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In vitro techniques to study the shoot-tip grafting of *Prunus avium* L. (cherry) *v*ar. Seeyahe Mashad

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Abstract

Development of an *in vitro* micrografting technique for *Prunus avium* L. (cherry) var. Seeyahe Mashad, which is one of the main stone fruits, was very important to rejuvenate adult tissues and mass propagation of disease-free plants. Seeds of *Prunus cerasus* L. (sour cherry) var. Albaloo Telkhehe were stratified, scarified and inoculated on gelled hormone-free medium. *In vitro* germinated seedling which emerged 3-4 weeks after inoculation were decapitated and used as rootstock. Five to six week *in vitro*-cultured meristematic apices and bud apices established from cherry with length 3-15 mm was also used as microscions. The technique of grafting and the effect of scion size and its origin on grafting success was studied. Grafting success was significantly dependent on the method of grafting and size of the scion. The highest percentage (65.5%) of successful grafts was obtained with apex graft (shoot-tip), with apical bud scion length greater than 6 mm. Success of side bud apices (wedge) grafts was 43%. As graft compatibility was satisfactory, graft union formed although very slowly during the first month. Contamination and necroses occurred more often *in vitro* conditions. Nutrient disorders and mineral deficiency symptoms were observed frequently when physiologically active meristematic apices were used. This technique have a good potential for mass propagation of diseases-free cherry var. Seeyahe Mashad.

Key words: Contamination, graft compatibility, graft union*,* *in vitro*, microrootstock, microscions, rejuvenation, micropropagation, *Prunus cerasus* L. viruses.

Introduction

Prunus avium L. (cherry) is native of Iran (around of the Caspian sea), and one of the main stone fruits being cultivated for long time. Among many indigenous and exogenous cherry varieties in Iran, variety Seeyahe Mashad is the most important, due to unique features of its fruit and flower. For example, fruits have the largest volume (8 cm^3) and size (8 g), black color, round shape and very good firmness and sweetness. Traditional propagation of cherry is by seed and budding. There are some problems with the plant cultivation, such as laborious, poor and ununiform germination, prolonged seedling emerging and disease susceptibility to mycoplasma [1].

There are many reports that cherry orchards are threatening due to graft-transmissible virus disorders (mycoplasma contaminations) [2, 3]. There are more than 40 virus or virus-like graft-transmissible pathogenic disorders such as: Plum pox virus; Prune dwarf virus; Prunus necrotic ring spot virus; Cherry line pattern virus; Cherry necrotic crook virus; Cherry rasp leaf virus; Cherry rosetting spot virus; Cherry twisted leaf virus; False cherry mottle leaf virus; and so on, which are affecting cherry in all over the world [4-7]. These disorders (mycoplasma contaminations) are mainly because of using traditional propagation method (budding). The viral diseases significantly decrease the life span and growth quality [8]. Consequently, infected trees show a gradual decline over time. In some cherry cultivars and rootstocks the infection is latent and even if symptoms occur they are not always displayed every year [2]. On leaves of several cultivars which grown in the north west of Iran (Dashte Moghan), chlorotic spots and rings as well as varying degrees of necrosis have been observed (Moeini per. com. 2004).

Since many woody species are unable to regenerate a whole plant from an *in vitro* cultured shoot-tip, Murashige and his colleagues proposed *in vitro* shoot tip grafting (micrografting) as an alternative for recovering virus-free plants [9]. Therefore, micrografting is being widely applied to address the propagation needs and to bring about rapid improvement in important plants. In addition, it can be used as a means of elimination of pathogens. Similarly, micrografting has been used to obtained virus free plants [10-14]. Navarro was the first pioneer of improved micrografting technique [15], which has been widely used by many authors for several woody plant species such as: *Citrus* spp., *Malus pumila* L. (apple), *Prunus armeniaca* L. (apricot), *Prunus amygdalus* L. (almond) and so on [16-19]. Furthermore, Parkinson and Yeoman studied the anatomical and physiological aspects (development of intercellular connections) of *in vitro* grafts [20]. Anatomical details (callus formation, healing cells and vascular junctions) for *in vitro* cleft grafts in *Vitis vinifera* L. (grapevine) was found by Cantos [21]. Similarly, feasibility and anatomical development of an *in vitro* cleft-graft of *Olea europea* L. (olive) was studied by Troncoso [22]. Successful techniques for grafting nodal explants of a range of Solanaceous species; *Acacia senegal* L. (gum Arabic); *Prunus persica* Batsch. (peach) and *Vitis vinivera* L. (grapevines) were developed [21, 23, 24].

