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Polyphenoloxidase activity and polyphenol levels in organically and conventionally grown peach (*Prunus persica* L., cv. Regina bianca) and pear (*Pyrus communis* L., cv. Williams)

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Abstract

Polyphenoloxidase (PPO) activity and total polyphenol content were tested in organically and conventionally grown whole fruits, peach (*Prunus persica* L., cv. Regina bianca) and pear (*Pyrus communis* L., cv. Williams), in order to evaluate the existence of a relationship between these parameters and of differences between fruits obtained with the two cultivation practices. Organic fruits were obtained on three different grounds: subterranean clover (sample A), spontaneous weed cover (sample B) and tilled (sample C). From the latter soil, the conventionally grown fruits were produced. All organic peach samples showed a highly significant ($P < 0.001$) increase in polyphenols (mg equivalents of tannic acid/100 g fresh sample) compared with conventional peaches, while, of the three organic pear samples, samples B and C displayed an increased polyphenol content with respect to the conventionally grown sample ($P < 0.05$). Activity of PPO (U.E./100 g fresh sample), extracted in appropriate conditions and tested towards 1 mM chlorogenic and caffeic acid, was significantly higher in most of the organic peach and pear samples analyzed with respect to the conventional samples.

1. Introduction

Enzymatic browning of fruit and raw vegetables is related to oxidation of phenolic endogenous compounds into highly unstable quinones, which are later polymerized to brown, red and black pigments (Martinez & Whitaker, 1995; Nicolas, Richard-Forget, Goupy, Amiot & Aubert, 1994).

The grade of browning depends on the nature and amount of endogenous phenolic compounds, on the presence of oxygen, reducing substances, metallic ions, on pH and temperature and on the activity of polyphenoloxidase (PPO), the main enzyme involved in the reaction (Goupy, Amiot, Richard-Forget, Duprat, Aubert & Nicolas, 1995; Sapers, 1989; Zawistowski, Biliaderis & Eskin, 1991). PPO is a copper-containing enzyme which acts on phenols in the presence of oxygen, catalysing the oxidation of *o*-diphenols into *o*-quinones (Haruta, Murata, Kadokura & Homma, 1999; Martinez & Whitaker, 1995).

Plant phenolics are readily oxidized by PPO, most often following tissue damage since PPO is suggested to act as a defensive enzyme (Mayer & Harel, 1990).

Although PPO has generally been recognized to be largely responsible for enzymatic browning of fruits and vegetables, it has not been clearly established, so far, whether a relationship among extent of browning, fruit polyphenol content and enzyme (PPO) activity exists (Gauillard & Richard-Forget, 1997).

PPO is a plastid enzyme in the higher plants generally located on the thylakoid membrane of chloroplasts such as a membrane-bound protein (Nicolas et al., 1994; Zawistowski et al., 1991). Indeed, strong non-ionic detergents (i.e. Triton X-100) are required to achieve a full extraction of the enzyme (Rocha, Pilar Cano, Galeazzi & Morais, 1998), although the existence of a readily soluble (non-membrane associated) form of the enzyme in the mitochondrial fraction of plant cells has also been reported (Barrett, Lee & Liu, 1991; Zawistowski et al.).

PPO, purified from several fruits and vegetables, exhibits multiple forms, at least in part as a consequence of artefacts caused by extraction procedures (incomplete

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release from membranes, denaturation, fragmentation) that can give rise to aggregated forms of the enzyme (Janovitz-Klapp, Richard & Nicolas, 1989). In Williams pears *Pyrus communis* L., cv. Williams), electrophoretic analysis of a crude extract of PPO indicated the presence of at least two isoenzymes and two latent forms of PPO (Gauillard & Richard-Forget, 1997).

Available experimental data on PPO activity show a high degree of variability, likely depending on kind of fruit, cultivar, stage of maturity, analytical methods and experimental extraction conditions (Barrett et al., 1991; Nicolas et al., 1994). The simultaneous presence of quinones in crude extracts of the enzyme and its endogenous phenolic substrates may make the situation even more complex, although minimization of quinone formation can be achieved by adding phenol-binding agents, such as polyethylene-glycol, soluble and insoluble polyvinyl(poly)pyrrolidone (PVP and PVPP), and reducing agents (diethyldithiocarbamate, ascorbic acid, mercaptoethanol or cysteine; Nicolas et al.).

