

RESEARCH RESULTS FOR THE YEAR 2009 ‘DEVELOPMENT OF PLUM POX VIRUS RESISTANT ‘FRENCH PRUNE’ (aka ‘IMPROVED FRENCH’) PLUM.

Ralph Scorza

OBJECTIVES

The objectives of this project are to 1) to develop genetically engineered clones of ‘French Prune’ that are highly resistant to PPV and 2) determine the level of susceptibility of ‘French Prune’ to PPV.

PROPOSED RESEARCH AND DEVELOPMENT

Since 1990, the USDA-AFRS Genetic Improvement Research Unit has been developing genetically engineered (GE) plum lines with different novel traits including resistance to plum pox virus (PPV). PPV is the most serious disease of plum, causing fruit deformation, premature fruit drop, and ultimately loss of productivity and tree death. PPV has devastated plum production in Europe. It spread to North and South America in the 1990s and continues to spread in Canada, Chile and is currently present in New York after being eradicated in Pennsylvania and Michigan. ‘HoneySweet’ a GE plum highly resistant to PPV, developed in our laboratory, is undergoing the deregulation process in the U.S. and has been approved by APHIS and FDA. In our laboratory we can consistently produce PPV resistant GE plums from seed and have plants in the greenhouse to test within 6 months. We propose to adapt this highly efficient seed transformation system to clonal material of ‘French Prune’. ‘French Prune’ will be genetically engineered with a piece of a PPV gene to induce a natural plant resistance mechanism called “gene silencing” to specifically destroy PPV. This is the mechanism that is responsible for the high level of resistance of ‘HoneySweet’ plum. Transgenic ‘French Prune’ lines will be tested in the containment greenhouse for resistance.

Based on our experience with previous transgenic plum lines we will utilize gene constructs with minimal intellectual property issues and constructs with the greater potential for consumer acceptability. Gene constructs will utilize only plant gene sequences (promoters and terminators) with the exception of the kanamycin antibiotic marker gene which is necessary at this stage of research.

Examination of the PPV susceptibility will be undertaken through inoculation of young propagated trees of ‘French Prune’ in an approved BL3 bio-containment greenhouse facility at USDA-ARS, Ft. Detrick, Maryland. Under these conditions vegetative tissue symptoms can be evaluated. Budwood will be sent to a European collaborator for field tests and observations of fruit symptoms. These fruit symptom observations will be reported to the CDFB in the future but will be beyond the scope of the proposed project timetable.

UUFunding cycle: Project funds for year one of the project were received by ARS and officially made available to R. Scorza on August 15, 2007. A postdoctoral research associate who was hired to work on the project began work on November 1, 2007. Year two funding was received in August 2008 and these funds supported work from November 2008 until December 2009. No funding was requested in 2009 because the delay in transfer of the funds into our program in 2007 placed us out of sync with the plum board’s funding cycle and the project utilized funds

from 2008 to support the project in 2009. We are now in sync with the CDPB funding cycle and the current represent results from January 15 through December 15, 2009.

RESULTS

Executive Summary of 2009 research results: During this cycle of R&D almost 5,000 'French Prune' leaf cultures were exposed to varying transformation treatments. As of December 2009 a few shoots continue to survive on kanamycin selection. The lack of vigorous growth of these shoots suggests that they may not be transformed. Nevertheless, these shoots will be analyzed for the presence of the PPV resistance transgene. These results indicate that transformation treatments must be expanded. From our experience this year and through consultations with a Russian researcher, Dr. Sergey Dolgov, who has successfully transformed plum shoots from leaves of a Russian plum variety, we have identified additional parameters must be evaluated and how they should be evaluated if project funding is continued.

A significant breakthrough in the regeneration process was achieved through the use of a regeneration enhancing gene. We feel that the use of this gene will significantly improve the production of transgenic 'French Prune' shoots.

We transferred 'French Prune' budwood to a collaborator in Romania and 'French Prune' trees, and most importantly fruit, can now be evaluated in the field for PPV symptomology. We have identified Pennsylvania PPV strains that produce strong symptoms on plum and these can now be used to evaluate symptoms of these strains in 'French Prune'. This will be important since these U.S. strains are most likely those that would enter California. These cannot be tested in Romania or other EU countries since new PPV strains cannot be brought into those areas.

Results Narrative:

Transformation of 'French Prune' with a PPV resistance gene: Approximately 3,800 'French Prune' leaf cultures were exposed to transformation treatments that included comparisons of *Agrobacterium* strains GV3101 vs. EHA 105 (*Agrobacterium* is the bacterium responsible for gene transfer), variations in the concentration, timing and length of exposure to kanamycin for selection, and trials of paramomycin as an antibiotic selection agent. While initial results were promising in terms of shoots growing under antibiotic selection, at the end of these trials no transgenic plants were recovered. Assays of the shoots that grew on kanamycin or paramomycin indicated that they were not transformed nor were they chimeric (with a mixture of transformed and non-transformed cells) (Fig. 1). This led us to investigate pretreatment of leaf pieces with the cytokinin growth regulator thidiazuron. In November 2009 we hosted in our laboratory Dr. Sergey Dolgov, a Russian researcher who has successfully transformed plum shoots from leaves of a Russian plum variety. The interaction with Dr. Dolgov led to changes in our *Agrobacterium* infection and selection protocols and to the pretreatment of leaf pieces with the auxin growth regulator IAA. Approximately 1,200 leaf pieces were subjected to these new protocols. From these, 71 shoots that regenerated from leaves survived the initial kanamycin selection. After 2 months of selection 13 'French Prune' shoots continue to survive, although none show vigorous growth that is associated with transformed shoots. These 13 shoots will be analyzed for transformation through PCR. Positive shoots will be further analyzed by DNA blotting. Based upon our experience and consultation with Dr. Dolgov there are a number of promising approaches to 'French Prune' transformation that remain to be explored.

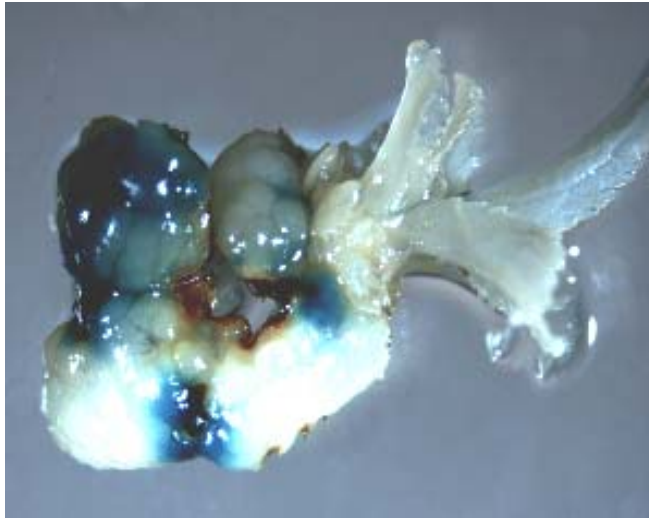


Figure 1. Transformation of leaf 'French Prune' (dark blue color) did not extend to the shoots in initial trials.

Increasing levels of 'French Prune' regeneration: While relatively high levels of regeneration of shoots from leaves of 'French Prune' have been obtained (64%) these rates are based on the number of leaves that regenerate shoots. Yet, the number of shoots produced per leaf is low (1-3 shoots per leaf). In an effort to increase the level of shoot regeneration Dr. Srinivasan has been working with genes that have the potential to induce high levels of regeneration from leaves. He has demonstrated that one of these genes when transferred into plum can increase the regeneration dramatically. In 'Bluebyrd' plum up to 100% of leaves have been induced to produce large numbers of shoots per leaf (Fig. 2) (data is still being collected from these trials). While not yet been applied to 'French Prune' this technology could dramatically affect transformation of this cultivar.



Figure 2. High rates of regeneration from leaves of 'Bluebyrd' plum in the presence of a regeneration-inducing gene.

Evaluation of susceptibility of 'French Prune' to PPV: The 'D' strain of PPV from Pennsylvania that was used to inoculate 'French Prune' in 2007-2008 did not produce clear leaf symptoms even though inoculated plants had a high level of infection (Fig. 5). The particular PPV strain used generally produces good symptom on peach but not on plum. Additional work by Drs. Vern Damsteegt and William Schneider USDA-ARS Ft. Detrick, Maryland in the 2009 project year has shown that there are several strains of Pennsylvania PPV that do produce severe symptoms on plum. 'French Prune' plants have been propagated for inoculation with these PPV strains.

Following APHIS approval to ship 'French Prune' budwood to Romania in winter 2008-2009 budwood was shipped in summer 2009 at a time requested by our Romanian collaborator. Budded trees are being exposed to field infection of PPV in Romania and leaf symptoms and fruit symptoms and infection levels will be evaluated in Romania to provide data on leaf and especially fruit symptomology of 'French Prune' infected with various strains of PPV under field conditions.

