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Changes in Cell/Tissue Organization and Peroxidase Activity as Markers for Early Detection of Graft Incompatibility in Peach/Plum Combinations

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Abstract. Changes in cell and tissue organization and in peroxidase activity have been analyzed to find early markers to predict graft incompatibility occurrence in peach/plum combinations (*Prunus persica*/*Prunus* spp.) at 5 months after grafting in the dormancy period. Different compatible and incompatible peach/plum grafts were grown for 5 months in a nursery. The cellular study of the graft interface revealed structural changes associated with graft incompatibility symptoms. The main structural features were cambium cell disorganization, less differentiation of vascular tissues, degeneration of phloem and xylem cells, and accumulation of phenols at the graft interface after 5 months of graft development. The peroxidase study was performed during dormancy and the vegetative growth period, and revealed a significant increase in peroxidase activity in the incompatible unions, with significant differences between compatible and incompatible grafts. Analysis of gel profiles of nonbudded rootstocks and scions revealed an anodal isoperoxidase band [relative front (Rf) = 0.48] present in scions and compatible rootstocks, and another isoperoxidase band (Rf = 0.53) only present in the incompatible rootstocks. Our results show that the analysis of cell organization to detect early structural events and the evaluation of peroxidase activity at graft unions constituted feasible and convenient methods for early diagnosis of graft incompatibility. Also, it was suggested that the presence of band Rf = 0.48 in plum rootstocks and peach cultivars could be used as a marker to predict graft compatibility for peach scions and plum rootstocks.

In the Mediterranean area, there is a widespread use of peach × almond hybrids (*Prunus persica* × *Prunus dulcis*) as peach rootstocks because they are tolerant and/or resistant to variety of biotic and abiotic stresses (Byrne et al., 1990; Felipe, 2009; Kester and Asay, 1986; Pinochet, 2009) and are generally graft-compatible with peach cultivars (Cambra, 1990; Moreno and Cambra, 1994; Moreno et al., 1994b). However, these rootstocks are known to be extremely vigorous (Wertheim and Webster, 2005; Zarrouk et al., 2005) and relatively sensitive to waterlogging (Okie and Weinberger, 1996). Because control of tree vigor is becoming a matter of increasing importance (Wertheim and Webster, 2005), and peach production is expanding to heavy soils, the use of plum rootstocks is a feasible solution (Beckman and Lang, 2003). Plum rootstocks are generally less vigorous (Moreno et al., 1995), and are more tolerant to waterlogging (Rowe and Beardsell, 1973) and root knot nematodes [*Meloidogyne* spp. (Pinochet et al., 1999)]. They are also tolerant to calcareous soils (Gogorcena et al., 2004; Jiménez et al., 2008), and they provide the possibility to overcome replanting problems (Nicotra and Moser, 1997). However, many peach cultivars exhibit graft incompatibility when grafted onto some plum rootstocks (Moreno et al., 1994a; Zarrouk et al., 2006). Manifestations of peach/plum incompatibility appear generally during the first summer after grafting, although symptoms can also be delayed for several years (Moreno et al., 1993), and are typically evident by translocated incompatibility symptoms (Zarrouk et al., 2006). They are expressed by tree growth cessation and premature tree defoliation (Herrero, 1951) caused by a phloem dysfunction (Moing and Carde, 1988; Schmid and Feucht, 1981) and biochemical alterations at the graft interface (Moing et al., 1987). Otherwise, some peach/plum combinations can exhibit symptoms of localized incompatibility after several years without any other symptoms. These localized symptoms are characterized by anatomical irregularities at the union interface (Zarrouk et al., 2006) and with breaks in cambial and vascular continuity (Mosse, 1962). The abnormal functioning of the newly formed cambium causes an invagination of the cambial zone, a differentiation of parenchymatous tissue in the place of xylem (Deloire and Hébant, 1983; Gur et al., 1968), and a lack

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of lignification of cells interlocked at the graft union (Hartman et al., 2002). The peroxidase enzyme is associated with differentiating xylem (Sterjiades et al., 1993), as well as with lignification processes (Harkin and Obst, 1973). In addition, this enzyme was reported to be implicated in process related to graft establishment (Deloire and Hébant, 1982; Feucht et al., 1983; Schmid and Feucht, 1985). Higher peroxidase activity in grafted rootstocks was related to graft incompatibility in *Prunus* species (Rodrigues et al., 2002), and it has been suggested that differences in peroxidase concentration between rootstock and scion may cause bad vascular connections (Santamour, 1992). Graft incompatibility of peach rootstocks is an important characteristic to be assessed in breeding programs. Nevertheless, traditional evaluation is time consuming because appearance of physiological symptoms or anatomical anomalies may take 1 to several years (Moreno et al., 1993). Many studies were carried out to control graft incompatibility in peach/plum combinations (Breen, 1975; Moing and Carde, 1988; Moing and Gaudillère, 1992; Moing et al., 1987; Moreno et al., 1994a). However, few studies describe a method to detect early graft problems. Ermel et al. (1999) identified 13 variables that potentially discriminate compatible from incompatible grafts by a histological study on 5-month-old pear/quince combinations (*Pyrus communis*/*Cydonia oblonga*). On the other hand, the peroxidase isozyme analysis of scion and rootstock to predict incompatibility before grafting was reported in chinese chestnut [*Castanea mollissima* (Santamour, 1988a)], red oak [*Quercus rubra* (Santamour, 1988b)], and pear (Gulen et al., 2002). When the peroxidase isozyme pattern of the scion matches with that of the rootstock, a graft resulted in a compatible union and in the restoration of vascular connection. In contrast, when it was different, callus formation was impaired and graft incompatibility occurred (Santamour, 1988c). Moreover, other studies with herbaceous species such as tomato (*Solanum lycopersicum*) show that peroxidase activity is implicated in the grafting process and that the enzyme is located mainly in the graft region (Fernández-García et al., 2004).

