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Evolution of Robusta Green Coffee Redox Enzymatic Activities with Maturation

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Oxidation reactions in coffee involve redox-sensitive polyphenols and appear to control the fragmentation of coffee storage proteins both in solution and during roasting. Coffee-specific nitrogenous flavor precursors may derive from this process. Accordingly, data converge to suggest that the redox status of the green bean before roasting might control the development of subsequent redox reactions during roasting. Consequently, we decided to identify biological events that may trigger or prevent oxidation during maturation of the coffee cherry and set the final redox status of the green bean. In a previous study, we observed that the sensitivity of green coffee to oxidative processes decreased along maturation. By using the very same samples originating from open-pollinated Robusta clones, we followed the activity of three essential redox enzymes: catalase (CAT), peroxidase (POD) and polyphenoloxidase (PPO). While CAT and POD activities increased with maturation, PPO activities decreased. Thanks to the identification of an atypical immature subclass, it appeared that CAT might be an essential factor in setting the final redox status of the green bean before the roasting event.

KEYWORDS: Green coffee; maturation; redox enzymes; catalase; peroxidase; polyphenoloxidase; redox status

INTRODUCTION

Chlorogenic acids (CGAs) form a family of esters derived from defined transcinnamic acids (caffeic and ferulic) and quinic acid. They are the most abundant polyphenols found in the coffee bean, which is one of the richest sources of CGAs in general (7−10 and 5−7.5% on a dry weight basis for Robustas and Arabicas, respectively) (*1*). Plant polyphenols are redox-sensitive and become oxidized as a consequence of oxidative stress, changing the redox status of the plant. The capacity of a plant to fight oxidative stress should then ultimately determine its redox status. As observed in our previous study, the sensitivity of the coffee cherry to oxidation decreased during maturation and might, after processing of the cherry, influence the sensory profile of cup coffee (*2*). Oxidation also appeared to control the fragmentation of coffee storage proteins both in solution and during roasting (*3*). Coffee-specific nitrogenous flavor precursors may derive from this process. Hence, the redox status of the green coffee bean before roasting might determine, to some extent, the chemistry triggered by the roasting processes itself.

Typical plant stressors are high light, drought, heat, mineral deficiency, low temperature, chilling, frost, wounding, UV-A, UV-B, ozone, insects, bacteria, fungi, viruses, herbicides, air pollution, acid rain, acid fog, acid morning dew, heavy metals, etc. It can be deduced from this long list that a plant's defense mechanisms must be constantly triggered. One of these mechanisms, as studied in *Salmonella typhimurium* and *Escherichia coli* used as model systems, implies the OxyR redox-sensitive protein, which belongs to the LysR-type family of transcriptional regulators (LTTR) (*4*, *5*). The crucial first step in redox regulation is the sensing of redox-signaling molecules such as superoxide ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$) and hydrogen peroxide (H_2O_2). Upon exposure to H_2O_2, the OxyR protein undergoes a thiol-disulfide switch, which then modulates its affinity to DNA and ultimately triggers the expression of several genes including the *KatG* encoding CAT. Hence, H_2O_2 appears to function as an intracellular second messenger. Its relative stability, small size, and ability to diffuse across cell membranes facilitate its action at locations far from its site of formation. CAT (CAT; EC 1.11.1.6) constitutes the most efficient and elaborate system available in both plants and animals to control H_2O_2 concentrations. It catalyzes the dismutation of H_2O_2 to water and oxygen. Each molecule of CAT displays four polypeptide chains, each composed of more than 500 amino acids, and nested within this tetrad are four porphyrin heme groups. CAT is common among organisms that can grow in the presence of air. In plants, CAT essentially occurs as multiple isoforms (*6*, *7*) with temporal and spatial specificities in their expression (*8*). They are located predominantly in peroxysomes (called glyoxysomes when specialized in the conversion of fatty acids into carbohydrates where β-oxidation of fatty acids generates a lot of H_2O_2), but also exist in the mitochondria and cytoplasm.

