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Simultaneous Micrografting, Rooting and Acclimatization of Micropropagated American Chestnut, Grapevine and Hybrid Hazelnut

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Summary

American chestnut, hybrid hazelnut and grapevine were cultured *in vitro* on Nas and Read Culture Medium. Micropropagated scions (shoots) were micrografted on *in vitro*-propagated unrooted rootstocks by hand. The scions (2–4 cm) and rootstocks (2–3 cm) were fixed using an elastic electric-wire tube or alginate gel beads. After grafting, the bases of rootstocks were dipped in 1000 ppm indole-3-butyric acid (IBA) solution for 5 s (grapevine) or 10 s (American chestnut and hybrid hazelnut). Grafted plantlets were then cultured in Jiffy peat plugs *in vitro* or placed in Jiffy peat plugs *ex vitro* and were gradually acclimatized. Scions of *in vitro* cultured grafts developed aerial roots and the grafting was unsuccessful. Two months after grafting, the survival of successful *ex vitro* grafts was ca. 50 % for grapevine, 70 % for hazelnut, and 80 % for American chestnut. The results indicate that, when grafted plants are preferred to own-rooted plants, micropropagated scion and rootstocks can simultaneously be grafted, rooted and acclimatized *ex vitro*.

Zusammenfassung

Simultanes Mikroveredeln, Bewurzeln und Akklimatisieren von mikrovermehrten amerikanischen Kastanien, Weinreben und Hybridhaselnüssen. Amerikanische Kastanien, Hybridhaselnüsse und Weinreben wurden *in vitro* auf Nas- und Read-Medium kultiviert. Mikrovermehrte Reiser (Sprosse) wurden von Hand auf *in vitro*-vermehrte unbewurzelte Unterlagen mikroveredelt. Zur Fixierung von Reis (2–4 cm) und Unterlage (2–3 cm) fanden entweder Ummantelungen von Elektrokabeln oder Alginatgelklemmen Verwendung. Nach der Veredlung wurden die Unterlagen 5 s (Wein) oder 10 s (Amerikanische Kastanie und Hybridhaselnuss) in 1000 ppm Indol-3-Buttersäure-Lösung (IBA) getaucht. Die veredelten Pflänzchen wurden anschließend auf Jiffy Torfblöcken *in vitro* oder *extra vitrum* mit schrittweiser Akklimatisierung kultiviert. Die Reiser der *in vitro*-kultivierten Veredelungen entwickelten Luftwurzeln und die Veredelung misslang. Bei den *extra vitrum*-Veredelungen betrug die Anwachsrate zwei Monate nach der Veredelung beim Wein 50 %, bei den Haselnüssen 70 % und bei den Kastanien 80 %. Die Ergebnisse zeigen, dass es bei Veredelungen möglich ist, die mikrovermehrten Reiser und Unterlagen gleichzeitig zu veredeln, zu bewurzeln und *extra vitrum* zu akklimatisieren.

Key words. Chestnut – grapevine – hazelnut – micrografting – micropropagation – rooting – acclimatization

Introduction

Micropropagation is the most powerful clonal propagation technique that offers unlimited production and rapid distribution of superior plants selected from breeding programs. All stages of *in vitro* culture have influence on the number and the quality of propagules but the ultimate success of a micropropagation program depends largely on successful rooting, hardening and acclimatization of plants leading to high survival rates and satisfactory plant growth *ex vitro*. However, in fruit culture, a high multiplication, rooting and survival rate of plants alone may not be sufficient for commercial propagation. In fruit culture, there are certain cases where the use of own-rooted plants must be avoided or where grafted plants are for various reasons superior to own-rooted plants. In such cases, the key to development of economically feasible micropropagation depends on efficient grafting (micrografting) of plants propagated *in vitro*.

We have developed micropropagation protocols for American chestnut, grapevine, and hybrid hazelnut. When these protocols are employed, great numbers of plants and high rooting and survival rates were obtained (unpublished data). However, for various reasons grafting of these species is obligatory or preferred.

