SHORT COMMUNICATION

CORRELATIONS BETWEEN *XANTHOMONAS ARBORICOLA* pv. *JUGLANDIS* SEVERITY AND ENDOGENOUS JUGLONE AND PHENOLIC ACIDS IN WALNUT

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SUMMARY

Endogenous phenolic compounds in walnut fruits were correlated with the severity of walnut blight caused by *Xanthomonas arboricola* pv. *juglandis* (*Xaj*) assessed in the field, to determine the possible role of phenolics in resistance to the disease. Healthy fruits of the cvs Franquette, Cisco, and Sampion with different susceptibilities to infection by *Xaj* were sampled from diseased trees three times during growth and analysed using HPLC with a PDA detector. An identical phenolic profile, consisting of juglone and six phenolic acids (ellagic, gallic, syringic, p-coumaric, caffeic, and chlorogenic), was detected in the studied cultivars. Juglone was the most abundant, ranging between 373 mg 100 g^{-1} and 5,074 mg 100 g^{-1} DW, compared to the least abundant caffeic and p-coumaric acids, which did not exceed 10 mg 100 g^{-1} DW. A negative correlation between the total amount of phenolics present in the fruit tissues and blight severity was found in all cultivars, indicating the role of these compounds in the fruit-bacteria interactions. As the major phenolic characterized by the strongest seasonal fluctuations, juglone seemed to have the main and negative relation with disease development during the year. Thus, its involvement into the defence mechanism of walnut against bacterial blight is strongly suspected. The same may apply to gallic acid, considering its seasonal variations with respect to disease incidence. Additional studies including *in vitro* determination of the anti-bacterial activity of some phenolics, and their response to artificial inoculation with *Xaj* seem desirable to clarify the role of phenolic compounds in walnut resistance against this bacterium.

Key words: common walnut, bacterial blight, phenolic compounds, defence mechanism.

Blight, one of the most serious and widespread diseases of Persian (English) walnut (*Juglans regia)*, causes significant crop losses in all areas of cultivation (Mulrean and Schroth, 1982; Ninot *et al*., 2002). This bacterial disease, caused by *Xanthomonas arboricola* pv. *juglandis* (*Xaj*), affects all the current-season tissues of the tree, i.e. buds, male inflorescences, pistilate flowers, leaves, non-lignified shoots, and fruits. Greatest economic losses occur when, following infection, the fruit mesocarp is destroyed, resulting in darkened shells and shrivelled kernels, thus reducing the marketability of the nuts (Teviotdale and Schroth, 1998).

Disease severity depends on the frequency and amount of spring rains, the leafing date of the cultivar (Woeste and McGranahan, 1992), the previous history of the disease within the orchard (Olson *et al*., 1997), as well as the soil characteristics (Charlot and Radix, 1997).

Wide variations are observed on disease incidence between and within cultivars, suggesting the existence of a combination of genetically and environmentally-mediated plant defence mechanisms against *Xaj*. Phenolic compounds may be crucial components of this defence (Benett and Wallsgrove, 1994; Radix *et al*., 1998).

As secondary metabolites, which occur abundantly in plants, phenolics belong to a large and heterogeneous group of biologically active non-nutrients (Schahidi and Naczk, 1995) which, among other functions, play an important role as cell-wall support and barrier against microbial invasion (Wallace and Fry, 1994; Strack, 1997; Treutter, 2001).

As a result of microbial attack, phenolics may accumulate as inducible low-molecular-weight compounds, which can be post-infectional or constitutive. In the first case, phenolics can rapidly accumulate upon attack, although they may already be present at low concentrations in the plant. In the second case, these compounds are already present in healthy tissues at concentrations high enough for defence, either as free elements or in conjugated forms, from which they are released after the attack (Strack, 1997). The involvement of phenolics in fruit defence against different pathogens has frequently been reported for apple infected by *Venturia inaequalis* (Mayr *et al*., 1997; Usenik *et al*., 2004; Mikulic Petkovsek *et al*., 2008). Within the family Juglandaceae, Cline and Neely (1984) investigated the relationship between phenolic contents and antracnosis caused by the fungus

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Gnomonia leptostyla of black walnut (*Juglans nigra*).