On the other hand, in routine assays, an accurate estimation of the amount and type of phenolic compounds is difficult to achieve because extraction methods do not always warrant total solubilization of phenolic compounds and do not comprise reactions that allow us to discriminate among different classes of phenolics (Nicolas et al., 1994). Nevertheless, several studies have demonstrated that the level of phenolic compounds in fruits is highly dependent on many external and internal factors, such as variety, stage of maturity, storage and environmental or genetic factors (Nicolas et al.). Among these, light, temperature, oxygen, ethylene, growth regulators, nutrients and pesticides have been demonstrated to affect phenolic metabolism (Amiot, Tacchini, Aubert & Nicolas, 1992; Amiot, Tacchini, Aubert & Oleszek, 1995; Daniel, Meier, Schlatter & Frischknecht, 1999; Lea & Beech, 1978).

The level of phenolic compounds in a plant has also been found to increase as a response to infection by phytopathogens (Lattanzio, De Cicco, Di Venere, Lima & Salerno, 1994), in agreement with the proposed role of these compounds in the plant defence mechanism. Infected plant tissues and resistant tissues have been found to be characterized by a common shift in the metabolic pattern that includes activation of phenol-oxidizing enzymes and peroxidases. Indeed, the degree of resistance has been related to the amount of phenolic compounds oxidized by phenolases (Ohazurike & Arinze, 1996).

In recent years, much attention is being paid to organic production, as an alternative form of agriculture, to obtain high-quality food in an environmentally compatible manner. However, a comparison between the nutritional quality of conventional and organic products of several foods revealed only minor differences (Woese, Lange, Boess & Bogl, 1997).

The application of pesticides and fertilisers has been previously reported to modulate the biosynthesis of phenolics in plants (Daniel et al., 1999; Lea & Beech, 1978; Nicolas et al., 1994). Therefore, the aim of this study was to compare the concentration of total phenolic compounds and the activity of PPO in organically and conventionally grown fruits (peach and pear). The existence of a relationship between these parameters and of differences between fruits obtained with the two cultivation practices was tested. Such differences may be useful for the definition of markers which allow us to distinguish between products of different origin.

2. Materials and methods

Whole fruits (cortex and peel) of peach (*Prunus persica*, cv. Regina Bianca) and pear (*P. communis* L., cv. Williams), either organically or conventionally grown, were obtained from the experimental orchard of the Istituto Sperimentale per la Frutticultura (ISF) at Rome, and were sent to the Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (INRAN, Rome), immediately after picking at commercial maturity stage.

Under the denomination of organic fruits are marked all those products of plant origin which are produced under controlled cultivation conditions in line with the provisions of the EC Regulation on organic forming [Verordnung (EWG) 2092/91 and updatings until 1488/97] as well as with related national laws. These fruits, unlike conventional fruits, are produced without the aid of chemical-synthetic pesticides and largely without the use of soluble mineral fertilisers, within a diverse range of crop rotation and extensive soil tillage. In organic tillage, fruits analyzed in this study also differed with type of ground on which plants were grown: subterranean clover (organic sample A), spontaneous weed cover (organic sample B) and tilled (organic sample C). From the latter type of ground, the conventional corresponding sample was also produced.

Immediately on arrival, parts of the fruit samples were lyophilized (for determination of total polyphenols) and parts were frozen at −40°C (for activity assays).

Total polyphenols were determined by the spectrophotometric method of Joslyn (AOAC, 1999).

The procedure for the determination of PPO activity consisted of two steps: extraction of the enzyme and assay of PPO activity. The experimental procedure was performed in order to optimize analytical extraction conditions of the enzyme and to evaluate the affinity of PPO towards two different substrates.