In grape, *Phylloxera* attacks the roots of grape vines (*Vitis vinifera*) and eventually kills the plant, but it does not affect the roots of native American grapes (*Vitis labrusca*). To avoid *Phylloxera* infection, *Vinifera* scions must be grafted onto *V. labrusca* rootstocks. Chestnut is naturally a difficult to root plant and thus propagated

by grafting. However, even if rooting could readily be achieved, in wet soils, where ink disease (i.e. phytophthora root rot) causes decline of growth or even death of plants, grafting of standard chestnut cultivars on *Phytophthora*-resistant rootstocks is preferred. Similarly, in mechanized hazelnut culture (i.e. in USA) single-trunk trees are preferred but most commercial cultivars naturally grow as multi-stemmed shrubs and produce basal shoots (sucker). Suckers are eliminated by multiple spray of herbicide every year. The herbicide application has a recurring cost and causes environmental concerns. Alternatively, suckering can be alleviated by grafting scions on non-suckering rootstocks. The later method may be preferred as it is less costly and it is environmentally sound.

Despite the fact that many fruit trees and rootstocks have been independently micropropagated, simultaneous *in vitro* culture of scions and rootstock plants followed by micrografting has rarely, if ever, been employed. Furthermore, in general, *in vitro* propagated scions have been micrografted on several months old seedling-origin rootstocks (PONSONBY and MANTELL 1993; BARBARA et al. 1995; DE PASQUALE et al. 1999). However, grafting of microscions on clonally propagated proven rootstocks is more preferable. Here we report simultaneous micrografting, rooting and acclimatization of micropropagated American chestnut, hybrid hazelnut and grapevine.

Materials and Methods

Plant materials

Micropropagation of hazelnut (*Corylus avellana* L. × *C. americana* Marsh) was according to NAS (2002). Micropropagation of chestnut *Castanea dentata* × *C. mollissima*) and grapevine are to be published somewhere else, therefore they will only briefly be described: Single axillary buds of grapevine and chestnut were cultured on Nas and Read Medium (NAS 2002) containing 0.2 mg BA (6-benzyladenine) L^{-1} + 0.01 mg IBA (indole-3-butyric acid) L^{-1} while those of hazelnut were cultured on the same medium containing 5 mg BA L^{-1} + 0.01 mg IBA L^{-1} for 35 days. Shoots developing from axillary bud explants were used in micrografting.

Although none of genotypes was necessarily developed to be a rootstock, in this study some genotypes (cultivars) were randomly used as rootstocks, while the others were used as scions. In hazelnut, genotype G-029-N was grafted on E-295-S, and genotype SS'-182 was grafted on E-093-S. In chestnut, genotype 711 was grafted on 907, and in grapevine, cultivar Chancellor was grafted on cultivar MN1047.

Micrografting, rooting and acclimatization

***In vitro* micrografting.** A splice micrografting (whip grafting) method illustrated in Fig. 1 (see *ex vitro* grafting) was employed *in vitro*. To secure graft union, sterile elastic electric-wire tubes (5–7 mm long, 1–2 mm diameter) or alginate gel beads were used as graft bandages. The alginate gel beads were made by dropping 2 % (w/v) sodium alginate (Sigma, A-0682) solution into a 100 mM $CaCl_2$ solution and keeping it for 30 min. Following the fixation of grafts, the bases of rootstocks were dipped in 1000 ppm IBA solution for 5 s (grape) or 10 s (hazelnut and chestnut), they were cultured into sterile Jiffy peat plugs (No 9), and then placed into sterile transparent plastic culture vessels (7.2 cm x 7.2 cm x 11 cm).

***Ex vitro* micrografting.** A splice micrografting (whip grafting) method was employed. A schematic illustration of grafting procedure is given in Fig. 1: First, to secure graft union, a microclamp was made by attaching two hard plastic wings to an elastic electric-wire tube (5–7 mm long, 1–2 mm diameter).

