THE EFFECT OF NUTRIENT MEDIA IN MICROPROPAGATION AND IN VITRO CONSERVATION OF WILD POPULATION OF MAHALEB CHERRY (PRUNUS MAHALEB L.)

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ABSTRACT

Shoot tips of Prunus mahaleb L. isolated from wild populations of Zejmen (Lezhe), promising as rootstocks for sweet cherry cultivars, were submitted to in vitro culture to test if micropropagation could be used for their rapid production. This study was carried out to determine the optimal nutrient media for micropropagation and to develop a suitable protocol for mid-term storage of Prunus mahaleb L. germplasm. For micropropagation were tested three different basal media MS, WPM and LP, all the three combined with 0.3 mg l⁻¹ BAP, 0.1 mg l⁻¹ IBA, 0.3 mg l⁻¹ GAs. The highest shoot length (5.53 cm) was observed on explants cultured on MS media, whereas this parameter was reduced on explants cultured on WPM and LP media (4.63 and 2.10 respectively). During subculture stage, MS and WPM media didn’t show statistical differences regarding to shoots number/explants and leaves number/explants. The rooting percentages of plantlets ranged from 10 to 90%, depending on NAA concentration in the rooting media. In order to find out a medium-term in vitro preservation protocol effect of reduced sucrose and MS salts concentrations and elimination of PGRs from nutrient media on a collection of 30 days old of in vitro wild mahaleb cherry nodal segments have been examined for different periods. The highest survival and regeneration percentage (respectively 93.36 % and 83.72 %) were found in cultures stored at ½ MS media without sucrose for the period of 3 months. The maximal time of conservation without subculture on reduced sucrose and MS salt (1/2 MS) concentrations is up to 5 months and in basal MS media without PGRs is up to 3 months. Hence the shoot tips of Prunus mahaleb L. can be successfully stored in vitro for medium terms at ½ MS media without sucrose.

Keywords: In vitro culture, micropropagation, mid-term storage, MS, LP, WPM media, Prunus mahaleb L.

INTRODUCTION

Prunus mahaleb (syn. Cerasus mahaleb L., St. Lucie cherry, also occasionally Rock cherry or Mahaleb cherry) trees occur in thickets and open woodland on dry slopes; in Central Europe at altitudes up to 1700 m a.s.l. and in highlands at 1200-2000 m a.s.l. in Southern Europe (Rushforth, 1999). It is drought resistant and can grow in very poor and calcareous soils, in full sun or partial shade (Guitian, 1994).

The plant is cultivated for its strongly fragrant flowers, throughout temperate regions of the world. The common use is as rootstock for sweet or sour cherry cultivars. A number of cultivars have been selected (Bean, 1976). In the first stage of the cherry rootstock breeding, the activity is focused on the collection of native mahaleb cherry (Prunus mahaleb L.) varieties (Hrotkó, 2004; Hrotkó et al., 2004). The common Prunus mahaleb L. rootstocks used for cherry propagation are becoming more unsatisfactory. Trees from grafted varieties are too large, fruit set is too late and harvest costs are too high (Edín et al., 1996).

Micropropagation is a suitable method for obtaining a large quantity of genetically homogeneous and healthy plant material which can be used for planting (Korgjika et al., 2002; Damiano et al., 2008). The rapid in vitro multiplication of cloned plants is desirable to shorten crossing programs in fruit tree breeding (Daaorden et al., 2004). 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 (Neveen et al., 2008). 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 (Kameswara, 2004). Slow growth storage via in vitro cultures has been reported in many species (Maqsood et al., 2010). The aim of this study is to determine the optimal nutrient media for micropropagation and to develop a suitable protocol for mid-term storage of Prunus mahaleb L. germplasm.

