New *in vitro* micrografting method for apple by sticking

INTERNATIONAL JOURNAL OF HORTICULTURAL SCIENCE

AGROINFORM Publishing House, Hungary

Dobránszki J.[1], Magyar-Tábori K.[1], Jámbor-Benczúr E.[2] and Lazányi J.[1]

[1]*Research Centre of University of Debrecen, H-4401 Nyíregyháza, P.O.Box 12., Hungary*

[2]*Szent István University, Department of Floriculture and Dendrology, H-1118 Budapest, Villányi út 35–43., Hungary*

Key words: micrografts, sticking, apple, cv. Royal Gala, *in vitro*

Summary: The requirements for *in vitro* micrografting in apple are described. *In vitro* multiplicated shoots of cv. *Royal Gala* were the sources of rootstocks and scions after different pre-treatment, respectively. Oxidative browning of cut surfaces could be inhibited by the use of antioxidant mixture during grafting process. Scion base cut in v-shape was stuck by 1% agar-agar solution into the vertical slit of rootstock. There was no any displacement and the rate of fused and further developed grafts was 95 percent. Agar-agar between the rootstock and scion made the transport of different materials possible and hold the graft units together until the fusion took place. Fusion was proved also by histological studies. Some of *in vitro* micrografts were planted and acclimatisated and the survival was 100 percent.

Introduction

Majority of fruit trees have been propagated by means of grafting. Grafting under *in vitro* conditions can give several advantages both for production and for research. *In vitro* micrografting has been often applied in the improvement and rejuvenation of several tree species *(Navarro & Juarez,* 1977, *Ke* et al., 1993, *Perrin* et al., 1994, *Mantell* et al.,1997). It can be a useful technique to obtain virus-free plants from virus-infected ones *(Navarro & Juarez,* 1977, *Huang & Millikan,* 1980). *In vitro* micrografts can be used in studying of some physiological connections between rootstock and scion and they can be used in *in vitro* resistance-tests to study how rootstock and scion exercise mutual influence. *In vitro* grafting method was established for raising of von developing plantlet of interspecific hybrid embryos in *Capsicum* spp. *(Fári & Csilléry,* 1984).

Several methods have been described for micrografting of tree species, such as using silicon and glass tubing, elastic trips, nylon bands, aluminium foil tube or placing the scion in a vertical slit in the rootstock *(Parkinson & Yeoman,* 1982, *Hossain,* 1987, *Mosella-Chansel,* 1979, *Ke* et al., 1993, *Abousalim & Mantell,* 1992). The first aim of these devices was to hold grafts together until fusion took place. The disadvantages of these techniques was the relatively low efficiency: the rate of fused grafts has rarely exceeded the 70 per cent *(Ramanayake & Kovoor,* 1999) and in some cases the graft junction could not be visible monitored to ensure good contact.

The aim of this study was to find a method that could give high efficiency and maintained good contact between scion and rootstock until fusion took place.

Material and methods

Preparation of plant material for grafting

The present study involved only homoplastic grafts using *in vitro* plant material of cultivar *Royal Gala.* For *in vitro* micrografting experiments *in vitro* shoots of cv. *Royal Gala* were multiplied at 4-week-intervals. Shoot multiplication was carried out on MS-medium *(Murashige & Skoog,* 1962) supplemented with 100 mg l^{-1} myo-inositol, 0.7% agar-agar, 3% saccharose and 0.5 mg l^{-1} 6-benzylaminopurine riboside (BAR) , 0.3 mg l^{-1} indole-3-butyric acid (IBA) and 0.2 mg l^{-1} gibberellic acid (GA_3). The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were grown at 22±2 °C with 16 h photoperiod provided by warm-white lamps (Tungsram) at a PPF of 105 µMol s^{-1} m^{-2}.

Before grafting scions and rootstocks were pre-treated. For pre-treatment 3-week-old *in vitro* shoots developed on the above mentioned multiplication medium were used after removing of bottom leaves.

Shoot tips about 30 mm in length excised from these proliferating axillary shoots were placed on S basal medium *(Jámbor-Benczúr & Márta-Riffer,* 1990) consisting of BM macroelements *(Jámbor-Benczúr & Márta-Riffer,* 1990),

Heller (1953) microelements, MS vitamins and was supplemented with 100 mg l^{-1} myo-inositol, 0.8% agar-agar, 3% saccharose and 0.5 mg l^{-1} 6-γ-γ-dimethylallylaminopurine (2-iP) and 0.1 mg l^{-1} indole-3-butyric acid (IBA). They were cultured under light conditions, used during multiplication for one week and then they were used as scions in grafting experiments.

