

Experimental approaches to *in vitro* grafting in *Prunus armeniaca* and *P. spinosa*

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Key words: apricot, black thorn, 'Caldesi', 5(6)-carboxyfluorescein, micropropagated shoots, toluidine, vascular elements.

Abstract: An *in vitro* grafting protocol was established with micropropagated shoots of the apricot cultivars Caldesi and Alb 3 (apricot selection from 'Cricot' x 'NJA53' cross) and the *P. spinosa* seedling 'PS123' (open pollination from a wild tree). Compatible combinations (homografts) were ascertained by considering vascular continuity between scion and stock, as determined by fast toluidine blue staining procedure, and by restoration of phloem connections as determined by application of 5(6)-carboxyfluorescein (CF) to the scion. Fifteen days after graft establishment, the differentiation of new xylematic tissue resulting in unification of the vascular elements between stock and scion was observed in all the genotypes which, moreover, showed restoration of active phloem. High correlation between the differentiation of new xylem and the differentiation of new phloem at the graft point was found. Graft-take, however, did not correlate with the scion-stock compatibility, in that heterograft-take was high but did not reflect xylematic continuity or phloematic transport, which were not established in any of the tested scion-stock combinations. The use of rooted shoots as rootstock resulted in unsuccessful graft technique.

1. Introduction

Grafting is the main propagation system in stone fruits. Nevertheless, graft incompatibility may suddenly occur in *Prunus* several years after grafting and without any external symptom. This phenomenon, classified as localised-type incompatibility, is very common in apricot cultivars which show different incompatibility degrees when grafted on seedlings on *P. cerasifera* rootstocks (myrabolan). In an attempt to study early signs of graft incompatibility in apricot, Errea *et al.* (1994) found such condition to be related to a lack of callus differentiation into cambium and vascular tissue. *In vitro* tissue culture has proven to be a tool for early testing for compatibility in fruit trees such as *Prunus*, *Citrus* and *Pyrus* by micrografting, internode associations and/or callus fusion (Jonard *et al.*, 1983; 1990; Musacchi *et al.*, 1997; Errea *et al.*, 2001). In *Prunus* spp., *in vitro* grafting can serve several functions as screening tool on rootstock populations coming from breeding programs, to investigate the factors involved in incompatibility and also for mass propagation of disease-free plant cultivars.

The present research was developed within a wider program aimed at finding a compatible rootstock for apricot and, in particular, to test black thorn (*Prunus spinosa*) plants, selected for low sucker formation. Black

thorn has been reported to give good results both in terms of uniform vegetative growth and yield when used as interstock for apricot (Djuric and Keserovic, 1999).

An *in vitro* autografting protocol using micropropagated shoots of the apricot cultivar Caldesi, Alb 3 (apricot selection from the cross 'Cricot' x 'NJA53') and the *P. spinosa* 'PS123' (wild selection from open pollination) was established. Compatible combinations (homografts) were determined by checking the graft-take and by anatomical studies to check either xylematic continuity of the function of phloem connections between the two individuals. Rooting ability of 'PS123' autografts was determined. 'Caldesi'/'PS123' and 'Alb 3'/'PS123' grafting combinations were also tested.

2. Materials and Methods

Shoot tissue cultures

Proliferating shoots of apricot 'Caldesi', 'Alb 3' and black thorn 'PS123' were maintained on agarised MB medium (Deogratias *et al.*, 1989) with 5.0 µM 6-benzyladenine (BA), 3% (m/v) sucrose, 0.6% m/v bacteriological agar (Acumedia manufactures, Baltimore, MD); pH was adjusted to 5.5 before autoclaving for 15 min at 121°C. 5.0 µM BA was used to produce actively proliferating shoots that were then transferred to 2.5 µM BA to get a large size of the stem as necessary to perform the grafts.

Cultures were routinely grown in Green-boxes®

(Duchefa), at 22-24°C, under Philips TDL 33 cool white fluorescent tubes, with irradiance of approximately 7.5 $\mu\text{mol s}^{-1} \text{m}^{-2}$ and 16 hr photoperiod. Shoots were transferred to fresh medium at 4-week intervals.

