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Full Length Research Paper

Lipolytic activity and chilling requirement for germination of some almond cultivars

Sellema BAHRI

Département de Biologie, Faculté des Sciences de Tunis, Université de Tunis El Manar, 2092, Tunis, Tunisia.
E-mail: sellemab@yahoo.fr. Tel: + 21671 812600 post 362. Fax: +216 71885 480.

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Traditionally, cold stratification for three weeks is commonly used for almonds (*Prunus amygdalus* Mill.) seeds germination. In this study, the effect of cold stratification in two almonds varieties: ''Achaak'' a Tunisian cultivar and ''Tuono'' an Italian cultivar was tested. The study revealed, for the first time, that only the Tunisian cultivar ''Achaak'' was able to start up its germination after 16 h of imbibition, at 26°C without any previous chilling. In these conditions, (i) hypocotyl elongation was not affected compared to the Controls, suggesting that the cold requirement of a seed is an adaptation to climatic factors; (ii) the ability of germination of 5-year-old Achaak seeds was not totally affected; (iii) the partially purified lipase showed a true neutral activity, detected at the 3-day-old germinating step. This is the first published data regarding the almond lipase identification.

Key words: Germination, imbibition, stratification, chilling, triacylglycerol lipase, *Prunus amygdalus* Mill.

INTRODUCTION

Almond is a nut which represents an important part of the diet of many people worldwide. This tree nut has been cultivated since ages. In Tunisia, the almond tree has been grown extensively since the Carthaginian era, 8th century B.C. (Jaouani, 1976). Almond tree is the second agricultural product in Tunisia after the olive tree with approximately 22 millions of trees covering more than 302 000 ha. Tunisian almond plantations are located throughout the country and are characterized by an important genetic diversity (Gouta et al., 2008; 2010). The central and southern agricultural area of the country, contribute 45% of the national production.

Almond seeds have been extensively explored. Many authors (Felipe, 1981; Bewley, 1997; Egea et al., 2003; Garćia-Gusano et al., 2005; Ghayyad et al., 2010; Rahemi et al; 2011) have used different techniques such as scarification, stratification, chilling or hormonal application to study their effects on almond seed germination and the break of dormancy. The present study focused on oil seed composition: the dicotyledonous seed contains between 53.7 to 65% of lipids. Oleic (59 to 78%) and linoleic (19 to 30%) have been reported as the main fatty acids in these oil seeds (Garcia-Olmedo and Marcos-Garcia, 1971; Soler et al., 1988). According to Martin–Carratala et al. (1999), nine triglycerides have been detected by high-performance liquid chromatography (HPLC) in diverse almond cultivars.

In oil seeds, triacylglycerol molecules (TAGs) are stocked during maturation in subcellular droplets called lipid bodies or oleosomes (Yatsu and Jacks, 1972). During the first steps of germination, this lipid reserve is rapidly mobilized and lipases (EC. 3.1.1.3) catalyse the initial reaction of oil degradation that converts TAGs to sugars and other metabolites (Bewley and Black, 1994). Lipase activity has been found in several seeds with lipid bodies such as castor bean (Huang, 1984) corn (Lin and Huang, 1983) or sunflower seeds (Bahri et al., 2012). But little is still known about plant lipases. This is probably due to their low activity *in vitro*. Recently, the gene encoding the castor bean acid lipase has been cloned and sequenced (Eastmond, 2004). In contrast, mammal and microorganism enzymes have been well studied (Verger and de Haas, 1976; Winkler et al., 1990; Brady et al., 1990). The aim of this paper is to search for optimal germinating conditions including chilling requirement and