BUDGET SUMMARY

Project funds received in 2008, utilized in 2009:

January 15, 2009 – December 31, 2009

Visiting foreign scientist salary and benefits	\$38,219.
--	-----------

Fringe benefits and Foreign Agricultural Service scientist support services	8,390.
--	--------

	46,609.
--	----------------

Recap of prior year results: ‘French Prune’ budwood was kindly provided by Dave Wilson Nursery and by the National Clonal Repository in Davis, CA. *P. domestica* rootstock was bud-grafted and ‘French Prune’ buds were allowed to produce shoots. During the initial stages of the project we were able to successfully establish shoots of ‘French Prune’ in vitro which is the first step in developing a clonal regeneration system.

As these shoots developed leaves were harvested for in vitro tissue culture (step 1, Fig. 3).

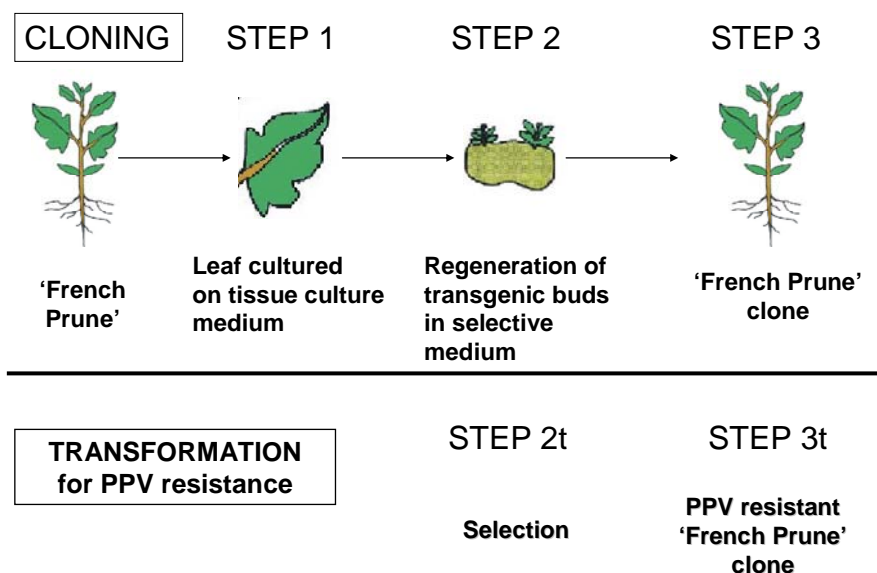


Figure 3. Scheme for the regeneration and transformation of ‘French Prune’

A series of factors known to affect regeneration from leaves were evaluated for ‘French Prune’ (step 2, Fig. 3). These included growth regulators (plant hormones), growth media nutrients, chemicals used to set up the gelling of the tissue culture medium and the ratio of hours darkness to light. Optimization of these factors has led to 64% of the cultured leaves producing one to several shoots each. These regenerated shoots were then exposed to a number of treatments to induce further shoot development and rooting. Following regeneration of shoots from leaves we developed protocols to root shoots and establish cloned plants of ‘French prune’ in the greenhouse (step 3, Fig. 3, and Fig. 4). This work demonstrated the success in cloning ‘French Prune’ from leaves which had not been previously reported for this cultivar. These results were published in 2009 (Petri and Scorza, 2009. *Annals of Applied Biology* 156:78-79).

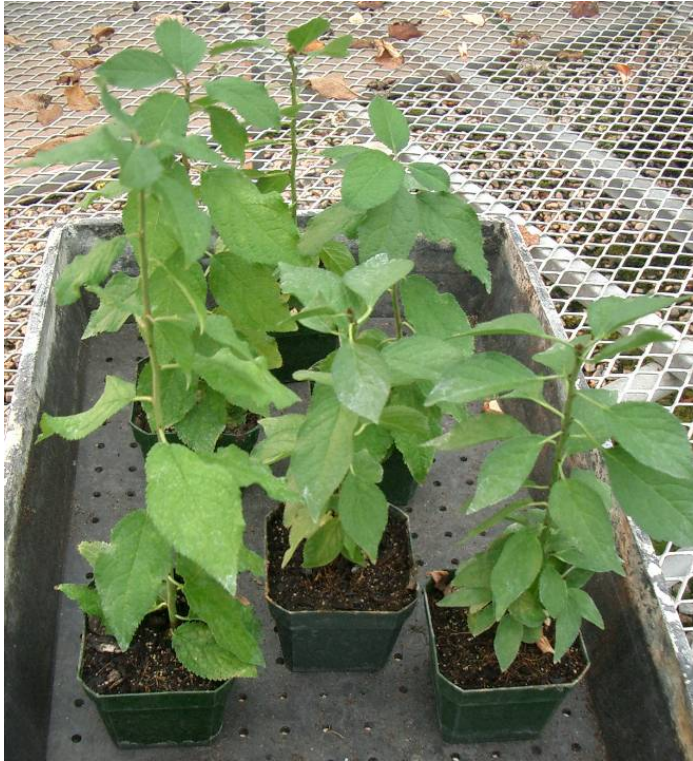


Figure 4. 'French Prune' plants growing in the greenhouse developed from in vitro cloning of leaves.

Transformation of 'French Prune' Step 2t: In order to discriminate those regenerated shoots that carry the transferred gene for PPV resistance it was necessary to use a gene specifically for selection so that only the shoots that are transformed will live on the culture medium with the selection agent. We tested a herbicide resistance gene for resistance to Basta (glufosinate) and a gene for resistance to the antibiotic kanamycin, both resistance genes have been approved for use in genetically engineered crops. We found that both were useful in plum for selecting leaf tissue in vitro that was transformed. Based on the long history of safe use for kanamycin, its EPA exemption from tolerance in raw agricultural commodities, and our success with its use in plum transformation from seed material we chose to concentrate on the kanamycin selection system. Additionally, although there is a general wariness of the use of antibiotic resistance markers in the EU, kanamycin resistance has been favorably reviewed for use by the European Food Safety Authority (EFSA) in two separate reports. [In the future we plan to test the possibility of eliminating the antibiotic resistance gene and produce transgenic 'French Prune' plants without selection. We can now do that using plum seed material which regenerates at much higher levels than do 'French Prune' leaves at this point in time.]

Using the kanamycin resistance selection system we have been able to achieve a transformation rate of 90% for in vitro cultured leaves of 'French Prune'. That is, 90% of the leaves that we exposed to transformation treatments produced areas of growth on the selection medium indicating that cells had taken up the genes and could then grow under antibiotic selection. Shoots were developed from these transformed leaves but these shoots subsequently died on the kanamycin-containing medium indicating that either, they were not originally transformed but

were able to survive for a short time on kanamycin or they were chimeras, that is, they were a mix of transformed and non-transformed cells and eventually succumbed to kanamycin. This work demonstrated the accomplishment of transformation step 2t (Fig. 1) but Step 3t was not completed and this was a major goal of the project in 2009.

Evaluation of susceptibility of ‘French Prune’ to PPV: In order to test the susceptibility of ‘French Prune’ to PPV and to evaluate leaf symptoms we inoculated plants with PPV Pennsylvania D strain under containment conditions at the USDA facility at Ft. Detrick Maryland. Molecular analyses (PCR) proved these plants to be infected with PPV (Fig. 5) but they did not produce clear PPV symptoms on leaves. These plants had been aphid inoculated with the D strain isolate from Pennsylvania. While this strain (PENN-3) produces severe symptoms on peach we find that in general it does not produce clear symptoms on plum. Nevertheless ‘French Prune’ was clearly susceptible to PPV allowing high levels of infection.

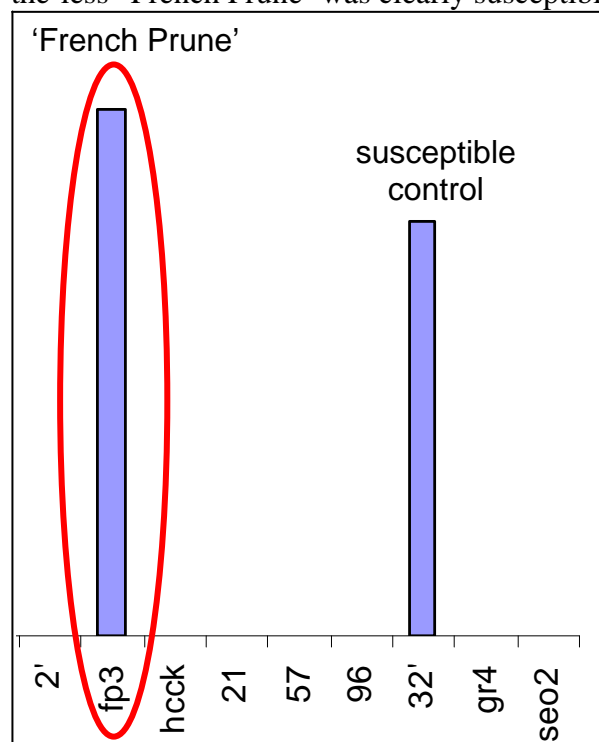


Figure 5. Quantitative PCR analysis of ‘French Prune’ PPV infection. Bars indicate a high level of infection in ‘French Prune’ and the susceptible control. Absence of bars indicates little or no infection of the resistant test plants.