The main objective of this work was to assess in peach/plum combinations grafted with traditional methods the use of histological examination and to evaluate peroxidase activity and isozyme profiles in wood during dormancy, as early indicators associated with graft incompatibility.

Materials and Methods

Plant material for histological studies. Two plum rootstocks showing different graft compatibility performance with the exigent nectarine (*P. persica*) cultivar Summergrand were used: 1) one clone of 'Pollizo de Murcia' under selection: 'PM 105 AD' (*Prunus insititia*), compatible with 'Summergrand' and 2) 'Myrobalan GF 3–1' (*Prunus cerasifera*), incompatible with 'Summergrand'. Hardwood cuttings of the two plum rootstocks were propagated in a seed plot. The following winter (2003–04), rooted cuttings were transferred and established in a nursery. Budwood of the cultivar Summergrand and rootstocks were collected from the *Prunus* collection, maintained at the Aula Dei Experimental Station (Zaragoza, Spain), and were T-grafted in situ on the two rootstocks on 19 Sept. 2004. Three types of combinations were carried out: compatible homografts ('Myrobalan GF 3–1'/'Myrobalan GF 3–1' and 'PM 105 AD'/'PM 105 AD'), a compatible heterograft ('Summergrand'/'PM 105 AD'), and an incompatible heterograft ('Summergrand'/'Myrobalan GF 3–1'). Grafts were collected on 17 Feb. 2005, 157 d after grafting (about 5 months later).

Histological examination. Three graft unions per combination were collected and each stem piece (3–5 mm in diameter) was cut transversally into four slices numbered from the upper part to the bottom (1, 2, 3, and 4). As a result, 12 slices per combination were obtained. Slices were fixed in 2.5% glutaraldehyde, containing 0.1% caffeine in 0.1 M phosphate buffer (pH 7.4), for 4 h at 4 °C, dehydrated in a graded ethanol series, and embedded in glycolmethacrylate resin Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) according to Ermel et al. (1999). For the light microscopy study, transverse semithin sections (2 μm) were obtained with a Leica RM2135 microtome (Leica Instruments, Nussloch, Germany) equipped with a tungsten carbide knife. Sections were stained with 0.05% Toluidine Blue O (O'Brien et al., 1965). Sections were observed under a bright field in a light microscope (Ernst Leitz, Wetzlar, Germany) equipped with a digital camera (DP10; Olympus Europe, Hamburg, Germany).

Plant material and methods for peroxidase activity. Two grafted combinations were used: 'Summergrand' grafted onto peach-based rootstocks and 'Summergrand' grafted onto plum-based rootstocks (Table 1). 'Summergrand' grafted on 'GF 677' was used as standard of compatibility for peroxidase activity studies. The hardwood cuttings of rootstocks were grown in a seed plot during the winter. Rooted cuttings of rootstocks were established in a nursery the following winter and were T-grafted in situ with budwood of 'Summergrand' collected from the *Prunus* collection orchard in September. For each sample, graft union portion consisting on bark and cambium (2 g) excised from the union zone of 4- to 5-month-old trees (January–February: dormant trees) and from 7- to 8-month-old trees (April–May: first-growing vegetative station) were ground using a mortar and a pestle in liquid nitrogen. Ten milliliters of extraction buffer (0.05 M Tris citrate, pH 8.0, 1 $g \cdot L^{-1}$ citric acid, 1 $g \cdot L^{-1}$ ascorbic acid, 10 $g \cdot L^{-1}$ polyethylene glycol (PEG) 4000, and 0.8% 2-mercaptoethanol) plus 1 g of polyvinylpolypyrrolidine (PVPP; Sigma-Aldrich, St. Louis) were then added to the frozen powder. The homogenization was made on ice at 4 °C. Samples were then centrifuged at 6000 g_n for 20 min at 4 °C as described by Arulsekar and Parfitt (1986). The clear supernatant representing the enzyme extract was recovered.

