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Relevance of endo- β -mannanase enzyme in coffee seed deterioration process

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The endo- β -mannanase acts on the hemicellulose fraction of the endosperm cell walls, mainly mannans and galactomannans. This process weakens cell walls and allows radicle protrusion during seed germination, but may also occur during the deterioration process. Thus, the aim of this research was to determine the activity of endo- β -mannanase enzyme in dry coffee seeds and in soaked seeds, evaluating its relationship between physiological qualities. Coffee seeds obtained by different processing methods (natural, fermented and demucilated) and drying (sun, shade and dryer) were used. Seed quality was evaluated by germination and tetrazolium tests, and the endo- β -mannanase enzyme activity was determined in dry seeds and after 10 days of soaking. From the results, it was concluded that there is significant inverse relationship between the physiological quality of coffee seeds and the expression of endo- β -mannanase, and seeds with lower percentages of germination and viability of embryos have a higher activity of the enzyme. After ten days of soaking, coffee seeds had higher expression of endo- β -mannanase as compared to the dry seeds for all treatments of fruit processing and drying.

Key words: *Coffea arabica* L., processing, drying, physiological quality.

INTRODUCTION

The germination of coffee seeds occurs slowly and unevenly and many factors have been suggested as likely responsible for these characteristics. Among them, a resistant endosperm layer surrounding the embryo may

contribute to germination delay. For germination to occur it is necessary that the embryo breaks the barrier imposed by the endosperm tissues surrounding the embryo. This tissue layer is called ‘cap region’ and it

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consists of high cellulose and hemicellulose polysaccharides content, conferring the embryo resistance and protection (Silva et al., 2005; Eira et al., 2006).

Therefore, in order for the germination process to occur, some enzymes play an essential role promoting cell walls weakening and softening of the cap region, allowing radicle protrusion. α -Galactosidase, β -mannosidase and endo- β -mannanase enzymes are usually considered as the main responsible for hydrolysis of mannans present in the cap region during germination of coffee seeds (Marracini et al., 2001; Nunes et al., 2006; Joet et al., 2013). These enzymes act in the hydrolytic degradation of cell walls, allowing radicle protrusion (Nonogaki et al., 1992; Silva et al., 2004). Cap endosperm weakening by endo- β -mannanase enzyme has been suggested as a prerequisite for the germination of tomato seeds (Mo and Bewley, 2003), *Datura ferox* (Arana et al., 2006), pepper (Caixeta et al., 2014) and coffee seeds (Silva et al., 2004).

There are two steps which characterize weakening of endosperm structures surrounding the coffee embryo; the first one increased cellulase enzyme activity and the second increased endo- β -mannanase activity (Schröder et al. 2009). The role of these enzymes is regulated by phytohormones as abscisic acid, which inhibits both enzyme and gibberellin, inducing endosperm degradation and consequently promoting germination (Bewley et al., 2012; Silva et al., 2004).

Different isoforms of endo- β -mannanase has already been identified to be linked to the germination process in coffee seeds. Four types of seeds were soaked (Silva et al., 2004) and eight different isoforms using germinated seeds (Marraccini et al., 2001). These results suggest that different endo- β -mannanase isoforms have varied functions during coffee seeds germination and subsequent seedling growth. However, little is known about the role of these enzymes in seeds with different quality levels as well as the presence of the enzyme in dry seeds.

Besides participating in the germination process, some studies have associated the activity of endo- β -mannanase enzyme to coffee seeds deterioration process, given the increased enzyme activity during storage, when seeds loose quality (Veiga et al., 2007). Therefore, the loss of quality in coffee seeds is related, among other factors, with increased activity of endo- β -mannanase enzyme.

Post-harvest treatments as processing and drying stand out among the factors, which may affect the quality of seeds and coffee beans. Recent results have shown that changes that occur in coffee seeds during the post-harvest operations influence the chemical composition and integrity of enzyme systems, affecting its physiological quality (Bytof et al., 2007; Saath et al., 2014; Taveira et al., 2015). Therefore, the aim of this

study was to determine endo- β -mannanase enzyme activity on dry and soaked coffee seeds, evaluating the relationship between this activity and the effects of processing and drying of seeds in physiological quality.

