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BRIEF COMMUNICATION

Rapid micropropagation of mature wild cherry

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Abstract

Explants taken from the mature vigorous tree of wild cherry (*Prunus avium* L.) were assayed for their organogenic capacity under various phytohormonal treatments. The highest rate of adventitious shoot multiplication was recorded at a combination of 0.5 mg dm^{-3} 6-benzylaminopurine (BAP) and 0.05 mg dm^{-3} thidiazuron (6.83 shoots per explant). No differences in multiplication rates were found among media supplemented with BAP, BAP + α-naphthaleneacetic acid (NAA) or BAP + indole-3-butyric acid (IBA). Shoot elongation was significantly affected by the concentration of BAP, regardless of auxin addition to medium. Up to 73 % of microshoots rooted after using 0.3 mg dm^{-3} IBA, otherwise the adventitious rooting occurred at reasonable frequencies in all auxin treatments. Regenerated plantlets were successfully hardened *ex vitro* and continued to grow after the transfer to soil. No morphological aberrations were observed in the regenerates.

Additional key words: adventitious shoot multiplication, *Prunus avium*, thidiazuron.

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Prunus avium L. is a fast-growing timber species, highly rated for its valuable hardwood over a short crop rotation of 55 - 70 years. Due to its timber and ecological importance, this species has been quite extensively planted over Europe and included in several gene conservation and genetic improvement programs of varying intensities in different European countries (Nicoll 1993, Turok *et al*. 1996).

Commercial propagation of wild cherry derives from seed which is variable and has to go through cold stratification in duration up to 5 months. Clonal propagation of mature superior genotypes may speed up conservation efforts or establishment of clonal plantations. However, conventional propagation by cuttings encounters difficulties in rooting. A development of reliable regeneration systems from mature tissues either via multiplication of adventitious shoots or somatic embryogenesis is a prerequisite to the genetic improvement of this species. In wild cherry, adventitious shoots were induced from leaf explants (Grant and Hammatt 2000) or roots (Druart *et al*. 1998), early stages of somatic embryogenesis were obtained from protoplast-derived cell cultures (David *et al*. 1992) while later stages were induced from immature zygotic embryos (Garin *et al*. 1997). In addition, shoot organogenesis was also reported for other species of the *Prunus* genus such as peach (Gentile *et al*. 2002), apricot (Escalettes and Dosba 1993), almond (Miguel *et al*. 1996), plum (Nowak *et al*. 2004), bird cherry (Chalupa 1983, Hammatt 1993), and black cherry (Hammatt and Grant 1998).

The objectives of the studies described in this paper were to develop an efficient and reliable method for *in vitro* micropropagation of mature wild cherry tree.

Slightly unfolded axillary buds taken from the mature plus tree of *Prunus avium* L. (height 33.75 m, breast girth 46 cm) were selected for tissue culture establishment. Twigs were first rinsed under tap water for 5 min to remove any surface dirt. Then explants were surface-sterilized using the following sequence: agitating in 0.1 % mercuric chloride solution with few drops of *Tween 20* as a wetting agent for 10 min, and three rinses for 5 min each in autoclaved distilled water. Bud scales were removed with a scalpel and explants were placed onto culture media. After 10 d, when the media in contact

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IBA - indole-3-butyric acid; NAA - α-naphthaleneacetic acid; TDZ - thidiazuron; WPM - woody plant medium.

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with the established plant material darkened, explants were transferred onto fresh media to minimize any deleterious effects of this dark exudate. Successfully established cultures were then cultured on media supplemented with 2 $cm^3\ dm^{-3}$ (v/v) plant preservative mixture (*PPM*, *Plant Cell Technology*, Washington, USA) to minimize the culture contamination. All cultured shoots were maintained in 100 cm^3 Erlenmeyer flasks with 28 cm^3 of medium.

Woody plant medium (WPM, Lloyd and McCown 1980) was supplemented with the cytokinin 6-benzylaminopurine (BAP) either individually or in combination with the auxins α-naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA), and solidified with agar (6 g dm^{-3} *Sigma*, St. Louis, MO). Thidiazuron (TDZ) was also applied individually as well as in combination with BAP. Sucrose (20 g dm^{-3}) was added as a carbon source. The pH of all media was adjusted to 5.6 - 5.8 with 1 M KOH or 1 M HCl before dispensing in culture tubes and autoclaving at 121°C and pressure 1.2 - 1.3 kg cm^{-2} for 20 min. Cultures were maintained at day/night temperatures of 25 ± 1/19 ± 1 °C under a 16-h photoperiod. Light was provided by cool white fluorescent tubes (*Belux*, Aschau im Chiemgau, Germany) located 45 cm above the shelf, a photon flux density was 50 µmol $m^{-2}\ s^{-1}$. Cultures were regularly sub-cultured every 4 weeks.

