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Micrografting of almond (*Prunus dulcis* Mill.) cultivars "Ferragnes" and "Ferraduel"

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ABSTRACT

The success of various *in vitro* micrografting techniques, establishment of the rootstock, size of the microscion, and the effects of culture medium on the grafted seedling development for almond cultivars "Ferragnes" and "Ferraduel" were studied. *In vitro* germinated wild almond seedlings developed from seeds were used as rootstocks. Shoot culture initiation was successfully achieved from the above almond cultivars by culturing mature shoot tips from forced nodal buds, about 3–5 mm, on 0.7 mg/L BA and 0.01 mg/L NAA containing a MS medium. The regenerated adventitious shoots from *in vitro* cultures were maintained and proliferated by sub-culturing on a fresh medium every three to 4 weeks. Regenerated shoot tips, which were micrografted onto *in vitro* seedlings, resulted in the restoration of shoot proliferation. The results indicated that the most successful method for the grafting of tested almond cultivars was slit micrografting. High levels of micrograft take were achieved with all ranges of scions (4–15 mm) obtained from the regenerated shoot tips. Slow growth and lack of axillary shoot development on the micrografts were noticeable when the micrografts were cultured on hormone-free germination medium. *In vitro* micrografted plantlets were successfully acclimatized and no problems were encountered with the establishment of micrografted plants *in vivo*. The developed technique has demonstrated a high potential for application in the micropropagation of almond cvs. "Ferragnes" and "Ferraduel" and thereby, represents a feasible method for the renewal of almond orchards in Turkey and elsewhere in the world.

1. Introduction

Almond (*Prunus dulcis* Mill.) is one of the major tree crops of the world (Kester et al., 1986). Conventional breeding of woody fruit species is slow and difficult due to the high levels of heterozygosity and long generation cycles (Ainsley et al., 2000). Since almond is a cross-pollinated species, a continuous genetic variation has occurred in this species over the ages, which has led to the formation of different types of trees in terms of crop development, fruit quality and yield, and tolerance to environmental stresses. To minimize the problem of enormous genetic variation and to obtain genetically identical populations, vegetative propagation via layering or cutting is applied. However, these techniques are inefficient due to the large number of problems faced in this fruit species in *in vivo* rooting of cuttings. Because of the difficulties encountered with the rooting of cuttings of almond cultivars, grafting and budding onto seedling rootstocks is the usual method used for vegetative propagation.

Traditional improvement of almond cultivars consists of controlled crosses among selected clones followed by seed isolation and germination, selection and vegetative propagation, all of which take long periods of time (Kester and Asay, 1975). However, this long and difficult process can be shortened by plant transformation techniques that need initially the development of an *in vitro* micropropagation technique. Although successful micropropagation protocols have been published for various *Prunus* species like Japanese plum (Rosati et al., 1980), peach (Hammerschlag, 1982), Japanese apricot (Harada and Murai, 1996) and wild cherry (Hammatt and Grant, 1997), the published reports, especially for adult almond *in vitro* culture (i.e., Miguel et al., 1996; Ainsley et al., 2000, 2001) still need to be improved due to the problems faced with *in vitro* rooting of micropropagated microshoots. Thus, improvement of *in vitro* rooting especially for paper shell almond cultivars, was attempted previously by assessing the influences of auxin type, concentration and exposure time together with shoot base shading and basal salt composition, and the inclusion of even

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phloroglucinol (PG) to the basal media (Ainsley et al., 2001) 60% rooting was achieved in both "Nonpareil" and "Ne Plus Ultra" cultivars with the insertion of microshoots for 12 h into a water-agar (0.6%, w/v) with 1.0 mM indole-3-butyric acid (IBA) followed by the incubation of microshoots in auxin-free basal media including 100.0 μM PG. The recalcitrant nature of rooting can also be overcome by applying a micrografting technique to the almond cultivars in the same manner as the rooting of *Persea americana* Mill. (Alfaro and Murashige, 1987) and *Sequoia sempervirens* (Arnaud et al., 1993) was achieved by utilizing this technique.

Micrografting was developed in the 1980s (Burger, 1985; Jonard, 1986) and consists of the placement in aseptic conditions of a miniaturized scion onto an *in vitro* or *in vivo* grown rootstock. The results of *in vitro* micrografting and the plant material deriving from it can be further cultivated in tissue culture conditions or acclimatized to outdoor conditions (Onay et al., 2003). Micrografting was also applied successfully to *Picea* species (Ponsonby and Mantell, 1993), *Hevea brasiliensis* (Perrin et al., 1994) and *Quercus* (Ewald and Naujoks, 1996) for the rejuvenation of adult tissues. The technique can also be used for production of virus-free plants (Wu et al., 2007) and assessment of graft incompatibility between scions and rootstocks (Burger, 1985; Navarro, 1988).

