Chapter

Long-Term Observation of *In Vitro*-Derived Malus Sylvestris (L.) Mill., the Path from the Bud to the Tree

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Abstract

The European wild apple *(Malus sylvestris* L.), a wild contributor to the domesticated apple, belongs to the endangered species in the Czech Republic. Thus, an efficient protocol was developed for *in vitro* plantlet regeneration using the post-dormant buds. The highest shoot induction was obtained on MS medium supplemented with 0.5 mg.l^{-1} BAP, 5 mg.l^{-1} GA and 0.1 mg.l^{-1} IBA. Shoot multiplication and elongation took place on the same medium with 0.2 mg.l^{-1} BAP and 0.1 mg.l^{-1} IBA. Indole-3-butyric acid at 0.5 mg. l^{-1} was most effective for rooting. The micropropagated plantlets were successfully acclimatized in greenhouse conditions and were transplanted into soil in forest. Finally, qualitative and quantitative parameters of tissue culture-derived plants were evaluated. Monitoring of *in vitro* plantings on experimental trials suggests that micropropagated wild apple trees retain the growth characteristics of generative individuals.

Keywords: acclimatization, establishment of *in vitro* culture, long-term forest trial, *Malus sylvestris* L., rooting, shoot multiplication

1. Introduction

M. sylvestris L., the only native wild apple species in Europe, belongs to the family Rosaceae. It is mainly pollinated by bees and flies, and thanks to its small and hard fruits, the trees are often called crab apples. This species occurs across Western and Central Europe, from southern Scandinavia to the Iberian Peninsula and from the Volga to the British Isles [1]. With a high up to 10 m, it grows in low-density populations and the growth habit looks like shrubs, more than trees. Under good growth conditions (high light requirements), the crab apple can live up to 80–100 years [2]. Though many different varieties of apples were developed over time, it was shown that old varieties have higher nutritional quality when compared to commercial ones [3–5].

In the Czech Republic, the wild apple belongs to the endangered species [6] because of its shrinking habitat, fragmentation of populations [7–9] and likelihood of a genetic admixture with domesticated apple (Malus domestica Borkh.). Recent

studies have underlined the significant contribution of wild apples to the cultivated apple genome, M. domestica Borkh., during the domestication history from the Central Asian progenitor *Malus sieversii* (Ldb.) M. Roem [10]. Because of hybridization, it can be very difficult to reliably identify 'true type' wild apples from existing hybrids based on morphological characteristics, such as the fruit width or the hairiness of leaves [2]. Easy hybridization of wild apple with the domesticated apple is due to the absence of prezygotic isolation mechanisms [11], which results in fitness reduction of the wild apple populations and can lead to their reduction or extinction. Moreover, due to human activities, such as forest clearing, industrialization, population increase and intensive agriculture, natural and artificial forest stand regeneration is almost impossible. To avoid loss of wild apple genetic resources, they must be conserved and the conservation strategy should be applied sustainably.

The solution to the problem of preservation and restoration of the gene pool of endangered wild apples could be the *in vitro* cultivation using the micropropagation method. Micropropagation is used to multiply a wide variety of plants by a number of tissues, cells or organ culture methods. It means the aseptic culture of small plant explant of tissue or organs, closed in vessel with defined culture media and under controlled conditions. It provides the large-scale production of disease-free seedlings within a short time and with limited space. In the Czech Republic, biotechnology approaches for *in vitro* conservation of different woody plant species are well established and widely used (e.g., see [12–17]). During the past years, many studies have been carried out using *in vitro* cultures of apples (for review, see [18]). A key objective of apple *in vitro* cultures is to multiply disease-free clones, suitable for rooting, acclimatization and planting. However, as in many other plant species, the medium composition, plant growth regulators requirement or specific growth conditions are cultivar-dependent [19].

Additionally, micropropagation enables the protection of endangered species, thanks to providing the plant material in a larger amount for plant breeding programs at specific sites [20]. However, not many available papers and reviews dealing with studies on tissue cultures of forest trees include subsequent *ex vitro* evaluation of their quantitative and qualitative traits. The aim of our study was to find the optimal composition of explant culture media used for initiation of culture, as well as long-term multiplication and rooting of wild apples *in vitro*. In addition, the growth parameters of *in vitro*-derived wild apple trees were evaluated.

