

**Coffee (*Coffea arabica* cv. Rubi) seed
germination: mechanism and regulation**

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Propositions (Stellingen)

- 1- Contrary to seed germination in tomato, the completion of coffee seed germination is the net result of embryo growth and endosperm weakening (*this thesis*).
- 2- Abscisic acid inhibits coffee seed germination by suppressing the increase in embryo growth potential and the second step of endosperm cap weakening (*this thesis*).
- 3- Coffee seed germination is both stimulated and inhibited by gibberellins at 'physiological' concentrations. (*this thesis*).
- 4- Accelerating coffee seed germination and seedling establishment by one month greatly reduces the labour and cost of establishing new coffee shrubs.
- 5- The study of the behavior of tree seeds during development, germination and storage is mandatory in the preservation of biodiversity.
- 6- Facts are the air of scientists. Without them you can never fly. *Linus Pauling*
- 7- Collaboration among developed and developing countries is an outstanding way of promoting scientific and technologic development.
- 8- The Brazilians should be more proud of their country.

These propositions belong to the PhD thesis entitled: Coffee (*Coffea arabica* cv Rubi) seed germination: mechanism and regulation.

Amaral da Silva
Wageningen, 26 June 2002

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**Coffee (*Coffea arabica* cv. Rubi) seed
germination: mechanism and regulation**

Edvaldo Aparecido Amaral da Silva

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Cover illustration: Coffee tree bearing unripe (green) and ripe (red) fruits that each contain two seeds.

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Preface

This work is indebted to a wide range of people.

First I would like to say thanks to Wageningen University and the Federal University of Lavras for giving me the unforgettable opportunity to pursue this PhD program in The Netherlands.

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Prof. Linus van der Plas is very much acknowledged for accepting me in the Laboratory of Plant Physiology as a PhD student under his supervision and for all the support during the last 4 years.

My special thanks goes to "meneer" Dr. Henk Hilhorst, for the bright supervision, for providing me with a good education during my stay in The Netherlands, for the opportunities given that allow me to expose myself to other scientists and to know other cultures. What you taught me is priceless.

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My gratitude goes to the people of the Seed Laboratory at the Federal University of Lavras for the continuous motivation, for helping me to achieve my objectives and for shipping the coffee seeds to The Netherlands every year. To all of you "Muito obrigado".

I would like to express my appreciation to the staff members and PhD students at the Laboratory of Plant Physiology and the Laboratory of Plant Cell Biology, and to my roommate José Marcio for the agreeable coexistence, support and help during my studies.

To the Brazilian community in Wageningen thank you very much for the support, friendship, help and patient.

Finally I could not have done this work without the support and understanding of my family, to my wife Claudia and my son Samuel for their comprehension..... and forgive me for my alienation during the last 4 years.

Amaral

*I dedicate this thesis
To my wife Claudia and my son Samuel.*

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CHAPTER 1

General Introduction

Introduction

Coffee is a member of the Rubiaceae family and the genus *Coffea*. *Coffea arabica* L. originates from Ethiopia at a high plateau (1300-1900 m) between 6° and 9° N, where the dry season lasts about four to five months with temperature extremes of 4° C and 31° C (Coste, 1992). There are more than 70 species of coffee in the world (Rena *et al.* 1994) but only two species are economically important: *Coffea arabica* L and *Coffea canephora* Pierre ex Froehner; 70% of the coffee traded in the world is arabica and 30% is robusta (*Coffea canephora*) (Rena *et al.*, 1994). Brazil is the major coffee producer, contributing 25% of the world production and the second consumer market in the world (MARA, 2000). Since its introduction in 1727 in Brazil, it has become one of the most important crops for its economic and social values. It represents a considerable source of income to the Brazilian economy and to the individuals involved in its production.

To satisfy coffee production chain demands and the consumers within Brazil and around the world, intensive breeding programs are undertaken to create new cultivars resistant to fungal disease and insects, and for incorporation of new trade values. In addition, new production and processing technologies are introduced every year, which have allowed an enormous improvement in coffee production in recent years. Although progress has been made, not many studies have been devoted to the improvement of seed quality for propagation, as opposed to grain quality.

Coffee seeds have a slow and asynchronous germination, which makes it difficult to obtain seedlings that are desirable for coffee production. Little work has been done to understand coffee seed germination and there is a lack of information concerning the regulation of the germination process. Therefore, studies on this level are essential for agricultural practices and further development of coffee production.

Germination Mechanism

According to Bewley (1997) there are 3 possibilities for radicle growth. The first possibility is that late during germination the osmotic potential (ψ_n) in the embryo cells becomes more negative due to solute accumulation and, thus, permitting the embryo to break through the envelope tissues for completion of germination. The second possibility is an increase of extensibility of the radicle cell walls during germination in response to the internal turgor pressure. Xyloglucan endotransglucosylase (XET) and expansins have been suggested to be involved in cell wall loosening, allowing cell expansion. Expansins may cause cell wall creep by loosening noncovalent bonds between cellulose and hemicellulose (Cosgrove, 1999). XET breaks the xyloglucan chains and allows the cellulose microfibrils to move apart, driven

by the internal cell turgor pressure (ψ_p) (Bewley, 1997). *Brassica napus* is an example of a seed where an increase in the turgor and cell wall extensibility of the embryo is a prerequisite for radicle protrusion (Schopfer and Plachy, 1985).

The third possibility is that the tissues enveloping the embryo weaken, allowing radicle growth. In seeds that show a severe constraint on radicle cell growth imposed by surrounding structures, the pressure potential (ψ_p) in the embryo as well as the turgor are insufficient to drive cell wall expansion. In this case weakening of the cell walls of the constraining tissues by action of hydrolases is required for decline of the mechanical resistance (Bewley, 1997). The current model for tomato seed germination from Toorop (1998) is presented in Figure 1.

Objectives

Coffee seed germination is slow and shows wide variation in the timing of emergence. The overall objective of this thesis was to unravel the mechanism of coffee seed germination as well as its regulation by abscisic acid and gibberellic acid.

More specifically, the objectives of this thesis are:

1. Structural analysis to investigate endosperm cell wall morphology and degradation during coffee seed germination and its significance to radicle protrusion;
2. Study of the involvement of enzymes required for endosperm cell wall degradation during germination, as well as control by abscisic acid of the germination process;
3. Understanding the role of endogenous and exogenous gibberellins in embryo growth and endosperm degradation during germination;
4. Study of the effect of exogenous abscisic acid on the cell cycle machinery in the coffee embryo during germination;
5. Cloning of endo- β -mannanase and β -mannosidase genes during coffee seed germination as well as the timing and location of endo- β -mannanase and β -mannosidase in the different seed parts during germination.

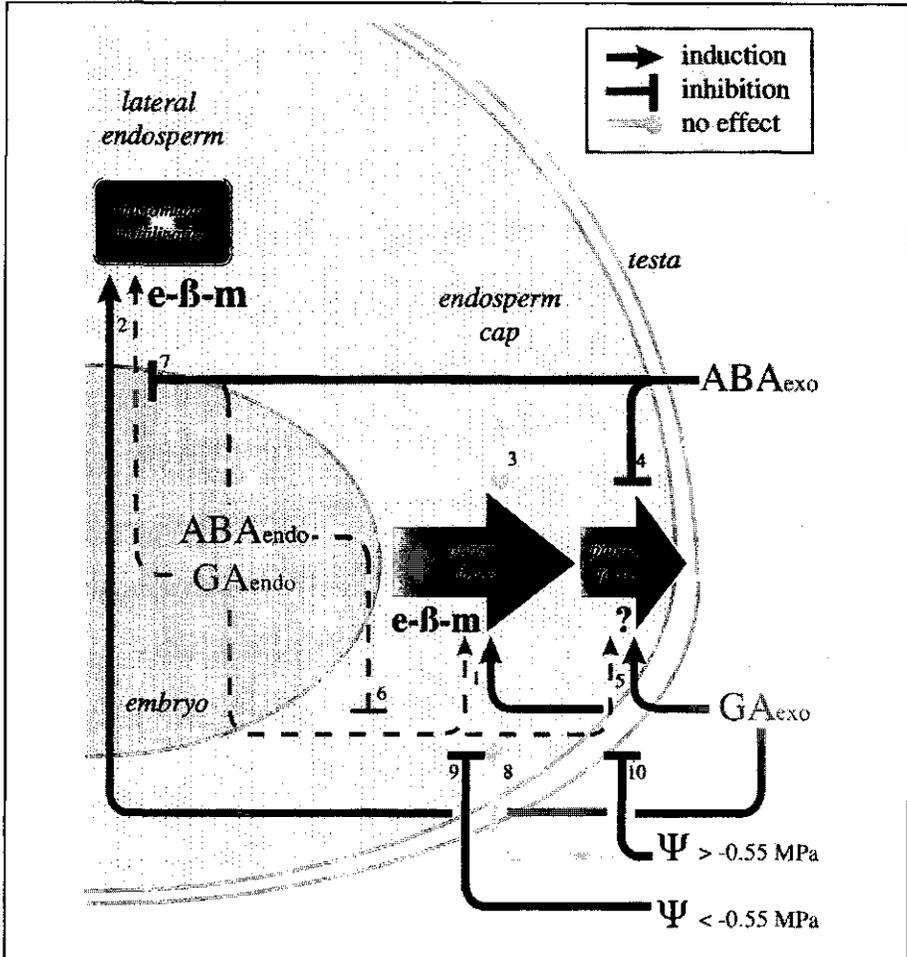


Figure 1. Model for the mechanism and regulation of tomato seed germination as proposed by Toorop (1998). The model is based on the concept that only endosperm weakening is required for radicle protrusion as the embryo has a water potential of approximately - 2 MPa. Endogenous GAs, synthesized in the embryo and secreted into the endosperm, induce the enzymatic degradation of cell walls in the endosperm cap (1) and lateral endosperm (2) and endogenous ABA inhibits these processes (6). Of the two-step degradation of the endosperm cap the first step, mediated by endo- β -mannanase, is induced by endogenous and exogenous GAs (1). The first step is not affected by ABA (3) but is inhibited by an external osmotic potential < -0.55 MPa (9). Degradation of the lateral endosperm through endo- β -mannanase is inhibited by ABA (7). The second step of endosperm softening is promoted by endogenous and exogenous GAs (5) and inhibited by ABA (4) and an external osmotic potential > -0.55 MPa (10), which did not affect the first step (8). The enzyme(s) involved in the second step of endosperm weakening are not known ("?").

Scope of the thesis

Chapter 1 - General Introduction

The origin and the importance of coffee is presented, as well as the problems occurring in germinating coffee seed, the objectives of the thesis as well as the thesis structure;

Chapter 2 - Morphology and anatomy of the coffee (*Coffea arabica* cv. Rubi) fruit and seed during germination.

Light microscopy and low temperature scanning electron microscopy (Cryo-SEM) were used to describe the different fruit and seed tissues and cells. Morphology and anatomy of endosperm, endosperm cap and embryo during coffee seed germination are presented. Certain aspects of cell and cell wall morphology were investigated in more detail through light microscopy and Cryo-SEM.

Chapter 3 - ABA regulates embryo growth potential and endosperm cap weakening during coffee (*Coffea arabica* cv. Rubi) seed germination.

The involvement of hydrolytic enzymes in endosperm cap degradation was investigated during coffee seed germination as well as the time and duration of these events in relation with the completion of germination. Additional investigations of the location of these events within the seed were done by tissue printing. Puncture force was measured to determine endosperm weakening. Different isoforms of endo- β -mannanase from the endosperm cap and rest of the endosperm were identified by using isoelectric focussing. Endogenous ABA levels of coffee embryos were determined and the possible role of this hormone is addressed.

Chapter 4 - Supra-optimal GA concentrations inhibit germination in coffee seed (*Coffea arabica* cv. Rubi) and leads to death of the embryo.

The role of endogenous GA and the inhibitory effect of exogenous GAs were studied during coffee seed germination.

Chapter 5 - ABA reduces the abundance of the microtubules and inhibits transversal organisation of the microtubules, embryo cell elongation and cell division during coffee (*Coffea arabica* cv. Rubi) seed germination.

The effect of exogenous ABA on DNA synthesis, β -tubulin accumulation and assembly of the microtubules were studied in coffee seed embryos during germination.

Chapter 6 - Cloning of endo- β -mannanase and β -mannosidase from germinating coffee seeds.

Endo- β -mannanase and β -mannosidase activities are determined in different seed parts during germination. Clones of both enzymes were isolated during germination and the alignment of the deduced protein sequences is presented.

Chapter 7 - General Discussion.

A final discussion and a schematic overview of the events occurring in the embryo, in the endosperm cap and in the rest of the endosperm during coffee seed germination are presented.

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CHAPTER 2

Anatomy and morphology of the coffee (*Coffea arabica* cv. Rubi) seed and fruit during germination

E.A. Amaral da Silva, Peter E. Toorop, Adriaan C. van Aelst and Henk W.M. Hilhorst

Abstract

The coffee (*Coffea arabica* cv. Rubi) fruit is a drupe containing two seeds. The coffee seed is comprised of an endosperm, embryo and spermoderm or "silver skin". The thickened cell walls of the endosperm are composed mainly of mannans with 2% of galactose. The endosperm contains both polygonal and rectangular cell types. The rectangular cell type was located adjacent to the embryo in the so-called internal endosperm whereas the polygonal cells were located in the external endosperm. The endosperm cap cells have smaller and thinner cell walls than the rest of the endosperm, which indicates that the region where the radicle will protrude is predestined in coffee seeds. Radicle protrusion in the dark at 30 °C was initiated around day 5 of imbibition and at day 10, 50% of the seed population showed radicle protrusion. The endosperm cap of the coffee seed changed during germination. Cell compression was followed by loss of cell integrity, appearance of a protuberance and occurrence of cell wall porosity. The observations indicated that embryo growth and changes in the endosperm cap region control radicle protrusion in coffee seed.