Apart from the results of Radix *et al.* (1998), who listed several factors that induce synthesis of phenolics in the fruits, and investigations of Solar *et al.* (2006a, 2009), and Matias *et al.* (2009), where some phenols were shown to be potential defence compounds against walnut blight, the knowledge on this subject is scanty.

The present study quantifies endogenous pre-infection phenolics in walnut fruits, and links them with blight severity assessed *in situ* (in the orchard) for determining the possible role of these compounds in the resistance to walnut blight.

Phenolic compounds were determined in healthy fruits on diseased trees of walnut cvs Franquette, Cisco, and Sampion. Under Slovenian climatic conditions, the fruits of cv. Franquette typically show very low susceptibility to blight, the fruits of cv. Cisco show medium susceptibility whereas the crop losses of cv. Sampion can exceed 80% (Ambrozic-Turk *et al.*, 1999).

Material for analyses was taken from two adult, 14-year-old trees per cultivar, grown on a flat land, at a spacing of 10x10 m within an experimental orchard of the Biotechnical Faculty of Ljubljana, located in Maribor (NE Slovenia, 46°32'N, 15°39'E, elevation 275 m).

Fruits were sampled on June 10 (SD-1), July 7 (SD-2) and August 31 (SD-3). Five fruits without visible symptoms of bacterial blight were collected randomly from the well exposed branches in the middle height of the canopy from the quadrant of single trees. Immediately after sampling, the fruits were immersed in liquid nitrogen and stored at -20°C. Before analysis, fruit samples were lyophilised.

The husk (exocarp + mesocarp, 1 mm thick) of each fruit was ground to a fine powder, 50 mg of tissue were placed into a test tube, to which 5 ml methanol containing 1% of 2,6-di-tert-butyl-4-methylphenol (BHT) were added prior to exposure to an ultrasonic bath for 45 min to extract the phenolic compounds. Samples were centrifuged for 7 min at 4,200 rpm, the supernatant was filtered through a Chromafil AO-45/25 polyamide filter (Macherey-Nagel, Germany) then transferred to a vial prior to injection into a HPLC (high performance liquid chromatography) system (Thermo Finnigan, USA).

The HPLC system incorporated a Surveyor quaternary LC pump equipped with the Surveyor photodiode array (PDA) detector and was controlled by the ChromQuest™ 4.0 Chromatography workstation software system. A Chromsep HPLC column SS (250x4.6 mm, Hypersil 5 ODS) protected with a Chromsep guard column SS (10x3 mm) (Chrompack, The Netherlands) was used. The chromatographic conditions (mobile phase, gradient program, operating temperature of column) were similar to those reported by Schieber *et al.* (2001). The column was operated at 25°C. Solvent A was 2% acetic acid in aqueous solution and solvent B was 0.5% acetic acid in aqueous solution and acetonitrile (ratio 1:1). The flow rate was 1.0 ml min^{-1} and the volume of the injected sample was 20 µl. For the analysis of phenolic acids, the gradient used was from initial 90% of solvent A to 45% of A in 50 min, then 0% of A in 60 min and a fresh 90% of A in 65 min at the end of the analysis. The total running time was 65 min. The equilibration treatment with 90% of A lasted 15 min and was performed during each analysis.

Phenolic compounds were detected at 280 and 320 nm in a spectrum of 220 to 360 nm. Their identification and quantification was done according to concentrations of a corresponding external standard. The concentrations were expressed in mg·100 g^{-1} dry weight (DW).

