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Cell wall modifications in chilling-injured plum fruit (*Prunus salicina*)

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Abstract

The aim of this study was to analyze the changes in cell wall pectins in normally ripening (juicy) and in chilling-injured plum fruit (*Prunus salicina* cv. Fortune) showing mealiness. Total cell wall neutral sugars and uronic acids, solubilization and depolymerization of pectins in water-, CDTA- and Na_2CO_3-soluble fractions of the cell wall (WSF, CSF and NSF, respectively), non-cellulosic neutral sugar compositions of these fractions, and the activities of the cell wall-degrading enzymes polygalacturonase (PG), pectin methylesterase (PME), 1,4-β-D-glucanase/glucosidase and β-galactosidase (β-gal) were determined. No differences in the total content of pectin and neutral sugars between normally ripening and chilling-injured fruit were detected. However, the mealy plums presented a higher level of tightly bound pectin (NSF) and a lower proportion of loosely bound pectin (WSF) than the juicy controls. Lower pectin depolymerization and reduced solubilization of neutral sugars in the WSF and CSF were also detected in the chilling-injured tissues, confirming an alteration in the normal ripening-associated pattern of polyuronide disassembly. While no differences were found in the activities of PG, PME and 1,4-β-D-glucanase/glucosidase between normally ripening and mealy fruit, the latter had reduced β-gal activity. This might have led to differential solubilization of polymers with galactan side chains, but further studies are required to determine if there is a causal relationship between these events. Overall, results indicated that the development of chilling injury symptoms in 'Fortune' plums is associated with abnormalities in cell wall metabolism, including a reduction in pectin solubilization and depolymerization and decreased ripening-associated modification of galactose-rich pectin polymers.

Keywords: Plum; Chilling injury; Internal breakdown; Mealiness; Gel breakdown; Cell wall

1. Introduction

Plums are highly perishable and low temperature storage is recommended to extend fruit postharvest life and maintain quality (Crisosto and Kader, 2000). However, extended cold storage leads to physiological disorders (Wang, 1990; Wang, 1993) and abnormal fruit ripening, reducing consumer acceptance (Crisosto and Kader, 2000). In many commodities the severity of chilling injury (CI) increases when the fruit is refrigerated for prolonged periods at close to 0 °C, but above fruit freezing point. In contrast, for plums, peaches and nectarines, CI symptoms develop more markedly when fruit are stored at temperatures in the range 2–8 °C (Crisosto et al., 1999; Nanos and Mitchell, 1991; Manganaris et al., 2006). These symptoms mainly develop during fruit ripening after cold storage, thus the problem is not noticed until the fruit reach customers (Crisosto et al., 1999).

The physiological basis of CI symptoms has been studied in detail in peach (reviewed in Lurie and Crisosto, 2005). Mealiness is the most prominent CI symptom and main factor that negatively affects peach fruit ripening after removal from cold storage. Mealiness is characterized by loss of juiciness and pectin gel formation. It is accepted that the textural changes occurring are associated with abnormal modifications in the activities of cell wall-degrading enzymes, generally leading to alterations in pectin metabolism (Brummell et al., 2004; reviewed in Lurie and Crisosto, 2005). It has been reported that when the fruit are stored at low temperature for extended periods, the normal increase in endo-PG activity does not occur during ripening and mealiness results (Ben Arie and Sonego, 1980; Zhou et al., 2000a,b). The degree of methyl-esterification of pectin also may be altered in mealy fruit (Ben Arie and Lavee, 1971; Lurie et al., 2003). A more recent study (Brummell et al., 2004) confirmed that the ripening-associated solubilization of high molecular weight pectins remains low, not showing the increase characteristic of juicy fruit. However, this report also showed that the nature of the chilling-injured fruit ripening pro-

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cess is more complex than simply an alteration in the balance between PG and PME, involving reduced disassembly of Ara and Gal-rich polysaccharides. These alterations in the normal metabolism of cell wall polysaccharides might affect the properties of the middle lamella leading to tissue breakage along enlarged air spaces, rather than across cells resulting in reduced availability of free juice upon tissue disruption.

