UNIVERSIDAD DE MURCIA

Escuela de Doctorado

TESIS DOCTORAL

Combinación de técnicas biotecnológicas para la eliminación de microorganismos en plantas de albaricoquero

Combination of biotechnological techniques for the elimination of microorganisms from apricot plants

AUTOR/A Cristian Pérez Caselles

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A mis padres,

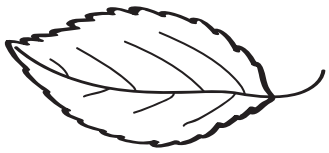
Index

Abbreviations

+ssRNA	Positive-sense single-stranded RNA
ACLSV	*Apple chlorotic leafspot virus*
AgNPs	Silver nanoparticles
ANOVA	Analysis of Variance
ApLV	*Apricot latent virus*
ApMV	*Apple mosaic virus*
ASGV	*Apple stem grooving virus*
ASSVd	*Apple scar skin viroid*
BA	6-benzyladenine
BAP	6-benzylamino-purine
BAP_R	6-benzylamino-purine riboside
BYMV	*Bean yellow mosaic virus*
Ca-PPV	'Canino' infected with PPV
CMV	*Cucumber mosaic virus*
CP	Coat Protein
CRM	Cryotherapy Recovery Medium
CSVd	*Chrysanthemum stunt viroid*
CVA	*Cherry virus A*
DKW	Driver and Kuniyuki Walnut
DMSO	Dimethyl Sulfoxide
dsRNA	Double-stranded RNA
ELISA	Enzyme-Linked Immunosorbent Assay
EPPO	European and Mediterranean Plant Protection Organization
FAOSTAT	Statistics of Food and Agriculture Organization of the United Nations
HLVd	*Hop latent viroid*
HSVd	*Hop stunt viroid*
IBA	Indole-3-Butyric Acid
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectroscopy
ihpRNA	Intron-spliced hairpin RNA
LM	Liquid Medium
LM-$CaCl_2$	Liquid Medium without Calcium Chloride

LN	Liquid Nitrogen
MAPA	Spanish Ministry of Agriculture, Fishing and Food
MeNPs	Metallic nanoparticles
MM1	Meristem Multiplication Medium 1
MM2	Meristem Multiplication Medium 2
MR-HSVd	'Mirlo Rojo' infected with HSVd
MS	Murashige and Skoog
PCA	Principal Component Analysis
PDV	*Prunus dwarf virus*
PNRSV	*Prunus necrotic ringspot virus*
PPV	*Plum pox virus*
PPV-D	Dideron strain of *Plum pox virus*
PPV-M	Marcus strain of *Plum pox virus*
PSTVd	*Potato spindle tuber viroid*
PTGS	Post-Transcriptional Gene Silencing
PVP	Polyvinylpyrrolidone
PVS2	Plant Vitrification Solution 2
PVS3	Plant Vitrification Solution 3
QL	Quoirin and Lepoivre
qPCR	Quantitative Polymerase Chain Reaction
RISC	RNA-Induced Silencing Complex
RM	Rooting Medium
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
siRNA	Small interference RNA
SSM	Semisolid Medium
SSM-$CaCl_2$	Semisolid Medium without Calcium Chloride
TEM	Transmission Electron Microscopy
TIS	Temporary Immersion System
ToRSV	*Tomato ringspot virus*
TRSV	*Tobacco ringspot virus*

Resumen



El albaricoquero (*Prunus armeniaca* L.) es una de las principales especies de *Prunus* cultivadas con mayor importancia económica en España. La Región de Murcia es la principal comunidad autónoma productora y exportadora de albaricoque. Sin embargo, el preocupante estado sanitario de las plantaciones tradicionales pone en riesgo la continuidad de la producción. El desarrollo de nuevos métodos de detección ha demostrado que el albaricoquero se ve afectado por diversas enfermedades causadas por virus y viroides. De entre todos ellos, destacan *Plum pox virus* (PPV), responsable de la enfermedad de la Sharka, y *Hop stunt viroid* (HSVd). El objetivo principal de esta tesis es el desarrollo de aproximaciones biotecnológicas que eliminen PPV y HSVd de variedades de albaricoquero.

La mejor estrategia contra los virus es conferir a la planta resistencias frente a estos patógenos. El CEBAS-CSIC ha desarrollado durante más de 30 años un programa de mejora de albaricoquero y entre sus objetivos principales se encuentra la obtención de variedades resistentes a PPV. Las nuevas variedades resistentes han sido evaluadas para determinados aislados de la cepa Dideron del PPV, sin embargo, su resistencia a cepas más agresivas como Marcus no se ha podido evaluar aún, ya que hasta el año 2024 esta cepa era cuarentenaria y su utilización no estaba permitida en investigación.

Se han desarrollado diferentes estrategias para producir plantas resistentes a Sharka mediante ingeniería genética utilizando construcciones moleculares basadas en diferentes genes del virus. Una de las más efectivas ha sido la construcción h-UTR/P1 que codifica un ihpRNA y confiere resistencia a las principales cepas de PPV mediante un mecanismo de silenciamiento génico post-trascripcional basado en la generación de pequeños ARN de interferencia (siRNA). El grupo de Biotecnología de Frutales del CEBAS-CSIC obtuvo ciruelos transgénicos resistentes a PPV utilizando dicha construcción. Una estrategia interesante para conferir resistencia al virus sería injertar plantas no transformadas sobre esos ciruelos transgénicos resistentes, ya que se ha demostrado que los siRNAs pueden desplazarse por todo el organismo a través del floema.

El objetivo de la Publicación I fue comprobar si la resistencia a PPV podía ser transmitida desde patrones de ciruelo transgénicos a variedades de albaricoquero no transgénicas injertadas sobre ellos. Se realizaron injertos de la variedad ‘Canino’ sobre cuatro patrones de ciruelos transgénicos: dos resistentes a PPV, uno tolerante y uno susceptible como control. Tras un invierno artificial en cámaras de frío, se infectaron los injertos mediante injerto de chapa. La presencia o ausencia de PPV fue evaluada cada ciclo vegetativo mediante RT-PCR. Los resultados demostraron que la resistencia al PPV basada en el silenciamiento de ARN podía transmitirse desde los patrones de ciruelo resistentes a PPV a los injertos de albaricoquero no transgénicos. La eficacia de la técnica aumenta tras sucesivos ciclos de crecimiento, alcanzando un 92,0% y 83,9% de plantas libres de PPV, sobre los dos patrones resistentes, tras cuatro ciclos vegetativos. Asimismo, se observó una acumulación de siRNAs de 24 nucleótidos en los injertos sobre patrones resistentes que no ocurrió en los injertos sobre el patrón susceptible. Esta aproximación proporciona a los albaricoqueros resistencia frente al PPV, permitiendo su recuperación frente a futuras infecciones. Además, podría ser una estrategia interesante en especies que carecen de fuentes naturales de resistencia y/o son recalcitrantes a la transformación. Finalmente, el uso de patrones transgénicos elimina la posibilidad de dispersión de los transgenes a través del polen, ya que esas plantas no florecerán. También disminuye la posible preocupación por el consumo de alimentos transgénicos, ya que la variedad comercial que produce el fruto no está transformada genéticamente.

Aunque la transmisión de la resistencia del patrón al injerto ha sido efectiva con albaricoquero, existe un veto, a nivel práctico, a la comercialización de plantas modificadas genéticamente

en la Unión Europea, lo que imposibilita adoptar esta estrategia para realizar plantaciones comerciales. Una alternativa a la transformación genética sería la utilización de material certificado libre de patógenos en las nuevas plantaciones, lo que ayudaría a evitar la difusión de enfermedades. Para ello es necesario contar con distintos protocolos biotecnológicos que permitan la eliminación de virus y viroides de variedades de albaricoquero. Asimismo, disponer de estos protocolos ayudaría a la preservación del germoplasma y permitiría el intercambio de material vegetal evitando el peligro de difusión de nuevas enfermedades.

El cultivo de meristemos, también conocido como rescate de meristemos, es la técnica que se ha utilizado tradicionalmente para el saneamiento de material vegetal. Consiste en la regeneración de plantas completas a partir de los meristemos apicales o axilares, que son zonas de tejido indiferenciado con alta capacidad de división celular. El éxito de este procedimiento radica en que las células meristemáticas suelen estar libres de microorganismos. En el Anexo II se estudió la eficiencia del rescate de meristemos en brotes de albaricoquero *in vitro* para eliminar PPV y HSVd, utilizando 'Canino' infectado con PPV (Ca-PPV) y 'Mirlo Rojo' infectado con HSVd (MR-HSVd). La evaluación por RT-PCR mostró que el cultivo de meristemos no fue capaz de eliminar PPV ni HSVd en ningún caso. Esto se debe a que existen patógenos muy virulentos capaces de infectar rápidamente la región meristemática. La combinación del cultivo de meristemos con otras técnicas es necesaria para aumentar la eficiencia de eliminación de PPV y HSVd en albaricoquero.

Se ha demostrado la capacidad antimicrobiana de las AgNPs frente a diversos microorganismos como bacterias, hongos o virus. La aplicación pulverizada de AgNPs sobre la planta como tratamiento preventivo a la infección por virus redujo o eliminó por completo la aparición de síntomas de enfermedades en diversos estudios con especies vegetales. Sin embargo, las AgNPs no se han utilizado anteriormente como tratamiento curativo. El objetivo de la Publicación II fue el desarrollo de un protocolo de micropropagación que permitiese la introducción de AgNPs en el tejido vegetal de albaricoquero. La plata metálica, medida con la técnica ICP-OES, no se incorporó a las plantas cuando se añadieron AgNPs al medio semisólido. Se desarrolló un protocolo de micropropagación de albaricoquero en medio líquido basado en un Sistema de Inmersión Temporal que utiliza biorreactores Plantform®. Las condiciones utilizadas durante la fase de proliferación fueron dos minutos de inmersión cada seis horas y tres minutos de aireación cada tres horas. El medio de cultivo líquido sin cloruros permitió la introducción de AgNPs en las plantas, siendo la concentración de plata metálica 23 veces mayor que en el control.

En la Publicación III se comprobó si las AgNPs afectaban la proliferación del albaricoquero y se examinó la concentración de AgNPs en el medio de cultivo con la que se conseguía una mayor concentración de plata metálica en la planta. Se cultivaron brotes de Ca-PPV y MR-HSVd en medio líquido sin cloruros con la adición de 0, 25, 50, 75 y 100 mg L^{-1} de AgNPs. El análisis ICP-OES determinó que la concentración de AgNPs que maximiza la concentración de plata metálica fue 50 mg L^{-1} en Ca-PPV (35,7 mg kg^{-1}) y 75 mg L^{-1} en MR-HSVd (35,5 mg kg^{-1}). Para evaluar la posible fitotoxicidad de las AgNPs en la micropropagación de albaricoquero se midió la proliferación, longitud del tallo, productividad, peso fresco y seco, superficie foliar y la concentración de nutrientes de la planta. Los resultados mostraron que la exposición a AgNPs tuvo un efecto hormético que condujo a un aumento de la proliferación, producción de biomasa y acumulación de nutrientes y no se observó fitotoxicidad en ninguna de las concentraciones ensayadas.

En la Publicación IV se estudió el efecto de las AgNPs sobre la eliminación de PPV y HSVd. Brotes de Ca-PPV y MR-HSVd *in vitro* se cultivaron en medio líquido sin cloruros con la adición

de 25, 50, 75 y 100 mg L^{-1} durante cuatro y ocho semanas. A la finalización de los tiempos de exposición, se realizó un rescate de meristemos de 1,5 milímetros. Los meristemos se cultivaron en medios de regeneración y crecieron hasta convertirse en brotes con supervivencias entre un 22,6% y 36,7% en MR-HSVd y un 15,1% y 42,2% en Ca-PPV. No fue posible la eliminación de HSVd siguiendo esta metodología. Sin embargo, se encontraron plantas libres de PPV en la mayoría de los tratamientos, alcanzando la mayor eficiencia en el tratamiento de 75 mg L^{-1} durante ocho semanas (75,0%).

La técnica más utilizada para la eliminación de virus en plantas es la termoterapia combinada con el cultivo de meristemos. Consiste en exponer a las plantas a regímenes de altas temperaturas y rescatar meristemos a la finalización de los tiempos de exposición. Asimismo, la etiolación es el proceso que ocurre en las plantas ante la ausencia de una fuente de luz provocando la elongación de los tallos, entre otras características. En la Publicación V se evaluó el efecto de la termoterapia, la etiolación y la combinación de ambas seguida de un cultivo de meristemos sobre la eliminación de PPV y HSVd de brotes de Ca-PPV y MR-HSVd *in vitro*. La termoterapia consistió en la alternancia de períodos de cuatro horas a 38 ºC y 32 ºC durante 30, 35, 40 y 45 días, tras una aclimatación de un día a 28 ºC y dos días a 30 ºC. En el tratamiento de etiolación, las plantas crecieron en condiciones de oscuridad durante ocho semanas. El tratamiento combinado consistió en 45 días de termoterapia, como se ha descrito anteriormente, y en condiciones de oscuridad. Al final de cada tratamiento se rescataron meristemos de 1,5 milímetros y se cultivaron en medios de regeneración. Se evaluó la presencia o ausencia de PPV o HSVd mediante RT-PCR en aquellos meristemos que se desarrollaron hasta brotes. El tratamiento combinado y el de 45 días de termoterapia consiguieron las mejores eficiencias de eliminación de PPV (75,0% y 66,7%, respectivamente). Se necesitaron al menos 40 días de termoterapia para obtener plantas libres de HSVd, aunque la mejor eficiencia se obtuvo tras 45 días de termoterapia (22,7%).

