applied sciences

MDPI

Article

Sanitation of Apple Cultivars from AP Phytoplasma and ApMV and ACLSV Viruses Using In Vitro Culture and Cryo-Knife Therapy in Liquid Nitrogen

Jiří Sedlák *, Matěj Semerák and Martina Rejlová

Research and Breeding Institute of Pomology Holovousy Ltd., 508 01 Holovousy, Czech Republic
* Correspondence: jiri.sedlak@vsuo.cz

Abstract: Systemic infections with phytoplasmas and viruses threaten the production of healthy plant material under the fruit species certification system. We tested the possibility of sanitation using in vitro culture and cryotherapy. The starting material of the cultivars Golden Delicious (clones A and B), Virginia Crab, and Panenské zlepšené was taken from in vivo plants that tested positive for apple proliferation phytoplasma. The Táborita cultivar was obtained from already established in vitro cultures that had tested positive for apple proliferation phytoplasma, apple mosaic virus, and apple chlorotic leaf spot virus. Cultivars Golden Delicious A, Virginia Crab, and Panenské zlepšené were sanitated from the phytoplasma in the first step, i.e., by sterilization and a subsequent transfer to in vitro conditions. Golden Delicious B remained infected with the phytoplasma, and both viruses, after the in vitro culture phase and together with Táborita, were subjected to cryotherapy by vitrification. In Golden Delicious B, three out of thirteen initial shoot tips regenerated after a liquid nitrogen treatment. Four mericlones were regenerated from 10 initial cryopreserved shoot tips of Táborita. None of the three pathogens were detected by PCR in the regenerated Golden Delicious B mericlones. On the contrary, in the case of Táborita, infection with all the pathogens was detected after regeneration. The results obtained indicate the potential applicability of in vitro cultivation techniques or, if necessary, subsequent cryopreservation as a method for sanitizing against systemic microbial contamination. However, further research on the relationship between pathogens and specific genotypes is needed.

Keywords: in vitro culture; cryopreservation; vitrification; apple; ApMV; ACLSV; apple proliferation; phytoplasma

Citation: Sedlák, J.; Semerák, M.; Rejlová, M. Sanitation of Apple Cultivars from AP Phytoplasma and ApMV and ACLSV Viruses Using In Vitro Culture and Cryo-Knife Therapy in Liquid Nitrogen. *Appl. Sci.* **2023**, *13*, 7527. https://doi.org/10.3390/app13137527

Academic Editor: Ying Zhai

Received: 19 May 2023
Revised: 16 June 2023
Accepted: 20 June 2023
Published: 26 June 2023

1. Introduction

Apples (*Malus domestica* L.) have long been the most economically important fruit crop in the conditions of the central European temperate climate zone. In the territory of the Czech Republic, 5864 hectares of production plantations of apple trees were registered in 2022, which represents almost half of the total area of commercial orchards. The importance of this species is also evidenced by the fact that the fruit's nursery sector produced a total of 1,288,554 apple trees, which is more than the production of trees of all major commercial stone fruit species in the last statistically processed year of 2021 [1]. The nursery production of fruit trees is a stabilized, economically significant branch of crop production with slightly growing potential.

However, apple trees can be infected with several viruses and phytoplasmas, often in mixed infections. The synergistic negative effect of these systemic pathogens then reduces fruit yields and quality, tree vigor, and fruit orchards' longevity. Trees infected with viruses and virus-like pathogens cannot be sanitized after planting in a permanent location. The infection is often systemic, and the economic damage persists to varying degrees throughout the lifetime of an individual tree in the production orchard [2]. Therefore, the

nursery production of healthy, pathogen-free apple-starting propagation material has a demonstrable benefit in increasing the competitiveness of growers by producing fruits of better quality and yield. Apple chlorotic leaf spot virus (ACLSV), apple mosaic virus (ApMV), and *Candidatus* Phytoplasma mali-causing apple proliferation (AP) are among the most important systemic infections of apple trees in Europe [2]. ACLSV is widespread and the most prevalent latent virus of apples in the Czech germplasm collection, with an infection rate exceeding 90% [3].

