Changes in Wall-Bound Phenolic Acids, Phenylalanine and Tyrosine Ammonia-Lyases, and Peroxidases in Developing Durum Wheat Grains (*Triticum turgidum* L. Var. Durum)

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Some aspects of phenolic metabolism have been followed during the development of durum wheat grain. Bound ferulic (FA) and *p*-coumaric (PCA) acids have been extracted after alkaline hydrolysis and quantified by high-performance liquid chromatography. They increased to reach a maximum during the hydrical step and then decreased rapidly during grain dehydratation. Changes in phenylalanine ammonia-lyase (PAL) (E.C. 4.1.1.5) and L-tyrosine ammonia-lyase (TAL) (E.C. 4.3.1.5) activities have been monitored all along the development of the grain. TAL activity was maximal 2 days before PAL, at the beginning of the hydrical step. The presence of a maximal peroxidasic activity at the end of the hydrical step should be linked to the decrease of alkaline-resistant bound forms of FA. These results may suggest a possible role of peroxidase in the progressive changes from ester-linked forms of phenolic acids to insoluble derivatives, resistant to alkaline hydrolysis.

Keywords: *Phenolic acids; ammonia-lyases; peroxidases; durum wheat grain; development*

INTRODUCTION

Durum wheat (*Triticum turgidum* L. var. Durum) is mainly cultivated to produce semolina and pasta. The yield depends on cultivation conditions and attacks by different pathogens. Among the diseases affecting the grain itself, black point is distinguished by areas more or less black (Miller *et al*., 1988), which is damaging to agroalimentary use (Dexter and Matsuo, 1982). Black point may derive from fungus attacks (King *et al*., 1981), but climatic factors and cultivation methods strongly influence the appearance and intensity of the disease (Dexter and Matsuo, 1982). In all cases, the blackening which distinguishes the disease results in damages on a cellular scale, probably due to the oxidative degradation of endogenous phenolic compounds. This is a phenomenon generally involved when browning appears in plants (Nicolas *et al*., 1993).

Ferulic (FA) and *p*-coumaric (PCA) acids are the main phenolic acids present in the cell wall of monocots and especially of Gramineae (McCallum, 1989; Hartley *et al*., 1990; Lam *et al*., 1992a; Rybka *et al*., 1993). Ferulic acid constitutes more than 90% of the total phenolic acids in wheat flour (Sosulski *et al*., 1982), and its blue autofluorescence allows its localization in the aleurone cell walls of wheat kernels (Jensen *et al*., 1982; Pussayanawin *et al*., 1988). In the bran of soft and durum wheat (Pussayanawin and Wetzel, 1987), phenolic acids occur in bound forms as conjugates with sugars or proteins, and the presence of feruloylated arabinoxylans has been reported in wheat grain (McDougall, 1993). In fact, the occurrence of FA and PCA in ester linkages with arabinoxylans, pectic polysaccharides, or xyloglucans is now well established, and various speculative proposals for covalent cross-links between hydroxycinnamic acids and cell wall polysaccharides have been made (Bacic *et al*., 1988; Iiyama *et al*., 1994).

Although cereal seedlings have long been used to study enzymology and regulation of phenolic biosynthesis (Creasy, 1987), no extensive results have been obtained up to now in the developing grain. McCallum and Walker (1990) have shown that phenylalanine ammonia-lyase (PAL; E.C. 4.1.1.5) activity was maximal during the early milk stage of soft wheat grain and then declined. In many cases, this enzyme, closely related to the physiological or developmental status of the plant, is coordinated with the presence of other enzymes associated with phenolic biosynthesis (Jones, 1984). Although it has been reported that the purified PAL also exhibited L-tyrosine ammonia-lyase (TAL; E.C. 4.3.1.5) activity (Nari *et al*., 1972), there are indications that some tissues contained distinct forms of the enzyme with differing PAL/TAL ratios (Hanson and Havir, 1981). Up to date, no data have been reported concerning variations of TAL activity during the development of durum wheat grain.