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POD (POD, EC 1.11.1.7) is also a heme-containing enzyme of major interest. It is known to oxidize various phenols and enediols substrates coupled to the consumption of H_2O_2 by a complex peroxidatic reaction cycle. POD may, however, convey multiple and opposite functions involving consumption and production of ROS. As such, it plays an essential role in cell wall stiffening and cell wall loosening (*9*, *10*, *11*), two important processes for plant growth.

PPO (PPO, EC 1.14.18.1) is a bifunctional copper-containing enzyme, also known as catechol oxidase, catecholase, diphenol oxidase, *O*-diphenolase, phenolase, cresolase, and tyrosinase. It triggers enzymatic browning by oxidizing phenols, usually impairing the appearance and the sensory and nutritional properties of raw material and food. In general, PPO and its substrates occupy different cellular compartments and interact upon membrane disruption. Hydroxylation of monophenols (cresolase activity) is an extra function shared by many PPO (*12*, *13*). All identified PPO do, however, convert *O*-dihydroxyphenols to *O*-benzoquinones (catecholase activity) (*14*). Oxygen is required as a second substrate to achieve either cresolase or catecholase activity. In coffee, PPO was identified and characterized in leaves and endosperm (*15*). PPO seems to be involved in plant resistance against diseases (*16*, *17*).

In this study, we investigated CAT, POD, and PPO activities in well-characterized green coffee subclasses that were previously defined (*2*). Criteria for segregation were the metabolic notion of maturation as determined by the color of the coffee cherries (classes), combined to the observed absence, presence, and color of the silver skin on the respective green beans (subclasses). The distribution of enzymatic redox activities among subclasses should help us to establish their potential involvement in determining the redox status of the green bean in relation to maturation. While CAT activity is expected to prevent oxidation of polyphenols, POD and PPO activities are expected to trigger oxidation of polyphenols.

To comply with typical harvesting procedures, the color of the cherry was used to define maturation classes. Arabicas are commonly "pick-harvested", a method where coffee cherries are handpicked according to color to harvest ripe cherries selectively. On the other hand, Robusta coffee is usually "strip-harvested", a method where coffee cherries are harvested without consideration of maturation (color). Hence, Robusta coffee generally includes a high percentage of immature beans potentially degrading cup coffee quality. Accordingly, this study is expected to provide some clues as to the impact of immature beans in Robusta coffee chemistry.

MATERIALS AND METHODS

Origin, Harvesting, and Preparation of Samples. *Harvesting in Relation to Maturation* (*Maturation Classes*). Robusta green coffee samples (*Coffea canephora* Pierre ex A. Froehner, *Rubiaceae*) were obtained from Cagayan, The Philippines. Cherries were harvested according to color from open-pollinated Robusta clones. Harvesting was completed on three consecutive days by manual picking of the cherries. Dark to red brown (over-ripe), yellow orange to red (red ripe) and light green (green mature) cherries were selectively harvested on the first, second, and third day of harvesting, respectively. Cherries with characteristic color were harvested regardless of size and shape.

Postharvest Treatment. Cherries were then processed by the dry method. The average duration of drying was 15 days. The exact drying period, however, varied according to maturation stages. Green cherries were dried longer than over-ripe ones. No sorting or pre-classification based on size and shape were made. In the absence of a huller for such small quantities, the beans were hulled manually, using a pestle and mortar.