Cryopreservation of the shoot tips taken from in vitro-grown explants has been widely used as a potential tool for plant material sanitation. Rather inconsistent results have been reported, dependent on specific pathogens and plant material, and different opinions have been expressed on the applicability of cryotherapy.

Numerous cryopreservation protocols have been published [4], of which vitrification is one of the most widely used. This procedure includes—in many variations—acclimatization, treatment with a loading solution (LS), the vitrification itself, freezing in liquid nitrogen, thawing, the unloading of the shoot tips with an unloading solution (ULS), and regeneration. To date, different compositions of plant vitrification solutions (PVS) have been used so far for different plant species [5].

The eradication of apple stem pitting virus (ASPV) and ACLSV from in vitro-grown apple cultures by cryopreservation was very successful [6]. An attempt to use cryotherapy for the apical buds of hop explants infected with ApMV did not yield results that would indicate the effectiveness of this approach [7], whereas ApMV was successfully eradicated for hazelnut [8]. For apple stem grooving virus (ASGV), present in apple rootstocks, exposure to liquid nitrogen was not effective unless preceded by thermotherapy. The combination of thermotherapy and cryotherapy has also been suggested for ACLSV [9]. The results of virus cryotherapy vary among apple cultivars [10], and different cryopreservation techniques may lead to different results [11].

On the other hand, cryopreservation can also be used for the opposite purpose—as a method to preserve some viruses. In support of this possibility, ASGV was shown to persist in the cryopreserved shoot tips of the apple cultivar Gala [12] be in agreement with the results obtained by Bettoni et al. [9].

Phytoplasmatic infections may also be targets for potential cryotherapy. *Candidatus* Phytoplasma aurantifolia was reported to be eliminated by the shoot tip cryopreservation of sweet potato cultures; it was observed that in the meristematic zone, the upper cell layers, free of phytoplasma, were less damaged during the freezing procedure than the lower layers, allowing the regeneration of healthy shoots after thawing [13]. Similar results were obtained in the case of the Chinese jujube for *Candidatus* Phytoplasma ziziphi [14].

In general, not much work has been published so far on phytoplasma eradication for economically important fruit species in temperate climates. In particular, AP phytoplasma causes considerable economic damage annually in the nursery sector and in central European apple plantations. Vegetative clonal propagation in the nursery fruit sector depends on the availability of high-quality plant starting material with proven health status. For this reason, two projects were carried out within the framework of the Czech National Agency for Agricultural Research with the aim of obtaining phytoplasma- and virus-free initial propagating material of mother plants for the certification system established in the Czech Republic.

Propagation by in vitro cultures is a modern way of producing plant material. Provided that effective methods of sterilization of the initial explants and suitable types of culture media are found, a large number of species and fruit crop cultivars can be propagated by these biotechnological methods. The advantage over classical nursery methods is the year-round cultivation under controlled laboratory conditions and, in particular, the possibility of targeted biotechnological therapeutic intervention in the case of systemic infections of the phytoplasma type.

In order to test the possibilities of AP phytoplasma sanitation using in vitro culture and cryo-knife therapy, we purposefully selected apple genotypes with different genetic

bases and from different production groups. These were two clones of the Golden Delicious variety as representatives of the main commercial segment of the apple market, two original Czech cultivars, Táborita (landrace) and Panenské zlepšené (Panenské české x Jonathan), and the Virginia Crab apple as a representative of the cider production segment. Phytoplasmas were the main focus of the monitoring of general health following the sanitation system presented in this article, but recovery from viruses was also monitored due to the expected entry of the resulting healthy plant material into the propagation certification scheme.

2. Materials and Methods

Experimental cultures of the Táborita cultivar infected with AP phytoplasma were established using actively growing explants from the in vitro microorganism gene bank maintained at the Research and Breeding Institute of Pomology (RBIP), Holovousy Ltd. (Czech Republic). The starting material of the apple cultivars Golden Delicious (clones A and B), Virginia Crab, and Panenské zlepšené was obtained from phytoplasma-infected plants growing in technical isolation of the RBIP within the in vivo microorganism bank. The plant material of these cultivars had been tested before the start of this project (Table 1).