The general objective of our work was to determine if the appearance of black point during ontogenesis and ripening of durum wheat grain could be related to endogenous phenolic compounds. In this general framework, we report here the first part of a study which compares certain aspects of phenolic metabolism in two durum wheat cultivars, Arbois and Primadur, known by French breeders to be respectively susceptible and moderately resistant to black point. Changes in esterified insoluble FA or PCA as well as in PAL, TAL, and peroxidase (PO) activities have been investigated during grain development and ripening.

MATERIALS AND METHODS

Plant Material. Two cultivars of durum wheat, Arbois and Primadur, were sowed at the end of December in the plain of Nîmes (France). The sampling started as soon as the grains contained 70% water and was repeated every 2 days during the main phases of development (Figure 1), until the complete ripening of grains. Master spikes were harvested at each sampling, and only the middle one-third part of the spikes was used (Gurnade and Malet, 1981). Fifty grains were dried for 48 h in an oven at 90 °C for estimation of dry weight. Another part of the sample (300 grains/70 spikes) was kept at −30 °C and freeze-dried before being reduced into powder and used for biochemical analysis. The whole grain, including testa,

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S0021-8561(95)00607-8 CCC: $12.00

pericarp, and endosperm, was used for this analysis. In any case, sampling was repeated three times, and all determinations have been conducted three times independently.

Extraction of Phenolic Acids. Soluble phenolic acids were extracted four times from the powder (500 mg) with 5 mL of methanol−acetone−water (7:7:6) containing 0.5% sodium disulfite, at 4 °C temperature. The pellet was dried to remove organic solvents and then used to extract insoluble phenolic acids according to the procedure of Krygier *et al.* (1982), slightly modified to avoid starch gelatinization. The residue was initially dispersed in 0.5 mL of 4 N NaOH and stirred for 4 h under nitrogen. Sodium borohydride (0.05 mg) was added to stabilize ferulic acid. The solution was then slightly acidified (pH 2) and centrifuged and the residue resuspended twice in water and centrifuged. The combined supernatants were concentrated under vacuum to 1 mL and washed four times with an equal volume of hexane to remove interfering lipids. Free phenolic acids were then extracted four times with diethyl ether. The combined extracts were evaporated to remove the organic solvent, and the residue was solubilized in methanol (200−500 μL).

The extracts (100 μL/injection) were analyzed in duplicate by reversed-phase HPLC using a VARIAN 2000 instrument equipped with a precolumn (Nucleosil, 3 μm, C18) coupled to an analytical C18 column (Machery and Nagel; 250 × 5 mm). Samples were eluted in a solvent consisting of C_2H_3N (acetonitrile) and H_2O (pH 2.6), according to a gradient of 10−80% C_2H_3N within 50 min with a 1 mL min^{-1} flow rate. The amounts of phenolic acids injected in the system were within a range where the absorption of the phenolic acids at 320 nm was linear with concentration.

Extraction and Determination of Enzymatic Activities. The freeze-dried powder (0.5 g) was homogenized at 0 °C for 20 min in chilled 0.05 M potassium phosphate buffer (1 mL, pH 6.6) with 1% poly(vinylpyrrolidone) (w:v) and 0.01% bovine albumin (w:v) and then centrifuged for 15 min (40000*g*). In order to remove extraneous phenolic compounds, the supernatant, stored on ice, was shaken for 30 min with a Dowex resin (1X2) (0.25 g) previously equilibrated with the extraction buffer. The extract was centrifuged to remove the resin, and the supernatant was used as a source of crude enzymes.

PAL and TAL activities were assayed during 10 min by UV spectrophotometry (290 nm), through the production of cinnamic (PAL) or *p*-coumaric (TAL) acids. The assay mixture consisted of crude enzyme (450 μL) plus L-phenylalanine (22.6 mg mL^{-1}) or L-tyrosine (0.7 mg mL^{-1}) in 0.05 M sodium borate buffer, pH 8.6 (250 μL). Triplicate assays were performed for each extract, and enzyme activities were calculated according to classical previous data (McCallum, 1989).

For estimation of peroxidase activity, the extract was prepared as described above, except that bovine albumin was not included. The assay mixture consisted of 0.1 M Tris-maleate buffer (pH 6.5) containing 40 mM guaiacol plus hydrogen peroxide (30 μL) and 10−200 μL of crude enzyme. Oxidation of guaiacol was followed at 470 nm.