Peroxidase activity was determined spectrophotometrically by following the increase in absorbance at 470 nm over 10 min in a spectrophotometer ultraviolet-2101PC (Shimadzu Scientific Instruments, Columbia, MD). The reaction mixture contained 50 mM potassium phosphate, pH 6.8, 10 mM hydrogen peroxide, 9 mM guaiacol, and 20 μL of enzyme extract in a total volume of 1 mL, as described by Fernández-García et al. (2004). The mean value of at least three replications was taken as indicator of peroxidase activity.

Determination of isoperoxidase profile by native polyacrylamide gel electrophoresis (PAGE). Bark and cambium tissues were excised from current-year shoot growth of 'Catherina' and 'Summergrand' scions, as well as non-budded *Prunus* rootstocks (Table 2), from 15-year-old trees established in the *Prunus* collection orchard of Aula Dei Experimental Station. Samples from three different plants were collected following the protocol described by Gulen et al. (2002). Other triplicate samples were also taken from the graft

Table 1. Rootstocks grafted with 'Summergrand' nectarine for the peroxidase activity study.

Rootstocks	*Prunus* species	Origin[z]	Compatibility[y]
Peach-based rootstocks			
'Barrier'	*P. persica* × *P. davidiana*	CNR, Italy	Compatible
'Cadaman® Avimag'	*P. persica* × *P. davidiana*	INRA, France	Compatible
'GF 677'	*P. dulcis* × *P. persica*	INRA, France	Compatible
Plum-based rootstocks			
'Adesoto 101'	*P. insititia*	CSIC, Spain	Compatible
'PM 105 AD'	*P. insititia*	CSIC, Spain	Compatible
'Ishtara® Ferciana'	(*P. cerasifera* × *P. salicina*) × (*P. domestica* × *P. persica*)	INRA, France	Compatible
'Damas GF 1869'	*P. domestica* × *P. spinosa*	INRA, France	Incompatible
'Krymsk-1'	*P. tomentosa* × *P. cerasifera*	KEBS, Russia	Incompatible
'Marianna 2624'	*P. cerasifera* × *P. munsoniana*	UC, USA	Incompatible
'Marianna GF 8–1'	*P. cerasifera* × *P. munsoniana*	INRA, France	Incompatible
'Myrobalan GF 3–1'	*P. cerasifera* × *P. salicina*	INRA, France	Incompatible

[z]CNR = Centro Nacionale della Recerca, CSIC = Consejo Superior de Investigaciones Científicas, INRA = Institut National de la Recherche Agronomique, KEBS = Krymsk Experimental Breeding Station, UC = University of California.
[y]Compatibility performance of rootstocks with 'Summergrand' nectarine.

Table 2. Cultivars and rootstocks used for the isoperoxidase profile study of bark tissues of non-budded plants.

Rootstocks	*Prunus* species	Origin[z]	Compatibility[y]
Peach and nectarine cultivars			
'Catherina'	*P. persica*	NJAES, USA	—
'Summergrand'	*P. persica*	FA-RN, USA	—
Peach-based rootstocks			
'Barrier'	*P. persica* × *P. davidiana*	CNR, Italy	Compatible
'Cadaman® Avimag'	*P. persica* × *P. davidiana*	INRA, France	Compatible
'GF 677'	*P. dulcis* × *P. persica*	INRA, France	Compatible
Plum-based rootstocks			
'Adesoto 101'	*P. insititia*	CSIC, Spain	Compatible
'PM 105 AD'	*P. insititia*	CSIC, Spain	Compatible
'Hiawatha'	*P. besseyi* × *P. salicina*	USDA, USA	Compatible
'Ishtara® Ferciana'	(*P. cerasifera* × *P. salicina*) × (*P. domestica* × *P. persica*)	INRA, France	Compatible
'Jaspi® Fereley'	(*P. salicina* × *P. cerasifera*) × *P. spinosa*	INRA, France	Compatible
'Marianna 2624'	*P. cerasifera* × *P. munsoniana*	UC, USA	Incompatible
'Marianna GF 8–1'	*P. cerasifera* × *P. munsoniana*	INRA, France	Incompatible
'Myrobalan B'	*P. cerasifera*	EM, UK	Incompatible
'Myrobalan 29 C'	*P. cerasifera*	GB, USA	Incompatible
'Myrobalan GF 3–1'	*P. cerasifera* × *P. salicina*	INRA, France	Incompatible

[z]CNR = Centro Nacionale della Recerca, CSIC = Consejo Superior de Investigaciones Científicas, EM = East Malling Research Station, FA-RN = F. Anderson, Merced, and Reedley Nursery (California), GB = Gregory Brother's (California), INRA = Institut National de la Recherche Agronomique, KEBS = Krymsk Experimental Breeding Station, NJAES = New Jersey Agricultural Experiment Station, UC = University of California, USDA = U.S. Department of Agriculture.
[y]Compatibility performance of rootstocks with 'Summergrand' nectarine.

unions of budded plants at approximately 5 months after grafting (Table 2). Protein extract was prepared as described for peroxidase activity. Isozyme separation analysis was carried out by electrophoresis in polyacrylamide gels. Gels were 0.75 mm thick with three layers: the spacer gel (4% of acrylamide), the upper resolving gel (6.3% of acrylamide), and the lower resolving gel (10% of acrylamide). Gel buffers for three layers and the tray buffer used were as described by Royo et al. (1997). Electrophoretic separation was carried out at 2 mA per well for 3 to 4 h at 4 °C.