In vitro grafting

Shoots of 'Caldesi', 'Alb 3' and 'PS123' were grafted to themselves (autografts) and 'Caldesi' and 'Alb 3' were grafted to 'PS123' as understock (heterografts), by cleft grafting (Westwood, 1993). Shoots from 2.5 to 3 cm in length were aseptically cut from the proliferating clusters and defoliated of the unfolded leaves; visible lateral buds were also removed from understocks. Because preliminary studies showed that 'PS123' autografts made on rooted stocks resulted in significantly less grafting success, unrooted shoots were used as stocks. Shoot tips of the stock were then excised and a cleft cut about 0.5 cm long was made using a scalpel (Fig. 1 A). The base of the scion was cut with a scalpel to fit the stock cleft, where it was inserted using sterile forceps (Fig. 1 B). The scion was held in place inside a 5-7 mm length of silicone rubber pipe (Fig. 1 C). Grafted shoots were placed into 30-ml disposable vessels (Coulter ZFG, pbi Int.) containing 10 ml regulator free basal MB (Fig. 1 D) and incubated as described above.

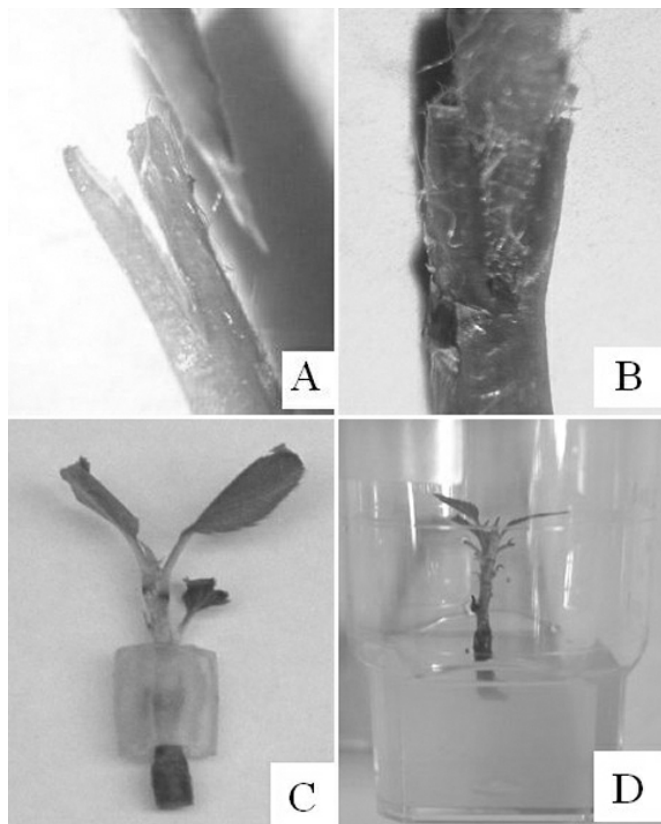


Fig. 1 - Cleft grafting technique phases. (A) Shoot tip of the stock is excised and a cleft cut about 0.5 cm long is made using a scalpel. (B) The base of the scion is inserted into the stock cleft. (C) The scion is held in place inside a 5-7 mm length of silicone rubber pipe. (D) An autograft growing for 15 days in a 30-ml disposable vessel, after graft union (graft-take) check.

Graft-take was initially monitored by manually checking the graft union after 15 and 30 days. Graft-take was expressed as percentage for 20 grafts of each combination.

Xylematic continuity

The establishment of vascular continuity between scion and stock was determined using a fast toluidine blue staining procedure. Grafts were longitudinally cut, 0.5 cm below and 0.5 cm under the graft union, with a stainless-steel razor blade to give two longitudinal graft sections of the same thickness which were immersed in 100% ethanol until complete decolouration. Sections were rinsed with distilled water and vacuum infiltrated with an aqueous solution of 0.005% toluidine blue then rinsed again. Specimens were immersed in an aqueous solution of 70% ethanol for 3 min at 50°C to remove the excess stain and after a final rinse in water they were ready for light microscope observation. Data were expressed as percentage of grafts showing xylematic continuity across the graft interface and each percentage was calculated on 5-10 samples.