La crioterapia consiste en la exposición de explantes a temperaturas ultrabajas (inmersión en nitrógeno líquido). En el Anexo I se estudió el efecto de la crioterapia sobre la eliminación de PPV en brotes de Ca-PPV *in vitro*. Se desarrolló un protocolo de crioterapia aplicado a meristemos basado en la técnica "vitrificación en gota" utilizando la solución vitrificante PVS2. Se rescataron meristemos de tres milímetros y se cultivaron tres días en medio de precultivo. A continuación, los meristemos se transfirieron a la "solución de carga" durante 20 minutos y PVS2 durante 75 minutos antes de introducirlos en nitrógeno líquido por una hora. Los meristemos que sobrevivieron el proceso se cultivaron en medios de regeneración y crecimiento hasta convertirse en brotes con una supervivencia del 6,0%. A pesar de la baja supervivencia, el protocolo permitió la eliminación de PPV con una eficiencia del 81,9%. No obstante, debido a la presencia de DMSO en la solución PVS2, sería conveniente evitar su uso desarrollando protocolos con diferentes soluciones vitrificantes o utilizando otras técnicas, como la "encapsulación-deshidratación".

Todas las publicaciones de esta tesis y el Anexo I tienen en común la producción de plantas de albaricoquero libres de algún patógeno. La primera publicación describe una estrategia basada en patrones transgénicos resistentes al virus de la Sharka que da lugar a plantas que eliminan el PPV silenciando parte de su genoma. El resto de las publicaciones y el Anexo I describen diferentes técnicas que, combinadas con el cultivo de meristemos, dan lugar a plantas de albaricoquero libres de PPV y/o HSVd. Además, las publicaciones II, III y IV están íntimamente relacionadas, pues mientras que en la II se estudia la manera de introducir la plata en la planta, en la III se determina el efecto de las AgNPs sobre la micropropagación y, finalmente, en la IV se estudia si las AgNPs son efectivas eliminando los patógenos estudiados.

Todo el trabajo presentado en esta tesis ha permitido llegar a las siguientes conclusiones:

1. El cultivo de una variedad de albaricoquero no transgénica sobre patrones de ciruelo transformados resistentes a Sharka, producidas silenciando mediante RNAi, es una estrategia válida y efectiva para transmitir la resistencia al PPV. Esta aproximación proporciona al albaricoquero resistencia al virus permitiéndole recuperarse de futuras infecciones. Asimismo, podría ser una estrategia interesante para especies que carecen de fuentes de resistencia naturales y son recalcitrantes a la transformación. Finalmente, la variedad comercial que produce el fruto no es transgénica, lo que evitaría el consumo de productos modificados genéticamente.

2. El cultivo de meristemos no es una técnica efectiva para la eliminación de PPV y HSVd en variedades de albaricoquero debido a su eficiencia muy baja o nula. Es necesario combinarlo con tratamientos adicionales para obtener plantas libres de patógenos.

3. El uso de AgNPs permite la producción de plantas de 'Canino' libres de PPV con una alta eficiencia tras el cultivo de meristemos de las plantas tratadas. Sin embargo, es necesario cultivar los brotes de albaricoquero en un medio líquido modificado (sin cloruros) utilizando un sistema de inmersión temporal. La adición de AgNPs al medio líquido modificado no tuvo un efecto perjudicial en la micropropagación de la planta, de hecho, se observó un efecto hormético que condujo a un aumento en la proliferación y producción de biomasa.

4. Los tratamientos de termoterapia seguidos del cultivo de meristemos permitieron la producción de plantas de albaricoquero libres de PPV y HSVd. Se necesitaron tiempos de exposición más largos para la eliminación del viroide en comparación con la del virus. El tratamiento de etiolación mostró una baja eficiencia en la erradicación de PPV. La combinación de termoterapia y etiolación no mejoró significativamente la eficiencia alcanzada por la termoterapia.

5. La aplicación de crioterapia a meristemos de albaricoquero mediante "vitrification en gota" utilizando PVS2 consiguió eliminar el PPV con una alta eficiencia. El procedimiento afectó significativamente la supervivencia de los meristemos de albaricoquero. Mejorar la supervivencia es esencial para utilizar este protocolo con el objetivo de criopreservar. Además, sería conveniente desarrollar nuevos protocolos que eviten el uso de DMSO, como la PVS3 o la técnica de "encapsulación-deshidratación".

6. Se ha eliminado eficazmente el PPV utilizando diversas estrategias, incluyendo termoterapia (66,7%), etiolación (18,7%), combinación de termoterapia y etiolación (75,0%), crioterapia (81,9%) y aplicando AgNPs (75,0%). La selección de la técnica más adecuada dependerá de varios factores, como el patógeno, la variedad o especie infectada, la disponibilidad de equipos o la cualificación del personal.

7. En esta tesis, solo se ha podido eliminar eficazmente el HSVd mediante termoterapia, ya que la aplicación de AgNPs y la etiolación no produjeron plantas libres de viroides.

Abstract

The apricot tree (*Prunus armeniaca* L.) is one of the main cultivated *Prunus* species with significant economic importance in Spain. Murcia is the leading region in apricot production and exports in Spain. However, the concerning health status of traditional orchards compromises the continuity of production. The development of new detection methods has demonstrated that apricots are affected by various diseases caused by viruses and viroids. Among them, *Plum pox virus* (PPV), responsible for Sharka disease, and *Hop stunt viroid* (HSVd) stand out. The main goal of this thesis is the development of biotechnological approaches to eliminate PPV and HSVd from apricot cultivars.

The best strategy against viruses in plants is to confer resistance to these pathogens. CEBAS-CSIC has been breeding apricots for over 30 years and one of its main objectives has been the development of PPV-resistant cultivars. The new resistant varieties have been evaluated for certain isolates of the Dideron strain of PPV. However, their resistance to more aggressive strains such as Marcus has not yet been assessed, as this strain was classified as a quarantine pathogen and was not allowed for research use until 2024.

Different strategies have been developed to produce Sharka-resistant plants through genetic engineering using molecular constructs based on different viral genes. One of the most effective approaches has been the h-UTR/P1 construct, which encodes an ihpRNA and confers resistance to major PPV strains via a post-transcriptional gene silencing mechanism through the generation of small interfering RNAs (siRNAs). The Group of Fruit Trees Biotechnology at CEBAS-CSIC obtained PPV-resistant transgenic plums using this molecular construct. A promising strategy to confer virus resistance would be grafting non-transformed plants onto these resistant transgenic plums, as it has been demonstrated that siRNAs can move throughout the organism via the phloem.

The objective of publication I was to determine whether PPV resistance could be transmitted from transgenic plum rootstocks to non-transgenic cultivars grafted onto them. 'Canino' apricot was grafted onto four transgenic plum rootstocks: two PPV-resistant, one tolerant, and one susceptible (control). After an artificial winter in cold chambers, the grafts were infected via chip budding. The presence or absence of PPV was evaluated in each vegetative cycle using RT-PCR. The results demonstrated that the resistance to PPV based on RNA silencing was transmitted from PPV-resistant plum rootstocks to non-transgenic apricot scions. The efficiency increased after successive growth cycles, reaching 92.0% and 83.9% of PPV-free plants on the two resistant rootstocks after four vegetative cycles. Additionally, a 24-nucleotide siRNA accumulation was observed in grafts on resistant rootstocks but not in those on the susceptible rootstock. This approach provides apricot trees with resistance to PPV, allowing them to recover from future infections. Moreover, it could be an interesting strategy for species that lack natural resistance and, additionally, it would allow the transfer of resistance to cultivars or species recalcitrant to transformation. Finally, the use of transgenic rootstocks eliminates the risk of transgene spread via pollen, as these plants will not flower. It also reduces concerns regarding genetically modified food consumption, as the commercial fruit-producing variety is not genetically modified.

Although transmission of resistance from rootstocks to grafts has been effective in apricot, the commercialisation of genetically modified plants is currently banned in the European Union, preventing the adoption of this strategy for commercial plantations. An alternative to genetic transformation would be the use of certified pathogen-free material in new plantations, as it would help to prevent the spread of the disease. Biotechnological protocols are needed to eliminate viruses and viroids from apricot cultivars. Furthermore, these protocols would aid

in germplasm preservation and allow the exchange of plant material while avoiding the risk of spreading new diseases.

Meristem culture, also known as meristem rescue, is the traditional technique used for cleaning plant material. It involves regenerating whole plants from apical or axillary meristems, which are undifferentiated tissue areas with a high cell division capacity. The success of this procedure lies in the fact that meristematic cells are usually free of microorganisms. In Annex II, the efficiency of meristem rescue in *in vitro* apricot shoots was assessed for eliminating PPV and HSVd, using PPV-infected 'Canino' (Ca-PPV) and HSVd-infected 'Mirlo Rojo' (MR-HSVd). RT-PCR evaluation showed that meristem culture was unable to eliminate PPV or HSVd in any case. This is due to the capacity of highly virulent pathogens for rapidly infecting the meristematic region. Combining meristem culture with other techniques is necessary to improve the efficiency of PPV and HSVd elimination in apricot.

The antimicrobial properties of silver nanoparticles (AgNPs) have been demonstrated against various microorganisms, including bacteria, fungi, and viruses. The application of AgNPs as a preventative treatment against viral infection reduced or completely eliminated disease symptoms in several plant species. However, AgNPs had not previously been used as a curative treatment. The objective of publication II was to develop a micropropagation protocol that would allow the introduction of AgNPs into plant tissue. Metallic silver, measured using ICP-OES, was not incorporated into plants when AgNPs were added to semisolid medium. An apricot micropropagation protocol based on liquid medium was developed using a Temporary Immersion System (TIS) with Plantform® bioreactors. The proliferation phase involved two-minute immersion every six hours and three-minute aeration every three hours. The liquid medium was modified by removing chlorides, which allowed the introduction of AgNPs into the plants, increasing metallic silver concentration 23-fold compared to the control.

In publication III, the effect of AgNPs on apricot proliferation was evaluated, and the AgNP concentration in the culture medium that maximised metallic silver uptake in plants was determined. Shoots of Ca-PPV and MR-HSVd were cultivated in chloride-free liquid medium with 0, 25, 50, 75, and 100 mg L^{-1} AgNPs. ICP-OES analysis determined that the AgNP concentration maximising metallic silver accumulation was 50 mg L^{-1} in Ca-PPV (35.7 mg kg^{-1}) and 75 mg L^{-1} in MR-HSVd (35.5 mg kg^{-1}). To assess possible AgNP phytotoxicity in apricot micropropagation, the proliferation rate, shoot length, productivity, fresh and dry weight, leaf area, and nutrient concentration were measured. The results indicated that AgNP exposure induced a hormetic effect, leading to increased proliferation, biomass production, and nutrient accumulation, with no phytotoxicity observed at any tested concentration.

The effect of AgNPs on PPV and HSVd elimination was investigated in publication IV. *In vitro* Ca-PPV and MR-HSVd shoots were cultivated in chloride-free liquid medium with 25, 50, 75, and 100 mg L^{-1} AgNPs for four and eight weeks. At the end of the exposure times, 1.5 mm meristems were rescued and cultured in regeneration media, developing into shoots with survival rates between 22.6% and 36.7% for MR-HSVd and 15.1% and 42.2% for Ca-PPV. HSVd elimination was not achieved using this methodology. However, PPV-free plants were obtained in most treatments, with the highest efficiency in the 75 mg L^{-1} treatment for eight weeks (75.0%).

The most widely used technique for virus elimination in plants is thermotherapy combined with meristem culture. This method involves exposing plants to high-temperature regime followed by a meristem rescue at the end of the exposure time. Likewise, etiolation is the process that occurs in plants in the absence of a light source, leading to stem elongation, among other characteristics.

In publication V, the effect of thermotherapy, etiolation, and their combination, followed by meristem culture, was evaluated for the elimination of PPV and HSVd from *in vitro* Ca-PPV and MR-HSVd shoots. Thermotherapy consisted in alternating four-hour periods at 38°C and 32°C for 30, 35, 40, and 45 days, following an acclimatisation phase of one day at 28°C and two days at 30°C. In the etiolation treatment, plants were grown in darkness for eight weeks. The combined treatment involved 45 days of thermotherapy, as previously described, under dark conditions. At the end of each treatment, 1.5 mm meristems were rescued and cultured in regeneration media. The presence or absence of PPV or HSVd was assessed using RT-PCR in meristems that developed into shoots. The combined treatment and the 45-days thermotherapy achieved the highest PPV elimination efficiencies (75.0% and 66.7%, respectively). At least 40 days of thermotherapy were needed to obtain HSVd-free plants, although the best efficiency was achieved after 45 days of thermotherapy (22.7%).

Cryotherapy involves exposing explants to ultra-low temperatures (immersion in liquid nitrogen). The effect of cryotherapy on PPV elimination in *in vitro* Ca-PPV shoots was examined in Annex I. A cryotherapy protocol was developed for meristems using the droplet-vitrification technique with the vitrification solution PVS2. Meristems of 3 mm were rescued and cultured for three days in preculture medium. The meristems were then transferred to the loading solution for 20 minutes and PVS2 for 75 minutes, before being immersed in liquid nitrogen for one hour. The surviving meristems were cultured in regeneration media until they developed into shoots, achieving a survival rate of 6.0%. Despite the low survival rate, the protocol achieved PPV elimination with an efficiency of 81.9%. However, due to the presence of DMSO in the PVS2 solution, it would be desirable to develop protocols with alternative vitrification solutions or employing other techniques, such as encapsulation-dehydration.