Gels were stained for peroxidase by 3-amino-9-ethyl carbazol solution (10%, w/v) according to Arulsekar and Parfitt (1986). Each band was identified by its relative front using Rf = 1.0, as the distance to the fastest band, and Rf = 0.0, as the starting point of the resolving gel, as it was described by Manganaris and Alston (1992). The Rf of bands was derived from mean values from at least three independent runs. Gels were scanned and photographs were taken, but they seldom showed all visible bands. Thus, zymograms were drawn from average Rf and intensity of bands. Bands that were not visible in the three runs were assumed as not detectable and are not included in this study.

Statistical analysis. Data were evaluated by analysis of variance with SPSS (version 13.0; SPSS, Chicago). The analysis of variance (ANOVA) was made at $P \leq 0.05$ and $P \leq 0.001$, and was used to assess the significance of

Table 3. Histological variables studied to assess changes in cell/tissue organization of the graft interface of 5-month-old grafts in plum/plum-compatible homografts and compatible and incompatible heterografts of peach/plum. Existence or absence of the variable is indicated by + or –, respectively.

	Compatible homografts		Compatible heterografts	Incompatible heterografts
	'PM 105 AD' / 'PM 105 AD'	'Myrobalan GF 3–1'/ 'Myrobalan GF 3–1'	'Summergrand'/ 'PM 105 AD'	'Summergrand'/ 'Myrobalan GF 3–1'
Interface				
Interface visible	+	+	+	+
Interface continuity	+	+	+	+
Presence of lacuna	–	+	–	–
Cambium				
Differentiated cambium[z]	+	+	+	+/–
Presence of overstaining cells	–	–	–	+
Vascular tissue				
Vascular connection continuity	+	+	+	–
Xylem and phloem tissue degeneration	–	–	–	+
Similarity[y] (%)	—	91%	100%	37%

[z]Cb* in Figs. 1 and 2.
[y]Percentage of similarity of traits was calculated using the homograft 'PM 105 AD'/'PM 105 AD' as a reference.

peroxidase activity and peroxidase isozyme profile. Isozyme band position was assessed by Carnoy (version 2.1; Laboratory of Plant Systematics, Katholieke Universiteit Leuven, Leuven, Belgium). Mean separation was effectuated by Duncan's test and results shown are mean values ± SE.

Results

Cell and tissue structural organization at the graft union. The rate of bud-take differs from homografts ('Myrobalan GF 3–1'/'Myrobalan GF 3–1' and 'PM 105 AD'/'PM 105 AD') to heterografts (compatible 'Summergrand'/'PM 105 AD' and incompatible 'Summergrand'/'Myrobalan GF 3–1') and oscillates from 70% to 100%. Graft interface was continuous in all combinations, indicating good graft establishment in the three types of grafts (Table 3). Lacuna was observed in the interface of the homograft 'Myrobalan GF 3–1', corresponding to the delay in callus bridge formation. Changes related to incompatibility between different types of combinations were mainly expressed in cambial and vascular tissues. The major differences found in the histological study at the interface level, related to cambium and vascular organization, are summarized in Table 3. Because the percentage of similarity of all traits investigated was high (90%–100%) between homografts and compatible heterografts, only data corresponding to heterografts are presented in Figs. 1 and 2.

The interface was identified by a line delimiting scion from rootstock tissues, which was strongly stained with Toluidine Blue O or by a layered zone between the two components, which stick the grafted tissues together. The over-stained line was slightly apparent and discontinuous in the compatible combination (arrowheads in Fig. 1, A and B). However, for incompatible combinations, the line was emergent and continuous (arrowheads in Fig. 2A, arrow in Fig. 2D). In the central part of the graft, the two partners cohered by a multiplication of callus cells (Figs. 1A and 2A).

Compatible combinations showed organized and homogenous arrangement of cambium cells in the contact interface (Fig. 1, A and B). In contrast, cambium cells in the incompatible interface showed a disorganized arrangement. Islands of