As for peaches, one of the most common symptoms in chilling-injured plums is the development of mealy texture (Crisosto et al., 1999, 2004). There are some data regarding the influence of ripening stage, position on the tree and cultivar, as well as the cold storage temperature on the severity and extent of CI symptoms in plum (Taylor et al., 1993a,b, 1994, 1995; Crisosto et al., 1999). However, there are significant differences between the cell wall modifications reported for chilling-injured peaches and plums. For instance, while reduced solubilization of pectins is associated with mealiness in peach, for plums no major differences in polyuronide solubilization were observed between normally ripening and chilling-injured fruit that are characterized by gel breakdown (Taylor et al., 1995). In addition, the biochemical characterization of the disorder in plums is still partial. For example, while peach mealiness has been shown to be associated with a substantial alteration in pectin depolymerization, in plum no such studies have been done. The objective of the present work was to characterize the changes in pectin solubilization, depolymerization and composition as well as the modification in some cell wall-degrading enzymes associated with plum mealiness development.

2. Materials and methods

2.1. Plant material

Plum fruit (*Prunus salicina* Lindell cv. Fortune) were harvested at commercial maturity stage according to fruit size and skin background color. Forty fruit were allowed to ripen at 20 °C for 4 d (juicy) while another 40 fruit were stored for 4 weeks at 5 °C (90% RH) and subsequently transferred to 20 °C for 4 d. Fruit stored at 5 °C developed CI symptoms, evident as mealiness, based on the perceived sense of dry texture when tasted and the lack of juice when squeezed. In order to analyze fruit with similar firmness a Fruit Texture Analyzer equipped with a 7.9-mm-diameter, flat-tipped probe was used to perform compression tests at a speed of 0.17 mm s^{-1}. The maximum force during the test was determined and fruit within the most common range of tissue firmness (4.1 ± 0.4 N) were selected for further analysis. Longitudinally cut wedge-shaped slices from each fruit were cut, frozen in liquid nitrogen and stored at −40 °C until use.

2.2. Preparation of cell walls

Fifty grams of fruit pulp tissue were homogenized with an Ultraturrax (IKA Werke, Janke & Kunkel GmbH & Co. KG, Staufen, Germany) in 200 mL of 95% ethanol and boiled for 30 min to extract low molecular weight solutes and to prevent autolytic activity. The insoluble material was filtered through glass fiber filters (Whatman GF/C) and sequentially washed with ethanol, chloroform:methanol (1:1, v/v) and acetone and allowed to dry at 37 °C, yielding the alcohol insoluble residue (AIR).

2.3. Neutral sugars (NS) and uronic acids (UA)

Ten milligrams of AIR were solubilized in H_2SO_4 as described by Ahmed and Labavitch (1977) and aliquots of the AIR solution were subsequently assayed for uronic acid (Blumenkrantz and Asboe-Hansen, 1973) and total sugars (Yemm and Willis, 1954). Results were calculated by using a standard curve of galacturonic acid (UA) or glucose (NS). Three independent samples were analyzed for each treatment, and results were expressed as grams of galacturonic acid or glucose equivalents per kg of AIR for UA and NS, respectively.

2.4. Fractionation of cell wall pectins

Fractions of different cell wall components were obtained by sequential chemical extraction of the cell wall material (AIR). Approximately 200 mg of AIR residue from each sample were suspended in 15 mL of water and stirred at room temperature for 12 h. The samples were then centrifuged at 6000 × *g* and 4 °C for 10 min, the supernatant was filtered through glass fiber filters (Whatman GF/C), and the pellet was washed with water. The filtrate and water washings were combined and designated the water-soluble fraction (WSF). The residue was then extracted for 12 h at room temperature with 15 mL of 50 mmol L^{-1} CDTA, pH 6.5 with stirring. The slurry was centrifuged and passed through fiber glass filters, as above, and the pellet was washed with CDTA solution. The combined filtrates were collected, extensively dialyzed against water and designated the CDTA-soluble fraction (CSF). The CDTA-insoluble pellet was then extracted with 15 mL of 50 mmol L^{-1} Na_2CO_3 containing 20 mmol L^{-1} $NaBH_4$ at 4 °C for 12 h. After filtration (as above) the filtrate obtained was neutralized with glacial acetic acid, extensively dialyzed against water and the sample was designated Na_2CO_3-soluble fraction (NSF).