During the winter of 2007-2008 we had a commitment from a long-time collaborator in Romania to test ‘French Prune’ for leaf and fruit symptom expression in a test plot in Romania. This plot contains the D, M, and REC (D+M recombinant) strains of PPV and would provide an excellent test of ‘French Prune’ reaction to PPV infection on leaves and fruit. APHIS approved shipping the budwood in the winter of 2008-2009.

RESEARCH ARTICLE

Factors affecting adventitious regeneration from *in vitro* leaf explants of 'Improved French' plum, the most important dried plum cultivar in the USA

C. Petri^{1,2} & R. Scorza³

1 Genetic Improvement of Fruit Crops Unit, USDA-ARS-Appalachian Fruit Research Station, Kearneysville, WV, USA

2 Departamento de Mejora Vegetal, CEBAS-CSIC, Campus de Espinardo, Espinardo (Murcia), Spain

3 USDA-ARS-Appalachian Fruit Research Station, Kearneysville, WV, USA

KeywordsEuropean plum; organogenesis; *Prunus domestica*; silver thiosulphate; thidiazuron.**Correspondence**R. Scorza, USDA-ARS-Appalachian Fruit Research Station, 2217 Wiltshire Road, Kearneysville, WV 25430, USA.
Email: Ralph.Scorza@ARS.USDA.GOV

Received: 22 April 2009; revised version accepted: 20 July 2009.

doi:10.1111/j.1744-7348.2009.00364.x

Abstract

An adventitious shoot regeneration protocol from *in vitro* leaves of the most important dried plum cultivar in the USA, 'Improved French', has been established. Factors affecting regeneration were studied in order to optimise regeneration. The proliferation medium in which the shoots, used as the source of leaf explants, were cultured had a strong influence on subsequent regeneration. Shoot regeneration was observed at a mean frequency of 52% when a Murashige-based and Skoog-based shoot culture medium with 3 µM *N*⁶-benzylaminopurine and 0.25 µM indole-3-butyric acid (IBA) was employed compared with shoot regeneration frequencies of less than 5% for a Quoirin-based and Lepoivre-based shoot culture medium, with 8.9 µM *N*⁶-benzylaminopurine and 0.49 µM IBA. The shoot regeneration medium contained α-naphthaleneacetic acid at 2.0–6.0 µM and thidiazuron at 4.5–15.0 µM. 2,4 Dichlorophenoxy-acetic acid at 9.0 µM was included in the medium but only for the first 4 days of culture. Shoot regeneration frequencies were positively related to thidiazuron concentration and significantly greater ($P < 0.05$) for 9–15 µM thidiazuron than for the media with 4.5 µM thidiazuron. Leaf explants, incubated in a 16-h-light/8-h-dark photoperiod or in the dark for 1 week followed by exposure to light, showed significantly more organogenic activity ($P < 0.01$) than was observed for leaves cultured in the dark for 2 or 3 weeks before they were transferred to the light. The utilisation of Bacto agar (0.7%) as the gelling agent increased organogenesis compared with media gelled with TC Agar (0.7%), or an agar–gellan gum blend (AgargelTM) (0.45%). The addition of the ethylene inhibitor silver thiosulphate at 60–120 µM also improved organogenesis. When all the studied factors were optimised, a regeneration rate of 65% was achieved. Rooting frequency of regenerated shoots was significantly increased ($P < 0.05$) by the use of full-strength Murashige and Skoog salts (40%) or 100 mg L⁻¹ phloroglucinol (53%) to the rooting medium.

Introduction

'Improved French', also known as 'French' or 'French Prune', is perhaps the most economically important European plum (*Prunus domestica* L.) in the world. It is particularly suited to drying and accounts for most of the

world's trade in dried plums (prunes). 'Improved French' is particularly important in California where it accounts for 99% of the dried plum production, which reached 180 000 tons in 2006 with a production value of US \$255 684 000 (USDA, 2006). California supplies 100% of

the USA and 60% of the world's dried plums (Boriss & Brunke, 2005). The origin of 'Improved French' is 'Prune d'Agen' imported from France in the mid-1800s (Hedrick, 1911). 'Improved French' is probably a seedling of 'Prune d'Agen' that was selected by Luther Burbank (J. Doyle, personal communication). 'Improved French' is similar to 'Prune d'Agen' in appearance and quality. 'Prune d'Agen' and the many budsports of 'Prune d'Agen' form the basis of the dried plum industry in France, which is the second largest producer after California (FAS, 2005). Virtually, all dried plums in world trade are 'Improved French' or budsports of 'Prune d'Agen', and new varieties must meet or exceed the quality standards of this closely related germplasm.

Conventional plum breeding, similar to breeding temperate fruit trees in general, is constrained by a long reproductive cycle, complex reproductive biology (such as self-sterility), polyploidy and a high degree of heterozygosity (Okie & Weinberger, 1996; Petri & Burgos, 2005). Trait improvements in plum such as delayed bloom, changes in ripening date and disease resistance must be developed within a defined set of quality parameters that include specific industrial and consumer-oriented traits such as sugar level, skin and flesh colour, pit size, flesh adhesion to the stone and moisture content for drying. As opposed to conventional breeding, a potential advantage of genetic engineering (GE) lies in the ability to target specific improvements in established cultivars. Genetic improvement through GE will likely require less time, labour and field space (Petri & Scorza, 2008). Fixation through the sexual cycle is unnecessary because cultivars are clonally propagated. In addition, the targeted improvement of established cultivars avoids the time and effort necessary for the establishment of a new cultivar in the market. Because most of the production of dried plums in the USA is based on 'Improved French' (USDA, 2006), the potential impact of genetic engineering this cultivar is significant.

For most plant GE protocols, adventitious shoot regeneration is one of the most important rate limiting steps. Fruit trees are especially recalcitrant for shoot regeneration (Petri & Burgos, 2005). Moreover, regeneration efficiency is highly genotype dependent and in some cases, regeneration protocols cannot be transferred between cultivars. There are few reports on *in vitro* adventitious shoot regeneration from clonal explants of European plum. In these reports, regeneration rates are low, 12–32% depending on the genotype (Bassi & Cossio, 1991; Escalettes & Dosba, 1993; Csányi *et al.*, 1999), or the varieties used as explant sources are generally of limited or of local importance (Nowak *et al.*, 2004; Mikhailov & Dolgov, 2007). In addition, none of these prior research groups worked with the 'Improved French' cultivar.

The present study was designed to explore the conditions required for the regeneration of shoots from leaf explants of 'Improved French' plum, and to maximise regeneration. Regeneration was investigated using a strategy that was successfully applied to regenerate apricot (*Prunus armeniaca* L.) from leaf explants (Petri *et al.*, 2008a). Parameters shown to generally affect shoot regeneration in fruit tree species were evaluated, including shoot proliferation medium (Burgos & Albuquerque, 2003), light (Leblay *et al.*, 1990; Korban *et al.*, 1992; Miguel *et al.*, 1996; Pérez-Tornero *et al.*, 2000; Gentile *et al.*, 2002; Espinosa *et al.*, 2006), gelling agent (Welander & Maheswaran, 1992; Chevreau *et al.*, 1997; Pérez-Tornero *et al.*, 2000; Burgos & Albuquerque, 2003) and growth regulators (Escalettes & Dosba, 1993; Declerck & Korban, 1996), including ethylene inhibitors (Escalettes & Dosba, 1993; Burgos & Albuquerque, 2003; Petri *et al.*, 2005).

Materials and methods

Establishment of shoots *in vitro* and maintenance of cultures

Young 10–15 cm long shoots from current season growth of 1 to 2-year-old greenhouse-grown 'Improved French' plum were the source of explants for establishing the *in vitro* cultures. Leaves were removed and shoots were cut into 1.5–2.0 cm long sections, each with one or two nodes. Sections were washed in deionised water with 0.025% LiquiNox® (Alconox, White Plains, NY, USA) for 5 min, and then rinsed three times with sterile deionised water. For surface disinfestation, the explants were immersed for 2 h in an aqueous solution of 0.01 M sodium dichloroisocyanurate (Sigma, St Louis, MO, USA) (Parkinson *et al.*, 1996). Explants were then rinsed three times with sterile deionised water and placed in shoot multiplication media. As shoots developed from the nodes of the stem explants, they were removed and subcultured to fresh medium. Every 3 weeks, new axillary shoots (>1 cm tall) were harvested and individually subcultured in fresh medium.

