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Micropropagation and *in vitro* Conservation via Slow Growth Techniques of *Prunus webbii* (Spach) Vierh: An Endangered Plant Species in Albania

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Abstract**—**Wild almond is a woody species, which is difficult to propagate either generatively by seed or by vegetative methods (grafting or cuttings) and also considered as Endangered (EN) in Albania based on IUCN criteria. As a wild relative of cultivated fruit trees, this species represents a source of genetic variability and can be very important in breeding programs and cultivation. For this reason, it would be of interest to use an effective method of in vitro mid-term conservation, which involves strategies to slow plant growth through physicochemical alterations of in vitro growth conditions. Multiplication of wild almond was carried out using zygotic embryos, as primary explants, with the purpose to develop a successful propagation protocol. Results showed that zygotic embryos can proliferate through direct or indirect organogenesis. During subculture, stage was obtained a great number of new plantlets identical to mother plants derived from the zygotic embryos. All in vitro plantlets obtained from subcultures underwent in vitro conservation by minimal growth in low temperature (4ºC) and darkness. The efficiency of this technique was evaluated for 3, 6, and 10 months of conservation period. Maintenance in these conditions reduced micro cuttings growth. Survival and regeneration rates for each period were evaluated and resulted that the maximal time of conservation without subculture on 4ºC was 10 months, but survival and regeneration rates were significantly reduced, specifically 15.6% and 7.6%. An optimal period of conservation in these conditions can be considered the 5-6 months storage, which can lead to 60-50% of survival and regeneration rates. This protocol may be beneficial for mass propagation, mid-term conservation, and for genetic manipulation of wild almond.

Keywords**—**Micropropagation, minimal growth, storage, wild almond.

I. Introduction

PRUNUS Webbii Vierh. trees occur at altitudes 0 – 700 m in dry or semidry woodlands in Central Asia, Balkan Peninsula, South Italy, etc. In Albania, it occurs in Përmet, Mallakastër, Butrint, Konispol, Delvinë, Himarë, Vlorë, Tepelenë, Përmet, Leskovik, Skrapar, etc. [1], [2]. Based in IUCN criteria, this specie is considered EN [1]. The wild almond can be used as rootstock for almond, nectarine and peach and for cultivated almond [3], [4]. This specie is characterized for containing compounds that have antitumor and anti-bacteriological properties [5] and stimulate respiration and improve digestion also [6].

The seeds have a stony endocarp and their germination is very difficult. This is a serious problem for propagation of wild almond [2], [7]. Micropropagation is a suitable method for obtaining a large quantity of genetically homogeneous and healthy plant material which can be used for planting [8], [9]. The rapid *in vitro* multiplication of clonal plants is desirable to shorten crossing programs in fruit tree breeding [10]. *In vitro* culture 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. For safe preservation, the *in vitro* slow growth storage method was developed and is considered an alternate solution for medium term storage of fruit germplasm [11]. The aim of medium term storage is to increase the interval period between subcultures by reducing growth. This might be achieved by the use of modified environmental conditions, modified culture medium, growth retardants, osmotic regulators and/or reduction of oxygen concentration [12]. Slow growth storage via *in vitro* cultures has been reported in many species [13].

The present study involved investigations of micropropagation and slow growth conditions for the conservation of *Prunus webbii* explants cultured *in vitro.*

II. Materials and Methods

A. Plant Material: Collection and Disinfection

Zygotic embryos were isolated from mature seeds under aseptic conditions using a stereo microscope. In order to establish the most appropriate protocol for *in vitro* cultivation, as primary explants were used zygotic embryos (fully isolated embryos and embryos with part of cotyledons). These explants were taken from wild almond trees of Fushë – Kruja orchards.

Before sterilization, the seeds were left for 12- 24 hours in tap water in order to alleviate the embryos isolation. Then was realized double sterilization with $HgCl_2$ 0.01% for 20 min before, and after removing seeds tegument. Thereafter, the explants were rinsed out three times with sterilized H_2Od [14].