MATERIALS AND METHODS

The study was conducted at the Seed Central Laboratory, Department of Agriculture in Universidade Federal de Lavras. *Coffea arabica* L. fruits were harvested in the cherry stage and subjected to three different types of processing: natural (seed kept in the fruits themselves), fermented (fruit mechanically peeled and seeds demucilated by fermentation in water during 24 h at 25°C) and demucilated (peeled fruit and mucilage removed, both mechanically). Three drying methods were used: in the sun, in the shade and in a drier at 35°C, until seeds reached moisture of $11 \pm 1\%$. After processing and drying, physiological quality and activity of endo- β -mannanase enzyme were assessed.

To assess the quality of seeds, germination test was conducted according to requirements from the Rules for Seed Analysis (Brasil, 2009), with results expressed in percentages. The tetrazolium test was conducted with four replicates of 25 coffee seeds, which were soaked in water for 36 h for extraction of embryos, and kept in 0.5% tetrazolium solution at 30°C for 3 h. The embryos were ranked into viable and non-viable according to location and extent of damage (Clemente et al., 2012).

Endo- β -mannanase enzyme activity was determined on 50 dry seeds and on coffee seeds soaked in water for 10 days, under constant temperature of 30°C. The seeds were grinded in a cooled mill at 4°C, with polyvinylpyrrolidone antioxidant (PVP). In microtubes containing 200 mg of ground seeds, 600 μ L of extraction buffer containing 0.1 M HEPES and 0.5 M NaCl (pH 8,0) plus ascorbic acid (5 mg of acid for every ml of buffer) was added. Then, the microtubes were vortexed for 1 min and centrifuged at 10,000 g for 30 min at 4°C. The supernatant was applied to the gel which was made with 6 ml LBG (*Locust Bean Gum*-Sigma), 0.24 g agarose (*Qbiogene*) and 24 mL buffer pH 5.0. Gel holders were covered with *Gelbond film* and the gel was applied to it. After solidification, the gel was stored in refrigerator for 24 h and then it was drilled with a hole punch of 2 mm. In each hole, was applied 2 μ L of enzyme extract in 3 replicates of each sample. The gel was kept in a germination chamber at 25°C for a period of 21 h in the dark, in a moist chamber (plastic trays lined with two layers of moistened paper towel and sealed with plastic wrap) for the enzyme to act. For reading, the gel was first washed in distilled water, then washed in buffer (gel buffer) for 30 min and washed again in distilled water. The gel was covered with Congo red dye 0.5% for 30 min and placed in ethanol for 10 min to remove the dye. After removing ethanol with distilled water, it was added, 1 M NaCl solution until visual observation of white halos in the holes containing the samples. At that time, a diameter measure of the samples was performed with a caliper. A standard curve generated by commercial endo- β -mannanase from *Aspergillus niger* (Megazyme) was used to calculate the activity of the endo- β -mannanase enzyme, performed according to Downie et al. (1994).

Physiological evaluations results were analyzed in a completely randomized design (3x3 factorial design), with three processing methods for fruits (natural, fermented and demucilated) and three drying methods (drying in the sun, shade and dryer) with four replicates. The endo- β -mannanase enzyme activity results were analyzed in a completely randomized design (3x3x2 factorial design) with three processing methods for fruits (natural, fermented and demucilated) three drying methods (drying in the sun, shade and in dryer) and two methods of preparing the seeds (dry and

soaked seeds) with three replications. Data were subjected to variance analysis. Means were compared using the Tukey test ($p > 0.05$).

RESULTS AND DISCUSSION

The variance analysis showed a significant effect between processing and drying factors on the germination tests results and significant effect of fruit processing on the embryos viability in the tetrazolium test. Coffee seeds from processing via fermentation, showed better physiological performance as compared to other treatments, regardless of the drying type (Table 1). Seeds submitted to mechanical depulping (demucilation) and dried in shade also showed similar results.