The extraction of PPO from peach fruits was performed using the method of Kader, Rovel, Girardin & Metche (1997). A 150 g sample of frozen (−40°C) fruits was homogenized with 200 ml of ice-cold (−18°C) solution of pure acetone/water/Triton X-100 (80:19:1,

v/v) in a Waring (Blendor) CB6 24CB blender (15.500 ***g***/min). The resulting homogenate was left at −25°C during 1 h, then filtered under vacuum through a glass filter on a Buchner funnel (G-3); the residue was washed several times using 250 ml of ice acetone (−18°C) until a white powder was obtained. The powder was dried (2 h) at room temperature and stored at −40°C until use for PPO assays.

Appropriate aliquots of powder were suspended in 0.005 mol/l sodium phosphate buffer pH 7.0 containing 0.005 mol/l cysteine and stirred at 4°C for 2 h. The suspension was centrifuged at 2000 rpm for 20 min (4°C) and the supernatant was recovered. This procedure was repeated twice, and the supernatants were collected and centrifuged at 12 000 rpm for 15 min (4°C); collected supernatants were finally used as crude extracts for activity assays.

The extraction of PPO from pears was performed using the procedure of Gauillard and Richard-Forget (1997), with some modifications. Crude samples were homogenised for 3 min with an Ultra Turrax T25 (I-Ka-Labor Technik) blender in 0.05 mol/l sodium phosphate buffer at pH 6.5, containing 0.02 mol/l ascorbic acid and 2% PVPP as a phenolic scavenger. The homogenates were stirred overnight at 4°C. The suspensions were centrifuged (2200 rpm, 15 min, 4°C) and supernatants were recovered. Further extraction of the sediments was carried out in the same buffer, by stirring in an external ice bath. The supernatants were collected and centrifuged (14 000 rpm, 15 min, 4°C) and the recovered supernatants were used for PPO activity assays.

Enzyme activity was tested towards chlorogenic acid and caffeic acid because they were found to be the best substrates for PPO extracted from several fruits, including pear (Gauillard and Richard-Forget, 1997; Kader et al., 1997; Nicolas et al., 1994).

The assay was performed for all samples by incubating different amounts of enzymes (50–200 μl) in the specific solution buffer: sodium acetate buffer (0.05 mol/l) at pH 4.0, and 0.05 mol/l sodium phosphate buffer at pH 6.5, were used for PPO obtained for peaches and pears, respectively.

The total reaction mixture was 3.0 ml. This contained 50, 100 or 200 μl of enzyme extract and complementary amounts of buffer in which 100 μl of 1 mmol/l substrate solution of caffeic acid (18 mg/100 ml) or chlorogenic acid (7 mg/100 ml) (Sigma Chemical Co., St Louis, MO, USA), dissolved in the same buffer used for activity assays, were added. The increase in absorbance at 400 nm for chlorogenic acid or at 420 nm for caffeic acid, for at least 5 min, was followed at 25°C with a Beckman DU7400 spectrophotometer (Beckman Instruments, Fullerton, CA, USA).

One unit of activity of PPO is defined as the amount of enzyme which caused an absorbance increase of 0.001 unit min^{-1} (initial rate) under the conditions of the assay (Kader et al., 1997). The results are reported as enzymatic activity in 100 g of fresh sample (U.E./100 g f.w.).

Data were subjected to analysis of variance. The significance of the differences between means was estimated by Student's *t*-test.

3. Results and discussion

Polyphenol content of conventional and organic peach and pear fruit samples is presented in Table 1. Polyphenols, measured as mg equivalents of tannic acid/100 g fresh sample, ranged from 19.6 to 29.0 for peach samples and from 41.4 to 56.1 for pear samples. All organic peach samples showed higher polyphenol levels than conventional peaches ($P < 0.01$), while polyphenol content of pear samples was higher in organic samples B and C than in conventional pears ($P < 0.05$; Table 1).