After the successful culture establishment, unfolded buds and shoot clumps were collected and treated with various phytohormones. Adventitious shoot regeneration and elongation were promoted by BAP, either alone or in combination with a low concentration of NAA or IBA. Multiple shoot cultures formed after 12 weeks and were regularly sub-cultured for the next 3 years without any significant loss of vitality. Shoot number observations were recorded on a 6 week basis, and shoots longer than 1.0 cm were counted. Microshoots, more than 1.5 cm long, were excised from shoot proliferating cultures and transferred to test tubes with 8 cm^3 of a half-strength rooting WPM, supplemented with IBA or combination of IBA and 2,4-dichlorophenoxyacetic acid (2,4-D). Thirty shoot tip microcuttings were cultured in each treatment. Rooting frequencies were scored after 5 weeks. Shoots in all rooting treatments were maintained under environmental conditions mentioned above. Rooted plantlets were transplanted to pots containing *Agroperlite*, peat and soil (1:2:2). Pots were kept in plastic containers with transparent covers. The regenerates were regularly sprayed with water and grown under high air humidity for 3 weeks and, afterwards, covers were opened gradually during another 2-week period. Then the acclimatized plantlets were potted in a soil mixture containing peat and soil (1:2) and kept under shade.

Nested analysis of variance (*ANOVA*), one-way *ANOVA*, and Duncan's multiple range tests were performed to analyze shoot number and shoot length rates. Likelihood ratio χ^2-test (*G*-test) was used to analyze rooting frequencies. Living sterile shoots and roots constituted the basis of the statistical analysis.

Wild cherry axillary buds were exposed to 16 different treatments. Within eight weeks the buds unfolded and elongated, afterwards the adventitious shoot multiplication was induced. The addition of phytohormones was crucial for multiple shoot culture formation but no significant differences in multiplication rates were found among media supplemented with BAP, BAP + NAA or BAP + IBA (Table 1). A very high mean rate of shoot multiplication (6.32 shoots per explant) was recorded on WPM augmented with 1.0 mg dm^{-3} BAP + 0.01 mg dm^{-3} NAA (Fig. 1). Treatments with high BAP concentrations produced significantly higher muliplication rates than treatments with low BAP concentrations. However, when increasing the concentration of BAP up to 3.0 mg dm^{-3} (data not shown), shoot number was not significantly enhanced, moreover shoots did not elongate, leaves were very short and started to twist gradually. Shoot lengths were also significantly affected by the concentration of phytohormones. Our results with fully mature wild cherry point out even higher shoot multiplication rates than those reported for younger, 8-year-old cherry rootstock Maxma-14 (Muna *et al*. 1999). The highest mean number of shoots per explant obtained by these authors on MS medium was 5.42 when using 4.44 µM BAP + 0.49 µM IBA while the combination of 4.44 µM BAP + 0.49 µM NAA produced in average 3.37 shoots per explant only. Our higher rates recorded under very similar phytohormone concentrations may be explained by different

Table 1. Effects of phytohormones on shoot multiplication and shoot length of *P. avium* explants cultured on WPM; means ± SE, n = 30 explants per treatment. Mean values followed by the same letters, a - f, (x - y in the case of separate TDZ treatments, respectively) are not significantly different at the 0.05 level of significance (Duncan's multiple range test).