Phloem transport

Restoration of phloem continuity between scion and stock was determined by application of 5(6)-carboxy-fluorescein (CF) to the scion using a modified procedure described by Schöning and Kollmann (1996). CF was solubilised in 0.3 M KOH and diluted with distilled water to obtain the final concentration of 0.5 mM; pH was adjusted at 6.3 with HCl 0.1 N. Grafts were placed vertically in Petri dishes containing the basal culture medium. Each scion was transversally cut 7 mm above the graft interface and 2 mm stem inserted into a silicone rubber pipe; 4 μl of tracer solution was injected into the pipe. Samples were incubated in the light at 100% RH at room temperature. Samples were removed after 1 hr, washed thoroughly under distilled water and 0.1-0.2 mm long cross-sections of both scion and stock were dissected, immersed in water and viewed with an epifluorescence microscope (Leica DMR). Sections showed a green-yellowish fluorescence under epifluorescence filter combination I3 (excitation 450-490 nm, dichroic mirror 510 nm, cut-off 520 nm), only when phloem transport was completely restored. Scion sections were used as control and only those grafts whose scion sections gave a clear fluorescent signal in the phloematic zone were used. Stock sections with either partially (Fig. 2 B) or completely restored (Fig. 2 A) phloem transport were scored as positive. Data were expressed as percentage of grafts showing restored phloem transport.

Rooting

Rooting ability of 'PS123' autografts, growing on regular-free MB medium, was checked by comparing rooting percentages with those obtained from 'PS123' proliferating shoots. Both proliferating shoots and

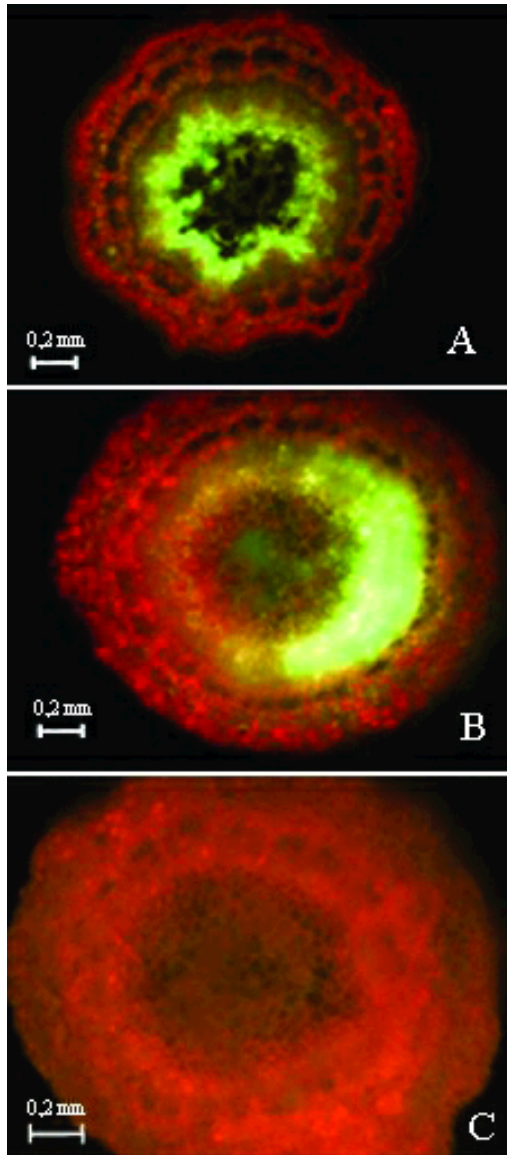


Fig. 2 - *P. spinosa* 'PS123' autograft stock section viewed with an epifluorescence microscope (Leica DMR) after application of 5(6)-carboxyfluorescein (CF) to the scion. Greenish/yellowish fluorescence of CF formed a continuous ring only when phloem transport was completely restored (A); Phloem transport partially restored (B); control (C). Samples were observed with epifluorescence filter combination I3 (excitation 450-490 nm, dichroic mirror 510 nm, cut-off 520 nm).

grafts were treated by basal dipping in 50% ethanolic solution of 0.0, 2.5, 5.0 and 7.5 mM indole-3-butyric acid (IBA) followed by culture on growth regulator-free MB medium.