The other group of multiplicated shoots were used as rootstocks. They were placed on rooting medium consisting of MS macro- and microelements at half strength supplemented with 0.5 mg l^{-1} B_1 vitamins 100 mg l^{-1} myo-inositol, 0.75% agar-agar, 2% saccharose, and 3.0 mg l^{-1} indole-3-butyric acid (IBA). The pH of the medium was adjusted to 5.5 with 1 N KOH. Shoots were cultured in darkness at 22 ±2 °C for one week and they were used then as rootstocks.

Grafting method

Rootstocks and scions were removed from the pre-treating media and aesculap (Aesculap AG., Germany) sterile surgical blades N°10 were used for cutting the plant material. The cut surfaces were immediately flooded with a mixture of citric acid (0.15 mg l^{-1}), ascorbic acid (0.1 mg l^{-1}) and gibberellic acid (0.1 mg l^{-1}). The rootstock was decapitated and all leaves were removed. A vertical slit was made on the stump and the scion base cut in a v-shape was fitted in this slit. Before fitting scion was dipped into fluid 1% agar-agar-solution (Bacteriological Agar, Reanal Finechemical Co.) or into 1% agar-agar-solution supplemented with 5 mg l^{-1} indole acetic acid (IAA) and so they were stuck to the vertical slit of rootstocks. After fitting two drops from agar-solution were dropped around the fitting site to hold the grafting strongly. As control treatment the scion was fitted in the vertical slit of rootstock without sticking by agar-agar. The stuck and fitted grafts were placed on rooting medium, which contained MS macro- and microelements at half strength supplemented with 50 mg l^{-1} myo-inositol, 2 ml l^{-1} Wuxal, 0.7% agar-agar, 3% saccharose and 0.1% activated charchoal. The pH of the medium was adjusted to 5.7 before autoclaving. Micrografting were cultured at 22±2 °C with 16 h photoperiod at a PPF of 105 µMol s^{-1} m^{-2}. Fusion between stock and scion was examined by electron-microscopy using longitudinal and cross sections of the graft junction. Sections were made at the end of the 6th week.

Five of the 7-week-old rooted graftings were planted in Jiffy-7 peat pellets from both treatment. After transfer the Jiffy-7s were placed under 100% relative humidity and incubated under light conditions as applied during *in vitro* culture of micrografts. After 2 weeks when roots began to grow out of the Jiffy-7s, graftings were transferred to pots (8 cm in diameter) covered with plastic bags. To acclimate the plants, after observing a new leaf, the corner of the plastic bags was cut off and one week later bags were removed.

Results

In the control treatment all the scions become displaced during the first two weeks after grafting *(Table 1)*. If grafting was made by sticking there was no any displacement *(Table 1)* and both the scion and the rootstock developed further by formation of new leaves *(Table 2)* and by development of root system, respectively. Root development could be observed on the half of grafts after the 3rd and on all the grafts after 6th week. It is assumable that root development started on more than 50 percent of grafts till the 3rd week but the roots were not visible because of the activated charchoal-content of the medium *(Figure 1)*.

Table 1 Comparison of grafting methods

Grafting method	Number of grafts	Displaced grafts	Undeveloped grafts	Fused grafts
Grafts without sticking	20	20	–	–
Grafts stuck by agar-agar	20	–	1	19
Grafts stuck by agar-agar containing IAA	20	–	1	19

At the end of the first week after sticking part of the rootstocks sprouted and had 1–2 axillary shoots. When agar-agar contained 5 mg l^{-1} IAA was used for sticking the number of grafts with axillary shoots on the rootstock was by 16 percent less compared to grafts stuck by agar-agar alone. At the end of the 3rd week there was no any difference in the number of grafts with axillary shoots on the rootstock between the two treatments. It was too high (84%) therefore axillary shoots from the rootstock were cut off. At the end of the 6th week 79% of grafts stuck by agar-agar had axillary shoots on the rootstock but no one of grafts stuck by IAA-containing agar-agar had any axillary shoot development *(Table 2)*.