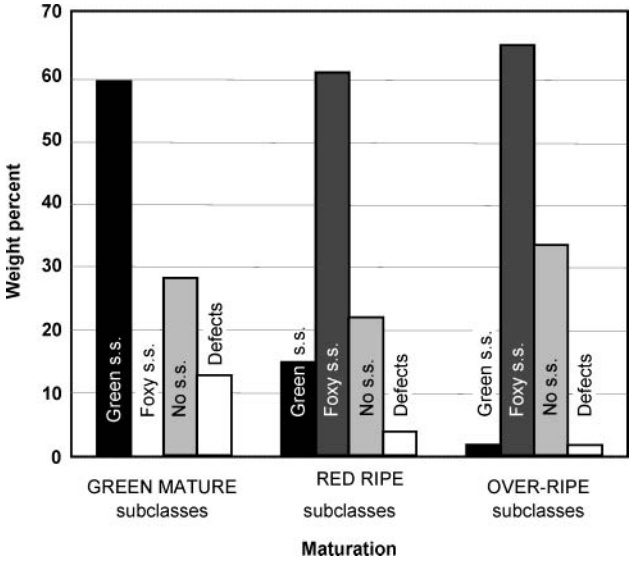

Figure 1. Quantitative distribution of subclasses within maturation classes (100 g green coffee/class). Classes were named green mature, red ripe, and over-ripe and correspond to light green, yellow orange to red, and red brown cherries, respectively. Segregation criteria for subclasses were absence of silver skin (no s.s.) or presence of silver skin (either green (green s.s.) or reddish-brown (foxy s.s.)). Broken and malformed beans (e.g., shell beans and black beans) were considered as defects.

Extra Sorting of Original Samples (*Subclasses*). A high degree of heterogeneity was observed in all three green coffee samples (maturation classes) received from The Philippines. Hence, we decided to create subclasses: two subclasses in relation to the color (green or red brown) of the integument, usually called silver skin; a subclass for the beans that did not harbor any silver skin, and a fourth for defective beans (classified under "defects", broken, misshaped (e.g., shell beans) and black beans) (**Figure 1**). We also investigated enzymatic activities in immature black beans. Accordingly, we created a new subclass with the black beans that originally belonged to the "green mature defects" subclass and was renamed "green mature black beans".

Enzymatic Activities. *Reagents.* Reagents were from the following sources: *O*-Dianisidine and 4-methylcatechol were from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade.

Extraction Procedure. Green coffee samples were frozen in liquid nitrogen and ground to a fine powder in a pre-cooled IKA laboratory mill (Janke & Kunkel, Staufen, Germany). For each sample, 1 g of green coffee powder was then extracted at 4 °C for 15 min with 5 mL of 0.1 M Na−Pi, pH 6.0. After centrifugation of the decanted samples (15 min at 15 000*g*), the supernatant was recovered and spin-desalted (5 min at 600 g) in small Sephadex G-25 columns, previously equilibrated in 10 mM Na−Pi, pH 6.0. The eluate (10 mM Na−Pi, pH 6.0) was directly used for the assays.

Protein content was determined with the Bio-Rad (Glattbrugg, Switzerland) microassay kit for soluble proteins.

Assays. All 3 enzymatic activities were determined spectrophotometrically, using H_2O_2 (CAT, POD), *O*-dianisidine (POD) and 4-methylcatechol (PPO) as substrates.

CAT activity was determined in a reaction mixture containing 0.2 mL of 500 mM Na−Pi buffer (pH 7.0), 0.1 mL of 490 mM H_2O_2, 0−0.2 mL of desalted extract and distilled water to a final volume of 1.0 mL. The reaction was initiated by the addition of the extract, and the decrease of absorbance at 240 nm was recorded with a spectrophotometer (Kontron Uvikon 930) for 10 min at 25 °C.

POD activity was determined in a reaction mixture containing 0.2 mL of 500 mM Na−Pi buffer (pH 6.0), 0.09 mL of 49 mM H_2O_2, 0.01 mL of *O*-Dianisidine (20 mg dissolved in 2 mL of ethanol), 0−0.05 mL of desalted extract and distilled water to a final volume of 1.0 mL. The reaction was initiated by the addition of the extract and increase of absorbance at 420 nm was measured for 5 min at 25 °C.