2. Material and methods

2.1 Plant material and *in vitro* culture establishment

M. sylvestris L. trees were carefully determined, not to be mistaken for domesticated apples M. domestica Borkh.

Juvenile branches with dormant leaf buds of the crown of M. sylvestris trees from central Bohemia were collected and cut into 10–15 cm long twigs. Twigs were immediately packed into the plastic bags and stored at 4°C before being cultured *in vitro*. To decrease microbial contamination, the twigs were cut into 5 cm long segments with buds, rinsed for 30 min in running water and surface disinfected with Tween®20 (2 drops/10 ml) for 20 min, followed by soaking in KORSOLEX for 20 min, rinsed with sterile distilled water for 20 min, incubated in $HgCl_2$ solution (1 $mg.l^{-1}$) for 15 min and subsequently immersed in distilled water three times for 15 min.

The surface disinfected bud explants (0.3–0.5 cm large) were placed in glass jars containing 100 ml of culture medium: MS medium [21] supplemented with 0.5 mg l^{-1} BAP, 0.1 mg l^{-1} IBA, 10 mg l^{-1} glutamine, 2 mg l^{-1} glycine, 30 g l^{-1} sucrose and 6 g l^{-1} agar. The pH value was adjusted to 5.8 before autoclaving at 121°C, 150 kPa for 20 min. The aseptic cultures were incubated in a growth chamber at 24 ± 1°C with a 16-h photoperiod (30 μE m^{-2} s^{-1}).

2.2 Shoot multiplication

New shoots from explant cultures were separated into stems approximately 2.0 cm long and transferred into shoot induction media. The medium used was MS medium containing 0.2 mg l^{-1} BAP, 0.1 mg l^{-1} IBA, 200 mg l^{-1} glutamine, 2 mg l^{-1} glycine, 200 mg l^{-1} casein, 30 g l^{-1} sucrose, 6 g l^{-1} agar and final pH adjusted to 5.8. Explants were cultured in the growth conditions as described above and repeatedly subcultured at a constant 4-week subculture interval.

2.3 Root induction, *ex vitro* acclimatization and hardening

Healthy shoots (1.5–2 cm) were excised and cultured on ¼ MS medium supplemented with 0.5 mg l^{-1} IBA, 10 g l^{-1} sucrose, 6 g l^{-1} agar and final pH adjusted to 5.8. Explants were cultured in the growth conditions as described above.

After 2–3 weeks, the rooted shoots were carefully washed with distilled water to remove agar and the rooted shoots were hardened in a culture room in conical planter Quick Pot T 35 with perforated bottom, filled with perlite (Perlite Praha spol. s.r.o., Czech Republic) and placed into the transparent plastic box fully closed. Plants were regularly watered with 1/2 strength liquid MS medium devoid of sucrose and phytohormones and diluted with distilled water in a ration 1:10. After 2–3 weeks, the plants were transferred into the planter Quick Pot T 60 containing soil (Zahradnický substrát a.s. Soběslav, Czech Republic): perlite (2:1) and kept in a bigger transparent plastic box. During 2 weeks, plantlets were adapted to lower moisture conditions by gradually tilting the upper part of the plastic box. Fully adapted plants were moved into greenhouse and subsequently transferred into the outdoor flower beds to obtain capable seedlings.

3. Results

3.1 Plant material

The wild apple trees are characterized by pressed buds, leaves and combs. Only in spring, the leaves can have inconspicuous hairs on the reverse side at the base of the thicker veins. The size of the leaves and fruits are also different, with clearly smaller size in the wild apple tree. *M. sylvestris* L. has leaves of up to 6.5 cm long, while the leaves of the domestic apple tree are 6–12 cm long. The fruits (pome) of the wild apple tree are 2–3.5 cm in diameter, while the apples of the domestic apple tree are at least 5 cm in size. The crown slices of flowers of the wild apple tree are also smaller and tend to be narrower [22].

