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Full Length Research Paper

Micrografting and micropropagation of olive (*Olea europea* L.) Iranian cultivar: Zard

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A protocol for micropropagation and micrografting of shoots of olive (cv. Zard) was developed in Iran, because this variety of olive (cv. Zard) had difficult in proliferation. Three-week-old seedlings were used as rootstock. Lateral meristems (1 to 1.5 cm long), taken from olive (cv. Zard) mother plants, were cut and grafted onto healthy olive seedlings, micrografts cultured on OM medium culture with Zeatin. 7 to 10 days after grafting, callus formation was observed and by 15 days after grafting a strong union developed. Survival of the micrografted plants was always high, especially after the third successive phase of micrografts. After micrograft establishment, shoot elongation or number of new regenerated buds improved as a consequence of the repeated micrograft procedure. Shoots, which were regenerated from these explants, were transferred to OM medium with 2ip, where they multiplicated. Every six weeks, upper segments plantlets were cut in one-bud explants and transferred to new media where they were developed again in plantlets with micropropagation rate 1:4 and lower segments were used for micrografting again after four months.

Key words: Micrografting, micropropagation, rootstock, regeneration.

INTRODUCTION

The olive-oil yield has remained almost the same in the last decades, indicating very little progress in the development of olive cultivation and down processing. The objectives of current olive *in vitro* culture research efforts are concerned mainly with genetic improvement (Canas et al., 1987; Canas and Benbadies, 1988), obtaining pathogen-free plants (Rugini, 1986), and cryopreservation of germplasm. However, to apply new biotechnologies, developing *in vitro* procedures with selected plant material is essential. The *in vitro* establishment of olive still remains quite difficult for many other cultivars, particularly those from mature plant material (Rugini and Fedeli, 1990).

The *in vitro* shoot-tip grafting technique was later improved by Navarro et al. (1975), as an alternative for recovering virus-free plants, rejuvenation of mature shoot materials (Franclet, 1983; Hackett and Murray, 1993). In addition to *in vitro* shoot-tip grafting techniques are also potentially useful, such as for the propagation of hard-to-root cultivars or for multiplication of *in vitro* clones. A suitable micrografting technique could provide a solution to the often difficult or limited regeneration of roots which is characteristic for *in vitro* olive tissues and explants (Rugini, 1986).

The *in vitro* culture of the Iranian olive cultivar “zard” from mature explants has been difficult in our laboratory due to frequent explant desiccation, and falling of the leaves. Furthermore, cutting taken from mature trees and culture under *in vitro* rooting conditions rarely produced roots. However, through the technique of micrografting, reported here for the first time in this variety, both the vigor of the cuttings and rooting competence of the regenerated shoots were restored, and complete plants

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were established in soil conditions and grown in the greenhouse. This methodology has been previously applied in woody plants (Munteuuis, 1991; Abousalim and Mantel, 1992; Huang et al., 1992; Perrin et al., 1994), and we show that it is now feasible to use *in vitro* cloning of selected mature olive cultivars which have been previously reported to be recalcitrant to vegetative multiplication.

MATERIALS AND METHODS

The mature material consisted of selected trees *Olea europea* L. cv. Zard, were obtained by rooted cuttings and grown 4 years under greenhouse conditions. Before explants were taken, a fungicide treatment was given to the trees for at least 2 months.

Portions (1 to 1.5 cm long) of terminal shoots comprising the apical and buds and taken from newly emerged laterals on branches, were used as explants to initiate *in vitro* cultures. To prepare the initial scions of the micrografts, the leaves from those portions were eliminated. Explants were previously surface-sterilized by soaking in a 10% sodium hypochlorite solution for 20 min and rinsed twice with sterile water.

Fruits were collected in September, and embryos were isolated and germinated. Bartolini and Leva (1994) have suggested, Croneiqi of seeds were better for germination. After 1 month of culture, seedlings originating from the *in vitro* culture of isolated embryos of (cv. Croneiqi), were germinated from seeds, hypocotyl segments used as rootstocks. Regenerated hypocotyls were decapitated cotyledons. For insertion of the scion wedge, a longitudinal incision of about 0.5 cm was made below the level of decapitation. To avoid undesirable growth from the rootstocks the results in a loss of vigor of the scions, the buds were carefully removed.

The micrografts, performed with scions, were done under lamina air flow by inserting the wedge of the scion into the longitudinal incision of the rootstock. Both rootstock and scion remained together by an open ring of plastic with an internal diameter of 5 mm. The micrografted plants were transferred to jars with solid culture medium. Regrafting was done at 6 weeks intervals, after taking a portion of the regenerated shoot from the first micrografting as the apical and subapical buds (approximately 1.5 cm long) and were used into a new hypocotyls (second micrograft).

Juvenile shoot segments and micrografted adult shoots were cultured in a OM medium with 8 mgl^{-1} zeatin (Pliego-Alfaro et al., 1987). These cultures were kept in a temperature-controlled chamber at 25°C and a 16 h photoperiod with a photon flux density of 30 to 40 µmol $m^{-2}s^{-1}$. The percentage of explants or micrograft survival and the length increment of the regenerated shoots were measured after 45 days of culture in the proliferation medium. The ANOVA was conducted and significant difference between means were tested by t-test at $p < 0.05$. The experiments were repeated two times.

