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European Plum *in Vitro* Regeneration from Different Organs

Farida Anwar Meerja[1,2] • Yuan Song[1,3] • Susan Sibbald[1] • Jayasankar Subramanian[4] • Lining Tian[1*]

[1] Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford Street, London, Ontario, N5V 4T3 Canada
[2] Department of Biology, University of Western Ontario, 1393 Western Road, London Ontario, N6A 3K7 Canada
[3] The College of Life Sciences, Lanzhou University, Lanzhou, China
[4] Department of Plant Agriculture, University of Guelph, Vineland, Ontario, N1G 2W1 Canada

Corresponding author: * **lining.tian@agr.gc.ca**

ABSTRACT

Plum (*Prunus domestica* L) is an important fruit crop in many countries. Several aspects were studied to improve plum *in vitro* regeneration. Three different media, namely, B5, WPM, and MS, were evaluated for shoot induction from hypocotyls. Use of B5 medium resulted in significantly higher regeneration efficiency compared to other types of media. Thidiazuron (TDZ) was found to be more effective for promotion of shoot induction than 6-benzylaminopurine (BA). Increase of concentration of TDZ did not show an increase in regeneration efficiency. Study was conducted to evaluate shoot induction from epicotyls of stored mature seeds. Shoots were induced from epicotyls of all 13 plum varieties evaluated. However, strong genotype difference exits for shoot development via epicotyls. Full plants were recovered from the shoots induced from epicotyls.

Keywords: epicotyls, hypocotyls, culture medium, micropropogation, plant growth regulators, *Prunus domestica*, regeneration
Abbreviations: BA, 6-benzylaminopurine; **IBA**, indole-3-butyric acid; **TDZ**, thidiazuron

INTRODUCTION

European plum (*Prunus domestica* L.) is an important fruit crop in the world (Okie and Ramming 1999; Bellini *et al*. 2002; Kaufmane *et al*. 2002; Szabo and Nyeki 2002; Capote *et al*. 2006). Plant regeneration and genetic transformation are useful tools for fruit tree improvement via biotechnology as these kinds of plants have long breeding cycles, high levels of heterozygosity, and high degrees of incompatibility, which make trait improvement via breeding difficult (Petri and Burgos 2005). Plum plant regeneration via hypocotyl culture was reported in several studies (Mante *et al*. 1991; Gonzalez-Padilla *et al*. 2003; Tian *et al*. 2006).

Agrobacterium-mediated genetic transformation via hypocotyls was also described (Scorza *et al*. 1994; Gonzalez-Padilla *et al*. 2003; Petri *et al*. 2008; Tian *et al*. 2009). Regeneration via other types of explants, such as leaves, was later reported (Bassi and Cossio 1991; Nowak *et al*. 1997, 2002; Mikhailov *et al*. 2008). Recently, genetic transformation using leaves as an explant was also reported (Mikhailov *et al*. 2008). However, essential Southern blot and other related molecular analyses were not conducted to confirm transformation in that study.

In spite of demonstration of plum regeneration and transformation, the plum regeneration efficiency, in general, has remained low. Also, genetic transformation efficiency of plum is low and has only been reported in a few varieties. Inefficiency of regeneration and low transformation can restrict plum improvement via biotechnology for various commercial varieties. Different factors, especially culture medium, plant growth regulator, which are usually the most important factors for plant regeneration, have not been well studied and optimized in plum. Also, some other potential explants for plant propagation, such as epicotyls, are not investigated in this crop. Continuous research in related areas will provide new knowledge on plum regeneration and is important for improvement of regeneration and transformation in this species.

In this report, we studied and optimized several important aspects of plant regeneration via hypocotyls. We also demonstrated efficient plant recovery from epicotyls via micropropagation for a large number of commercial plum varieties.

MATERIALS AND METHODS

Explant preparation

Mature plum fruits of different varieties were collected from Vineland Station, Ontario, Canada. Mesocarps (flesh) were removed and the endocarps were cracked to release seeds. Seeds were sterilized with 15% commercial bleach and 20 µl of Tween-20 per 100 ml solution for 15 min and rinsed with sterile water. Seeds were then soaked in sterile water overnight for later easier dissection. The seed coat was removed and cotyledons were split open. The embryonic axis was separated from cotyledons. The embryo was dissected into epicotyl, hypocotyls, and radicle. The radicle was discarded. Hypocotyl was cut into three slices of 0.5-1 mm in thickness. Epicotyls and hypocotyl slices were introduced into the culture media.

