In vitro culture establishment and multiplication of the *Prunus* rootstock 'Adesoto 101' (*P. insititia* L.) as affected by the type of propagation of the donor plant and by the culture medium composition

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Abstract

The establishment of new in vitro cultures from mature woody plants is often a difficult task due to the little growth of initial explants. Since the explant origin plays an important role, in this work the effect of the origin of the explants (micropropagated or conventionally propagated plants) on both establishment and multiplication of the in vitro cultures has been studied using different culture media. Best results during establishment were obtained with explants taken from micropropagated plants. The multiplication rate of new cultures was strongly affected by the type of propagation of the mother plants. Thus, while the cumulative number of shoots increased sharply in cultures originated from micropropagated plants, cultures originated from cutting-derived plants showed only a moderate increase. Culture medium composition influenced the multiplication rate. After 9 subcultures, a significantly lower number of shoots was found on QL medium than on MS or on WP. The positive effect of micropropagation of donor plants on the establishment and multiplication of new

in vitro cultures is discussed in terms of a possible reinvigoration during in vitro culture.

Keywords:
Apparent rejuvenation, reinvigoration, micropropagation, cuttings, culture medium composition

Introduction

Woody plants raise frequent propagation difficulties when using conventional techniques. Tissue culture can relieve this problem since it has been reported that plants may acquire higher rooting capabilities after continuously subculturing in vitro (Howard et al., 1989; Jones and Hadlow, 1989; Webster and Jones, 1989; Hammatt and Grant, 1993; Grant and Hammat, 1999). Besides, this type of ‘rejuvenation’ can influence other aspects of plant propagation, such as the ability to initiate new healthy in vitro cultures. During the establishment of new cultures from mature plants, the explants often show slow growth and low survival rates; however, juvenile explants taken from young grafts displayed better in vitro growth than those taken from adult cashew plants (Thimmappaiah et al., 2002), and had higher multiplication rates in Fagus (Meier and Reuther, 1994). This different performance of juvenile vs. mature explants has been related with the contents of phenolic compounds in chestnut (Mato et al., 1994). Thus, rejuvenation has been used to facilitate in vitro culture of explants from mature plants, mainly grafting buds into juvenile rootstocks (Pliego-Alfaro and Murashige, 1987; Meier and Reuther, 1994; Sanchez et al., 1997; Thimmappaiah et al., 2002). Similarly, a partial rejuvenation was also

obtained by intensive pruning, what stimulates the sprouting of the basal buds that could retain juvenile characters, increasing both in vitro proliferation and maintenance of culture lines of filbert (Diazsala et al., 1994), as well as affecting the endogenous polyamine contents in hazelnut leaves and buds (Rey et al., 1994).

No direct relation between growth regulators and rejuvenation was found, when they were included in the culture medium (see George, 1993, for a review). However, both internal concentration and external applications of growth regulators were related with juvenile traits. Thus, juvenile tissues contain higher IAA levels as a consequence of high concentrations of auxin protectors (Mato et al, 1994). On the other hand, exogenously applied cytokinins improved in vitro performance of mature explants of chestnut in terms of establishment, multiplication and rooting (Sanchez et al., 1997).

Recently, different factors of *Prunus* micropropagation have been studied as the effect of subculture frequency (Grant and Hammatt, 1999), the effect of different carbohydrates (Harada and Muray, 1996; Nowak et al., 2004), the comparison of different iron sources in the culture medium (Molassiotis et al., 2003), the effect of different combinations of growth regulators (Pruski et al., 2000), the application of mycorrhiza for pathogen protection, and the performance of micropropagated plants after their transfer to soil (Hammerschlag and Scorza, 1991; Hammat, 1999; Marín et al., 2003).

Different culture media have been used in Prunus with a genotype-dependent response as in apricot and almond (Perez-Tornero and Burgos, 2000; Channuntapipat et al., 2003). In addition to media composition, the concentration of salts may play an important role, as in cherry, in which half concentration MS macronutrients resulted in more growth than full or double concentration (Ruzic et al., 2003). Nevertheless, culture medium can affect in vitro growth in different ways, depending on the culture stage, so it would be interesting to study the effect of commonly used culture media in a particular Prunus species along the micropropagation phases.

Here we studied the effect that previous micropropagation of donor plants has on obtaining suitable explants to establish new in vitro cultures. This study confirmed that micropropagated plants could acquire this juvenile character, as it was previously stated for rooting capability. We compared the effect of the type of propagation of pruned mother plants (cuttings vs. micropropagation) on the establishment and multiplication of new in vitro cultures in three different culture media.

