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IN VITRO CONSERVATION OF *PRUNUS AVIUM*, AN ENDANGERED SPECIES IN TUNISIA, USING A PLANT EXTRACT OF *RUBUS FRUTICOSUS* COMPARED TO INDOLE-3-BUTYRIC ACID

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ABSTRACT

Prunus avium L. is the rarest forest fruit tree in northwestern Tunisia, with strong socio-economic, agronomic, and commercial potential. The aim of this study was to provide more information about the domestication of this species by using an inexpensive *in vitro* culture technique for micropropagation of meristems. The plant extract used in this study was *Rubus fruticosus*. The results showed that the best concentration of the extract of *R. fruticosus* applied for the budding of meristematic microcuttings was 15 g/L, with a 100% success rate. The maximum number of adventitious shoots (7.15 ± 0.35) was obtained for the meristematic microcuttings at a concentration of 15 g/L of the extract. These results indicated that the extracted *R. fruticosus* plant promotes the neoformation of adventitious shoots and leaves. Rhizogenesis was strongly favored by the addition of 1 mg/L of IBA and 15 g/L of plant extract to the MS medium, which leads to the formation of roots for the meristematic micro-cuttings with percentages of about 80.36% and 53.32%, respectively. The current investigation proved that *in vitro* micropropagation could constitute a good alternative to the regeneration of this rare forest fruit species and may be applicable to other endangered plants.

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INTRODUCTION

Wild cherry (*Prunus avium* L.) is an autochthonous, fast-growing tree species that spreads in a large area from Europe to northern Africa and western Asia. Its trees reach a height of 15 to 30 meters. This tree species is of great significance not only in fruit production but also in the furniture industry and cabinet making. Cherry wood is a hardwood widely used and valued in the industry due to its color, texture, and natural luster (Katičić et al., 2015).

The timber of wild cherry is highly valued in Europe due to its reddish color, comparable to tropical trees such as mahogany, and for the firmness of the wood, combined with fast and fine-grained growth (Russell, 2003; Quamabusch et al., 2017). Wild cherry is a popular forest tree, usually characterized by dispersal distribution. Its importance derives not only from its high-quality wood products but also from its extraordinary ecological and naturalistic importance, which makes it an essential component of sensitive and threatened ecosystems, such as mixed deciduous forests (Jdaidi et al., 2022).

Vegetative propagation of these certified genotypes via *in vitro* propagation enables high multiplication rates and stable clonal plant material (Meier-Dinkel, 2007). Improvement of wild cherry with conventional propagation methods could be inadequate and a very

slow process. It is obstructed by the fact that finding sufficient quantities of natural seed may be difficult (Tančeva Crmarić and Kajba, 2016; Xi et al., 2022). The same authors have shown that certain biotechnological *in vitro* methods may be an alternative that accelerates the process and ensures genetic stability. Micropropagation of explants derived from adult genotypes is the fastest and best method of wild cherry improvement (Gururajet et al., 2007; Sharma and Vashistha, 2015).

Micropropagation is an efficient method that is widely used for the multiplication of standard rootstocks and new plant cultivars (Vujovice et al., 2012). Through *in vitro* clonal propagation techniques, plants can be propagated rapidly with a high level of efficiency, maintaining a large number of elite plant materials characterized by genetic uniformity (Gao et al., 2010; Debnath et al., 2012).

During the *in vitro* multiplication stage, several experimental treatments were applied to establish the optimal culture media, suitable culture vessels, adequate types of microcuttings, and the gelling agent that would provide economical proliferation rates and a high number of shoots suitable for acclimatization. For multiplication, generally, MS medium containing plant growth regulators was used, especially BAP at various concentrations, mainly 1-3 mg/L, auxins (IBA) at low concentrations, and GA3. For *in vitro* rooting, medium supplemented with auxins (especially IBA) and without plant growth regulators was tested (Clapa et al., 2013; Arikan et al., 2014). The process of acclimatization is crucial to *in vitro* plant propagation because it involves a gradual transition from artificial culture conditions to the natural habitat.