Keywords: Coffee seed, light microscopy, cryo-scanning electron microscopy, morphology, anatomy, germination.

Introduction

The coffee (*Coffea arabica* cv. Rubi) fruit is a drupe containing two seeds. The coffee seed is comprised of an endosperm, embryo and spermoderm or "silver skin". The thickened cell walls of the endosperm are composed mainly of mannans with 2% of galactose.

Seed germination "begins with the water uptake by the seed (imbibition) and ends with the elongation of the embryonic axis, usually the radicle" (Bewley and Black, 1994). Therefore, the end of the germination process in coffee seeds corresponds with protrusion of the radicle through the endosperm.

For radicle protrusion to occur the expansion force or "thrust" of the embryo must exceed the mechanical restraint of the surrounding layers of tissue, i.e. endosperm and seed coat. In a number of endosperm retaining species it has been shown that weakening of the endosperm through hydrolytic degradation of the cell walls allows the radicle to overcome endosperm resistance. Less attention has been paid to the (expansion) growth of the embryo (Bewley, 1997).

The majority of the work that has been published on coffee seed considered germination as emergence of the seedling from the soil. Therefore, germination *sensu stricto* has never been studied in detail in coffee seed.

Studying the anatomy and morphology of life processes in plants (and seeds) may give clues pertaining to the nature of physiological and biochemical processes. Such a study may give directions as to which lines of physiological studies should be pursued (Toorop *et al.*, 2000; Nijse *et al.*, 1998).

The objective of this work was to characterize germination in coffee seed at the time of radicle protrusion through the endosperm. For a general morphological overview of the germination process we used conventional light microscopy whereas for a more detailed structural analysis of cells and cell components cryo-scanning electron microscopy was employed.

Material and methods

Seed source. Coffee seeds from *Coffea arabica* L. cultivar Rubi were harvested in 1997 in Lavras-MG-Brazil. The fruits were mechanically depulped, fermented and the seeds were dried to 12% of moisture content and stored at 10° C during the experiment.

Germination conditions. Seed coats were removed by hand and the seed surface was sterilized in 1% of sodium hypochlorite for 2 minutes. Subsequently, seeds were rinsed in water and imbibed in demineralized water. Seeds were placed in 94-mm Petri dishes on filter paper (no. 860, Schleicher & Schuell, Dassel, Germany) in 10 ml of water. During imbibition

seeds were kept at $30 \pm 1^\circ \text{C}$ in the dark (Huxley, 1965; Valio, 1976). At least 3 seeds were taken randomly every day during germination for light microscopy and low temperature scanning electron microscopy studies.

Light microscopy. The entire imbibed seeds were sectioned using a microtome (Reichert, Austria). The sections of 20-30 μm thickness from the endosperm cap and the rest of the endosperm were first transferred to demineralized water and then fixed in liquid Kaiser's glycerol gelatin (Merck, Germany) for observations. Observations were made in a Nikon Optiphot microscope in bright field mode. Photographs were taken with a digital Panasonic Colour Video Camera or a Sony CCD Camera DKR 700. Images of the coffee fruit, seeds and embryo were taken by using a Leica binocular.

Cryo-scanning electron microscopy. Coffee seeds were prepared for Cryo-Scanning Electron Microcopy (Cryo-SEM). The seeds were longitudinally sectioned with a razor blade and mounted on a cup shaped holder with tissue freezing medium. After mounting, the samples were plunge-frozen and stored in liquid nitrogen for subsequent cryo-planing and observations. Cryo-Planing, which attempts to produce flat surfaces for observations in Cryo-SEM, was performed using a cryo-ultramicrotome with a diamond knife, according to Nijssen *et al.*, (1999). For observations the specimens were heated up to -90°C , sputter-coated with platinum and placed in the cryostat of the scanning electron microscope (JEOL 6300 Field emission SEM). Observations were made at -180°C using a 2.5-5kV accelerating voltage. Digital images were taken and printed. Alternatively, the seeds were freeze-fractured with a cold scalpel knife, heated up to -90°C , partially freeze-dried and sputter-coated with 5 nm of Pt.

Results and discussion

Coffee fruit and seed morphology

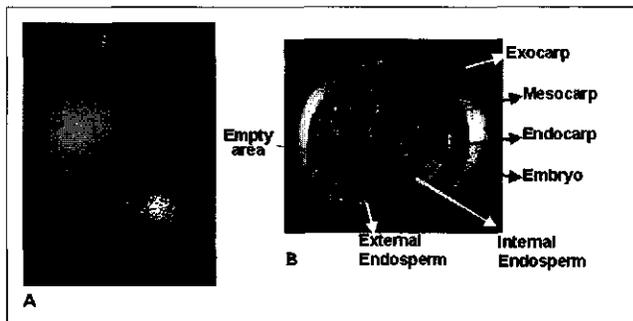


Figure 1 A: Green coffee (*Coffea arabica* cv. Rubi) fruit; **B:** Transversal section of coffee fruit showing the internal structures.

The coffee fruit (*Coffea arabica* L.) is a drupe containing two seeds (Fig. 1 A and B). The thick exocarp is easily removed, revealing the soft mesocarp. The outer cover of the seed is formed by a hard

pale brown endocarp that becomes the “parchment” after drying (Fig. 2 A). The endocarp contains an enclosed seed, which has a thin, green testa known as the “silver skin” after drying, and which is a remnant of the perisperm tissue (Mendes, 1941; Chin and Roberts, 1980). Transversal sections of the coffee fruit or seeds showed a longitudinal empty area in

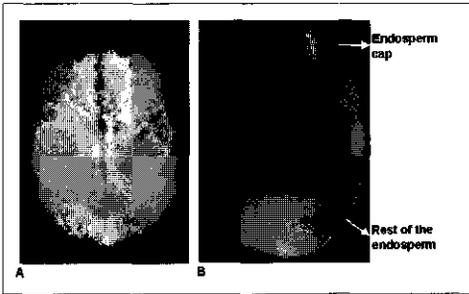


Figure 2 A: Dry coffee seed (*Coffea arabica* cv. Rubi) with endocarp attached to the endosperm. **B:** Imbibed coffee seed (*Coffea arabica* cv. Rubi) at 6 days of imbibition with endocarp removed, showing the endosperm cap (ec) as well as the rest of the endosperm (lateral endosperm). Observe the appearance of a protuberance in the endosperm cap and remnants of the spermoderm or “silver skin” at the bottom of the seed surface.

the endosperm that is filled by the endocarp and the “silver skin”(Fig. 1 B). The coffee seed is comprised of an endosperm, embryo and spermoderm or “silver skin” (Fig. 1 B). Measurements made in a large number of seeds indicated that the seeds are 10-18 mm long and 6,5-9,5 mm wide (Dedecca, 1957).

The endosperm tissue is divided in a hard external endosperm and soft internal endosperm (Dedecca, 1957; Fig. 1 B) and has a high content of polysaccharides (Wolfrom *et al.*, 1961). The cell walls are composed of cellulose and hemicellulose (Wolfrom and Patin, 1964). The main hemicellulose in coffee seeds is an insoluble mannan (Wolfrom *et al.*, 1964). The endosperm is extremely hard because the hemicellulose is deposited in a very thick cell wall. Coffee mannans contain 2% of galactose, probably as a side chain of the mannan backbone (Bewley and Black, 1994). The part of the endosperm in front of the radicle tip is called endosperm cap and the lateral endosperm is also called 'rest of the endosperm' (roe; Fig. 2 B).

The fully differentiated coffee embryo is enveloped by the soft endosperm tissue (Krug and Carvalho, 1939; Mendes 1941). The embryo is very small and does not have much storage reserves deposited. It depends entirely on the endosperm to develop into a seedling

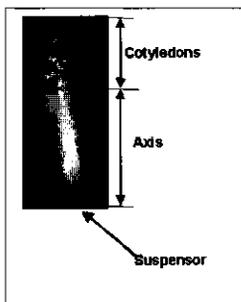


Figure 3 Imbibed coffee embryo (*Coffea arabica* cv. Rubi) isolated at 7 days of imbibition in water, showing the cotyledons, the embryonic axis and remnants of the suspensor at the radicle tip.

(Giorgini and Campos, 1992). It is 3 to 4 mm long and is composed of an axis and two cotyledons (Fig. 3); it is localised close to the convex surface of the seed (Rena *et al.*, 1986). During embryo development hypocotyl formation is preceded by the formation of the cotyledons, but embryo development takes place after endosperm development (Arcila-Pulgarín and Orozco-Castaña, 1987). Polyembryony, more than one embryo per seed, and empty seeds have been observed in coffee seed at a frequency of 1,2% (Mendes, 1944).

Germination characteristics

Coffee seeds germinate slowly (Rena *et al.*, 1986). Seedling emergence from the soil starts 50 to 60 days after sowing in the warmer periods of the year (Maestri and Vieira, 1961). When temperatures are lower the emergence period may increase to 90 days (Went, 1957). Following germination, the coffee cotyledons grow by absorbing the endosperm and turn green (Wellman, 1961). The first seed parts to emerge from the soil are the cotyledons, characterizing epigeal germination, and 3 to 4 weeks are required for the cotyledons to completely deplete the endosperm and be free from any residual endosperm (Huxley, 1964).

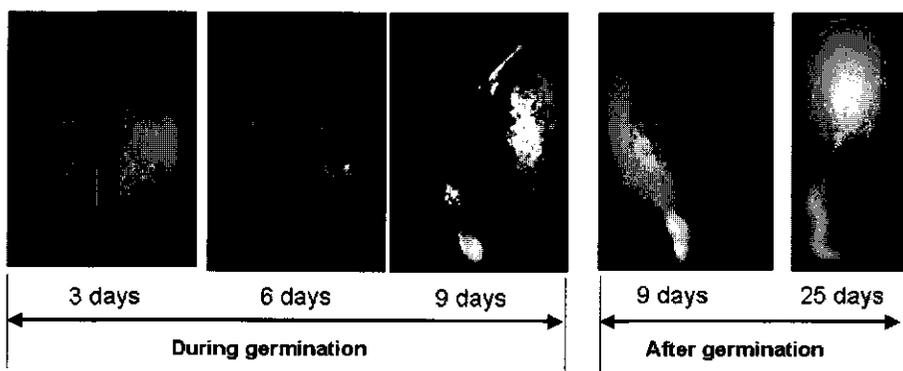


Figure 4 Germination *sensu stricto* of the coffee seed (*Coffea arabica* cv. Rubi). A fully imbibed seed is shown at day 3 of imbibition with no visible protuberance; a protuberance is visible from day 6 of imbibition onwards and radicle protrusion starts at day 9. Following germination, the radicle grows and the endosperm remains attached to the cotyledons. The cotyledons will completely dissolve the endosperm before they become green and autotrophic.

Radicle protrusion in coffee seeds under optimal conditions (30 °C, in the dark) started around day 5 or 6 and at day 10 of imbibition 50% of the seed population displayed radicle protrusion. At day 15 of imbibition most of the seeds had shown radicle protrusion. Obviously, germination is faster under optimal conditions when environmental effects such as variation in day-night temperatures and soil water potential are absent. In addition,

germination under field conditions is defined as seedling emergence from the soil; radicle protrusion has already been completed some time before emergence. The development of the germination process *sensu stricto* in coffee seed is presented in figure 4.

Structural description of the endosperm and embryo during germination

The main hemicellulose in the cell walls of coffee seeds is an insoluble β -(1 \rightarrow 4) D-mannan with 2% of galactose present in the side chains that may serve as a carbohydrate reserve (Wolfrom *et al.*, 1961; Bewley and Black, 1994). The galactose units are also found in arabinogalactans in the coffee seed (Wolfrom and Patin, 1965). The coffee seed belongs to the group of seeds that have a relatively high amount of mannans (Wolfrom *et al.*, 1961).

Proteins, lipids and minerals are also present in the cytoplasm of the endosperm cells and could be another source of reserves (Dentan, 1985). Endosperm cells have rectangular and polygonal cell types (Fig. 5 A and B). The rectangular cells are located adjacent to the embryo and are observed in the region of the internal endosperm whereas the polygonal cells were located in the external endosperm (Fig. 5 A and B). The cells of the rest of the endosperm displayed thick cell walls, indicating the source of reserves. These cell walls are probably degraded following germination to provide a source of energy to the growing seedlings.

A different morphology was observed in the cells of the endosperm cap. These cells are smaller and the cell walls are thinner than the cells of the rest of the endosperm that has thicker cell walls with thin-walled regions (Fig. 5C, 6 C and D). Plasmodesmata have been observed in the primary pit-field of the coffee endosperm walls (Dentan, 1985). The difference in cell size and in cell wall morphology between endosperm cap and rest of the endosperm indicates that the region where the radicle will protrude is predestined in coffee seeds to allow embryo growth, although it may not exclude the requirement of endosperm cap degradation prior to radicle protrusion in order to facilitate radicle protrusion.

During the first 3 days of germination the endosperm cells expand probably as a result of the water uptake. At day 3 of imbibition the cells are apparently turgid indicating that phase 2 of the germination process has been attained. However, at day 6 of imbibition, still cells that were not fully imbibed, often surrounded by fully imbibed cells, are visible in the endosperm cap and in the rest of the endosperm (Fig. 6 C and D). There were more fully imbibed cells in the endosperm cap than in the rest of the endosperm. Apparently, the thickness of the cell walls and probably also the accumulation of solutes are very important in controlling water uptake during germination.