B. Media Composition for in vitro Cultivation

Stage I – Organogenesis or Embryogenesis Induction

For organogenesis induction, MS basal [15] was used and two different treatments regarding PGRs content were tested:

- PGRs free MS media;
- MS media supplemented with cytokinin 6 benzylaminopurine (BAP) 1 mg/l and auxin indole-3-butyric acid (IBA) 0.1 mg/l;

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Stage II – Subculture

All plantlets regenerated from Stage I were inoculated in basal MS media supplemented with BAP 0.7 mg/l, naphthalene acetic acid (NAA) 0.01 mg/l and GA_3 0.1 mg/l;

All media were enriched with sucrose 3% and agar 0.7%. The pH of the media is adjusted to 5.6 prior to autoclaving.

C. Chamber Conditions for in vitro Cultivation

The explants in every developmental stage were grown in the growth chamber at temperature of 25° ± 2° C in a 16 h light/24 h regime with cool, white fluorescent light.

D. In vitro Conservation

Combination of low temperature and light regime was the technique used for *in vitro* conservation purposes. The proliferated shoots were incubated at 4°C in dark conditions and the cultures were stored in these conditions for different periods (3, 6, 10 months). The media under these conditions was the same with the multiplication medium. There were made three replications and there were at least 15 shoots in each replication for each conservation period. Survival of the cultures was assessed on the basis of criteria as suggested by [16] as dead and brown shoots are considered as unsurvived, while those with vigorous growth and having healthy leaves are considered survived.

E. Statistical Analysis

All experiments were repeated at least three times. Data collections in experiment were evaluated by computer using the statistical evaluation program JMP 7.0.

III. Results and Discussions

A. Explants Proliferation and Organogenesis Induction

The germination of zygotic embryos and roots development is observed after three days of cultivation, meantime the leaves developed after 6 – 7 days (Figs. 1 (a), (b)). Because of juvenile properties, the embryos possess a great regeneration potential and therefore are considered optimal explants for *in vitro* micropropagation purposes. The results for organogenesis' induction in two types of nutrient media are presented in Table I.

TABLE I
ORGANOGENIC RESPONSE DURING EMBRYO CULTURE OF WILD ALMOND FOR BOTH TYPES OF EXPLANTS INOCULATED IN TWO DIFFERENT NUTRIENT MEDIA

Type of nutrient media	Fully isolated zygotic embryo		Embryos with part of cotyledons	
	Roots and leaves	Only leaves	Roots and leaves	Only leaves
Basal MS media without PGRs	18% ± 1.3 St dev ± 4.1	57.6% ± 2.3 St dev ± 7.3	63.5% ± 2.3 St dev ± 7.3	18.8% ± 1.5 St dev ± 4.9
Basal MS media with PGRs	0% ± 0 St dev 0	54.1% ± 2.4 St dev ± 7.67	29.9% ± 1.6 St dev ± 5.1	55.5% ± 2.4 St dev ± 7.6

From the results are observed differences not only related with explants type, but even in their reaction in different induction media. The explants that show better response are the embryos isolated with part of cotyledons for both media tested. Characteristic of both explants is callus formation, especially in the contact zone with the nutrient media. Callus formation results in a high percentage during cultivation in basal MS media with PGRs (Fig. 1 (c)).

In this case, the regeneration of new shoots occurs via direct and indirect organogenesis. Maybe this is due to the presence of phytohormones or PGRs in the media. Their effect is added to the regulatory role that play the endogenous hormones, proteins and sugars deposited in the mature embryo.

More effective resulted from basal MS media without PGRs, especially when are inoculated fully isolated zygotic embryos. Even for the other type of explants (zygotic embryos with part of cotyledons), organogenesis induction resulted in a high percentage in such media. In many cases, when using mature zygotic embryos, PGRs or phytohormones are not necessary because the embryo has a considerable size and is in an autotrophic phase. As reported by other authors [17], there is no specific need for additional amounts of PGRs in the nutrient media for a large broad of wild plants.

