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Lipase activity and antioxidant capacity in coffee (*Coffea arabica* L.) seeds during germination

Sonia Patui[a], Luisa Clincon[a], Carlo Peresson[a], Marco Zancani[a], Lanfranco Conte[b], Lorenzo Del Terra[c], Luciano Navarini[c], Angelo Vianello[a], Enrico Braidot[a,*]

[a] Department of Agricultural and Environmental Sciences, Unit of Plant Biology, University of Udine, via delle Scienze 91, 33100 Udine, Italy
[b] Department of Food Science, University of Udine, via Sondrio 2/A, 33100 Udine, Italy
[c] Illycaffè s.p.a, via Flavia 110, 34147 Trieste Italy

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ABSTRACT

In this paper, lipase activity was characterized in coffee (*Coffea arabica* L.) seeds to determine its involvement in lipid degradation during germination. The lipase activity, evaluated by a colorimetric method, was already present before imbibition of seeds and was further induced during the germination process. The activity showed a biphasic behaviour, which was similar in seeds either with or without endocarp (parchment), even though the phenomenon showed a delay in the former. The enzymatic activity was inhibited by tetrahydrolipstatin (THL), a selective and irreversible inhibitor of lipases, and by a polyclonal antibody raised against purified alkaline lipase from castor bean.

The immunochemical analysis evidenced a protein of ca. 60 kDa, cross-reacting with an anti-lipase antibody, in coffee samples obtained from seeds of both types. Gas chromatographic analyses of free fatty acid (FFA) content confirmed the differences shown in the lipolytic activity of the samples with or without parchment, since FFA levels increased more rapidly in samples without parchment. Finally, the analyses of the antioxidant capacity showed that the presence of parchment was crucial for lowering the oxidation of the lipophylic fraction, being the seeds with parchment less prone to oxidation processes.

1. Introduction

Coffee is an economically relevant commodity, internationally traded as green coffee [1]. The *Coffea* genus contains around 100 species, being *C. arabica* and *C. canephora* the most important ones for commercial purposes. The coffee plant is an evergreen shrub or small tree, native to the forests in the Ethiopian mountains. Its white and bisexual flowers form a short inflorescence called glomerule. The fruit is classified as a drupe, consisting of a smooth skin or exocarp, a soft yellowish pulp or mesocarp, and a greyish-green fibrous endocarp surrounding the seeds, which are commonly called coffee beans. The exocarp becomes transiently yellow and then red at the final stage of development. The mesocarp is rich in reducing sugars, sucrose and water. The endocarp (also called parchment, or "pergaminho") is a hard and lignified tissue, protecting coffee seeds against digestive enzymes from the gut of frugivorous animals [2].

Coffee seeds represent vital organs, in which various metabolic reactions take place during post-harvest processing, e.g. germination-related metabolism and stress metabolism [3]. Coffee seeds are classified as intermediate seeds [4], able to withstand considerable drying in comparison to recalcitrant seeds. Such seeds cannot tolerate extreme water loss as in the case of orthodox ones [5]. Indeed, coffee beans can tolerate desiccation down to a water content of about 7–12%, although further drying leads to rapid loss in viability [3,4]. Similarly, the coffee embryo is very sensitive to low temperature, and it is heavily damaged when seeds are kept to temperature lower than 25 °C. In particular, it is demonstrated that the lower the storage temperature, the more critical the seed water content becomes [6]. Seed storage for medium periods at 25 °C is possible if environment relative humidity is maintained around 50%, while for conservation at freezing temperatures, a lower content in coffee seed moisture and hermetic conditions are required [7].

Both intermediate and recalcitrant seeds are characterized by the lack of dormancy and partial desiccation, features that are associated to an appreciable metabolism, which makes the seeds ready

Abbreviations: FFA, free fatty acids; TAGs, triacylglycerols; ROS, reactive oxygen species; DAI, days after imbibition; DGGMR, 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin)-ester; THL, tetrahydrolipstatin; MAGs, monoacylglycerols; DAGs, diacylglycerols.

* Corresponding author. Tel.: +39 0432 558792; fax: +39 0432 558784.
E-mail addresses: sonia.patui@uniud.it (S. Patui), luisa.clincon@uniud.it (L. Clincon), carlo.peresson@uniud.it (C. Peresson), marco.zancani@uniud.it (M. Zancani), lanfranco.conte@uniud.it (L. Conte), Lorenzo.DelTerra@illy.com (L. Del Terra), Luciano.Navarini@illy.com (L. Navarini), angelo.vianello@uniud.it (A. Vianello), enrico.braidot@uniud.it (E. Braidot).

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to germinate. In coffee seeds, the germination-related processes have been monitored by the expression of enzymes related to germination, like isocitrate lyase, and by β-tubulin accumulation, as markers of cell cycle activity [8]. The extent and time-course of this metabolism depends strongly on post-harvest processing; indeed, germination-related reactions are activated earlier during "wet" processes in comparison to "dry" ones [3,8]. Another aspect of coffee processing is represented by the unusual maintenance of the parchment in coffee seeds after "wet" processes. In this case, green coffee beans exhibit high viability for longer periods, if compared to the hulled beans obtained by "typical wet" and "dry" processes, where the parchment is usually removed [9].

In addition, this maintenance of viability seems to be positively correlated to the preservation of green coffee quality during prolonged storage [9]. Indeed, the active metabolism of green coffee seeds could have a negative impact on the quality features of commercial beans through the deterioration of well-known precursors of coffee flavour, such as free amino acids and free sugars, which could lead to a poor final cup quality [9,10]. During green coffee storage, the "off-flavours" are also produced by the oxidation processes acting on the lipid fraction [9,11]. Oxidative stress, lipid hydrolysis, phospholipid loss and decrease in concentrations of two crucial antioxidant compounds, such as glutathione and ascorbate, are involved in the ageing of coffee seeds stored at 20 °C and at intermediate relative humidity (81%) [5]. Therefore, the control of oxidative processes is an essential aspect, not only for storage but also for germination of coffee seeds, since the embryo viability could be affected by the uncontrolled release of free fatty acids (FFA).