Table 2 Formation of new leaves on the scion and axillary shoots on the rootstock

Grafting method	Average number of new leaves on the scion per graft			Number of grafts with axillary shoots on the rootstock		
	1st week	3rd week	6th week	1st week	3rd week	6th week
Sticking by agar-agar	2	3.2	7.6	7	16	15
Sticking by agar-agar containing IAA	1.6	3.9	8.0	4	16	0

The two phases of fusion described by Jeffree and Yeoman (1983) on tomato could be observed in 6-week-old grafts independent on IAA-content of sticking agar-agar. Either formation of elongated cells generated from the peripherical tissue of stock and scion was visible at the graft junction *(Figure 2 A., B., Figure 3 A., B.)* or total fusion of cells could be detected *(Figure 2. C., D., Figure 3 C., D.)*.

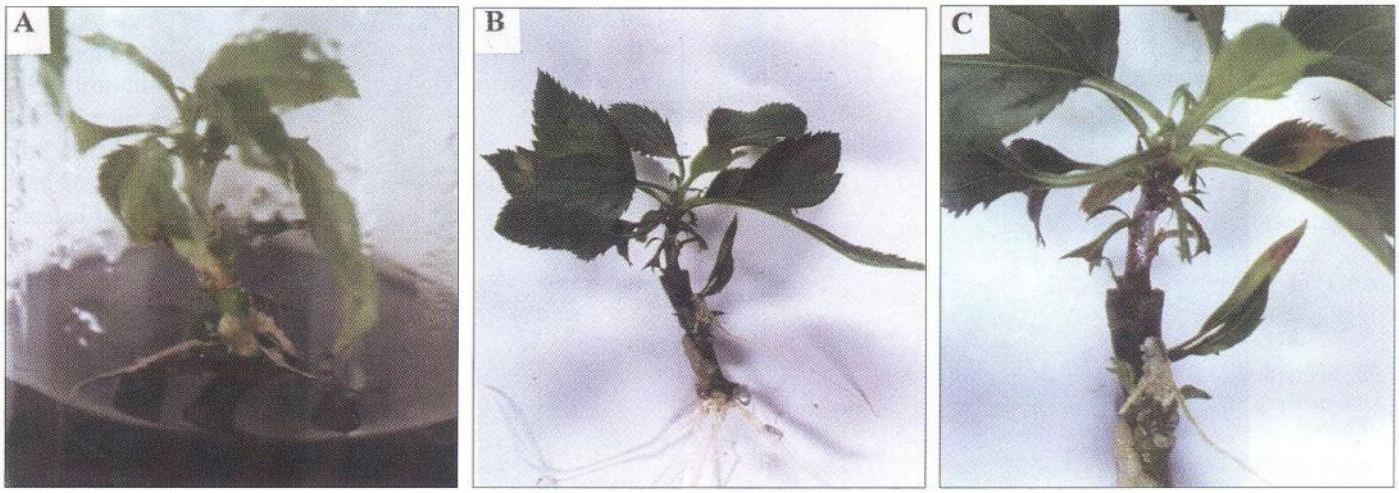

Figure 1 Six-week-old apple micrografts; A: on rooting-medium. B: removed from jar. C: The grafted part of the plant.