Juvenile branches with axillary buds were collected during the early spring. In agreement with Ref. [23], buds collected during spring and summer seasons produced a significantly higher percentage of explant establishment and were less contaminated than buds collected during autumn or winter.

3.2 Shoot propagation from the buds

As for surface sterilization of buds, different methods have been described. The disinfection procedures include washing in sodium hypochlorite (NaOCl) solution [24], mercuric chloride ($HgCl_2$) solution [19], 75% alcohol followed by $HgCl_2$ solution [25] or calcium hypochlorite [$(Ca(OCl)_2$] solution [26]. In our experiment, we used an $HgCl_2$ solution (1 mg.l^{-1}), which is highly efficient for surface sterilization of buds from field-grown trees.

As in other plant species, the optimal basal medium is often cultivar-dependent in *Malus* species [27]. Additionally, Kabylbekova et al. [28] have used response surface methodology (RSM) and showed that each apple cultivar requires a different composition of mineral nutrition for its optimal growth. Similarly, the selection of the optimal plant growth regulators is also genotype-dependent, as has been shown in the study of different apple scions and rootstocks [29, 30]. Thus, different media types for bud induction and shoot development were tested. The MS medium with 0.5 mg l^{-1} BAP, 0.1 mg l^{-1} IBA, 10 mg l^{-1} glutamine and 2 mg l^{-1} glycine was the most effective medium, based on monitoring following parameters: percentage of contamination, percentage of necrotic explants and percentage of explants with shoot initiation. In our experiment, plant growth regulators BAP and IBA were used for culture establishment and also for shoot multiplication, consistently with the studies from Refs. [31] or [28]. However, application of meta-topolin [32] or TDZ [33] in the growth medium was also described in apples.

3.3 Shoot proliferation

Newly developed shoots from bud explants were transferred into shoot induction media (**Figure 1**). Based on determination of multiplication parameters, that is, multiplication index (the number of newly formed shoots per initial shoot tip) and length of lateral shoots, MS medium consisted of 0.2 mg l^{-1} BAP, 0.1 mg l^{-1} IBA, 200 mg l^{-1} glutamine, 2 mg l^{-1} glycine and 200 mg l^{-1} casein was chosen. Same as in our experiment, Sota et al. [34] have used MS medium for shoot multiplication of wild apples. However, the BAP concentration was quite higher than in our experiment (1 mg.l^{-1} BAP), and instead of IBA, α-Naphthaleneacetic acid (NAA) was used. Additionally, the application of other growth nutrient media has also been reported [27, 35].

3.4 *In vitro* rooting, acclimatization and hardening

Well-multiplied shoots were subjected to MS medium lacking cytokinins but supplemented with auxins. All the treatments resulted in root production. The highest rooting percentage and roots per cultured shoot were obtained on ¼ MS supplemented with 0.5 mg l^{-1} IBA, 10 g l^{-1} sucrose and 6 g l^{-1} agar. Although some authors found that NAA [36] or IAA [37] is more effective for rooting, the application of IBA showed the best results in our experiment.

The rooted shoots obtained from the best treatment (**Figure 2**) were removed from the rooting medium and the plantlets were then transferred to planter with perlite and watered with liquid MS medium. After 2 weeks, plantlets were transferred into bigger planter containing a mixture of perlite and soil and gradually adapted to lower moisture conditions. Finally, fully adapted plants (**Figure 3**) were transferred into greenhouse.

Figure 1.
In vitro shoot multiplication of M. sylvestris L.

3.5 Qualitative and quantitative traits of wild apples at experimental trials

The study was conducted at the Oldřichov (425 m a.s.l., central Bohemian Highlands) in 2003 and at Polná II (550 m a.s.l., Czech-Moravian Highlands) in 2007 in the Czech Republic. At both experimental sites, row planting with 2 × 2 m spacing was used and the plots were fenced off for the entire monitoring period. The subject of the evaluation of quantitative traits was survival rate, height and diameter at breast height (DBH). The DBH was measured using a millimetre calliper, and the height was determined using a measuring rod and a Vertex III ultrasonic altimeter (Haglöf Sweden AB, Langsele, Sweden). As for qualitative traits, trunk shape, forkness, branching angle, branch thickness and vitality were established. The qualitative traits were determined according to the manual in **Table 1**.