Thirty days after the treatment, samples were scored for root formation and data expressed as percentage. Each percentage was calculated on 10 samples and the experiment was performed in triplicate.

Statistical analysis

Data were analysed by the χ^2 test or Anova and, in this case, data expressed in percentage were first subjected to Kolmogorov-Smirnov non parametric test of normality. Correlations were analysed according to Pearson's two-tailed test. Statistical analysis was performed at 5% level using SPSS 11.0 for Windows package by SPSS inc.

3. Results

In vitro grafting

Data from this research show that the early cytological events leading to graft adhesion, as a consequence of the healing process, followed a genotype independent trend and both autograft and heterograft-take percentages showed a linear and positive trend with time (Figs. 3 and 4). Graft-take started soon and 15 days after grafting average percentages scored for each genotype (38.3% for black thorn 'PS123', 50.0% for apricot 'Alb 3' and 36.7% for apricot 'Caldesi') did not differ significantly. Regarding the heterografts, the percentages were 25 and 50% for 'Alb 3'/'PS123' and 'Caldesi'/'PS123', respectively.

Xylematic continuity

Specimens stained with toluidine blue produced polyphenols and the lignified walls of tracheary elements typically stained blue in colour (Fig. 5).

Fifteen days after graft establishment, the differentiation of new xylematic tissue leading to unification of

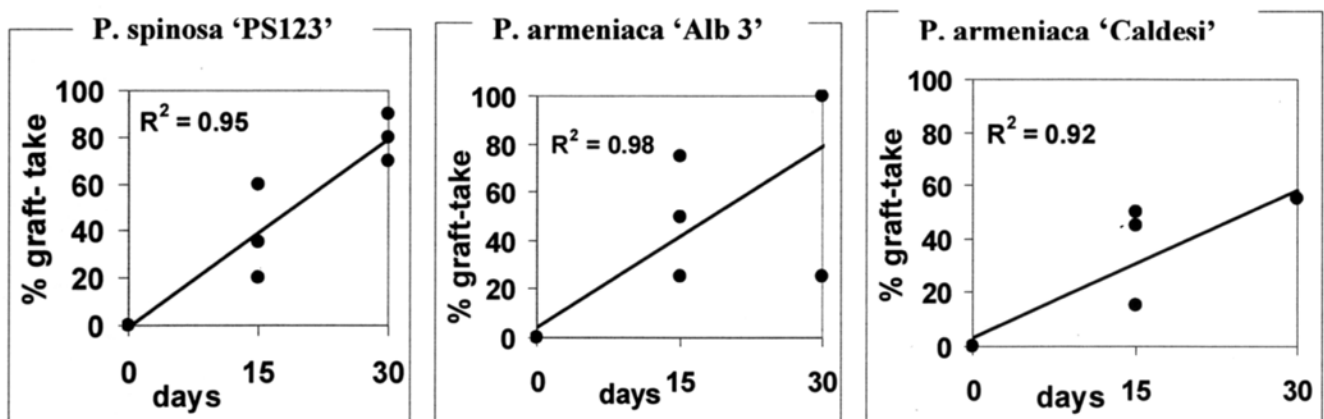


Fig. 3 - Trend lines and coefficients of determination ($P < 0.001$) on time course of percentage of autografts positive to manual test for graft adhesion (graft-take). Each percentage was calculated on 20 samples and each experiment was repeated three times.