All publications and Annex I share the goal of producing pathogen-free apricot plants. The first publication describes a strategy based on transgenic rootstocks resistant to Sharka virus, resulting in plants that eliminate PPV by silencing part of its genome. The remaining publications and Annex I detail various techniques which, when combined with meristem culture, produce PPV and/or HSVd-free apricot plants. Moreover, publications II, III, and IV are closely related. Publication II investigates the method of introducing silver into the plant, publication III assesses the effect of AgNPs on micropropagation, and publication IV examines whether AgNPs are effective in eliminating the studied pathogens.

The results of this thesis have led to the following conclusions:

1. Cultivating a non-transgenic apricot cultivar onto transformed plum rootstocks resistant to Sharka, obtained through RNAi-mediated silencing, is a valid and effective strategy to transfer the resistance to PPV to the apricot scion. This approach provides the apricot tree with resistance to the virus, allowing it to recover from future reinfections. Additionally, it could be an interesting strategy for species that lack natural resistance sources and are recalcitrant to transformation. Finally, the commercial variety producing the fruit is not transgenic, which helps address public concerns regarding the consumption of genetically modified products.

2. Meristem culture is not an effective technique for the elimination of PPV and HSVd in apricot cultivars due to its very low or negligible efficiency. It needs to be combined with additional treatments in order to produce pathogen-free plants.

3. Using AgNPs allows the production of PPV-free ‘Canino’ plants with high efficiency after rescuing meristems from treated plants. However, it is necessary to culture apricot shoots in modified liquid medium (without chlorides) using TIS. The addition of AgNPs to the modified

liquid medium did not have a detrimental effect on the plant micropropagation; instead, a hormetic effect was observed, leading to increased proliferation and biomass production.

4. Thermotherapy treatments followed by meristem culture enabled the production of PPV- and HSVd-free apricot plants. Longer exposure times were necessary for viroid elimination than for virus eradication. The etiolation treatment showed low PPV eradication efficiency. The combined thermotherapy and etiolation treatment did not significantly improve the efficiency achieved by thermotherapy.

5. The application of shoot tip cryotherapy using droplet-vitrification with PVS2 successfully eliminated PPV with high efficiency. Cryotherapy procedures significantly affect meristem survival from apricot plants. Improving meristem survival would be essential for an efficient cryopreservation protocol. Furthermore, developing new protocols that avoid the application of DMSO, such as using PVS3 or the encapsulation-dehydration technique, would be desirable.

6. PPV has been effectively eliminated using several strategies, including thermotherapy (66.7%), etiolation (18.7%), a combination of thermotherapy and etiolation (75.0%), cryotherapy (81.9%), and AgNPs application (75.0%). Selection of the most appropriate approach will depend on different factors such as pathogen, infected cultivar/species, equipment available or staff expertise.

7. HSVd has been effectively eliminated only using thermotherapy in this thesis where AgNPs and etiolation did not produce viroid-free plants.

Introduction

1. Apricot: taxonomy, physiology and economic impact

Stone fruits are a group of species belonging to the family *Rosaceae*, subfamily *Prunoideae*, and genus *Prunus*. The main cultivated species of this genus ranked by global production volume in 2022 (Figure 1A) are: peach (*Prunus persica* (L.) Batsch), plum (*Prunus domestica* L. and *Prunus salicina* Lindl), almond (*Prunus dulcis* (Mill.) D.A. Webb), apricot (*Prunus armeniaca* L.), and cherry (*Prunus avium* L.). The apricot tree originates from Central Asia and its cultivation has now spread across all five continents. Its fruit is a drupe, round, fleshy, yellow or orange in colour with a red blush, and is highly valued for both fresh consumption and processing (Agustí, 2010).

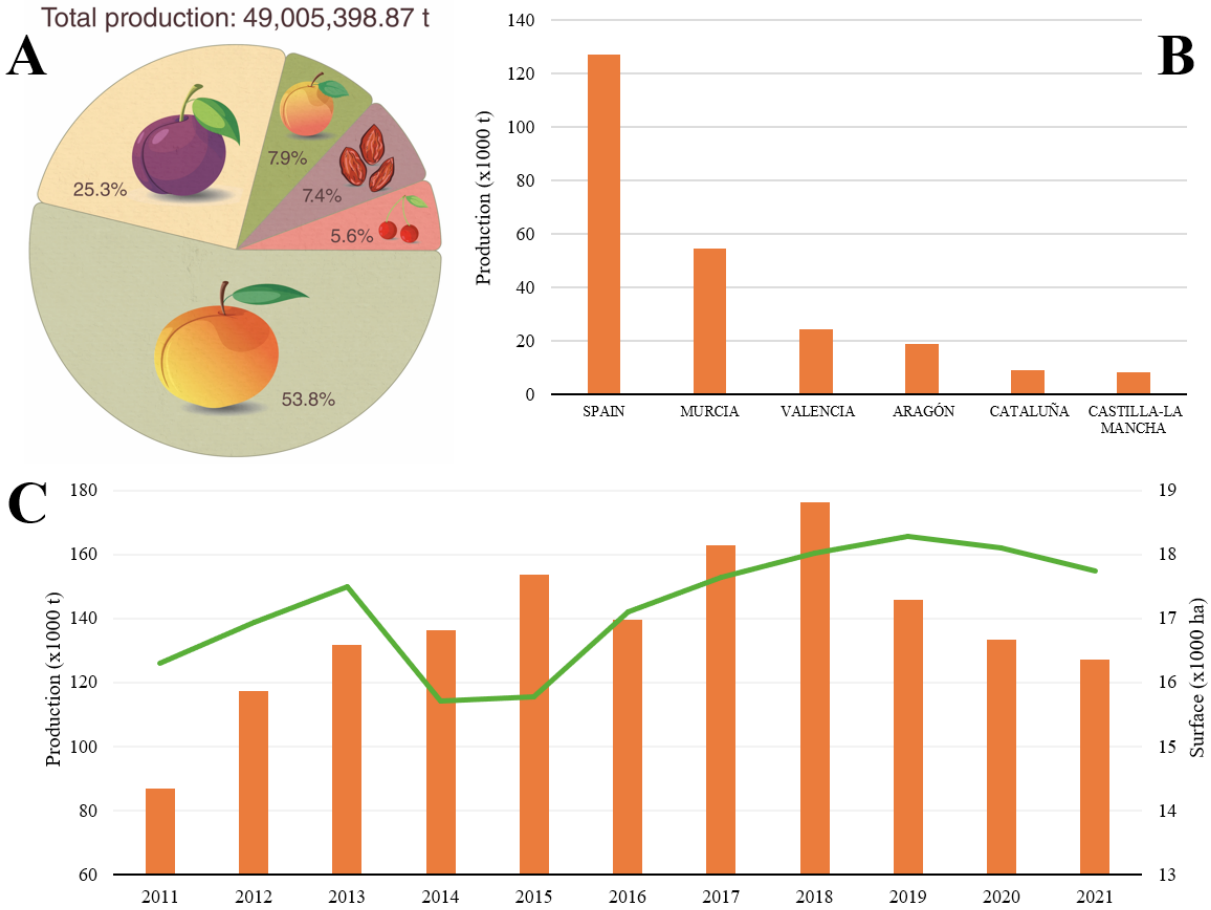


Figure 1. A) Representation of global stone fruit production in 2021 (FAOSTAT, 2024). B) Apricot production in the main Spanish regions in 2021 (MAPA, 2024). C) Evolution of apricot production (bars) and cultivated area (green line) in Spain between 2011 and 2021 (MAPA, 2024).

Spain has a strong tradition of apricot cultivation, as it offers various areas with ideal soil and climate conditions to meet the needs of these species, ensuring consistent, early, high-quality yields with good returns (Egea & Ruíz, 2014). The Region of Murcia is the leading Spanish area in production, with 54,510 tonnes, achieving one of the highest yields (Figure 1B). In 2021, Spain was the 8th largest producer globally, with a production of 127,218 tonnes. Until a few years ago, Spain ranked among the top five global producers. This decline is attributed to negative trends in both production and cultivated area (Figure 1C) as well as the emergence of new market competitors, such as Iran and Pakistan.

2. *In vitro* culture of apricot

In vitro culture is a biotechnological technique involving the cultivation of plant organs, tissues, cells, or embryos in an artificial culture medium under controlled and sterile conditions. *In vitro* culture has a wide range of applications, such as germplasm conservation, micropropagation, genetic transformation, and the production of pathogen-free plants (Cheong, 2012).

2.1. Micropropagation

Micropropagation is a technique for obtaining many genetically identical plants from a single individual. George & Debergh (2007) describe micropropagation in four stages (Figure 2).

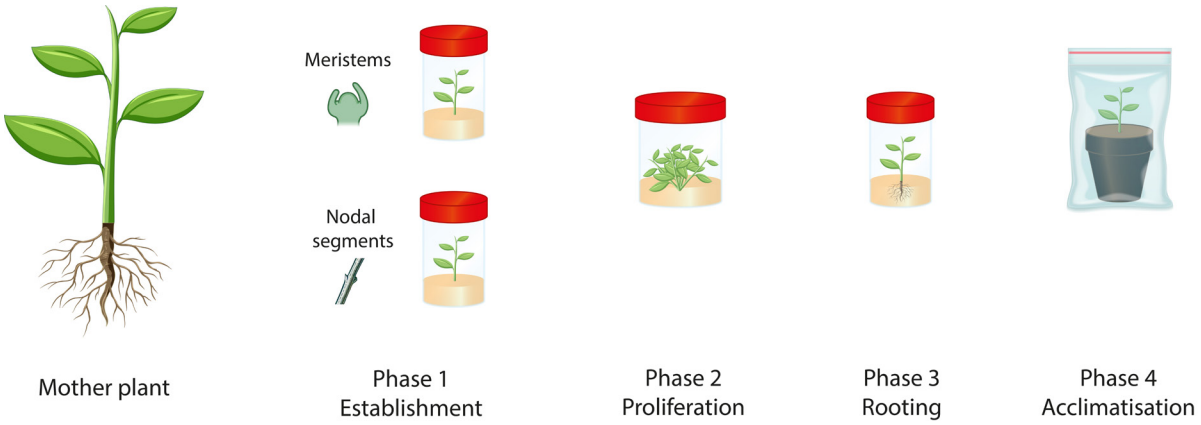


Figure 2. Stages of Micropropagation.

Firstly, a healthy mother plant with the desired characteristics is selected to introduce the plant material *in vitro*. Phase 1, or the establishment phase, involves selecting explants from the mother plant (usually nodal segments or meristems), sterilising them, and placing them on an *in vitro* culture medium under aseptic conditions. The plant material undergoes disinfection treatments under controlled conditions to minimise exogenous contamination and maximise the survival and quality of the explants (Debergh & Maene, 1981).

Phase 2, or the proliferation phase, consists of the multiplication of shoots from the original explant. Using growth regulators (cytokinins play a significant role) induces the formation and development of multiple vegetative buds, resulting in mass production of clonal material (George & Debergh, 2007). Phase 3, or the rooting phase, entails transferring the shoots to a specific culture medium that promotes root development. Auxins are typically used to stimulate the formation of the root system. Finally, Phase 4, or the acclimatisation phase, involves transferring the rooted plants to natural conditions. This requires the gradual adaptation of the plants to external light, temperature, and humidity to ensure their survival and development (George & Debergh, 2007).

Micropropagation plays a key role in the production of woody plants, offering numerous advantages over conventional vegetative propagation (Rathore et al., 2004). The main advantages of apricot micropropagation include the rapid production of genetically uniform plants at any time of the year, space optimisation, genetic material preservation, and the propagation of improved rootstocks and cultivars.

The Fruit Tree Biotechnology Group at CEBAS-CSIC has extensive experience in the *in vitro* culture of apricots and has developed efficient protocols for the different micropropagation

stages of this species: introduction of plant material (Pérez-Tornero & Burgos, 2007), proliferation (Wang et al., 2013), rooting and acclimatisation (Pérez-Tornero & Burgos, 2000).

2.2. Temporary Immersion System

An alternative to the traditional semisolid agar-based culture is the Temporary Immersion System (TIS). The TIS is an *in vitro* cultivation system that involves the intermittent immersion of explants in liquid medium under controlled conditions. The containers housing the explants are known as bioreactors. Bioreactors are usually automated and ensure aseptic as well as optimal environmental growth conditions (Vidal & Sánchez, 2019).

There are numerous types of bioreactors, depending on the type of immersion (continuous or temporary), the presence of aeration, as well as their structure and design (Georgiev et al., 2014). One of the most widely used today is Plantform® (Welander et al., 2014). This bioreactor is separated in two compartments: an upper chamber where the explants are placed and a lower chamber that holds the liquid medium. The nutrition of the explants occurs when a filtered air source is introduced into the lower chamber, moving the liquid medium into the upper chamber, immersing the explants. The key feature of Plantform® is that it allows an additional source of filtered air to enter the upper chamber, pushing the medium back into the lower chamber while simultaneously enabling drying the explants. Aeration of explants reduce hyperhydration alterations, particularly in sensitive species such as apricot (Pérez-Tornero et al., 2001).

3. Pathogens affecting apricots

The apricot tree is severely affected by various diseases caused by viruses and viroids. The development of detection methods (nucleic acid-based PCR and high-throughput sequencing) has enabled the identification of new pathogens infecting this species. Table 1 shows the main pathogens affecting apricot trees, organised by family, genus, and their characteristics. It includes species from various families of both, viruses and viroids. Most pathogens have a +ssRNA genome and the primary transmission system is through vegetative propagation.

Among viruses and viroids infecting apricots in this thesis we have focused on two that cause important damage in apricot production: *Plum pox virus* (PPV) and *Hop stunt viroid* (HSVd). The following sections provide a detailed description of these pathogens.