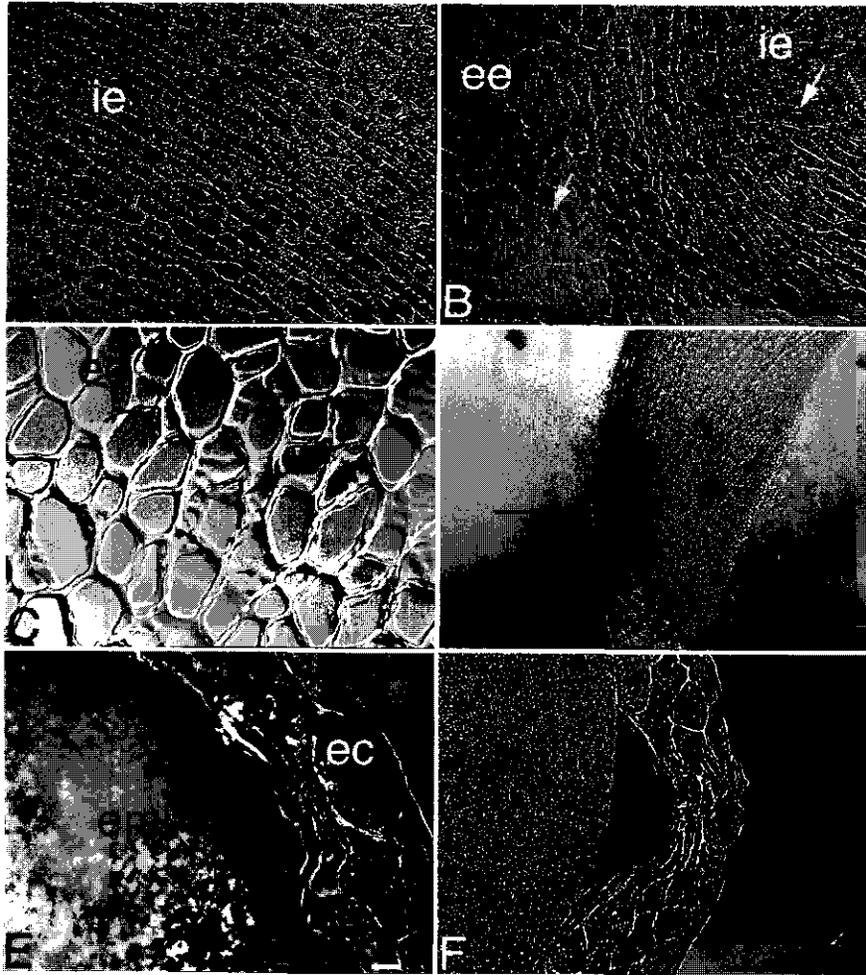


Figure 5 Light microscopy images of the endosperm cap and rest of the endosperm of coffee seed during germination. A: Cells of the rest of the endosperm adjacent to the embryo during imbibition (bar indicates 100 μm). Note the uniformity of the cell size in this region. B: Cells of the rest of the endosperm during imbibition showing the border between internal endosperm (ie) and external endosperm (ee) (bar indicates 100 μm). Note that the cells of the internal endosperm adjacent to the embryo are rectangular and will be the first cells to be consumed, following germination. The cells of the external endosperm (ee) have a polygonal shape; these cells will be consumed later. C: Higher magnification of the external endosperm region of the rest of the endosperm showing thin-walled areas (bar indicates 10 μm). D: Endosperm cap of a 9 day-imbibed seed showing remnants of a suspensor at the radicle tip (dark spot) (bar indicates 100 μm). E: Endosperm cap (ec) region and embryo (em) at 6 days of imbibition showing compressed cells in the endosperm (bar indicates 10 μm). F: Endosperm cap (ec) region and embryo (em) in a 10-day imbibed seed showing loss of cell integrity just before radicle protrusion (bar indicates 100 μm).

In the endosperm cap region, various compressed cells walls were evident prior to radicle protrusion at day 6 of the imbibition process (Fig. 5 E). The remnants of the suspensor were observed at the endosperm cap just prior to radicle protrusion (Fig. 5 D) and were also observed in the endosperm cap region outside of the seed surface when the protuberance appeared. As germination proceeded the compressed cells lost their integrity just before radicle protrusion (Fig. 5 F). Compressed cells and loss of cell integrity coincided with the appearance of the protuberance observed in the endosperm cap preceding radicle protrusion (Fig 4, 6B). Endosperm cell walls surrounding the embryonic axis, just below the radicle tip, also showed compressed cells, possibly caused by the lateral expansion of the embryo inside the endosperm during germination (not shown).

Obviously, compressed cells, loss of cell integrity and appearance of the protuberance were the result of embryo growth inside the endosperm prior to radicle protrusion that may be driven by embryo cell expansion, elongation or division. In the embryo cells many intercellular spaces were observed (Fig. 6 E). At day 9 of imbibition presence of nuclei, nucleoli, protein bodies and large central vacuoles were observed (Fig. 6 E). Apparently, the vacuoles are fused to form a large central vacuole prior to radicle protrusion in the embryo cells, since in earlier stages of imbibition more vacuoles were observed in individual embryo cells. Plastids that may contain starch as a source of reserves for the growing coffee embryo were observed at day 9 of imbibition (Fig. 6 E).

Concomitantly with the morphological changes in the endosperm cap region we observed the development of porosity in the cell walls at day 9 of imbibition (Fig. 6 F). For tomato seeds it has been shown that these pores are caused by the evaporation of water during the freeze-drying process, indicating the absence of cell wall components and coinciding with a decrease in the force required to puncture the endosperm, as well as an increase in endo- β -mannanase activity (EC 3.2.1.78) (Toorop *et al.*, 2000). Therefore, this porosity of the cell walls in the endosperm cap of coffee seed indicated also that cell wall degradation took place during coffee seed germination, possibly to weaken the endosperm cap in order to facilitate radicle protrusion. Furthermore, degradation of the rest of the endosperm (lateral endosperm) during germination may not be ruled out. Thus, our observations indicate that endosperm degradation in the coffee seed is important during germination, not only to weaken the endosperm cap but also as a source of reserve materials during seedling establishment when endosperm degradation also takes place in rest of the endosperm. Previous work in coffee seed has suggested that mobilization of mannan-rich cell walls is a post-germinative phenomenon since endo- β -mannanase activity responsible for endosperm mobilization of the mannan polymers was detected only after radicle protrusion (Giorgini and Comoli, 1996; Marraccini *et al.*, 2001).

Light microscopy observations did not show the presence of an aleurone layer surrounding the endosperm that could be the source of hydrolytic enzymes. In addition, incubation of endosperm slices in tetrazolium (2,3,5 triphenyltetrazolium chloride) solutions at 30°C for 16 hours, showed a positive reaction (data not shown). This indicates that coffee endosperm cells are alive and may be themselves the source of the hydrolytic enzymes present within the endosperm rather than being dependent on a specialized aleurone cell layer as source of enzymes. Finally, the results indicate that both embryo growth and changes in the endosperm tissue control germination in coffee seed.

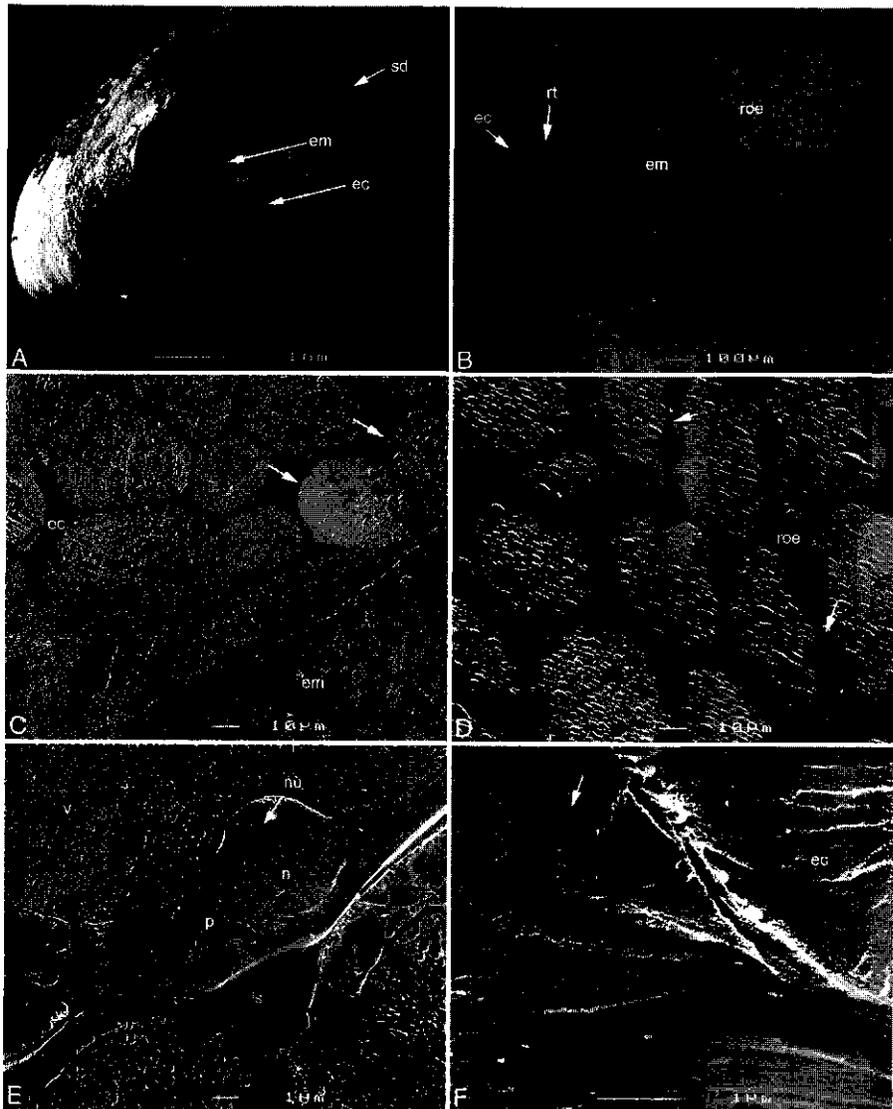


Figure 6 Low temperature scanning electron microscopy of a coffee seed. A: Transversal section of the endosperm cap from a 9 day-imbibed seed. Observe the embryo (em), endosperm cap (ec) and remnants of the spermoderm (sd) or silver skin at the convex seed surface. B: Longitudinal section of a 6 day-imbibed seed, showing the endosperm cap (ec) and rest of the endosperm (roe). Mark the localization of the embryo (em) and the radicle tip (rt) within the endosperm prior to radicle protrusion, and the lateral expansion of the embryo causing a protuberance. C: Endosperm cap of a 6-day imbibed seed showing the thinner cell walls. Observe that some cells are not completely hydrated (arrows), surrounded by fully hydrated cells of endosperm cap (ec) and embryo (em). D: Cells of the rest of the endosperm (roe) of 6-day imbibed seeds. Note that these cell walls are thicker than the cell walls of the endosperm cap region (Fig. 6 C) and are apparently not hydrated. Again thin-walled regions can be observed locally (arrows) E: Embryo cells in a 9-day imbibed seed showing intercellular space (is) and a large vacuole (V). At the cell periphery nuclei (n), nucleoli (nu) and plastids (p) are visible. F: Endosperm cap cell wall of a 9-day imbibed seed (prior to radicle protrusion), showing porosity, indicating cell wall degradation.

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CHAPTER 3

ABA regulates embryo growth potential and endosperm cap weakening during coffee (*Coffea arabica* cv. Rubi) seed germination

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Abstract

The mechanism and regulation of coffee seed germination were studied in *Coffea arabica* cv. Rubi. The coffee embryo grew inside the endosperm prior to radicle protrusion and ABA inhibited the increase in pressure potential. There were two steps of endosperm cap weakening. An increase in cellulase activity coincided with the first step and an increase in endo- β -mannanase activity with the second step. ABA inhibited the second step of endosperm cap weakening presumably by inhibiting the activities of at least two endo- β -mannanase isoforms. The increase in the activity of endo- β -mannanase and cellulase coincided with the decrease in the force required to puncture the endosperm and with the appearance of porosity in the cell walls as observed by low temperature scanning electronic microscopy. Tissue printing showed that endo- β -mannanase activity was spatially regulated in the endosperm. Activity was initiated in the endosperm cap whereas later during germination it could also be detected in the rest of the endosperm. Tissue printing revealed that ABA inhibited endo- β -mannanase activity in the endosperm cap, but not in the rest of the endosperm. ABA did not inhibit cellulase activity. There was a transient rise in ABA content in the embryo during imbibition, suggesting that also endogenous ABA may control embryo growth potential and the second step of endosperm cap weakening during coffee seed germination.

Keywords: coffee seed, endosperm weakening, abscisic acid, endo- β -mannanase, cellulase, cryo-scanning electron microscopy, puncture force.

Introduction

The coffee (*Coffea arabica* L.) embryo is enveloped by an endosperm tissue (Krug and Carvalho, 1939; Mendes, 1941). The fully differentiated embryo lies inside an embryo cavity, is 3 to 4 mm long and is composed of an axis and two cotyledons (Rena and Maestri, 1986). The endosperm is surrounded by the endocarp that resembles a seed coat (Chin and Roberts, 1980). The coffee endosperm is composed of a hard greenish tissue with polyedric cells, is isodiametrically divided in a hard external endosperm and a soft internal endosperm (Dedecca, 1957), and belongs to the nuclear type (Mendes, 1941). The endosperm cells have very thick walls that are crossed by plasmodesmata (Dentan, 1985). These cell walls are composed of cellulose and hemicellulose (Wolfrom and Patin, 1964). The main hemicellulose is an insoluble β -(1 \rightarrow 4) D-mannan with 2% of galactose present in the side chains (Wolfrom et. al., 1961). The galactose units are also found in arabinogalactans in the coffee seed (Wolfrom and Patin, 1965). The coffee seed belongs to the group of seeds that have a relatively high amount of mannans (Wolfrom et al., 1961).