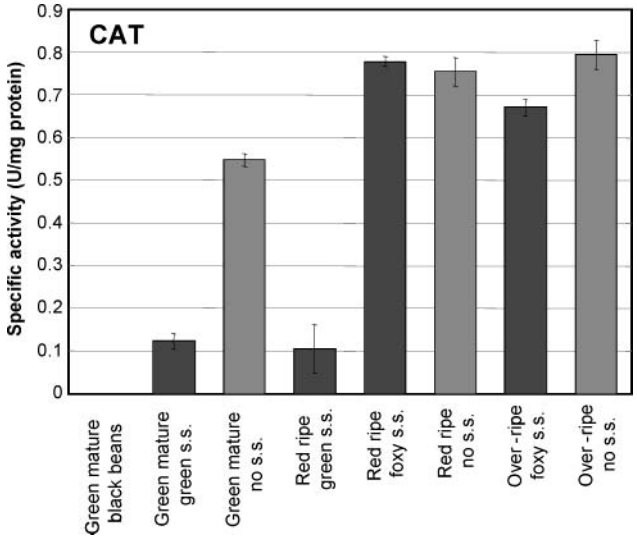

Figure 2. CAT activity profile in Robusta green coffee subclasses. Subclasses (dark gray bars for silver skin and light gray bars for no silver skin subclasses) were arranged according to increasing levels of maturation (from left to right).

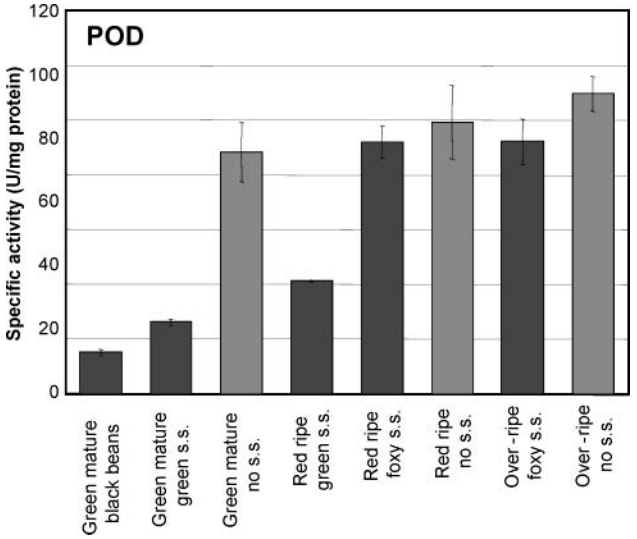

Figure 3. POD activity profile in Robusta green coffee subclasses. Subclasses (dark gray bars for silver skin and light gray bars for no silver skin subclasses) were arranged according to increasing levels of maturation (from left to right).

PPO activity was determined in a reaction mixture containing 0.2 mL of 500 mM Na−Pi buffer (pH 6.0), 0.5 mL of 40 mM 4-Methylcatechol, 0−0.3 mL of desalted extract and distilled water to a final volume of 1.0 mL. The reaction was initiated by the addition of the extract and increase of absorbance at 400 nm was recorded for 10 min at 25 °C.

Specific enzyme activities were reported as arbitrary units (U/mg protein). One unit of enzyme activity was defined as the amount of enzyme causing an increase or respectively a decrease of absorbance of 0.1 unit per min at the corresponding wavelength. Enzymatic activity assays were performed in triplicate.

RESULTS AND DISCUSSION

Enzymatic activities were recorded in green coffee samples corresponding to all the Robusta subclasses that had been previously generated (*2*) (**Figure 1**). A new subclass was, however, created by selecting immature black beans found among beans of the "defects" subclass of the green mature class. This subclass was named "green mature black beans". In Brazil, immature black beans are referred to as "café verde-preto" and are known to severely affect cup coffee quality (*18*).