3.1. *Plum pox virus*

PPV belongs to the *Potyviridae* family and the *Potyvirus* genus. Its genome consists of a +ssRNA with helical symmetry, comprising approximately 9,800 nucleotides (Figure 3A and 3B; García et al., 2014). This genome encodes a polyprotein that is processed into various functional proteins related to RNA replication (VPg, Nlb), structural components like the capsid (CP), or virus mobility and transmission (6K1, 6K2, Cl) (Figure 3C; Sochor et al., 2012). PPV exhibits significant genetic diversity, which García et al. (2024) classify into ten different strains based on their biological, serological, and genomic sequence characteristics: Dideron (D), Marcus (M), El Amar (EA), Cherry (C), Recombinant (Rec), Turkey (T), Winona (W), Cherry Russian (CR), Cherry Volga (CV), and Ancestor (An). To date, the only endemic strain present in Spain is the PPV-D or common type, although a few isolated outbreaks of PPV-M have been detected. These outbreaks have been eradicated swiftly.

Table 1. Main viruses and viroids affecting cultivated apricots. Adapted from Rubio et al. (2017) and Szabó et al. (2024).

Family	Genus	Species (Abbreviation)	Genome	Particle shape	Transmission	Vectors
Secoviridae	*Cheravirus*	*Cherry rasp leaf virus* (CRLV)	+ssRNA	Icosahedral	1	
Bromoviridae	*Ilarvirus*	*Prune dwarf virus* (PDV)	+ssRNA	Icosahedral	1, 2, 3	
		Prunus necrotic ringspot virus (PNRSV)	+ssRNA	Icosahedral	1, 2, 3	
		Apple mosaic virus (ApMV)	+ssRNA	Icosahedral	1	
		American plum line pattern virus (APLPV)	+ssRNA	Icosahedral	1	
Closteroviridae	*Ampelovirus*	*Plum bark necrosis stem pitting-associated virus* (PBNSPaV)	+ssRNA	Filamentous	1	
Potyviridae	*Potyvirus*	*Plum pox virus* (PPV)	+ssRNA	Filamentous	1, 4	Aphids
Betaflexiviridae	*Capillovirus*	*Cherry virus A* (CVA)	+ssRNA	Filamentous	1	
	Foveavirus	*Apricot latent virus* (ApLV)	+ssRNA	Filamentous	1	
	Robigovirus	*Cherry green ring mottle virus* (CGRMV)	+ssRNA	Filamentous	1	
		Cherry necrotic rusty mottle virus (CNRMV)	+ssRNA	Filamentous	1	
		Cherry rusty mottle associated virus (CRMaV)	+ssRNA	Filamentous	1	
		Apple chlorotic leafspot virus (ACLSV)	+ssRNA	Filamentous	1	
		Apricot pseudo chlorotic leafspot virus (APCLSV)	+ssRNA	Filamentous	1	
	Trichovirus	*Cherry mottle leaf virus* (CMLV)	+ssRNA	Filamentous	1, 4	*Eriophyid* mites
		Peach mosaic virus (PcMV)	+ssRNA	Filamentous	1, 4	*Eriophyid* mites
	Prunevirus	*Apricot vein clearing associated virus* (AVCaV)	+ssRNA	Filamentous	1	
Pospiviroidae	*Hostuviroid*	*Hop stunt viroid* (HSVd)	ssRNA	Circular	1	
Avsunviroidae	*Pelamoviroid*	*Peach latent mosaic viroid* (PLMVd)	ssRNA	Circular	1	

Transmission: 1, Vegetative; 2, Pollen; 3, Seeds; 4, Vectors.

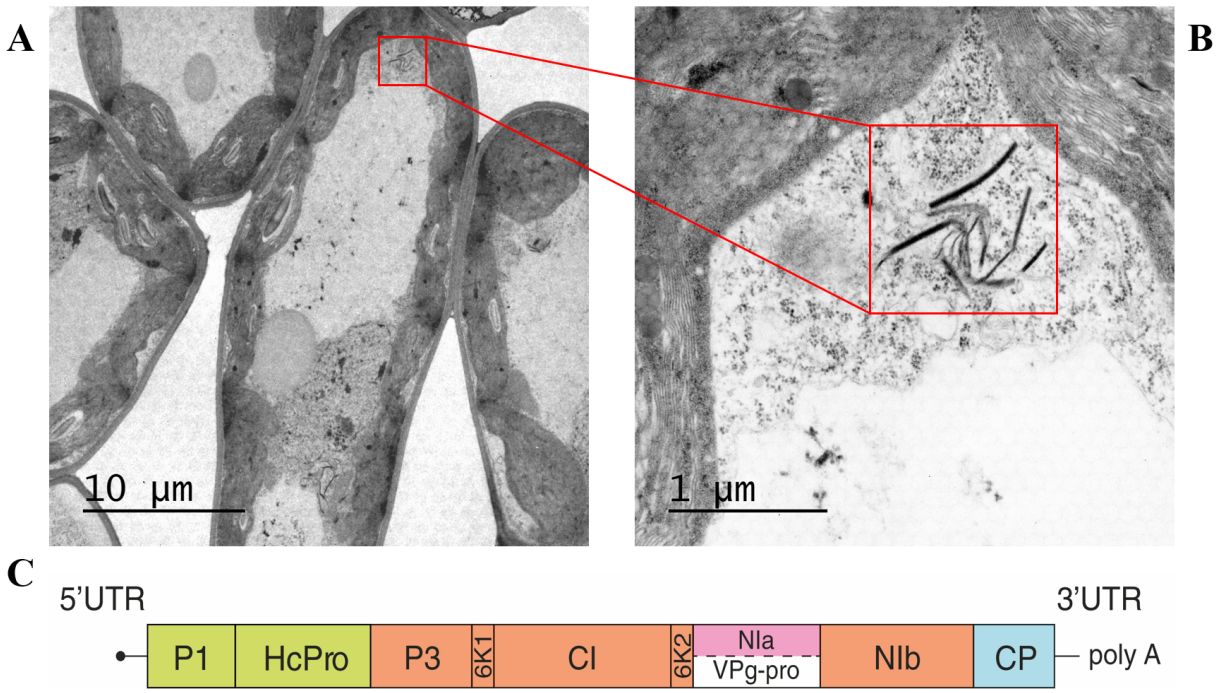


Figure 3. PPV viral particles captured at 4k (A) and 25k (B) using a transmission electron microscope. Schematic representation of the PPV genome (modified from García et al., 2014) (C).

PPV is the most feared pathogen affecting stone fruit trees, as it is the causative agent of Sharka disease. This disease is considered the most severe in stone fruit trees due to the significant losses it causes and its high transmission efficiency (Jones, 2021). In apricot, symptoms appear as chlorotic spots and rings on leaf surfaces, fruit deformities, and ring-shaped necrotic spots on the surface of the stones. In this species, damage can render up to 100% of the production unmarketable.

Reliable methods for detecting PPV are crucial for disease control. Visual inspection of symptoms is the most economical method but the least reliable, due to potential confusion with symptoms caused by other factors and the irregular distribution of the virus, which may result in absent or overlooked symptoms. Therefore, visual inspections must be complemented with sampling and more accurate techniques. Serological methods based on the ELISA (Enzyme-Linked Immunosorbent Assay) technique remain the most common for PPV detection due to their relatively low cost, capacity to process large sample volumes, and reliability in detection (García et al., 2024). However, nucleic acid-based detection techniques, such as RT-PCR (Reverse Transcription Polymerase Chain Reaction), are more sensitive than serological tests (Vidal et al., 2012). Currently, high-throughput sequencing techniques have become established as powerful tools for PPV screening and precise isolate characterisation by acquiring complete or nearly complete viral genome sequences (Barath et al., 2022; Rott et al., 2017).

Disease control is carried out through traditional methods, including the removal of inoculum sources, the use of healthy propagation material, vector control, and proper care and cleaning of field tools. Additionally, cultivating resistant cultivars is the most effective method for controlling Sharka, particularly in endemic regions. This is one of the primary goals of breeding programmes. However, advancements in genetic engineering, such as RNA silencing-mediated antiviral defences targeting viral RNA, may provide an alternative solution to the scarcity of natural resistance sources against certain PPV strains in *Prunus* species (García et al., 2024).

3.2. *Hop stunt viroid*

HSVd belongs to the family *Pospiviroidae* and the genus *Hostuviroid*. It was first identified in hops (Sasaki & Shikata, 1977). However, this pathogen has been detected in a wide range of hosts, including stone fruit trees (Astruc et al., 1996). HSVd consists of a circular single-stranded RNA molecule, 295–303 nucleotides in length, depending on the isolates and sequence variants. Despite its simplicity, HSVd contains a Conserved Central Region (CCR) specific to the genus and a Terminal Conserved Hairpin (TCH) but lacks a conserved terminal region (Hataya et al., 2017). HSVd isolates can be classified into three groups (plum-type, hop-type, and citrus-type). However, several phylogenetic studies suggest two additional groups with recombinant origins (plum–citrus type and plum–hop/Cit3 type) (Márquez-Molins et al., 2020).

Although HSVd infection was initially thought to be latent in apricot (Astruc et al., 1996), Amari et al. (2007) described the disease "degeneration," characterised by external fruit deformities, including roughness and loss of organoleptic qualities. Regarding detection and identification methods for HSVd, the most widely used technique today is RT-PCR, as it is more sensitive than tissue printing hybridisation or techniques based on polyacrylamide gel electrophoresis (PAGE) (Márquez-Molins et al., 2020).

The pathogen is primarily transmitted mechanically, although seed transmission is also possible. Therefore, effective disease control relies on proper cultural practices, such as disinfecting tools and using HSVd-free plant material in new plantations and grafted stock. Additionally, it is recommended to remove infected plants and their surroundings due to the pathogen's latency and long incubation periods (Hataya et al., 2017).

4. Importance of a plant sanitation protocol and certification programme

Viruses that infect stone fruit trees cause severe problems, compromising tree health and reducing both the quality and quantity of production. There are not any conventional methods to protect plants against viruses. To prevent viral infections in trees, cultural techniques such as vector control and the use of healthy propagation material (both rootstocks and cultivars) are applied.

According to the regulation BOE-A-1995-14422, certified apricot trees must be free of the following pathogens: *Agrobacterium tumefaciens*, *Pseudomonas syringae pv. syringae, P. syringae pv. morspronorum*, PPV, *Apple chlorotic leafspot virus* (ACLSV), *Apple mosaic virus* (ApMV), *Apricot latent virus* (ApLV), *Prune dwarf virus* (PDV), and *Prunus necrotic ringspot virus* (PNRSV), among others. This regulation does not address HSVd, although it is now widespread across the country.

Although there are recommendations to use pathogen-free fruit trees, certification standards for apricot are not mandatory. However, there is growing interest from both national and international nurseries, as well as from fruit tree breeders, in having certified plant material. For this reason, it is crucial to develop efficient protocols using various methodologies capable of producing virus- and viroid-free apricot trees.

5. Virus and viroid elimination in apricot

A protocol to produce virus-free plants would be useful to preserve plant germplasm, control viral diseases, exchange breeding materials or import novel cultivars. Table 2 summarises studies on the elimination of viruses and viroids in apricot. Several therapies can be used for this purpose, and they can be performed either *in vivo* or *in vitro*.

Although *in vivo* methods have achieved efficient results, *in vitro* protocols are now preferred for sanitising plant material. *In vitro* culture allows better control of environmental variables, enables the application of specific therapies, facilitates the isolation and culture of small meristems, reduces the risk of reinfection, and requires less space. Despite these advantages, producing virus-free plants from infected trees remains a laborious process (Figure 4).

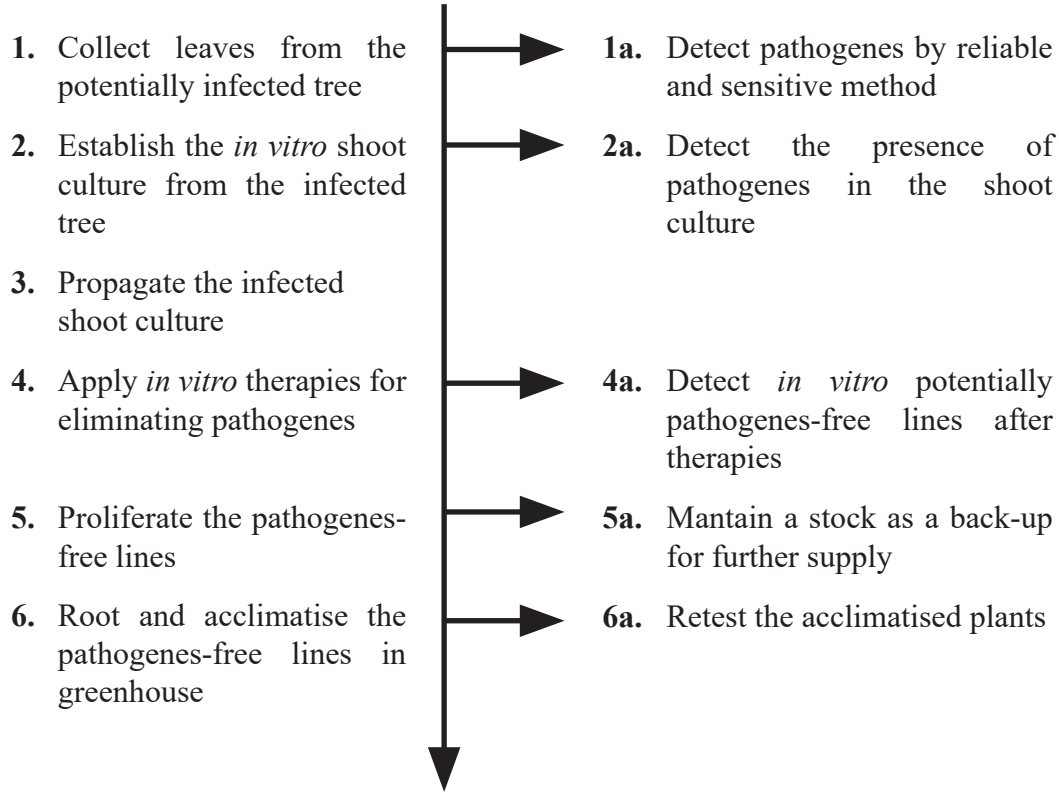


Figure 4. Production of pathogen-free plants from infected trees.