To achieve high survival rates throughout the acclimatization stage, ideal cultural conditions must be maintained. Plantlets planted *in vitro* in root-friendly conditions are typically employed for acclimatization (Ružić and Lazić, 2006; Fira et al., 2009; Najaf-Abadi and Hamidoghli, 2009; Clapa et al., 2013; Arikan et al., 2014). Therefore, although seedling propagation and other traditional methods have their share of problems, using *in vitro* methods for cherry tree propagation can result in the production of healthy, uniform plants in a much shorter period of time. However, when using tissue culture methods, the genetic stability of cloned plants during micropropagation is influenced by various factors, but bud meristems are undoubtedly the most suitable organ for preserving genetic characteristics of a cultivar. The use of vegetative bud-meristem is the most common method of viral removal programs in plants, especially fruit trees (Acero et al., 2019; Dominguez-Rodriguez et al., 2021).

The aim of this study was therefore to compare the efficiency of *in vitro* cultivation of meristimatic microcuttings of *P. avium* and to evaluate the influence of the plant extract of *R. fruticosus* compared to indole-butyric acid (IBA) on the capacity of microcuttings to perform buds and roots. In addition, it aimed to evaluate the effect of the association between the plant extract and the IBA on the development of the aerial and root parts.

MATERIEL AND METHODS

Plant material

Microsections of explants (2 to 4 cm long) of *P. avium* were collected at the end of the growing season from selected local old trees growing for more than 40 years in the region of Ain Saida, Tunisia (N36°52'25''-E008°41'47'), on December 15, 2021. The microsection explants were kept in an icebox during their transportation to the laboratory of the Sylvo-Pastral Institute of Tabarka for *in vitro* micropropagation. The microsections of explants carry a large number of pathogens. Disinfection of plant material was carried out using a chlorine-based solution at a concentration of 100 to 200 ml/L for 15 to 20 minutes.

Preparation of *Rubus fruticosus* plant extracts

Blackberries, often termed “Brambles”, are a diverse group of species and hybrids in the genus *Rubus*. They belong to the family Rosaceae. *Rubus* is one of the most diverse genera of flowering plants in the world and is adapted to a wide range of environments. Blackberries are native to Asia, North Africa, Europe, and North and South America (Abdalla and Mostafa, 2015). It is a shrubby tree with an erect, semierect, or creep-grown habit, and most cultivars have sharp branches (Pamfilet et al., 2010; Aly et al., 2022).

We start by bringing the *R. fruticosus* root that is growing at the lab's end. After that, we dry the roots for 48 hours at 60°C in an oven (Figure 1). Using an electric grinder, we process the roots until they are powdered. We then weigh five grammes and fifteen grammes of powder. Next, we combine each amount in a container with one litre of distilled water. The two solutions are then heated while being stirred, and they are then kept in a location away from light for a whole day. Using filter

paper, we separate the two solutions. Ultimately, we are left with two *R. fruticosus* extracts (5 g/L and 15 g/L).

Cultural conditions

The micropropagation medium used was MS medium (Murashige and Skoog, 1962), supplemented with 20 g/L sucrose and solidified with 8 g/L agar. Before adding the latter, the pH of the medium was adjusted to 5.7. Disinfection was done by autoclaving at 121°C for 20 minutes. The microsections of *P. avium* were put into flasks (25 ml) on (MS) media (base environment of Skoog and Murashige) without and with the plant extract of *R. fruticosus* (5 and 15 g/L). The improvement of the rooting of the microsections was made by transplanting microsections on the same MS with either indole butyric acid (IBA) at different concentrations (0.5 and 1 mg/L) or a plant extract of *R. fruticosus* (5 and 15 g/L).

The flasks containing the transplanted material were placed in a culture chamber with a photoperiod of 14 to 16 hours, a temperature of 24°C ± 2°C during the day and 22°C at night, and a light intensity of 41 µMol (photons) $s^{-1}m^{-2}$. Every treatment was carried out three times. For every repeat and treatment, ten explant microsections were utilized.

Acclimatization of plants

After three weeks, the rooted plants were moved to an 8-cm-diameter plastic pots with a rooting medium consisting of earth, peat, and perlite (2:1:1) (v/v). The pots were then placed in a controlled growth room at 22°C. To maintain high humidity, the plants were covered with plastic bags and removed for a few minutes each day. After about five weeks, the bags were completely removed.

Statical analysis

The statistical significance was based on the analysis of variance (ANOVA) carried out using the "SAS" program (software version 9.4). The Student test at the 5% threshold was used for the comparison of the means.