There was no significant interaction of factors on the viability of embryos in tetrazolium test, only seen in the processing methods. Regardless of the drying method employed, seeds obtained through fermentation and mechanical depulping showed higher embryo viability results in the tetrazolium test as compared to the coffee beans processed by natural method (Table 2).

Higher values was found in the viability of embryos than in the germination test. The tetrazolium test is performed in a shorter time, thus reduces possible adverse factors, for example, attack of microorganisms, in which case can occur in the germination test affecting the test evaluation, and it does not happen in the tetrazolium test. Thus, small differences can be found between the results of both tests (Clemente et al., 2012; Krzyzanowski et al., 1999). On the other hand, differences between results of the germination test and the tetrazolium test have been observed when evaluating the physiological quality of coffee seeds (Dussert et al., 2006; Coelho, 2015). In these studies, the differences between these results are more common in poorer quality seeds and have been attributed to increased endosperm sensitivity to stress conditions than embryos.

Regarding the types of processing, there were better results in physiological quality of coffee seeds coming from pulped and demucilated fruits, which can be related to the effect of processing on the chemical and enzymatic composition of the seeds, as already observed in other studies of coffee beans. Superior drink characteristics were observed in pulped coffee as compared to natural coffees (Selmar et al., 2006; Bytof et al., 2007). These authors observed that in coffee processed using a wet method, which showed better quality, there is greater expression of the ICL gene, which codes for the isocitrate lyase enzyme, and this expression increases more rapidly during the processing of pulped coffees as compared to expression in natural coffees. The isocitrate lyase enzyme and endo- β -mannanase are related to the germination process.

As for the endo- β -mannanase enzyme activity results in the dry seeds and soaked seeds, there was a significant

Table 1. Coffee seed germination percentage under different methods of processing and drying.

Processing	Germination (%)		
	Drying		
	Sun	Shade	Dryer
Natural	28.0 ^{cA}	42.0 ^{bA}	40.0 ^{bA}
Fermented	86.0 ^{aA}	81.0 ^{aA}	74.0 ^{aA}
Desmucilated	67.0 ^{bB}	86.0 ^{aA}	75.0 ^{aB}
CV (%)	13.34		

The average followed by different letters (lowercase in the column and capital in line) are statistically different from each other, at 5% significance by Tukey test.

Table 2. Percentage of viability of embryos in coffee seeds in tetrazolium test under different processing methods.

Processing	Tetrazolium
	Viability (%)
Natural	80.33 ^b
Fermented	97.33 ^a
Desmucilated	94.33 ^a
CV (%)	4.45

The average followed by different lowercase letters is statistically different from each other, at 5% significance by Tukey test.

interaction between processing and drying factors. With the results presented in Table 3 and Figure 1, it is observed that in coffee seeds from natural processing, there was increased activity of endo- β -mannanase enzyme in relation to other types of processing. Furthermore, the soaked seeds also have increased activity of endo- β -mannanase than dry seeds.

In studies on the regulation of coffee seeds germination, Silva et al. (2005) observed increased activity of endo- β -mannanase and β -mannosidase enzymes to 8 days of soaking. After seed germination, indicated by the root protrusion, the enzyme activity reduced.