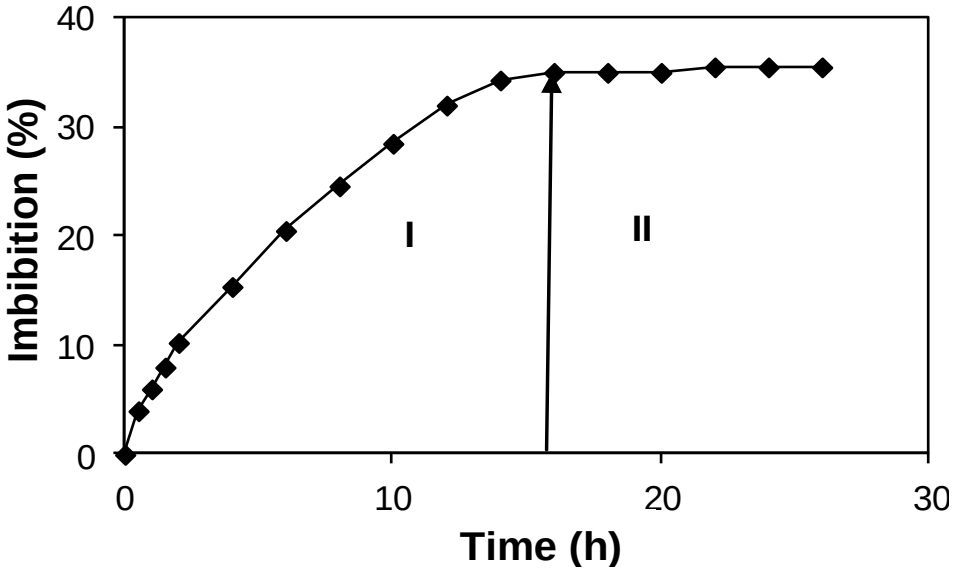

Figure 1. Almonds cv ‘‘Achaak’’ seeds water uptake kinetics. Phase I: imbibition phase, Phase II: germination phase stricto sensu. Imbibition was calculated as follows: % Imbibition = ((Fresh weight of seed - dry weight of seed)/(Fresh weight of seed)) × 100.

to study lipolytic activity for a local almond cultivar.

MATERIALS AND METHODS

Plant materials

Samples of almond seeds (*Prunus amygdalus* Mill., var. ‘‘Achaak’’), both just after harvest and 5-year-old aged seeds, were used for the present study. This Tunisian cultivar is well adapted to drought. Var ‘‘Tuono’’ syn. ‘‘Mazetto’’ an old introduction Italian cultivar was used just after harvest. Seeds were provided by the ‘Olive Tree Institute’ (Sfax-Tunisia). After removing the exocarpe, the selected seeds were soaked at room temperature in running water, seminal tegument was also removed. Seeds were sterilized with 2% thirame fungicide and two different trials were carried out. In the first set of controls, seeds were stratified in sterilized sandpits at 4°C for 3 weeks, and then germinated in Petri dishes, containing moist layers of cotton and filter paper, in the dark at 26±1°C. In the second one, seeds did not undergo the pre-required cold treatment and were directly germinated in the dark at 26±1°C.

Almond seeds fractionation

We adapted the method of Qu et al. (1986) to prepare almonds soluble lipase extracts. The supernatant obtained after centrifugation at 10 000 g (S_{10}) was used as the soluble enzymatic fraction.

Lipase activity measurement

The enzymatic activity was measured by a colorimetric method (Hirayama and Matsuda, 1972; Beisson et al., 2000). The substrate (olive oil) was purified according to the method by Hulanicka et al. (1964) controlled by thin layer chromatography and emulsified in a 10% gum arabic. The reaction was carried out in a shaker water bath. Lipase activity was measured at 514 nm against an appropriate blank. Palmitic acid (1 mM) was used as standard.

Protein determination

The protein content of the supernatant (centrifuged at 10 000 g) was determined using the Bradford (1976) method. All manipulations were carried out at 4°C. All assays were performed in triplicate.

RESULTS

Germination conditions: Imbibition step

As for most seeds, germination begins with imbibitions; the almonds seeds were soaked in water for 24 h. Figure 1 shows two phases: the initial imbibition phase (phase I) in which an intense seed’s tissues water absorption took place for 16 h (phase I), followed by the stabilization of the tissue hydratation (phase II) corresponding to the germination phase *stricto sensu*..