The following standards were used for quantification of phenolic compounds: syringic acid (4-hydroxy-3,5-dimethoxy benzoic acid) and gallic acid from Merck (Germany), chlorogenic acid (5-caffeoylquinic acid) and ellagic acid from Sigma (USA), *p*-coumaric acid and caffeic acid from Fluka (Switzerland) and juglone (5-hydroxy-1,4-naphthoquinone) from Aldrich (USA). Butylated hydroxytoluene (BHT) dissolved in methanol, used in the extraction solution, was obtained from Sigma (USA). Acetonitrile, methanol, and acetic acid used as eluents in HPLC system were from Merck (Germany). Water used for sample preparation and analyses was double distilled and purified with a Milli-Q water purification system (Millipore, USA).

Parallel with the analysis of phenolic compounds, the severity of *Xaj* infection of the fruits was visually assessed *in situ* (in the orchard) on the same trees from which the samples for phenolic determination were taken, simultaneously with the fruit sampling for phenolic determination. Twenty fruits (five per each side of the quadrant), grown on well-exposed branches at mid-height of the canopy, were collected from each tree and examined at each sampling date.

The severity of *Xaj* infection was estimated as percentage of watersoaked and necrotic tissue as shown in Fig. 1, allocating each examined fruit into one of six severity classes. The external disease severity was calculated according to the modified Towsend-Heuberger (1943) model:

$$P = \frac{\sum_{0}^{i} (n.v)}{i.N} \cdot 100\%$$

where: P = external *Xaj* severity (%); n = number of fruits in each class; v = numeric value of the severity class (0, 1, 2, 3, 4, 5); i = the highest severity class (5); N = total number of examined fruits (20).

A multifactor analysis of variance (ANOVA) was carried out to determine differences in the phenolic compounds among the cultivars and the sampling dates and their interaction. ANOVA was also used to determine differences in the degree of damage of fruits related to

Table 1. The content of six phenolic acids and juglone (mean ± SE in mg·100 g^{-1} dry weight) in walnut fruits of the cultivars Sampion, Cisco and Franquette determined on three sampling dates (SD).

Cultivar	SD	Ellagic acid	Gallic acid	Syringic acid	*p*-coumaric acid	Caffeic acid	Chlorogenic acid	Juglone
Sampion	1	826.3±35.8 c	135.3±9.4 b	38.8±2.1 c	4.0±0.4 b	7.3±0.4 b	49.4±1.8 b	5074.8±355.4 c
	2	214.6±16.8 b	17.9±1.1 a	15.6±0.9 b	0.8±0.04 a	1.5±0.1 a	3.4±0.3 a	1072.9±121.4 b
	3	22.7± 4.2 a*	3.1±0.7 a	4.8±0.9 a	1.8±0.3 a	0.75±0.1 a	3.6±1.4 a	394.3±31.6 a
Cisco	1	164.4±11.0 b	121.4±12.6 b	17.5±0.4 b	4.7±0.6 b	3.4±0.2 b	18.8±0.3 b	1834.3±205.4 b
	2	64.6±11.7 a	8.9±1.0, a	5.3±0.9 a	1.5±0.3 a	2.2±0.4 b	2.1±0.3 a	372.8±39.5 a
	3	211.9±11.2 c	2.2±0.4 a	3.1±0.4 a	1.2±0.1 a	0.3±0.0 a	2.5±0.5 a	460.1±50.5 a
Franquette	1	190.5±3.1 c	164.4±6.2 b	20.7±1.2 c	9.3±0.5 b	7.4±0.3 b	11.4±0.9 c	1868.5±66.7 c
	2	61.3±3.6 a	28.9±2.5 a	11.5±0.8 b	0.8±0.05 a	1.7±0.1 a	2.4±0.4 a	1007.5±134.3 b
	3	144.7±6.6 b	2.9±2.5 a	3.3±0.4 a	1.0±0.1 a	0.2±0.1 a	7.3±0.4 b	438.6±56.8 a

a* - Means marked with the same letter do not differ statistically significant according to Duncan Multiple Range Test at p≤0.05. Differences among means of phenolics contents within the same cultivar on three sampling dates were denoted.

the cultivar and the estimation date. The statistical differences between the means at a 95% confidence level were calculated using the Duncan Multiple Range Test (DMRT), using the statistical program Statgraphics Plus 4.0. (Manugistics, USA).