Table 1. Phytoplasma detection in initial in vivo plants by PCR testing.

Sample	DNA Internal Control Ø Ct	AP Phytoplasma Ø Ct
Golden Delicious A	12.29	14.99
Golden Delicious B	11.09	15.09
Virginia Crab	11.94	15.69
Panenské zlepšené	12.40	16.05

Ø Ct—average PCR cycle threshold value (samples run in triplicate); AP—apple proliferation.

The scions were cut at the end of the dormancy phase and then kept in water under laboratory conditions. At the time of first sprouting, the apical parts of buds (5–10 mm) containing a meristematic area were excised, and the covering scales were peeled off. The resulting explants were then immersed in 0.15% of $HgCl_2$ for 1 min. Following sterilization, they were rinsed twice with demineralized water. The buds were dried on sterile filtration papers in a laminar flow cabinet, and 10 explants of each cultivar were planted on a growth medium in Erlenmeyer flasks.

The composition of the cultivation medium was based on Murashige and Skoog [15]. Two plant growth regulators were added: 1.5 mg/L of 6-benzylaminopurine and 0.1 mg/L of indole-3-butyric acid. The medium was solidified with 8.5 g/L of a Difco Bacto agar and autoclaved at 121 °C and 200 kPa for 20 min.

The material was maintained in a cultivation room in a 12 h photoperiod and 22 ± 2 °C. All manipulations with explants were performed under sterile conditions in a laminar flow cabinet. The material was transferred to a fresh medium every day due to leakage and oxidation of polyphenolic substances manifested by the browning of explants and the medium at the beginning of cultivation. Consequently, the subcultivation interval was extended to 1 month until a sufficient number of shoots was obtained.

Prior to cryopreservation, random mixed samples consisting of stem and leaf material from 4–5 mericlones were collected from the Erlenmeyer flasks containing the in vitro cultures of all apple cultivars tested in order to perform PCR detection of AP phytoplasma. If positive, the material was also tested for the presence of ApMV and ACLSV. For Golden Delicious A, 2 samples were collected for phytoplasma testing. For Golden Delicious B, 4 samples were taken for phytoplasma testing and 1 for virus testing. For Virginia Crab, 1 sample was collected for phytoplasma testing, and for Táborita, 2 samples were collected for phytoplasma testing and 1 for virus testing.

Briefly, the total DNA was isolated from 100 mg of leaves ground in liquid nitrogen using an Exgene Plant SV isolation kit (GeneAll Biotechnology, Seoul, Republic of Korea),

according to the manufacturer's instructions. For virus diagnostics, RNA was isolated from 50 mg of pulverized leaves ground in liquid nitrogen using a Ribospin Plant kit (GeneAll Biotechnology), following the manual. The RNA was then reverse-transcribed using M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). For real-time PCR detection, a qPCR 2x Blue Master Mix (Top-Bio) was used under the following general conditions: samples run in triplicate; final volume, 20 µL; final concentration of primers/probes, 0.5 µM; template, 2 µL of isolated DNA/cDNA. AP phytoplasma and virus detection was carried out in a Rotor-Gene Q (Qiagen) cycler utilizing the same temperature profile: denaturation, 94 °C/5 min; cycling, 45× (94 °C/20 s, 58 °C/20 s with fluorescence reading, 72 °C/20 s).

Primers and probes were designed using Geneious Prime software 2023.1 (Biomatters Ltd., Auckland, New Zealand);

AP phytoplasma detection:

primers 5′-GCAGCTGCGGTAATACATGG, 5′-GAATTCCACTTGCCTCTATCCAA, and a probe 6-FAM-5′-AGTTCAACGCTTAACGTTGTGATGCTAT.

ApMV detection:

primers 5′-CGAAACTTACCCACGAAAGGATG, 5′-CATCTTCGCGATGAACCCTCTC, and a probe 6-FAM-5′-TCCTTAARGCTCGAATCYAAAGAGCAG.