Two media, used previously with *P. domestica*, were used for shoot multiplication: JS, as described by Mikhailov & Dolgov (2007) or shoot growth medium (SGM), as described by Gonzalez-Padilla *et al.* (2003). JS medium consisted of Quoirin and Lepoivre (QL) salts (Quoirin & Lepoivre, 1977) supplemented with 100 mg L⁻¹ myoinositol, 0.1 mg L⁻¹ thiamine HCl, 0.5 mg L⁻¹ nicotinic acid, 0.5 mg L⁻¹ pyridoxine HCl, 2.0 mg L⁻¹ glycine, 3% (w/v) sucrose, 8.89 µM N⁶-benzylaminopurine (BA) and 0.49 µM indole-3-butyric acid (IBA). SGM consisted of three fourth strength of Murashige and Skoog (MS) salts (Murashige &

C. Petri & R. Scorza

Skoog, 1962) supplemented with 100 mg L⁻¹ myoinositol, 0.1 mg L⁻¹ thiamine HCl, 0.5 mg L⁻¹ nicotinic acid, 0.5 mg L⁻¹ pyridoxine HCl, 2.0 mg L⁻¹ glycine, 2% sucrose (w/v), 3.0 μM BA and 0.25 μM IBA.

Bacto agar (Becton, Dickinson and Company, Sparks, MD, USA; Cat. No. 214010) at 0.7% (w/v) was used as the gelling agent for both media, and the pH was adjusted to 5.8 before autoclaving at 121°C for 20 min.

Cultures were maintained in a growth room with a 16-h-light/8-h-dark photoperiod (45–50 μE m⁻²s⁻¹) at 24 ± 1°C.

General strategy for regeneration

The first four apical, expanding leaves from 3-week-old axillary shoots were placed in sterile water and swirled to randomise. Leaves were blotted dry on sterile filter paper, cut transversely three or four times across the midrib without fully separating the segments and cultured with the adaxial side in contact with the regeneration medium.

Regeneration strategy and medium were as described by Petri *et al.* (2008a). Briefly, shoot regeneration medium (SRM) consisted of QL macronutrients, Driver and Kuniyuki micronutrients, vitamins and organic compounds (Driver & Kuniyuki, 1984) 3% (w/v) sucrose and 0.7% (w/v) TC Agar (Phytotechnology Laboratories, Shawnee Mission, KS, USA: Cat. No. A175). SRM was supplemented with 9.0 μM thidiazuron (TDZ), 4.0 μM α-naphthaleneacetic acid (NAA) and 9.0 μM 2,4-dichlorophenoxy-acetic acid (2,4-D). After 4 days in the dark, explants were placed in SRM without 2,4-D and supplemented with 60-μM silver thiosulphate (STS).

The medium was adjusted with KOH to pH 5.7, autoclaved at 121°C for 20 min and then dispensed into 8.5 cm × 1.5 cm sterile plastic Petri dishes (~25 mL each).

After explants were positioned on the medium, the dishes were sealed with Parafilm®, and incubated in the dark at 24 ± 1°C. After 2 weeks in the dark, explants were transferred to the light with a 16-h photoperiod (45–50 μE m⁻² s⁻¹) at 24 ± 1°C.

Leaf explants were maintained in the same Petri dish (not transferred to fresh medium) during the entire experiment. This shoot regeneration protocol was followed, with the modifications indicated below, for all of the experimentation. Adventitious shoot regeneration was evaluated weekly, starting 3 weeks after the leaf explants were placed on the regeneration medium and continuing until adventitious buds stopped appearing for 2 weeks in a row, approximately 9–10 weeks from the beginning of the experiment, at which point, final data were collected on the shoot regeneration frequencies and yields.

Factors affecting regeneration from leaves of 'Improved French' plum

Effect of the shoot proliferation medium

Leaf explants from axillary shoots cultured for 3 weeks on fresh JS or SGM shoot multiplication media were collected and placed on SRM for subsequent regeneration.

Effect of the gelling agent

Leaves from shoots maintained in SGM were cultured on SRM with 0.7% (w/v) TC Agar (Phytotechnology Laboratories, Shawnee Mission, KS, USA: Cat. No. A175), 0.7% (w/v) Bacto agar (Voigt Global Distribution Inc., Lawrence, KS, USA: Cat. No. 214010) or 0.45% (w/v) Agargel™ (Sigma-Aldrich, St. Louis, MO, USA: Cat. No. A3301). Agargel™ is a blend of agar and gellan gum (Sigma Phytigel™).

All the three gelling agents were added at the concentration recommended by the manufacturers for plant tissue culture, and this resulted in similar apparent media firmness.

Effect of the dark period

Leaves from shoots cultured in SGM were cultured on SRM under four light or dark treatments: a 16-h photoperiod (45–50 μE m⁻² s⁻¹) at 24 ± 1°C or complete darkness for 1, 2 or 3 weeks (at 24 ± 1°C) before transfer to a 16-h photoperiod (45–50 μE m⁻² s⁻¹) at 24 ± 1°C.

Effect of growth regulator concentrations

Leaves from shoots cultured in SGM were placed on SRM with the following combinations and concentrations of growth regulators: 15.0 μM TDZ with 6.0 or 4.0 μM NAA, 12.0 μM TDZ with 6.0 or 4.0 μM NAA, 9.0 μM TDZ with 4.0 or 2.0 μM NAA and 4.5 μM TDZ with 4.0 or 2.0 μM NAA. These combinations were designed to test a range of cytokinin:auxin ratios in the SRM (approximately from 4:1 to 1:1), at different concentration levels.

Bacto agar (0.7%) was used as the gelling agent, and leaves were cultured in the dark for 1 week before transfer to the light.

Effect of ethylene inhibitor (STS)

Leaves from shoots cultured in SGM were cultured on SRM, solidified with 0.7% Bacto agar. After 4 days, when leaf explants were transferred to SRM without 2,4-D, SRM was supplemented with 0, 30, 60 or 120 μM STS.

STS stock solution was prepared by mixing a 0.465 mM AgNO₃ solution with a 1.865 mM Na₂S₂O₃ solution (ratio 1:4), following procedures described by Burgos & Albuquerque (2003). STS was added to the culture medium prior to autoclaving.

Isolation of regenerated buds, rooting and acclimatisation

When regenerated buds reached approximately 0.5 cm in diameter they were isolated from the leaf explants with a small portion of the callus underneath and placed in SGM. When adventitious shoots reached 2–3 cm long, they were transferred to rooting medium (RM), as described by Gonzalez-Padilla *et al.* (2003). The basal RM consisted of half of the strength of MS salts, with SGM organics (vitamins and sucrose), 0.1 μM kinetin and 5.0 μM NAA. Different modifications of RM were tested: full-strength MS salts or addition of 100 mg L^{-1} phloroglucinol (PG) or addition of 2.5 μM IBA instead of 5.0 μM NAA. Bacto agar at 0.7% (w/v) was used as the gelling agent. Shoots were cultured under a 16-h-light/8-h-dark photoperiod ($45\text{--}50 \mu\text{E m}^{-2} \text{s}^{-1}$) at $24 \pm 1^\circ\text{C}$. Roots appeared after 3–5 weeks, and when roots reached approximately 1-cm long, shoots were ready for acclimatisation.

For acclimatisation, procedures described by Petri *et al.* (2008b) were followed. Briefly, shoots rooted in RM were washed in sterile water to eliminate agar residues, and then transferred to 7.6-cm² peat pots containing Metro-Mix 510 potting medium (SUNGRO Horticulture, Bellevue, WA, USA). The potted plantlets were introduced into zipper-seal plastic bags which were sealed and maintained completely closed in the tissue culture growth room with a 16-h-light/8-h-dark photoperiod ($45\text{--}50 \mu\text{E m}^{-2} \text{s}^{-1}$) at $24 \pm 1^\circ\text{C}$. After 2 weeks, the plastic bags were fully opened. Following 2 weeks in open plastic bags, during which time plants were watered with deionised water as needed, the plants were sprayed with an aqueous solution (1:20, v/v) of Anti Stress 2000 (Polymer Ag, Fresno, CA, USA) and cultured under ambient greenhouse conditions of 12–16 h photoperiod and temperatures ranging from 22°C to 26°C .

Experimental design and data analysis

At least five Petri plates were evaluated for each treatment, each plate containing seven leaves. Experiments were repeated twice. For the rooting studies, experiments were repeated three times with a minimum of 10 shoots per treatment.

Regeneration and rooting percentages and number of shoots per regenerating explant were compared with maximum likelihood ANOVA and, when necessary, specific contrasts of maximum likelihood were designed (SAS, 1988). Bonferroni LSD means procedure was used for mean values separation following ANOVA (SAS, 1988).

Results

Although shoot multiplication data were not collected, observations indicated that the rates of culture growth were equivalent across both micropropagation media (JS and SGM) with about two to three shoots being produced per original shoot per 3-week culture period. However, the shoots cultured on JS medium were clearly shorter and had smaller leaf size and were significantly ($P < 0.001$) less competent for shoot regeneration under the experimental conditions employed. Less than 5% of the leaf explants from the shoot cultured in JS medium produced shoots ($1.4 \pm 1.4\%$) compared with mean shoot regeneration frequencies of $51.4 \pm 8.4\%$ for the leaves of shoots cultured in SGM.