2.5. Neutral sugar composition

Tubes containing aliquots from the WSF, CSF and NSF were blown-dried with air in a water bath at 40 °C. After that the samples were hydrolyzed in 2 mol L^{-1} trifluoroacetic acid (Albersheim et al., 1967), and converted to alditol acetates (Blakeney et al., 1983) for gas chromatographic analysis of neutral sugar composition. The derivatized samples were dissolved in acetone and 1 μL-aliquots were injected into a gas chromatograph fitted with a 30 m × 0.25 mm DB-23 capillary column (J&W Scientific, Folsom, CA, USA) and a flame ionization detector. Temperature in the injector was 250 °C and a linear oven temperature gradient (initial temperature 160 °C, 0 min; the oven increased at 4 °C/min to 250 °C) was used to improve separation. The different alditol acetates were identified by comparison with standards containing myo-inositol (internal standard), rhamnose (Rha), fucose (Fuc), arabinose (Ara),

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xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glc). Neutral sugars were expressed as mol%.

2.6. Size-exclusion chromatography

The WSF, CSF and NSF were dialyzed (Spectrapor 1, SpectrumLabs, Rancho Dominguez, CA; MW cutoff 8 kDa) against water for 1 d at 4 °C and lyophilized. Samples were dissolved in 200 mM ammonium acetate, pH 5.0, chromatographed on an HW65F (fractionation range 1×10^4 to 1×10^6 Da; Tosoh Bioscience, Tokyo, Japan) SEC column (3 cm × 30 cm) eluted with 200 mM ammonium acetate, pH 5.0. Fractions (2 mL) were collected at a flow rate of 16.7 μL s^{-1} and held in a water bath (50 °C) for 4 h to volatilize the NH_4OH, which can interfere with colorimetric assays. Fractions were assayed for UA as described in Section 2.3.

2.7. Cell wall enzyme assays

Twenty grams of frozen tissue were homogenized in an Ultraturrax (IKA Werke, Janke & Kunkel GmbH & Co. KG, Staufen, Germany) with 60 mL of buffer containing 50 mmol L^{-1} sodium acetate, 1 mol L^{-1} NaCl and 10 g L^{-1} polyvinylpolypirolidone (PVPP), pH 5.5. The homogenate was stirred for 3 h at 4 °C, centrifuged (10,000 × *g*, 30 min, 4 °C). The supernatant was collected, dialyzed overnight against 50 mmol L^{-1} sodium acetate, pH 5.0 and subsequently used for assaying the enzyme activities of polygalacturonase (PG, E.C. 3.2.1.67), 1,4-β-D-glucanase/glucosidase and β-galactosidase (β-gal, E.C. 3.2.1.23).

PG was assayed in a mixture containing 400 μL of 50 mmol L^{-1} sodium acetate buffer pH 5.0, 400 μL of 0.15% (w/v) polygalacturonic acid and 400 μL of the enzyme extract. The mixture was incubated at 40 °C and aliquots (200 μL) were taken up to 8 h and released galacturonic acid was measured with the 2-cyano-acetamide assay (Gross, 1982). Results were expressed as mol s^{-1} kg^{-1} of fresh tissue. For 1,4-β-D-glucanase/glucosidase, a mixture containing 400 μL of 50 mmol L^{-1} sodium acetate buffer pH 5.0, 400 μL of 0.2% (w/v) carboxy-methyl-cellulose and 400 μL of the enzyme extract was prepared. The mixture was incubated at 40 °C, aliquots (200 μL) were taken up to 8 h and assayed for reducing sugar assay, as previously described. The enzyme activity was expressed as mol s^{-1} kg^{-1} of fresh tissue.

β-Gal activity was assayed in a mixture containing 400 μL of 50 mmol L^{-1} sodium acetate buffer pH 5.0, and 400 μL of the enzyme extract and 200 μL of 10 mmol L^{-1} *p*-nitrophenyl-β-D-galacto-pyranoside. The reaction mixture was incubated at 40 °C, aliquots (200 μL) were taken at different times and discharged into 600 μL of 0.4 mol L^{-1} Na_2CO_3. The change of optical density at 400 nm was followed. The enzyme activity was expressed as mol s^{-1} kg^{-1} fresh tissue.