Materials and methods

One-node explants of the Prunus rootstock ‘Adesoto 101’ (*Prunus insititia* L.) were taken in spring from trees propagated either by cuttings or by micropropagation that had been severely pruned every winter. After washed in running tap water, the surface of the explants was disinfected with HgCl2 (0.05%) for 15 min and then rinsed 3 times in sterile distilled water. Explants (48 one-node explants per treatment) were placed on 15 ml medium contained in

33 ml glass tubes sealed with polypropylene caps. Three kinds of medium were used: MS medium (Murashige and Skoog, 1962), WP (Lloyd and McCown, 1980), and QL (Quoirin and Lepoivre, 1977). All three media were supplemented with 0.5 µM IBA , 5 µM BA, 30 g·l-1 sucrose, and 7 g·l-1 agar (Bacto-agar, Difco, Fisher Scientific). The pH was adjusted to 5.6 before autoclaving. Explants were cultured at 22º C under a photoperiod of 16h of cool-white fluorescent light (35 µmol×m-2×s-1). The explants were examined weekly and those that exhibited healthy expanding leaves were scored and the percentages of established cultures, after an initial period of 8 weeks, were recorded. Subsequently, shoots that arose from nodes were transferred onto 30 ml fresh medium in 100 ml glass culture vessels (Sigma Chemical Co., St. Louis MO, USA). As a result, a variable number of culture lines were multiplied depending on the combination of treatments. Thus, 16,14 and 16 culture lines were maintained and multiplied respectively for MS, WP and QL when the cultures were originated from trees propagated by cuttings, whereas they raised to 20, 25 and 22 culture lines from micropropagated trees as average. Every culture line, derived from a growing node, was identified and transplanted to a fresh medium at 4-weeks intervals. New shoots were cut off and placed again in the same medium, and the number of shoots was scored. A cumulative number of shoots per line after 9 subcultures was obtained as a measurement of the multiplication rate, while the percentage of lines per treatment that showed continuous growth after that period indicated the survival of the culture lines. The whole experiment was repeated three times on different dates.

Data analysis

A completely randomized design with two treatments (type of propagation of donor plants and culture medium composition) and three repetitions was applied. Two-factors analysis of variance (ANOVA) and Duncan's multiple range test were performed to analyse the cumulative number of shoots at the 9th subculture, as well as the transformed percentages (arcsine transformation) of both, the establishment of new cultures and the survival of culture lines. SPSS statistical software (SPSS Inc., Chicago, USA) was used.

Results

Establishment of new culture lines in vitro

Most of the nodes exhibited growth soon after the culture initiation, showing some bud swelling and leaf expansion. The number of explants that showed healthy growth increased sharply during the first 30-40 days in all culture media and plant origin, and then continued displaying a slight increase or, in some cases a decrease, as in cutting derived cultures on either WP or QL (Figure 1). Buds taken from micropropagated plants grew faster even in a higher amount (up to 63.9% of explants at the end of the initial phase) than those taken from plants propagated by cuttings (up to 43.8%) in all three culture media tested, (Figure 1) and this effect was statistically significant ($P<0.05$, Table 1). Therefore, the initial growth of explants during the establishment of in vitro culture was significantly affected by the type of propagation of mother plants. On the other hand, culture medium composition affected the percentage of establishment of new cultures in vitro, since it was higher in WP than in QL or MS (Table 1), either in micropropagated or in cutting derived cultures. However, these differences were not statistically significant (Table 1).

Multiplication of culture lines

The cumulative number of shoots of each combination of treatments increased with time in all cases but at different rates (Figure 2). The propagation technique of donor plants influenced shoot production during the multiplication phase since micropropagated derived cultures produced more shoots than cutting-derived cultures in any subculture of the multiplication phase. Cultures derived from micropropagated trees produced more shoots than those derived from cuttings and this effect was maintained in every culture medium (Figure 2, Table 1). After 9 subcultures the statistical analysis of the multiplication rate (as the number of cumulated shoots per culture line) showed significant differences between micropropagated and cutting-derived cultures ($P \leq 0.001$, Table 1). Cultures derived from micropropagated trees produced an overall average of 17.3 shoots per culture line, while cultures derived from cutting-trees produced only 9.9 shoots per culture line. On the other hand, the composition of the culture media had also an effect on shoot production, thus, explants cultured in MS or WP media developed significantly more shoots than explants cultured in QL ($P<0.01$, Figure 2, Table 1), with 17.0, 13.5 and 8.5 shoots per culture line respectively, as overall averages, while no significant differences were found between MS and WP following Duncan's multiple range test.