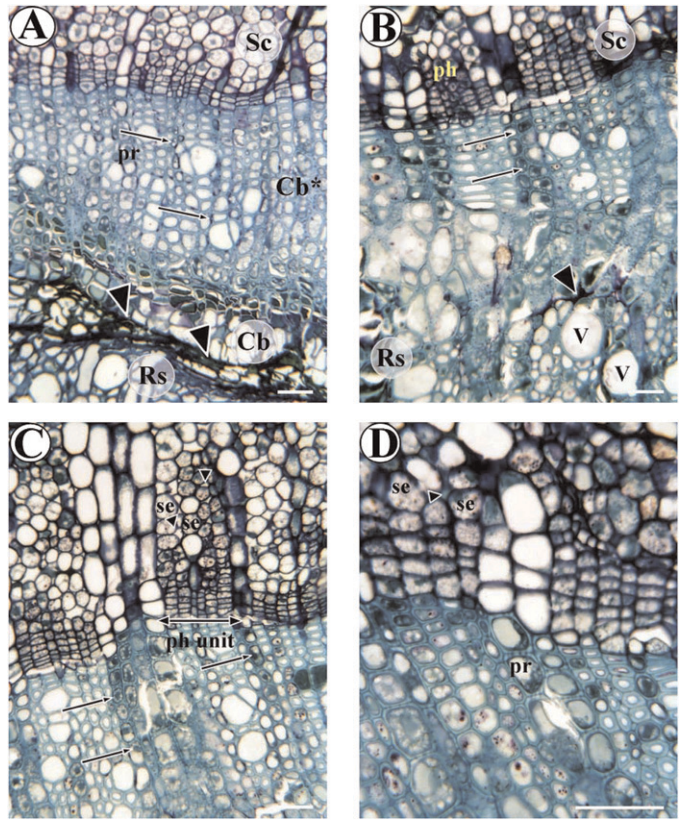

Fig. 1. Graft interface structure of peach/plum ('Summergrand'/'PM 105 AD')-compatible combination in transversal sections of grafts. (**A**) Compatible overstaining line delimited stock from scion (arrowheads). Proliferation and differentiation in new vascular cells of cambium between the two grafted components. Arrows indicate tracheary elements. (**B**) Reabsorption of overstaining line (arrowhead). Differentiation of wood rays in graft interface (arrows). (**C**) Complete differentiation of scion phloem delimited by adjacent rays (ph unit). Wood rays differentiation (arrows). (**D**) Details of the scion phloem structure; Cb = cambium, Cb* = cambium differentiated into vascular tissues, ph = phloem, pr = wood rays (= parenchymatous rays = xylem rays), Rs = rootstock, Sc = scion, se = sieve elements, V = vessels; bar = 20 µm.

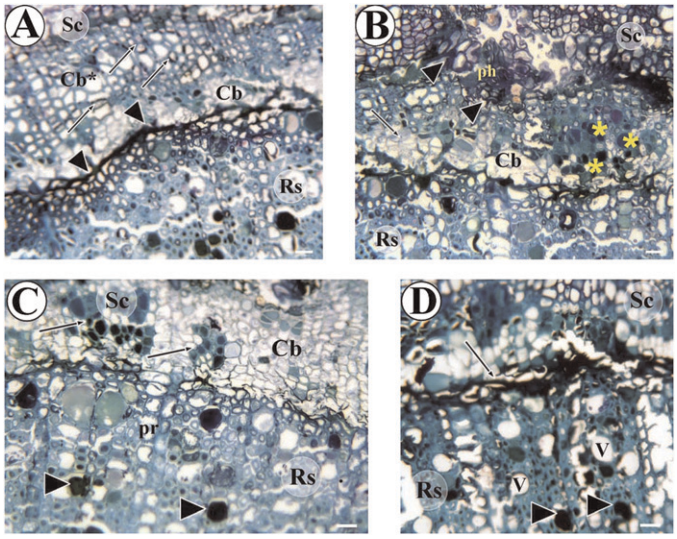

Fig. 2. Transversal sections of graft interface structure of peach/plum ('Summergrand'/'Myrobalan GF 3–1')-incompatible combination. (**A**) Incompatible continuous overstaining line delimited stock from scion (arrowheads). Proliferation and differentiation in new vascular cells of cambium between the two grafted components. Cb* indicates that cambium is being differentiated into vascular cells. Arrows indicate the presence of tracheary elements. (**B**) Cell cambium disorganization (arrow). Cambium cell vacuoles stained in blue-green color showing the presence of phenolic compounds (asterisks). Phloem disorganization and degeneration (arrowheads). (**C**) Cambium before differentiation into vascular tissues. Phenolic compound accumulation in the cambial region (arrows). Wood ray proliferation (pr). Tylosis reaction (arrowheads). (**D**) Vast degeneration of vessels by tylosis reaction (arrowheads). Overstaining line persisting between two components (arrow); Cb = cambium, Cb* = cambium differentiated into vascular tissues, ph = phloem, pr = wood rays (= parenchymatous rays = xylem rays), Rs = rootstock, Sc = scion, V = vessels; bar = 9 μm.

dark-staining phenolic compounds in vacuoles of cambium cells were regularly observed at the graft interface (asterisk in Fig. 2B, arrows in Fig. 2C). Invagination of the cambium was slightly observed in incompatible grafts (data not shown). In some areas of incompatible grafts, a lesser amount of new differentiated vascular cells was noted compared with compatible interfaces (Cb* in Figs. 1A and 2A, Cb in Fig. 2C). Sieve elements were easily identified in compatible grafts and showed a good vascular connection establishment and proper phloem ray arrangement and structure (phloem unit in Fig. 1C, "se" and arrowhead in Fig. 1D). In apart, in the incompatible grafts, we observed phloem structural disorders (arrowheads in Fig. 2B) and discontinuities (see absence of vascular connection continuity in Table 3).