5.1. Meristem culture

The meristem culture, or meristem rescue, is a widely used technique for pathogen elimination in both woody and herbaceous plants. It consists of isolating small portions (between 0.2 and 5 mm) from apical or axillary shoots tips, containing as few leaf primordia as possible (Bettoni et al., 2024), and cultivating them in specific media for regeneration and growth.

Meristems are a group of cells capable of developing and regenerating all plant tissues and organs. Most plant viruses are excluded from meristematic cells, suggesting the presence of an antiviral barrier against movement and/or replication. However, the underlying mechanisms that prevent pathogen colonisation of meristems remain largely unknown (Bradamante et al., 2021).

Table 2. Biotechnological approaches applied in apricot for pathogen eradication.

Therapy	System	Pathogen	Methodology	Meristem Size (mm)	Survival (%)	Efficiency (%)	Reference
Thermo-	*In vivo*	PPV	30-35 ºC for 8 weeks followed by meristem culture	1-2	35	74	Koubouris et al., 2007
		PPV, PDV PNRSV	37 ºC (light) and 35ºC (dark) for 45 days followed by meristem culture	3	66.7-75	60-100	Krizan & Ondrusiková, 2009
		PPV	37 ºC (gradual increase from 20 ºC) for 15 or 22 days and placed in greenhouse	-	0-100	0-100	Polák & Hauptmanová, 2009
	In vitro	ACLSV	37 ºC between 15-18 days followed by meristem rescue	1-2	80-93	66-100	Gella & Errea, 1998
		HSVd	28 ºC for 1 day, 30 ºC for 2 days, and 32/38 ºC every 4 hours for 60 days followed by meristem rescue	0.2-0.5	0	-	Karimpour et al., 2020
		PPV	35-37 ºC for 20 days (gradual increase 1 ºC/day) followed by meristem rescue	5	28	82	Koubouris et al., 2007
		PPV, PDV, PNRSV	37 ºC (light) and 35 ºC (dark) for 45 days followed by meristem culture	3	30-50	50-100	Krizan & Ondrusiková, 2009
		ACLSV, ApMV, TRSV	From 24 to 38 ºC (gradual increase 2ºC/day) and 38 ºC for 7 days followed by meristem culture	1.1-2	60-80	90-100	Khafri et al., 2024
		PPV	37 ºC for 3, 4, 5, 6, or 7 weeks	Unknown	0	-	Seker et al., 2015
Chemo-	*In vitro*	ACLSV, ApMV, TRSV	Culture with 25 mg/L ribavirin for 4 weeks followed by meristem culture	1	5-10	60-80	Khafri et al., 2024
		PPV	Shoot tips culture with 1 mg/L of 8-azaguanine for 30 days	Unknown	0	-	Seker et al., 2015
		PPV	Shoot tips culture with 1 mg/L of ribavirin for 30 days	Unknown	80	100	Seker et al., 2015
		PPV	Shoot tips culture with 1 mg/L of quercetin for 30 days	Unknown	100	100	Seker et al., 2015
		PPV	Culture with 5 or 10 mg/L of ribavirin for 12 weeks	-	100	100	Hauptmanová & Polák, 2011
Cryo-	*In vitro*	ACLSV, ApMV, TRSV	Preculture: 1 mg/L BA, 5% DMSO and 2% proline at 4 ºC for 1 day. PVS2 for 40 min. Frozen in two steps: -40 ºC and LN for 1 hour. Water bath (1 min at 40 ºC) and 1.2M sucrose for 30 min	1.1-2	5-10	60-80	Khafri et al., 2024
		PPV	Preculture: 1 mg/L BA, 5% DMSO and 2% proline at 4 ºC for 1 day. PVS2 for 40 min. Frozen in two steps: -40 ºC and LN for 1 hour. Water bath (1 min at 40 ºC) and 1.2M su-crose for 30 min	Unknown	10	100	Seker et al., 2015
Cold-	*In vitro*	HSVd	4 ºC in darkness for 60 days followed by meristem rescue	0.2-0.5	4.6-12.2	0	Karimpour et al., 2020
Electro-	Mix	ACLSV, ApMV, TRSV	1M NaCl, 30-40mA, 100V for 15 minutes followed by disinfection and meristem culture	1.1-2	3.3-54.5	40-60	Khafri et al., 2024

The main hypothesis is that meristematic cells do not possess a fully developed vascular system, which restricts pathogen movement through the phloem and plasmodesmata (Gosalvez-Bernal et al., 2006). Additionally, the high cell multiplication rate in this region demands intense metabolic activity, which could reduce the concentration of factors required for viral replication (Dolenc et al., 2000; Navarro et al., 2019). Recently, Incarbone et al. (2023) described a broad-spectrum antiviral RNAi pathway, activated by salicylic acid, that inhibits viral replication and keeps meristematic cells pathogen-free.

Despite evidence suggesting the presence of strong barriers against invading pathogens, some viral species can infect the meristematic region. This could be because antiviral barriers are always active, and certain viruses have evolved mechanisms to suppress them, or because these barriers are only activated upon infection, and some viruses do not trigger them when invading the meristem (Bradamante et al., 2021).

The Fruit Biotechnology Group at CEBAS-CSIC has an *in vitro* collection of apricot cultivars, including 'Canino' infected with PPV (Ca-PPV) and 'Mirlo Rojo' infected with HSVd (MR-HSVd). The cultivation systems routinely used to maintain and propagate plant material include the traditional semisolid medium culture with agar (SSM) and liquid medium culture using a TIS based on Plantform® bioreactors. Preliminary experiments were conducted to determine whether meristem culture, by itself, is effective in eliminating PPV and HSVd from apricot plants after cultivation in both SSM and TIS (see Annex II).

From our results meristem culture has no efficiency in eliminating PPV and HSVd from *in vitro* apricot cultivars (see Annex II). Meristem culture does not always guarantee pathogen elimination, as certain viruses and viroids can rapidly infect the meristematic region, significantly reducing the effectiveness of this technique (Laimer & Barba, 2011). The application of additional therapies in combination with meristem culture is recommended to enhance pathogen elimination efficiency (Bettoni et al., 2024). Some of the most used techniques including thermotherapy, cryotherapy, chemotherapy, or electrotherapy are described below.

In this thesis several therapies have been implemented in combination with meristem culture to enhance pathogen elimination efficiency in apricot.

5.2. Thermotherapy

Thermotherapy involves exposing plants to high-temperature regimes. Although the mechanism by which this technique is effective is not fully understood, it is based on the principle that pathogens are more sensitive to heat than plant cells. Viral RNA degradation or silencing, reduced replication, and/or restricted movement towards meristematic cells are among the main hypotheses (Wang et al., 2018). Generally, thermotherapy treatments are combined with meristem culture after the exposure time to high temperatures. This is one of the most widely used methods for pathogen elimination in apricot (Table 2).

Some studies have been conducted on thermotherapy followed by *in vivo* meristem rescue. Koubouris et al. (2007) eliminated PPV with 74% efficiency and 35% survival. Similarly, Krizan & Ondrusiková (2009) eliminated PPV, PDV, and PNRSV with efficiencies ranging from 60% to 100%, while maintaining survival rates between 66.7% and 75%. Despite these promising results, *in vitro* culture is now the preferred approach for this technique. *In vitro* culture offers a greater quantity of homogeneous plant material available year-round and requires less space (Szabó et al., 2024). Additionally, treatment duration is reduced, and pathogen elimination

efficiencies are improved (Koubouris et al., 2007). Moreover, pathogen-free plants can be proliferated more quickly, and a stock can always be maintained. More examples of pathogen elimination through *in vitro* thermotherapy are listed in Table 2.

The success of a thermotherapy protocol depends on identifying the optimal temperature and exposure time to maximise plant survival while ensuring effective pathogen elimination. Generally, higher temperatures and longer exposure times result in higher pathogen elimination efficiency but lower plant survival rates (Panattoni et al., 2013; Szabó et al., 2024). To improve the survival of *Prunus* species, Cieslinska (2007) recommended a gradual adaptation to high temperatures before starting heat treatment. Additionally, alternating cycles of high and low temperatures has been shown to improve pathogen elimination efficiency (Howell et al., 2001, Stein et al., 1991).

5.3. Chemotherapy

Chemotherapy involves exposing plants to antiviral compounds. It is a straightforward technique, as shoots or meristems are cultured in media supplemented with the antiviral compound for a defined period and transfer new regenerated material to fresh and antiviral-free medium (Bettoni et al., 2024). The factors to be defined in a chemotherapy protocol are the type and concentration of the antiviral agent, the exposure time, and the explant used (Magyar-Tábori et al., 2021).

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, also known as Virazole) is the most used antiviral in chemotherapy. It is a guanosine analogue that integrates into RNA, inhibiting viral RNA synthesis. However, it has multiple mechanisms of action depending on the specific pathogen (Loustad-Ratti et al., 2016). Other antivirals, such as quercetin and 8-azaguanine, have also been used in apricot (Table 2).

Exposure to high concentrations of ribavirin (> 40 mg L^{-1}) causes phytotoxicity in *Prunus* species (Paunovic et al., 2007). The application of 25 mg L^{-1} ribavirin for four weeks to apricot plants resulted in survival rates of only 5–10% in meristems rescued after exposure, but an elimination efficiency of 60–80% for *Tobacco ringspot virus* (TRSV), ApMV, and ACLSV (Khafri et al., 2024). Reducing the concentration to 10 mg L^{-1} while increasing the exposure time to 12 weeks achieved 100% survival of the treated plants as well as 100% elimination of PPV (Haupmanová & Polak, 2011). Seker et al. (2015) applied 1 mg L^{-1} of ribavirin directly to apricot shoot tips for 30 days, achieving 80% survival and 100% PPV elimination. Despite the high efficiency of these treatments, the cost of antivirals may compromise their use for eliminating plant pathogens in a sustainable manner.

5.4. Nanotherapy

Nanotherapy is a novel and original concept that describes the use of nanomaterials as virucidal agents. Among the different groups of nanomaterials, metallic nanoparticles (MeNPs) have been shown to have antimicrobial properties against a wide range of pathogens (Salem & Fouda, 2020; Schröfel et al., 2014), as well as other applications within the field of agriculture (Alfosea-Simón et al., 2025), particularly silver nanoparticles (AgNPs).

AgNPs have proven to be an effective tool in regulating antiviral activity against numerous viruses affecting animal species (Vijayaram et al., 2023). Furthermore, several studies have

applied AgNPs as a spray on plants before, during, or shortly after virus infection, observing a significant reduction or complete elimination of disease symptoms with high efficiency (Alfosea-Simón et al., 2025). Further research into the mechanism of action of AgNPs is necessary, as the reasons behind their effect remain unclear.

In this thesis, studies have been conducted using AgNPs with the aim of eliminating pathogens affecting apricot plants, ensuring that the treatment has a curative effect.

5.5. Cold therapy

Cold therapy involves exposing plants to low temperatures (generally between 2 and 6 °C) for extended periods, as the effects of the treatment are usually gradual. Cold therapy is commonly used in combination with other techniques such as meristem rescue or chemotherapy (El-Dougdoug et al., 2010; Hu et al., 2024).

Cold therapy is particularly beneficial for treating plants that cannot tolerate high temperatures, as it avoids the thermal stress that could damage sensitive tissues. Furthermore, it is especially effective against viroids that accumulate rapidly in meristematic tissue and are difficult to eliminate through thermotherapy (Barba et al., 2017).

El-Dougdoug et al. (2010) were the first to successfully eliminate HSVd from a stone fruit species, specifically peach. They applied a temperature of 4 °C for three months, followed by meristem rescue, achieving a survival rate of 25% and an efficiency of 18%. However, Karimpour et al. (2020) was unable to eliminate HSVd from apricot when applying 4 °C for two months followed by meristem culture (Table 2).

5.6. Cryotherapy

Cryotherapy is a biotechnological technique based on cryopreservation techniques since ultralow temperatures (-196°C with liquid nitrogen) are used to eliminate pathogens (Wang et al., 2006). The most commonly used explants in these procedures are shoot tips. The mechanism of action is based on the physiological differences between meristematic and differentiated cells. Differentiated cells are larger, have bigger vacuoles, and a lower nucleocytoplasmic ratio than meristematic cells. During exposure to ultralow temperatures, intracellular liquids crystallise, leading to cell death by damaging membrane structures (Bettoni & Volk, 2024; Feng et al., 2013; Wang et al., 2009).

Various techniques have been developed to improve survival rates in cryotherapy procedures. Vitrification involves transforming intracellular liquid into an amorphous or glassy state using cryoprotectants (Fahy et al., 1984). Using a vitrification protocol, Brison et al. (1997) were the first to report a successful case of pathogen elimination through cryotherapy. They eliminated PPV from an interspecific *Prunus* rootstock, achieving survival rates between 11% and 70% and efficiency rates between 45% and 60%, depending on meristem size. Using the same protocol, other authors have successfully eliminated viruses from apricot plants (Table 2). Khafri et al. (2024) achieved survival rates between 5% and 10% and eliminated ACLSV, ApMV, and TRSV with efficiencies ranging from 60% to 80%. Seker et al. (2015) reported a survival rate of 10% and successfully eliminated PPV with 100% efficiency.