Seed germination starts when the expansive force of the embryonic radicle exceeds the mechanical restraint of the surrounding tissues (Hilhorst et al., 1998). The possible causes for embryo growth are lowering of its osmotic potential (ψ_{π}), thus raising the pressure potential (ψ_p) in the radicle cells, relaxation of the radicle cell walls, weakening of the tissues surrounding the embryo or a combination of these causes (Bewley and Black, 1994). In celery, *Anemone coronaria* and in *Fraxinus* seeds the embryo also grows inside the endosperm before radicle protrusion (Steinbauer, 1937; Bullowa et al. 1975; Jacobsen et al., 1979; van der Toorn, 1992). In lettuce seed Takeba (1980) found an accumulation of free amino acids in the growing axes that would be high enough to increase the growth potential of non-dormant lettuce seed. However, Weges (1991) did not find a solute accumulation prior to radicle protrusion in lettuce seed that would decrease the osmotic potential, allowing radicle protrusion. In *Brassica napus* embryos an increase of turgor and cell wall extensibility is required for radicle protrusion (Schopfer and Plachy, 1985).

Weakening of the tissues in front of the radicle tip has been proposed to be a prerequisite for radicle protrusion in tomato seed (Haigh and Barlow, 1987; Groot and Karssen, 1987), muskmelon (Welbaum et al., 1995), *Datura ferox* (de Miguel and Sanches, 1992) and pepper (Watkins et al., 1983). In tomato and muskmelon seeds embryo water uptake is restricted by the endosperm during germination and lowering of the osmotic potential or an increase of embryo turgor have never been observed before radicle protrusion (Haigh and Barlow, 1987; Welbaum

and Bradford, 1990). In *Datura ferox* the increase in embryo growth potential was insufficient to allow germination (de Miguel and Sanches, 1992). In tomato seed endo- β -mannanase (E.C.3.2.1.78) activity correlated with weakening of the endosperm cap (Groot *et al.*, 1988; Toorop *et al.*, 2000). Endo- β -mannanase activity also correlated with porosity in the endosperm cap cell walls, as observed by cryo-scanning electronic microscopy (cryo-SEM) and with a decrease in the required puncture force (Toorop *et al.*, 2000). Other enzymes such as polygalacturonase (Sitrit *et al.*, 1999), cellulase (Leviatov *et al.*, 1995) and arabinosidase (Bradford *et al.*, 2000) have also been shown to increase in activity during tomato seed germination. Also in muskmelon seed cellular degradation and weakening occurred concomitantly with the decrease in puncture force (Welbaum *et al.*, 1995). In *Datura* spp scanning electron micrographs and analyses of endosperm cell wall polysaccharide composition showed morphological changes in the micropylar endosperm before radicle protrusion (Sánchez *et al.*, 1990). In pepper seeds the endosperm cap displayed compressed cells and loss of integrity before radicle protrusion (Watkins *et al.*, 1985) as well as a decrease in the required puncture force (Watkins *et al.*, 1983). However, endo- β -mannanase activity was only detected after radicle protrusion (Watkins *et al.*, 1985).

Cell wall hydrolytic enzymes have previously been studied in coffee seed. These include, α -galactosidase (EC 3.2.1.22) (Petek and Dong, 1961; Shadaksharaswamy and Ramachandra, 1967), cellulase (EC 3.2.1.4), (Takaki and Dietrich, 1980 and Giorgini, 1992) and endo- β -mannanase, (Giorgini and Comoli, 1996 and Marracini *et al.*, 2001). However, there is little information about enzyme activity in relation to the germination mechanism and its regulation.

Abscisic acid (ABA) is known to induce dormancy and inhibit seed germination (Bewley and Black, 1994). In lettuce seed endogenous ABA inhibits endo- β -mannanase activity (Dulson *et al.*, 1988) and cellulase activity (Bewley, 1997). In fenugreek and carob seeds ABA suppresses the activity of endo- β -mannanase in the endosperm (Kontos *et al.*, 1996). In tobacco β -1,3-glucanase (EC 3.2.1.39) correlates with endosperm rupture and ABA delays this rupture (Leubner-Metzger *et al.*, 1995). In the endosperm cap of tomato seed ABA does not inhibit cellulase (Toorop, 1998 and Bradford *et al.*, 2000) and endo- β -mannanase activity (Toorop *et al.*, 1996; Still and Bradford, 1997) but radicle protrusion is prevented. In the embryo of *Brassica napus*, Schopfer and Plachy (1985) have shown that ABA inhibited cell wall loosening. In coffee seed Valio (1976) found that endogenous ABA-like substances and exogenous ABA caused inhibition of germination through inhibition of embryo growth. However, the role of ABA during coffee seed germination has not been described in clear detail.

The aim of the present work is to determine the targets and mechanism of the ABA controlled inhibition of coffee seed germination.

Materials and methods

Seed source. Coffee seeds from *Coffea arabica* L. cultivar Rubi were harvested in 1998 in Lavras-MG-Brazil, depulped mechanically, fermented and dried to 12% of moisture content and shipped to The Netherlands where they were stored at 10° C.

Germination conditions. The seed coat was removed by hand and the surface sterilised in 1% of sodium hypochlorite for 2 minutes. Subsequently, seeds were rinsed in water and imbibed in 10 ml demineralized water or ABA solution in a concentration of 1000 μ M, 100 μ M or 10 μ M (racemic mixture; Sigma, St. Louis, Mo., USA). Seeds were placed in 94-mm Petri dishes on filter paper (no. 860, Schleicher & Schuell, Dassel, Germany). During imbibition seeds were kept at 30 \pm 1° C in the dark (Huxley, 1965; Valio, 1976). ABA solutions were prepared by dissolving the compound in 1 N of KOH followed by neutralisation with 1 N of HCl. Fluridone solution was prepared by dissolving the compound in 0.1% of acetone until complete dissolution. Control experiments showed that the acetone concentration used did not affect germination. The germination percentage was recorded daily.

Imbibition curve. Intact seeds were imbibed as described above and the fresh weight was measured daily.

Embryo growth. Twenty embryos from water-imbibed seeds were isolated by cutting the endosperm with a razor blade. Embryo length was measured by using callipers. After length measurement the embryos were separated in embryonic axes and cotyledons and these were measured again.

Water potential (ψ) measurements. The water potential (ψ) and osmotic potential (ψ_{π}) of coffee embryos from seeds imbibed in water or in ABA solutions were measured by using a calibrated thermocouple psychrometer (Model HR-33T, Wescor, USA) C-52 sample chamber (Wescor, USA). Samples were equilibrated for 40 minutes and 2 readings were taken before starting the experiments to ensure that equilibrium had been attained. Cooling time was 45 seconds. The C-52 chamber was placed in an airtight glove box kept at 100% relative humidity by a stream of water-saturated air at a constant temperature of 25 \pm 1° C. Embryos were isolated as described above and placed in the C-52 chamber for measurements. Three replications of 5 embryos were used for the measurements. After measurement of the water potential the embryos were put in liquid nitrogen for determination of the osmotic potential (ψ_{π}). After 2 hours in liquid

Results

Germination. Radicle protrusion started at 5 days of imbibition in water. ABA at 1000 μM completely inhibited germination. Lower concentrations of 100 μM and 10 μM allowed germination for 36% and 49%, respectively (Fig. 1). Fluridone, an inhibitor of carotenoid biosynthesis that also inhibits ABA accumulation (Li and Walton, 1990), accelerated radicle protrusion significantly at a optimal concentration of 50 μM . In the presence of fluridone the seeds required 8.9 days to reach 50 % of germination, whereas in water the seeds required 9.9 days (Fig. 1). ABA (1000 μM) in the presence of 50 μM of fluridone did not allow radicle protrusion (Fig. 1).

Imbibition curve. The fresh weight of intact seeds during imbibition increased (phase I) to reach a plateau (phase II) at day 3 of imbibition, and remained constant until day 15 of imbibition (Fig. 2).

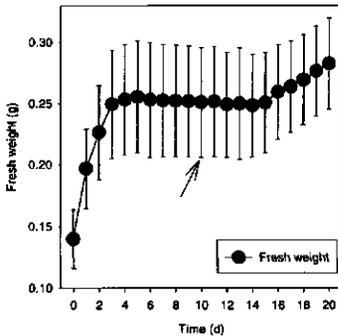


Figure 2 Imbibition curve during coffee seed germination. Data points are average of 100 seeds; error bars indicate standard deviations. Arrow indicates radicle protrusion of 50% of the seeds.

Embryo growth. The embryo grew inside the endosperm before radicle protrusion. In water-imbibed seeds there was a significant increase in both the length of the embryonic axes and of the cotyledons (Fig. 3) when 50% of the seed population had germinated. The increase in embryo length was 1.06 mm (35%) until 10 days of imbibition ($P < 0.01$). The increase in length of the embryonic axis was around 0.73 mm (36%) until 10 days of imbibition and in the cotyledons 0.33 mm (34%).

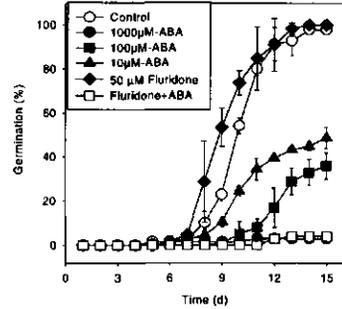


Figure 1 Germination of coffee seeds in water, 1000 μM , 100 μM or 10 μM of ABA, 50 μM of Fluridone or 50 μM of Fluridone + 1000 μM ABA. Data points are average of 4 replications of 25 seeds; error bars indicate standard deviation.

Figure 3 Length of embryo, axis and cotyledons from coffee seeds imbibed in water. The embryos were isolated immediately before measurement. Data points are average of 20 embryos; error bars indicate standard deviation.

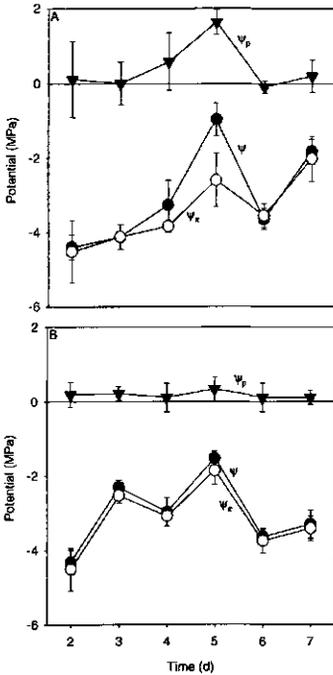
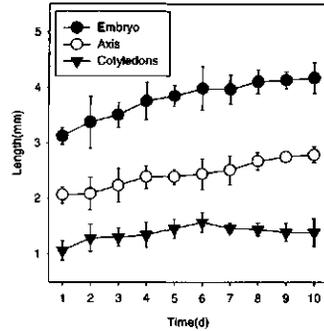


Figure 4 Water potential Ψ (●), osmotic potential Ψ_{π} (○) and pressure potential Ψ_p (▼) of coffee embryos isolated from water-imbibed (A) seeds and from seeds imbibed in 1000 μM of ABA (B); error bars indicate standard deviation.

Water potential measurements.