Effect of temperature during germination

In order to test the effect of temperature on almonds seeds germination, two almond varieties were used: ‘‘Achaak’’ a Tunisian cultivar and ‘‘Tuono’’ an Italian cultivar, were put to germinate without pre-required cold treatment, at a temperature of 26°C. Hypocotyl growth was hand measured. At the first germination day, Achaak hypocotyl elongation increased from 1.9 to 29 mm at the end of germination. While for “Tuono” the hypocotyl

Table 1. Almond seeds hypocotyl elongation at 26°.

Parameter	Germination day					
	1	2	3	4	5	6
Tuono Hypocotyl (mm)	-	-	-	-	-	2
Achaak Hypocotyl (mm)	1.9	2	4	15	22	29

Figure 2. Germination steps of almonds (cv ‘‘Achaak’’) without previous chilling treatment.

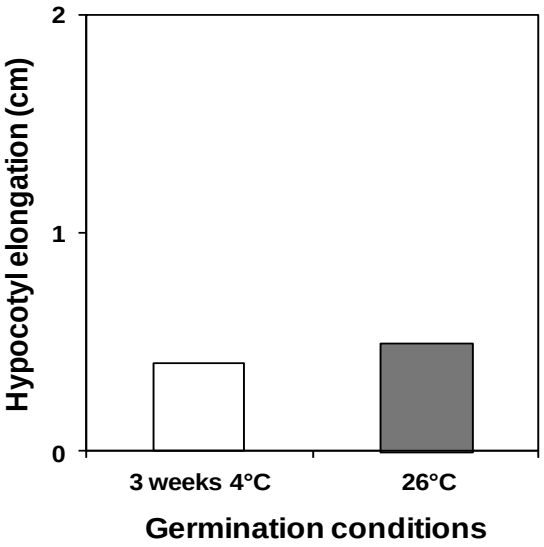

Figure 3. Three-day-old hypocotyl elongation in cold pre-required and without previous chilling germination conditions.

remains unchanged until 6 days after which an elongation of 2 mm (Table 1) was noted. This difference of response between the two cultivars testifies that (i) “Tuono” seems to need cold to activate its germination and as a consequence has chilling requirement. (ii) “Achaak” is able to start up its germination without pre-required cold conditions (Figure 2) since the germination rate reaches 85% (data not shown) without cold treatment conditions, at 26°C. (iii) hypocotyl elongation for Achaak was not affected compared to controls (Figure 3).

Ability of germination for Achaak 5-year-old seeds

Furthermore, the germination without chilling treatment was tested for a 5-year-old ‘‘Achaak’’ seeds, properly stored in a dry place. It was observed that these aged seeds were still viable and could germinate. After 5 days at 26°C, the presence of the first set of leaves (Figure 4) strongly suggest that the ability of germination for ‘‘Achaak’’ aged seeds was not totally affected.

Evidence of a true lipase activity in almond soluble fractions

The S_{10} extract lipolytic activity of the harvested Achaak cultivar was tested as described previously. As shown in