Characteristic of basal MS media with PGRs was that in both types of explants was observed a minimal percentage of plantlets with roots and leaves, and a very high percentage of those only with leaves. Maybe this is due to the high ratio cytokinin/auxin in the nutrient media.

As reported by other authors, in proliferation of proembryos of *Eruca sativa* [18], *Phaseolus coccineus* [19], and *C. bursa-pastoris* [20] better results are observed when the embryo is isolated with a part of cotyledon. Furthermore, the presence of cotyledonal pieces during *in vitro* cultivation of avocado (*Persea americana* mill.) does not reduce the germination percentage and the regenerated plantlets are morphologically identical to mother plants [21]. The role of PGRs in callus formation during embryo *in vitro* culture is considered by other authors an interesting process because in such way, in many plant species, is stimulated the formation of somatic embryos using zygotic embryos as initial explants [22].

TABLE II
BIOMETRIC PARAMETERS, MEANS AND STANDARD DEVIATIONS OBSERVED DURING SHOOTS PROLIFERATION AND ELONGATION

	20 days	30 days	40 days
Shoots length	1.05 ± 0.29 St dev 0.93	1.44 ± 0.32 St dev 1.02	1.89 ± 0.37 St dev 1.17
Leaves number	2.20 ± 0.53 St dev 1.68	5.20± 0.55 St dev 1.75	7.10 ± 0.92 St dev 2.92

B. Multiplication Via Subcultures

The regenerated plantlets from Stage I are inoculated in subculture medium. During first days of cultivation, the explants did not show signs of proliferation, but later (2 – 3 weeks) it is observed the organogenic response which is characterized by an increase in leaves number and shoots length (Table II).

Most of explants reacted normally giving a great number of leaves and lateral shoots (Fig. 1 (d)), but in some cases were observed signs of hyper hydration. Such plantlets do not survive and were eliminated (Fig. 1 (e)).

Except new shoots formation, during subcultures was observed callus formation in shoot basis and new shoots were developed from this callus. Micropropagation coefficient resulted very high and this is due to the presence of BAP cytokinin, whose function is apical dominance interruption and new shoots formation. Similar observations for the role of cytokinins in micropropagation are reported by other authors too [23]-[25].

During subcultures of shoots proliferation and elongation is observed the influence of interaction of low doses of three types of PGRs in basal media (BAP, IBA and GA_3). Other authors [26] accentuate that for micropropagation purposes of wild almond shoots, positive effect have only high doses of BAP (1 mg l^{-1}).

Wild almond propagation is the object of a lot of studies in regard to find a universal nutrient media specific for this specie [27]. These authors have raised the hypotheses that the composition of such media must imitate seed composition. For this purpose, they have analyzed a new specific media using NAS nutrient media as a basis [28]. Using mature explants from different cultivars of wild almond, the new tested media is compared with several basal nutrient media such: MS, WPM, DKW and NRM. MS and WPM are found suitable for subculture stage. These results are similar with those in the present study.