Wood vascular connections were observed in both combinations (Table 3), but they were more evident in compatible combinations with the presence of tracheae elements in new vascular cells (arrows in Figs. 1A and 2A). Note the degeneration of vessels in xylem in the incompatible combinations, indicating a tylosis reaction (arrowheads in Fig. 2, C and D). At this stage of graft development, it was difficult to identify the cells that emerged from the scion and those emerging from the stock, for both graft combinations. Only when it was present did the over staining line allow us to determine the graft interface.

Peroxidase activity. As shown in Table 4, incompatible combinations of 'Summergrand' grafted on 'Damas GF 1869', 'Krymsk-1', 'Marianna 2624', 'Marianna GF 8–1', and 'Myrobalan GF 3–1' rootstocks exhibited higher peroxidase activities during dormancy and vegetative growth periods when compared with compatible grafts. Peroxidase activity of incompatible combinations significantly differs from compatible combinations ($P \leq 0.05$). Graft combinations that first showed some symptoms of translocated incompatibility at the beginning of the summer exhibited the highest peroxidase activity at dormancy and over the vegetative period (Table 4). This was the case for the combination 'Summergrand'/'Damas GF 1869' that maintained the highest peroxidase activity, with such a tendency being maintained during the vegetative period. With compatible and incompatible combinations, the rate of peroxidase activity during dormancy was also higher than during the vegetative growth season (Table 4).

Peroxidase isozyme profile. Profiles of isoperoxidase of nonbudded scions and rootstocks revealed some differences among *Prunus* rootstocks (Fig. 3). Conversely, the isoperoxidase profiles of peach and nectarine scions used in this study

Table 4. Peroxidase activity in dormancy and vegetative periods of graft union of 'Summergrand' nectarine grafted on different *Prunus* rootstocks.

Rootstock	Peroxidase activity [mean ± SE (μmol·min^{-1}·g^{-1} FW)]		Compatibility[z]	P[y]
	Dormancy period	Vegetative period		
Peach-based rootstocks				
'Barrier'	17.7 ± 0.3 a[x]	4.3 ± 0.6 a	Compatible	***
'Cadaman® Avimag'	18.6 ± 0.1 a	4.4 ± 0.1 a	Compatible	***
'GF 677'	18.5 ± 0.1 a	4.7 ± 0.1 a	Compatible	***
Plum-based rootstocks				
'Adesoto 101'	18.6 ± 0.1 a	4.7 ± 0.4 a	Compatible	***
'PM 105 AD'	18.0 ± 0.3 a	3.9 ± 0.1 a	Compatible	***
'Ishtara® Ferciana'	18.3 ± 0.4 a	4.4 ± 0.3 a	Compatible	***
'Damas GF 1869'	25.0 ± 0.5 c	12.6 ± 0.3 d	Incompatible	***
'Krymsk-1'	24.4 ± 0.3 bc	7.4 ± 0.1 c	Incompatible	***
'Marianna 2624'	24.1 ± 0.3 bc	6.4 ± 0.3 b	Incompatible	***
'Marianna GF 8–1'	23.6 ± 0.5 b	6.6 ± 0.1 bc	Incompatible	***
'Myrobalan GF 3–1'	23.8 ± 0.1 b	7.1 ± 0.5 bc	Incompatible	***

[z]Compatibility performance of rootstocks with 'Summergrand' nectarine.
[y]Mean separation between the two treatment periods was performed by t test at $P \leq 0.05$ (n = 3); *** indicates significance at $P \leq 0.001$.
[x]Mean separation within columns by Duncan's multiple range tests at $P \leq 0.05$ (n = 3).