Phytohormones [mg dm^{-3}]		Number of shoots [$explant^{-1}$]	Shoot length [cm]
WPM basal	0	1.59 ± 0.14d	1.65 ± 0.08def
BAP	0.2	3.63 ± 0.26c	1.83 ± 0.09bcde
	0.4	4.58 ± 0.61abc	1.89 ± 0.09bcd
	0.5	4.96 ± 0.63abc	1.53 ± 0.07f
	0.7	4.97 ± 0.45abc	1.56 ± 0.09ef
	1.0	6.15 ± 1.01a	1.51 ± 0.08f
BAP+IBA	0.2+0.01	3.42 ± 0.37c	2.03 ± 0.12ab
	0.4+0.01	4.07 ± 0.38bc	2.23 ± 0.11a
	0.5+0.01	4.73 ± 0.43abc	1.83 ± 0.10bcde
	0.7+0.01	4.95 ± 0.72abc	1.71 ± 0.09cdef
	1.0+0.01	5.00 ± 0.73abc	1.43 ± 0.09f
BAP+NAA	0.2+0.01	3.36 ± 0.24c	1.95 ± 0.09bc
	0.4+0.01	4.63 ± 0.53abc	1.87 ± 0.08bcd
	0.5+0.01	5.07 ± 0.59abc	1.63 ± 0.07def
	0.7+0.01	5.63 ± 0.93ab	1.61 ± 0.07def
	1.0+0.01	6.32 ± 0.93a	1.54 ± 0.06f
TDZ	0.2	4.83 ± 0.28y	-
	0.5	5.40 ± 0.35y	-
BAP+TDZ	0.5+0.05	6.83 ± 0.61x	-

culture medium (WPM) and different genotype we used, ten times lower concentration of auxin applied in our experiments did not play a significant role. The older age of our tree did not show any disadvantage in shoot multiplication rates.

We also wanted to compare shoot multiplication using TDZ. The highest shoot multiplication rate of all treatments (6.83 shoots per explant) was recorded at the combined use of 0.5 mg dm^{-3} BAP with 0.05 mg dm^{-3} TDZ (Table 1, Fig. 2). In terms of shoot number per explant, TDZ alone was not as efficient as in combination with BAP, when the presence of BAP strengthened the effect of TDZ. For wild cherry, Hammatt and Grant (1998) reported that increasing concentration of TDZ from 4.4 µM to 22.2 µM led to enhanced number of regenerated shoots per explant (from 0.4 to 0.7). After improving regeneration protocol, the authors observed the formation and regeneration of 3.5 shoots per explant for their most responsive genotype when using WPM supplemented with 4.4 µM TDZ, 0.54 µM NAA and surfactant Tween-20 (Grant and Hammatt 2000). Gentile *et al*. (2002) harvested in average 5.5 shoots per explant from the most responsive genotype of 6-year-old peach after 2 months of culturing on media supplemented with various concentrations of TDZ. Nowak *et al*. (2004) reported up to 13.75 adventitious buds per explant in *Prunus domestica* growing on modified MS medium with enhanced concentration of glucose (6 %) and supplemented with 7.5 µM TDZ and 0.9 µM 2,4-D. On the other hand, the increased concentration of glucose had a deleterious effect on explants undergoing differentiation. In comparison to the above results reported for various *Prunus* species, our numbers of shoots per explant in mature *P. avium* point out very high shoot multiplication rates. With regard to shoot lenghts, the observed data on the highest lenghts and variation are fully consistent with those described by Muna *et al*. (1999) for the cherry rootstock Maxma-14.

Adventitious rooting from stem microcuttings (Fig. 3) was recorded at a frequency of at least 43.3 % for all auxin treatments (Table 2). The highest rooting percentage (73.3 %, *G*-test value 58.97 at the 0.001 level of significance) was achieved after using 0.3 mg dm^{-3} IBA. In addition, a spontaneous adventitious rhizogenesis

Fig. 1. Shoot multiplication on WPM supplemented with 1.0 mg dm^{-3} BAP + 0.01 mg dm^{-3} NAA.
Fig. 2. Shoot multiplication on WPM supplemented with 0.5 mg dm^{-3} BAP + 0.05 mg dm^{-3} TDZ.
Fig. 3. Adventitious root formation after 5 weeks on a half-strength WPM supplemented with 0.3 mg dm^{-3} IBA.
Fig. 4. Acclimatized 2-year-old regenerates showing normal growth.

Table 2. Effect of auxins on rooting of excised shoots after 5 weeks of culture on a half-strength WPM; *n* = 30 shoots per treatment.