MATERIAL AND METHODS

Plant material: collection and disinfection

Cultures of P. mahaleb L. isolated from wild populations of Zejmen (Lezhe), were established from apical and lateral buds removed from adult field-grown trees. The plants were collected during February and March. Most often shoot tips and meristems are the explants of choice due to their genetic stability. Plant material used to introduce P. mahaleb L. cultivars is obtained by collecting the active explants between January and March, when buds were starting to swell from shoots in dormancy. Active shoots were cut in two- or three-node sections. Two types of disinfection reagents were used: HgCl₂ and NaOCl. The stem sections were washed carefully with water and than were shaken for 5 min. in 70% ethanol, followed by 20 min. treatment with HgCl₂ 0.01% or NaOCl 0.3% and two drops of Tween 20. Finally stem sections were rinsed three times with sterile distilled water. Explants size is not as important for micropropagation as purposes as for obtaining disease-free plants. The buds were dissected up to 3 mm by removing the outer scales and showed no sign of contamination after over one year of continued culture.
Media composition for in vitro cultivation

**Proliferation and subculture media:** Three nutrient media were tested MS medium (Murashige and Skoog, 1962), LP medium (Quoirin and Lepoivre, 1977) and WPM medium (Lloyd and McCown, 1980), all the three combined with 0.3 mg L⁻¹ BAP, 0.1 mg L⁻¹ IBA; 0.3 mg L⁻¹ GA₃ and supplemented with 3% sucrose and solidified with 0.55% agar. The pH of the media was adjusted 5.7 – 5.8 before autoclaving. After a month, the developed buds were transferred to fresh media for further multiplication. Measurements of proliferation (%), length of the shoots and leaves number were taken.

**Rooting media:** When the explants derived from MS medium during proliferation and subculture stage reached 2 – 4 cm in length, they were transferred to rooting media. Three variants of rooting medium were analyzed:

- Rooting media I: ½ MS macronutrients, MS micronutrients, MS vitamins containing 0.1 mg L⁻¹ NAA;
- Rooting media II: ½ MS macronutrients, ½ MS micronutrients, MS vitamins with 0.1 mg L⁻¹ NAA;
- Rooting media III: MS macronutrients, MS micronutrients, MS vitamins with 2 mg L⁻¹ NAA.

Rooting response was evaluated after 4 – 5 weeks of culture.

**Plantlets acclimatization:** Rooted plantlets were transferred after 3 – 4 weeks on rooting media to a mixture of soil, peat and perlite (2:1:1) (v/v), in 7 cm diameter plastic pots and placed in a controlled growth chamber at 20°C. The plantlets were covered with plastic bags in order to maintain high humidity by removing them periodically for some minutes day after day. The bags were removed completely after about four weeks.

**In vitro chamber conditions:**

The culture in the proliferation stage was grown in the growth chamber at temperature of 25°C ± 2°C in a 16 h/8 h light/dark regime with cool, white fluorescent light of intensity 43.4 μmol m⁻² s⁻¹.

For in vitro conservation, two different methods of minimal growth are tested:

- Effect of reduced sucrose and MS salts concentrations: The cultures are transferred onto ½ MS media without sucrose and supplemented with the same rate of other components as in the multiplication media. The incubation conditions are the same as in the multiplication stage.
- Absence of phytohormones or growth regulators in the growth media: The cultures are transferred onto MS media without growth regulators or phytohormones and supplemented with the same rate of other components as in the multiplication media. The incubation conditions are the same as in the multiplication stage. The cultures are stored in these conditions for different periods (3, 4, 5 months) for each method tested. For each method are at least 15 shoots in each replication. Survival of the cultures is assessed on the basis of criteria as suggested by Reed (1992) as dead and brown shoots are considered as unsurvived while those with vigorous growth and having healthy leaves are considered survived.

**Statistical analysis**

All experiments were repeated at least twice. Data collections in experiment were subjected to analyses of variance and evaluated by computer using the statistical evaluation program JMP 7.0.

**RESULTS**

**In vitro cultivation**

**Explants disinfection:** The explants isolated from developing buds, after the surface sterilization with HgCl₂ 0.01% for 20 min resulted in the highest percentage of developed explants (85%). Contamination rates were about 15% for primary explants and less than 2% for subcultures. Sodium hypochlorite in the concentration 0.3% was not effective in disinfecting explants derived from field-grown adult trees. The contamination rates in this case were about 80%. Must be noted that the explants are isolated from the field trees with high contamination and it is required the disinfection with the most powerful reagents such as mercury chloride.