In modern cryotherapy procedures, most of the freezable water is removed before immersion in liquid nitrogen. This is achieved by exposing naked or encapsulated meristems to highly concentrated vitrification solutions, such as PVS2 (Sakai et al., 1990) or PVS3 (Nishizawa et al., 1993), or by air-drying. Droplet-vitrification involves placing meristems in a small drop of cryoprotectant solution on a strip of aluminium foil inside a cryotube before immersion in liquid nitrogen (Sakai & Engelmann, 2007). Encapsulation-dehydration consists of protecting the shoot tips in a calcium alginate capsule followed by controlled dehydration through exposure to laminar airflow before immersing in liquid nitrogen (Bettoni et al., 2018).

Volk & Bonnart (2020) reported a droplet-vitrification protocol for *Prunus* species cryopreservation. Soliman (2013) developed an encapsulation-dehydration protocol for apricot, achieving a survival rate of 60.4% after two years of storage in liquid nitrogen. However, the efficiency of these protocols in pathogen elimination has not been assessed.

5.7. Electrotherapy

Electrotherapy is a technique that uses controlled electric currents to eliminate plant pathogens. It is based on the ability of electricity to affect pathogens without significantly damaging plant cells. There are two application methods: direct (connecting the explant directly to the electrodes) and indirect (immersing the explant in electrophoresis buffer). The indirect method is generally used, applying intensities (5–100 mA) or voltages (5–15 V) for a short period (5–60 minutes), followed by sterilisation and *in vitro* cultivation of the treated explants (Adil et al., 2022). Treatment conditions must be optimised according to the genotype, explant, and pathogen.

The mechanism of action of electrotherapy is not fully understood, although it has been suggested that viral nucleoproteins may denature or lose virulence due to the temperature increase during treatment (Lozoya-Saldaña et al., 1996). The denaturation of viral particles and the inactivation of the specific nucleoprotein involved in cell-to-cell movement through plasmodesmata and long-distance movement via the plant's vascular system by means of an electric current would prevent the spread of viral particles to healthy cells (Raj et al., 2022). Additionally, exposure to electrical stimuli may induce stress responses in plants, such as the production of reactive oxygen species (ROS).

Thermotherapy has been successfully used to eliminate viral diseases in both herbaceous plants (Adil et al., 2022) and woody plants, such as olive (Mirzaei et al., 2024). Regarding apricot, Khafri et al. (2024) achieved survival rates of up to 54.5% and virus elimination efficiency for ACLSV, ApMV, and TRSV ranging between 40% and 60% (Table 2).

5.8. Genetic engineering

New biotechnological tools allow the direct modification of plant genomes to confer specific traits that either block the virus's life cycle or mitigate its effects. "HoneySweet" is the most well-known example in the fight against PPV through genetic engineering techniques (Scorza et al., 2016). "HoneySweet" is a transgenic plum cultivar developed by introducing a fragment of the PPV genome, specifically the complete CP gene. This cultivar has demonstrated near-total resistance to PPV under both controlled conditions and long-term field trials, with no known variants of the virus capable of overcoming this resistance (Singh et al., 2021; Zagrai et

al., 2021). The mechanism conferring resistance to PPV is known as Post-Transcriptional Gene Silencing (PTGS).

PTGS is a key mechanism in plant defence against viruses. The process begins when the plant detects double-stranded RNA (dsRNA). This dsRNA is processed by an enzyme called Dicer-like (DCL), which cuts it into small fragments (21–25 nucleotides) known as small interfering RNAs (siRNAs). These siRNAs are incorporated into a protein complex called the RNA-Induced Silencing Complex (RISC), which uses them as a guide to recognise and degrade complementary mRNA molecules, thereby eliminating viral particles (Zhao & Guo, 2022).

In addition to transgenic constructs derived from the CP gene, effective results have been achieved with the h-UTR/P1 construct, which encodes an ihpRNA containing the first 733 nucleotides of the PPV-M ISPaVe44 isolate genome (Di Nicola-Negri et al., 2005). Furthermore, this construct is resistant to the major PPV strains (Di Nicola-Negri et al., 2010). It was used to develop transgenic plums that showed resistance to PPV both *in vitro* and in greenhouse trials (García-Almodóvar et al., 2015).

Although genetic engineering techniques are an efficient tool for transferring virus resistance, they are often labour-intensive and costly in species that are recalcitrant to regeneration or transformation. However, it is known that siRNAs derived from the PTGS mechanism, induced locally, can spread to adjacent cells and even systemically throughout the organism via the phloem (Kalantidis et al., 2008).

The RNA-mediated silencing has been shown to move through the vascular system over long distances raising the possibility that a transgenic rootstock could transfer its virus resistance to a non-transgenic scion through grafting (Arpaia et al., 2020; Rubio et al., 2017). Although this is a promising strategy, contradictory results have been found in previous studies with *Prunus*. Zhao & Song (2014) described a certain degree of resistance to PNRSV after grafting a non-transgenic sweet cherry onto a transgenic cherry rootstock. However, Sidorova et al. (2021) reported that resistance to PPV was not transmitted to non-transgenic plum, despite the accumulation of a high level of virus CP-specific siRNA.

This strategy could be useful for conferring resistance to species that are difficult to transform, such as apricot (Petri et al., 2015), and would help address concerns associated with the spread of transgenes in the environment and the consumption of transgenic foods (Arpaia et al., 2020).

Objectives

The main objective of the present PhD Thesis is to develop biotechnological protocols to eradicate virus and viroid from apricot cultivars. To achieve the principal aim, the following specific objectives are addressed:

1. Transfer PPV resistance from transgenic plum rootstocks to wild type apricot scions (Publication I).

2. Determine *in vitro* culture conditions with silver nanoparticles (AgNPs) that allow the incorporation of silver into plant tissues (Publication II).

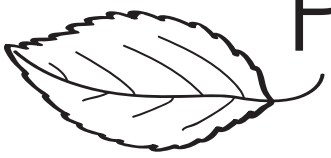
3. Study the effect of AgNP concentration on the micropropagation of apricot cultivars and silver uptake (Publication III).

4. Eliminate PPV or HSVd by adding AgNPs to the proliferation medium followed by meristem culture (Publication IV).

5. Apply thermotherapy and/or culture in the dark followed by meristem rescue to eliminate PPV or HSVd (Publication V).

6. Apply shoot tip cryotherapy using Droplet-Vitrification with PVS2 to produce PPV-free apricot plants (Annex I).

Scientific publications



Publication I

Trans-grafting *Plum pox virus* resistance from transgenic plum rootstocks to apricot scions

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Cristian Pérez-Caselles performed RT-PCR evaluations and assisted Northern blot evaluation in the laboratory, contributed to data analysis and revised the manuscript.

ABSTRACT

Introduction: Trans-grafting could be a strategy to transfer virus resistance from a transgenic rootstock to a wild type scion. However contradictory results have been obtained in herbaceous and woody plants. This work was intended to determine if the resistance to Sharka could be transferred from transgenic plum rootstocks to wild-type apricot scions grafted onto them.

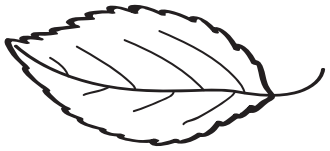
Methods: To this end, we conducted grafting experiments of wild- type apricots onto plum plants transformed with a construction codifying a hairpin RNA designed to silence the PPV virus and studied if the resistance was transmitted from the rootstock to the scion.

Results: Our data support that the RNA-silencing-based PPV resistance can be transmitted from PPV-resistant plum rootstocks to non-transgenic apricot scions and that its efficiency is augmented after successive growth cycles. PPV resistance conferred by the rootstocks was robust, already occurring within the same growing cycle and maintained in successive evaluation cycles. The RNA silencing mechanism reduces the relative accumulation of the virus progressively eliminating the virus from the wild type scions grafted on the transgenic resistant PPV plants. There was a preferential accumulation of the 24nt siRNAs in the scions grafted onto resistant rootstocks that was not found in the scions grafted on the susceptible rootstock. This matched with a significantly lower relative accumulation of hpRNA in the resistant rootstocks compared with the susceptible or the tolerant ones.

Discussion: Using transgenic rootstocks should mitigate public concerns about transgenes dispersion and eating transgenic food and allow conferring virus resistance to recalcitrant to transformation cultivars or species.

Keywords: resistance, rootstock, scion, sharka, trans-grafting

Publication II



How to Get More Silver? Culture Media Adjustment Targeting Surge of Silver Nanoparticle Penetration in Apricot Tissue during *in Vitro* Micropropagation

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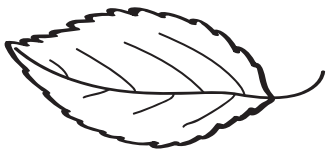
Cristian Pérez-Caselles performed the experiments in the laboratory and contributed to the experimental design, data analysis and the writing of the work.

ABSTRACT

The use of silver nanoparticles (AgNPs) is increasing nowadays due to their applications against phytopathogens. Temporary Immersion Systems (TIS) allow the micropropagation of plants in liquid media. This work aims to develop an effective protocol for apricot micropropagation in TIS and to study the necessary conditions to introduce AgNPs in apricot plants, as well as the effect of its application on proliferation-related parameters. AgNPs were introduced in different media at a concentration of 100 mg L^{-1} to test the incorporation of silver to plant tissues. Silver content analysis was made by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). The effect of initial shoot density and the addition of AgNPs on micropropagation were evaluated after four weeks in culture on TIS. Productivity, proliferation, shoot-length and leave surface were measured. The better micropropagation rate was obtained with 40 initial shoots, 2 min of immersion every 6 h and 3 min of aeration every 3 h. To introduce AgNPs in apricot plants it is necessary to culture them in liquid media without chloride in its composition. These results will contribute to the development of an *in vitro* protocol for virus inhibition by AgNPs application. This depends on the introduction of Ag nanoparticles within the plant tissues, and this is not possible if AgNPs after interaction with Cl^- ions precipitate as silver chloride salts.

Keywords: TIS; AgNPs; liquid media; virucide; micropropagation

Publication III



The Effect of Silver Nanoparticle Addition on Micropropagation of Apricot Cultivars (*Prunus armeniaca* L.) in Semisolid and Liquid Media

Cristian Pérez-Caselles [1], Lorenzo Burgos [1], Inmaculada Sánchez-Balibrea [1], Jose A. Egea [2], Lydia Faize [1], Marina Martín-Valmaseda [1], Nina Bogdanchikova [3], Alexey Pestryakov [4] and Nuria Alburquerque [1,*]

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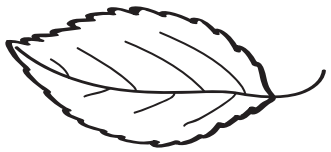
Cristian Pérez-Caselles performed the experiments in the laboratory and contributed to the experimental design, data analysis and the writing of the work.

ABSTRACT

Silver nanoparticles (AgNPs) are novel compounds used as antimicrobial and antiviral agents. In addition, AgNPs have been used to improve the growth of different plants, as well as the *in vitro* multiplication of plant material. In this work the effect of AgNPs on *in vitro* growth of 'Canino' and 'Mirlo Rojo' cultivars, as well as the leaf ion composition, are studied. Different concentrations of AgNPs (0, 25, 50, 75 and 100 mg L^{-1}) were added to two culture systems: semisolid medium with agar (SSM) in jars and liquid medium in temporary immersion system (TIS). Proliferation (number of shoots), shoot length, productivity (number of shoot x average length), leaf surface, fresh and dry weight were measured. Additionally, the silver and other ion accumulation in the leaves were evaluated by inductively coupled plasma optical emission spectroscopy (ICP-OES) analysis. The productivity of 'Canino' and 'Mirlo Rojo' decreased when increasing the concentration of AgNPs in the semisolid medium. However, the use of AgNPs in the TIS improved the proliferation and productivity of 'Canino' and Mirlo Rojo', increasing biomass production, and the concentration of nutrients in the plants, although these effects are genotype-dependent. TISs are the best system for introducing silver into shoots, the optimum concentration being 50 mg L^{-1} for 'Canino' and 75 mg L^{-1} for 'Mirlo Rojo'. Principal component analysis, considering all the analyzed ions along the treatments, separates samples in two clear groups related to the culture system used. The use of bioreactors with a liquid medium has improved the productivity of 'Canino' and 'Mirlo Rojo' in the proliferation stage, avoiding hyperhydration and other disorders. The amount of metallic silver that penetrates apricot plant tissues depends on the culture system, cultivar and concentration of AgNPs added to the culture medium. Silver ion accumulation measured in the shoots grown in the TIS was higher than in shoots micropropagated in a semisolid medium, where it is barely detectable. Furthermore, AgNPs had a beneficial effect on plants grown in TIS. However, AgNPs had a detrimental effect when added to a semisolid medium.

Keywords: agar culture; AgNPs; clonal propagation; nanobiotechnology; proliferation; temporary immersion system



Publication IV

Nanobiotechnology for efficient *Plum pox virus* elimination from apricot plants

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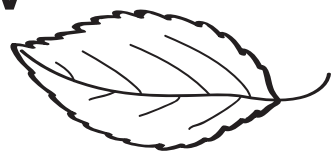
Cristian Pérez-Caselles performed the experiments and RT-PCR evaluations in the laboratory and contributed to the experimental design and the writing of the work.