Finally, for PME (PME, E.C. 3.1.1.11) 20 g of frozen tissue were homogenized in with 60 mL of 1 mol L^{-1} NaCl containing 10 g L^{-1} PVPP. The homogenate was stirred for 3 h at 4 °C, centrifuged (10,000 × *g*, 30 min, 4 °C). The supernatant was collected, adjusted to pH 7.5 with 10 mmol L^{-1} NaOH, extensively dialyzed against water, and used for assaying the enzyme activity according to Hagerman and Austin (1986). The activity was determined in a mixture containing 1.3 mL of 0.5% (w/v) pectin (72% methoxyl content), 400 μL of 0.01% bromothymol blue in 3 mmol L^{-1} phosphate buffer (pH 7.5) and 1.3 mL of enzymatic extract. The mixture was incubated at 40 °C and the changes in absorbance at 620 nm were measured.

For each enzyme analyzed, two extracts per treatment were prepared and determinations of enzyme activities were performed in duplicate for each independent replication.

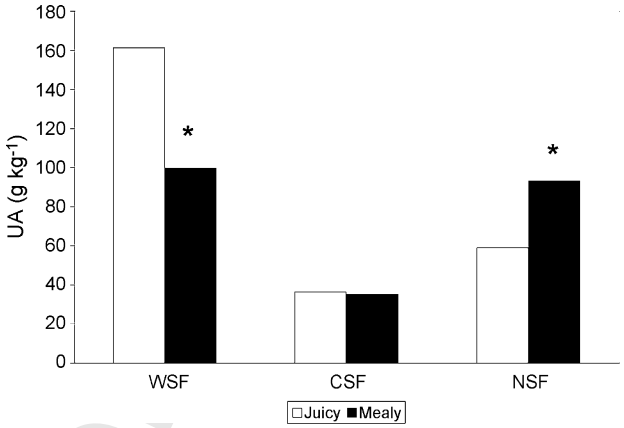

Fig. 1. Uronic acids in the WSF, CSF and NSF pectins of juicy and mealy 'Fortune' plums. The asterisks show differences between treatments for a given storage time at a level of significance of $P \leq 0.05$.

2.8. Statistical analysis

Experiments were performed according to a randomized block design. Data were analyzed using ANOVA, and the means were compared by the LSD test at a significance level of 0.05.

3. Results and discussion

3.1. Pectin and neutral sugar solubilization

Fruit ripened at 20 °C for 4 d without prior cold storage softened normally and showed juicy appearance. In contrast the fruit stored at 5 °C for 4 weeks and subsequently transferred at 20 °C for 4 d were soft but lacked juice when squeezed (i.e., they were mealy). No differences were detected in the levels of total UA and NS in the AIR of juicy and mealy fruit. Uronic acids were 333 ± 19 and 343 ± 27 g kg^{-1} AIR in normally ripening and chilling-injured tissue, respectively. The corresponding values for total neutral sugars were 408 ± 36 and 394 ± 25 g kg^{-1} AIR for juicy and mealy fruit. However, when the cell wall pectins were extracted with a series of aqueous solvents a clear difference was observed between tissues that had ripened normally or were chilling-injured. A higher proportion of pectin was isolated in the WSF from the juicy tissue AIR than from the mealy tissue AIR (Fig. 1). The CSF represented a lower proportion of the total wall pectins than did the WSF and no differences between control and chilling-injured fruit were detected. The amount of uronic acids isolated in the NSF showed an opposite pattern to

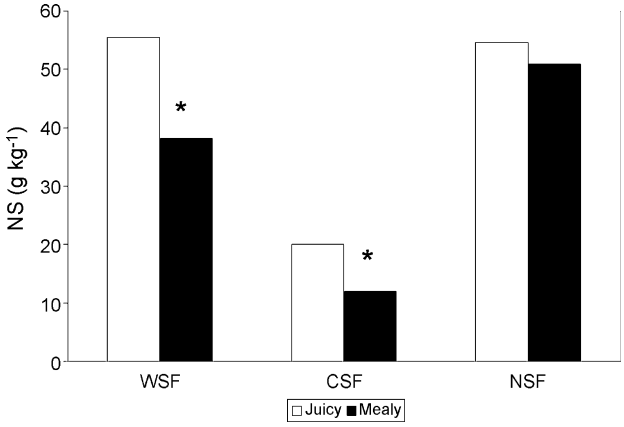

Fig. 2. Neutral sugars in the WSF, CSF and NSF pectins of juicy and mealy 'Fortune' plums. The asterisks show differences between treatments for a given storage time at a level of significance of $P \leq 0.05$.

that found for the WSF. That is, the mealy fruit AIR that had been extracted with water and CDTA contained a higher amount of NSF pectin than did the same AIR preparation from juicy fruit.