The leaves were obtained from the *in vitro* shoot cultures regenerated from hypocotyls. In this experiment European plum cultivars Vanette, Stanley, and Veeblue were used. The leaves were collected from young plants that were obtained after four weeks of rooting. Only four uppermost fully expanded leaves from an *in vitro* plant were collected for the experiments.

Medium and shoot induction

Vanette is a commercial plum variety which showed moderate regeneration among different plum varieties (Tian *et al*. 2006). This variety thus can better represent different varieties and was used in the studies of the effects of media and plant growth regulators. The three media used for experiments were MS medium (Murashige and Skoog 1962), B5 medium (Gamborg *et al*. 1968), WPM (Lloyd and McCown 1981). Each medium was supplemented with 100 mg L^{-1} of *myo*-inositol; 0.4 mg L^{-1} of thiamine HCl,

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Research Note

0.5 mg L^{-1} of nicotinic acid and 0.5 mg L^{-1} of pyridoxine. These three media were supplemented with the same amount of plant growth regulators, indole-3-butyric acid (IBA) at 2.5 µM, and cytokinin-TDZ (thidiazuron) at 7.5 µM, and 25 g L^{-1} sucrose. The pH values of the media were adjusted to 5.9 prior to adding 7 g L^{-1} agar. The medium was sterilized at 121°C for 20 min and cooled to 50°C to add plant growth regulators before dispensing into 100×25 mm plates. The culture was maintained at 25 ± 1°C with a 16-h photoperiod supplied by fluorescent Sylvania "Cool White" light having a photosynthetic photon flux of about 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The explants were transferred to fresh media every three weeks. A minimum of 150 explants were used per each medium.

Cytokinin study

B5 medium was used in study of plant growth regulators on shoot induction. Two different cytokinins, 6-benzylaminopurine (BA) and TDZ, were evaluated for regeneration induction. Different levels of cytokinins were used in the study: TDZ: 3.7, 7.5, 15 µM and BA: 2.2, 4.5, 9.0 µM. More than 150 explants were used on each cytokinin treatment. The culture plates were placed in the same culture conditions. The explants were transferred to a fresh medium every three weeks maintaining their respective TDZ and BA concentrations.

Plant recovery

Shoots induced from explants were transferred to the rooting medium consisting of half strength MS medium supplemented with 2.5 µM IBA, 10 g L^{-1} sucrose and 7 g L^{-1} agar. Rooted plantlets were transferred to Magenta vessels containing the same rooting medium for further development. Plantlets that were 4-5 cm in height were transferred to a commercial potting mix (Pro-mix™) and plants were recovered in a greenhouse with temperature from 18 to 25°C.

RESULTS AND DISCUSSION

Previously, MS was the medium used in plum regeneration via hypocotyls (Mante *et al*. 1991; Gonzalez-Padilla *et al*. 2003; Tian *et al*. 2006). In this research, three different types of media, namely, MS, WPM, and B5, were evaluated for shoot induction from hypocotyls. Results showed that although regeneration could be induced in all three media, use of different media could result in different regeneration efficiencies (**Fig. 1A**). The percentage of explants which showed regeneration in B5 medium was significantly higher than that in WPM and MS. Also, the average number of shoots developed per explant on B5 was significantly higher compared to those in other two media (**Fig. 1B**). The results indicate that use appropriate medium is important to achieve high regeneration.

The investigation was in stepwise to evaluate plant growth regulators on regeneration using B5 medium which showed highest regeneration among three media evaluated.