After 2 weeks in the dark, leaf explants enlarged and showed some bleaching. Calli appeared on the cut edges and midribs or in association with vascular tissue (Fig. 1a). Buds started appearing after 4 weeks from the beginning of the experiment (Fig. 1b) and after a total of 8–9 weeks no additional buds regenerated. Buds developed from calli and appeared from both adaxial and abaxial sides of the explants (Fig. 1b and Fig. 1c). Occasionally buds formed in clusters (Fig. 1d). Bud viability was high after isolation and placement on SGM (83.7% of the isolated buds survived and elongated).

Regeneration rates were significantly affected by the TDZ concentration ($P < 0.01$), but not by NAA. Separation of means by the Bonferroni LSD method revealed that highest regeneration rates were obtained with 15.0 and 12.0 μM TDZ (Table 1), although often, when 15.0 μM TDZ was added to SRM, buds appeared chlorotic (Fig. 1e). The number of shoots per regenerating explant was not significantly affected by growth regulator concentrations (Table 1).

The type of gelling agent also showed a significant influence on regeneration ($P < 0.01$). Although, regeneration rates were similar with TC agar or AgargelTM, regeneration was increased almost threefold when Bacto agar was used (Fig. 2).

'Improved French' leaves, incubated under a 16-h photoperiod or in the dark for 1 week before transfer to the 16-h photoperiod, showed significantly more organogenic activity ($P < 0.01$) than when explants were cultured in the dark for longer than 1 week (Fig. 3). Although calli appeared for all treatments, an increase in callus formation was observed as the dark period length was increased.

Specific statistical contrasts that compared each STS treatment with the non-STs control showed significant differences in the regeneration percentages when 60 μM ($P < 0.05$) or 120 μM ($P < 0.01$) was added to SRM (Fig. 4). Buds often appeared chlorotic when the medium

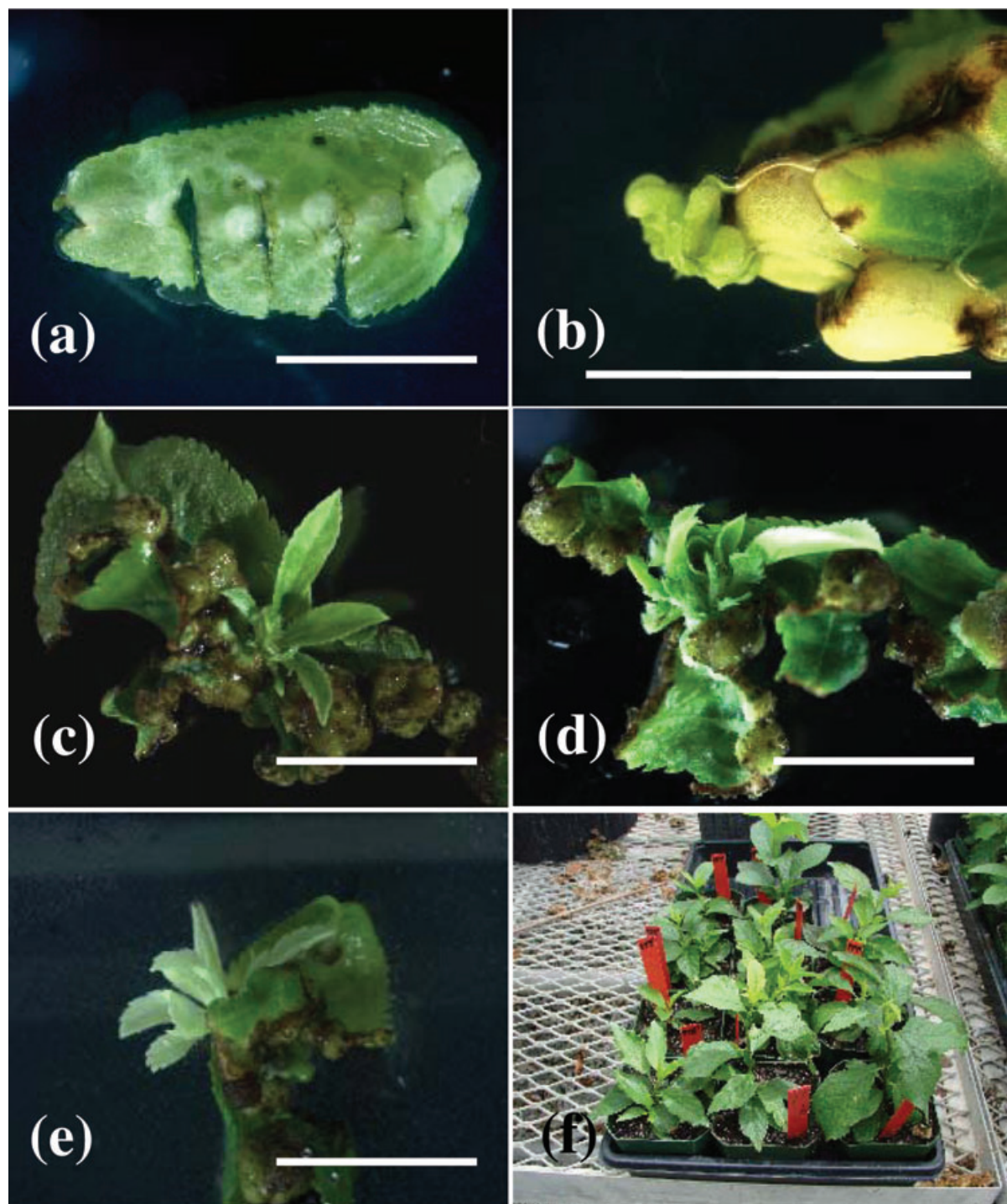


Figure 1 (a) Leaf explant when transferred to light after 2 weeks of culture in dark. (b) Bud regenerating from callus tissue at 4 weeks from the beginning of the experiment. (c) Regenerating shoot from 'Improved French' leaf. (d) Cluster of shoots. (e) Regenerating shoot from leaves cultivated in medium with $15.0 \mu\text{M}$ thidiazuron. (f) 'Improved French' regenerated plantlets cultivated in a greenhouse. Bars indicate 1 cm. Nine to ten weeks were allotted for shoot regeneration. The explants were maintained on the same plates of culture medium for the entire duration of the experiment excluding the transfer to 2,4 dichlorophenoxy-acetic acid free media at the 4-day point (see Materials and methods).

Table 1 Effect of thidiazuron (TDZ) and naphthaleneacetic acid (NAA) on adventitious shoot regeneration from 'Improved French' leaf explants^a

Growth Regulators (μM)		Number of Explants	Percentage of Regenerating Leaves (mean \pm SE)	Number of Shoots per Regenerating Leaf (mean \pm SE)
TDZ	NAA			
15.0	6.0	70	48.6 \pm 6.0 ab	1.58 \pm 0.17
15.0	4.0	70	64.3 \pm 5.7 a	1.62 \pm 0.11
12.0	6.0	70	47.1 \pm 6.0 ab	1.78 \pm 0.17
12.0	4.0	70	55.7 \pm 5.9 a	1.72 \pm 0.14
9.0	4.0	140	36.4 \pm 4.1 bc	1.57 \pm 0.13
9.0	2.0	70	45.7 \pm 6.0 ab	1.87 \pm 0.19
4.5	4.0	70	20.0 \pm 4.8 c	1.57 \pm 0.27
4.5	2.0	70	30.0 \pm 5.5 bc	1.29 \pm 0.12

^aAny two mean values with no letter in common are significantly ($P = 0.05$) different by the Bonferroni LSD method. 2,4 Dichlorophenoxy-acetic acid (9.0 μM) was added to the medium during the first 4 days in culture, and then explants were transferred to medium without 2,4 dichlorophenoxy-acetic acid and supplemented with 60 μM silver thiosulphate. Nine to ten weeks was allotted for shoot regeneration. The explants were maintained on the same plates of culture medium for the entire duration of the experiment excluding the transfer to 2,4 dichlorophenoxy-acetic acid free media at the 4-day point (see Materials and methods)