RESULTS AND DISCUSSION

Impact of *R. fruticosus* plant extract on aerial part proliferation

The influences of the concentration of the plant extract on microsections of *P. avium* are shown in Table 1. For the microcuttings, the concentrations of 5g/L and 15g/L resulted in 85% and 100% bud burst, respectively (Figure 2).

Figure 1: The roots of *Rubus fruticosus* used for the preparation of plant extract.

Figure 2: Bud burst of *Prunus avium* microsections in M6 medium.

The number of adventitious shoots varied from 5.41 ± 0.42 in the microsections cultivated in a medium containing 5 g/L of *R. fruticosus* extract to 7.15 ± 0.35 in those cultivated with 15 g/L. According to the obtained results, adding the plant extract to the culture medium improved caulogenesis. It appeared from this study that the plant extracts behaved as phytohormones for the development of the aerial part of the microsections.

These results are in agreement with those of Naddaf et al. (2021), who propagated *P. avium* using IBA-enriched medium, which produced the highest number of meristem shoots per explant culture.

In previous studies, MS and 1/2 MS were the main media for the *in vitro* propagation of wild cherry (Quambush et al., 2016; Naddaf et al., 2021). The protocols developed for the micropropagation of wild cherry and other species of the genus *Prunus* are determined by genotype but, in fact, are not broadly used (Ružić and vujovic, 2008).

Stage of acclimatization and rooting

For this species, inducing rooting appeared to be quite

challenging. Therefore, during the proliferation and subculture phases, two nutritional rooting media with varying concentrations of auxin (IBA) and *R. fruticosus* plant extract, incorporated into MS universal medium, were compared. Rhizogenesis was observed after the cultures were on the rooting media for five weeks, with the explants responding in various ways.

Explants grown in medium M6 exhibited the best results when combined with 1.0 mg/L of IBA and 15 g/L of *R. fruticosus* plant extract; however, in these cases, the rooting percentage (80.36%) was excessively high. Similarly, significant rooting percentages were demonstrated by rooting media M3 (1.0 mg/L IBA and 0 g/L *R. fruticosus* plant extract) and M5 (0.5 mg/L IBA and 15 g/L *R. fruticosus* plant extract), showing rooting percentages of 47.78% and 69.03%, respectively (Table 2 and Figure 3).

Table 1: Effect of the plant extract of *R. fruticosus* on the development of the aerial part of the microsections of *P. avium.*

Medium	Bud burst (%)	Number of adventitious shoots
M1 (0 gL^{-1})	33 c	2.23 ± 0.12 b
M2 (5 gL^{-1})	85 a	5.41 ± 0.42 a
M3 (15 gL^{-1})	100 a	7.15 ± 0.35 a

Different letters indicate significant differences at 5% level; Values representing Mean ± SE

Table 2: Effect of the plant extracts of *R. fruticosus* and IBA on the development of the root part of the microsections of *P. avium.*

Medium	Rooting rate (%)	Average length of roots (cm)	Average number of roots per plant
M1 (0 g/L PE + 0 mg/L IBA)	19.65 c	0.63 ± 0.12 d	2.06 ± 0.24 c
M2 (0 g/L PE + 0.5 mg/L IBA)	42.45 b	1.32 ± 0.03 b	3.67 ± 0.36 b
M3 (0 g/L PE + 1 mg/L IBA)	74.78 a	4.64 ± 0.23 a	5.66 ± 0.42 a
M4 (5 g/L PE + 0 mg/L IBA)	39.56 b	1.78 ± 0.90 b	3.26 ± 0.16 b
M5 (15g/L PE + 0.5 mg/L IBA)	69.03 a	3.58 ± 0.07 a	5.23 ± 0.16 a
M6 (15 g/L PE + 1 mg/L IBA)	80.36 a	5.79 ± 1.03 a	6.32 ± 0.15 a

Different letters indicate significant differences at 5% level; Values representing Mean ± SE

Figure 3: Acclimatization of *Prunus avium* L. in peat and perlite (A and B). Plants rooted after the acclimation (C).

The efficacy of IBA in rooting wild cherry cuttings has been previously demonstrated (Jdaidi et al., 2021). Zhou et al. (2010) utilized IBA concentrations of 4.92 and 7.38 µM, resulting in approximately 100% rooting in *P. persica in vitro*.