Survival of culture lines

While most of the established cultures continued growing during the multiplication phase, the growth of some culture lines declined and they eventually died, mainly when the culture lines derived from trees propagated by

cuttings and cultured on MS or QL (Table 1). However, differences in the percentages of survival of the culture lines (an overall average of 64.7% in cutting-derived culture lines vs. 85.5% in micropropagation-derived cultures) were not statistically significant.

No significant interaction between the method of propagation and the composition of the culture medium has been found either in the culture establishment or in the multiplication and survival of the culture lines.

Discussion

The data contained in this work clearly indicate that the type of propagation of the donor plants affected the establishment of new cultures and the multiplication rates of the culture lines. Plants obtained by micropropagation were a better source of explants to establish new in vitro cultures than those propagated by cuttings. The positive effect of micropropagation suggests that the formerly micropropagated Adesoto 101 plants remained apparently rejuvenated after being transferred to soil and affected their in vitro cultures thereafter. To our knowledge, this has not been described for the establishment of new cultures; however, an apparent rejuvenation that improved rooting capability of cuttings had been observed in micropropagated apple, pear, cherry and plum trees, as well as in rhododendron plants (Howard et al., 1989; Jones and Webster, 1989; Marks, 1991; Webster and Jones, 1992; Grant and Hammat, 1999).

Partial rejuvenation, or re-invigoration, is related with a period of culture under in vitro conditions (Howard et al, 1989; Devries and Dubois, 1994) affecting growth and development of tissues; thus, the number of shoots per culture and the ability of shoots to produce adventitious roots increased with the age of a culture line in the cherry rootstock F12/1 (Hammatt and Grant, 1993; Grant and Hammat, 1999), but the length of the culture period to induce juvenile characters is not predetermined, since a variable number of subcultures for different culture lines was required, as in an adult clone of grape (Mullins et al., 1979). These changes can be associated with physiological differences already described between juvenile and adult tissues, thus, the polypeptide contents appeared to reflect the ontogenetic age of chestnut tissues (Amomarco et al., 1993), and higher polyphenol contents were found in juvenile tissues of chestnut (Mato et al., 1994). Furthermore, juvenile tissues of grape showed a lower concentration of abscisic acid than adult tissues (Langilier and Fournioux, 2000). However, in vitro culture affects not only the acquisition of juvenile traits, but also mature traits as it was described for in vitro induced flowering, under certain conditions, after a long-term culture of pear shoots (Harada and Murai, 1998). This apparent paradox may be explained since separate features of juvenility are supposed to be independently controlled, as pointed out by George (1993) using the different characteristics of juvenility reported in different species that have been micropropagated.

The composition of the culture medium has influenced the growth of new in vitro cultures, and this effect is caused by the salt composition of the media since the rest of the components remained unchanged. WP was the medium that

promoted a better establishment of the cultures, but MS supported higher multiplication rates. This effect seems to be related with an optimization of different phases of micropropagation with different culture media. In contrast, MS was better than WP for both explant establishment and multiplication of chokecherry (*Prunus virginiana* L.) (Zhang et al., 2000), and a similar effect was found in the culture establishment of mature wild cherry (Hammatt and Grant, 1997). Culture medium performance also depends on the genotype, thus, in almond AP medium performed better in the establishment of cultures of the cultivar Nonpareil, whereas MS medium was preferred for the cultivar Ne Plus Ultra (Channuntapipat, 2003).

Intensively pruned plants derived from micropropagation or propagation cuttings were used in this work, and this pruning treatment has possibly improved the establishment and multiplication of explants taken from adult plants, which showed here relatively high values. It was reported that intensive and repetitive pruning promoted physiological changes that affected the endogenous polyamine content in hazelnut leaves and buds (Rey et al., 1994) and that these changes were related with juvenility and rejuvenation.

In conclusion, micropropagated plants are a better source of explants than plants propagated by cuttings to initiate and multiply new in vitro cultures. This can be due to a possible partial rejuvenation of in vitro propagated plants. On the other hand, the growth and development of explants in culture is affected by the salt composition of the culture medium in a different way depending of the micropropagation phase.

Acknowledgements

This research was made possible by financial support of Spain's CICYT grants no. AGF98-0277-C04-01*,* AGL2001-2414-C04-01 and AGL 2002-03231, and CONSI+D –DGA P012/2001.

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Table 1. Percentages of both in vitro culture establishment and culture line survival, and cumulated number of shoots per culture line of in vitro cultures of the clonal rootstock Adesoto 101 (*Prunus insititia*) grown on three culture media and initiated from explants taken from micropropagated or from cutting-derived trees. Each value is the average of three separate experiments.

