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Short communication

Characterization of *Prunus domestica* L. *in vitro* regeneration via hypocotyls

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Abstract

Several important aspects of regeneration of European plum from hypocotyl explants were studied. Multiple shoots were induced and full plants were recovered for a large number of plum varieties. This indicates that European plum species is, in general, very responsive to *in vitro* regeneration from hypocotyls. Shoot organogenesis could be induced from both mature and immature seed explants but regeneration efficiency was higher when immature seeds were used. Rooting efficiency for varieties with low rooting tendency could be greatly increased by addition of naphthaleneacetic acid in the medium. Primary shoots, when sub-cultured on fresh induction medium, produced multiple shoots at a high frequency and such multiplication could continue for many cycles. The secondary new shoots could be induced for various plum varieties. Plant recovery from the secondary shoots was as efficient as that from the primary shoots. This new system may be an alternative for plum transformation with a potential for increasing transformation efficiency. The system can be used for propagation of transgenic lines and other genetic clones of various varieties.

Keywords: Embryonic axes; Immature seeds; *Prunus*; Shoot organogenesis

1. Introduction

Genetic engineering has been reported in an increased number of fruit plants for various trait improvements (Ko et al., 2000; Ravelonandro et al., 2000; Costa et al., 2002; Belfanti et al., 2004; Broothaerts et al., 2004; Manshardt, 2005). Successful application of this technology has greatly increased the potential for genetic improvement for many fruit plants. European plum (*Prunus domestica*) is an important fruit species worldwide (Okie and Ramming, 1999). Genetic transformation of European plum via *Agrobacterium* was described before (Mante et al., 1991); however, transformation was reported with only one or two varieties and the transformation efficiency was very low. Studies on various aspects, such as selection schemes, different *Agrobacterium* strains, different transformation vectors, and alternative selectable markers did not result in a transformation improvement over the years (Mante et al., 1991; Scorza et al., 1994; Gonzalen-Padilla et al., 2003; Sibbald et al., 2006). Currently, the transformation efficiency in this species still remains very low, with a range of 0–4.2% and an average of only 1.2% (Gonzalen-Padilla et al., 2003), and transformation for many important plum varieties has not been developed yet. In previous reports, important aspects of plum *in vitro* regeneration were not studied nor optimized. The lack of knowledge and in-depth understanding of *in vitro* regeneration can be a limitation and obstacle to develop efficient transformation technologies and for efficient plant regeneration for other applications.

In this report, we studied several important aspects of regeneration of European plum using hypocotyls. The objective of this study was to gain a better knowledge and understanding of plum *in vitro* regeneration. The information from the study can be used for plant regeneration of different plum varieties and provide a useful base to develop transformation technologies for plum varieties.

2. Materials and methods

European plum (*P. domestica* L.) fruits were collected from Vineland, Ontario, Canada. Thirteen plum varieties, namely,

Abbreviations: IBA, indolebutyric acid; NAA, naphthaleneacetic acid; TDZ, thidiazuron

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V70033, California Blue, Vanette, Bluefre, Bluebell, V72481, Italian, Victoria, Stanley, Shropshire Damson, Veeblue, President, and V72511 were evaluated for *in vitro* regeneration. The varieties chosen represent fruits with different maturity times from plum grown in Canada and some regions of USA. Maturity dates range from early maturing (approximately 13 weeks after flowering) to late maturing (approximately 21 weeks after flowering). The selection includes an industry standard variety (such as Stanley), old traditional blue plums (such as Italian and Bluefre), and small fruit (such as Shropshire Damson). Thus, plum varieties selected in the study represent all major varieties in plum growth- and market-season and have the major characteristics of plum fruits. However, as plum is open-pollinated, plum varieties may not be the true varieties in a strict genetics sense. For the effect of development stage on regeneration study, fruit was sampled at six times throughout the season ranging from 8 weeks before maturity until 1 week post-maturity.

Fruit flesh was removed and the stones were washed with tap water, cleaned with a sodium hypochlorite solution (0.05%), and rinsed under running water for 5 min. Stones were dried on a lab bench at room temperature (20–25 °C) for 3–4 days and then stored at 4 °C.

The stones were cracked open carefully and the seeds were collected, disinfected with a 0.5% sodium hypochlorite solution containing 0.005% Tween 20 for 15 min and rinsed three times with sterile water. Seeds were then soaked in sterile water at room temperature overnight to soften tissue texture for easier dissection.

The embryonic axis was excised from cotyledons and the hypocotyl was carefully removed. The hypocotyl was cut into three slices across the axis (each was about 0.5–1 mm thickness; Fig. 1A) under a dissection microscope and slices were cultured on induction medium. Induction medium consisted of MS salts (Murashige and Skoog, 1962), 555 μM inositol, 1.2 μM thiamine HCl, 4.1 μM nicotinic acid, 2.4 μM pyridoxine HCl, 2.5 μM indolebutyric acid (IBA), 25 g L^{-1} sucrose, 7 g L^{-1} Bactoagar (Difco), and was adjusted to pH 5.9 before autoclaving for 30 min (in 1 L containers). The medium was allowed to cool and 7.5 μM thidiazuron (TDZ) added before pouring approximately 35 mL into 100 mm × 25 mm Petri dishes. About 20 hypocotyl segments were placed in each Petri dish. Explants on this induction medium were incubated at 25 °C under a 16-h photoperiod with a Photosynthetic Photon Flux of 50 μmol m^{-2} s^{-1}. Shoot induction was evaluated after 1 month in culture.