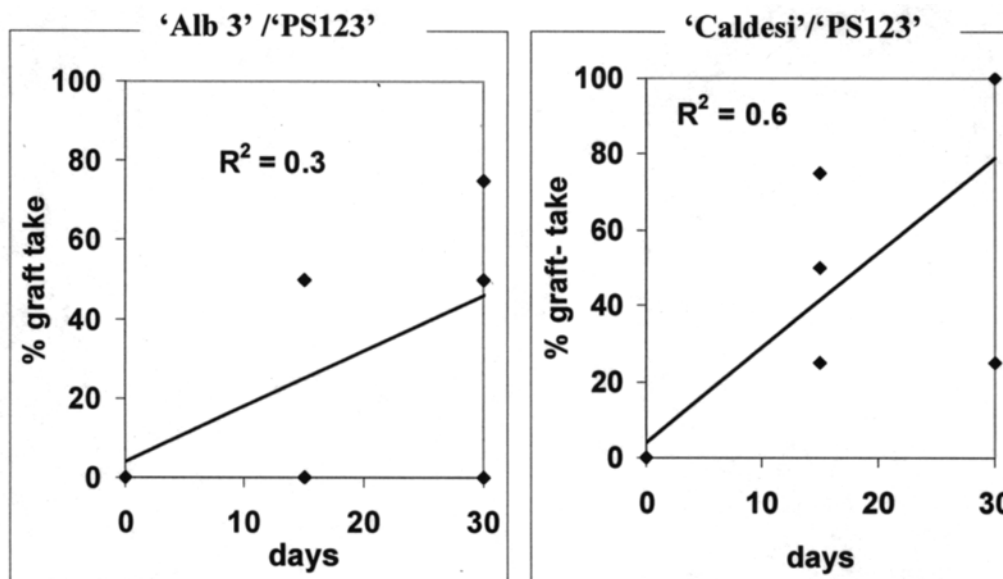


Fig. 4 - Trend lines and coefficients of determination ($P < 0.001$) on time course of percentage of heterografts positive to manual test for graft adhesion (graft-take). Each percentage was calculated on eight samples and each experiment was repeated three times.

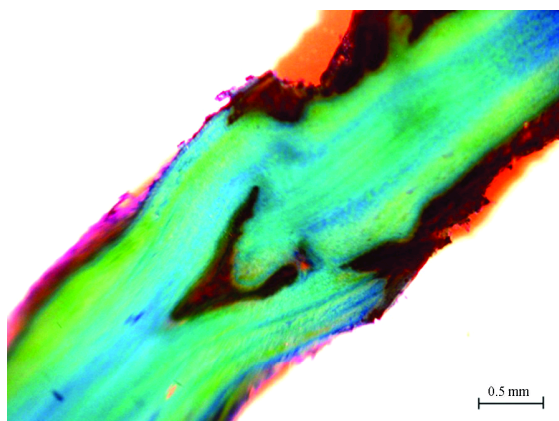


Fig. 5 - Xylematic connections in *P. armeniaca* 'Alb 3' autograft. Longitudinal section of the union area, stained with toluidine blue, showing the lignified walls of tracheary elements typically blue in colour 15 days after graft establishment.

the vascular elements of the stock and scion was observed in all the genotypes (Fig. 6). After 45 days, average percentages of autografts showing xylematic restoration, 60.0% for black thorn 'PS123', 40.0% for apricot 'Alb 3' and 43.3% for apricot 'Caldesi', did not show significant differences. Data from heterografts (not shown) indicated that xylematic continuity was not restored in any of the considered combinations.

Phloem transport

CF is a fluorescent tracer strictly confined to the phloem and consequently the restoration of transport across the graft interface can be monitored by its distribution. The time course of phloem regeneration is