Furthermore, micrografting techniques have been successfully practiced by many other researchers, for example, Alfaro and Murashige for *Persea americana* Mill. (avocado) ; Ponsonby and Mantell for *Picea* species; Arnaud for *Sequoia* and Ramanayake and Thimmappaiah for *Anacardium occidentale* L. (cashew) [25-29]. Thus, a suitable micrografting technique could provide a solution

to the often difficult or limited regeneration of roots which is characteristic for *in vitro* cherry (variety of Seeyahe Mashad) tissues and explants.

Materials and Methods

The technique has the following steps: rootstock preparation, scion preparation, grafting procedure, growing grafted plants *in vitro*, and transfer to soil.

Rootstock preparations: Rootstocks were prepared from sour cherry (*Prunus cerasus* L. var. Albaloo Talkhehe) seeds (a wild variety of sour cherry). Initially, mature seeds were stratified in refrigerator for one month and scarified (soften) in concentrated sulfuric acid for 10 min, followed by thorough washing in strolled distilled water. Seeds were surface sterilized by immersion in 70% ethanol (2 min) followed by agitation for 20 min in 50% solution of sodium hypochlorite (NaOCl) chlorine (4% available chlorine) with one or two drops of Tween-20. After rinsing three times for three min in sterile distilled water, the seeds were inoculated into pre-sterilized screw cap bottles (200 ml capacity, 5.5 cm x 10.5 cm OD x H) containing 40 ml of gelled hormone-free medium, and incubated in the dark in a growth chamber (25°C) for germination. Seedlings emerged after three to four week inoculation, and they were decapitated (2 cm from the tip) and used as rootstock. Axiliary buds, if any, at the cotyledon junction were removed.

Scion preparation: Scions were obtained from apical meristem, dormant or physiologically active, taken from juvenile (4-year old) plant grown in the greenhouse, besides, apical meristems aseptically taken from five to six week *in vitro*-cultured shoots of cherry (*Prunus avium* L. var. Seeyahe Mashad). Meristems nodal cuttings from tree were soaked for a few seconds in 70% ethanol, and then put for 15 min in 12% sodium hypochlorite (NaOCl) chlorine with drops of Tween-20. The culturing medium was a MS[30] basal medium containing 0.5 mg l^{-1} 6-benzylaminopurine (BAP), 0.5 mg l^{-1} indole-3-butyric acid (IBA), 0.1 mg l^{-1} gibberellic acid (GA3), 30 g l^{-1} sucrose and 7 g l^{-1} agar (Difco Bacto agar). The pH medium was adjusted to 5.8 ± 0.1 by addition of KOH (N) before autoclaving at 108 kPa and 120°C for 20 min. Plants were maintained in a culture room at 19-25°C with a 12-h photoperiod and illumination of 50 µmol $m^{-2} s^{-1}$ for one month. After four weeks, apical buds and meristematic apices with length 3-15 mm were prepared and used as microscions. The proximal end of the scion shoot was cut in a "V" shape (wedge).

Grafting procedure: *In vitro* seedling rootstocks were decapitated at about 25 to 40 mm height and 1.5 to 5.0 mm diameter at 50 days *in vitro*. Grafting was done aseptically by inserting a piece (3-15 mm) of the scion. A downward central incision of 5-10 mm was made and the wedge of microscion was inserted gently and firmly into the vertical split on the decapitated seedling. Similarly, for side grafting, a slanting incision at an angle of 45° was made on the decapitated seedling (Thimmappaiah's method) [29]. Different micrografting methods, i.e. shoot-tip (apex) grafting and side (wedge) grafting, were tested with different size of scions. For comparing the mode of grafts, 70 grafts were made of which 40 were of shoot-tip grafts and 30 were of side grafts. Forty grafts were made with scion of length 3-6 mm and 30 grafts with scion length of 6-15 mm. The union process was completed after 10-12 days.