CAT and POD Activity Profiles. H_2O_2 is a strong mediator of oxidative stress responses (*19*). It might also be integrated in regulation processes driving maturation of coffee cherries (*20*, *21*). In relation to the processing of H_2O_2 by CAT and POD, we first consider the maturation subclasses that displayed a silver skin. We observed a dramatic increase of CAT and POD activities at middle maturation (from green mature to red-ripe green beans, corresponding originally to light green and yellow orange to red cherries, respectively) (**Figures 2 and 3**). At a late maturation, over-ripe green beans (corresponding to red brown cherries) displayed CAT and POD activities similar to red ripe beans, but only to the subclass that displayed a foxy silver skin as compared to the green silver skin subclass. Actually, we observed a near 8-fold increase in CAT activity moving from the red-ripe green to the red-ripe foxy silver skin subclass (**Figure 2**)! In parallel, POD activity also increased, but less abruptly (about 2-fold, **Figure 3**). The red-ripe green silver skin subclass displayed a CAT activity level similar to the green mature green silver skin subclass, which belongs to an early maturation stage. Accordingly, this red ripe subclass resembled the green mature subclass as to its CAT expression behavior (**Figure 2**). Its green silver skin testifies that pigmentation of the pulp must have been weaker than that for the foxy silver skin subclasses. The red brown color of foxy silver skin subclasses most likely originated from the red pigments produced by the pulp during maturation. Thus, the red ripe green silver skin subclass almost certainly comprises the less pigmented cherries of the red ripe class (the yellow orange cherries, as compared to the red cherries). If we now take into account that activities were calculated as specific activities per milligra of protein and that levels of storage proteins increased during maturation (*22*), one can estimate that the overall rise of CAT and POD activities along the maturation process may even be under-estimated. According to our data, sensitivity of coffee beans to oxidative stress and its consequences should dramatically lessen with maturation. If we now consider the no silver skin subclasses, we observed that they all, including the least mature one (green mature no silver skin), displayed considerable CAT and POD activities (**Figures 2 and 3**). Hence, silver skin and no silver skin subclasses strongly differed at an early maturation stage as to their respective CAT and POD activities and their capacity to fight oxidative stress. This feature was not observed at advanced maturation stages (**Figures 2 and 3**). High CAT activities displayed by no silver skin subclasses might, in fact, explain the characteristic absence of silver skin. CAT, by destroying H_2O_2, should prevent oxidative cross-linking of polyphenols with various molecules at the silver skin interface. Due to its lower adherence, the silver skin easily leaves the bean during postharvest processing generating a "no silver skin" bean. The more advanced maturation subclasses all expressed strong CAT activities, but not all were devoid of silver skin (**Figures 2 and 3**). Early expression of high CAT activities might then be essential to avoid adherence of the silver skin, suggesting that oxidative processes (formation of quinones and subsequent polymerization reactions) are not, or are only partially, reversible.

While both CAT and POD activities reduce H_2O_2 to water, enzymatic oxidation of phenols is specifically coupled to peroxidatic reaction cycles, favoring subsequent polymerization processes and affecting the redox status of the bean. Besides the typical peroxidatic cycle, POD exhibits oxidase and hydroxylating activities involved in polymerization and depolym-

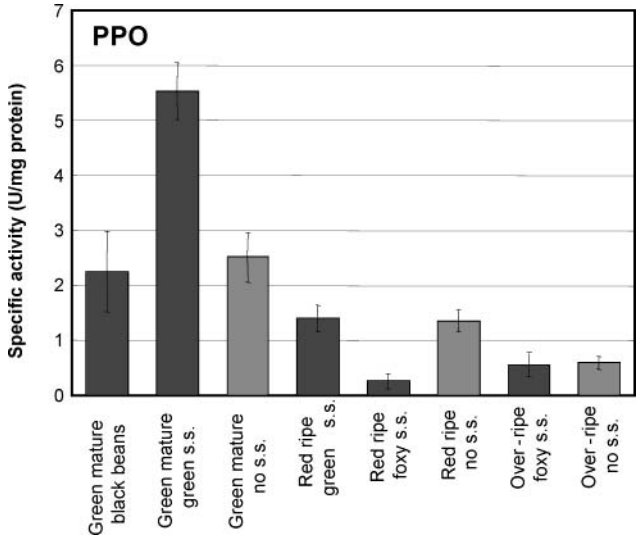

Figure 4. PPO activity profile in Robusta green coffee subclasses. Subclasses (dark gray bars for silver skin and light gray bars for no silver skin subclasses) were arranged according to increasing levels of maturation (from left to right).

