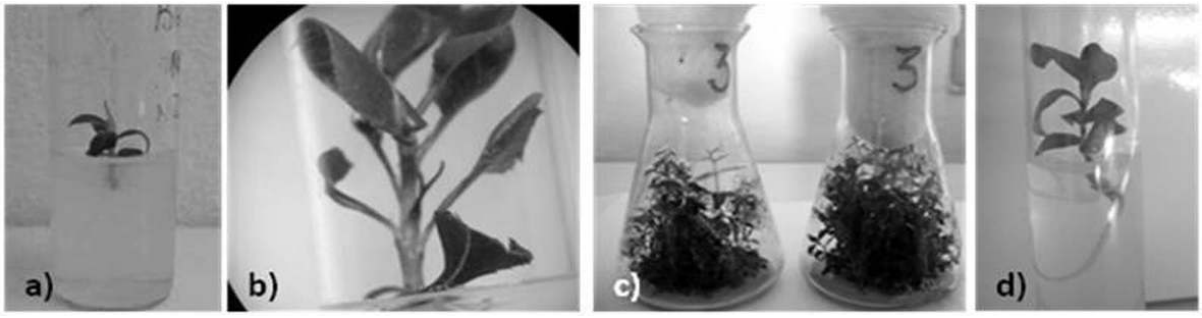

Fig. 3. Diverse fasi di micropropagazione di mirto: (a) germoglio di mirto in terreno MS con BAP 0.65 mg l^{-1}, ANA 0.01 mg l^{-1}, GA_3 0.1 mg l^{-1}; (b) nuova piantina pronta per la subcoltura; (c) un gran numero di piantine nella fase di subcoltura (lo stesso terreno MS con quello della proliferazione) and (d) piantina di mirto, radicata in terreno MS con AIB 0.1 mg l^{-1}.

Fig. 3 - *Different micropropagation stages of myrtle: (a) inoculated bud in MS medium supplemented with BAP 0.65 mg l^{-1}, NAA 0.01 mg l^{-1}, GA_3 0.1 mg l^{-1}; (b) new plantlet ready for subculturing; (c) a great number of plantlets in the subculture stage in the same MS medium of proliferation stage) and (d) rooted myrtle plantlet in MS medium with IBA 0.1 mg l^{-1}.*

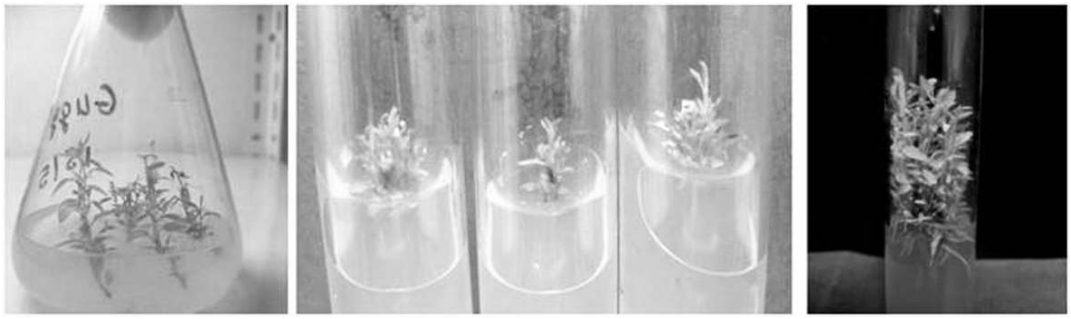

Fig. 4 - Diverse fasi di micropropagazione di giuggiolo: (a) germogli di giuggiolo al termine della fase di proliferazione in MS terreno con BAP 1 mg l^{-1}, AIB 0.5 mg l^{-1}; (b) piantine in fase di subcoltura nello stesso terreno MS di fase di proliferazione e (c) piantine di giuggiolo, radicate in terreno MS con AIB 0.1 mg l^{-1}.

Fig. 4 - Different micropropagation stages of Zizyphus jujube*: (a) jujube plantlets at the end of proliferation stage in MS medium with BAP 1 mg l^{-1}, IBA 0.5 mg l^{-1}; (b) plantlets in subculture stage in the same MS medium of proliferation stage and (c) rooted jujube plantlets in MS medium with IBA 1 mg l^{-1}.*

coefficient is very high, respectively 5.2 for myrtle and 11.1 for jujube. The auxin IBA (0.1 mg l^{-1}) is considered an important factor for rooting of shoots of the two species of myrtle (fig. 3d) and jujube (4c).

Shoots of *Pyrus pyraster* L. and *Pyrus amygdaliformis* Vill. reacted differently when cultured onto MS and WPM medium (tab. 3). The nutrient medium affects significantly the organogenic response of the explants (fig. 5a-d). During proliferation and subculture stage, WPM nutrient medium resulted optimal in comparison with MS nutrient medium for both species under investigation (fig. 5a, b). Rooting