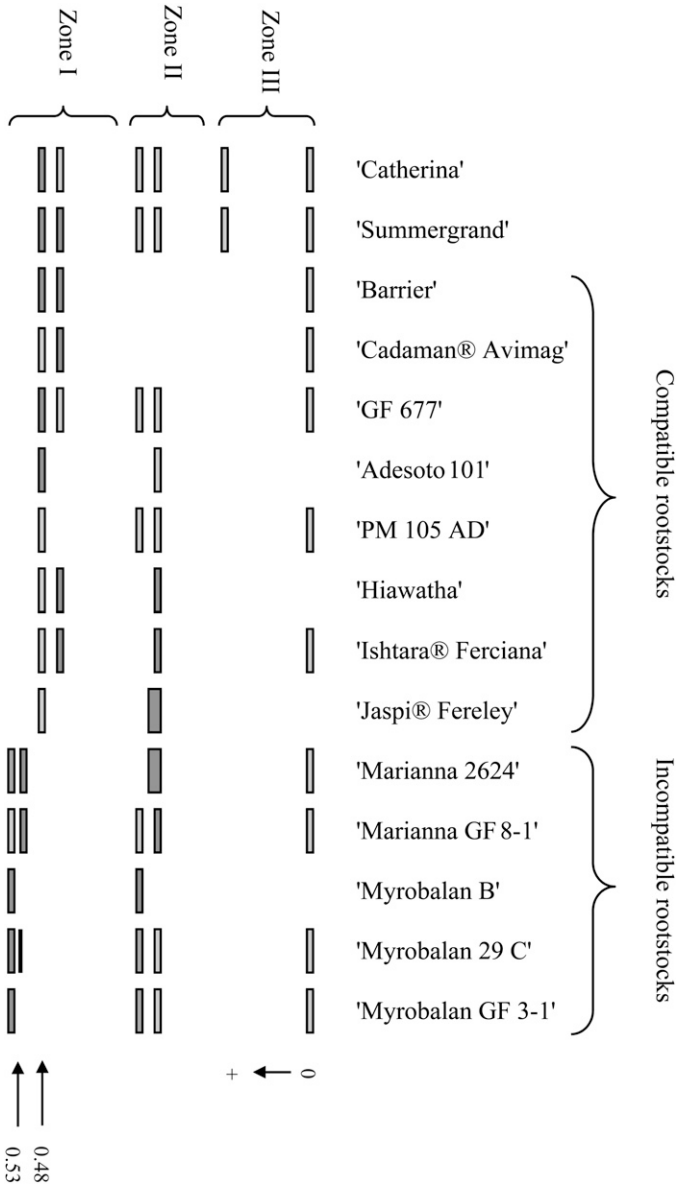

Fig. 3. Zymograms for peroxidase of nonbudded samples of 'Catherina' peach and 'Summergrand' nectarine scions, and compatible and incompatible *Prunus* rootstocks. Isozyme profile is divided in three zones of activity: Zone I, Zone II, and Zone III from the most anodal (+) to the most cathodal one (–). Arrow represents migration of the proteins in the gel. Only the anodal zone (Zone I) is considered in this study because these peroxidases may be related to graft compatibility.

were identical. To facilitate the study, isozyme profile was divided in three zones of activity: Zone I, Zone II, and Zone III, from the most anodal to the most cathodal one. Only the anodal zone (Zone I) was considered in this study because these peroxidases could regulate lignification and may be related to graft compatibility (Buchloh, 1960; Catesson et al., 1986). Analysis of gel profiles of Zone I showed an isoperoxidase band (Rf = 0.48) that was present in peach and nectarine cultivars, as well as in the compatible peach- and plum-based rootstocks. Another isoperoxidase band (Rf = 0.53) was present only in the incompatible plum-based rootstocks (Fig. 3). In addition, we studied the isozyme profiles of the graft union only in compatible ('Summergrand'/'PM 105 AD') and incompatible heterograft ('Summergrand'/'Myrobalan GF 3–1'). Results showed that the phenotype was additive in Zone I in compatible and incompatible combinations used in this study (data not shown). In the incompatible combination, the isozyme profile showed that in Zone II, one band of the scion disappeared when it was grafted on the 'Myrobalan GF 3–1' rootstock (data not shown).

Discussion

In this study, we describe a method for early graft incompatibility detection at dormancy based on cellular organization and peroxidase activity assessment and profile studies.

Our results confirmed that there are visible differences in the structural organization of cells and tissues of compatible versus incompatible grafts in 5-month-old peach grafts. Differences were exclusively located at the scion/rootstock interface mainly in cambium and vascular components, as has already been mentioned by other authors (Ermel et al., 1997, 1999). In line with previous reports for woody (Deloire and Hébant, 1982; Ermel et al., 1997, 1999) and herbaceous species (Moore and Walker, 1981; Tiedemann, 1989; Wang and Kollmann, 1996), stock and scion were delimited by an over-stained line. The arrangement of the cambium cells was less organized in graft interface of the incompatible combinations according to previous reports about apricot [*Prunus armeniaca* (Errea et al., 2001)] and pear (Ermel et al., 1997). This supports that cambium cell disorganization is an early indicator of graft incompatibility.

In 5-month-old unions, differentiated phloem was observed, while xylem differentiation was incomplete in compatible and incompatible combinations. Previous studies on conifers in *Abies sachalinensis* reported the capacity of cells to initiate the formation of phloem before cambial reactivation and xylem differentiation (Oribe et al., 2003). In contrast with the findings of Moing and Carde (1988) on peach/plum combinations, but in line with reports for pear (Moore, 1984) and herbaceous species (Moore and Walker, 1981), phloem tissue degeneration and disorganization in some graft interface areas were observed in the incompatible grafts (ph in Fig. 2B). Wood vascular connection was observed in the compatible combinations. In turn, as it was already reported in pear (Espen et al., 2005), incompatible grafts showed less tracheary elements (Fig. 2A).