To take potential benefit of leaves and buds on rooting ability of rootstocks, a slanting cut was made at the rootstock in such a way that there will be one or two leaves left on the rootstock (Fig. 1, (1)). A corresponding slanting cut was made at the base of the scion (2). After the cuts, the rootstocks were 2–3 cm long and scions were 2–4 cm long. The microclamp was wide opened by pressing on its wings, and then rootstock and scion were pushed into the clamp (3). The cut surfaces of rootstock (4) and scion (5) were placed together so that the cambial regions were in contact.

To induce rooting, the base of grafted rootstocks (6) were dipped in 1000 ppm IBA solution for 5 s (grapevine) or 10 s (hazelnut and chestnut), and then they were cultured directly into non-sterile Jiffy peat plugs (No 9). All shoots transplanted into peat plugs were shifted to the greenhouse (22–26 ± 3 °C) and then transplanted into pots containing a mixture of 60 %

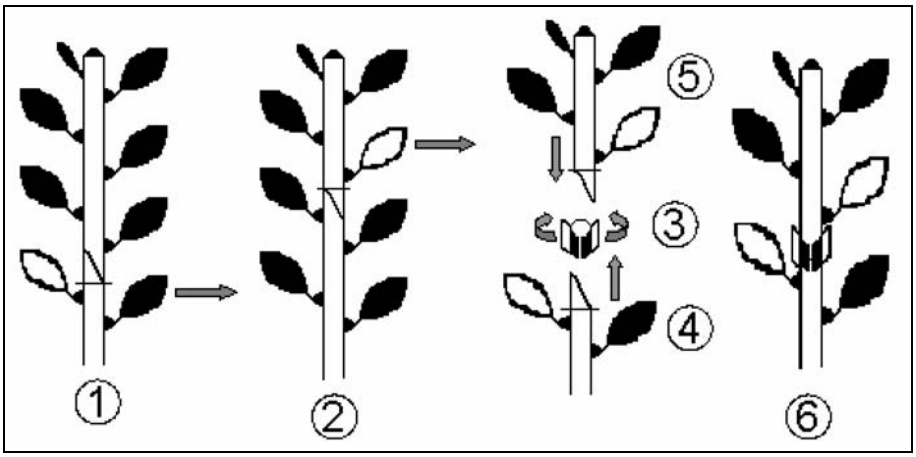


Fig. 1. A schematic illustration of the micrografting procedure. Microshoot used as the rootstock (1), microshoot used as the scion (2), micrografting clamp used to hold rootstock and scion together (3), rootstock (4) and scion (5) given slanting cuts so that they can be placed together, a micrograft ready for root induction (6).



Fig. 2. A: *In vitro* micrografted hazelnut. Notice the aerial roots (arrows) on the upper part (scion) of the graft. B: *In vitro* micrografted hazelnut using alginate gel beads as grafting bandage. The scion looks healthy for three weeks but the graft healing is poor.

peat and 40 % perlite. Shoots transplanted to pots were covered with transparent plastic cups and pots were placed on a bench shaded with cheesecloth. The illumination was for 16 h/ day (13 to 14 h day light at 125–145 μmol s^{-1} m^{-2} + 2 to 3 h at 40 μmol s^{-1} m^{-2} from 400 W metal halide lamps). After three weeks the plastic cups and after four weeks the cheesecloth were removed and plants were left to grow under the same greenhouse that was shaded (300–420 μmol s^{-1} m^{-2} day light).

Results and Discussions

In vitro grafting was time consuming and unsuccessful. When elastic tubes were used, the base of some scions formed aerial roots (Fig. 2A). When alginate beads were used, within the three weeks following the grafting procedure scions remained healthy-looking but no sign of graft healing was observed (Fig. 2B). Apparently, the high humidity in the culture vessels allowed scions to survive but prevented the complete healing of graft union. Therefore, *in vitro* micrografting was discontinued.

In *ex vitro* micrografting, assembling of microclamps was the most time-consuming part of the work. When pre-fixed microclamps were used, preparing scion and rootstocks, placing scion and rootstocks together, dipping the base of grafted rootstocks in IBA solution and placing them into peat plugs took only 60–90 s. Most of the unsuccessful grafts were observed within the first week following the grafting procedure. When scion and rootstocks were poorly placed, the scion started to wilt as early as three days after grafting. Although not all grafts were successful, virtually all rootstocks rooted and survived *ex vitro*.