ACLSV detection:

5′-GGTGAGAGGCTCTATTCACATCTTG, 5′-CAGAATAAATTCTGGAGCTTTTCACC, and a probe 6-FAM-5′-TCGCAGAAGGGGATATTCCAATTGCT.

The presence of chloroplast 16S rDNA and the mitochondrial Nad5 transcript was used as an internal control for AP phytoplasma and virus detection, respectively. The relative load of a pathogen was related to the level of an internal control using the formula:

$$2^{(\text{Ct [internal control]} - \text{Ct [pathogen]})}.$$

Cultivars Golden Delicious B and Táborita, which were positively tested for phytoplasma and viruses, were cryopreserved. Cryopreservation of both cultivars was carried out according to a modified protocol published by Höfer [16]. The cultures were transferred to an incubator (PHCbi MIR-154) to grow under short photoperiod conditions (8 h: 22 °C, light; 16 h: −1 °C, darkness) after 2 month-growth in the cultivation room. After 12 days of acclimatization in the incubator, shoot tips (2 mm in length) were excised under a stereoscopic microscope. Excised shoot tips were put in Petri dishes on a growth medium containing 5 vol% of dimethyl sulfoxide (DMSO) and kept on this medium for 2 days in the incubator. In the middle of this period, they were transferred to a fresh medium to avoid adverse effects of the medium and explant browning. The tips were then immersed in 1 mL of LS containing 2 M of glycerol and 0.5 M of sucrose in a liquid growth medium in cryovials for 30 min. The LS was then exchanged for 0.75 mL of PVS2 containing 4 M of glycerol, 2 M of DMSO, 2.7 M of ethylene glycol, and 0.2 M of sucrose in a liquid growth medium. After 30 min, the vials were immersed in liquid nitrogen and stored in liquid nitrogen for 1 week.

Thawing was performed by immersing the cryovials in a 40 °C water bath for 1 min. The PVS2 was replaced with 1 mL of ULS, containing 1.2 M of sucrose in a liquid growth medium. After 20 min, the shoot tips were dried on filter paper and planted in Petri dishes with the growth medium. They were kept in darkness for 1 week and then transferred to standard conditions in the cultivation room. The regenerating material was gradually replanted to Erlenmeyer flasks and kept on the standard growth medium with a subcultivation interval of 1 month. When a sufficient amount of biomass was reached, a mixed sample, consisting of all shoots derived from the successfully regenerated tips, was taken for both cultivars in order to perform PCR testing. The presence of all three initial pathogens was detected in both cultivars after cryotherapy.

3. Results

Of Golden Delicious A and B, Virginia Crab, and Panenské zlepšené, only Golden Delicious B tested positive for AP phytoplasma after the establishment of the in vitro cultures. This result shows that the dissection of the apical part of the explant combined with the sterilization process was able to eliminate the AP phytoplasma infection in three of the total four apple genotypes. PCR testing of Táborita, after multiplication from the initial in vitro culture kept in the microorganism bank, confirmed the presence of phytoplasma. ApMV and ACSLV were also detected in both cultivars. In the Táborita cultivar, all three pathogens were present at high levels. In the case of Golden Delicious, AP phytoplasma and ACLSV were also present at high levels, while the test result for ApMV was only slightly positive (a Ct value of 29, Table 2).

Table 2. Detection of phytoplasma and viruses in the explant cultures before and after cryotherapy by PCR testing.

Sample	DNA Internal Control Ø Ct	AP Phytoplasma Ø Ct	RNA Internal Control Ø Ct	ApMV Ø Ct	ACLSV Ø Ct
Before cryotherapy					
Golden Delicious A (1)	15.82	Negative	-	-	-
Golden Delicious A (2)	13.47	Negative	-	-	-
Golden Delicious B (1)	10.74	15.74	-	-	-
Golden Delicious B (2)	9.97	14.81	-	-	-
Golden Delicious B (3)	10.13	15.03	-	-	-
Golden Delicious B (4)	11.89	16.73	-	-	-
Golden Delicious B (5)	-	-	17.58	29.00	13.95
Virginia Crab	9.12	Negative	-	-	-
Panenské zlepšené (1)	11.03	Negative	-	-	-
Panenské zlepšené (2)	11.93	Negative	-	-	-
Táborita (1)	11.12	14.75	-	-	-
Táborita (2)	9.97	13.66	-	-	-
Táborita (3)	-	-	17.00	13.42	14.04
After cryotherapy	-	-	-	-	-
Golden Delicious B	10.00	Negative	16.74	Negative	Negative
Táborita	10.01	30.88	16.35	14.73	13.90

Ø Ct—average PCR cycle threshold value (samples run in triplicate); AP—apple proliferation; ApMV—apple mosaic virus; ACLSV—apple chlorotic leaf spot virus.