Propagation method	Culture Medium	Establishment (%)	Cumulated shoots per culture line	Culture line survival (%)
Cuttings	MS	25.0	11.7	55.0
	WP	43.8	11.4	84.2
	QL	28.1	5.1	50.1
Micropropagation	MS	37.5	22.4	87.7
	WP	63.9	15.7	94.5
	QL	54.5	12.0	68.9

ANOVA	F-value	P-value	F-value	P-value	F-value	P-value
Propagation method	6.3	0.017*	15.6	0.001***	3.8	0.060
Culture Medium	2.9	0.066	6.5	0.005**	2.9	0.069
Method x Medium	0.17	0.840	1.1	0.332	0.45	0.640

366 Figure captions

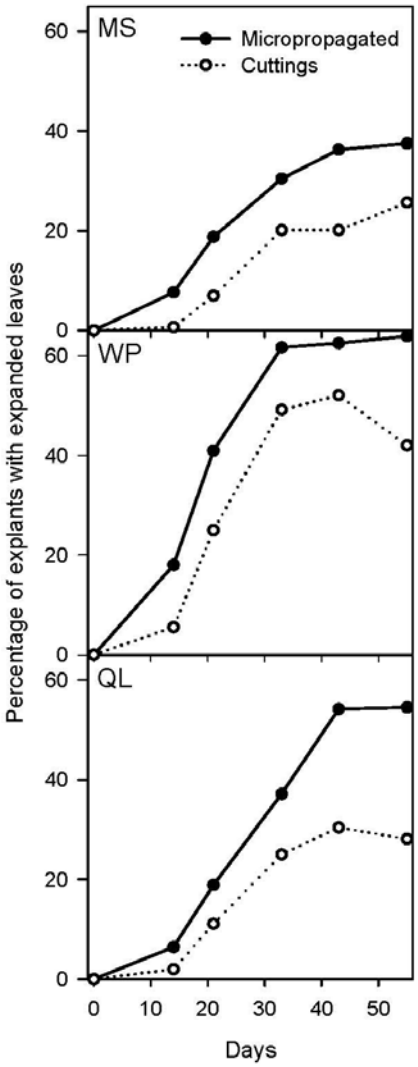
367

368 Figure 1. Evolution of the percentages of one-node explants grown on three
369 culture media, showing expanding leaves during the establishment of new in
370 vitro cultures of the clonal rootstock Adesoto 101 (*Prunus insititia*) previously
371 micropropagated or propagated by cuttings.

372

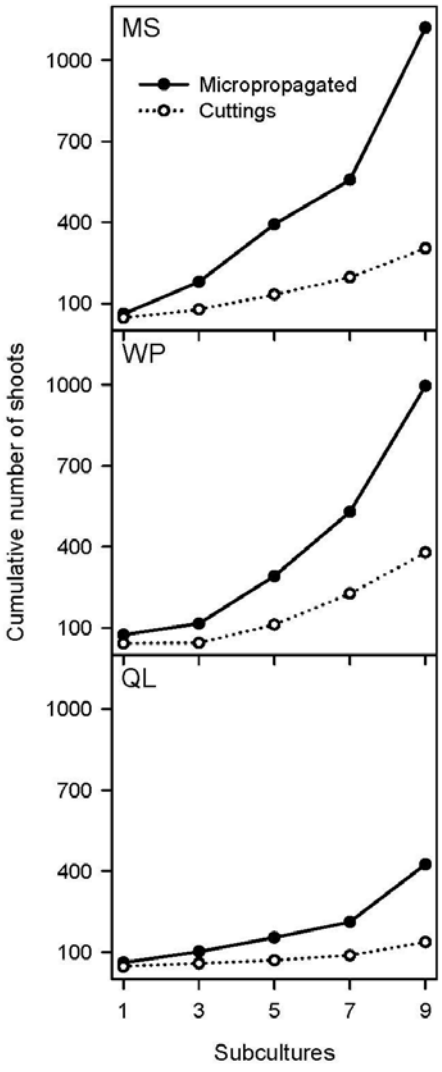
373 Figure 2. Evolution of the cumulative number of shoots per treatment during
374 nine subcultures on three culture media, and initiated from explants taken from
375 micropropagated or from cutting-derived trees of the clonal rootstock Adesoto
376 101 (*Prunus insititia*).

377


MS
Micropropagated
Cuttings
WP
QL
Percentage of explants with expanded leaves
Days
0
10
20
30
40
50
0
20
40
60

378

379


MS
Micropropagated
Cuttings
WP
QL
Cumulative number of shoots
100
400
700
1000
1
3
5
7
9
Subcultures

380