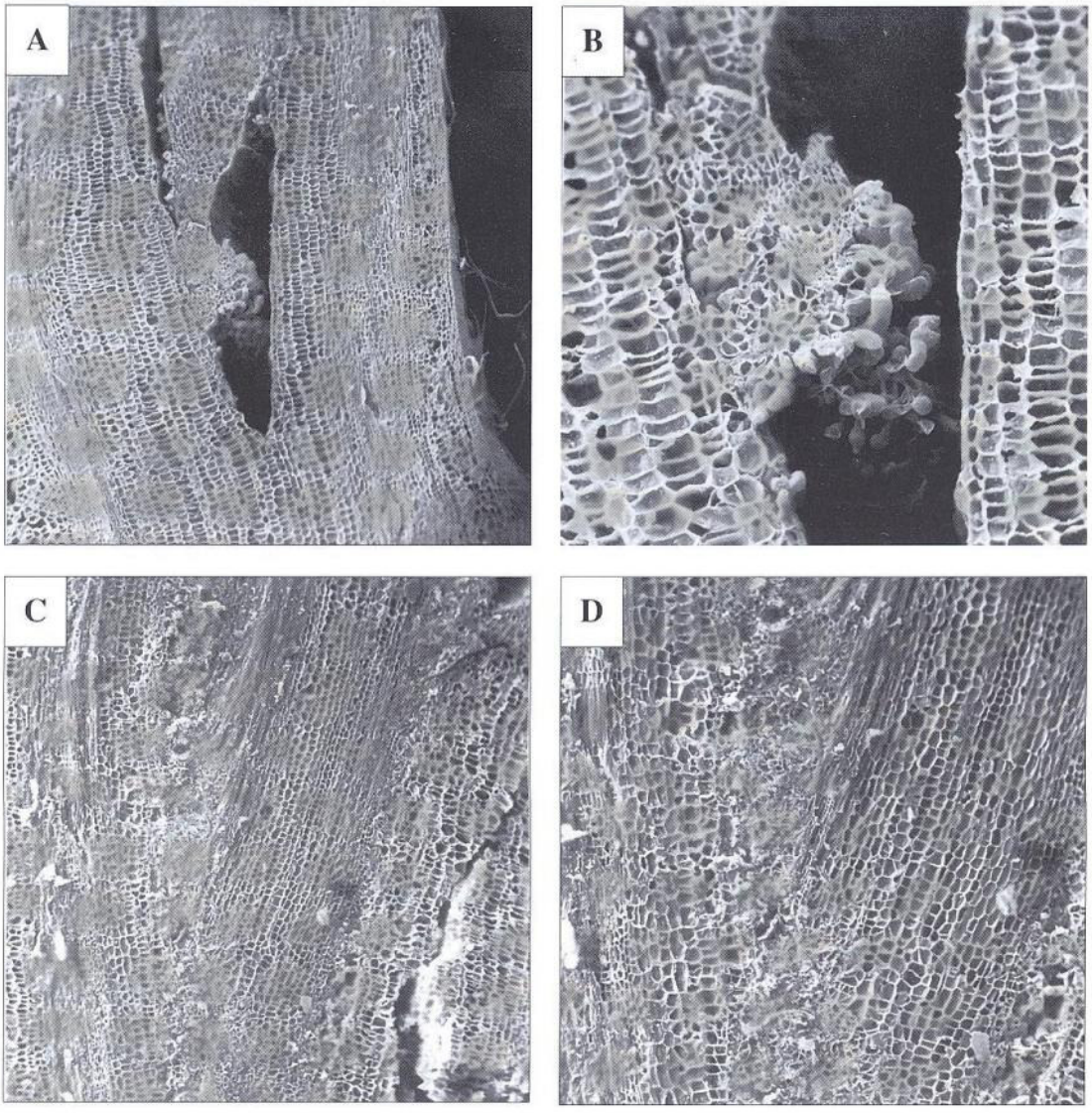

Figure 2 Longitudinal sections of graft junction. A (30 x) and B (100 x): Formation of elongated cells. Cells adjacent to the cut surfaces of stock and scion are stimulated to divide with formation of callus. This activity seems to be more pronounced in the scion than in the stock. C (30 x) and D (50 x): Total fusion of cells generated from the peripherical tissue of stock and scion. A mechanical union between surfaces can be seen.