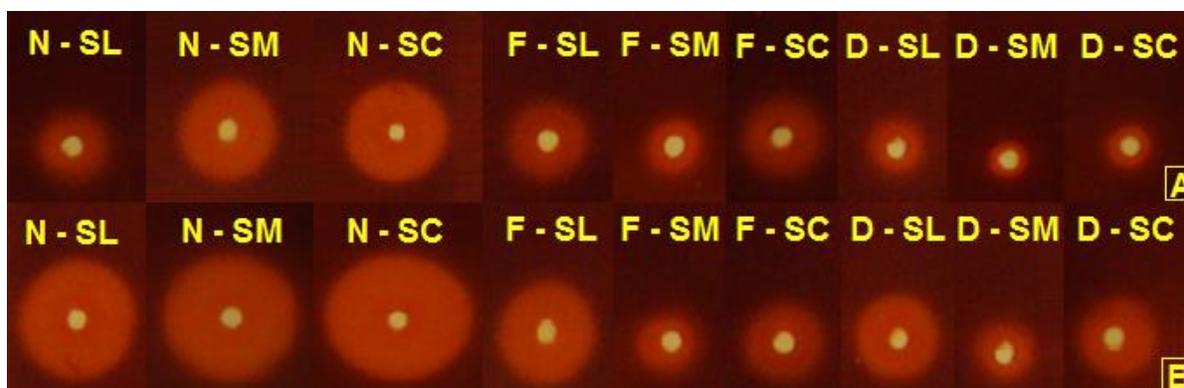
In this study, the major enzymatic activity of the endo- β -mannanase was linked to loss quality in coffee seeds, as better physiological performance results were observed in seeds that were withdrawn from the fruit that had mesocarp removed by mechanical demucilation or by fermentation with water and dried in shade, which presented greater endo- β -mannanase enzymatic activity. This shows an inverse relation between physiological quality of coffee seeds and the endo- β -mannanase activity.

In the study of Silva et al. (2005), the endo- β -mannanase enzyme activity was determined during

Table 3. Endo- β -mannanase activity ($\mu\text{mol}\cdot\text{min}^{-1}$) in soaked and dry coffee seeds obtained from different methods of processing and drying.

Processing	Drying					
	Drying		Sun		Shade	
	Soaked	Dried	Soaked	Dried	Soaked	Dried
Natural	8.406 ^a	5.518 ^a	5.138 ^a	1.722 ^b	9.191 ^a	4.670 ^a
Depulped	2.502 ^b	1.970 ^b	4.451 ^b	2.816 ^a	1.579 ^b	1.372 ^b
Desmucilated	2.196 ^b	1.264 ^c	2.453 ^c	1.442 ^b	1.787 ^b	0.933 ^b
CV (%)	6.83					

The average followed by different lowercase letters in column are statistically different from each other, at 5% significance by Tukey test.

**Figure 1.** Endo- β -mannanase enzyme activity, in agarose gel, in coffee beans (*Coffea arabica*) subjected to different types of processing: natural (N), fermented (F), demucilated (D); and to different methods of drying sun (SL), shade (SM) and dryer (SC). Dry seeds (A) and Soaked seeds (B).**Table 4.** Activity of endo- β -mannanase ($\mu\text{mol}\cdot\text{min}^{-1}$) soaked seeds and dry coffee seeds obtained by different methods of processing and drying, dried and soaked.

Drying	Processing					
	Desmucilated		Depulped		Natural	
	Soaked	Dried	Soaked	Dried	Soaked	Dried
Sun	2.453 ^{Aa}	1.442 ^{Ba}	4.451 ^{Aa}	2.816 ^{Ba}	5.138 ^{Ac}	1.722 ^{Bb}
Shade	1.787 ^{Ab}	0.933 ^{Bb}	1.579 ^{Ac}	1.372 ^{Ac}	9.191 ^{Aa}	4.670 ^{Ba}
Dryer	2.196 ^{Aab}	1.264 ^{Bab}	2.502 ^{Ab}	1.970 ^{Bb}	8.406 ^{Ab}	5.519 ^{Ba}
CV (%)	6.83					

The averages followed by the same uppercase letter in row and lowercase letter line in column do not differ statistically from each other, at 5% significance by Tukey test.

germination process, when it acts to weaken the cell walls and soften the endosperm region where root protrusion occurs. It is noteworthy that during the deterioration process degradation of cell walls by action of endo- β -mannanase also occurs and is thus related to the deterioration of seeds.