Growing grafted plants in vitro: The prepared micrografts were cultured on hormone-free gelled MS medium containing half major nutrients, 30 g l^{-1} sucrose and 7 g l^{-1} agar. Forty ml medium was dispensed into 200-250 ml wide mouth screw cap bottles (14.5 cm x 5.5 cm, H x OD) fitted with polypropylene caps and autoclaved at 108 kpa and 121°C for 20 min. The cultures were incubated, initially for a week, in low light (3 µmol $m^{-2} s^{-1}$) and then at increased photon flux density (55 µmol $m^{-2} s^{-1}$) provided by "cool white" tubular fluorescent lamps (Phillips) giving a 16/8 h photoperiod. All axillary buds and shoots that developed on the rootstock were pinched off regularly.

Transfer to soil: Micrografts were maintained under *in vitro* conditions for 10-12 weeks after which grafting success was assessed and the grafted plants were transplanted out into small plastic pots containing two parts of sand and one part perlite and covering it with 200 gauge polybags. Later the survival rate and growth appearance were determined. Data was analyzed by ANOVA and means subjected to LSD test at 5% level using NEVA [31].

Results

Grafting success was significantly (P=0.05) dependent upon the method of grafting and the size of the scion. Although, the initial percentage of successful grafts was about 75%, this rate decreased at the end of the experiment (Table 1). The highest percentage (65.4%) of successful grafts was obtained with apex (shoot-tip) graft which scion was bud apical with length greater than 6.0 mm. Success of side bud apical (wedge) graft was 43% (Table 1). Scion of grafted cherry developed very slowly and reached less than 20 mm length after four weeks culture (Fig. 1). Union of stock and scion followed by opening of leaves was taken as graft success (Fig. 2). In other words, graft union, as graft compatibility index, was satisfactory, but it was very slow (healing) during the first month. Rootstock on the average was vigorous and grew more rapidly than scion, and grafting union formed very slowly (Fig. 3). Growth quality and quantity (appearance, rate of leaf opening, leaf area and No.), comparing to root growth, was slow at the stage of transfer grafted plant to *in vivo* (soil). In other words, grafted plant was highly root vigorous (Fig. 3). This growth contradiction is not due to graft incompatibility.

Table 1. Effect of size and origin of scion and method of grafting on the success of cherry (*Prunus avium* L.) micrografting.

Mode of grafting	Size and origin of scions (mm)	No. of grafting	Contam*. %	Necrosis %	Success %
apex	meristematic apices 3-6	12	00[++]	26[b]	17.7[a]
"	bud apices 3-6	8	48[cd]	18.9[ab]	33[b]
side	meristematic apices 6-15	8	21.5[b]	44[c]	34[b]
"	bud apices 6-15	10	16[a]	00[++]	43[c]
apex	meristematic apices 6-15	12	00	26[b]	54[d]
"	bud apices 6-15	8	21[b]	13.5[a]	65.4[e]
side	meristematic apices 3-6	10	39.5[c]	45[c]	16[a]
"	bud apices 3-6	10	39.5[c]	65[d]	10[+]

* Contamination LSD (P = 0.05)
+Omitted from statistical analyses; only one surviving explant
++data missed

Figure 1. Initial status *in vitro* cherry (*Prunus avium* L.var. Seeyahe Mashad) micrografted.

Figure 2. *In vitro* micrografted cherry (*Prunus avium* L.var. *Seeyahe Mashad*) (one months old).

Figure 3. The growth of scion and rootstock of untransformed micrografting cherry (*Prunus avium* L.var. Seeyahe Mashad). Rootstock on the average was vigorous and grew more rapidly than scion. Grafting union formed very slowly.

Discussion

Though contamination and necrosis were high, the poor success rate was mainly due to excessive collogenesis observed on more than 50% of the apices and vitrification on more than 30% of the explants. As the size of the meristems increased from 5 to 10 mm, the success rate of the grafts was improved and reached 32 to 45%, despite the persistence of collogenesis and vitrification. However, we observed that these grafts desiccated rapidly, especially when top grafting was applied.

Using inactive dormant apices material, contamination increased, and using physiologically active apices, grafting was hampered by production of phenolic compounds in the graft union, which oxidized and provoked the necrosis of the apices. Nutrient disorders and mineral deficiency symptoms was observed frequently when physiologically active meristematic apices were used. Aseptic grafting, where scions and rootstocks were initiated *in vitro*, could be an efficient method for micropropagation of cherry. The technique presents several advantages and could offer serious opportunities for rapid mass propagation for healthy plant materials.

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