Despite the beneficial effects of IBA, the plant extract from *R. fruticosus* facilitated the full rooting of *P. avium* developed shoots. Full-strength MS salts yielded long roots with thick, medium-involved subroots, while half or quarter strength resulted in hairy roots and subroots forming on the upper surface of the MS salt medium, prolonging the rooting period. Initial investigations by Fira et al. (2010), Clapa et al. (2013). and Arikan et al. (2014) supported these findings. According to Mir et al. (2010), shoots obtained under *in vitro* conditions were utilized as materials for rooting. Different concentrations of IBA (1.0, 2.0, 3.0, and 4.0 mg/L) were employed for *in vitro* rooting of shoots. After 40 days of culture, half of all

explants (50%) grown on half-strength MS medium supplemented with 2.0 mg/L IBA exhibited roots, with the maximum number and length of roots obtained being 1.20 ± 0.02 and 3.0 ± 0.22 cm, respectively.

Auxins are the dominant plant growth regulators linked to the formation of root systems. *In vivo*, IBA is the most commonly used auxin for rooting (Zamanipour et al., 2019; Vanja et al., 2020). The positive effect of IBA on *in vivo* rooting has been reported in many studies (Kumar et al., 2020). As the peach rootstock series is mainly propagated through micropropagation, a successful protocol for 'Gisela 6' was reported by Aghaye et al. (2013) and Surropoulou et al. (2013).

This protocol achieved a rooting percentage exceeding 80.36% with a concentration of IBA of about 1 mg/L, which is correlated with the present results. In addition, a positive effect of IBA was also reported on the rooting of peach rootstock (Hasan et al., 2010). *R. fruticosus L. is* commonly known as a berry fruit plant that is considered a suitable species for vegetative propagation under *in vitro* conditions (Fira et al., 2014).

A biotechnological alternative to obtaining large quantities of healthy plants is the isolation of meristematic tissue, since this is generally free of viruses because its active cell division reduces differentiation of vascular tissues (García-Gonzáles et al., 2010). Thus, meristematic tissue culture is an appealing technique to eliminate pathogenic bacteria, fungi, and viruses carried by adult plants.

However, a number of constraints need to be overcome in order to facilitate meristem isolation and establishment in *in vitro* conditions, including reducing the release of phenolic compounds from the tissues into the culture medium and appropriate environmental conditions, such as suitable temperatures (Ko et al., 2009). The vegetative propagation method studied in this study presents the best and fastest genotype propagation method. Ljubojevic *et al.* (2013) proved that root stocks such as dwarf *P. avium* are a promising material due to their anatomical, morphological, and physiological parameters. This propagation method has provided a sufficient number of rootstocks with special combining abilities, which are currently being tested in field grafting trials.

CONCLUSION

The production of wild cherry plants from meristems must be carried out using an *in vitro* culture method. In addition to the rapid growth of a large number of new shoots, it produces healthy plants due to the relatively good success of meristem culture and its integration into cherry production. In the light of obtained results, it seems that the application of plant extract of *R. fruticosus* at a concentration of 15 g/L seems to improve caulogenesis and rhizogenesis in these microcuttings. The combination of 15g/L of *R. fruticosus* plant extract and 1 mg/L of IBA is therefore the most suitable tool to improve the multiplication rate of meristimatic microcuttings of *P. avium*. This strategy can constitute an alternative to the direct production of wild cherry plants from those resulting from *in vitro* culture, in particular by making it possible to reduce the cost. This study aims to determine the application of a new plant growth hormone extracted from the roots of *R. fruticosus*. The results showed that 15 g/L of this plant extract allows the proliferation of the aerial and underground parts of the microsections of *P. avium*. This thorough method for *in vitro* development of *Prunus avium* meristem rooting, and acclimation could be used to quickly and efficiently create a large number of clonal plants from used raw materials. It could also be used to cultivate cultivars selected for certain qualities' commercial value or to preserve genetic heritage varieties.

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AUTHORS' CONTRIBUTIONS

Nouri Jdaidi, Houcine Selmi, Foued Aloui, and Abbes Chaabane conceived the idea, designed the study, performed experiment, collected and anlalyzed the data, wrote the draft and edited the manuscript.

DECLARATION INTEREST

Authors declare no conflict of interest.

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