An identical phenolic profile was found throughout the studied cultivars (Table 1). The husk tissue contained juglone (a quinone) and six phenolic acids, i.e. ellagic, gallic, and syringic from the group of hydroxybenzoic acids, and p-coumaric, caffeic, and chlorogenic acid belonging to the hydroxycinnamic acids. To date, juglone is the more frequently detected phenolic compound in fleshy walnut husks, in which between two and nine different phenolic acids have also been quantified (Binder *et al*., 1989; Radix *et al*., 1998: Mahoney *et al*., 2000; Stampar *et al*., 2006; Solar *et al*., 2006a; Cosmulescu *et al*., 2010).

Juglone was the most abundant among all the studied phenolics, determined on three sampling dates and three cultivars. It ranged from 373 mg 100 g^{-1} DW in cv. Cisco at SD-2 to 5,074 mg 100 g^{-1} DW in cv. Sampion at SD-1 (Table 1). In our initial study of six walnut cultivars (Solar *et al.*, 2006a), the juglone content in green husks was lower compared to the content determined in this study, ranging between 24 mg 100 g^{-1} (cv. Hartley, 30th May) and 1,817 mg 100 g^{-1} DW (cv. Franquette, 21st June). The last value agrees with the data by Radix *et al.* (1998) who reported that the June-July content of juglone in the husks of cv. Franquette growing in permeable soils, was up to 2,200 mg 100 g^{-1} DW.

Among the six phenolics, juglone was followed by ellagic, gallic, syringic, chlorogenic, p-coumaric, and caffeic acids, in order of concentration. The content of ellagic acid was three to ten-fold lower than that of juglone. Caffeic and p-coumaric acids were present at very low levels, not exceeding 10 mg 100 g^{-1} DW, regardless of the cultivars and sampling dates. Compared to previous findings, walnut husks contained between 21 and 65 times less p-coumaric acid than leaves (Pereira *et al*., 2007) and kernel pellicles, but six to nine times more than the kernel storage tissue (Colaric *et al*., 2005).

The contents of individual phenolic compounds were significantly cultivar dependent ($p = 0.02$), as reported also for walnut leaves (Amaral *et al*., 2004; Pereira *et al*., 2007), kernels (Colaric *et al*., 2005; Gómez-Caravaca *et al*., 2008) and shoots (Claudot *et al*., 1997; Solar *et al*., 2006b). For individual phenolic, the largest intercultivar variation was found in juglone, ellagic and chlorogenic acid, ranging between 2,267 and 6,542 mg 100 g^{-1} DW (juglone), between 397 and 1,064 mg 100 g^{-1} DW (ellagic acid), and between 21 and 56.4 mg 100 g^{-1} DW (chlorogenic acid), respectively (Table 1). This finding is partly in agreement with Matias *et al.* (2009) who observed a high intercultivar variability of chlorogenic acid and juglone, whilst ellagic acid proved to be the less variable compound in walnut husks.