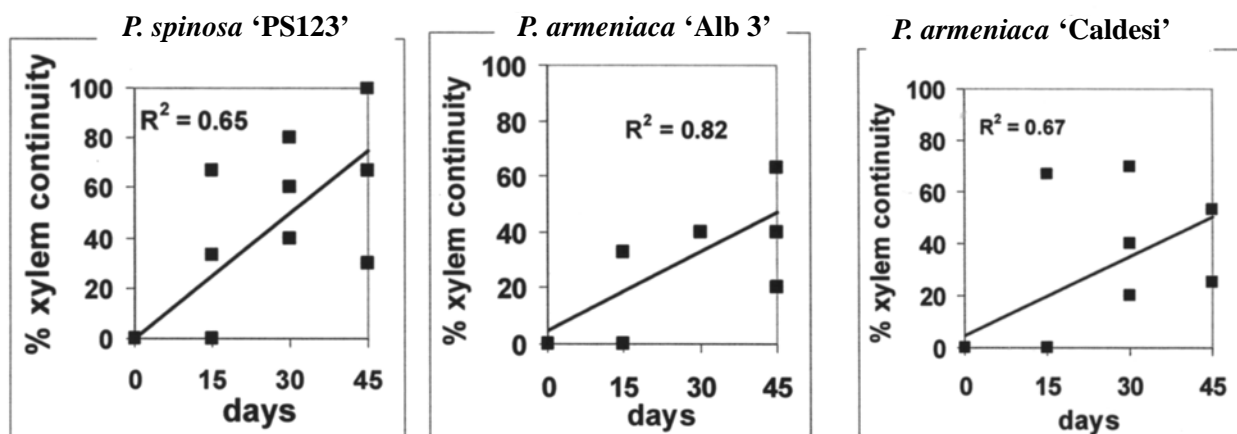


Fig. 6 - Trend lines and coefficient of determination ($P < 0.001$) on time course of percentage of autografts showing xylematic continuity establishment, as determined by fast toluidine blue staining procedure. Each percentage was calculated on 5-10 samples and each experiment was repeated three times.

shown in figure 7 where the percentages of autografted samples having both complete and partial phloematic transport are related to time of culture. Similar to xylematic continuity, it is possible to see 15 days after grafting that all the genotypes had initiated the phloem restoration process and, interestingly, in ‘Alb 3’ autografts only complete phloematic transport was detected. In Table 1, average percentages of autografts showing any type of phloematic transport, complete or partial, are reported. Genotype had a significant influence particularly on speed of vascular connection recovery. In fact, after 30 days black thorn ‘PS123’ scored the highest percentage, 73%, of grafts positive to FC and this value is very close to its maximum, 77.8%, scored after 45 days; ‘Caldesi’ had a similar trend but with lower absolute percentages, 50% after 30 days and 55.6% after 45 days. ‘Alb 3’ was the slowest genotype to recover, as after 30 days the percentage of autografts showing phloematic transport was 20% while the maximum percentage, 55.6%, was reached only after 45 days. Significant correlations between xylematic continuity and phloematic transport were found by the Pearson’s two-tailed test, regardless of genotype (Fig. 8). Results from heterograft combinations showed that phloematic transport was not restored (data not shown).

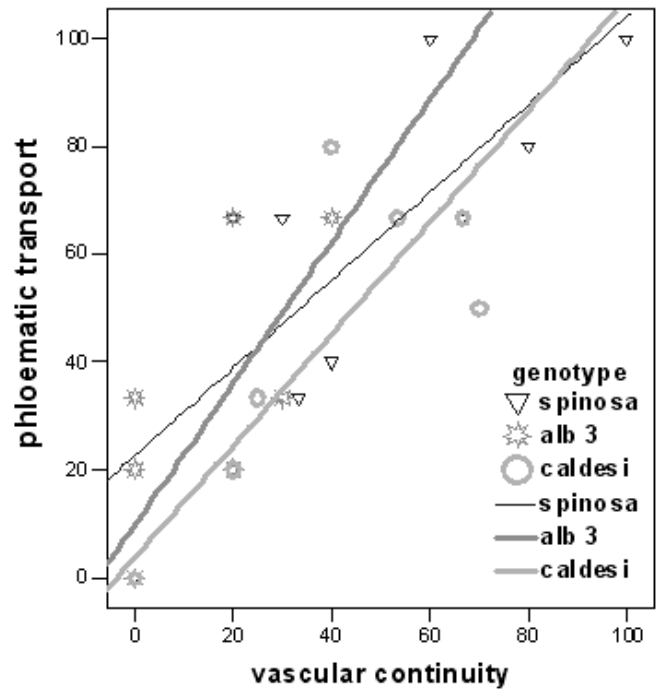


Fig. 8 - Correlation between percentage of autografts showing vascular continuity (toluidine) and restoration of phloematic transport (fluorescein) as determined by the Pearson’s two-tailed test at 0.05 and 0.01 level. $R^2 = 0.7$ (*P. spinosa*), $r^{**} = 0.8$ (‘Alb 3’), $r^{**} = 0.9$ (‘Caldesi’).