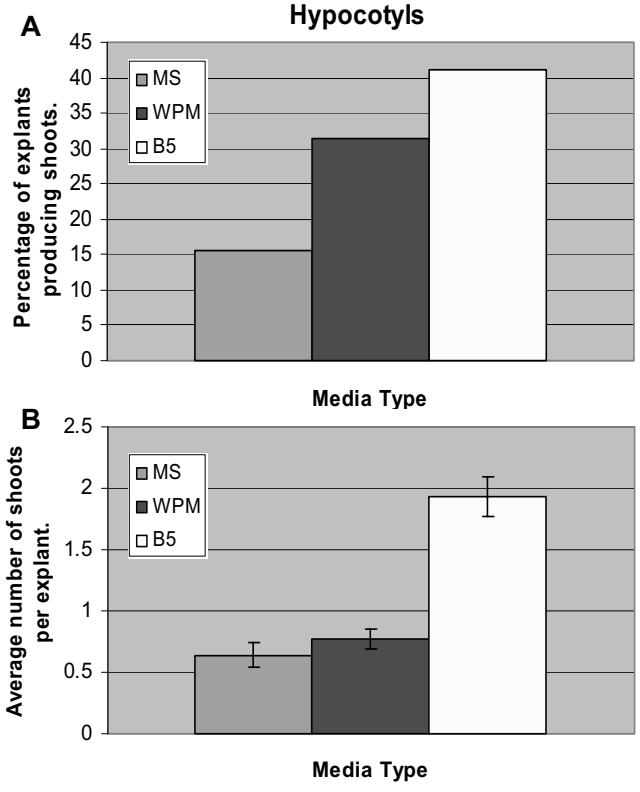

Fig. 1 Shoot regeneration from hypocotyls on MS, WPM, B5 media. (**A**) Percentage explants which produced shoots. (**B**) Average number of shoots per explant.

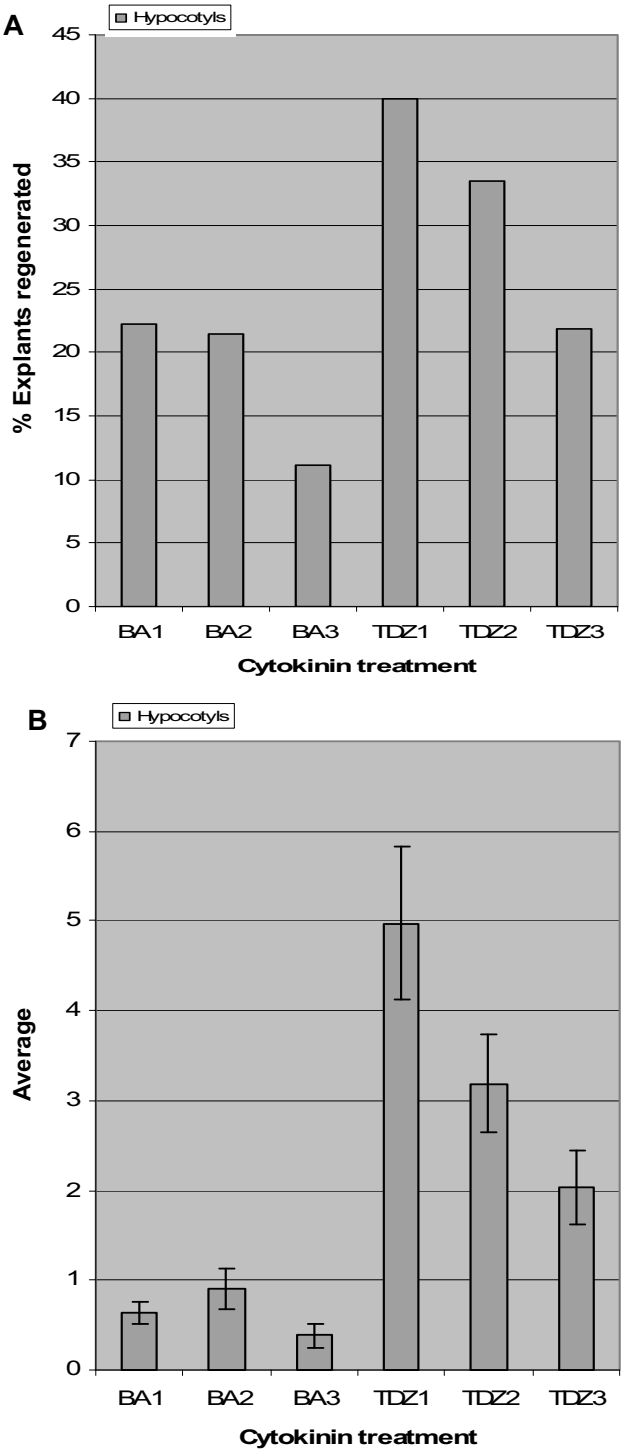

Fig. 2 Shoot regeneration from hypocotyls in B5 medium containing TDZ and BA at different levels. (**A**) Percentage explants which produced shoots. (**B**) Average number of shoots per explant.