Auxins	[mg dm^{-3}]	Rooting frequency [%]
Control	0	0
IBA	0.1	70.0
	0.3	73.3
	0.5	63.3
	1.0	60.0
IBA + 2,4-D	0.1 + 0.1	43.3
	0.3 + 0.3	43.3
	0.5 + 0.5	46.7
	1.0 + 1.0	60.0

occurred even during the adventitious shoot multiplication on media without auxin (for example WPM supplemented with 0.5 mg dm^{-3} BAP + 0.05 mg dm^{-3} TDZ). Such situations may speed up the production of rooted wild cherry plantlets. However, the rooting percentage was not observed in frequencies 90 % or even 100 % as reported for *P. persica* × *P. amygdalus* PR 204/84 rootstock (Fotopoulos and Sotiropoulos 2004). The regenerates with fully expanded leaves and well-developed roots were acclimatized in simple plastic boxes with transparent covers that were subsequently lifted to reduce humidity. The acclimatized plantlets were potted in a soil mixture, successfully overwintered under cold greenhouse conditions, and afterwards established in soil. Although Piagnani *et al*. (2002) found the somaclone with a reduced apical dominance among regenerated plantlets of *P. avium* cv. 'Hedelfinger', we did not observe any abnormal morphological variation or growth characteristics in the regenerates *ex vitro* (Fig. 4). After 2 years, the mean height of trees was 55.5 cm.

References

Chalupa, V.: Micropropagation of conifer and broadleaved forest trees. - Commun. Inst. Forest. Czechosl. **13**: 7-39, 1983.

David, H., Domon, J.M., Savy, C., Miannay, N., Sulmont, G., Dargent, R., David, A.: Evidence for early stages of somatic embryo development in a protoplast-derived cell culture of *Prunus avium*. - Physiol. Plant. **85**: 301-307, 1992.

Druart, P., Delporte, F., Brazda, M., Ugarte-Ballon, C., Da Camara Machado, A., Da Camara Machado, L.M., Jacquemin, J., Watillon, B.: Genetic transformation of cherry trees. - Acta Hort. **468**: 71-76, 1998.

Escalettes, V., Dosba, F.: *In vitro* adventitious shoot regeneration from leaves of *Prunus* spp. - Plant Sci. **90**: 201-209, 1993.

Fotopoulos, S., Sotiropoulos, T.E.: *In vitro* propagation of the peach rootstock: the effect of different carbon sources and types of sealing material on rooting. - Biol. Plant. **48**: 629-631, 2004.

Garin, E., Grenier, E., Grenier-De March, G.: Somatic embryogenesis in wild cherry (*Prunus avium*). - Plant Cell Tissue Organ Cult. **48**: 83-91, 1997.

Gentile, A, Monticelli, S., Damiano, C.: Adventitious shoot regeneration in peach [*Prunus persica* (L.) Batsch]. - Plant Cell Rep. **20**: 1011-1016, 2002.

Grant, N.J., Hammatt, N.: Adventitious shoot development from wild cherry (*Prunus avium* L.) leaves. - New Forests **20**: 287-295, 2000.

Hammatt, N.: Micropropagation of fastigiate bird cherry (*Prunus padus* L.) and adventitious shoot formation from leaves. - J. hort. Sci. **68**: 975-982, 1993.

Hammatt, N., Grant, N.J.: Shoot regeneration from leaves of *Prunus serotina* Ehrh. (black cherry) and *P. avium* L. (wild cherry). - Plant Cell Rep. **17**: 526-530, 1998.

Lloyd, G.B., McCown, B.H.: Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. - Proc. int. Plant Propag. Soc. **30**: 421-427, 1980.

Miguel, C.M., Druart, P., Oliveira, M.M.: Shoot regeneration from adventitious buds induced on juvenile and adult almond (*Prunus dulcis* Mill.) explants. - *In Vitro* cell. dev. Biol. Plant **32**: 148-153, 1996.

Muna, A.-S., Ahmad, A.-K., Mahmoud, K., Abdul-Rahman, K.: *In vitro* propagation of a semi-dwarfing cherry rootstock. - Plant Cell Tissue Organ Cult. **59**: 203-208, 1999.

Nicoll, F.: Genetic improvement of cherry for farm woodlands. - Quart. J. Forest. **87**: 187-194, 1993.

Nowak, B., Miczynski, K., Hudy, L.: Sugar uptake and utilisation during adventitious bud differentiation on *in vitro* leaf explants of 'Wegierka Zwykla' plum (*Prunus domestica*). - Plant Cell Tissue Organ Cult. **76**: 255-260, 2004.

Piagnani, C., Iacona, C., Intrieri, M.C., Muleo, R.: A new somaclone of *Prunus avium* shows diverse growth pattern under different spectral quality of radiation. - Biol. Plant. **45**: 11-17, 2002.

Turok, J., Eriksson, G., Kleinschmit, J., Canger, S. (compilers): Noble Hardwoods Network. - IPGRI, Rome 1996.