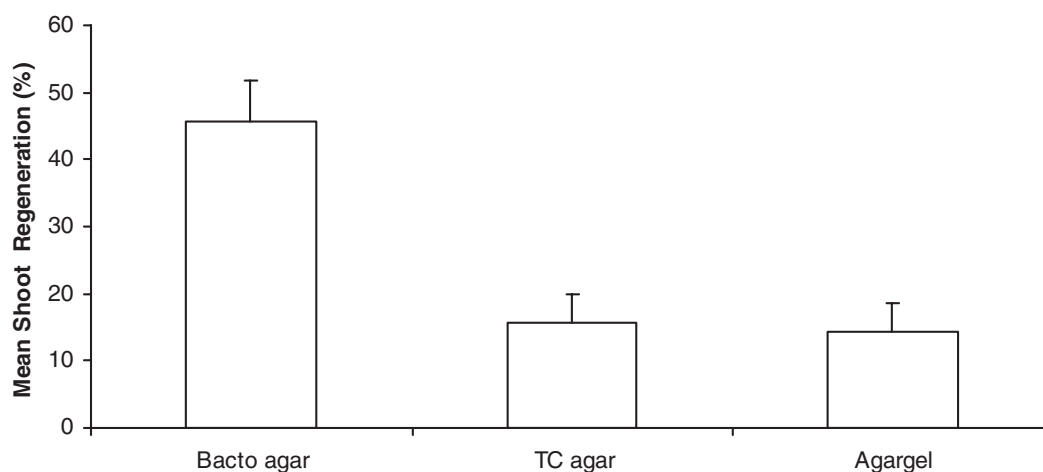


Figure 2 Effect of different gelling agents on adventitious shoot regeneration from leaf explants of plum convertible 'Improved French'. A total of 210 explants were used in this study. Bars indicate standard errors. Nine to ten weeks were allotted for shoot regeneration. The explants were maintained on the same plates of culture medium for the entire duration of the experiment excluding the transfer to 2,4 dichlorophenoxy-acetic acid free media at the 4-day point (see Materials and methods).

was supplemented with 120 μM STS, as well as when 15.0 μM TDZ was applied (Fig. 1e).

Rooting efficiency was very low when RM, as described by Gonzalez-Padilla *et al.* (2003), was used (Table 2). The addition of full-strength MS salts or 100 mg L^{-1} PG to RM increased rooting efficiency significantly for 'Improved French' shoots (Table 2). Substitution of 5.0 μM NAA by 2.5 μM IBA did not improve rooting rates compared to RM. No visual differences between the root system (length, diameter, etc.) of shoots rooted in the different RM modifications were observed. All 17 shoots that were used to test the acclimatisation step survived (Fig. 1f).

Discussion

Our results agree with the previous reports showing that the medium in which shoots (used as the source of explants) were cultured had a significant influence on subsequent regeneration (Antonelli & Druart, 1990; Burgos & Albuquerque, 2003). In this study, significant differences in regeneration capacity of leaves were found depending on the shoot proliferation medium used. The main difference between the two shoot proliferation media used was the concentration of BA, almost threefold lower than that used in the JS medium (3.0 μM in SGM vs 8.89 μM). This may explain why axillary shoots were, on average, shorter with smaller leaves when shoots were

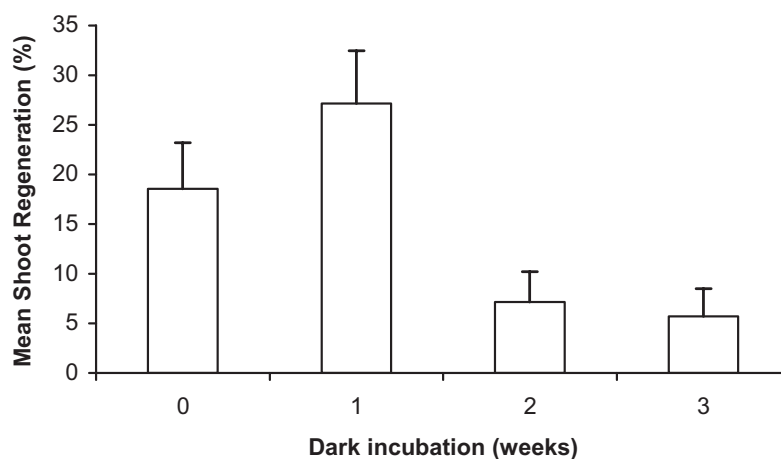


Figure 3 Influence of dark period on the regeneration percentages from leaves of 'Improved French'. A total of 280 explants were used in this study. Bars indicate standard errors. Nine to ten weeks were allotted for shoot regeneration. The explants were maintained on the same plates of culture medium for the entire duration of the experiment excluding the transfer to 2,4 dichlorophenoxy-acetic acid free media at the 4-day point (see Materials and methods).

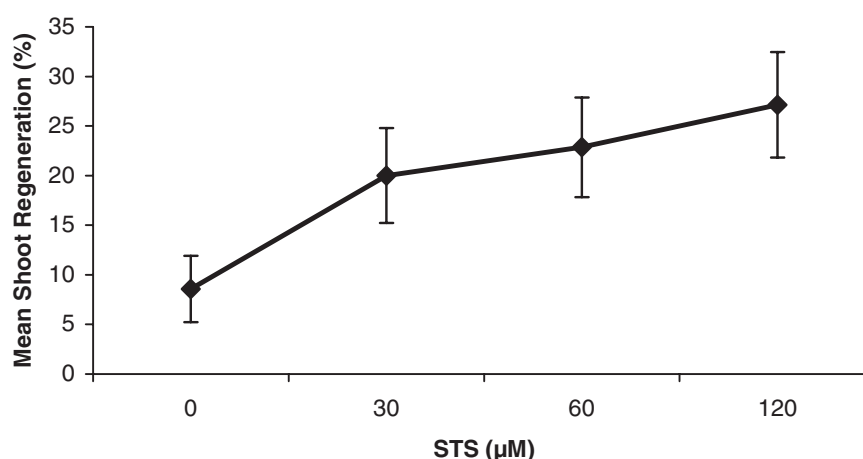


Figure 4 Effect of different concentrations of silver thiosulphate on adventitious regeneration from 'Improved French' leaf explants. Regeneration was calculated as the average of 70 explants for each treatment. Vertical bars represent standard errors. Nine to ten weeks were allotted for shoot regeneration. The explants were maintained on the same plates of culture medium for the entire duration of the experiment excluding the transfer to 2,4 dichlorophenoxy-acetic acid free media at the 4-day point (see Materials and methods).

cultured in JS medium compared with shoots cultured in SGM. This agrees with results obtained in *Prunus canescens* where shoot regeneration occurred satisfactorily on leaves collected from shoots grown in an elongation medium, without BA, but not on those taken from shoots cultured in a proliferation medium with 4.4-µM BA (Antonelli & Druart, 1990). In addition, significant increases in shoot regeneration from apricot leaves were obtained when shoots were cultured in a proliferation medium with a lower BA concentration (1.78 vs 3.31 µM) (Burgos & Albuquerque, 2003). The authors hypothesised that

propagation media affect the physiological status of the plant material, an important factor in regeneration.

This TDZ-based regeneration medium produced good results from 'Improved French' leaf explants. TDZ has been used successfully for plum regeneration from leaf explants, ranging from 12.5% to 65.5% for the cultivars 'Damas de Toulouse' (Escalettes & Dosba, 1993) or 'Wegierka Zwycla' (Nowak *et al.*, 2004) respectively. Although high regeneration efficiency has been reported when BA was used in the regeneration medium (80%) for the plum cultivar 'Startovaya' (Mikhailov & Dolgov, 2007), for the cultivar 'Damas de Toulouse'

Table 2 Rooting of 'Improved French' *in vitro* shoots using different media composition^a

Rooting Medium (RM)	Number of Shoots	Rooting Rate (%)	Number of Roots per Shoot
RM ^b	48	20.8 b	2.0
RM full MS Salts	30	40.0 a	2.3
RM 100 mg L ⁻¹ PG	30	53.3 a	2.2
RM 2.5 µM IBA	30	20.0 b	1.3

IBA, indole-3-butyric acid; MS, Murashige and Skoog salt; PG, phloroglucinol.

^aAny two mean values with no letter in common are significantly ($P = 0.05$) different by the Bonferroni LSD method.

^bAs described by Gonzalez-Padilla *et al.* (2003). See Materials and methods for composition.

no adventitious regeneration was achieved when BA was added, and only TDZ promoted shoot regeneration (Escalettes & Dosba, 1993). TDZ has been more effective than BA for inducing shoot regeneration from leaves of other *Prunus* species such as *P. armeniaca* (Pérez-Tornero *et al.*, 2000), *P. avium* (Bhagwat & Lane, 2004) and *P. serotina* (Hammatt & Grant, 1998; Espinosa *et al.*, 2006). Pérez-Tornero *et al.* (2000) also found that regeneration percentages were affected only by the TDZ concentration, but not by NAA.

The optimum TDZ concentration for plum has been shown to vary depending on the cultivar from 3.0 µM (Escalettes & Dosba, 1993) to 7.5 µM (Nowak *et al.*, 2004). Even though regeneration rates were slightly higher when 15.0 µM TDZ was added to SRM, 12.0 µM TDZ (with 4.0 µM NAA) was the optimum concentration as regenerated buds appeared healthier. While cytokinins are well known for antisenesescence activity, it has been demonstrated that 13.0 µM BA induced programmed cell death in carrot and *Arabidopsis* cell-suspension cultures and in *Arabidopsis* plants, probably by accelerating the senescence process (Carimi *et al.*, 2003). Low bud viability (Petri *et al.*, 2008a) or production of swollen or fasciated shoots (Huetteman & Preece, 1993; Bosela & Michler, 2008) has been reported in other woody plants, related to high TDZ levels.