The appearance of the infected cultures varied: Golden Delicious showed no significant symptoms in growth and shoot morphology, except for smaller and narrower leaves compared to the uninfected in vitro cultures of this cultivar (Figure 1a). Táborita showed stunted growth, dwarfed undeveloped leaves, and extreme lateral shoot proliferation (Figure 1b).

After the cryopreservation and subsequent thawing, five out of thirteen Golden Delicious shoot tips and five out of ten cryopreserved Táborita shoot tips were regenerated (Figure 2). Three Golden Delicious tips and four Táborita tips that showed a high ability to form viable shoots were selected for culture establishment. A shoot size sufficient for PCR testing was achieved after five months and five consecutive subcultures.

The regenerated Golden Delicious shoot tips produced visually healthy shoots with apparently larger leaves than before the cryopreservation (Figure 3a). The Táborita cultures did not thrive after thawing; although it was possible to obtain a sufficient amount of material for PCR testing, the viability of all the shoots declined over the following months, followed by the gradual death of all the material (Figure 3b).

(**a**) (**b**)

Figure 1. Initial in vitro cultures of: (**a**) Golden Delicious; (**b**) Táborita.

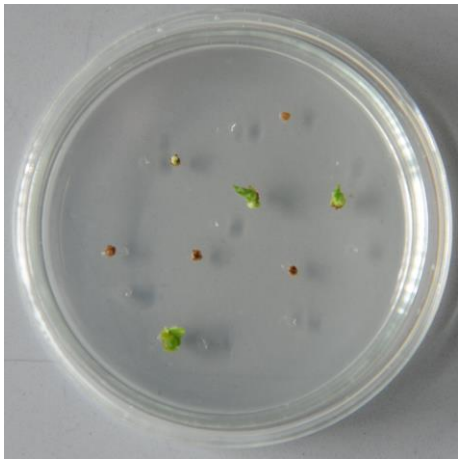
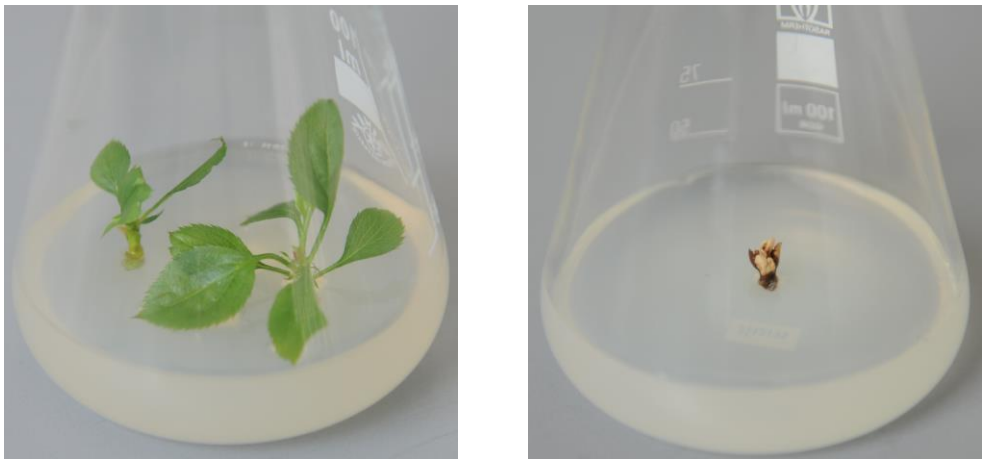


Figure 2. Regenerating shoot tips 4 weeks after thawing.