RESULTS

Biochemical parameters have been followed during several years (between 1990 and 1993). Results were comparable when meteorological variations were taken into account. To simplify graphical presentation, only 1991 results are presented here.

Grain development (Figure 1). No major differences were observed between the two cultivars concerning the main phases of their development. During a first period, dry matter and water content accumulated rapidly in the grain. Between June 5 and 21, while the water content became constant, the increase of dry matter was linear. According to Geslin and Jonard (1948), this corresponds to the hydrical step associated with the phase II of grain development. After June 21, the water content decreased progressively and the dry matter accumulation stabilized. This last period was associated with the grain ripening.

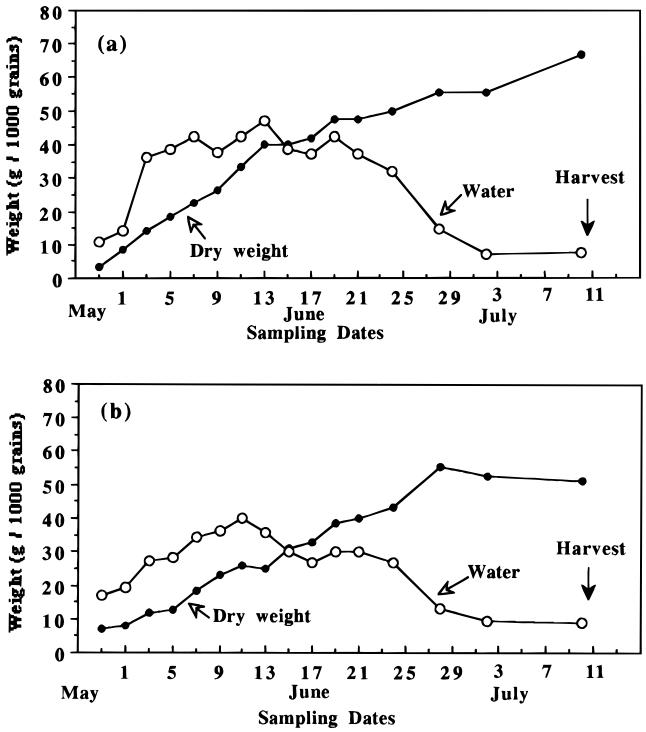

Figure 1. Variations of water content and dry weight in the grain of two durum wheat cultivars during development: (a) cv. Arbois and (b) cv. Primadur. The hydrical step (see text) appears at the same period (June 5−21) for the two cultivars. Standard errors averaged 3% of the mean for all determinations.

Changes in FA and PCA Contents. Because the content of soluble phenolic compounds in wheat grain is always very small, our results concern phenolic acids released after alkaline hydrolysis. FA was always the major acid (98% of total phenolic acids), and only a little amount of PCA was observed. FA and PCA contents present a maximum during the hydrical step (Figure 2), this maximum being obtained more or less early according to the acid and the cultivar.

Changes in PAL, TAL, and PO Activities. Significant levels of PAL and TAL activities were found during grain development and showed one or two maximums during the hydrical step. They were in the same level of magnitude for the two enzymes. Later on, these activities decreased progressively and could not be detected in mature seeds (Figure 3). The highest TAL activity occurred a few days before the maximum of PAL activity. Significant higher PO activities occurred during the hydrical step (Figure 4) with only a weak residual level in mature seeds.

Comparison between Cultivars. Comparison of FA content revealed significant differences between cultivars: Arbois always exhibited a higher peak than Primadur. Moreover, levels of PAL, TAL, and PO activities were always smaller in Primadur grains.

DISCUSSION

Variations in water content and dry matter accumulation (DW) in durum wheat grains during their development were similar to those already described for soft wheat (Gurnade and Malet, 1981; Schnyder *et al.*, 1993). The phase II of grain development generally takes place 12−35 days after anthesis and is defined as the hydrical step. This period, associated with protein storage and starch accumulation in endosperm, results from a DW decrease in external pericarp and a DW increase in internal pericarp, testa, and endosperm