The lipid fraction in green *C. arabica* seeds represents a significant part of dry matter, ranging from 13 to 17%, consisting mainly of triacylglycerols (TAGs) [11], which are responsible for the major aroma in roasted beans [12]. Since the lipid fraction is a typical substrate for degradation reactions, the content of FFA is positively correlated to increases of temperature, oxygen content and moisture, during storage for 18 months [11]. The role of lipid hydrolysis in the behaviour of coffee seeds during storage has been further confirmed by the negative correlation observed between coffee seed viability and FFA content [13]. Nevertheless, it is not yet clear how environmental conditions could affect lipid metabolism during storage and the initial phases of germination. As a consequence of the features described above, the storage behaviour of coffee beans depends on the result of various and complex factors, acting at different times.

Considering the crucial role of lipid metabolism during both seed germination and post-harvest processing, these aspects appear to be scarcely investigated [14]. In particular, to our knowledge, lipase activity in coffee seeds has been only preliminarily identified. Lipases are ubiquitous enzymes belonging to the class of serine hydrolases (triacylglycerol acylhydrolases EC 3.1.1.3) with a Ser-His-Asp triad in their active site. They mainly catalyze the hydrolysis of ester bonds in monoacylglycerol, diacyglycerol and TAGs into FFA and glycerol at the oil/water interface. In plants, these enzymes play a relevant role in the mobilization of reserves (stored as TAGs) during oilseed germination [15,16].

A further aspect involved in both germination and storage is the control of the levels of reactive oxygen species (ROS). As recently proposed, low levels of ROS are present in ungerminated dormant seeds, while during germination ROS act as signalling molecules leading to seed dormancy release. Nevertheless, the homeostasis of ROS should be strictly controlled since their overproduction leads to oxidative damage in both germinating and stored seeds [17].

In this work lipase activity was examined in both ungerminated and germinating *C. arabica* L. seeds, either in presence or absence of the parchment. In addition, the antioxidant properties of germinating coffee seeds were analyzed, aiming at identifying possible correlations between the antioxidant protection and the lipid degradation processes.

2. Materials and methods

2.1. Plant material and seed germination

Coffee seeds (*C. arabica* L.) harvested in Colombia were provided by illycaffè spa, Trieste, Italy, as a single bulk, stored in polypropylene plastic bags for a maximum of two months at room temperature. The seeds were provided with the parchment and then were divided into two lots: the first had the parchment manually removed (− parchment), while the second still conserved the endocarp (+ parchment). The seeds were kept in water for 7 days at 28 °C in the dark. After imbibition, the seeds were sown in perlite at 28 °C in the dark and daily watered. At each sampling day (0, 3, 7, 10, 12, 15, 18, 21, 24, 28 days after imbibition, DAI), about 40 ungerminated or germinating seeds were collected, the parchment was manually removed when present, and the seeds were finally frozen in liquid nitrogen and stored at −80 °C. The experimental design consisted of three independent replicates for all the considered variables. The replicates were performed using the same seed lot for three progressive sowing within two months.

2.2. Acetone powder preparation

Ten g of frozen coffee beans was ground by a blender (Ika Werke, Staufen, Germany) to obtain a fine powder. Then, the powder was stirred for 4 h at 4 °C in 50 ml of chilling acetone (−20 °C), and subsequently centrifuged at 1900 × *g* (SS-34 rotor, Sorvall) for 15 min. The pellet was dried under nitrogen, resuspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.4 M sucrose, homogenized with an Ultra-Turrax (Ika Werke, Staufen, Germany), and finally centrifuged at 11,900 × *g* (SS-34 rotor) for 20 min. The supernatant was filtered through cotton gauze and stored at −80 °C. This preparation was used to evaluate the lipase activity, protein content, and to perform SDS-PAGE and Western blotting.

2.3. Assay of lipase activity

Lipase activity was assayed by a colorimetric method, using a kit from Randox (Lipase, Crumlin, UK). The assay was based on the hydrolysis of a specific substrate (1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin)-ester) (DGGMR): this chromogenic compound is cleaved by the catalytic action of lipases into 1,2-o-dilauryl-rac-glycerol and glutaric acid-(6-methyl resorufin) ester, an unstable intermediate. The latter decomposes spontaneously in alkaline solution to form glutaric acid and methylresorufin, a coloured compound that was determined photometrically by a Multilabel plate reader at 570 nm (Wallac 1420, PerkinElmer Waltham, MA, USA). The reaction mixture was composed by 5 μl (approx. 7.5 μg protein) of extract, 155 μl of buffer and the reactions were started by the addition of 40 μl of DGGMR. The analysis was carried out for 2 h at 30 °C. The enzymatic activity was expressed as nmol methylresorufin mg^{-1} protein min^{-1}, using an extinction coefficient $\varepsilon = 54 \times 10^3\ M^{-1}\ cm^{-1}$ at 570 nm.

The analysis of lipase activity inhibition was performed using tetrahydrolipstatin (THL) and an anti-lipase antibody (Ab) raised against the lipase of castor bean (*Ricinus communis* L.) (Agrisera, Cernusco sul Naviglio, Milan, Italy). THL (up to 300 μM) and anti-lipase Ab (up to 0.6 ng) were added to the reaction mixture in the assay and incubated for 30 min at 30 °C.