ABSTRACT

Metallic nanoparticles have antimicrobial, virucidal, and anticancer activities and have been widely applied in medicine. In plants, silver nanoparticles have been used as preventive treatments in the greenhouse to reduce viral titers and symptoms. This work investigates the effect of Argovit™ AgNP formulation on apricot plants infected with *Plum pox virus* or with *Hop stunt viroid*. Meristems were rescued from plants treated with different Argovit™ concentrations and two exposure times. Although viroid-free plants were not obtained, a very high efficiency in eliminating the Sharka virus is reported with a maximum cleaning efficiency (75 %) after 8 weeks of exposure at 75 mg L^{-1} AgNP. To the best of our knowledge, this is the first time that silver nanoparticles application is used, combined with meristem culture, to produce virus-free plants and opens a new path to the elimination of viruses from plants.

Keywords: Silver nanoparticles, Plant pathogenes, *In vitro* culture, Meristem rescue, Temporary Immersion System

Publication V



Production of HSVd- and PPV-free apricot cultivars by *in vitro* thermotherapy followed by meristem culture

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Cristian Pérez-Caselles performed the experiments and RT-PCR evaluations in the laboratory and contributed to the experimental design and the writing of the work.

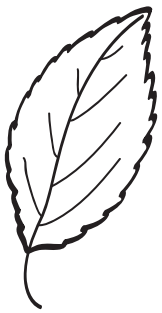
ABSTRACT

Background: The production of virus-free apricots (*Prunus armeniaca* L.) is essential for controlling viral diseases, exchanging breeding materials without the risk of spreading new diseases, and preserving plant germplasm. *Plum pox virus* (PPV) is the most devastating disease of the *Prunus* genus and *Hop stunt viroid* (HSVd) is prevalent in most apricot-growing regions. It was evaluated whether thermotherapy, etiolation, or a combination of both followed by meristem culture could effectively eliminate PPV and HSVd from 'Canino' and 'Mirlo Rojo' apricot cultivars *in vitro*.

Results: In the thermotherapy treatments, shoots were exposed to 38ºC and 32ºC, alternating every four hours, for 30, 35, 40, and 45 days. Before this, shoots were acclimated to heat for one day at 28ºC and two days at 30ºC. Etiolation experiments consisted of eight weeks of culture in dark conditions. A combination of 45 days of thermotherapy, as described previously, and etiolation was also performed. At the end of each treatment, 1.5 mm meristems were cultured, and developed as potential independent pathogen-free lines. The presence or absence of pathogens was analysed by RT-PCR. The 45 days of thermotherapy and the combined thermotherapy and etiolation treatments resulted in the highest percentages of PPV-free plants (66.7 and 75.0%, respectively). At least 40 days of thermotherapy were required to obtain HSVd-free plants, although the best efficiency was achieved at 45 days (22.7%).

Conclusions: In this study, we have developed an effective *in vitro* thermotherapy protocol that eliminates PPV and HSVd from apricot cultivars. This is the first report where a thermotherapy protocol eliminates HSVd in *Prunus* species.

Keywords: Etiolation, Heat treatment, *Hop stunt viroid*, *Plum pox virus*, *Prunus*



Conclusions

In this PhD thesis, we applied several biotechnological tools to eliminate PPV and HSVd from apricot cultivars. Based on the results obtained we concluded the following:

1. Cultivating a non-transgenic apricot cultivar onto transformed plum rootstocks resistant to Sharka, through RNAi-mediated silencing, is a valid and effective strategy to transfer the resistance to PPV to the apricot scion. This approach provides the apricot tree with resistance to the virus, allowing it to recover from future reinfections. Additionally, it could be an interesting strategy for species that lack natural resistance sources and are recalcitrant to transformation. Finally, the commercial variety producing the fruit is not transgenic, which helps address public concerns regarding the consumption of genetically modified products.

2. Meristem culture is not an effective technique for the elimination of PPV and HSVd in apricot cultivars due to its very low or negligible efficiency. It needs to be combined with additional treatments in order to produce pathogen-free plants.

3. Using AgNPs allows the production of PPV-free 'Canino' plants with high efficiency after rescuing meristems from treated plants. However, it is necessary to culture apricot shoots in modified liquid medium (without chlorides) using TIS. The addition of AgNPs to the modified liquid medium did not have a detrimental effect on the plant micropropagation; instead, a hormetic effect was observed, leading to increased proliferation and biomass production.

4. Thermotherapy treatments followed by meristem culture enabled the production of PPV- and HSVd-free apricot plants. Longer exposure times were necessary for viroid elimination than for virus eradication. The etiolation treatment showed low PPV eradication efficiency. The combined thermotherapy and etiolation treatment did not significantly improve the efficiency achieved by thermotherapy.

5. The application of shoot tip cryotherapy using droplet-vitrification with PVS2 successfully eliminated PPV with high efficiency. Cryotherapy procedures significantly affect meristem survival from apricot plants. Improving meristem survival would be essential for an efficient cryopreservation protocol. Furthermore, developing new protocols that avoid the application of DMSO, such as using PVS3 or the encapsulation-dehydration technique, would be desirable.

6. PPV has been effectively eliminated using several strategies, including thermotherapy (66.7%), etiolation (18.7%), a combination of thermotherapy and etiolation (75.0%), cryotherapy (81.9%), and AgNPs application (75.0%). Selection of the most appropriate approach will depend on different factors such as pathogen, infected cultivar/species, equipment available or staff expertise.

7. HSVd has been effectively eliminated only using thermotherapy in this thesis where AgNPs and etiolation did not produce viroid-free plants.

Annexes

Annex I

Efficient shoot tip cryotherapy for *Plum pox virus* eradication from 'Canino' apricot cultivar

1. Introduction

Cryotherapy is a pathogen elimination method based on cryopreservation techniques (Wang et al., 2006). Cryopreservation involves the long-term storage of plant material (usually shoot tips) at ultra-low temperatures (-196 ºC in liquid nitrogen), because biological and physicochemical processes within plant cells are halted under these conditions (Benelli et al., 2013; Sakai, 1995).

Shoot tips are the most used explant in cryotherapy procedures. On the one hand, meristematic cells are often pathogen-free (Bradamante et al., 2021). On the other hand, physiological differences between meristematic and differentiated cells allow their survival during cryotherapy procedures. Differentiated tissue cells are larger in size, have bigger vacuoles, and a lower nucleocytoplasmic ratio than meristematic cells. During immersion in liquid nitrogen, intracellular liquid crystallisation causes lethal and irreversible damage due to the rupture of membrane structure by ice crystals (Bettoni & Volk, 2024; Feng et al., 2013; Wang et al., 2009). Therefore, following cryotherapy, cells which most likely harbour pathogens are eliminated, while younger cells with dense cytoplasm may withstand the process.

To ensure success in cryotherapy procedures, lethal damage to meristematic cells must be avoided. This requires proper dehydration and vitrification. Vitrification is the process by which intracellular water transitions into an amorphous or "glassy" state using cryoprotectants (Fahy et al., 1984). Brison et al. (1997) were the first to report efficient pathogen eradication using a two-step vitrification protocol with Plant Vitrification Solution 2 (PVS2) to eliminate *Plum pox virus* (PPV) from an interspecific *Prunus* rootstock. This method involved an initial temperature decrease to -40 ºC using a programmable freezer, followed by immersion in liquid nitrogen.

Droplet-vitrification is among the new 'one-step freezing' methods. In this technique, after proper dehydration and vitrification of the meristems, they are placed in a small droplet of cryoprotective solution on a strip of aluminium foil before being immersed in liquid nitrogen (Sakai & Engelmann, 2007).

The Fruit Biotechnology Group at CEBAS-CSIC owns an *in vitro* collection of apricot cultivars, including 'Canino' infected with PPV (Ca-PPV). The aim of this study is to develop an efficient shoot tip cryotherapy protocol by using droplet-vitrification to eliminate PPV from Ca-PPV shoots.

2. Materials and methods

2.1. Plant material

Ca-PPV shoots were used for cryotherapy experiments. *In vitro* stocks are maintained in a growth chamber at 23 ± 1 ºC under a 16/8h light/dark photoperiod (56 µmol m^{-2} s^{-1}). The Semisolid Medium (SSM) used for proliferation was described by Wang et al. (2013) (Table 1). Plants are subcultured every four weeks in 500 mL glass jars, with approximately 12 shoots per container.

Table 1. Media used for shoot tip cryotherapy recovery, meristem growth and apricot multiplication and rooting. Adapted from Pérez-Caselles et al. (2025).

	CRM	MM1	MM2	SSM	RM
Macronutrients	modified QL	QL	QL	QL	QL
$CaCl_2 \cdot 2\ H_2O$ (mM)	-	-	-	3.0	1.5
Micronutrients	DKW	DKW	QL	DKW	DKW
Vitamins	DKW	DKW	a	DKW	DKW
Sucrose (% w/v)	-	-	-	3.0	2.0
Sorbitol (% w/v)	2.0	2.0	2.0	-	-
Meta-topolin (µM)	1.05	1.05	-	2.1	-
BAP (µM)	-	-	6.66	-	-
BAP_R (µM)	0.56	0.56	-	1.12	-
Adenine (µM)	14.8	14.8	-	29.6	29.6
IBA (µM)	0.05	0.05	0.05	0.05	3.0
Phloroglucinol (mM)	-	-	-	0.8	0.8
Agar (% w/v)	0.6	0.7	0.7	0.7	0.7

All media were adjusted to pH 5.7 and autoclaved at 121 ºC for 20 minutes.
Modified QL: QL macronutrients without adding NH_4^+.
a: 277.53 µM myo-inositol, 3.77 µM thiamine, 8.12 µM nicotinic acid, 0.41 µM biotin, 0.07 µM p-aminobenzoic acid, 0.27 µM riboflavin, and 2.28 µM Ca-pantothenate.
References: CRM (this annex), MM1 (Pérez-Caselles et al., 2025), MM2 (Pérez-Tornero et al., 1999), SSM (Wang et al., 2013), RM (Pérez-Tornero & Burgos, 2000), QL (Quoirin & Lepoivre, 1977), DKW (Driver & Kuniyuki, 1984).

2.2. Shoot tip cryotherapy and recovery

Shoot tip cryotherapy procedures were performed as described by Volk & Bonnart (2020), with modifications. Apical or axillary shoot tips (3 mm long) containing between two and four leaf primordia were isolated from 4-week-old Ca-PPV shoots and were cultured in Preculture Medium (0.3 M sucrose, 0.4 M proline, 1 mM glutathione (reduced form), 0.1 mM salicylic acid, 1/2 strength MS, and 0.8% w/v agar at pH 5.7) for three days at 23 ºC with low light intensity (no direct light incidence) (Figure 1A).

Shoot tips were removed from the Preculture Medium and placed in Loading Solution (2 M glycerol, 0.4 M sucrose, and 1/2 strength MS salts at pH 5.8) for 20 minutes at 22 ºC (Figure 1B). The Loading Solution is removed and replaced with PVS2 at 0 ºC for 75 minutes (Figure 1C). PVS2 was described by Sakai et al. (1990) and consisted of 0.4 M sucrose, 30% w/v glycerol, 15% w/v Ethylene-glycol, 15% w/v DMSO (dimethyl sulfoxide), and 1/2 strength MS at pH 5.8. The shoot tips are then placed onto a thin drop of PVS2 on foil strips (Figure 1D), plunged into liquid nitrogen (Figure 1E) and transferred to cryotubes (Figure 1F). Cryotubes are immersed in liquid nitrogen for at least one hour (Figure 1G).

Shoot tips are warmed by removing the foil strips from the cryotubes and then placing the foil strips into Unloading Solution (1/2 strength MS + 1.2 M sucrose at pH 5.7) for 20 minutes at room temperature (Figure 1H). Shoot tips are then plated onto Cryotherapy Recovery Medium (CRM, Table 1) after removing excess moisture (Figure 1F).