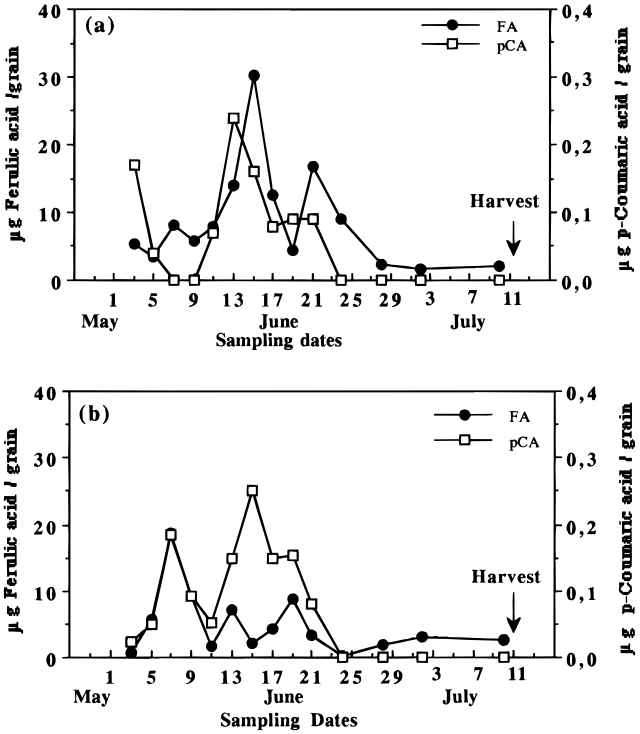

Figure 2. Changes in ferulic and *p*-coumaric acid contents during development of cv. Arbois (a) and Primadur (b). Standard errors averaged 5% of the mean.

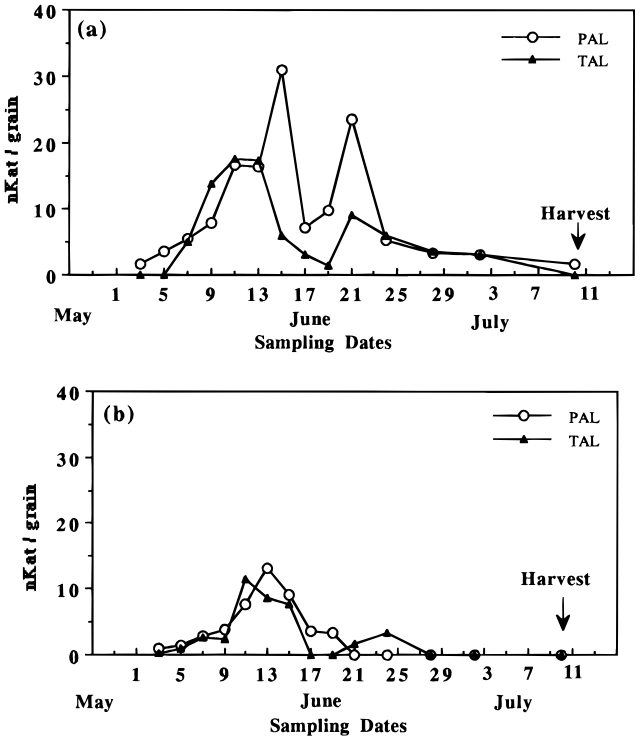

Figure 3. Changes in PAL and TAL activities during development of cv. Arbois (a) and Primadur (b). Standard errors averaged 5% of the mean.

(Housley *et al.*, 1982). The following ripening phase is characterized by a rapid dehydratation in all tissues, causing many perturbations (Bewley and Black, 1995).

Our results indicate FA is mainly present as bound forms in cell walls, likely in the aleurone layer as previously shown in earlier studies (Fulcher *et al.*, 1972; Rybka *et al.*, 1993). It is likely that the maximal FA content shown at the end of phase II is due to the protein-rich cell layer development. However in our conditions, the global FA variations differed slightly

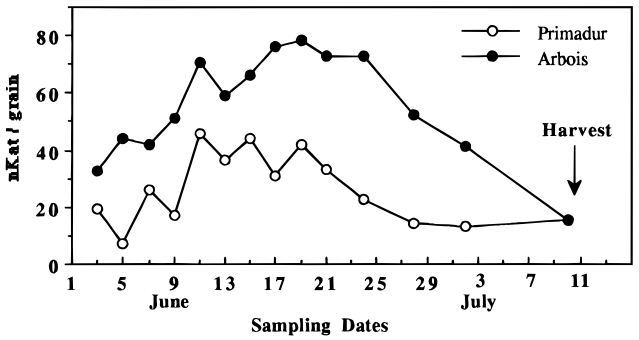

Figure 4. Peroxidase activity during development of cv. Arbois (a) and Primadur (b). Standard errors averaged 3% of the mean.