**Proliferation and subculture stage:** The shoots number per explants (SN) was affected by the type of media (Tab. 1, Fig. 1a, b). Explants cultured on MS and WPM media showed the highest shoot number/explants (3.00 and 2.80 respectively) compared to the explants cultured on LP media (1.50), after 4 weeks of culture. The results showed that the highest leaves number/explants (LN) (9.50) was recorded on explants cultured on MS media, value that wasn’t statistically different from those cultured on WPM media (7.80), whereas leaves number/explants was smaller (4.20) on explants cultured on LP media. The mean shoot length (SL) was also affected by the type of media. The highest shoot length (5.53 cm) was observed on explants cultured on MS media, whereas this parameter was reduced on explants cultured on WPM and LP media (4.63 and 2.10 respectively).

Even in this stage, shoots number/explants, shoots length/explants and leaves number/explants were affected by the type of media. SN and LN values weren’t statistically different between the explants cultured on MS and WPM media, whereas SL values were statistically different (Tab. 1).

<table>
<thead>
<tr>
<th>Culture stage</th>
<th>Nutrient media</th>
<th>Shoot number (SN)</th>
<th>Shoot length (SL)</th>
<th>Leaf number (LN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation</td>
<td>MS</td>
<td>3.00 ± 0.55 A</td>
<td>5.53 ± 0.16 A</td>
<td>9.50 ± 1.00 A</td>
</tr>
<tr>
<td></td>
<td>WPM</td>
<td>2.80 ± 0.25 A</td>
<td>4.63 ± 0.12 B</td>
<td>7.80 ± 0.51 A</td>
</tr>
<tr>
<td></td>
<td>LP</td>
<td>1.50 ± 0.22 B</td>
<td>2.10 ± 0.25 C</td>
<td>4.20 ± 0.53 B</td>
</tr>
<tr>
<td>Subculture</td>
<td>MS</td>
<td>3.80 ± 0.49 A</td>
<td>5.80 ± 0.31 A</td>
<td>13.90 ± 1.23 A</td>
</tr>
<tr>
<td></td>
<td>WPM</td>
<td>4.00 ± 0.53 A</td>
<td>4.94 ± 0.15 B</td>
<td>11.50 ± 0.98 A</td>
</tr>
<tr>
<td></td>
<td>LP</td>
<td>2.10 ± 0.27 B</td>
<td>4.30 ± 0.24 B</td>
<td>7.60 ± 0.56 B</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rooting media I (%)</th>
<th>Rooting media II (%)</th>
<th>Rooting media III (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 ± 2.33</td>
<td>10 ± 1.92</td>
<td>30 ± 2.91</td>
</tr>
</tbody>
</table>

**Note:** Values represent mean ± standard error. Means followed by the same letter within the column do not differ significantly (P ≥ 0.05) according to a Tukey’s Honestly Significant Difference test.

**Rooting and acclimatization stage:** Rooting induction appears very difficult, especially regarding to trees species. For this reason, three nutrient rooting media containing different concentrations of auxin, α-naphthalene acetic acid, NAA and macro- and micronutrients, presented in the universal medium MS were compared for the explants derived from MS medium during proliferation and subculture stage. After 3 weeks of the culture on the rooting medium, rhizogenesis was observed. The explants reacted differently in three types of rooting media (Fig. 1c, d, e). The mineral and NAA concentration of the culture media affect rooting percentage and roots characteristics. Best results were observed in explants cultured on I rooting media where the percentage of rooting appeared to be too high (90% ± 2.33). The two other media II and III rooting media showed lower rooting percentage, respectively 10% ± 1.92 and 30% ± 2.91 (Tab. 1). At the ultimate case (rooting media III), 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 (Fig. 1c). Acclimatization was affected directly by rooting conditions. Survival was best when plantlets were transferred to pots after a short period of root emergence on rooting media (Fig. 1f). Maintenance on rooting media gave longer roots but resulted in poor survival. Acclimated plantlets are able to pass in the permanent place in the greenhouse.

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Germlasm mid-term storage

In Table 2 and Graphics 1, 2 are presented and analyzed the results of survival percentage and regeneration percentage for both methods of conservation tested in three different periods (3, 4 and 5 months). The regeneration percentage is calculated after transferring the survived shoots in the nutrient media used for micropropagation purposes.