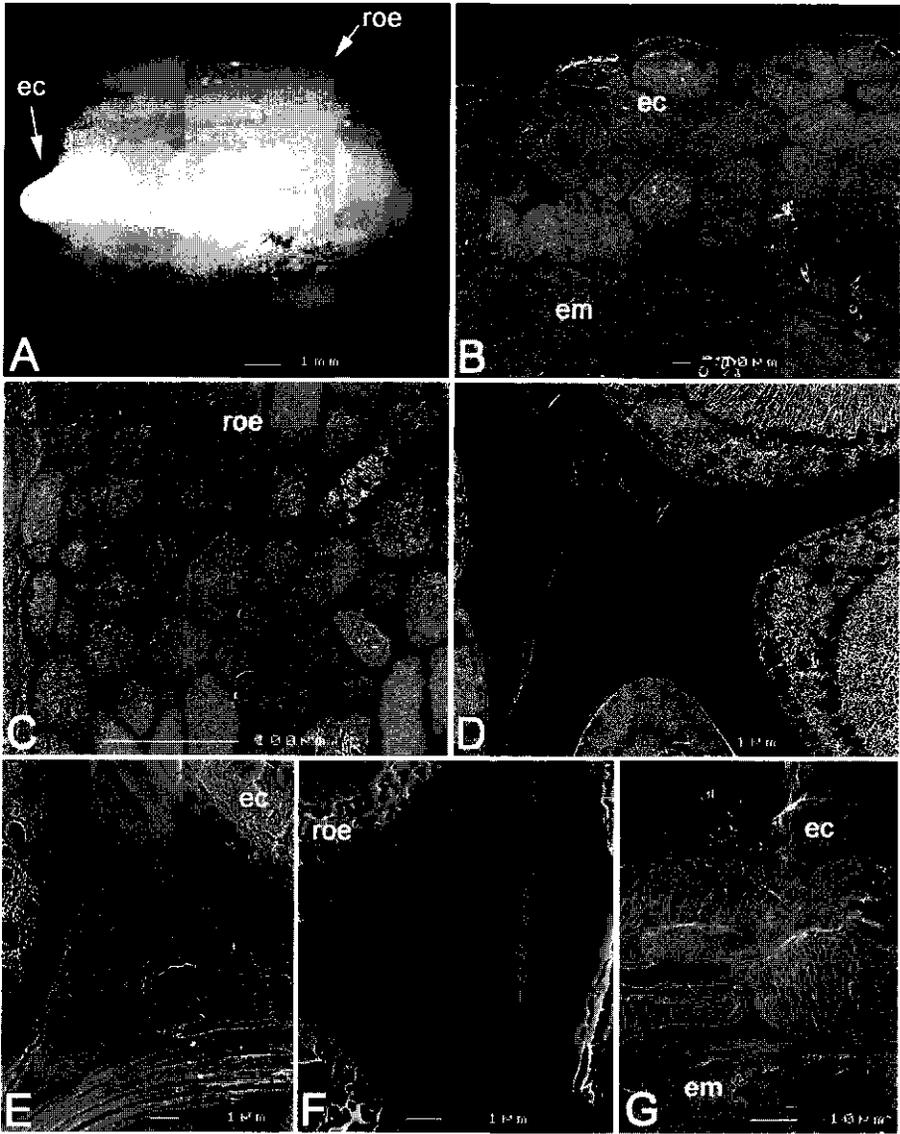
Psychrometric measurements were started at 2 days of imbibition. The embryo water potential was -4.40 MPa and increased to -0.96 MPa at 5 days of imbibition. The osmotic potential increased from -4.50 MPa at 2 days of imbibition to -2.59 MPa at 5 days of imbibition. Consequently, the pressure potential increased from 0.11 MPa to 1.62 MPa at 5 days. At 6 days of imbibition there was a decrease in both the water and osmotic potential. The water potential decreased from -0.96 MPa to -3.64 MPa and the osmotic potential from -2.59 MPa to -3.55 MPa. The pressure potential also decreased from 1.62 MPa to around 0 MPa. After 6 days of imbibition water potential and osmotic potential increased again (Fig. 4a).

In ABA the embryo water potential increased from -4.31 MPa to -1.53 MPa at 5 days of imbibition and the osmotic potential from -4.50 MPa to -1.85 MPa at 5 days of imbibition. At 6 days of imbibition there was a decrease in water potential from -1.53 MPa to -3.63 MPa. The osmotic potential also decreased from -1.85 MPa to -3.84 MPa. No change in pressure potential in ABA-imbibed seeds was observed (Fig. 4b); values were always slightly above zero.

of endo- β -mannanase (pI 4.5, pI 6.5 and pI 7.0). In ABA-imbibed seeds two isoforms were completely inhibited in the endosperm cap (pI 4.5 and pI 6.5). There was an extra isoform that seemed to be specific for the rest of the endosperm (pI 5.5) since this isoform was not observed in the endosperm cap (Fig. 8). ABA inhibited only one isoform in the rest of the endosperm (pI 4.5).

Endosperm structure during germination. The endosperm cap expanded during imbibition prior to radicle protrusion. Endosperm expansion prior to radicle protrusion has been described in other species as a protuberance (Werker, 1997). The protuberance observed in the endosperm cap was detected after five days of imbibition (Fig. 9A); it was inhibited by ABA and increased in size until radicle protrusion. Three to four cell layers were observed in the endosperm cap in front of the radicle tip (Fig. 9B). Cryo-SEM revealed compressed cells and loss of integrity of endosperm cap cells before radicle protrusion, coinciding with the protuberance (Fig. 9G). The endosperm cap showed thinner-walled cells than the rest of the endosperm (Fig. 9B and C). Concomitantly with the occurrence of the protuberance porosity in the walls of the endosperm cap was observed (Fig. 9E) as well as in the rest of the endosperm (Fig. 9F), but no porosity was observed earlier during imbibition (Fig. 9D). From day 3 to 9 of imbibition the number of cell layers in the endosperm cap showing porosity increased. There was a gradient in porosity from higher porosity in the cell walls close to the embryo to lower porosity in cell walls close to the epidermal cells. In ABA-imbibed seeds the endosperm cap also showed the same gradient in porosity as observed in water-imbibed seeds. In the rest of the endosperm porosity was also observed in ABA-imbibed seeds from day 6 onwards (results not shown). Initially, the pores appeared in the cell walls that were close to the embryo and at day 9 of imbibition the first cell wall layer adjacent to the embryo was completely eroded.

Figure 9 A. Coffee seed after 5 days of imbibition in water with indication of endosperm cap (ec) and the rest of the endosperm (roe). Note the occurrence of a protuberance. B. Scanning electron micrograph of the endosperm cap (ec) at 3 days of imbibition in water; embryo(em). C. Scanning electron micrograph of the rest of the endosperm (roe) at 3 days of imbibition in water. Note that the cell walls are thicker than in the endosperm cap. D. Scanning electron micrographs of the endosperm cap (ec) at 2 days of imbibition in water. No porosity was detected. E. Scanning electron micrograph of the endosperm cap (ec) at 6 days of imbibition in water. Highly porous cell walls can be observed. F. Scanning electron micrograph of the rest of the endosperm (roe) at 6 days of imbibition in water. Porosity can be observed throughout the cell walls. G. Scanning electron micrograph of the endosperm cap (ec) at 9 days of imbibition in water. Cells appear compressed and show loss of integrity.



activities ($r^2 = 0.86$ for endo- β -mannanase and $r^2 = 0.83$ for cellulase). Endosperm weakening prior to radicle protrusion has also been demonstrated to occur in muskmelon (Welbaum *et al.*, 1995), *Capsicum annuum* (Watkins and Cantiliffe, 1983) and *Datura ferox* (de Miguel and Sanches, 1992) which also coincided with the occurrence of enzyme activity in the endosperm. However, we can not exclude that the embryo growth during coffee seed germination may also contribute to the second phase of the endosperm cap weakening.

ABA only inhibited the second step of endosperm cap weakening as well as endo- β -mannanase activity. Thus, the first phase of the decrease in the required puncture force cannot be attributed to endo- β -mannanase activity whereas the second phase may be under control of this enzyme. Isoelectric focussing showed that there were three different isoforms of endo- β -mannanase in the endosperm cap of coffee seed and that ABA inhibited at least two of them (pI 4.5 and pI 6.5). This suggests that there are endo- β -mannanase isoforms that may have a decisive role during the second step of endosperm cap weakening. In the rest of the endosperm ABA inhibited only one isoform (pI 4.5). In total we observed four different isoforms of endo- β -mannanase in the endosperm of coffee seed whereas Marracini *et al.*, (2001) observed more isoforms. This difference may be due to the fact that we used seeds prior to radicle protrusion in isoelectric focusing studies whereas these authors used 28-days imbibed seeds *i.e.* after radicle protrusion. Different isoforms of endo- β -mannanase are also present in tomato seeds during germination (Toorop *et al.*, 1996; Nonogaki *et al.*, 1998).

The first step of endosperm cap weakening was not inhibited by ABA and ABA did not inhibit cellulase activity. Indeed, the increase in cellulase activity coincided with the first phase of decrease in puncture force in ABA-imbibed seeds. The presence of cellulase has previously been demonstrated in coffee seed (Takaki and Dietrich, 1980; Giorgini, 1992). Tissue printing demonstrated that cellulase activity was present throughout the endosperm during imbibition and no differences were observed with and without ABA. Also in tomato seeds ABA did not inhibit cellulase activity (Toorop *et al.*, 1998). A cDNA having high homology with known β -1-4 glucanases was isolated from radicle and endosperm cap of tomato seeds prior to radicle protrusion and ABA had no effect on its expression (Bradford, 2000). Tomato seeds also show a biphasic endosperm cap weakening (Toorop *et al.*, 2000). During the first phase the decrease in required puncture force correlated with an increase of endo- β -mannanase activity and the occurrence of ice crystal-induced porosity in the cell wall as observed by scanning electron microscopy. During the second phase endo- β -mannanase activity and required puncture were

uncoupled in ABA-imbibed seeds. Thus, tomato seeds show a similar behaviour in endosperm weakening but a dissimilar pattern of endo- β -mannanase activity as compared with coffee seeds

Cryo-SEM studies showed that the endosperm cap cells were compressed and lost integrity before radicle protrusion. Evidently, growth of the embryo inside the endosperm caused the occurrence of the protuberance, as well as the compression of cells in the endosperm cap and loss of cell integrity. Cryo-SEM also showed porosity in the endosperm cap and in the rest of the endosperm before radicle protrusion. There was a progressive increase in porosity before radicle protrusion in the endosperm cap and in the rest of the endosperm. The same trend, however, albeit at lower levels, was observed in ABA-imbibed seeds. The porosity in the endosperm cap coincided with the decrease in required puncture force, increase in cellulase and endo- β -mannanase activity, and with the occurrence of specific endo- β -mannanase isoforms in the endosperm cap and in the rest of endosperm. In tomato seeds the development of porosity in the endosperm cap coincided with the increase in endo- β -mannanase activity and the overall decrease in required puncture force (Toorop *et al.*, 2000). Also in *Datura* spp. eroded cell walls were present in the micropylar endosperm before radicle protrusion (Sánchez *et al.*, 1990). Moreover, the coffee endosperm cap cell walls are thinner compared with the cell walls in the rest of the endosperm. The same structural difference has been described in the endosperm cap of tomato (Hilhorst *et al.*, 1998), muskmelon (Welbaum *et al.*, 1990) and in *Datura* species (Sánchez *et al.*, 1990).

There was a transient rise in ABA content in coffee embryos around day two of imbibition and a second peak around day 5 (Fig. 11). The lowering in ABA content to near zero values at day 8 of imbibition coincided with radicle protrusion (50% of the seed population). It shows that ABA is synthesized *de novo* in the embryo during coffee seed imbibition and is degraded or leached out thereafter. Fluridone, an inhibitor of ABA biosynthesis (Li and Walton, 1990) significantly advanced radicle protrusion. Therefore, ABA biosynthesis during coffee seed imbibition may contribute to the slow radicle protrusion observed in coffee seeds. We hypothesise that ABA controls the embryo growth potential during germination, and the second step of endosperm cap weakening by inhibiting two isoforms of endo- β -mannanase.

In conclusion, embryo growth and weakening of the endosperm cap control coffee seed germination and ABA inhibits seed germination by controlling the second step of endosperm cap weakening and the increase in pressure potential in the embryo.

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Abstract

The mechanism of inhibition of coffee (*Coffea arabica* cv. Rubi) seed germination by exogenous gibberellins (GAs) and the requirement of germination for endogenous GA were studied. Exogenous GA₄₊₇ inhibited coffee seed germination. The response to GA₄₊₇ showed two sensitivity thresholds: a lower one between 0 and 1 μ M and a higher one between 10 and 100 μ M. However, radicle protrusion in coffee seed depended on the *de novo* synthesis of GAs. Endogenous GAs were required for embryo cell elongation and endosperm cap weakening. Incubation of coffee seed in exogenous GA₄₊₇ lead to loss of embryo viability and dead cells were observed by low temperature scanning microscopy only when the endosperm was surrounding the embryo. The results described here indicate that the inhibition of germination by exogenous GAs seems to be caused by factors that are released from the endosperm during or after its weakening, causing cell death in the embryo leading to inhibition of radicle protrusion.

Keywords: Gibberellins, endo- β -mannanase, β -mannosidase, cell death, puncture force, coffee seed, germination.

Introduction

Gibberellins (GAs) are an absolute prerequisite for seed germination of many species (Bewley, 1997). For example, GA-deficient mutants of *Arabidopsis* and tomato do not germinate in the absence of exogenous GA (Koorneef and van der Veen, 1980; Groot and Karssen, 1987). Tetcyclacis and paclobutrazol are inhibitors of GA-biosynthesis and may thus prevent seed germination (Karssen, *et al.* 1989; Rademacher, 2000) as at their effective concentrations these inhibitors do not exert side effects. Addition of exogenous GAs completely reverts the inhibitory effect of tetcyclacis and paclobutrazol, e.g. in *Arabidopsis* (Debeaujon and Koorneef, 2000).

There is ample evidence that GAs induce endosperm degradation by stimulating cell wall hydrolase activity. In pepper seeds GA₄₊₇ induced the decrease in the required force to puncture the endosperm cap (Watkins and Cantliffe, 1983) and stimulated endosperm degradation (Watkins *et al.*, 1985). In celery seeds gibberellic acid induced changes in the cell wall structure in the endosperm (Jacobsen *et al.*, 1976). In tobacco seeds GA₄ induced β -1-3 glucanase activity in the micropylar endosperm, which corresponded with endosperm rupture (Leubner-Metzger *et al.* 1996). Endo- β -mannanase and β -mannosidase, both involved in hydrolysis of galacto-mannans, were induced in the micropylar endosperm of *Datura ferox* by GA (Sánchez and de Miguel, 1997). In the tomato seed GA, possibly from the embryo, triggered weakening of the endosperm cap, induced degradation of the endosperm cell walls and allowed radicle protrusion (Groot and Karssen, 1987; Groot *et al.*, 1988). Groot *et al.* (1988) have shown that the activity of endo- β -mannanase, β -mannosidase and α -galactosidase was enhanced in GA deficient mutant seeds (*gib-1*) treated with exogenous GA₄₊₇. In the absence of GA only α -galactosidase could be detected but no endo- β -mannanase and β -mannosidase.