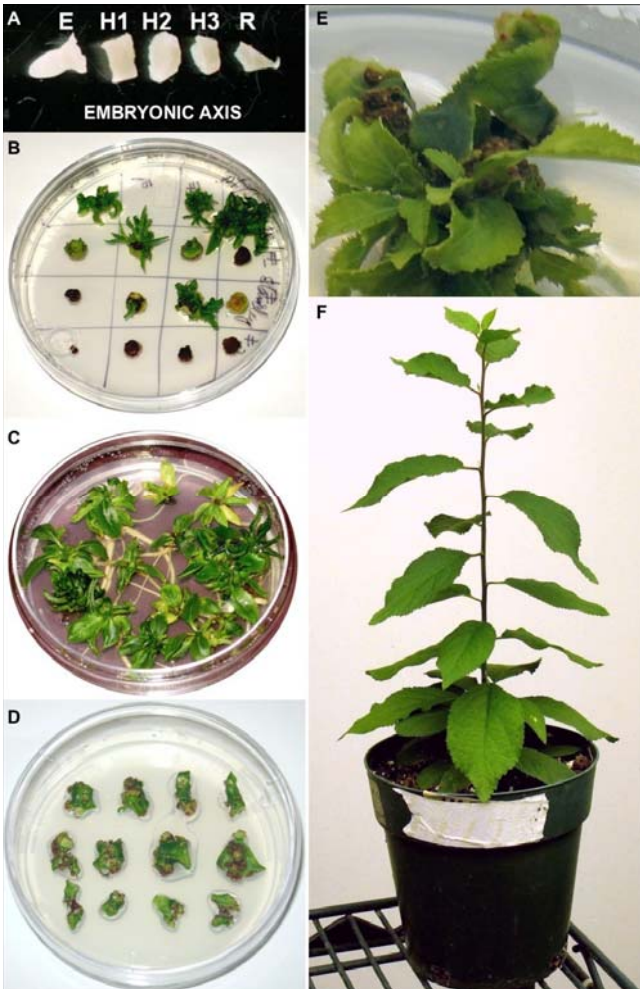

Fig. 3 Shoot regeneration of European plum. (**A**) Embryonic axis; (**B**) Shoot induction from hypocotyls; (**C**) Root development from induced shoots; (**D**) Soot induction from leaves; (**E**) Shoot growth and development from a leaf explant; (**F**) Plant recovery in a greenhouse.

Two cytokinins, namely TDZ and BA, were tested as they were widely used in the regeneration of *Prunus* species (Hammatt *et al*. 1998; Bhagawat *et al*. 2004; Espinosa *et al*. 2006; Tian *et al*. 2007). Results showed that the percentage of explants that produced shoots and the average number of shoots per explant with TDZ treatment was significantly higher from the corresponding results obtained with BA (**Fig. 2**). Similar finding was also reported in some other *Prunus* species (Hammat *et al*. 1997; Bhagawat *et al*. 2004; Tian *et al*. 2007). It was seen that both 3.7 and 7.5 µM TDZ concentrations were not significantly different. However, the shoots inducted with these lower concentrations of TDZ had relatively better regeneration than 15 µM TDZ concentration. Plum regeneration via hypocotyls is shown in **Fig. 3**.

Epicotyls have not been studied for plum plant propagation before. A total of 13 plum varieties were used to study plant propagation via epicotyl culture. All the varieties tested showed shoot development from epicotyl culture. However, shoot development from epicotyls varied significantly among varieties (**Fig. 4**). The shoot development frequency ranged from low at 1.89% to high at 57.69% (**Fig. 4**). This indicates there is a high level of genotype difference regarding shoot induction from epicotyls. Similar genotype difference was also observed in regeneration via hypocotyls (Tian *et al*. 2006). The overall shoot development frequency from epicotyls appeared to be higher than shoot regeneration from hypocotyls (Tian *et al*. 2006). Some varieties, such as Shropshire Damson, responded poorly to *in vitro* culture from epicotyls. This variety also showed low regeneration when hypocotyls were used as explants (Tian *et al*. 2006). Apparently, genotype has played a role for shoot development from both hypocotyls and epicotyls. The shoots from slices of epicotyls probably developed from pre-existing axillary meristem tissues but some shoots may have developed from cell dedifferentiation as lower part tissues of epicotyls are adjacent to upper part of hypocotyls which showed higher levels of regeneration (Mante *et al.* 1991; Tian *et al*. unpublished results). Histological study is needed to reveal the origination of shoot development from epicotyls. When the primary shoots were transferred to fresh induction medium, the secondary shoots could be induced from the lower part tissues of the primary shoots. The new shoot development can repeat by placing the shoots to new media (not shown). Induction of new shoots from the primary shoots can be useful for germplasm and clone preservation. Shoots induced from epicotyls can be transferred into soil and full plants can be recovered in a greenhouse. Shoot development from epicotyls, repeating new shoot development from the primary shoots and plant recovery from epicotyls demonstrated in this study provides another avenue for plum propagation.

Table 1 Shoot induction from leaves of European plum.