Several studies indicate that phenolic compounds contribute to plant resistance to either mechanical stress, such as wounds made by insects or lesions determined during harvest, or biological infections by fungi, bacteria and viruses (Lattanzio et al., 1994; Ohazurike & Avinze, 1996; Lucarini et al., 1999). Therefore, the increase in polyphenol content observed in organic peaches and in samples B and C of organic pears may support the hypothesis (Daniel et al., 1999; Lea & Beech, 1978; Nicolas et al., 1994) of an enrichment in plant defence mechanisms against infestations through an increase in endogenous polyphenols when external pesticides of common use in conventional agriculture are lacking. In many plants, regulation of phenolic metabolism has been observed to depend on several factors (Daniel et al.). Among external factors, the use of pesticides and fertilisers has been found to be responsible for a significant decrease in phenol content in apple fruits (Lea & Beech; Nicolas et al.).

Parallel analysis of PPO activity towards different substrates revealed substantial differences among most of the fruit samples. Results obtained for conventional and organic peach samples are shown in Table 2. A higher PPO activity ($P < 0.001$), towards both caffeic and chlorogenic acid, was observed for organic sample

Table 1
Polyphenol content (mg tannic acid/100 g fresh weight) of conventional and organic fruit samples[a]

Sample	Conventional	Organic		
		A	B	C
Peach	19.6±2.1 a	27.9±0.4 b	29.0±1.7 bc	23.2±0.9 d
Pear	48.2±0.8 a	41.4±3.2 b	51.1.1±1 c	56.1±1.7 d

[a] Values are the average of 10 determinations±S.D. Within the same row, different letters indicate significant differences.

A compared with the conventional fruit, in agreement with the increase in total polyphenols measured between the two samples (Table 1). Also, PPO activity towards chlorogenic acid of organic peach sample C was higher ($P < 0.05$) than that of the conventional sample (Table 2), in line with the increase in polyphenols observed for this sample (Table 1).

A correlation between polyphenol content and PPO activity in peach fruits of the same cultivar has previously been reported (Lee, Kagan, Jaworski & Brown, 1990) and was also observed, with the exception of organic sample B, in this study (Tables 1 and 2).

Table 3 shows the results of PPO activity determinations on conventional and organic (A, B, C) pear samples. The best substrates of PPO in pears have been shown to be 5-caffeic acid derivatives, such as 5′-caffeoylquinic acid (chlorogenic acid); (Amiot et al., 1995; Gauillard & Richard-Forget, 1997; Mosel & Herrmann, 1974). Chlorogenic acid was found to account for more than 70% of total hydroxy-cinnamoyl derivatives in pear samples (Amiot et al.).

Irrespective of the sample, when chlorogenic acid was used, activity of PPO was the highest, as already reported by Gauillard and Richard-Forget (1997) on the basis of a comparison of kinetic parameters toward different substrates obtained with purified pear PPO. A relatively high activity was also measured with caffeic acid (Table 3), in agreement with the results obtained with other fruits (Kader et al., 1997). Activity of PPO towards chlorogenic acid was significantly higher in organic samples B ($P < 0.05$) and C ($P < 0.001$) than in the conventional pear sample. With caffeic acid, it was similarly higher in organic samples B and C than in the conventional fruit ($P < 0.05$). In the case of either organic sample B or C, the increase in PPO activity observed in comparison with the conventional sample paralleled the increase in total polyphenol content (Table 1). A decrease in total polyphenol content and in the activity of PPO, although not significant, was measured in organic sample A (Table 3). Because all the pear samples used in this study showed a comparable stage of maturity, as far as specific parameters such as pH, malic acid, citric acid and soluble sugars content is concerned, the different behaviour of sample A compared with samples B and C cannot be ascribed to a different stage of ripeness of fruits.

PPO, like other oxidases, is an enzyme involved in several metabolic changes and reactions of fruit tissue responsible for tissue browning. Both polyphenol content and activity of PPO have been found to be influenced by maturity stage and postharvest storage of most fruits, as well as by several external and internal factors affecting phenolic metabolism (Amiot et al., 1992; Daniel et al., 1999; Lea & Beech, 1978; Nicolas et al., 1994). PPO activity is also known to be very sensitive to experimental conditions such as extraction conditions and pH used for the assay and it can also vary from one preparation to the other.