The first attempts to rejuvenate mature material by micrografting of *in vitro* mature scions onto hybrid almonds was carried out by Martinez-Gomez and Gradziel (2001), who grafted buds of "Nonpareil" seedlings onto different rootstocks with bud survival varying from 30% to 90%, while Ghorbel et al. (1998) micrografted apical buds from *in vitro* shoots of the same cultivar with 60–80% success. *In vitro* micrografting in which almond scions (1.5 cm long) were micrografted on rootstock stems and cultured on a rooting medium with 50% and 65% survival for "Nonpareil 15-1" and "Ne Plus Ultra", respectively, was also investigated by Channuntapipat et al. (2003). Although several studies have been published regarding *in vitro* micropropagation or micrografting of almond cultivars, until now, there is no published integrated protocol for the mass commercial production of selected almond cultivars. The regeneration protocols described in these publications tend to be difficult to replicate and unsatisfactory for other commercially important almond cultivars. Thus, development of a suitable micrografting technique can provide a solution to overcome rooting difficulties of plant species like *in vitro* proliferated almond tissues and explants. Likewise, when considering the application of genetic transformation to the almond, it is also important to develop protocols for efficient regeneration of plants from adult trees. There is also an evident need to develop a workable micrografting system with higher survival rates which could be used to rejuvenate and/or invigorate almond cultivars. This paper presents a protocol for the successful micrografting of the cultivars "Ferragnes" and "Ferraduel" both of which are widely grown by the almond industry.

2. Materials and methods

2.1. Establishment of in vitro rootstocks

Mature dry seeds from naturally grown wild bitter almond trees found in Diyarbakır Province of southeastern Turkey were used to raise *in vitro* seedlings for rootstocks. Mature kernels, from which the outer pericarp and shells had been removed, were surface-sterilized by immersion in a 10% (v/v) commercial bleach solution for 10 min. The seed coats were then removed, and the kernels were washed three times with sterile distilled water. After washing the intact kernels, half kernels with embryos and isolated embryos were cultured in Magenta GA 7 vessels (Chicago Corp.) containing 50 mL MS (Murashige and Skoog, 1962) medium including B5 vitamins (Gamborg et al., 1968), 30 g/L sucrose, 1.0 mg/L BA and 7 g/L agar (Sigma). Cultures were incubated in a growth room with light at 40 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density (fluorescent lamps, 75W) at plant level and a photoperiod of 16 h at 25 ± 2 °C. Fourteen-day-old *in vitro* seedlings were decapitated above the cotyledons, and used as rootstocks for grafting. At the end of the 14th day *in vitro* culture period, observations were made for a total of 30 seedlings regarding stem diameter (mm), stem height (mm), length of roots (mm) and percentage (%) of seed germination.

2.2. Establishment of in vitro shoot cultures for scion source

Mature plant material was collected from 6-year-old almond trees from an orchard in Diyarbakır Province of southeastern Turkey. Fifteen to 20 mm shoot tips were surface-sterilized for 10 min in 10% commercial bleach. After four to five rinses in sterile distilled water, shoot tips were cultured in Magenta GA 7 vessels containing 50 mL MS medium with Gamborg vitamins supplemented with 0.7 mg/L BA and 0.01 mg/L NAA (Rugini and Verma, 1982) and 30 g/L (w/v) sucrose to induce shoot proliferation. The pH of the medium was adjusted to 5.7 using 0.1N NaOH and/or 0.1N HCl, prior to autoclave at 121 °C for 25 min. All the cultures were placed and maintained for at least 3 weeks in a growth room under a 16 h photoperiod (40 $\mu mol\ m^{-2}\ s^{-1}$) with day and night temperatures of 25 ± 2 °C. The regenerated shoots from the forced shoot tips were micropropagated and subcultured every 3 weeks for more than 1-year period. After 2 or 3 weeks, shoot tips and meristematic apices of 4, 8 and 15 mm length were prepared and used as microscions.

2.3. Micrografting method

Stainless razor blades mounted on a handle were used for cutting the plant material. The following grafting procedures were used: (1) *Slit micrografting* in which the rootstock was decapitated to remove all leaves and a vertical slit was made on the stump. The scion base, cut in v-shape, was then fitted in to the slit; and (2) *Wedge micrografting on the stump* in which the rootstock was decapitated to remove all leaves. A wedge was cut in the stump and the scion base cut in a v shape, was gently fitted into the wedge.

2.4. Effects of scion size on success of the micrografts

In order to evaluate the effects of scion size on micrograft success, microscions of the following sizes were used: 4, 8, 15 mm long. Semi-solid MS medium supplemented with 30 g/L sucrose was used to maintain the micrografts *in vitro*.

2.5. Effects of media on the micrograft development

Due to the results of the experiments regarding the two micrografting procedures, the slit method was utilized in this micrografting trial. Micrografts were cultured in three different media: (1) proliferation MS medium with Gamborg vitamins containing 0.5 mg/L BA and 0.1 mg/L IBA, 30 g/L sucrose and 7 g/L agar; (2) rooting MS medium with Gamborg vitamins containing 0.5 mg/L IBA and 0.1 mg/L BA, 30 g/L sucrose and 7 g/L agar; and (3) a hormone-free MS medium consisting of Gamborg vitamins, 30 g/L sucrose and 7 g/L agar.

2.6. Acclimatization

The successful micrografts were removed from the culture vessels (Magenta GA 7), and rinsed with tap water to remove remaining agar from the root system. Subsequently, they were transplanted into individual commercial plastic pots filled with a autoclaved mix of sand and perlite [2:1 (v/v)], covered with a beher glass