Regarding the drying method (Table 4), there was increased activity of endo- β -mannanase enzyme in seeds

dried in the sun, and lower activity in seeds dried in a mechanical dryer for seeds demucilated mechanically or demucilated via fermentation (de-pulped). As for seeds kept in the fruits themselves (natural process), there was an accentuated activity of the enzyme in the seeds dried in shade, followed by those dried in a mechanical dryer. According to Borém et al. (2008), natural coffee grains require longer exposure to air drying than the depulped

so that a reduction of moisture can occur. The same occurs in coffee beans drying process and may lead to denaturation of some enzymes (Saath et al., 2014). On the other hand, longer drying time can provide increased activity of some antioxidative enzymes involved in response to neutralize reactive oxygen species produced under stressful conditions caused by the slower drying, as observed by Dussert et al. (2006). The same was observed in this study, where greater activity of endo- β -mannanase enzyme was observed in treatments whose seeds have been subjected to stress conditions such as seeds dried in the fruits themselves.

Regarding the soaking of coffee beans, there is increased activity of the endo- β -mannanase enzyme in comparison with the dry seeds (Table 4). According to Silva et al. (2004) and Farias et al. (2015), immediately after the start of imbibition, isoforms of endo- β -mannanase enzyme was observed, occurring even before radicle protrusion, in the cap region surrounding the radicle, other isoforms of the same enzyme were detected later in other regions of the endosperm, with progressive increase in enzyme gene expression along the germination time.

In a study using seeds harvested in cherry and green cane maturation stage, increased activity of endo- β -mannanase enzyme was observed in coffee beans harvested in the cherry stage, when seeds had better physiological quality as compared to green cane stage (Veiga et al., 2007). Furthermore, the authors observed a gradual increase in enzyme activity during storage of these seeds, when loss of quality was detected.

During storage, there is a reduction in seed quality due to a deterioration progress, which, among other events of degradation of cell walls and damage to membrane systems, occurs. Endo- β -mannanase enzyme acts to break down mannans present in cell walls (Silva et al., 2004). Thus, the increased activity of this enzyme in poorer quality seeds can be related to the advance of deterioration process, as noted in the seeds that had a worse physiological quality.

The endo- β -mannanase enzyme is a preexistent enzyme but is also synthesized "again" when the seeds are soaked (Ren et al., 2008). In this study, it is possible to see that the expression of endo- β -mannanase is noticeably higher in seeds soaked for 10 days in all treatments when compared with the dry seed treatments (Table 3 and Figure 1).

Iglesias-Fernández et al. (2011) assessed the expression of four genes involved in the expression of endo- β -mannanase enzyme (*AtMAN7*, *AtMAN6*, *AtMAN2* and *AtMAN5*) in *Arabidopsis thaliana* seeds subjected to periods of 0, 10, 20, 24, 30, 36, 42 and 48 h germination, by Real Time PCR. The *AtMAN5* gene showed higher expression in dry seeds, and after 24 h of soaking the expression of this gene reduced drastically. The *AtMAN2*, *AtMAN6* and *AtMAN7* genes showed elevated

expression until the period of 42 h of germination; however, within 48 h, expression was substantially reduced, showing that in fact, the activity of endo- β -mannanase participates in walls breaking processes to allow germination. Right after the radicle protrusion, the enzyme activity reduces. On the other hand, the enzyme activity may vary between dry and soaked seeds, with dry seeds having lower endo- β -mannanase activity as compared to the enzyme activity in soaked seeds (Dirk et al., 1995). This fact is due to activation of the enzyme during the germination process. Thus, with soaking, synthesis of enzymes occurs to act on the molecules to break energy production necessary for embryo growth. Previous studies have demonstrated that peak activity of this enzyme in germination occurs near the root protrusion (8-9 days) in the cap endosperm of coffee seeds (Silva et al., 2005).

Analyzing all these results, it can be suggested that the action of endo- β -mannanase enzyme in coffee seeds is complex and is associated with different physiological events from the process of maturation, germination, until the loss of quality with deterioration. Therefore, there is need for additional studies, including molecular studies to identify all different isoforms of the enzyme as well as the functions of each one.

Conclusion

There is an inverse relationship between the physiological quality of coffee seeds and the expression of endo- β -mannanase. After ten days of soaking, coffee seeds had higher expression of endo- β -mannanase as compared to the dry seeds. Endo- β -mannanase enzyme activity in *C. arabica* L. seeds is related to the deterioration process.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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