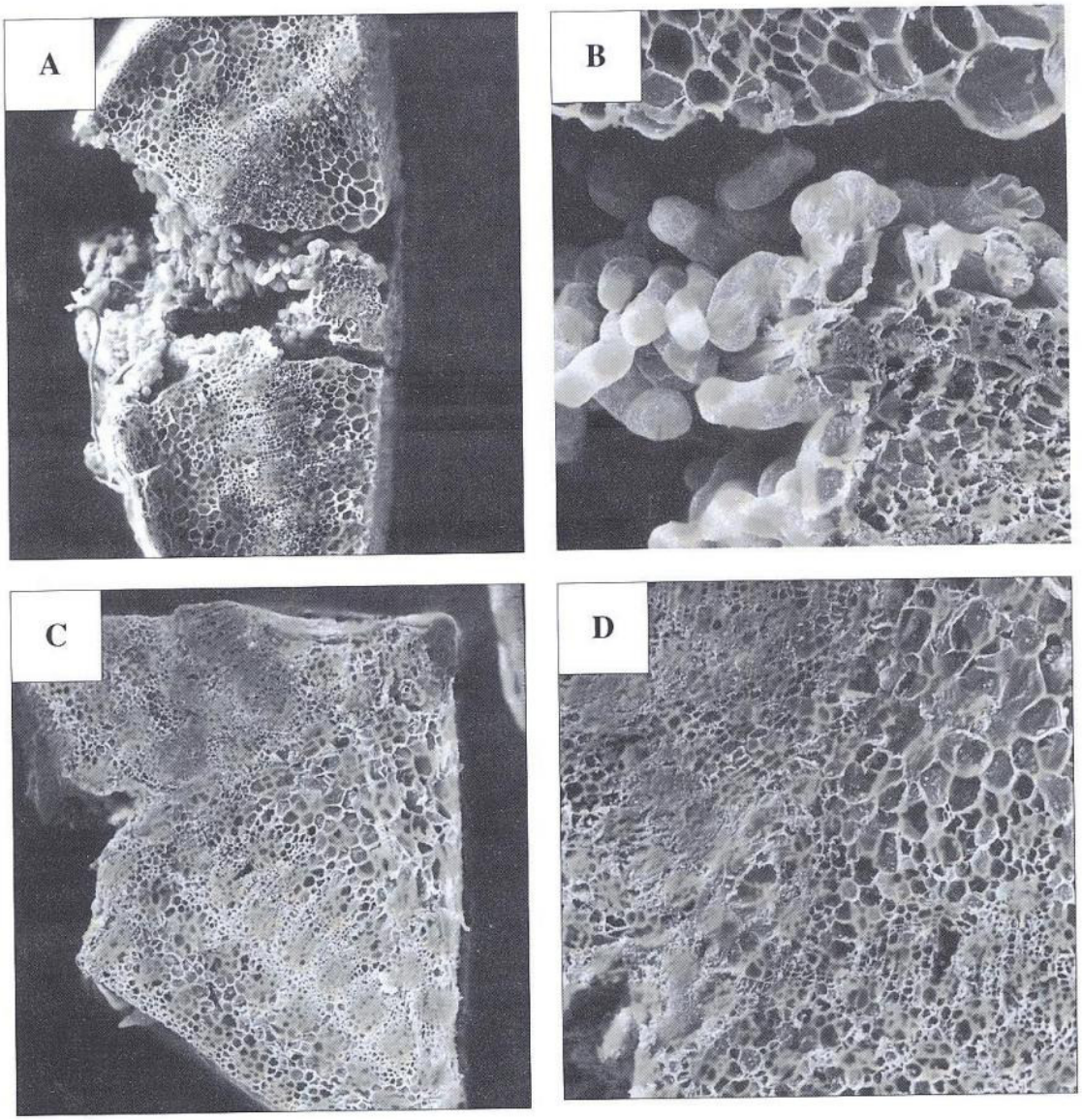

Figure 3 Cross sections of graft junction. A (50 x) and B (250 x): Formation of elongated callus cells generated from cut surfaces of stock and scion. The cells expanding into the space between stock and scion. C (50 x) and D (125 x): The gap between stock and scion is bridged by cells and total fusion of tissues can be seen.

After planting and acclimatisation of graftings, the rate of survival was 100 percent. Several new leaves developed on the scions and the stem length of scions reached at least 10 cm after 4-week-long acclimatisation.

Discussion

Because of incompatibility and/or poor contact between rootstock and scion, phenolic browning of cut surfaces and poor root development, *in vitro* micrografts of fruit plants often fail *(Fuji & Nito,* 1972, *Moor,* 1984, *Ramanayake & Kovoor,* 1999).

In the present study we tried to eliminate all of these problems and establish an effective method for *in vitro* micrografting of apple. Heteroplastic incompatibility could be avoided by using homoplastic grafts similar to results of *Ramanayake & Kovoor* (1999). Phenolic browning due to oxidation of phenols inhibits the growth and development of new cells and causes the death of scion. Floating of cut surfaces in the mixture containing citric acid and ascorbic acid could control this process and could inhibit the oxidative browning. Failing of micrografts because of poor root development was avoid by the induction of root development on rootstocks during pre-treatment.

The primary aim of this study was to find a method that maintained good contact between scion and rootstock until fusion took place. Sticking of scion into vertical slit of rootstock by agar-agar made the transport of different materials between scion and rootstock possible till the fusion, which can be seen from the further development of the scion *(Table 2)*. Agar-agar can maintain the good contact between the stock and scion. The other benefit of using of agar-agar was, that it could hold grafts together till the

fusion, independently on the size of stem diameter and the graft junction could be visibly monitored.