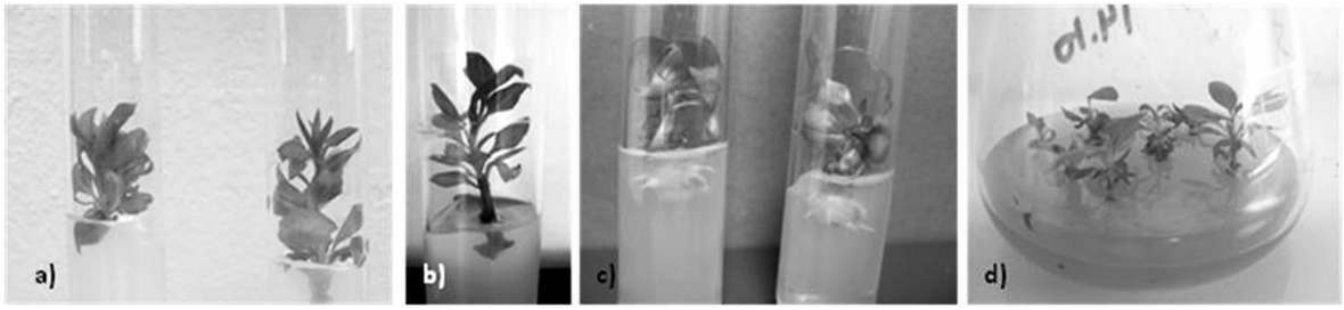

Fig. 5 - Diverse fasi di micropropagazione di *Pyrus pyraster* e *P. amygdaliformis*: (a) germogli di pera selvatica in fase di proliferazione in terreno WPM con BAP 1 mg l^{-1}, ANA 0.1 mg l^{-1}, GA_3 0.1 mg l^{-1}; (b) germogli di pera di mandorle in fase di proliferazione in terreno WPM con BAP 1 mg l^{-1}, ANA 0.1 mg l^{-1}, GA_3 0.1 mg l^{-1}; (c) piantine radicate di *P.* pyraster in terreno MS con AIB 0.1 mg l^{-1} e (d) piantine radicate di *P. pyraster* in terreno MS con AIB 0.1 mg l^{-1} e sequestrene 138.

Fig. 5 - Different micropropagation stages of Pyrus pyraster and P. amygdaliformis*: (a) wild pear buds in proliferation stage in WPM medium with BAP 1 mg l^{-1}, NAA 0.1 mg l^{-1}, GA_3 0.1 mg l^{-1}; (b) almond leaved-pear buds in proliferation stage in WPM medium with BAP 1 mg l^{-1}, NAA 0.1 mg l^{-1}, GA_3 0.1 mg l^{-1}; (c) rooted plantlets of* P. pyraster *in MS medium with IBA 0.1 mg l^{-1} and (d) rooted plantlets of* P. pyraster *in MS medium with IBA 0.1 mg l^{-1} and sequestrene 138.*

induction appears very difficult, especially regarding to trees species. For this reason, two variants of rooting medium were tested, both containing the auxin IBA 0.1 mg l^{-1}, but differing by the iron source (FeNaEDTA and FeNaEDDHA). The iron source resulted very important, and after 4 weeks of the culture on rooting medium, rhizogenesis was observed. The medium enriched with FeEDTA induced a high number of roots, but they were too short (fig. 5c). Plantlets cultivated onto medium enriched with FeNaEDDHA (Sequestrene 138) rooted faster and longer and dark green coloured roots developed (fig. 5d). In both rooting media, *P. pyraster* L. explants were tested and showed better performance in comparison with *P. amygdaliformis* Vill. explants, with a rooting rate of 81 % and 43 %, respectively.

In vitro storage

The method of minimal growth in low temperature (4°C) in darkness gives positive results in the short – medium term storage of plant material up to 10 months for *Myrtus communis* L., *Pyrus pyraster* L. and *P. amygdaliformis* Vill., and up to 14 months for *Zizyphus jujube* Mill. This method was shown the most effective method (tab. 4), 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). The response of *in vitro* explants to various storage periods was estimated as survival value (number of survival explants in relation to the total number of buds placed in conservation). The other methods of minimal growth, which involve the modification of nutrient media: (i) reduction of MS salts and sucrose elimination; (ii) phytohormones elimination from nutrient media; resulted effective for a conservation method up to 5 months for the examined species (tab. 4).

Discussions and conclusions

The work of our research group in the field of *in vitro* culture during nearly two decades has evidenced that in Albania has advancement of the use of biotechnological methods for the conservation of germplasm of threatened, autochthonous and with economic value plant species. New *in vitro* techniques offer facilities for storage of extensive plant collections using minimal space and long or medium-term conservation with regeneration possibility. The micropropagation and *in vitro* conservation method is a convenient way for the international exchange of germplasm of valuable species. The conserved collections of endemic, threatened and wild relatives of cultivated species represent a source of genetic variability and can be important in plant breeding and cultivation or for wild population recovery.

Riassunto

I genotipi selvatici di specie da frutto coltivati sono una importante fonte di variabilità genetica per programmi di miglioramento genetico. La conservazione *in vitro* è un metodo idoneo per conservare una grande quantità di materiale vegetale geneticamente omogeneo. Il materiale vegetale iniziale ha avuto origine da apici e internodi di piante di *Myrtus communis* L., *Punica granatum* L., *Zizyphus jujube* Mill., *Pyrus pyraster* L., *Pyrus amygdaliformis* Vill. in collezione nell'Orto Botanico di Tirana. Sono stati esaminati diversi protocolli di micropropagazione e conservazione. In tutte le specie, la temperatura di 4°C, abbi-

nata a un ridotto regime luminoso, è risultata più efficace per la conservazione a medio termine.

Parole chiave: specie "minori", micropropagazione, conservazione *in vitro*

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