erization reactions (*11*). POD activities rely on the presence of H_2O_2. Consequently, CAT must exert a very tight control on POD activities in general, and in particular, during oxidative stress. Interestingly, the immature black beans ("café verde-preto") displayed no CAT activity at all (**Figure 2**). As suggested by their denomination, black beans are only found among immature beans, which are characterized by very low CAT activity levels. Their dark color may thus be ascribed to the occurrence of unhampered H_2O_2-dependent oxidative processes. Furthermore, immature black beans are known to severely affect coffee cup quality (*19*), pointing toward a possible link between oxidative status and flavor.

Among the 4 subclasses that were tested in our previous study (*2*) for quantitative losses of CGAs during aerobic incubations of green coffee suspensions (borate buffer, 0.05 M, pH 8.5), the least mature samples (the two green silver skin subclasses including the red ripe green silver skin) displayed the strongest losses. Now, we observed that these very same subclasses harbored very low CAT activities (**Figure 2**). This leads us to believe that high CAT activities displayed by more mature and "no silver skin" subclasses may efficiently protect CGAs against oxidation during oxidative stress. Indeed, strong CAT activities were detected in the respective sodium borate green coffee suspensions upon addition of H_2O_2 (data not shown). Hence, H_2O_2 appears to be an essential mediator of CGA oxidation in green coffee.

PPO Activity Profiles. PPO activities strongly decreased along maturation from green mature to over-ripe (**Figure 4**). At the green mature stage, the no silver skin subclass displayed about half of the PPO activity of its silver skin counterpart (**Figure 4**). The immature black beans subclass, however, displayed lower PPO activity than the green mature subclass green silver skin (**Figure 4**). Most probably, a significant fraction of PPO was inactivated by POD- and PPO-produced quinones, which in turn inhibit PPO (*23*). For the remaining subclasses, decreasing enzyme levels rather than increasing enzyme inhibitions are likely to explain reduced PPO activities upon maturation. Some decrease, however, may be partially ascribed to increasing levels of storage proteins along maturation. Mature subclasses should then be less prone to oxidation upon membrane disruption, a prerequisite for PPO to meet its substrate. For the reasons discussed above, the use of PPO activities as quality indicators as proposed by De Amorim and Silva (*24*) can be misleading. Action of PPO requires the disruption of substrate- and PPO-specific compartmentalization. It supposes harsh conditions in the field and/or harsh postharvest treatments. As mentioned by Mazzafera and Robinson (*15*), low quality may be attributed to membrane damage rather than to PPO per se. In our study, samples were all processed under optimal and similar (if not identical) conditions. In fact, evolution of PPO activities upon maturation as measured in green coffee was similar to what was observed in coffee fruit endosperm (*15*). This again suggests that PPO activity remains essentially silent in healthy and adequately processed beans.

H_2O_2 is central to plant defense mechanisms (*19*) and may also play an important role in various steps of coffee processing (*3*). In this study, we suggest that CAT and H_2O_2 are essential players in setting the redox status of the green bean during maturation. High CAT activities also corresponded to high POD activities, indicating that a fast and strong POD response may arise upon CAT saturation or failure (release of inhibitors, for instance). Hence, a single overwhelming oxidative event may lead to rapid and strong polymerization of CGAs. One has also to take into account that low CAT activities might contribute to set coffee characteristics, which pertain to (strip-harvested) Robustas, and more specifically, to immature beans that display a strongly adherent silver skin.

Because PPO activity is launched upon damaging of the beans, enzymatic browning and low in vitro activities should be considered as a marker for inadequate treatment of the crop during harvest, processing, and storage. In healthy beans, however, PPO appears to play only a secondary role with respect to quality and redox status.

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