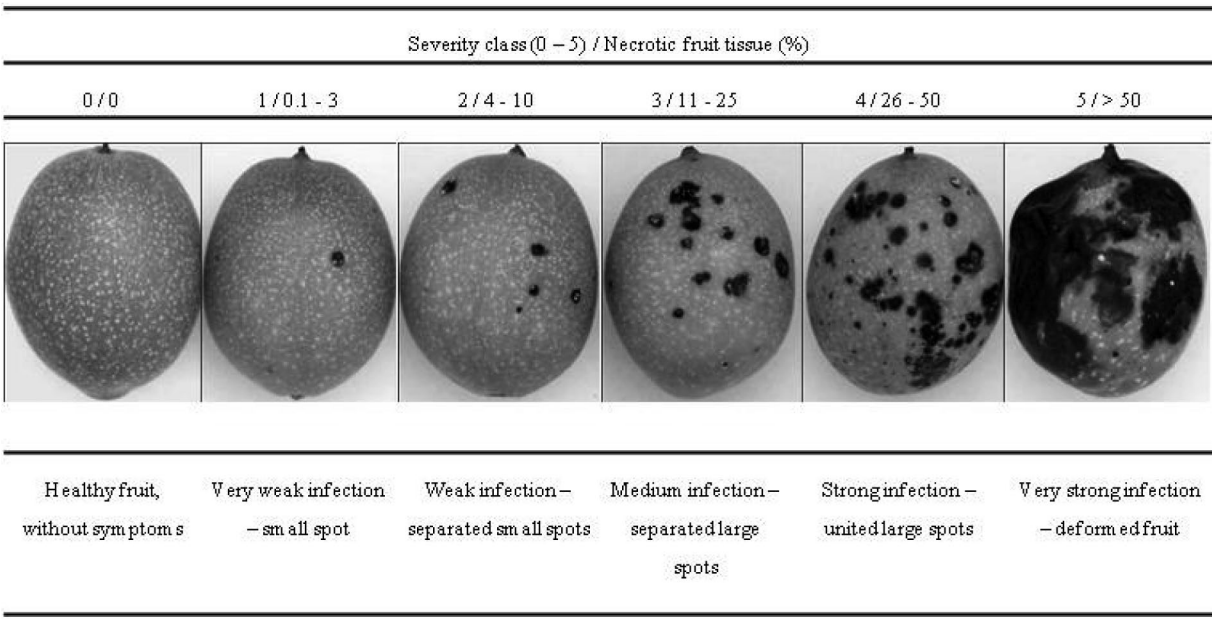

Fig. 1. Necrotic walnut husk tissue caused by *Xanthomonas arboricola* pv. *juglandis* corresponded to six classes of the disease severity. Naturally infected material.

Furthermore, the contents of individual phenolics were markedly affected by the ontogenetic stage of the fruit and its interaction with the cultivar ($p = 0.01$ and 0.02, respectively). In general, a statistically significant decrease in the phenolic contents from SD-1 towards SD-2 was observed. This seasonal decrease was cultivar- and phenolic-related, and ranged between 1.5-fold (caffeic acid, cv. Cisco) and 14.5-fold (chlorogenic acid, cv. Sampion). The observed trend compared favourably with the results of previous investigations on walnut fruits (Radix *et al.*, 1998; Stampar *et al.*, 2006; Solar *et al.*, 2006b; Matias *et al.*, 2009), as well as with the seasonal dynamics of phenolics in walnut leaves (Claudot *et al.*, 1992; Mahoney and Molyneux, 2004; Amaral *et al.*, 2004; Solar *et al.*, 2006a).

Orchard assessment of disease severity showed that at the first SD (June 10th) only small, water-soaked and well separated spots were found. Cumulatively, they did not affect more than 10% of the fruit surface (Fig. 2A, B, C), and there were no differences in symptom expression and disease severity between the cultivars studied. One month later (second SD, July 7th), cv. Sampion showed a mean of 48% of the fruit surface affected whereas the incidence was 30% in cv. Cisco and 10% in cv. Franquette. At the third SD (August 31st), only cv. Franquette showed a siginificant increase in disease severity (from 11% to 31%). At that time, *ca.* 30% of the fruits of cvs Franquette and Cisco were infected, whereas in cv. Sampion the infection exceeded 50% of the fruits, many of which showed deformations due to bacterial blight (Fig. 1, severity class 5).

In order to prove the involvement of phenolic compounds in walnut defence mechanisms against bacterial blight, the disease severity, assessed in the orchard, was correlated with the contents of phenols in developing visually healthy fruits.