Plant variety	Medium	Number of explants (leaves)	% Regeneration
Vanette	B5	62	1.60
	WPM	61	3.30
	MS	60	1.70
Stanley	B5	37	5.40
	WPM	36	0
	MS	36	8.3
	Q&L	36	0
Veeblue	B5	37	0
	WPM	36	0
	MS	36	0
	Q&L	36	5.53

Experiments were carried out to study regeneration using European plum leaves. Three varieties, namely, Vanette, Stanley, and Veeblue were used in the study. All the cultivars studied showed response to *in vitro* regeneration (**Table 1**). However, different cultivars exhibited different levels of regeneration and response differed in different media. The percentage of Vanette explants that produced shoots in B5, WPM, and MS was 1.61, 3.28, and 1.67%, respectively. For Stanley and Veeblue, regeneration was studied in four different nutrient media (B5, WPM, MS, Q&L). The regeneration efficiency with Stanley explants in B5, WPM, MS, and Q&L was 5.40, 0.00, 8.30, and 0%, respectively. No regeneration was observed in Veeblue for B5, WPM and MS media. However in Q&L medium, 5.53% regeneration efficiency was obtained. Leaves were less responsive to *in vitro* regeneration compared to that of using hypocotyls and epicotyls. Similar work was reported by Mikhilov *et al*. (2008) with other cultivars. They obtained between 2–7% regeneration in Etude cultivar and around 15–17% in Startovaya cultivar. As the response is low for all the treatments, it is difficult to study variety and medium difference for leave regeneration in current study. Large numbers of explants are needed to fully evaluate these aspects.

This report shows that plant regeneration of European plum can be achieved via using different types of explants, including epicotyls, hypocotyls and leaves. The regeneration efficiency differs in different varieties and using different media and growth regulators. The research results can be useful for various applications for plum trait improvement.

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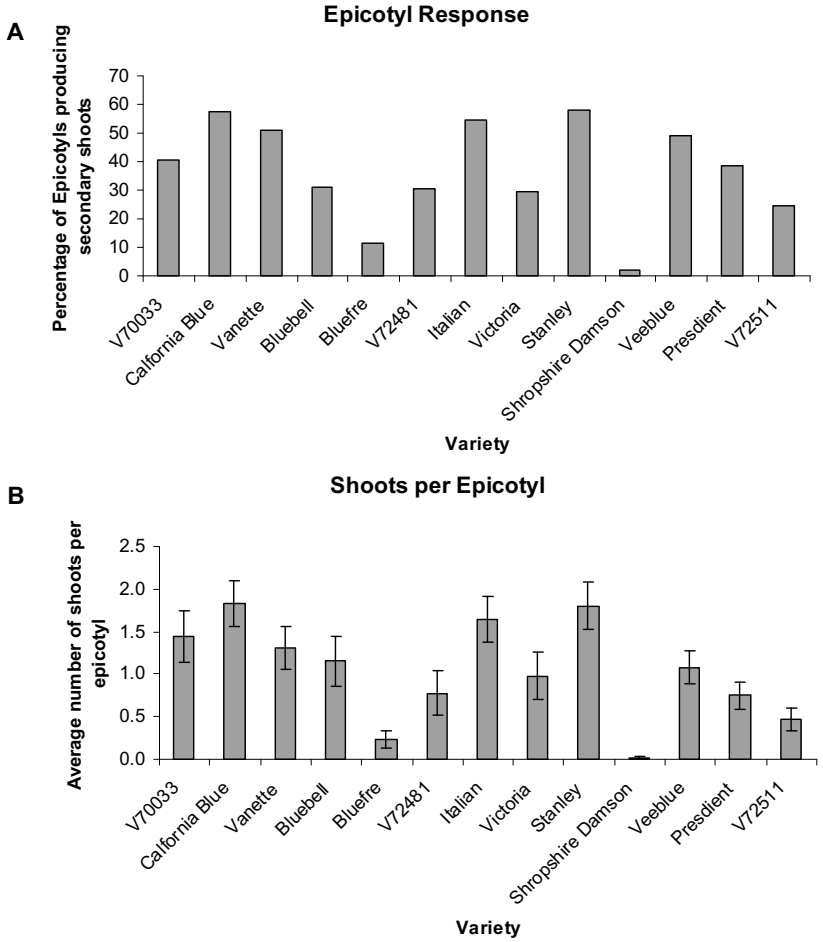

Fig. 4 Shoot development from epicotyl culture of different European plum varieties. (**A**) percentage regeneration from epicotyl explants of different varieties. (**B**) Average number of shoots developed from epicotyl explants.

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