Shoots induced from explants were excised and transferred to a medium consisting of half strength MS medium supplemented with 2.5 μM IBA, 10 g L^{-1} sucrose and 7 g L^{-1} agar, in a 100 mm × 25 mm Petri dish for rooting (basal rooting medium). Rooted plants were transferred to Magenta vessels (Magenta Corp., Chicago, IL) containing the same rooting medium for further development.

Naphthaleneacetic acid (NAA) effect on plum rooting was studied in four genotypes that were difficult to root in basal medium, namely, Vanette, Bluebell, President, and V72511. Shoots of similar size for all of the varieties were placed on the basal rooting medium and a modified medium containing 5 μM NAA and 0.01 μM kinetin instead of 2.5 μM IBA. Rooting efficiency was evaluated 5 weeks after shoots were placed on rooting media.

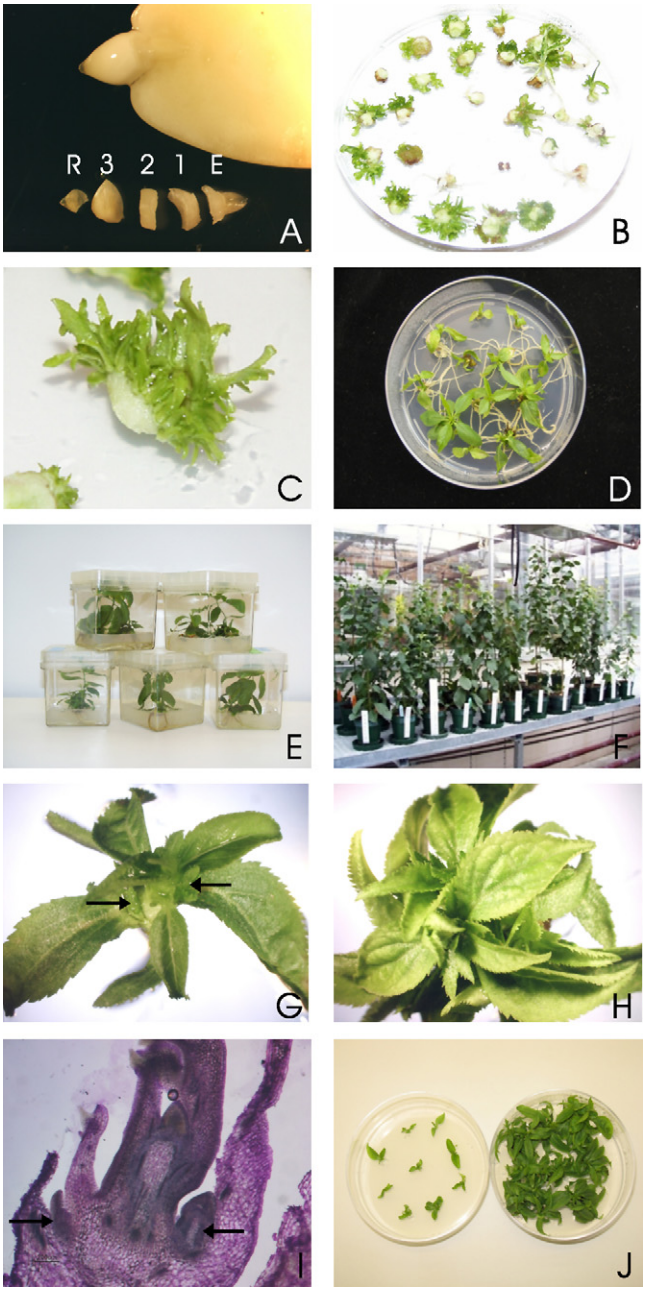

Fig. 1. Regeneration of plum from hypocotyls and new shoot development from primary shoots. (A) Hypocotyl explant preparation (E: epicotyl; 1–3: hypocotyl slices; and R: radicle). (B) Shoot development 1 month after the explants were introduced into the culture. (C) Closer view of shoot induction and development on a hypocotyl slice. (D) Efficient root development on modified rooting medium. (E) Plantlet development in Magenta containers. (F) Plant recovery of different plum varieties in the greenhouse. From left: Vanette, Italian, California Blue, V70033, Bluebell, Victoria, V72511, Stanley, V72481, Veeblue, President, and Bluefre. (G) Secondary shoot development from a primary shoot 10 days after the primary shoot was placed on fresh induction medium. New shoot development was indicated by arrows. (H) New shoot growth from primary shoots after 1 month. (I) Dissection study of secondary shoot initiation. New shoots were developing from axillary meristem tissue. (J) High frequency of new shoot induction and development from primary shoots.