From the obtained data result that the highest survival and regeneration percentage is found in cultures stored in ½ MS media without sucrose (respectively 93.36% and 83.72%) for the period of 3 months. The maximal time of conservation without subculture on reduced sucrose and MS salt (1/2MS) concentrations is up to 5 months and in basal MS media without PGRs is up to 3 months.

With increase in storage period, survival rate as well as regeneration is reduced significantly. Also are observed significant statistical differences in survival and regeneration rates of shoots for each method tested (Graph 1).

Table 2 Survival and regeneration percentage of wild mahaleb cherry explants conserved with different methods of minimal growth, for different periods

<table>
<thead>
<tr>
<th>Prunus mahaleb L.</th>
<th>Survival percentage</th>
<th>Regeneration percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 months</td>
<td>4 months</td>
</tr>
<tr>
<td>½ MS media without sucrose</td>
<td>93.36 ± 2.14</td>
<td>74.10 ± 0.73</td>
</tr>
<tr>
<td>Basal MS media (without PGRs)</td>
<td>65.00 ± 3.00</td>
<td>32.00 ± 2.08</td>
</tr>
</tbody>
</table>

Note: Values represent mean ± standard error.

DISCUSSION

Better results obtained in in vitro culture of Prunus mahaleb explants in MS medium comparing to LP and WPM ones could be related to different composition of these media. The major differences in macronutrients among these three basal media are in ammonium and nitrate ion concentrations and total ion concentration. Full-strength MS medium has higher values of ammonium and nitrate, while WPM and LP are low ammonium mediums. MS medium has even potassium nitrate as a major nitrogen source, while LP and WPM medium have calcium nitrate. Beside this, some micronutrients are present only in the MS medium. Effectiveness of MS media in micropropagation of Prunus sp. is also reported from other authors (Sedlák et al., 2008; Shatnawi et al., 2009; Ružić et al., 2008; Gurel et al., 1999; Namli et al., 2011; Marino et al., 1989; Muna et al., 1999). Meanwhile, from other studies resulted that LP basal media gives better results (Lamrioui et al., 2009; Hasan et al., 2010) and others report WPM basal media more effective (Yao et al., 2011; Harada et al., 1996; Liu et al. 2010).

Positive effect of lower doses of one of the auxin, “inductor” of rhizogenesis, α-naphthaleneacetic acid, NAA, is reported in the studies of some authors on the in vitro rooting of apple plantlets (Nemeth, 1981; Monter, 1992). Inside a species in the level of different varieties is demonstrated that the use of higher concentration of auxins (2-3 mg l⁻¹) favors the development of callus and limits root formation. As result, the use of the lower doses than 0.5 mg l⁻¹ is recommended.

Effectiveness for minimal growth conservation reducing MS salt concentration in basal media without sucrose is also reported from other authors during conservation of Vitis sp. (George, 1996) Pyrus sp. (Ahmed et al., 2009; Moriguchi et al., 1989), Coffea sp. (Desbrunais et al., 1992) etc. Plant growth could be reduced even if PGRs concentration in nutrient media is under optimal levels (Gunning et al., 1985). Reducing PGRs concentration resulted effective in conservation of Fragaria sp. (Jungnickel, 1988), meantime eliminating them from nutrient media is reported as an optimal storage method for this specie (Reed et al., 1995). This storage method is also reported for conservation of Ramonda sp. (Kongjika et al., 1998).

CONCLUSION

The most optimal nutrient medium is considered Murashige & Skoog medium (MS) supplemented with MS vitamins and combined with 0.3 mg l⁻¹ BAP; 0.1 mg l⁻¹ IBA; 0.3 mg l⁻¹ GA₃, which favors the buds development in the first stage of in vitro culture. Comparing the data of the response of the explants in three different proliferation media is observed the difference in shoots number, leaves number and shoot...
length parameter. 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. Best results on rooting percentage were observed in explants cultured on I rooting medium containing 1/2 MS macronutrients, MS micronutrients, MS vitamins supplemented with 0.1 mg 1⁻¹ NAA.

Conservation via reduction of MS salt concentration and sucrose elimination from nutrient media resulted effective for mid-term storage periods up to 5 months. With increase in storage period, survival rate as well as regeneration is reduced significantly.

REFERENCES