Also in tomato GA stimulated embryo growth (Karssen *et al.* 1989; Karssen and Laçka, 1986), possibly by enhancing the embryo growth potential. GA has been shown to stimulate elongation in hypocotyls of dark-grown lettuce seedlings (Katsu and Kamisaka, 1981) and in *Arabidopsis* GA controls cell elongation in light- and dark-grown hypocotyls (Cowling and Harberd, 1999).

Contrary to many reports on the stimulatory effect of GA during seed germination and tissue elongation, GA₃ inhibited radicle protrusion (Valio, 1976; Takaki and Dietrich, 1979a, 1979b; Takaki and Dietrich, 1980) and radicle emergence in coffee seed (Maestri and Vieira, 1961). This inhibition was proposed to be caused by mannose, a degradation product of the hydrolysis of mannans (Takaki and Dietrich, 1980). Coffee endosperm cell walls are composed mainly of mannans (Wolf from *et al.*, 1961). Mannose has been show to inhibit ATP

Enzyme activity in the supernatant was assayed using 75 μ l MacIlvaine buffer pH 5.0, 15 μ l 10 mM p-nitrophenyl- β -D-mannopyranoside dissolved in MacIlvaine buffer, pH 5.0 and 60 μ l enzyme extract. After incubation for 2 h at 37 °C the reaction was stopped by adding 75 μ l 0.2 M Na₂CO₃. The yellow color produced was measured at OD₄₀₅ in a microtiter plate reader. The enzyme activity was expressed as p-nitrophenol released (nmol sec⁻¹) per endosperm cap.

Tetrazolium stain. Embryos were isolated and incubated in 0.1% (w/v) of 2,3,5 triphenyltetrazolium chloride (Sigma) at 30 °C in the dark for 16 hours according to Dias and Silva, (1986). The tetrazolium salts are used to measure the activity of dehydrogenase enzymes as an index of the respiration rate and seed viability, distinguishing between viable and dead tissues (Copeland and McDonald, 1995).

Cryo-scanning electron microscopy. Seeds from water-, GA₄₊₇- and mannose-imbibed seeds were prepared for cryo-scanning electron microscopy (cryo-SEM). The embryos were mounted on aluminum rivets with a drop of tissue freezing medium (Tissue Tek, Sakura, The Netherlands). After mounting, the samples were plunge-frozen in liquid propane and stored in liquid nitrogen for subsequent cryo-planing and observations. The embryos were cryo-planed at -90° C in a cryo-ultramicrotome (Reichert-Jung Ultracut E/FC4D) with a diamond knife (8 mm wide; 45°, Drukker International, The Netherlands) according to Nijse and van Aelst (1999). The planed surfaces were freeze dried for 3 minutes at -89 °C and 10⁻⁴ Pa and sputter-coated with platinum in an Oxford CT1500 HF cryo transfer unit. The surfaces were photographed in a cryo-SEM (JEOL 6300 F) at -180 °C and 2.5-5.0 kV using a digital imaging system.

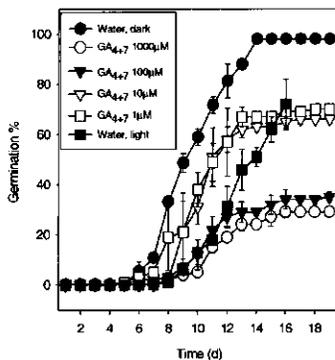


Figure 1 Germination of coffee seeds in water, in the dark or light, and in various concentrations of GA₄₊₇ in the dark. Data points are averages of 4 replications of 25 seeds each; error bars indicate standard deviation.

Statistical analysis. Statistical analyses were performed by using the general linear model (SPSS 10.0.5).

Results

Radicle protrusion of coffee seeds started at day five of imbibition in water (da Silva *et al.* 2002) and light partially inhibited coffee seed germination (Fig. 1). GA₄₊₇ inhibited radicle protrusion in a concentration dependent manner. However, the dose response relationship was not linear or log-linear as expected. Rather, a two step inhibition was observed with high and low sensitivity thresholds: 1 and 10 μ M

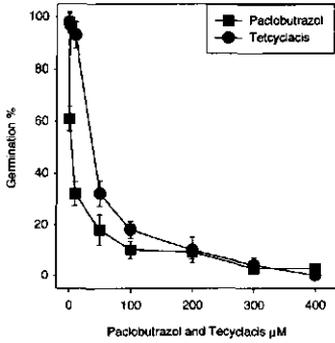


Figure 2 Germination of coffee seeds in paclobutrazol and in tetcyclacis. Data points are averages of 4 replications of 25 seeds each; error bars indicate standard deviation.

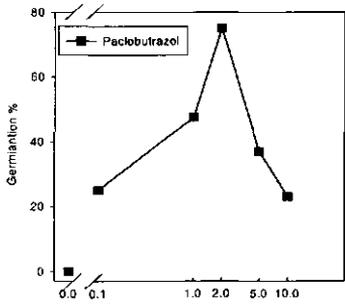
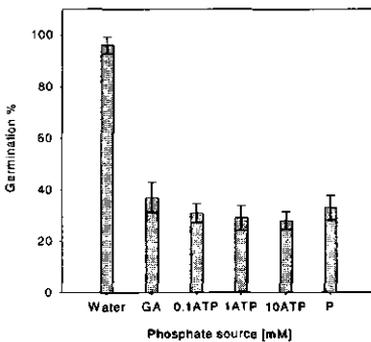


Figure 3 Germination of coffee seeds in various GA₄₊₇ concentrations in the presence of 300 μM paclobutrazol. Data points are averages of 4 replications of 25 seeds each; error bars indicate standard deviation.



GA₄₊₇ resulted in a reduction of the maximal germination by 35% whereas 100 and 1000 μM GA₄₊₇ lead to a further reduction by 30%. Apparently, the concentration thresholds for inhibition were between 0 and 1 μM and 10 and 100 μM, respectively.

GA₄₊₇ was more effective than GA₃ in inhibiting radicle protrusion and was used in all experiments (results not shown). The inhibition of radicle protrusion by exogenous GAs was only observed in coffee seed. The same GA₄₊₇ solution was used to germinate tomato seed and no inhibition of germination was observed but there GA₄₊₇ increased the speed of radicle protrusion (results not shown). Tetcyclacis and paclobutrazol, inhibitors of GA biosynthesis (Rademacher, 2000), completely inhibited germination at concentrations of 400 μM and 300 μM, respectively (Fig. 2). Application of exogenous GA₄₊₇ overcame the inhibition and allowed germination, which excluded the possibility of side effects during imbibition, and confirmed the requirement for GA-biosynthesis of coffee seed germination (Fig. 3). The dose-response curve displayed a narrow optimum of approximately 2 μM of GA₄₊₇ at the paclobutrazol concentration used. Germination in ATP or KH₂PO₄ + K₂HPO₄ did not overcome the inhibitory effect of exogenous gibberellins during coffee seed germination (Fig. 4).

Figure 4 Germination of coffee seeds in 0.1, 1 and 10 mM ATP and 20 mM of P (KH₂PO₄ + K₂HPO₄) in the presence of 100 μM of GA₄₊₇. Compare to germination in water and in 100 μM of GA₄₊₇ ("GA"). Data points are averages of 4 replications of 25 seeds each; error bars indicate standard deviation.

day 5 (Fig. 9A). In GA_{4+7} -imbibed seeds endo- β -mannanase activity in the endosperm cap from seeds was substantially higher (3-10 fold) than in water-imbibed seeds until 8 days of imbibition. After that, the activity decreased to the level of water-imbibed seeds at day 10 and was maintained at that level in the non-germinating seeds. Endo- β -mannanase activity was almost completely inhibited in tetcyclacis and paclobutrazol-imbibed seeds at all imbibition intervals.

β -mannosidase activity. The activity of β -mannosidase in the endosperm cap followed the same trend as endo- β -mannanase activity. In water-imbibed seeds the activity also increased before radicle protrusion. In GA_{4+7} the activity was higher (2-6 fold) until 8 days of imbibition and decreased thereafter. Slightly lower levels of β -mannosidase activity were detected in tetcyclacis and in paclobutrazol (Fig. 9B) as compared to the water control.

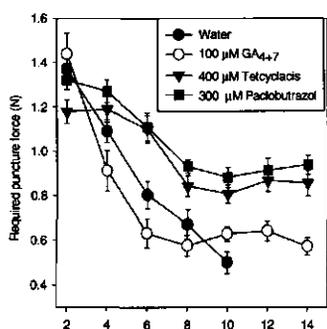


Figure 8 The required puncture force of water-, tetcyclacis-, GA_{4+7} - and paclobutrazol-imbibed seeds before radicle protrusion. Data points are averages of 30 measurements; error bars indicate standard error of mean.

compartmentalization (Fig 10 C and D). The dead or dying cells were surrounded by intact cells. The cell contents of the collapsed cells could be observed in the intercellular spaces of the hypocotyl cells (Fig. 10 D). The groups of dead cells were located in the epidermis, cortex and in the vascular region. Water- and mannose-imbibed seeds only showed normal turgid cells (results not shown).

Tetrazolium stain. Embryos lost their viability when the seeds were imbibed in GA solutions. This was particularly pronounced in the axes. The axes showed a brown color, confirming that the tissue had died. In the control the hypocotyls showed an intense red color, indicating that the embryos were respiring and alive (Fig.10 A).

Cryo-scanning electron microscopy. Since tetrazolium staining demonstrated that the embryonic axes of GA_{4+7} -imbibed seeds were dead, we visualised that particular region with cryo-SEM. The hypocotyl regions contained patches of deteriorated cells (Fig.10 B). Higher magnification revealed that the cells in this region had collapsed and/or had lost cellular

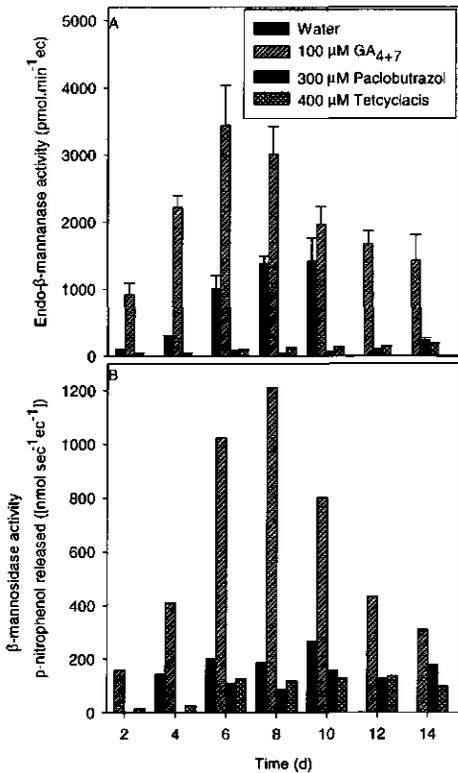


Figure 9 Endo-β-mannanase (A) and β-mannosidase (B) activities in water, 100 μM of GA₄₊₇, 300 μM of paclobutrazol and 400 μM of tetracyclis. Data represent 3 replications of extracts from 10 endosperm caps isolated from seeds prior to radicle protrusion. After radicle protrusion the seeds do not have endosperm cap. Error bars indicate standard deviation.

Discussion

Action of GA

GA₄₊₇ substantially inhibited germination of coffee seeds at a concentration as low as 1 μM. The response to GA₄₊₇ exhibited two sensitivity thresholds: a lower one between 0 and 1 μM and a higher between 10 and 100 μM. This may be caused by a heterogeneous population, consisting of sub-populations of seeds, displaying different sensitivities to the hormone. However, previous dose-response experiments with ABA never indicated any heterogeneity of the seed batch. The two steps of inhibition of germination may also be caused by two sites or mechanisms of inhibition with different sensitivities. When GA-biosynthesis was blocked by tetracyclis or paclobutrazol, applied GA₄₊₇ stimulated germination up to the optimum of 2 μM after which it became inhibitory. Clearly, the amount of applied GA adds up to the

endogenously synthesized hormone. From these data we estimate that the amount of GAs synthesized in the seed is in the order of a few μM of exogenous GA equivalents. We do not know to what percentage applied GAs are taken up by the coffee seeds. The optimal range of GA concentrations for germination appeared to be very narrow (Fig. 3).

Our results indicate that germination of coffee seeds depends on *de novo* synthesis of GAs. This has been shown for a large number of species, including *Arabidopsis* and tomato (Karssen *et al.*, 1989; Nambara *et al.*, 1991). However, to our knowledge coffee is the only species that displays inhibition of germination by GAs at physiological concentrations.

The site of GA action has been proposed to be both in the endosperm and in the embryo (Karssen *et al.*, 1989). In tomato seeds GA induces both embryo growth (Karssen *et al.*, 1989) and endosperm cap weakening (Groot *et al.*, 1997; Groot *et al.*, 1998).

by reactive oxygen species. Our results showed that the hypocotyl turned brown in 100 μM of GA_{4+7} , an indication of oxidation stress and/or the absence of sufficient 'reducing power'. Thus, what triggers cell death in the endosperm cap during or after its degradation may affect the embryo as well, leading to cell death and, consequently, inhibition of radicle protrusion.

In the coffee seed exogenous GA may speed up germination related processes, e.g. endosperm cap weakening. It is possible that under these conditions normal cell death of the endosperm occurs too early, with respect to embryo growth. The embryo would then be affected by the damaging components from the endosperm cells. In other words, too much GA disregulates the synchronization of germination processes occurring in the embryo and endosperm.