Gelling agents have been shown to influence growth *in vitro*, depending on the type (Chevreau *et al.*, 1997), manufacturer and concentration (Debergh, 1983). Also, levels of impurities differ, and the nutrient status of the medium may be affected (Mackay & Kitto, 1988). Previous studies in apple (Welander & Maheswaran, 1992), pear (Chevreau *et al.*, 1997) and apricot (Pérez-Tornero *et al.*, 2000; Burgos & Albuquerque, 2003) demonstrated that gelling agents significantly affect regeneration. The three gelling agents used in this study have different ionic composition. In general, agars contain

less mineral impurity than Gellan gum, a component of AgargelTM. In addition, TC Agar, a purified grade agar, would be expected to contain less mineral nutrient impurity than Bacto agar. This may explain the present results. The use of Gellan gum increased the regeneration rate for all genotypes tested in apple (41% vs 14% with agar) (Welander & Maheswaran, 1992), and in pear, reaching 95% for one cultivar versus 52% with agar (Chevreau *et al.*, 1997). In another report, Gellan gum significantly decreased adventitious regeneration in apricot, compared with agar and AgargelTM (Pérez-Tornero *et al.*, 2000). The purest gelling agent assayed, purified agar, increased regeneration from 20% to 40%, but only for one of the two apricot cultivars tested (Burgos & Albuquerque, 2003). According to the manufacturer, Bacto agar has been optimised for calcium and magnesium contents, and detrimental ions such as iron and copper have been reduced for bacterial culture (<http://www.bd.com/ds/technicalCenter/inserts/Agars.pdf>). As the effect of gelling agent seems to be genotype dependent, Bacto agar was more appropriate for 'Improved French' than the other gelling agents tested.

A dark period has been shown to be a key factor in organogenesis for different fruit tree species, with the optimum dark period varying between 1 and 4 weeks (Leblay *et al.*, 1990; Korban *et al.*, 1992; Miguel *et al.*, 1996; Pérez-Tornero *et al.*, 2000; Gentile *et al.*, 2002; Espinosa *et al.*, 2006). In apricot, higher regeneration occurred with 2 or 3 weeks (30%) compared with 1 or 4 weeks (<10%) of dark incubation before light treatment (Pérez-Tornero *et al.*, 2000). Our results do not support the need for a longer dark period and follow previous results for *P. domestica* hypocotyl explants (Mante *et al.*, 1989; Gonzalez-Padilla *et al.*, 2003). With leaves explants, no dark or a 1-week dark period produced the best results. It has been reported that the dark period can influence endogenous levels of growth regulators such as indole-3-acetic acid (IAA) (Lopez-Carbonell *et al.*, 1992). Some reports attributed the positive effect observed on adventitious shoot regeneration of the dark induction to an increase in the IAA levels during this period which then interacted with exogenously applied growth regulators (Korban *et al.*, 1992; Miguel *et al.*, 1996). The use of high cytokinin:auxin ratios induces shoot organogenesis (Hamill, 1993). The increase in the endogenous auxin levels during the dark period could have a detrimental effect on shoot regeneration from 'Improved French' leaf explants cultured in SRM, because it could lead to a low cytokinin:auxin ratio.

Beneficial effects of ethylene inhibitors on organogenesis have been widely reported (Songstad *et al.*, 1988; Chi *et al.*, 1990; Chraïbi *et al.*, 1991; Ma *et al.*, 1998; Bais *et al.*, 2001), but only a few previous reports (Escalettes &

C. Petri & R. Scorza

Dosba, 1993; Burgos & Albuquerque, 2003; Petri *et al.*, 2005; Liu & Pijut, 2008) describe the effect of ethylene inhibitors, including Ag⁺, on organogenesis from *Prunus*. In all these reports, Ag⁺ significantly stimulated adventitious regeneration.

The ethylene inhibiting effect of STS (Ag⁺) in plant tissues is believed to act through the attachment of Ag⁺ to ethylene binding sites (Beyer, 1979). The optimum STS level has been shown to be genotype dependent, varying between 30 and 60 µM, for two apricot cultivars (Burgos & Albuquerque, 2003), or between 60 and 80 µM, for two black cherry genotypes (Liu & Pijut, 2008). We found 120 µM as the optimum concentration for 'Improved French'. In future studies, higher STS concentrations should be tested in order to determinate if the optimum concentration was 120 µM or higher, but the chlorotic aspect observed in buds that regenerated in medium with 120 µM STS may indicate proximity to toxic levels and higher concentrations might have a detrimental effect on adventitious shoot regeneration.

Our results suggest that the rooting process is genotype dependent because 91% of shoots of the plum cultivar 'Bluebyrd' rooted when placed in RM (Gonzalez-Padilla *et al.*, 2003) versus only 21% of the 'Improved French' shoots evaluated in this study. PG is a rooting cofactor that has been previously reported to stimulate rhizogenesis in other fruit tree species (Jones & Hatfield, 1976; James & Thurbon, 1981; Hammerschlag *et al.*, 1987). In peach, the addition of 100 mg L⁻¹ PG significantly increased rooting efficiency for all genotypes tested, that is 67% and 100% with PG versus 6% and 35% without PG for the cultivars 'Redhaven' and 'Jerseyqueen' respectively (Hammerschlag *et al.*, 1987). This agrees with our results showing that PG significantly increased root formation.

Conclusions

Our results, together with previous reports, indicate that adventitious shoot regeneration from leaf explants was highly genotype dependent. Several factors and conditions must be studied in order to establish an efficient protocol for each cultivar. The best results for 'Improved French' were obtained when 3-week old leaves, from shoots cultured in SGM proliferation medium, were collected and placed on SRM supplemented with 9.0 µM 2,4-D (only during the first 4 days), 12.0 µM TDZ, 4.0 µM NAA, 120 µM STS, 0.7% Bacto agar, and cultured in the dark for 1 week before transfer to a 16-h photoperiod. This study provides the possibility for GE of the most important dried plum cultivar in the USA.

Factors affecting regeneration from leaves of 'Improved French' plum

Acknowledgements

Funding for this project from the California Dried Plum Board is gratefully acknowledged. The authors also acknowledge the statistical advice provided by Lorenzo Burgos and John G. Phillips, and the technical assistance of Ahn Silverstein and Mark Demuth. Mention of a trademark, proprietary product or vendor does not constitute a guarantee or warranty of the product by the US Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

References

- Antonelli M., Druart P. (1990) The use of a brief 2,4-D treatment to induce leaf regeneration on *Prunus canescens* Bois. *Acta Horticulturae*, **280**, 45–50.
- Bais H.P., Sudha G., Ravishankar G.A. (2001) Influence of putrescine, silver nitrate and polyamine inhibitors on the morphogenetic response in untransformed and transformed tissues of *Cichorium intybus* and their regenerants. *Plant Cell Reports*, **20**, 547–555.
- Bassi G., Cossio F. (1991) In vitro shoot regeneration on "Bluefre" and "Susina di Dro" prune cultivars (*Prunus domestica* L.). *Acta Horticulturae*, **289**, 81–82.
- Beyer E.M. (1979) Effect of silver ion, carbon dioxide, and oxygen on ethylene action and metabolism. *Plant Physiology*, **63**, 169–173.
- Bhagwat B., Lane W.D. (2004) In vitro shoot regeneration from leaves of sweet cherry (*Prunus avium*) 'Lapins and Sweetheart'. *Plant Cell, Tissue and Organ Culture*, **78**, 173–181.
- Boriss H., Brunke H. (2005) *Commodity Profile: Dried plums (prunes)*. University of California, CA: Agricultural Marketing Resource Center.
- Bosela M.J., Michler C.H. (2008) Media effects on black walnut (*Juglans nigra* L.) shoot culture growth in vitro: evaluation of multiple nutrient formulations and cytokinin types. *In Vitro Cellular and Developmental Biology-Plant*, **44**, 316–329.
- Burgos L., Albuquerque N. (2003) Low kanamycin concentration and ethylene inhibitors improve adventitious regeneration from apricot leaves. *Plant Cell Reports*, **21**, 1167–1174.
- Carimi F., Zottini M., Formentin E., Terzi M., Lo Schiavo F. (2003) Cytokinins: new apoptotic inducers in plants. *Planta*, **216**, 413–421.
- Chraïbi K.M., Latche A., Roustan J.P., Fallot J. (1991) Stimulation of shoot regeneration from cotyledons of *Helianthus annuus* by the ethylene inhibitors, silver and cobalt. *Plant Cell Reports*, **10**, 204–207.
- Chevreau E., Mourgues F., Neveu M., Chevalier M. (1997) Effect of gelling agents and antibiotics on adventitious bud