Analysis of the neutral sugars associated with the pectin-rich WSF, CSF and NSF, indicated a reduced solubilization of pectin-associated NS in water or CDTA in the mealy fruit relative to the fruit that had ripened to a juicy texture (Fig. 2). This is different from previous reports where no major changes in pectin solubilization were found in plum fruit characterized by lack of juiciness (Taylor et al., 1995). Increased solubility of pectins during fruit ripening and softening has been shown to occur in an array of fruit (Brummell, 2006). The water-soluble fraction is typically thought to include polymeric material that has been solubilized from the cell wall by metabolic processes or was only loosely associated with the wall prior to ripening, whereas the CDTA- and Na_2CO_3-soluble fractions are generally considered to be enriched for ionically and covalently bound pectins, respectively. Ripening-associated increases in WSF and CSF often have been shown to be reflected in a decrease in NSF (Carrington et al., 1993). Redgwell et al. (1997) showed that during plum fruit ripening there is a clear increase in cell wall swelling and pectin solubilization. The results for 'Fortune' plums indicate that, as in peach, CI symptoms are correlated with an alteration in the solubilization of cell wall pectins.

3.2. Pectin depolymerization

Pectin depolymerization is a common change accompanying fruit ripening, although pectin solubilization as described above is not always accompanied by depolymerization (Vicente et al., 2007). Although the changes are not as dramatic for plums as they are for other fruit, Redgwell et al. (1997) have shown that pectin polymer size is reduced during ripening as the fruit soften. Previous reports suggested that gel breakdown in plum is associated with modifications in the gelling properties of polyuronides (Taylor et al., 1995). However, no attempts were made in that work to determine if the disorder were associated with alterations in pectin depolymerization. In the present work, fruit developing mealy symptoms were accompanied by

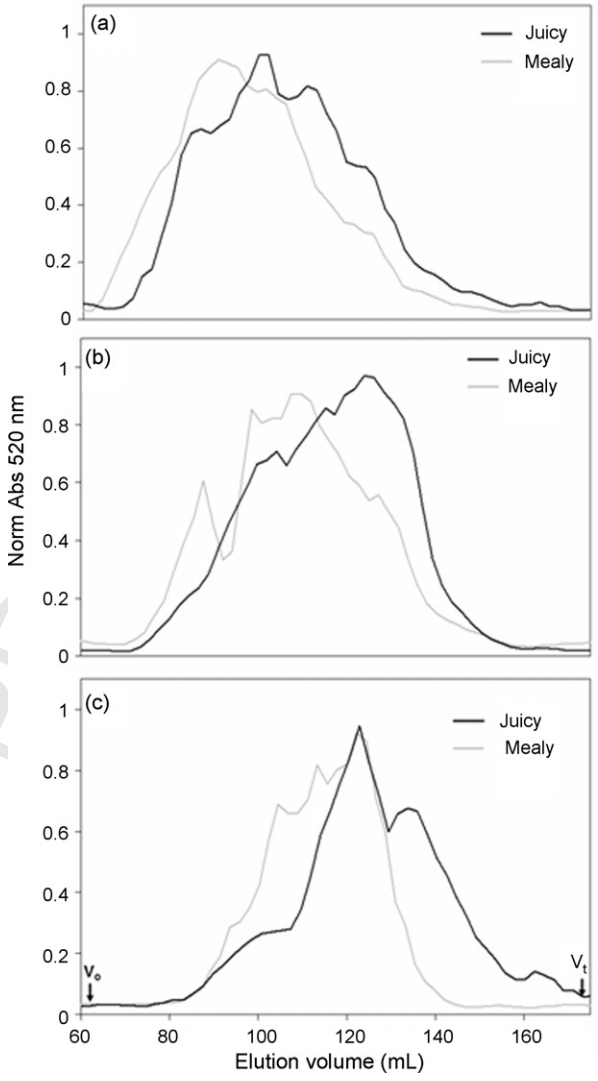

Fig. 3. Size exclusion chromatography profiles of pectin polymers of juicy and mealy 'Fortune' plums. The samples were fractionated on HW65. Column fractions (2 mL) were assayed for uronic acid content. V_o: void volume; V_t: total volume. (a) WSF; (b) CSF; (c) NSF.

reduced depolymerization in all pectin fractions compared to ripening-associated pectin changes observed in normally ripening fruit (Fig. 3). This is consistent with observations reported for chilling-injured (mealy) peach fruit where symptoms are also associated with decreased depolymerization of cell wall pectins (Brummell et al., 2004).