At June 10th (SD-1), when fruits showed an appreciable increase of their size, only between 6 and 8% of the husk tissue of all three cultivars was affected by *Xaj* (Fig. 2). At this time (SD-1), the highest phenolic content was observed. Total content of the seven phenolics that could be identified by HPLC analysis, i.e. six phenolic acids and juglone (PaJ), was 6,137 mg 100 g^{-1} DW for cv. Sampion, 2,273 mg 100 g^{-1} DW for cv. Franquette and 2,165 mg 100 g^{-1} DW for cv. Cisco (Fig. 2). A strong decrease in PaJ content occured during further fruit development in the season. At the beginning of July (SD-2), the PaJ was between two (cv. Franquette) and 4.7 times (cvs Cisco and Sampion) lower than at SD-1. During the same course of time, a rapid increase in the infection was noted in cvs Cisco and Sampion, but the increase in cv. Franquette was negligible. Assessment of the disease severity in the orchard showed that 30% (cv. Cisco) and 48% (cv. Sampion) of the hust tissue was infected (Fig. 2). Such a marked advance of the disease could mainly be attributed to the decrease in juglone content, representing more than 80% of the cumulative phenolic acids and juglone (PaJ) content, which declined by up to 4.9 times. These results are consistent with the negative correlation between juglone in walnut fruits and external disease severity caused by walnut blight reported by Matias *et al.* (2009). In our case, juglone was shown to be present in visually healthy walnuts prior to natural infection, while Matias *et al.* (2009) detected it as a postinfectional defence compound in artificially inoculated fruits.

At the end of August, three to four weeks prior to fruit maturation (SD-3), both the PaJ and juglone quan-

tities significantly decreased in cvs Sampion and Franquette (Fig. 2), and increased in a statistically insignificant way in cv. Cisco. In cv. Franquette, the decrease of phenolics was followed by a significant increase in disease severity, and a reverse relationship between the juglone content and disease severity was again observed. By contrast, the disease severity of cv. Sampion fruits did not increase after SD-2 in spite of the decreased contents of juglone and PaJ, respectively. This suggests that other phenolics may act as defence compounds against bacterial blight in walnut fruits.

Our results show that an anti-bacterial activity of gallic acid might be suspected in addition to that of juglone. Markedly higher (7.5 and 13.6 fold) gallic acid contents, noted in cvs Sampion and Cisco at SD-1 compared to the contents determined at SD-2, might have caused the significantly lower disease severity of those fruits observed in the orchard at SD-1 (Fig. 2). A similar relationship was also observed for cv. Franquette, in which blight severity increased from 6 to 10%, and the gallic acid content decreased by 5.7 times at the SD-2, in comparison with SD-1 (Fig. 2, Table 1).

Fruits of the very sensitive cv. Sampion, contained twice the PaJ than the fruits of the low-sensitive cv. Franquette, and even 2.4 times more than the medium-sensitive cv. Cisco (Table 1). With apple trees it was found that the cultivars resistant to scab disease usually had more constitutive phenolics than the susceptible ones (Picinelli *et al.*, 1995; Mikulic-Petkovsek *et al.*, 2009). Our results show that such a relationship is not so obvious when the sum of juglone and six hydroxycinnamic acids in walnut fruits is taken into account. It seems that the seasonal alterations in phenolic compounds had a stronger impact on disease severity than their pre-infection contents. Such a close correlation between the seasonal fluctuations of some phenolics, and the sensitivity to blight had previously been observed when rejuvenated annual shoots of walnut were studied (Solar *et al.*, 2006b).

To the best of our knowledge, the interaction between specific phenolic compounds synthesized in walnut fruits and susceptibility to bacterial blight, has herein been determined quantitatively for the fist time. Since phenolic synthesis is site-specific (Mayr *et al.*, 1997; Treutter, 2001), and some fungi may cause symptoms similar to those by *Xaj* (Belisario *et al.*, 2002; Giraud *et al.*, 2009; Moragrega and Özkatan, 2010), further investigations are necessary to support the hypothesis that walnut blight susceptibility is correlated with phenolic content. *In vitro* determination of the anti-bacterial activity of juglone and six phenolic acids against a strain of *Xaj* as well as the changes in the contents of phenolic compounds after artificial inoculation with the bacterium, could add important information on the role of phenolics in the walnut resistance mechanisms.