- regeneration from in vitro leaves of pear. *In Vitro Cellular and Developmental Biology-Plant*, **33**, 173–179.
- Chi G.L., Barfield D.G., Sim G.E., Pua E.C. (1990) Effect of AgNO₃ and aminoethoxyvinylglycine on in vitro shoot and root organogenesis from seedling explants of recalcitrant *Brassica* genotypes. *Plant Cell Reports*, **9**, 195–198.
- Csányi M., Wittner A., Nagy A., Balla I., Vértessy J., Palkovics L., Balázs E. (1999) Tissue culture of stone fruit plants: basis for their genetic engineering. *Journal of Plant Biotechnology*, **1**, 91–95.
- Debergh P.C. (1983) Effects of agar brand and concentration on the tissue culture medium. *Physiologia Plantarum*, **59**, 270–276.
- Declerck V., Korban S.S. (1996) Influence of growth regulators and carbon sources on callus induction, growth and morphogenesis from leaf tissues of peach (*Prunus persica* L. Batsch). *Journal of Horticultural Science*, **71**, 49–55.
- Driver J.A., Kuniyuki A.H. (1984) In vitro propagation of Paradox walnut rootstock. *HortScience*, **19**, 507–509.
- Escalettes V., Dosba F. (1993) In vitro adventitious shoot regeneration from leaves of *Prunus* spp. *Plant Science*, **90**, 201–209.
- Espinosa A.C., Pijut P.M., Michler C.H. (2006) Adventitious shoot regeneration and rooting of *Prunus serotina* in vitro cultures. *HortScience*, **41**(1), 193–201.
- Foreign Agriculture Service (FAS). (2005) *Dried Fruit (Dried Plum-Prune) Situation in Selected Countries*. <http://www.fas.usda.gov/htp/horticulture/dried%20fruits/01-31-05%20Prune%20Situation%20Report.pdf> [accessed on 6 October 2009].
- Gentile A., Monticelli S., Damiano C. (2002) Adventitious shoot regeneration in peach [*Prunus persica* (L.) Batsch]. *Plant Cell Reports*, **20**, 1011–1016.
- Gonzalez-Padilla I.M., Webb K., Scorza R. (2003) Early antibiotic selection and efficient rooting and acclimatization improve the production of transgenic plum plants (*Prunus domestica* L.). *Plant Cell Reports*, **22**, 38–45.
- Hamill J.D. (1993) Alterations in auxin and cytokinin metabolism of higher plants due to expression of specific genes from pathogenic bacteria: a review. *Australian Journal of Plant Physiology*, **20**, 405–423.
- Hammatt N., Grant N.J. (1998) Shoot regeneration from leaves of *Prunus serotina* Ehrh. (black cherry) and *P. avium* (wild cherry). *Plant Cell Reports*, **17**, 526–530.
- Hammerschlag F.A., Bauchan G.R., Scorza R. (1987) Factors influencing in vitro multiplication and rooting of peach cultivars. *Plant Cell, Tissue and Organ Culture*, **8**, 235–242.
- Hedrick U.P. (1911) *The Plums of New York*, Volume 3, part 2, p. 616, Eighteenth Annual Report. New York: New York Department of Agriculture.
- Huetteman C.A., Preece J.E. (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue and Organ Culture*, **33**, 105–119.
- James D.J., Thurbon I.J. (1981) Phenolic compounds and other factors controlling rhizogenesis in vitro in the apple rootstocks M.9 and M.26. *Zeitschrift für Physiologie*, **105**, 11–20.
- Jones O.P., Hatfield S.G.S. (1976) Root initiation in apple shoots cultured in vitro with auxins and phenolic compounds. *Journal of Horticultural Science*, **51**, 495–499.
- Korban S.S., O'Connor P.A., Elobeidy A. (1992) Effects of thidiazuron, naphthaleneacetic acid, dark induction and genotype on shoot organogenesis from *Malus* leaves. *Journal of Horticultural Science*, **67**, 341–349.
- Leblay C., Chevreau E., Raboin L.M. (1990) Adventitious shoot regeneration from in vitro leaves of several pear cultivars (*Pyrus communis* L.). *Plant Cell, Tissue and Organ Culture*, **25**, 99–105.
- Liu X., Pijut P.M. (2008) Plant regeneration from in vitro leaves of mature black cherry (*Prunus serotina*). *Plant Cell, Tissue and Organ Culture*, **94**, 113–123.
- Lopez-Carbonell M., Alegre L., Prinsen E., Van Onckelen H. (1992) Diurnal fluctuations of endogenous IAA content in aralia leaves. *Biologia Plantarum*, **34**, 223–227.
- Ma J.H., Yao J.L., Cohen D., Morris B. (1998) Ethylene inhibitors enhance in vitro root formation from apple shoot cultures. *Plant Cell Reports*, **17**(3), 211–214.
- Mackay W.A., Kitto S.L. (1988) Factors affecting in vitro shoot proliferation of French tarragon. *HortScience*, **113**, 282–287.
- Mante S., Scorza R., Cordts J.M. (1989) Plant regeneration from cotyledons of *Prunus persica*, *Prunus domestica*, and *Prunus cerasus*. *Plant Cell, Tissue and Organ Culture*, **19**, 1–11.
- Miguel C.M., Druart P., Oliveira M.M. (1996) Shoot regeneration from adventitious buds induced on juvenile and adult almond (*Prunus dulcis* Mill.) explants. *In Vitro Cellular and Developmental Biology-Plant*, **32**, 148–153.
- Mikhailov R.V., Dolgov S.V. (2007) Transgenic plum (*Prunus domestica* L.) plants obtained by *Agrobacterium*-mediated transformation of leaf explants with use of various selective agents. *Acta Horticulturae*, **738**, 613–623.
- Murashige T., Skoog F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum*, **15**, 473–497.
- Nowak B., Miczynski K., Hudy L. (2004) Sugar uptake and utilisation during adventitious bud differentiation on in vitro leaf explants of 'Wegierka Zwyczajka' plum (*Prunus domestica*). *Plant Cell Tissue and Organ Culture*, **76**, 255–260.
- Okie W.R., Weinberger J.H. (1996) Plums. In *Fruit Breeding. Vol 1. Tree and Tropical Fruits*, pp 559–607. Eds J. Janick and J.N. Moore. New York: John Wiley & Sons, Inc.
- Parkinson M., Prendergast M., Sayegh A.J. (1996) Sterilisation of explants and cultures with sodium

C. Petri & R. Scorza

- dichloroisocyanurate. *Plant Growth Regulation*, **20**, 61–66.
- Pérez-Tornero O., Egea J., Vanoostende A., Burgos L. (2000) Assessment of factors affecting adventitious shoot regeneration from in vitro cultured leaves of apricot. *Plant Science*, **158**, 61–70.
- Petri C., Alburquerque N., Pérez-Tornero O., Burgos L. (2005) Auxin pulses and a synergistic interaction between polyamines and ethylene inhibitors improve adventitious regeneration from apricot leaves and *Agrobacterium*-mediated transformation of leaf tissues. *Plant Cell, Tissue and Organ Culture*, **82**, 105–111.
- Petri C., Burgos L. (2005) Transformation of fruit trees. Useful breeding tool or continued future prospect? *Transgenic Research*, **14**, 15–26.
- Petri C., Scorza R. (2008) Peach. In *A Compendium of Transgenic Crop Plants: Transgenic Temperate Fruits and Nuts*, pp 79–92. Eds C. Kole and T.C. Hall. Oxford, UK: Blackwell Publishing.
- Petri C., Wang H., Alburquerque N., Faize M., Burgos L. (2008a) *Agrobacterium*-mediated transformation of apricot

Factors affecting regeneration from leaves of 'Improved French' plum

- (*Prunus armeniaca* L.) leaf explants. *Plant Cell Reports*, **27**, 1317–1324.
- Petri C., Webb K., Hily J.-M., Dardick C., Scorza R. (2008b) High transformation efficiency in plum (*Prunus domestica* L.): a new tool for functional genomics studies in *Prunus* spp. *Molecular Breeding*, **22**, 581–591.
- Quoirin M., Lepoivre P. (1977) Etude de milieux adaptés aux cultures in vitro de *Prunus*. *Acta Horticulturae*, **78**, 437–442.
- SAS (1988) *SAS/STAT User's Guide*. Release 6.03. 1st edn. Cary, NC: SAS Institute, Inc.
- Songstad D.D., Duncan D.R., Widholm J.M. (1988) Effect of 1-aminocyclopropane-1-carboxylic acid, silver nitrate, and norbornadiene on plant regeneration from maize callus cultures. *Plant Cell Reports*, **7**, 262–265.
- United States Department of Agriculture (USDA) (2006) *California Prune (dried plum) Acreage Report*. Sacramento, CA: USDA, NASS, California Field Office.
- Welander M., Maheswaran G. (1992) Shoot regeneration from leaf explants of dwarfing apple rootstocks. *Journal of Plant Physiology*, **140**, 223–228.