3.3. Cell wall enzyme activities

The development of mealiness in peaches has been correlated with alterations in gene expression and activity of enzymes involved in cell wall degradation (Brummell et al., 2004; reviewed in Lurie and Crisosto, 2005). For instance 1,4-β-D-glucanase/glucosidase activity and mRNA level are increased following chilling temperature storage, while fruit from treatments that do not cause or delay the appearance of mealiness do not display this increase (Zhou et al., 2000a). Other studies showed that mealy texture in peaches correlates with an

Table 1
Polygalacturonase (PG), 1,4-β-D-glucanase/glucosidase and β-galactosidase (β-gal) activities in juicy (normally ripening) and chilling-injured (mealy) plum fruit

Enzyme activity	Fruit tissue	
	Juicy	Mealy
PG (mol s^{-1} kg^{-1})	1.54×10^{-8}	1.53×10^{-8}
1,4-β-D-Glucanase/glucosidase (mol s^{-1} kg^{-1})	2.50×10^{-8}	2.38×10^{-8}
β-Gal (mol s^{-1} kg^{-1})	1.95×10^{-8}	1.30×10^{-8}*

Differences in the activities of these enzymes extracted from juicy and chilling-injured (mealy) tissues were detected only in the case of β-gal. The asterisk indicates differences at a significance level of 0.05.

imbalance in the ratio of PG and PME leading to an increased proportion of larger size de-esterified pectins with increased gel-forming tendencies (Ben Arie and Sonego, 1980; Zhou et al., 2000a,b).

In the present work no differences in 1,4-β-D-glucanase/glucosidase (Table 1), PG (Table 1) and PME (data not shown) activities were observed between juicy and mealy fruit. Several alternatives could account for the lack of differences in total PG activity (i.e., the sum of the reducing end generation caused by both endo- and exo-PG) even when clear differences in pectin depolymerization between juicy and mealy fruit were observed. First, endo-PG activity is most likely to be involved in the polyuronide size downshifts presented in Fig. 3 and endo-PG's activity in generation of reducing ends could be small relative to exo-PGs action in the PG assay used here. It could also be hypothesized that the action of other pectin-depolymerizing enzymes, which have received much less attention than PGs, including pectate lyase (Marín-Rodríguez et al., 2002; Trainotti et al., 2003) and/or rhamnogalacturonan hydrolase (Mutter et al., 1998), might also be important contributors to pectin depolymerization in ripening fruit. Furthermore, differences in the architecture and porosity of the cell wall between tissues could potentially determine modifications in the accessibility of cell wall degrading agents to their substrates and ultimately an increased *in muro* activity.

Galactose is a major neutral sugar in the cell walls of several fruit including plums. The neutral sugar components of branched pectins play a critical role in cell wall structure, and removal of NS-rich side chains has been regarded as an essential part of pectin solubilization (Dawson et al., 1992). This would include potential impacts on wall porosity and architecture as discussed above. β-galactosidases remove β-linked Gal residues from pectin side-chains (Brummell and Harpster, 2001). Interestingly the activity was reduced in mealy tissues presenting levels that were 60% relative to the activity measured in softening juicy fruit (Table 1).