In the present study, xylem vessel degeneration was noted in the incompatible combinations (Fig. 2, C and D) and was characterized by a tylosis reaction (Fahn, 1982). Tylosis impairs water transport and mineral element assimilation by injuring vessels (Sperry, 2003), which may explain the reduction of growth of incompatible trees (Soumelidou et al., 1994; Zarrouk et al., 2006). Our results support the suggestion of using vascular tissue disorders in graft interface between scion and stock at 5 months after grafting, as an indicator for early assessment of graft incompatibility. The abnormal accumulation of phenols at the incompatible graft interface was previously reported in cherry [*Prunus avium* (Gebhardt and Feucht, 1982; Usenik and Stampar, 2001)], pear (Ermel et al., 1999), apricot (Errea et al., 1994, 2001), pepper [*Capsicum annuum* (Deloire and Hébant, 1982)], and, more recently, in *Upaca kirkiana* (Mng'omba et al., 2008). Our results (Fig. 2, B and C) suggest that accumulation of phenolic compounds in the cambium cell interface of incompatible peach/plum combinations is also related to graft incompatibility and may avoid the establishment of the union between scion and rootstock by

inhibiting the lignification of cells (Gur and Blum, 1973) and consequently impairing a good union.

The dramatic presence of phenolic compounds in the incompatible graft interface may explain the high peroxidase activity found in the graft union because peroxidases are related to the oxidation of phenolic compounds (Takahama and Oniki, 1997). Differences in peroxidase activity between compatible and incompatible combinations were previously commented upon cherry during the first 3 weeks after grafting and its restoration 7 to 8 weeks after grafting (Schmid and Feucht, 1985). However, we observed that differences in peroxidase activities were maintained 5 to 7 months after grafting until the next vegetative period (Table 4). The higher peroxidase activity in the incompatible graft unions compared with compatible ones may explain cell degeneration in the graft interface and may justify the histological and vascular anomalies of connections found in the present study. Copes (1970) related cell necrosis in douglas-fir (*Pseudotsuga menziesii*) to the increase of peroxidase activity. Moreover, Gebhardt and Feucht (1982) associated the increase of peroxidase activity in cherry to a delay on xylem and phloem differentiation. The peroxidase activities of the incompatible 'Summergrand'/'Damas GF 1869' combination were significantly higher than the other values reported for incompatible combinations during dormancy and the vegetative period. This incompatible combination was the first to show an acute translocated incompatibility at the beginning of the summer. This supports the close relationship between peroxidase activity and the level of pear/quince compatibility as already observed by Musacchi et al. (2002). This finding suggested the determination of peroxidase activity at the graft union as an indicator for early detection of graft incompatibility.

The significant differences ($P \leq 0.001$) in peroxidase activities between dormancy and vegetative growth periods (Table 4) found herein can probably be explained by their role on defense mechanisms against frost stress (Citadin et al., 2002; Szalay et al., 2005). Dissimilarities in the isoperoxidase composition between stock and scion might result in abnormal lignification and lack of vascular connection at the graft union (Santamour, 1988c). Results of the present study are in accordance with Santamour (1988a, 1988b, 1988c). Peroxidases are a large family of isozymes with an extreme range of isoelectric point, serving a multitude of functions. Evidence showed that the activity of four basic isoperoxidases in castor bean [*Ricinus communis* (Bruce and West, 1989)] and one basic isoperoxidase in the pear/quince graft combination have correlated to lignin deposition (Gulen et al., 2002).

Therefore, in the present study, it was suggested that the absence of a band with Rf = 0.48 in the incompatible rootstocks (Fig. 3) might be associated with graft incompatibility in peach/plum combinations. This band was only present in compatible rootstocks and scions. Several isoperoxidases are involved in the production of different structural lignins that can have different binding characteristics (Santamour, 1988c) and can be associated with abnormal peroxidase activity at the graft interface, as observed for incompatible unions. The preliminary study on the isozyme profile of the union showed a difference between compatible and incompatible grafts in Zones I and II. It was found that in the compatible union ('Summergrand'/'PM 105 AD'), the isoperoxidase profile in Zone I was an addition of scion and stock isozyme. In the incompatible union of 'Summergrand'/'Myrobalan GF 3–1', isozymes profiles were additive in Zone I, but one band, present in both genotypes, disappeared in Zone II.

Conclusion

In the present investigation, it was possible to suggest a histological analysis and biochemical methods to detect early graft incompatibility in peach/plum combinations. The main symptoms found affecting cell organization were cambium cell disorganization, less differentiation of vascular tissues, degeneration of phloem and xylem cells, and accumulation of phenols at the graft interface in 5-month-old incompatible trees. In addition, peroxidase activity may be a good index for assessing graft incompatibility in the dormancy period, as well as in the vegetative growth period.

Isoperoxidase patterns of nonbudded scions and stocks can be used as a tool to predict early graft incompatibility and appeared to be promising because it would eliminate the need to wait for the appearance of symptoms of visual and anatomic abnormalities. Further studies are required to demonstrate the real implication of specific bands in the graft compatibility-incompatibility process.

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