In Oldřichov, grafters of wild apples were planted together with *in vitro*-derived plantlets (**Figure 4**). For statistical evaluation, data from 2010 to 2018 were used

Figure 2.
In vitro rooting of M. sylvestris L.

Figure 3.
Fully adapted in vitro-derived M. sylvestris L. plantlets.

Trunk shape		Forkness		Branching angle		Branch thickness		Vitality	
1	Straight	1	Stem continuous to the crown	1	Horizontal	1	Thin (up to 10% DBH)	1	Highly vital
2	Slightly curved on one side	2	Fork in the upper third of the tree's height	2	Ascending	2	Medium thick (10–25% DBH)	2	Vital
3	Strongly curved on one side	3	Fork in the second third of the tree's height	3	Overhanging	3	Thick (over 25% DBH)	3	Less vital
4	Trunk at least 2x Slightly ace-curved	4	Fork in the lower third of the tree					4	Declining tree
5	Significantly bent trunk	5	Repeated multiple forkness						

Table 1.
The list of qualitative traits and their descriptors.

Figure 4.
M. sylvestris L. trees grown at Oldřichov research plot in 2010.

(**Table 2**). The mortality of tissue culture plantlets was zero, while grafters' mortality reached 24%, mostly due to withering of the trees. ANOVA did not show any significant differences in height between *in vitro*-derived plantlets and grafters ($\alpha = 0.05$). However, in 2018, grafters were significantly higher than tissue culture plantlets, according to ANOVA, which could be caused by the growth rate of rootstock. No differences were observed between qualitative traits (**Table 2**). The trunk shape reached

		2010			2018										
		Height (m)			Height (m)				DBH (cm)			TrSh	Bra	BrAng	BrTh	Vit
Group	Count	Mean	Median	StD	Count	Mean	Median	StD	Mean	Median	StD	Median	Median	Median	Median	Median
In vitro	15	3.03	3.25	0.90	15	6.89	7.35	2.95	5.24	5.80	1.61	3	3	2	2	1
Grafters	195	2.79	3.30	1.45	148	8.01	8.63	2.80	7.26	7.50	1.56	3	3	2	2	1
All	210	2.80	3.28	1.41	163	7.91	7.40	1.66	7.08	8.40	2.82	3	3	2	2	1

Table 2.
Average growth parameters of Malus sylvestris L. plantlets at Oldřichov research plot. DBH - diameter of breast height, TrSh – trunk shape, Bra – Branching, BrAng – branching angle, BrTh – branch thickness, Vit - vitality and StD – standard deviation.