RESULTS AND DISCUSSION

After surface sterilization, percentages of contamination in the *in vitro* cultures were very low (3 to 5%). The fungicide pretreatment given to the mature olive trees before their *in vitro* introduction was essential for obtaining aseptic cultures. Without this pretreatment, a higher concentration of sodium hypochlorite was used, but this induced oxidation of the explants.

When portions of mature olive shoots were directly introduced *in vitro* with either the proliferation or the rooting medium, micropropagation was not achieved, rooting was low and frequent leaf abscission and explant desiccation occurred (Lambardi et al., 2003). However, in olive trees as in other-woody species, maturation is linked to a decline of vegetative vigor and rooting competence (Franclet, 1983), factors which greatly interfere with organogenic manipulation. Fortunately, mature plant material could be successfully established *in vitro* through micrografing. Survival of the micrografted plants was always very high, especially after the third successive phase of micrograft. After 45 days of *in vitro* growth conditions (Figure 1a), graft success was 80 to 85%. The survival rate for the transplanted grafted plants following 7 to 10 days of hardening was 90% (Rugini, 1986) (Table 1).

The results of experiments showed that the application of a layer of moist nutrient agar around the graft area to prevent dehydration of the microscion and rootstock was not necessary, since the rate at which the graft union formed was quick enough to heal the wounds and prevent moisture loss. A moisture of ring at graft area was beneficial in conventional grafting. The firm placement of the microscion onto the rootstock to ensure good contact was essential for the formation of the graft union.

Ten days after grafting, the first cellular unions (callus) between rootstock and scion were observed, Troncoso et al. (1999) showed callus, between rootstock and scion. The callus tissues grew rapidly and by 15 days after grafting developed into a strong union (Figure 1b). Often at the point union of the scion and rootstock callus tissues was observed, the callus provides the initial pathway for water until vascular connections (Wu et al., 2007).

It was possible to observe shoots with two-four leaves, and very vigorous shoots developed from the micrografted scion after 6 weeks of culture. After micrograft establishment, shoot elongation or number of new regenerated buds improved as a consequence of the repeated micrograft procedure. Two or three microshoots differentiated from the basal part of micrografts and they did not remain short (> 20 mm), collectively forming a cluster. A significant increase in length of shoots from grafted plants was recorded after the second micrografting. There was a significant increased in the length of axillary shoots, but decreased in the number of axillary shoots (Table 1). Increasing length of microshoots from serially grafted plants were significantly different from that of mature cuttings. Amiri et al. (2006) observed scion grafted cherry developed slowly and reached less than 20 mm length after 4 weeks culture.

According to Pierik (1990) in the absence of reliable biochemical and molecular markers, shoot vigor expressed as an increase in stem elongation and the restoration of high rooting capacity can be used to assess

A B

Figure 1**.** (a) The survival of *in vitro* plantlets serially grafted three times. (b) The callus healing formation along the cut-walls of the scion.

Table 1**.** Effects of three successive micrografts.

Successive micrografts	Surviving cultures (%)	Length of shoots per micrograft (cm)	Number of branching per micrograft
First micrograft	45	3.78± 0.2	3.6±0.1
2nd micrograft	67	4.47±0.1	3.78±0.15
3rd micrograft	83	5.20±0.2	4.38±0.24

Data taken after micrografts spent 45 days in OM medium with 8 mgl^{-1} Zeatin.

reinvigoration in woody plants. Reinvigoration was evident as a progressive increase in the reactivity of primary nodes and axillary shoots in response to successive grafting cycles (Giovannelli et al., 2000). Grafting is one of the most commonly used techniques to stimulate reinvigoration in mature tree (Hackett and Murray, 1993; Sanchez et al., 1997). In our systems, repeated grafting had a reinvigorating effect, because the best *in vitro* growth of primary nodes from grafted plants remained significantly higher than of nodal explants from microcutting explants. A similar reinvigoration phenomenon has been observed in *Sequoia sempervirens* (D. Don) Endl. (Huang et al., 1992). In microshoots from serially grafted plants, increased branching capacity and decreased apical dominance were achieved after vigorous and elongated shoots with expanded leaves were induced on OM medium culture containing 2-ip (Figure 2). Grafting on juvenile rootstock has been successfully used to reinvigorate adult plants for subsequent use *in vitro* clonal propagation system (Fouret et al., 1985; Munteuuis, 1991). In *Citrus*, a plant species most extensively studied and among the several thousand plants recovered by shoot-tip grafting, a reversion to the juvenile phase with mature plants as source of shoot tips was never observed (Navarro, 1990). However, in Pinus pinaster and other forest tree species, some micrografted plants showed an apparent reversion to the juvenile phase (Huang et al., 1992). It is important to emphasize here that normally, juvenile reversion is considered within the scope of the proliferation and

Figure 2**.** Elongation of microshoots and proliferation capacity grafted plantlets.

rooting ability (Revilia et al., 1996).

In vitro micrografting may be a useful tool for the early diagnosis of graft in compatibilities, which are known to be more common when the genetic distance between scion and rootstock is increased (Danthu et al., 2004).

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