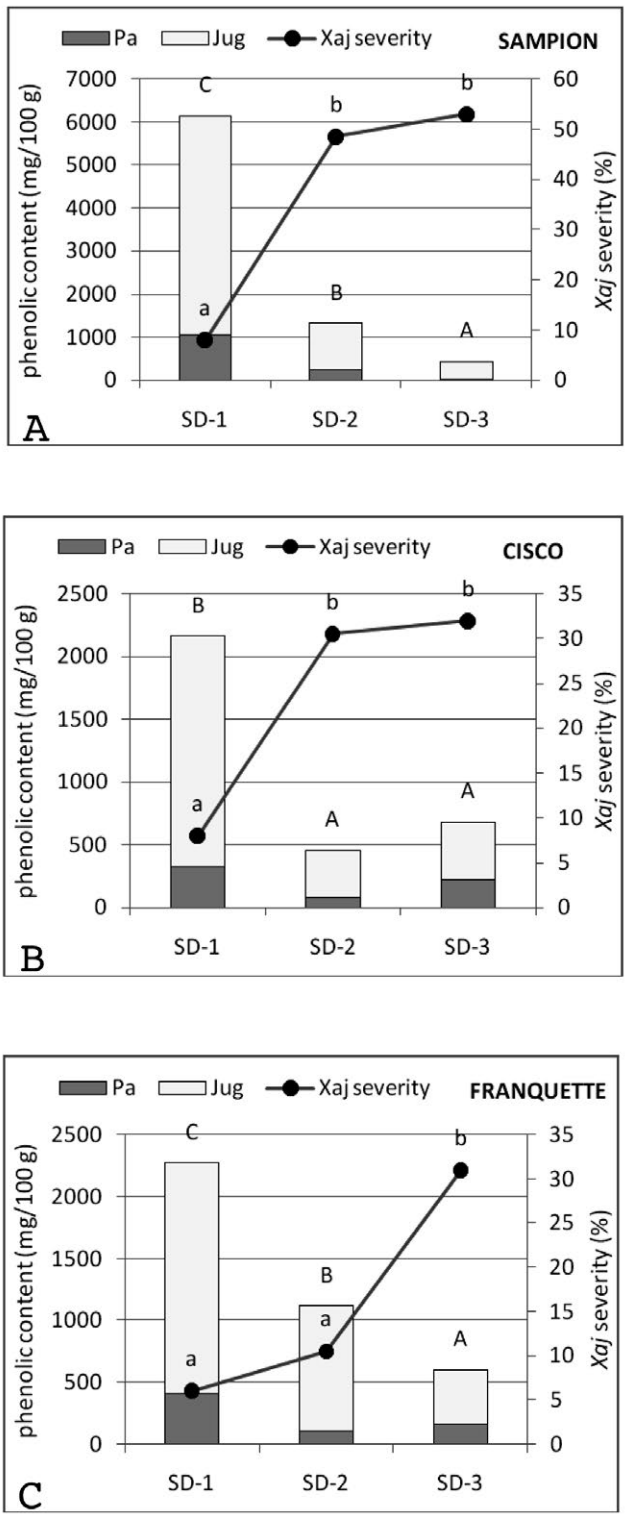

Fig. 2. Mean contents of seven phenolic compounds, viz. six phenolic acids (Pa) and juglone (Jug) (indicated by bars), and walnut blight severity as determined on developing visually healthy fruits on diseased trees (indicated by line) in cultivars Sampion (A), Cisco (B), and Franquette (C) at three sampling dates: June 10th (SD-1), July 7th (SD-2) and August 31st (SD-3). Different capital letters (A) denote statistically significant differences between summed phenolic content (Pa+Jug) in three cultivars ($p \leq 0.05$, DMRT), whilst different low letters (a) denote statistically significant differences in the disease severity between the three sampling dates within each cultivar.

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