Electron-microscopy carried out at the end of the 6th week proved the appearance and fusion of elongated cells adjacent to the vascular region *(Figure 2–3)*. The development of scion under *in vitro* conditions and later under acclimatisation indicated the further development: establishment of vascular continuity from newly formed cells.

Parkinson & Yeoman (1982) reported that IAA applied apically in graft unions enhances the vascular differentiation. In this study IAA was added into the agar-agar solution used for sticking the grafts together. However, differences between grafting methods with IAA or without IAA could not be detected by histological study carried out at the end of the 6th week.

The rate of complete fusion and survival of grafting was 95%. High percentage of succesful grafts proved that sticking of grafts by agar-agar can be used very efficiently in *in vitro* micrografting process. Further improvement of this technique is under way in our laboratory to avoid development of axillary shoots on rootstock with the use of rootstock cultivars.

Acknowledges

This work was supported by OTKA (Project No. T-030103).

References

Abousalim, A. & Mantell, S. H. (1992): Micrografting of pistachio (Pistachia vera L. Mateur). Plant Cell Tissue and Organ Culture. 29: 231–234.

Fári M. & Csilléry G. (1984): *In vitro* grafting of a non developing plantlet of *Capsicum frutescens* L. x *Capsicum cardenasii* Heiser and Smith hybrid produced by embryo culture. Capsicum Newsletter, 3: 51.

Fuji, T. & Nito, N. (1972): Studies on compatibility of grafting of fruit trees. I. Callus fusion between root stock and scion. J. Japan. Soc. Hort. Sci. 41: 1–10.

Heller, R. (1953): Recherches sur la nutrition minerale das tissues vegetaux cultives in vitro. Ann. Sci. Nat. Bot. Veg. 14: 1–223.

Hossain, M. A. (1987): Tissue culture techniques for studies of graft compatibility/incompatibility in higher plants. Proceedings of the Regional Workshop on Tissue Cultur of Tropical Crop Plants. University of Dhaka, Bangladesh, 1987 (Islam, A.L. and Haque, M. M., eds.) 103–107.

Huang, S. & Millikan, D. F. (1980): In vitro micrografting of apple shoot tips. Hortscience. 15(6): 741–743.

Jámbor-Benczúr E. & Márta-Riffer, A. (1990): In vitro propagation of Philodendron tuxtlanum (Bunting) with benzylaminopurine. Acta Agronomica. Hung. 39: 341–348.

Jeffree, C. E. & Yeoman, M. M. (1983): Development of intracellular connections between opposing cells in graft union. New Phytol. 93: 491–509.

Ke, S., Cai, Q. & Skirvin, R. M. (1993): Micrografting speeds growth and fruiting of protoplast-derived clones of kiwi fruit (Actinidia deliciosa). J. Hort. Sci. 68: 837–840.

Mantell, S. H., Bogetti, B., Bessa, A. M. S., Lemos, E. P., Abdelhadi, A. & Mneney, E. E. (1997): Micropropagation and micografting methods suitable for safe international transfer of cashew. In: Abstracts of papers and posters. International Cashew and Coconut Conference. Dar es Salaam. Government of Tanzania, Agricultural Research Institutes of Nalienele (cashew) and Mikocheni (coconut), 24.

Mosella-Chansel, L. (1979): L'utilization de l'apex caulinaire comme moyen d'élimination de 2 types de virions chez le Pecher. Thése d'Ingénieur de Sciences Agronomique et Phytotechniques USTL, Montpellier, France.

Murashige, T. & Skoog, F. (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497.

Navarro, L. & Juarez, J. (1977): Tissue culture techniques used in Spain to recover virus-free Citrus plants. Acta Hort. 78: 425–435.

Perrin, Y., Lardet, L., Enjalric, F. & Carron, M. (1994): Rajeunissement de clones matures d' Hevea brasiliensis Mull. Arg. par micrograffege in vitro. Can. J. Plant Sci. 74: 623–630.

Parkinson, M. & Yeoman, M. M. (1982): Graft formation in cultured explanted internodes. New Phytol. 91: 711–719.

Ramanayake, S. M. S. D. & Kovoor, A. (1999): In vitro micrografting of cashew (Anacardium occidentale L.). J. Hort. Sci. & Biotech. 74: 265–268.