On the other hand, previous studies on phenolic composition and polyphenol oxidase activity of peach, pear and apple fruits have shown that, at commercial maturity, major differences were observed only among different cultivars (Amiot et al., 1995; Lee et al., 1990; Nicolas et al., 1994). However, conventional and

Table 2
Polyphenoloxidase activity of conventional and organic peach samples[a]

	Caffeic acid		Chlorogenic acid	
	Unit min^{-1}/ml extract	Unit min^{-1}/100 g f.w.	Unit min^{-1}/ml extract	Unit min^{-1}/100 g f.w.
Conventional	11.1±1.2	2351.9±236.4 a	11.2±1.2	2111.3±160.0 a
Organic A	14.4±0.5	3071.8±102.7 b	20.7±0.8	3393.4±102.0 b
Organic B	6.9±1.9	1693.5±404.4 a	5.3±0.6	1286.7±120.0 c
Organic C	10.8±1.0	2123.7±176.10 a	12.7±1.1	2635.1±191.2 d

[a] Values are the average of at least six determinations±S.D. Within the same column, different letters indicate significant differences.

Table 3
Polyphenoloxidase activity of conventional and organic pear samples[a]

	Caffeic acid		Chlorogenic acid	
	Unit min^{-1}/ml extract	Unit min^{-1}/100 g f.w.	Unit min^{-1}/ml extract	Unit min^{-1}/100 g f.w.
Conventional	3.9±1.0	637.9±55.2 a	4.7±1.8	1011.7±93.4 a
Organic A	2.0±0.3	299.5±78.4 b	5.9±0.7	925.5±109.9 a
Organic B	4.7±0.8	800.1±69.6 c	8.9±1.0	1386.4±146.8 b
Organic C	5.7±0.8	890.1±62.7 cd	17.7±0.5	3157.6±155.8 c

[a] Values are the average of at least six determinations±S.D. Within the same column, different letters indicate significant differences.

organic fruits of the same cultivar were compared in this study. Therefore, the parallel increase between PPO activity and polyphenols, measured in most of the organic compared with conventional peach and pear fruits, and especially that observed between organic samples C and the respective conventional samples (both grown on a similarly–tilled-soil), is likely to be relevant. The relationship demonstrated between polyphenols and PPO activity adds support to the hypothesis that these compounds act together as a system with a role in the overall antioxidant defence system developed by the plant (Zawistowski et al., 1991).

In particular, the increase measured between organic and conventional samples might be the result of changes in phenolic metabolism in plants grown under organic cultivation conditions as a consequence of the absence of chemical-synthetic pesticides and of most of the readily soluble mineral fertilisers used in conventional cultural practices.

Characterization of conventional and organic apple fruits and tomatoes aimed at the identification of compounds developed by plant tissues as part of their defence mechanisms was recently carried out (Lucarini et al., 1999). The results of the study indicated higher levels of both total polyphenols and specific phenolic acids (caffeic and *p*-coumaric acid) in organic than in conventional apples and of ascorbic acid and lutein in organic than in conventional tomatoes.

Changes in the level of phenolics and in the amount and activity of oxidative enzymes, especially phenol oxidase, have been implicated as part of the mechanism of disease resistance that would be realized through inhibition of the polygalacturonase of the pathogen by oxidized phenolics (Ohazurike & Arinze, 1996). It is also possible that biochemical defences are present all the time in a heathy plant, although observed variations in susceptibility with age seem to indicate that they could be developed at particular stages (Amiot et al., 1995; Ohazurike & Arinze).

Because conventional and organic fruit samples of either peach or pear analyzed in this study appeared to be quite homogeneous, as far as external physical characteristics (such as size, weight or colour) are concerned and without physical defects and pathogen contamination, at least as judged by visual observation, the origin of the observed changes requires longer-term field trials as well as a close comparison of results coming from different studies, to be ascertained unequivocally.

If the results of this study are confirmed, parameters such as the activity of PPO or the phenolic levels will be selected as specific endogenous markers for the characterization of organic versus conventional plant products. Further studies, consisting in the concomitant examination of several endogenous compounds with different antioxidant mechanisms, are currently in progress.

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