A hypothesis of the ecological relevance of GA-inhibited germination

Arguments in favor of a possible ecological significance of this phenomenon are 1) The narrow optimum for GA-stimulated germination. This enables "fine tuning" of the response within a narrow range of GA concentrations and enables the seed to respond to small changes in its environment; 2) Light inhibits germination of coffee seeds (Valio, 1976 and Fig. 1). This makes sense in an ecological context since *Coffea arabica* is originally classified as a shadow plant (Rena and Maestri, 1986). Light induces GA-biosynthesis in seeds (Hilhorst and Karssen, 1992; Toyomasu *et al.*, 1993). To avoid germination under full sun light coffee seeds may have developed this inhibiting mechanism. It is likely that under natural conditions embryonic cell death does not occur. At lower levels factors that contribute to cell death may inhibit cellular processes rather than kill the cells.

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CHAPTER 5

ABA reduces the abundance of microtubules and inhibits their transversal organisation, embryo cell elongation and cell division during coffee (*Coffea arabica* cv. Rubi) seed germination

E. A. A. da Silva, P. van Ekeren, P. E. Toorop, A.A.M. van Lammeren and Henk W. M. Hilhorst

Abstract

We studied the growth of the coffee (*Coffea arabica* L. cv. Rubi) embryo during and following germination of water- and ABA-imbibed seeds. The coffee embryo grows first by increase in cell width followed by longitudinal growth. These events coincided with reorientation and increase in abundance of the microtubules and with accumulation of β -tubulin in the embryonic axis during and following germination. DNA synthesis was activated during imbibition and cell division started prior to radicle protrusion and its rate increased following germination. ABA decreased the number of microtubules, inhibited the longitudinal growth of the embryo cells, the reorganization of the microtubules and cell division in the embryonic axis. However, ABA neither inhibited swelling of the embryo cells nor organellar and nuclear DNA replication.

Keywords: coffee, cell expansion, cell elongation, cell division, microtubules, β -tubulin, abscisic acid.

Introduction

Seed germination can be defined as the event that starts with water uptake by the seed and is terminated with the radicle protrusion (Bewley and Black, 1994). Many biochemical and molecular events take place during imbibition. Among these events DNA replication is necessary at the beginning of the imbibition process for DNA repair (Osborne, 1983). DNA synthesis associated with cell division is a late event during imbibition (Bewley, 1997). However, β -tubulin accumulation, assembly of microtubules, nuclear DNA synthesis and cell division have been shown to occur during imbibition of tomato seeds prior to radicle protrusion (de Castro *et al.*, 1995, 2000). Coffee (*Coffea arabica* L.) seeds contain an endosperm tissue that envelops the embryo (Krug and Carvalho, 1939; Mendes, 1941; Chapter 2). The differentiated embryo lies inside an embryo cavity (Rena and Maestri, 1986; Chapter 2), has a length of 3 to 4 mm and consists of a radicle, an axis and two small cotyledons (Mendes, 1941). During coffee seed germination the embryo grows inside the endosperm before radicle protrusion by increasing the pressure potential and cell wall extensibility in the embryo cells (Chapter 2 and Chapter 3). Abscisic acid (ABA) is known to inhibit seed germination in many species (Bewley and Black, 1994). During germination ABA acts both in the embryo and in the endosperm (Hilhorst, 1995). ABA inhibits cell wall loosening in the coffee embryo (Chapter 3) as shown to occur in *Brassica napus* (Schopfer and Plachy, 1985).

In the embryo of apple ABA inhibited the transition of nuclei to the G2 phase of the cell cycle and, consequently, cell division (Bouvier-Durand *et al.*, 1989). However, ABA did not inhibit DNA repair in embryos of *Avena fatua* (Elder and Osborne, 1993). The coffee embryo displays growth during germination but it is unknown whether this growth is caused by cell elongation, cell division or both. In coffee seeds ABA inhibited the increase in turgor and, thus, inhibited coffee embryo growth during germination (Valio, 1976; Chapter 3). Microtubules play a crucial role in both cell elongation and cell division (Goddard *et al.*, 1994). Here we address the question whether inhibition of germination by ABA is targeted at the assembly and organisation of microtubules. For this we have used light microscopic analysis, immuno-histochemical detection of DNA synthesis, visualization of the microtubular cytoskeleton, flow cytometry, and detection of β -tubulin accumulation by Western blotting in water- and ABA-imbibed seeds.

pH, 6.9), for 4 hours and subsequently rinsed in 1:5 diluted MSB for 4 x 15 minutes. During fixation the embryos were submitted to vacuum.

The embryos were dehydrated in an ethanol series and embedded in butylmethacrylate according to Baskin *et al.* (1992). To avoid oxidation during sample preparation ascorbic acid (0.1% w/v) was added. For the analysis of the microtubular cytoskeleton the embryos were isolated and plunge-frozen in liquid propane and transferred to freeze substitution medium containing water free methanol and 0.1% glutaraldehyde. The cryo-tubes containing the embryos and freeze-fixation medium were incubated in a freeze

substitution unit (Cryotech-Benelux). After freeze substitution the freeze fixation medium was replaced by ethanol followed by embedding in butylmethacrylate and UV polymerisation at -20 °C according to Baskin *et al.* (1992). Sections were made either for the detection of DNA synthesis (incorporated BrdU) or for the visualization of the microtubular cytoskeleton (β -tubulin). Mouse anti- β -tubulin (Sigma) dilution 1:200 v/v and anti-BrdU (Amersham) dilution 1:1 v/v were applied. In both cases, the second antibody used was goat anti-mouse IgG conjugated with FITC (Molecular Probes) diluted 1:100 v/v. Nuclear DNA was counterstained with 1 mg ml⁻¹ propidium iodide (PI) (Molecular Probes, Eugene, OR, USA).

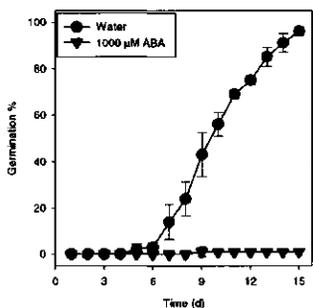


Figure 1 Germination of coffee seeds in water and in 1000 μ M of ABA. Data points are average of 100 seeds; error bars indicate standard deviation.

Statistical analysis. Statistical analyses were performed by using the general linear model (SPSS 10.0.5).

Results

Germination. Radicle protrusion of the first seed started at day 5 of imbibition in water. ABA at 1000 μ M completely inhibited germination (Fig. 1). Hydroxyurea, a nuclear DNA synthesis inhibitor (Górnik, *et al.*, 1997) did not inhibit germination up to a concentration of 10 mM (Fig. 2).

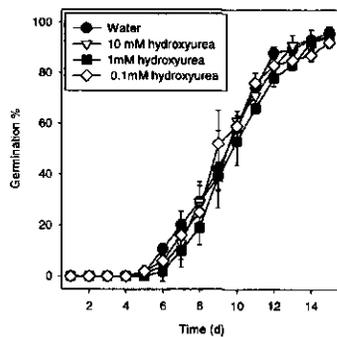


Figure 2 Germination of coffee seeds in water and in different concentrations of hydroxyurea. Data points are average of 100 seeds; error bars indicate standard deviation.

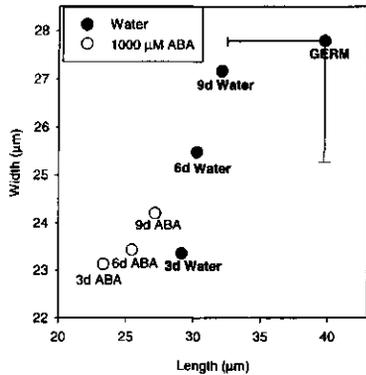


Figure 3 Changes in dimensions of the cells of the embryonic axis upon imbibition in water during and following germination (GERM) (●), and in 1000 μM ABA (○). The embryonic axis was divided in 10 equal parts and the cells in the regions 3, 7 and 9 were measured from days 3, 6 and 9 of imbibition. Data points represent the average cell length plotted against average cell width from 150 cells in μm. Bars indicate maximal standard deviation. Note the difference in scale at the X-axis and Y-axis.

Light microscopy. Coffee embryo axes were taken for light microscopy studies since this part of the coffee embryo appeared to contribute most to coffee embryo growth during germination (Chapter 3, 4). The embryonic axis was divided into 10 equal parts; microscopic analysis showed that cell growth occurred evenly in these 10 regions (data not shown). Therefore, three regions in the axis were taken for further analysis viz. regions 3, 7, and 9 from the cortex region. In water-imbibed seeds the cells initially increased both in length and width, and had the tendency to increase in length later during germination (Fig. 3). This indicates that the cells first grew about isodiametrically and then longitudinally (Fig. 3). Embryo growth at the moment of radicle protrusion was mainly controlled by cell elongation, since the increase in width from day 9 of imbibition to the moment of radicle protrusion was only 3% (Fig. 3). In ABA-inhibited

seeds the points representing 3, 6 and 9 days of imbibition showed little differences. (Fig. 3). Here, the cells kept their isodiametric shape. This implies that ABA effectively reduced cell elongation observed in water-imbibed seeds.

DNA replication. Flow cytometric analysis of dry control seeds showed a very low peak of 4C nuclei content, which indicates that most of the cells were in the G₁ phase of the cell cycle (Fig. 4). The number of 4 C nuclei showed a significant increase up to 6 days of imbibition in water (P < 0.001). At day 9 of imbibition in water the number of 4 C nuclei was the same as at day 6. After radicle protrusion the amount of 4C nuclei increased to almost twice the amount observed at day 6 or day 9 (Fig. 4). The same trend was also found in ABA-imbibed seeds until day 6. At day 9 of imbibition there was a slightly but significantly lower level of 4C nuclei in ABA-inhibited seeds (P < 0.001). No

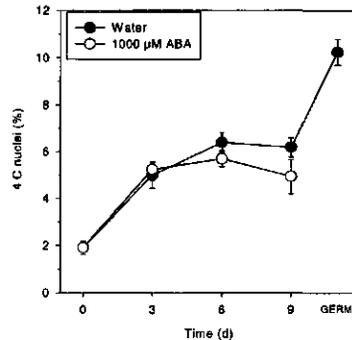


Figure 4 Frequency of nuclei of embryo cells with 4C DNA contents expressed as percentage of the total number of nuclei (2C+4C) during imbibition of the coffee seed in water (●) or in 1000 μM ABA (○). Error bars indicate standard deviation.

radicle protrusion was observed when the seeds were imbibed in ABA (Fig. 1).

DNA synthesis. BrdU incorporation was observed from 3 days of imbibition in water-imbibed seeds, mainly in the radicle tip (Fig 5 A). Until 6 days of imbibition nuclear DNA replication was limited whereas organellar DNA synthesis seemed to increase mainly at day 6 (Fig. 5 B), although only few dividing cells were observed (Fig. 5 F). At day 9 of imbibition nuclear DNA replication was relatively high compared to organellar DNA synthesis, which was very low or completely absent at that moment (Fig. 5 C). After radicle protrusion there was nuclear DNA replication both in the radicle tip and in the embryonic axis (Figs. 5 D and E) and also cell division was observed (Fig. 5 G). ABA did not inhibit organellar DNA and nuclear DNA synthesis (Fig. 5 H).

β -tubulin accumulation. The relative amount of β -tubulin in water-imbibed seeds increased from day 3 onwards to radicle protrusion. β -tubulin was also observed in ABA-imbibed seed but at much lower levels (Fig. 6).



Figure 6 Western blotting of β -tubulin in extracts from coffee embryos during and following germination of water- and ABA-imbibed seeds. Pure bovine brain tubulin (10 and 30 ng) was used as a control.

Configuration of microtubular cytoskeleton. Embryos were analysed for microtubular cytoskeleton at 0, 3, 6 days of imbibition and at 9 days both prior to and after radicle protrusion. At day 0 the cells exhibited green fluorescent granules and no microtubules were observed. At day 3 of imbibition the green fluorescent granules had partially disappeared and microtubules were formed (Figs. 7 A and B). Only a few cells showed a transversal organisation of the microtubules in the embryo axis at day 3 of imbibition (Fig. 7 B). From day 6 of imbibition onwards, but still before radicle protrusion, more cells showed microtubules that were transversally organised, and preprophase bands, mitotic spindles and phragmoplasts were observed, confirming the beginning of cell division (Figs. 7 C, D and E).

Cell division was also observed with propidium iodide staining at 6 days of imbibition (Fig. 5 F). The transversal organisation of the microtubules was also observed in the epidermal cells and outer cortex cells before (Fig. 7 F) and after radicle protrusion (Fig. 7 G). The microtubules also became more abundant from the beginning of imbibition until after radicle protrusion (Figs. 7 B, D, F and G). In ABA-imbibed seeds, microtubules were observed initially at low levels (Fig. 7 H), they remained randomly organised at 6 days of imbibition (Fig. 7 I). In addition, microtubules greatly reduced the number or disappeared at 9

days of imbibition in ABA (Fig 7 J). When the ABA-imbibed seeds were rinsed in demineralised water and the seeds were then imbibed in water for 9 days, microtubules reappeared (Fig. 7 K).