(**a**) (**b**)

Figure 3. Regenerated in vitro cultures 10 months after thawing: (**a**) Golden Delicious; (**b**) Táborita.

None of the infections found in the original material, i.e., AP phytoplasma, ApMV, and ACSLV, were detected in the regenerated Golden Delicious cultures. On the other hand, in the case of the Táborita cultivar, the tests showed that all the pathogens were still present, although AP phytoplasma was present at very low levels (a Ct value of 30.9), and ApMV and ACSLV also showed a moderate relative decrease (Table 2).

4. Discussion

The fact that the in vitro cultures of the three cultivars, which were derived from phytoplasma-infected plants, tested negative shows the potential of the in vitro explant excision and transfer for phytoplasma elimination. At the beginning of sprouting, the pathogen may not yet have been present in the actively dividing, intensively growing apical parts of the plant shoots, which were used for in vitro culture establishment. We hypothesize that the efficiency of this procedure may depend on the cultivar, the position of the initial scions and buds on the plant, or the time of the in vitro transfer.

The appearance of the initially infected Golden Delicious and Táborita explants suggests that in vitro cultures of different apple cultivars differ in their ability to cope with the infections studied. The poor growth of Táborita could have been caused by the susceptibility of this genotype to the pathogens present in high concentrations in the initial plant material. The relatively healthy and vigorous state of the infected Golden Delicious is rather contradictory to reports by previous researchers that AP phytoplasma causes the stunted growth of cultures derived from this cultivar [17].

The key mechanism of cryotherapy is the selective survival of unique meristematic cells with small vacuoles in the shoot tips, which are more resistant to dehydration and subsequent damage caused by ice crystal formation during freezing due to their distinct morphological structure compared to differentiated cells [13]. In most cases, systemic infections are unable to penetrate the intensively dividing meristematic tissue at the apical tip of the plant. Cryotherapy then cuts off/destroys infected differentiated cells on the principle of a cryo-knife, leaving only healthy cells in the pathogen-free meristematic area, which are capable of further division, differentiation, and regeneration of whole plant organs [4].

In agreement with Romadanova et al. [10], we observed different efficiencies of virus cryotherapy for the two genotypes studied. However, the fact that the initial plant material of the Táborita cultivar was obtained from a long-term-maintained in vitro gene bank must also be taken into account. Long-term maintenance of less intensively growing infected material in the form of explant cultures may have led to pathogen penetration into the meristem. In the case of Táborita, this phenomenon would disrupt the above-mentioned sanitation principle of cryotherapy. On the other hand, the vigorous growth of the Golden Delicious explants may have ensured that the meristematic zone remained virus-free.

The results of the PCR tests were indirectly supported by the observed morphology of the explants: in Golden Delicious, a significant enlargement of the leaves was visually observed, which could be related to an improvement of the health status due to the elimination of the pathogens studied, whereas the growth vigor of Táborita did not improve and even worsened after cryopreservation. We hypothesize that even a fraction of the meristematic cells may have been damaged during cryopreservation, resulting in a phytohormone imbalance and decreased differentiation potential. This fact, together with the effect of persistent systemic pathogens, may have caused the subsequent growth deterioration.

We confirmed the possibility of using cryotherapy for phytoplasma eradication, proposed by Wang et al. [13,14], for apple cultures. However, the results suggest that sanitation does not work equally for different cultivars, and further studies are needed. The possibility of specific interaction between the systemic pathogen and the particular apple genotype as a host may explain the difference in the success rate of the sanitation procedure.

The possible uneven distribution of phytoplasma or virus particles in the plant explants must also be taken into account when performing diagnostics after sanitation procedures, subsequent regeneration, and in vitro cultivation. Repeated testing with a longer time interval or even after planting on a permanent site of mother trees in the nursery will be necessary to intercept the possible re-accumulation of virus particles from low levels of infection to the level of detection.

Our work on the recovery from phytoplasmas in fruit tree species using a cryo-knife can be considered original. Until now, this cryo-eradication mechanism has been mainly

applied to the phytoplasma sanitation of herbaceous species or shrubs [13,14]. Our results represent possible ways to achieve recovery from systemic infections for the genus *Malus*.