The numbers of performed and successful grafts two months after grafting procedure are given in Table 1, and a successful graft after a one-month acclimatization period is presented in Fig. 3. The grafting was most successful, when scion and rootstock were in similar diameter. If scion and rootstocks were in close diameters, they could firmly be placed together. If the diameters of scion and rootstocks were significantly different from each other, the microclamp did not fas-

Table 1. The numbers of performed micrografts in micropropagated chestnut, grapevine and hazelnut, and the numbers of successful micrografts survived *extra vitrum* two months after grafting procedure. Numbers represent combined data of two experiments conducted one month apart.

Scion	Rootstock	Number performed grafts	Number successful grafts
G-029-N	E-295-S (hazelnut)	25	18 (72 ± 5%)
S'-182	E-093-S (hazelnut)	25	17 (67 ± 5%)
711	907 (chestnut)	20	16 (80 ± 5%)
'Chancellor'	MN1047 (grape)	30	14 (47 ± 5%)



Fig. 3. A micrografted hazelnut plant. Photo was taken one month after micrografting followed by root induction and acclimatization.

ten firmly and the graft remained loose. Thus the success of grafting was largely determined by the variability in diameter of materials used. In chestnut, the apical (scion) and basal (rootstock) part of microshoots were in close diameter and the greatest successful grafting rate (80 %) was obtained with that species. In hazelnut, the scion and rootstock were somewhat in similar diameter but occasionally they were too slim and could not firmly be placed together in the microclamp tubing. Despite, the loose placement of scion and rootstocks, in hazelnut the rate of successful grafts was about 70 %. Grafting was the least successful with grapevine (47 %). This was because the scions (apical part of shoots) of grape were usually slim while the rootstocks (basal part of shoots) were usually thick. As a result, one side of the microclamp did not fasten firmly and the graft remained loose.

With three species that were used, the graft union was usually smooth and no apparent swelling or excessive callusing was observed. Although we did not detach microclamps from grafts, it seemed that two or three weeks were sufficient for graft union to heal completely. *Ex vitro* growth of plants within the first three weeks was limited but after four weeks, plants started to grow fast and microclamps fell down freely.

The success of micrografting in grapevine, using rooted microrootstocks and under aseptic conditions has been reported to vary from 70 % to 90 % (TANNE et al. 1993). To the best of our knowledge, micrografting of *in vitro* propagated hazelnut and chestnut has not been reported. Because of high relative humidity and nutrient rich culture environment, micropropagated plants possess an *in vitro*-induced phenotype that usually causes high plant mortality *ex vitro*. In the current study, virtually all rootstocks rooted and survived *ex vitro* and they could be re-grafted in a conventional way when they reached a certain size. Therefore, although there is still room for improving the grafting technique (i.e. by wedge grafting), considering the potential adverse effects of *in vitro*-induced phenotype and acclimatization on grafting, the rates of successful micrografts that we report here for American chestnut, hazelnut and grapevine may be satisfactory.

Micrografting has been used widely to eliminate (MURASHIGE et al. 1972; BARBARA et al. 1995) or diagnose viruses (TANNE et al. 1993), to diagnose graft incompatibility mechanisms early (JONARD et al. 1990), to rejuvenate aged tissues for enhancing micropropagation (PONSONBY and MANTEL 1993), to shorten the time required for transfer of *in vitro*-derived plants to the field (KE et al. 1993), to rescue *in vitro* regenerants of species that normally do not have satisfactory root system (DE PASQUALE et al. 1999.), to recover difficult to root regenerants (GULATI et al. 2001) and to recover non-rooting transgenic plants (LUO and GOULD 1999). In addition to the known uses of micrografting, our results indicate that scion and rootstocks can be micropropagated, and then they can simultaneous be micrografted, rooted and acclimatized successfully.

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