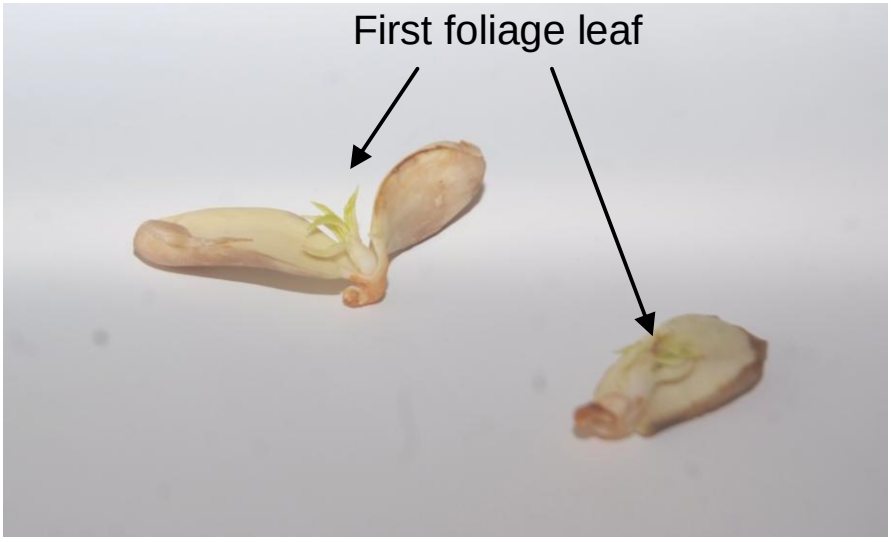

Figure 4. 5-year-old almonds cv ‘‘Achaak’’ ability of germination without pre-required cold treatment. Aged seeds were soaked in water during 16h00 then placed at 26°C. First foliage leaf was obtained after 5 days.

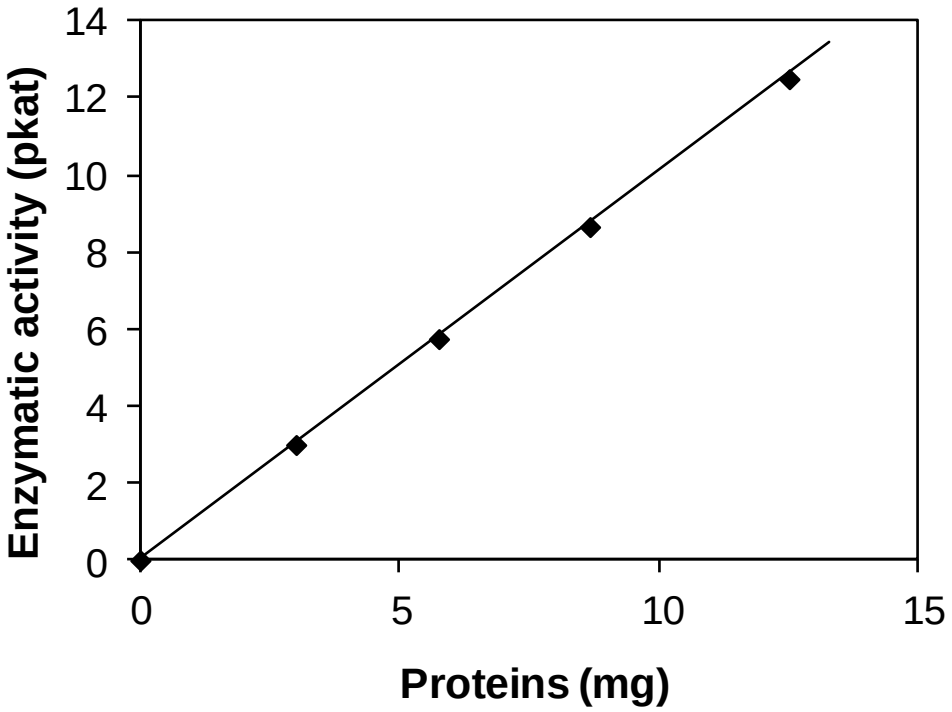

Figure 5. Lipase activity of almond seedlings soluble fraction (S_{10}) without chilling treatment.

Figure 5, by using purified oil (50 mM) as substrate, an increasing curve was obtained with the addition of the soluble enzymatic extract demonstrating the presence of a true lipase activity in S_{10} seedlings extracts. The enzymatic activity was detected at pH 7.5 for investigating the capacity of the enzyme to hydrolyse TAGs under conditions usually found *in vivo*.