During the testing, the use of mixed samples was necessary to obtain a sufficient amount of material for the DNA/RNA isolations. Our approach was also based on the fact that a negative result of a mixed sample implied the absence of the pathogen in each of the individual shoots. A smaller representation and an unequal number of initial samples of each cultivar result from the difficulty of the in vitro propagation of the several apple genotypes used in our study. However, even the acquisition of a sanitated explant and its successful regeneration to ex vitro conditions leads the clonally propagated fruit tree species to the subsequent propagation of large numbers of healthy plants by conventional grafting or inoculation in the nursery.

In the case of material that has undergone cryotherapy, it is also advisable to check the morphological stability several years after the cryotherapy treatment, especially in the case of fruit characters. A temperature range that is physiologically lower than the standard growth temperatures or a higher concentration of cryoprotective solutions in the growth medium may result in a higher frequency of somatic mutations. For example, a decrease in DNA methylation has been reported in apples after cryopreservation [18].

5. Conclusions

The results presented in this study contribute to knowledge in the field of the sanitation of apple cultivars by in vitro cultures combined with subsequent cryotherapy in liquid nitrogen. In the past, the problem of the health status of starting planting material has been significantly underestimated. We expect that the obtained virus- and phytoplasma-free basic apple plants of Golden Delicious clones A and B, Virginia Crab, and Panenské zlepšené will be included in the established system of the certification of healthy planting material. The study will continue with tests of ex vitro-transferred mother plants with a time interval of two years to verify that the sanitation procedure eradicates the pathogens completely and pushes the virus and phytoplasma concentration temporarily below the detection limit of used diagnostic PCR methods.

Solving the problem of the sanitation of fruit tree varieties and obtaining phytoplasma-free plant material, especially varieties indigenous to particular countries, is a condition for the operation of technical isolates as a basic component of each national certification system. Consequently, the competitiveness of the entire commercial nursery industry and fruit production industry also depends on this result. The results and the persistence of AP infection in the Táborita variety indicate the need for further research and the inclusion of other therapeutic procedures, which could be, for example, chemotherapeutic interventions with substances of an antibiotic nature.

Author Contributions: Conceptualization, J.S. and M.S.; methodology, J.S. and M.S.; validation, J.S. and M.S.; formal analysis, M.S., J.S. and M.R.; investigation, M.S., J.S. and M.R.; resources, J.S.; data curation, M.R., M.S. and J.S.; writing—original draft preparation, M.S., J.S. and M.R.; writing—review and editing, J.S. and M.S.; visualization, M.R.; supervision, J.S.; project administration, J.S.; funding acquisition, J.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Agriculture of the Czech Republic, the National Agency for Agricultural Research, grant numbers QK21020395 and QK1910065.

Data Availability Statement: Data available on request.