	2008				2017											
	Height (m)				Height (m)				DBH (cm)			TrSh	Bra	BrAng	BrTh	Vit
Clone	Count	Mean	Median	StD	Count	Mean	Median	StD	Mean	Median	StD	Median	Median	Median	Median	Median
J1	9	1.14 [a, b]	1.25	0.34	9	5.03 [a, b]	4.9	1.00	4.16	4.05	0.93	3	4	2	3	1
J2	10	1.21 [a, b]	1.12	0.29	10	4.88 [a, b]	4.95	0.68	3.96	3.93	1.01	3.5	3	2	3	1
J4	23	0.98 [b]	0.96	0.21	23	3.86 [b, c]	3.7	0.78	3.14	3.05	1.14	3	3	2	3	1
J5	18	1.11 [a, b]	1.10	0.23	17	4.35 [a, b, c]	4.6	0.72	3.99	4.1	1.11	3	3	2	3	1
J6	50	1.15 [a, b]	1.15	0.29	50	3.82 [c]	3.75	1.07	3.48	3.35	1.71	3	3	2	3	1
J7	18	0.99 [b]	1.01	0.27	18	3.94 [b, c]	4.05	0.61	3.26	3.4	1.23	3	3	2	3	1
J8	12	1.13 [a, b]	1.23	0.39	12	5.21 [a]	4.9	0.61	4.43	4.28	0.61	3	3.5	2	3	1
J9	10	1.20 [a, b]	1.22	0.20	10	4.82 [a, b, c]	4.8	1.03	3.83	4.1	1.39	3	3.5	2	3	1
J10	14	0.89 [b]	0.87	0.33	14	4.25 [a, b, c]	4.15	1.09	3.84	4.28	1.70	3	3	2	3	1
J12	20	1.10 [a, b]	1.06	0.22	20	4.36 [a, b, c]	4.5	0.88	3.27	3.3	1.37	3	3	2	2	1
J13	19	1.10 [a, b]	1.15	0.22	19	4.37 [a, b, c]	4.4	0.77	3.53	3.55	1.18	3	3	2	3	1
J26	20	1.33 [a]	1.40	0.27	20	4.25 [a, b, c]	4.4	1.06	3.68	3.45	1.62	3	4	2	3	1
J28	13	1.14 [a, b]	1.22	0.20	13	3.98 [b, c]	3.8	0.78	4.51	4.7	1.63	3	3	2	3	1
J30	14	1.12 [a, b]	1.18	0.25	14	4.25 [a, b, c]	4.4	0.94	4.27	4.45	1.33	3	3	2	3	1
All	250	1.11	1.12	0.28	249	4.24	4.30	0.96	3.69	3.75	1.42	3	3	2	3	1

Table 3.
Average growth parameters of Malus sylvestris plantlets on Polná II research plot. DBH - diameter of breast height, TrSh - trunk shape, Bra - branching, BrAng - branching angle, BrTh - branch thickness, Vit - vitality and StD - standard deviation. Values followed by the same letter(s) (a-c) within the same column are not significantly different according to Tukey's test ($P < 0.05$).

Figure 5.
Malus sylvestris L. trees grown at Polná II research plot in 2011.

the third degree (strongly curved on one side), same as forkness (forks in the second-third of the tree's height). The second degree was observed in the branching angle (ascending branches) and in the branch thickness (medium thickness with 10–25% DBH). The vitality reached the first degree (highly vital).

The growth characteristics of *in vitro*-derived wild apple plantlets recorded from years 2008 and 2017 grown at Polná II research plot are given in **Table 3**. The mortality was very low, only one individual in clone J5 died between the monitored years. ANOVA showed significant differences in height of wild apples in both years ($\alpha = 0.05$), whereas no differences were observed in DBH. Among the qualitative traits, only small variances were noted. The trunk shape is on the third degree (strongly curved on one side) with the exception of clone J2, where the trunk was at least 2x slightly ace-curved. Forkness reached mostly the third degree. Only for clones J1 and J26, the fourth degree of forkness (forks in the lower third of the tree) was noted, and for clones J8 and J9, an intermediate stage between the third and the fourth degree was noted. All the wild apple clones had ascending branches (the second degree of branch angle). For clone J12, medium branch thickness was recorded (10–25% DBH), whereas all other clones reached the third degree of branch thickness (strong thickness with over 25% DBH). All tested clones were highly vital (**Figure 5**).

The comparison of our results with domestic or foreign studies is very problematic. There is a lack of evidence about the growth characteristics of wild apples, due to their low abundance in nature and difficult determination. Moreover, wild apples grown in our research plots are residual individuals selected in the Czech Republic, with different qualitative traits and growth potential.

4. Conclusions

The results of the presented study indicated that our methodology for the micropropagation of M. sylvestris L. from dormant bud could be efficiently used for *in*

vitro conservation of endangered wild apples. Moreover, according to long-term experimental trials, *in vitro*-derived plantlets show quality and balanced growth, comparable with grafters. The growth characteristics of wild apples and grafters on the research plots at Oldřichov and Polná II will be still evaluated in the future.

In this regard, implementation of *in vitro* conservation of endangered wild apple M. sylvestris L. in practice can speed up the process of protection and reproduction.

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Conflict of interest

The authors declare no conflict of interest.

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