Optimum germinating step for lipase activity

Lipid breakdown was investigated starting with 1-day-old

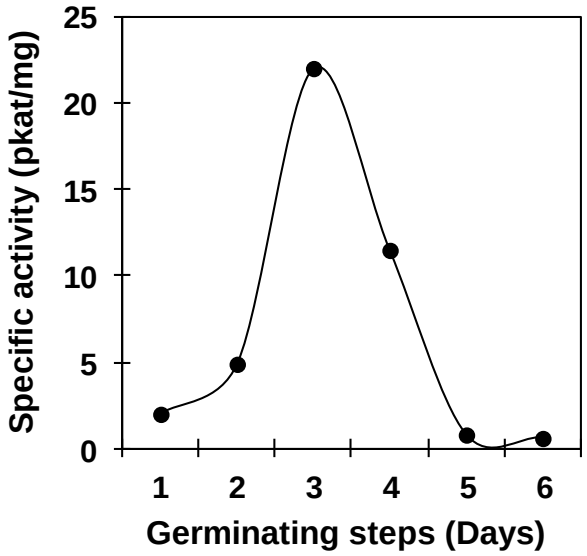

Figure 6. Optimum enzymatic activity during germinating steps.

to 6-day-old almond (*P. amygdalus* Mill. var ''Achaak'') germinating seedlings. The presence of an optimum lipase activity at the 3-day-step of germination (Figure 6) was observed at pH 7.5. However, the detected activity in this fraction was low, with a specific activity of 22 pkat/mg.

DISCUSSION

Usually, germination process begins with water uptake or imbibition. The corresponding curve in Figure 1 shows a classical pattern of water uptake. In the initial phase I which lasts for about 16 h, the release of a large volume of gas and a rapid leakage of substances like sugars and amino acids was noticed. This phase is followed by the stabilization of the tissue hydratation (phase II) corresponding to the germination phase *stricto sensu* in which other metabolic events took place like lipid reserves mobili-zation in preparation for germination (Bewley and Black, 1982, 1994; Mazliak, 1998). The present result suggests that almond seeds should be soaked for 16 h before germination phase *stricto sensu*. After imbibition, many cellular and metabolic changes occured which helped the embryonic axis emerges from the seeds and then complete germination (Bewley, 1997).

Stratification is the process where seeds are subjected to both cold and moist conditions. This method was used to remove endoendormancy (Seeley and Damavandy, 1985). Typically, temperatures must be between 1 and 5°C. For many species, the effective temperature for cold stratification is about 5°C (Stokes 1965). Diverse stratification studies showed the complementary effect of high temperatures on the germination of seeds previously stratified at cold temperatures (Frisbey and Seeley, 1993; Seeley et al., 1998; García-Gusano et al., 2005).

In temperate regions, like Tunisia, heat treatment adapted to a local early flowering cultivar ''Achaak'' almonds seedlings, seems to enhance germination. Although, it is a well known fact that the release of dormancy is not under the control of a single factor for germination (García-Gusano et al. 2009), a correlation was established between chilling requirements for germination and blooming time in almond (Kester et al., 1973; Alonso et al., 2005). The old local cultivar ''Achaak'' known for its low chills units need for flowering (Egea et al., 2003) is adapted to the Tunisian latitude and climate conditions. Furthermore, it does not need an absolute dependence upon low temperature for removing their dormancy even for aged seeds.

On the contrary, the late blooming Italian cultivar ''Tuono'' cannot germinate until this cultivar has fulfilled its chilling requirement. For this cultivar, the late flowering major gene could be involved in germination as described by Socias i Company (1998).

Despite the important number of studies related to dormancy process, many mechanisms are not well understood yet: the cold requirement of a seed could be an adaptation to the climatic factors (Bewley and Black, 1982) and the ability of germination, a viable evolutionary strategy (Kevin and Dyer, 2001).

While imbibed, seeds are metabolically active (Bewley, 1997). Stored TAGs breakdown is initiated by lipase. For the first time, a true lipase was detected at pH 7.5 under non traditional germination conditions (Figure 5). Neutral lipase activity was optimal at three-day-old step (Figure 6). This result is in agreement with that of sunflower (Bahri et al., 2011) and castor bean whose optimum activity peaked on day 4 (Hills and Beevers, 1987). However, the detected activity is weakly expressed as compared to animals and microorganisms lipases.

Finally, the study shows some important results that represent a good strategy for a better understanding of the Tunisian cultivar germination conditions and the molecular characterization of almond seedling lipase with the perspective of improving almonds production and storage.

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