3.4. Neutral sugar composition

Galactose was found to be the most abundant non-cellulosic NS of plums comprising over 50% of the total NS. Since galactosidases cause complete loss of galactose from the wall it might have been expected that mealy fruit showing a clear reduction in the enzyme activity might have presented a galactose content that was greater than that of normally ripened fruit. However, no significant differences were found between juicy

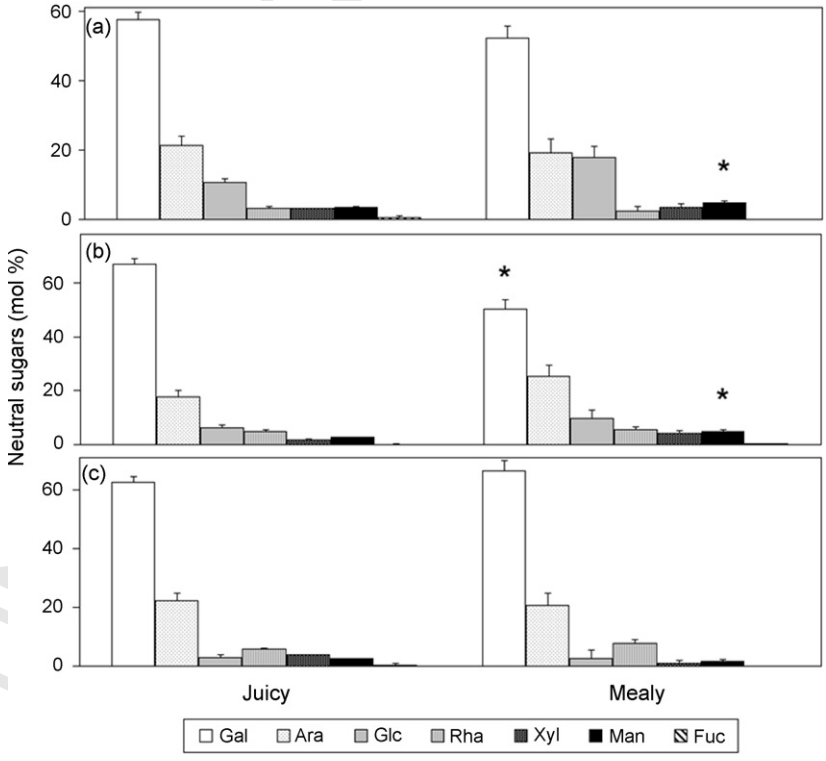

Fig. 4. Neutral sugar composition in the (a) WSF, (b) CSF and (c) NSF pectins of normally ripening (juicy) and chilling-injured (mealy) 'Fortune' plums.

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and mealy 'Fortune' plums (data not shown). In order to determine differences in specific neutral sugar solubilization, the neutral sugar compositions of loosely (WSF), ionically (CSF) and tightly bound pectins (NSF) were measured. The main modification observed was a lower proportion of galactose in the CSF of mealy tissues (Fig. 4). One possible interpretation linking the reduced β-gal activity observed in mealy fruit with the modifications observed in galactose metabolism, is that decreased processing of side chains from pectin polymers with varying amounts of galactan substitution might result in minor total galactose loss but that, in turn, might lead to differential polyuronide solubilization due, possibly, to a reduced porosity of walls from mealy fruit. However, it cannot be excluded that the differences observed in Gal-rich polymers solubility are not related to the measured β-gal activity differences which occur in the same developmental time-frame but are an unrelated event.

4. Conclusion

Chilling injury is a disorder limiting plum shelf life. The symptoms have been clearly described in the literature (Crisosto et al., 1999, 2004; Taylor et al., 1993a,b, 1994) and the apparent lack of juice has been associated, as in peaches, with increased formation of pectin gels. The present work shows that as in peach (Brummell et al., 2004), plum cv. Fortune mealy texture is characterized by decreased pectin depolymerization and is associated with a reduction in the proportion of water-soluble pectins, perhaps a direct reflection of the increased level of pectins that are more tightly bound to the cell wall. A difference between peach and plum is that while in peach Ara-rich pectin metabolism is altered; in plum, reduced solubilization of Gal and not Ara-rich polymers is associated with mealy texture. Mealiness also correlated with decreased β-gal activity while no differences in other assayed enzymes (PG, PME or 1,4-β-D-glucanase/glucosidase) were observed between juicy and mealy tissues. Whether these modifications are primary factors leading to the CI symptoms of 'Fortune' plums or represent a consequence and late manifestation of an earlier imbalance of cellular metabolism in fruit stored under low temperatures is not clear from the data reported herein. However, the results suggest that the development of CI symptoms in 'Fortune' plums is associated with an alteration of cell wall metabolism including a reduction in pectin solubilization and depolymerization and, perhaps, decreased cleavage of galactose rich-pectins.

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