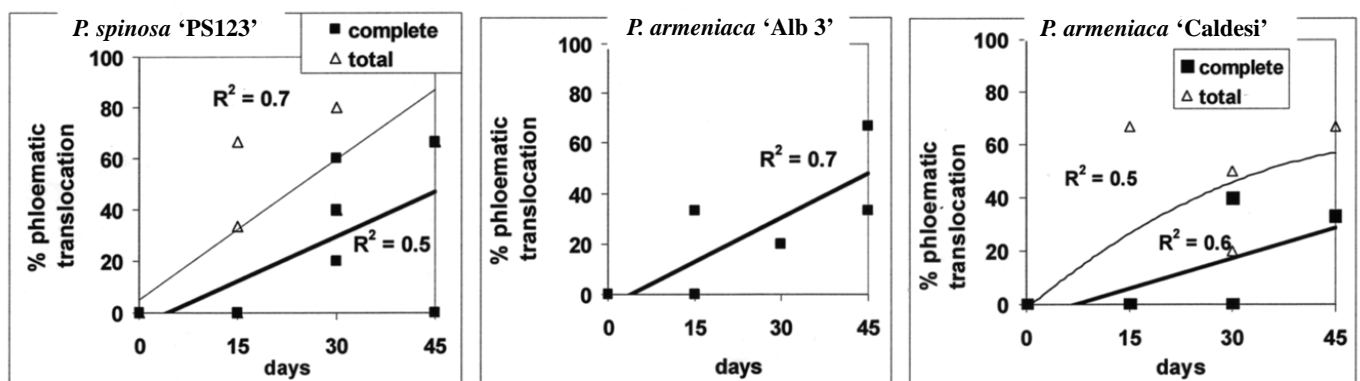


Fig. 7 - Trend lines and coefficients of determination ($P < 0.001$) on time course of percentage of autografts showing stock sections with completely restored phloem transport and stock sections showing any type of phloem transport. *P. armeniaca* ‘Alb 3’ restored only complete phloem transport. Each percentage was calculated on 5-10 samples and each experiment was repeated three times.

Table 1 - Percentages of autografts positive to fluorescein at each time point

Days	<i>P. spinosa</i>		<i>P. armeniaca</i>	
	‘PS 123’ (%)		‘Alb 3’ (%)	‘Caldesi’ (%)
15	33.3 a		11.1 a	22.2 a
30	73.3 a		20.0 b	50.0 ab
45	77.8 a		55.6 a	55.6 a

In each row, means with the same letters are not different according to the χ^2 test; $P = 0.05$.

Rooting

Rooting ability of autografts was dramatically low when compared to the stock proliferating shoots (Fig. 9). In fact 70% rooting was obtained by shoots basal dipping in 5 mM IBA, while the same treatment failed on autograft, which reached the maximum value, 20%, when IBA concentration increased to 7.5 mM. Nevertheless, 50% rooting ability was recovered by transferring stock autografts to a medium containing 2.5 μ M BA for 30 days prior to rooting treatment (data not shown).