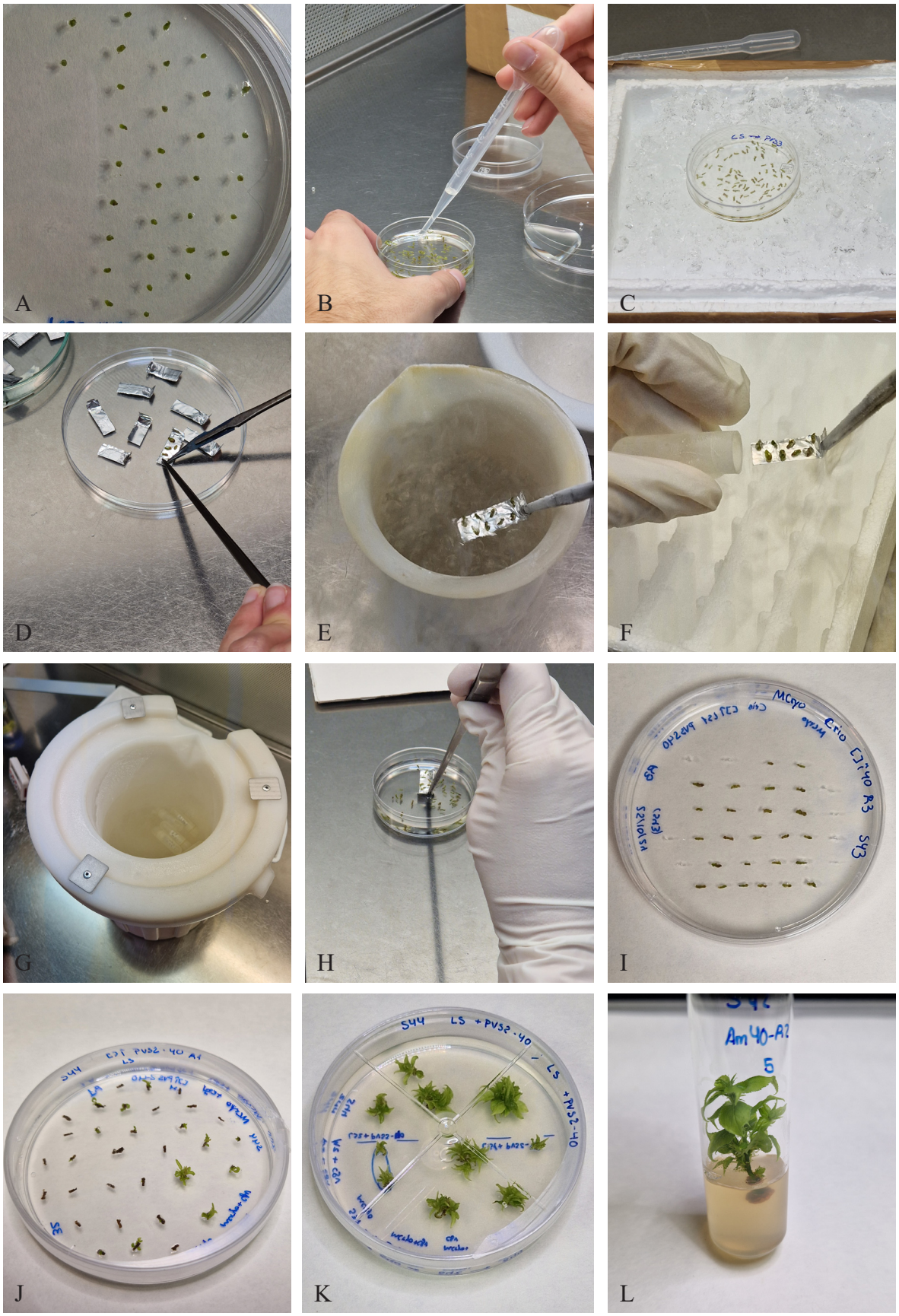


Figure 1. Isolated meristems (3 mm) from apical or axillary buds of Ca-PPV shoots and cultured in the preculture medium (A). Meristems individually transferred to the loading solution for 20 minutes (B) and PVS2 on ice for 75 minutes (C). Meristems placed on an aluminium foil strip (D), frozen them by immersion in liquid nitrogen (E), inserted into the cryotube (F), and kept in liquid nitrogen for one hour (G). Meristems are then thawed in the unloading solution for 20 minutes (H) and, after removing excess moisture, transferred to CRM (I). Meristems after 7 (J), 11 (K), and 15 (L) weeks of culture.

2.3. Meristem growth, shoot development, *in vitro* rooting and acclimatisation

Shoot tips were cultured in CRM (Table 1) for three weeks, the first four days with low light intensity. Shoot tips were cultured horizontally and slightly embedded in the culture medium, ensuring that most of the explant remained in contact with the medium. With the help of a binocular, those sprouted meristems were transferred to fresh CRM medium for four weeks. Afterwards, meristems were cultured another four weeks in Meristem Multiplication Medium 2 (MM2, Table 1). Then, they were subcultured to SSM (Table 1) every four weeks. The time necessary from shoot tip isolation to have a shoot as in Figure 1L was 15 weeks.

The rooting medium (RM, Table 1) for apricot was described by Pérez-Tornero et al. (2000). Before placing the shoots in this medium, their bases are immersed in 4.9 mM IBA to induce root formation, and their apices in 44.4 µM BAP_R to prevent necrosis. After four weeks, rooted shoots are acclimatised. The roots are rinsed with distilled water, to remove agar residues, and the plants are transferred to 300 mL pots containing a 2:1 (v/v) mixture of peat and perlite. They are fertilised with 20 mL of Hoagland & Arnon (1950) solution and placed in a zip-lock bag. The plants are then transferred to a greenhouse with controlled conditions and natural light. In the greenhouse, the zip-lock bag is gradually opened and eventually removed once the plant has been successfully acclimatised.

2.4. Virus detection

The presence or absence of PPV in the shoots derived from meristems was evaluated using RT-PCR as described by Pérez-Caselles et al. (2025). Each surviving meristem from the cryotherapy procedures that grew to a shoot was considered a potentially PPV-free line. Three RT-PCR evaluations were carried out, the first one 15 weeks after meristem rescue, another one after 12 additional weeks, and finally, the third evaluation, on the acclimated lines in the greenhouse. A line was considered PPV-free when the virus was consistently not found in any of the three evaluations.

3. Results and discussion

Figure 2 shows that the cryotherapy treatment significantly reduced meristem survival (6.0%) compared to the control (83.0%). The control results correspond to meristem rescue without applying any additional therapy (see Annex II). Although low survival rates are characteristic of cryotherapy protocols, the results of this study are particularly low compared to similar studies (Feng et al., 2013; Wang et al., 2022). However, other authors who applied vitrification protocols to apricot plants reported similar survival rates between 5–10% (Khafri et al., 2024) and 10% (Seker et al., 2015). This suggests that this species may be sensitive to cryotherapy procedures.

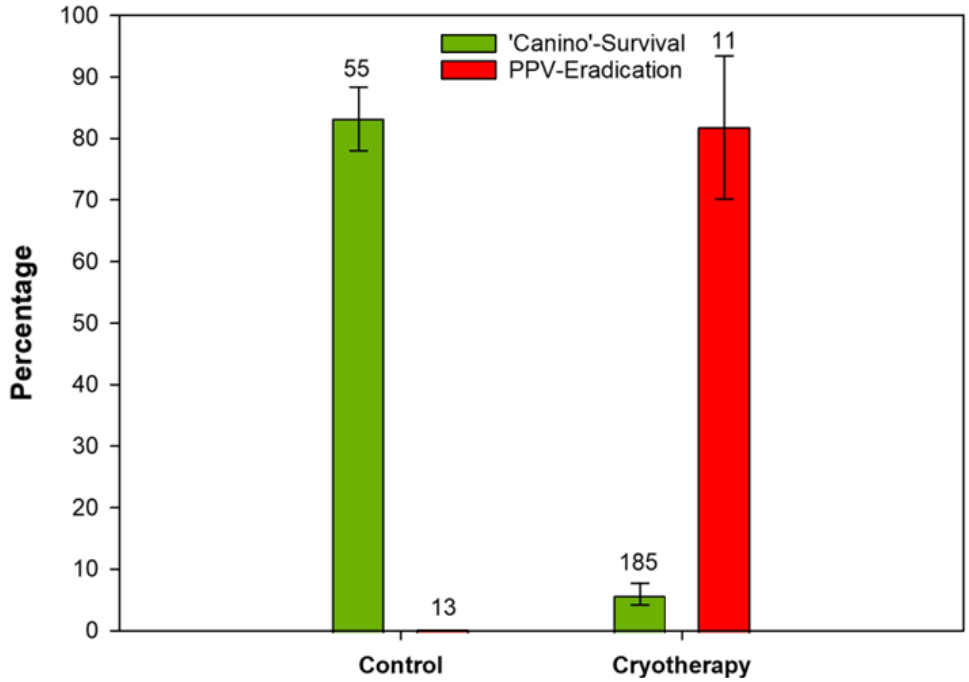


Figure 2. Effect of cryotherapy treatment on the survival rate (%) of shoot tips and the efficiency (%) of PPV elimination. Error bars represent the standard error. The numbers above the green bar indicate the total number of isolated shoot tips, while those above the red bar indicate the number of evaluated lines.

The cryotherapy treatment achieved an 81.9% efficiency in PPV elimination (Figure 2). These results correspond to the *ex vitro* evaluation. The first *in vitro* evaluation showed 100% efficiency. However, in the second *in vitro* evaluation, two cases of false positives were detected, the efficiency being 81.9%. The third evaluation (*ex vitro* evaluation) confirmed the results of the second assessment. These results align with those obtained in other cryotherapy procedures, which typically achieve high efficiency. Regarding other cryotherapy protocols for apricot, Khafri et al. (2024) obtained efficiencies ranging from 60 to 80%, while Seker et al. (2015) achieved 100%.

The size of the isolated meristems is a critical factor affecting meristem survival and efficiency of virus elimination, as larger meristems have a higher chance of containing differentiated cells infected by pathogens (Wang et al., 2009). However, due to the mechanism of action of cryotherapy, larger meristems with some primordial leaves can be used without compromising the efficiency of the technique (Feng et al., 2013). In this study, apricot was found to be highly sensitive to cryotherapy procedures. Therefore, it was necessary to use 3 mm-length meristems without removing the youngest primordial leaves, ensuring a more vigorous meristem while minimising explant manipulation.

Initially, MM1 (Table 1) was used as the meristem recovery medium following cryotherapy procedures. After the first experiments, it was observed that the aerial parts of the shoot tips necrosed after cryotherapy, while the portion in contact with the culture medium remained alive for a longer time. For this reason, shoot tips were cultivated lying flat and embedded in the culture medium. To facilitate the embedding of explants in the medium, the agar concentration was reduced to 6% w/v. Reducing the agar concentration in a medium with high cytokinin levels can lead to damage caused by hyperhydration. To prevent this issue, the NH_4^+ ion concentration was reduced (Bouza et al., 1992; Ziv, 1991). The CRM medium is, therefore, the MM1 medium with these modifications (Table 1).

Ensuring the viability and quality of cryopreserved plants is essential for the conservation of plant genetic resources. However, it is also important to confirm that plants developed from shoot tips are genetically stable and identical to the mother plant (Wang et al., 2021). Although DMSO provides protective effects for cells during the freezing process, it can be toxic when used at high concentrations or for prolonged exposure times (Kaya & Souza, 2017). For this reason, it is desirable to avoid the use of DMSO in cryotherapy procedures. An alternative is to use Plant Vitrification Solution 3 (PVS3, Nishizawa et al., 1993), which consists of 50% w/v sucrose and 50% w/v glycerol. Additionally, one of the most widely used cryotherapy techniques is Encapsulation-Dehydration. This method involves protecting the shoot tips in a calcium alginate capsule followed by controlled dehydration through exposure to laminar airflow before immersing in liquid nitrogen (Bettoni et al., 2018).

4. Conclusions

In this study, an efficient shoot tip cryotherapy protocol was developed using the dehydration-vitrification technique with PVS2 to eliminate PPV in apricot plants. Despite the success achieved, further optimisation of the protocol would be beneficial to improve meristem survival if intended for cryopreservation. Additionally, the development of new protocols that avoid the application of DMSO, such as using PVS3 or the encapsulation-dehydration technique, would be desirable.

Annex II

Inefficiency of meristem culture in the elimination of PPV and HSVd from apricot cultivars

This annex describes preliminary experiments conducted to demonstrate if meristem rescue from infected *in vitro* shoots was enough to produce pathogen-free apricot plants.

Methodology used

Shoots from Ca-PPV and MR-HSVd cultivars were grown in both SSM (Wang et al., 2013) and TIS (Pérez-Caselles et al., 2022). After a four-week cultivation cycle, meristem rescue was performed on shoots from both Ca-PPV and MR-HSVd cultivated in SSM and TIS. Meristems of 1.5 mm were isolated from both apical and axillary buds. Each isolated meristem was considered a potentially pathogen-free independent line. Meristems were grown and evaluated as described previously by Pérez-Caselles et al. (2025). The variables analysed in this study were meristem survival and the efficiency of pathogen elimination following meristem rescue.

Main results obtained

Meristem survival was similar between cultivars (Ca-PPV and MR-HSVd) regardless of the cultivation system used (Table 1). In SSM, survival rates reached 90.2% for Ca-PPV and 90.4% for MR-HSVd, while in TIS, survival was slightly lower, with values of 83.0% and 83.7%, respectively. This indicates that meristem rescue enables the establishment of apricot lines with high survival rates, independent of the cultivation system used. Regarding treatment efficiency, *in vitro* RT-PCR evaluation results (Figure 1) showed that no lines had eliminated PPV or HSVd in either SSM or TIS.

Table 1. Effect of meristem rescue on survival (%) and efficiency (%) of Ca-PPV and MR-HSVd.

Cultivar	Cultivation system	Meristem rescue and growth			*In vitro* evaluation	
		N	L	Survival (%)	Evaluated lines	Efficiency (%)
Ca-PPV	SSM	51	46	90.2	13	0
	TIS	53	44	83.0	15	0
MR-HSVd	SSM	52	47	90.4	13	0
	TIS	49	41	83.7	15	0

N: number of shoot tips isolated; L: number of established lines.

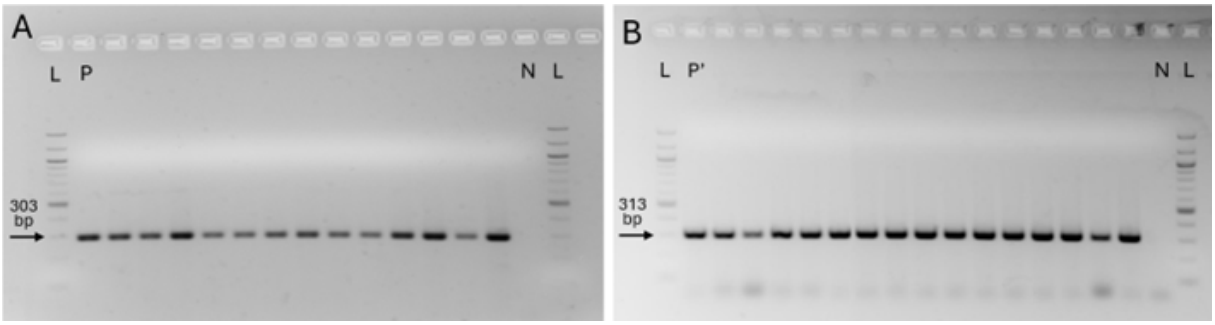


Figure 1. Electrophoretic analysis of RT-PCR products for HSVd (A) and PPV (B) detection. L: 100 bp ladder; P: HSVd positive control; P': PPV positive control; N: negative control; the rest of the lanes are independent apricot lines, each from a different meristem.

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