Acknowledgments: We thank Alexandra Slámová for the media preparation, Jiřina Rachotová for the establishment of the initial cultures, Radek Čmejla for the supervision of the virus and phytoplasma diagnostics, and František Švec for the English proofreading.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Nemcova, V.; Buchtova, I. *Situační a Výhledová Zpráva Ovoce*; Ministry of Agriculture: Praha, Czech Republic, 2022.
2. Hadidi, A.; Barba, M.; Candresse, T.; Jelkmann, W. *Virus and Virus-Like Diseases of Pome and Stone Fruits*; American Phytopathological Society: St. Paul, MN, USA, 2011.
3. Hassan, M.; Polak, J.; Paprstein, F. Detection and distribution of four pome fruit viruses in germplasm collection in the Czech Republic. In Proceedings of the 20th International Symposium on Virus and Virus-Like Diseases of Temperate Fruit Crops, Antalya, Turkey, 22–26 May 2006; International Society for Horticultural Science: Leuven, Belgium, 2008; p. 113. [CrossRef]
4. Jiroutova, P.; Sedlak, J. Cryobiotechnology of Plants: A Hot Topic Not Only for Gene Banks. *Appl. Sci.* **2020**, *10*, 14. [CrossRef]
5. Zamecnik, J.; Faltus, M.; Bilavcik, A. Vitrification Solutions for Plant Cryopreservation: Modification and Properties. *Plants* **2021**, *10*, 17. [CrossRef]
6. Bettoni, J.C.; Souza, J.A.; Volk, G.M.; Dalla Costa, M.; da Silva, F.N.; Kretzschmar, A.A. Eradication of latent viruses from apple cultivar 'Monalisa' shoot tips using droplet-vitrification cryotherapy. *Sci. Hortic.* **2019**, *250*, 12–18. [CrossRef]
7. Nukari, A.; Laamanen, J.; Uosukainen, M.; Lemmetty, A. Comparison of Virus Eradication of Apple Mosaic Virus from Hop by Encapsulation-Dehydration Cryotherapy and Meristem Culture Methods. In Proceedings of the 2nd International Symposium on Plant Cryopreservation, Fort Collins, CO, USA, 11–14 August 2013; International Society for Horticultural Science: Leuven, Belgium, 2014; Volume 1039, pp. 113–119.
8. Kaya, E. Comparison of three different techniques for eradication of Apple mosaic virus (ApMV) from hazelnut (*Corylus avellana* L.). *J. Plant Protect. Res.* **2021**, *61*, 11–19. [CrossRef]
9. Bettoni, J.C.; Fazio, G.; Costa, L.C.; Hurtado-Gonzales, O.P.P.; Al Rwahnih, M.; Nedrow, A.; Volk, G.M.M. Thermotherapy Followed by Shoot Tip Cryotherapy Eradicates Latent Viruses and Apple Hammerhead Viroid from In Vitro Apple Rootstocks. *Plants* **2022**, *11*, 18. [CrossRef]
10. Romadanova, N.V.; Mishustina, S.A.; Gritsenko, D.A.; Omasheva, M.Y.; Galiakparov, N.N.; Reed, B.M.; Kushnarenko, S.V. Cryotherapy as a method for reducing the virus infection of apples (*Malus* sp.). *CryoLetters* **2016**, *37*, 1–9. [CrossRef]
11. Wang, M.R.; Bao, J.H.; Ma, X.Y.; Xie, L.L.; Zhu, L.Y.; Liu, M.Z.; Lu, X.; Zhang, D.; Wang, Q.C. Vitrification Cryo-Foil for Apple Cryopreservation and the Seesaw Effect Between Shoot Recovery and Virus Eradication. *Research Square* **2021**. [CrossRef]
12. Wang, M.R.; Yang, W.; Zhao, L.; Li, J.W.; Liu, K.; Yu, J.W.; Wu, Y.F.; Wang, Q.C. Cryopreservation of virus: A novel biotechnology for long-term preservation of virus in shoot tips. *Plant Methods* **2018**, *14*, 10. [CrossRef]
13. Wang, Q.C.; Valkonen, J.P.T. Efficient elimination of sweetpotato little leaf phytoplasma from sweetpotato by cryotherapy of shoot tips. *Plant Pathol.* **2008**, *57*, 338–347. [CrossRef]
14. Wang, R.R.; Mou, H.Q.; Gao, X.X.; Chen, L.; Li, M.F.; Wang, Q.C. Cryopreservation for eradication of Jujube witches' broom phytoplasma from Chinese jujube (*Ziziphus jujuba*). *Ann. Appl. Biol.* **2015**, *166*, 218–228. [CrossRef]
15. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]
16. Hofer, M. Conservation Strategy of Genetic Resources for Strawberry in Germany. *Acta Hortic.* **2011**, *908*, 421–430. [CrossRef]
17. Bisognin, C.; Ciccotti, A.; Salvadori, A.; Moser, M.; Grando, M.S.; Jarausch, W. In vitro screening for resistance to apple proliferation in Malus species. *Plant Pathol.* **2008**, *57*, 1163–1171. [CrossRef]
18. Hao, Y.J.; Liu, Q.L.; Deng, X.X. Effect of cryopreservation on apple genetic resources at morphological, chromosomal, and molecular levels. *Cryobiology* **2001**, *43*, 46–53. [CrossRef]