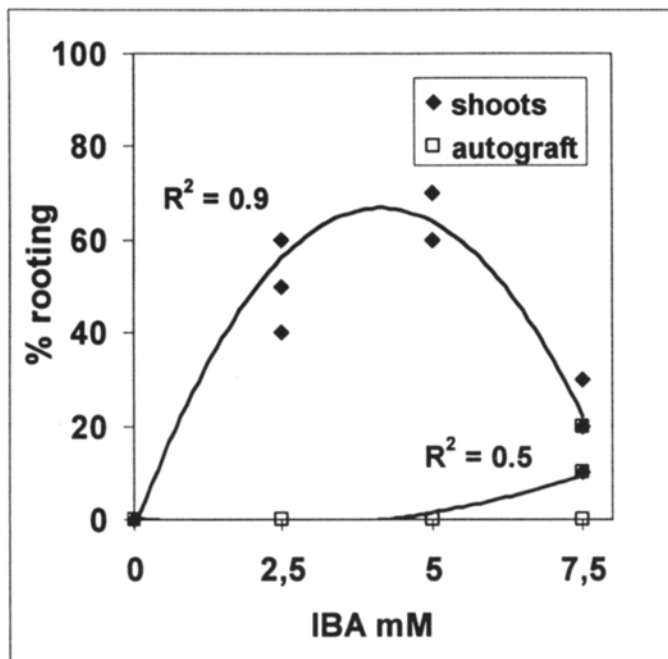


Fig. 9 - Effect of indole-3-butyric acid (IBA) on rooting ability of *P. spinosa* 'PS123' shoots and autografts. Each percentage was calculated on 10 samples and each experiment was repeated three times. $P=0.00$.

4. Discussion and Conclusions

The delayed manifestation of graft incompatibility symptoms is one of the main obstacles to rootstock selection programmes for *Prunus* spp. and the use of tools for early detection of compatibility is critical for both plant growers and breeders. Early testing of graft compatibility is particularly urgent for apricot culture in order to find reliable rootstocks with good adaptive traits for specific environments (soils) to overcome production irregularities.

For all the genotypes, within 15 days of graft establishment, both xylematic continuity and phloematic transport provided useful evidences of graft affinity. The use of fast toluidine blue staining procedure combined with application of 5(6)-carboxyfluorescein (CF) can give, in a relatively short time, information on the *in vitro* graft status of both vascularization and phloem transport restoration.

Early assessment of vascularization and phloem transport is important in evaluating rootstocks compatibility because histological investigations have revealed the presence of poor vascular connections, phloem degeneration and vascular discontinuity in incompatible grafts (Errea *et al.*, 2001; Errea and Borruey, 2004).

CF was successfully used *in vitro* to test internode vascular reconnection in both compatible and incompatible pear and quince combinations (Espen *et al.*, 2005). Under our conditions, considering the high correlation found between new xylem and phloem differentiation at the graft point, either the epifluorescence

or the toluidine test alone could be used as probe for the success of the graft.

Graft-take data did not match with the other data related to scion-stock compatibility; in fact heterograft-take percentages were high but did not reflect at all xylematic continuity and phloematic transport, which were never established in any of the tested scion-stock combinations. This is not surprising as, working on *in vivo* grafts, Errea *et al.* (1994) reported that the initial adhesion occurs in both compatible and non compatible *P. armeniaca* and *P. cerasifera* combinations. Moreover, the peculiar conditions of tissue culture could contribute to speed up the initial coalescence compared to *in vivo* grafts (Wang and Kollmann, 1996). Incompatibility between *P. armeniaca* 'Alb 3' and 'Caldesi' and the stock *P. spinosa* 'PS123' was evidenced by the complete failure of the transport functions through the eterografts, as ascertained in the course of this research.

Rooting efficiency is a key step of *in vitro* grafting technique and growth regulators play an important role. Under our conditions, BA stimulated 'PS123' autografts rooting but its addition to rooting medium had to be postponed just prior to the rooting induction phase to avoid residual lateral bud sprouting from the rootstock. On the other hand, using rooted shoots as rootstock resulted in unsuccessful grafts. This could be due to active cytokinin production by adventitious roots apex that may unbalance auxin, limiting its role in xylem formation; the large majority of genes involved as an integral part of tracheary differentiation process are, in fact, induced by auxin (Pesquet *et al.*, 2005).

The present research has shown that *in vitro*-grafting can be a powerful, time-saving method for early screening of scion-rootstock compatibility in *Prunus* species.

Acknowledgements

Authors wish to thank Gianni Sacchi and his staff for providing lab facilities to monitor phloem transport.

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