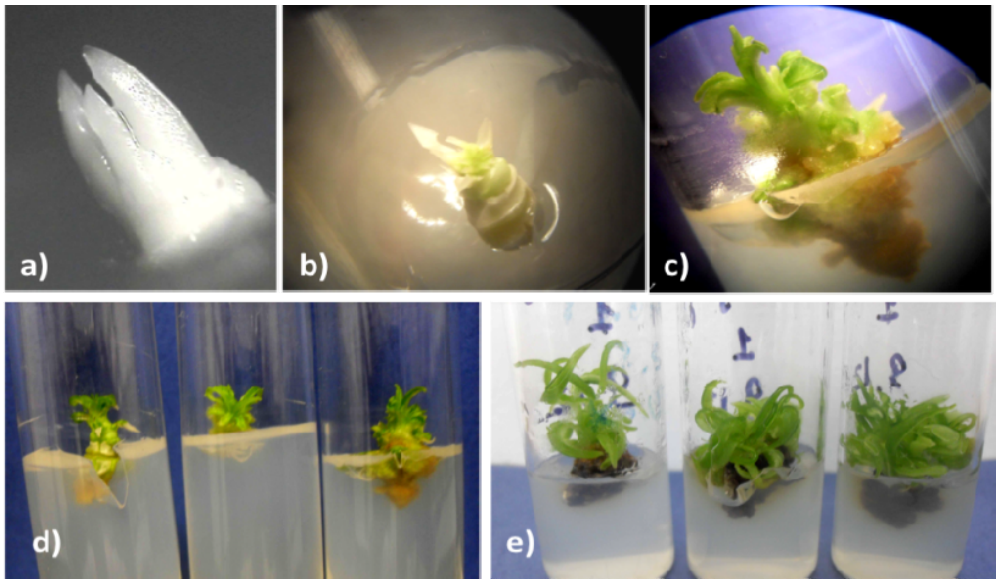

Fig. 1 Micropropagation of wild almond: a) Fully isolated zygotic embryo b) Embryos with part of cotyledons c) Shoots and roots regeneration after callusogenesis; d) Plantlets with lateral shoots regenerated after Stage I e) Hyper hydrated plants developed during subcultures

C. In vitro Conservation

The response of *in vitro* cultured shoots stored for 3, 6 and 10 months at 4°C in darkness is assessed on the basis of survival and regeneration rates. The survival and regeneration rate of shoots for each period of conservation differed significantly (Table III).

The maximum survival percentage is recorded for the 3 months conservation period, specifically 70.6%. Data presented in Table III show that up to 42.6% of explants remain healthy and green after 6 months storage at 4°C in darkness, while the lowest survival rate (15.6%) is observed for the 10 months conservation period.

Almost similar pattern is observed for the parameter of regeneration percentage. The highest regeneration rates are recorded for 3-month storage, which are significantly different for the other storage periods. The cultured shoots stored for 10 months presents the greatest decrease in both parameters.

With increase in storage period, survival rate as well as regenerability is reduced significantly. In the cultures, which are stored for the period of 10 months, their survival and regenerating percentage are greatly reduced. The present investigation revealed that the higher survival rates had positive effect on the regeneration percentage of the shoots.

TABLE III
SURVIVAL AND REGENERATION RATES OF EXPLANTS CONSERVED FOR DIFFERENT PERIODS

	3 months	6 months	10 months
Survival	70.6 ± 2.023 St. dev. 3.51	42.6 ± 2.02 St. dev 3.51	15.6 ± 3.18 St. dev 5.51
Regeneration	75 ± 2.08 St. dev 3.60	42 ± 2.64 St. dev. 4.58	7.6 ± 1.20 St. dev. 2.082

Low temperature has been successfully applied to *in vitro* cultures of various plants species for short and medium term storage. Other authors [29] preserved micro shoots of wild pear (*Pyrus syriaca*) through slow growth (low temperature) technique. In a previous study, temperature in the range of 5°C

to 10°C has been found suitable for short term *in vitro* storage of meristem cultures of several temperate species [30]. These results confirmed the findings of other authors who reported that meristem cultures of pear [31], [32], apple [30] and apple rootstocks [33], [34] can be stored *in vitro* at low temperatures. Therefore, it is concluded that the shoots of *Prunus webbii* (Spach) Vierh. can successfully be preserved *in vitro* by lowering culture temperatures for short to medium term storage.

IV. Conclusions

In vitro embryo culture results effective for micropropagation of wild almond plants. Basal MS media without PGRs resulted more effective for organogenesis induction. Plant regeneration resulted in a high percentage when were used embryos with part of cotyledons as primary explants. Maximum survival and regeneration rates were obtained during conservation on 4°C in darkness for 3 months of conservation. With increase in storage period, survival rate as well as regenerability was reduced significantly.

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