with genotype, the cultivar Primadur exhibiting a significant FA peak in the beginning of phase II when the aleurone layer is not completely differentiated (Morrisson *et al.*, 1975). The high FA level present during the second phase of wheat development may protect aleurone cells against endogenous hydrolytic enzymes, as demonstrated in spinach (Fry, 1983). The role of PCA in cell wall cross-linking is less clear, although it is esterified to polysaccharides (Mueller-Harvey and Hartley, 1986) and mainly ester- and ether-linked to lignin (Scalbert *et al.*, 1985; Lam *et al.*, 1992b).

Decrease in phenolic acid concentrations during ripening of soft wheat grain has been previously reported (McCallum and Walker, 1990), and a similar change is observed in durum wheat. This decrease in oxidizable phenolic acids should result from the contact with oxidative enzymes occurring during the breakdown of cellular structure in the pericarp and testa at the end of milk stage and during further maturation. The decrease in the wall-bound FA and PCA contents should be correlated with the formation of alkali-resistant bonds occurring in cross-linked polymers in cell walls (Scalbert *et al.*, 1985; Lam *et al.*, 1992b; Iiyama *et al.*, 1994). It has been suggested previously that the variations in PO activity during the grain development should be the consequence of differential evolution of PO in different tissues (Kruger and Laberge, 1974). These variations closely resemble those observed for FA, which suggests PO participates in phenolic oxidation and in the formation of covalent cross-links between phenolics and wall polymers (Siegel, 1993; McDougall, 1993; Iiyama *et al.*, 1994). Depending on whether these links are degradated or not by alkali treatment, PO should control the level of FA and PCA susceptible to be liberated with this treatment.

Although it is not possible to completely exclude the supply of phenolics in the phloem sap, the presence of FA and PCA in the wheat grain likely results from their biosynthesis *in situ*, as suggested by the high PAL and TAL levels observed during the hydrical step. The regulatory role of these enzymes in phenolic biosynthesis is now classical (Creasy, 1987; McCallum and Walker, 1990), and the fair relationship between the variations in PAL and TAL activity on the one hand and in FA and PCA levels on the other hand allows the extension of this conclusion to durum wheat.

The shift between the maximums observed in TAL and PAL activities may suggest these enzymes act, in fact, sequentially during phenolic biosynthesis. At first, this observation has to be extented to a larger number of cultivars and related to the avaibability of the two substrates concerned, phenylalanine and tyrosine. Furthermore the physiological meaning of such an observation remains unclear.

Our results strongly suggest an active phenolic metabolism during grain ontogenesis of durum wheat, with regard to both biosynthesis and degradation. Although the successive phases of grain development are very similar between the two cultivars studied, significant differences have been shown, especially concerning FA accumulation level and most enzyme activities. They always appear to be higher in cv. Arbois which is known to be more susceptible to black point. Nevertheless, it would be too quick to draw a definitive conclusion concerning the relations between phenolic metabolism and susceptibility of durum wheat to black point. Esterified forms of FA, as quantified here after alkaline treatment, likely represent only a part of the cell wall-bound FA, and a better study of alkali-resistant bound phenolic polymers has to be done to explain correctly the differences between cultivars and the decrease observed during grain ripening. Furthermore, a larger scale of cultivars is now being studied, in addition to the setup of experimental conditions allowing to control the initiation of black point in durum wheat.

ABBREVIATIONS USED

PAL, phenylalanine ammonia-lyase; TAL, tyrosine ammonia-lyase; PO, peroxidase; FA, ferulic acid; PCA, *p*-coumaric acid; DW, dry weight.

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Received for review September 11, 1995. Revised manuscript received March 29, 1996. Accepted April 19, 1996.[⊗] This research was supported in part by grants from a MRE-IRTAC agreement (no. 90 G 0759).

JF950607C

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1996.