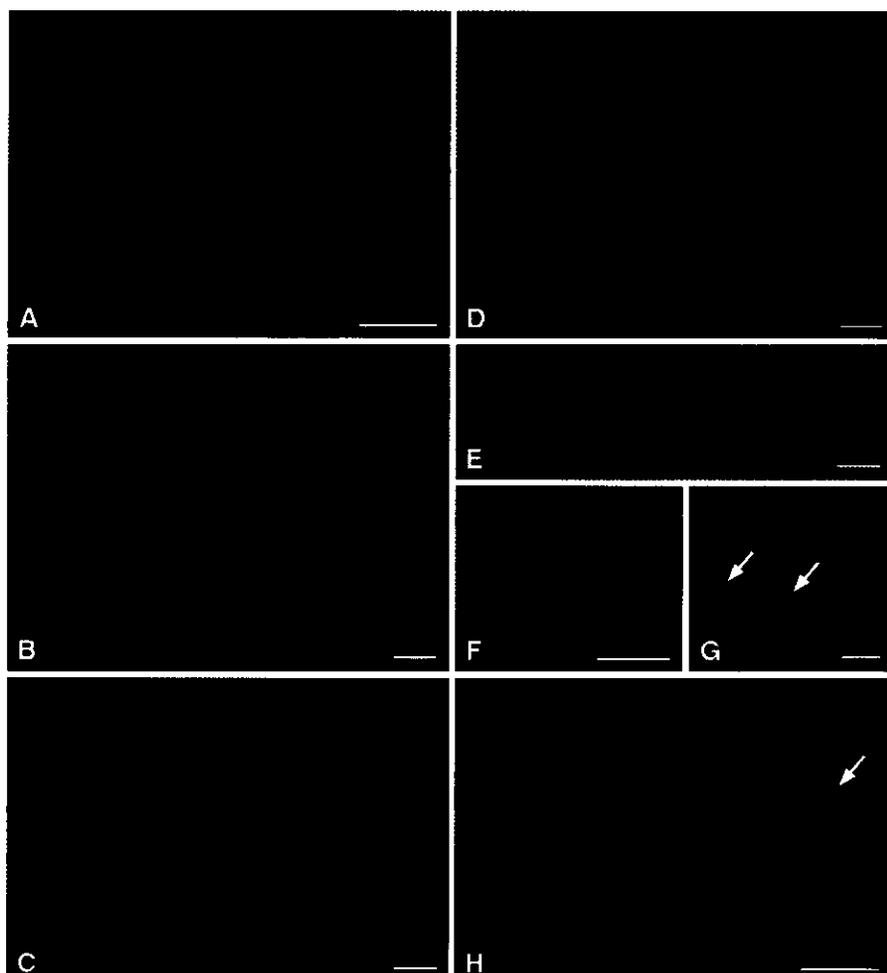


Figure 5 A-H Fluorescence micrographs of longitudinal sections of coffee embryos from water-imbibed seeds during and following germination (A-G) and of embryos from ABA-imbibed seeds (H). Nuclei show red fluorescence as the result of staining with propidium iodide. Nuclei showing green fluorescence are labelled with FITC, which indicates BrdU incorporation. **A** - Radicle tip region of a 3-day water-imbibed seed, showing BrdU incorporation after a 3-h pulse labelling. **B** - Radicle tip of a 6-day water-imbibed seed showing nuclei and organelles labelled with BrdU, indicating the occurrence of nuclear and organellar DNA synthesis. **C** - BrdU labelling in the radicle of a 9-day water-imbibed seed. **D** and **E**- Radicle tip and embryonic axis after radicle protrusion. Note that there are more nuclei labelled with BrdU in the radicle tip than at 9 days (C). **F** and **G** - Radicle region showing cell division at 6 days (F) and after radicle protrusion in water-imbibed seeds stained with propidium iodide (arrows). **H** - Embryo radicle tip from ABA-imbibed seeds showing organellar and nuclear DNA synthesis. Note the absence of DNA replication in the suspensor cells (arrow). Bars indicate 10 μ m for figure F, 20 μ m for figures A and H, and 50 μ m for figures B, C, D, E and G.

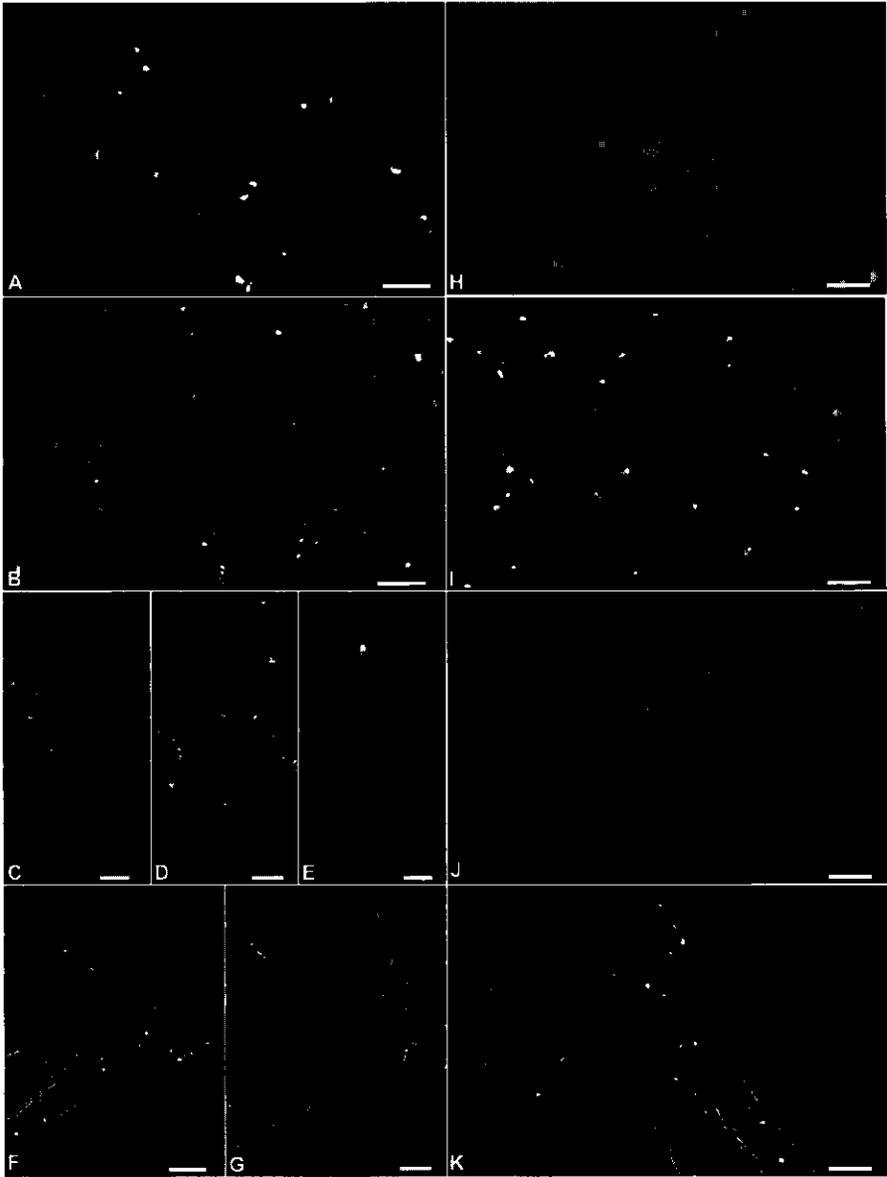


Figure 7 Fluorescence micrographs of longitudinal sections of embryos from water- and ABA-imbibed seed during and following germination. **A** - Axis region of dry embryo (0d of imbibition). Dots represent clusters of preserved β -tubulin **B** - Embryonic axis region from 3 days imbibed seeds in water showing low microtubule abundance and random microtubular organisation. **C**, **D** and **E** - Embryo axis region from seeds imbibed in water for 6d, showing transversal microtubular orientation in the outer cortex cells, and mitotic figures before radicle protrusion. **F** - Embryonic axis region from 9d water-imbibed seeds showing transversal microtubular orientation in the epidermal cells. **G** - Embryonic axis region after radicle protrusion showing transversal microtubular orientation and higher abundance of microtubules. **H** - Embryo axis region from 3d imbibed seeds in ABA showing low abundance of microtubules. **I** - Embryonic axis region from 6d ABA-imbibed seeds, showing random orientation of the microtubules. **J** - Embryo axis region from 9d imbibed seeds in ABA showing low abundance of microtubules. **K**- Embryonic axis region from 9d ABA-imbibed seeds that were rinsed in demineralised water after which the seeds were placed in water again. Note that the microtubules reappeared as in water-imbibed seeds. Bars indicate 2 μ m for all figures.

Discussion

The coffee embryo grows inside the endosperm before radicle protrusion, by increasing the pressure potential and cell wall extensibility (da Silva *et al.*, 2002; Chapter 3). Light microscopic analysis indicated that the embryonic axis grew initially by about isodiametric cell expansion, followed by cell elongation during and following germination. The initial isodiametric growth coincided with a predominantly random orientation of the microtubules and the subsequent longitudinal growth with a predominantly transversal orientation. In addition, the microtubules became more abundant during and following germination, in both epidermal and cortex cells. Apparently, the random orientation of the microtubules is a transition state, preceding the transverse orientation during coffee embryo elongation. The transition in microtubule orientation and abundance also coincided with the increase in β -tubulin. This implies that assembly of microtubules, at least partially, depends on *de novo* synthesis of β -tubulin during imbibition.

In Chapter 3, it has been shown that ABA inhibited the embryo growth potential by inhibiting cell wall loosening. Schopfer and Plachy (1985) have demonstrated that ABA inhibited cell wall loosening in *Brassica napus*. Here ABA allowed the isodiametric swelling of the coffee embryo cells during the first phase of the germination process. Indeed, ABA did not inhibit water uptake (data not shown) implying that this initial cell expansion was probably driven only by the water uptake. Thus, the suppression of embryo growth by ABA is targeted at the phase of predominantly longitudinal growth of the embryo cells during germination. Along with the isodiametric growth in ABA-imbibed seeds, the microtubules were randomly organised and their abundance decreased. Ishida and Katsumi (1992) found a similar organisation of microtubules in the embryonic hypocotyl cells of growing cucumber

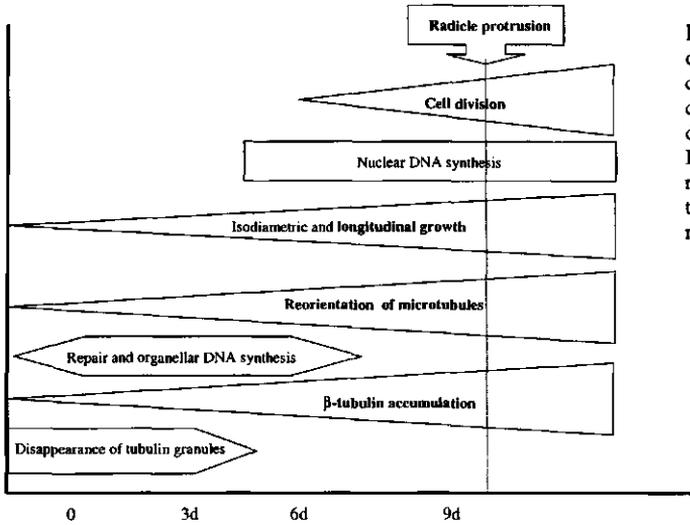


Figure 8 Schematic diagram of the cellular events in coffee embryos during germination. Bold characters represent the events that are ABA regulated.

seedlings treated with ABA. Therefore, the results found here indicate a relation between cell morphogenesis and microtubule orientation during embryonic axis elongation.

Quantification of the DNA levels by flow cytometry showed a 2C peak in dry control embryos (data not shown). DNA synthesis increased during imbibition as more nuclei stained positively for BrdU, and the occurrence of a 4C peak indicated DNA replication. Cell divisions were not observed during the first 3 days of imbibition. These results indicate that most of the embryonic cells are arrested in the G1 phase of the cell cycle during seed maturation and that upon imbibition there was an increase in the number of cells in the G2 phase of the cell cycle. Tomato seeds also showed DNA synthesis and an increase in the number of G2 cells during imbibition (Bino *et al.*, 1992; de Castro *et al.*, 2000). The significant increase in 4C nuclei from dry embryos to 6 d of imbibition, both in water and ABA, is likely due to nuclear DNA replication whereas BrdU incorporation also includes "cytoplasmic" DNA synthesis. Therefore, DNA synthesis (organellar and nuclear) found early during imbibition in the coffee embryo is probably DNA repair, necessary to repair damages caused during maturation drying and rehydration (Bewley, 1997), whereas DNA duplication at later stages of imbibition is a preparation for cell division.

The lag phase observed in the number of 4C nuclei from 6 d to 9 d of imbibition in water-imbibed seeds may indicate that the S phase of DNA replication in coffee embryos was relatively long. In ABA-imbibed embryos the amount of 4C nuclei was similar to that of the control but after 6d it was slightly, but significantly, lower. Thus, ABA appears to have an inhibitory effect on DNA duplication as preparation for cell division which becomes predominant after 6 d of imbibition. We hypothesise that ABA action is targeted mainly to specific proteins involved in the cell cycle machinery that are associated with cell division but

not with repair or organellar DNA synthesis. The DNA repair mechanism seems to be very well conserved and independent of the presence of ABA during imbibition. Elder and Osborne (1993) have suggested that continuous DNA synthesis (presumably DNA repair) is important to maintain genome integrity, allowing a continuous but slow replacement of DNA in dormant or ABA treated embryos of *Avena fatua*.

The presence of 4C nuclei in water-imbibed seeds correlated with accumulation of β -tubulin and with mitotic figures in the embryonic axis prior to and after radicle protrusion. In tomato seeds, the presence of 4C nuclei and β -tubulin expression correlated with the presence of mitotic cells in the embryo as well as with the progression of germination (de Castro *et al.*, 2000). To our knowledge cell division prior to radicle protrusion in the coffee embryo has never been described before. However, cell division is not a prerequisite for radicle protrusion in coffee seed, since inhibition of DNA synthesis but not DNA repair (Górnik *et al.*, 1997), by hydroxyurea did not inhibit radicle protrusion in coffee seed. Cell division is likely to be necessary for further (post-germinative) embryo growth.

In conclusion, embryo growth during and following germination in coffee seed was a result of cell expansion and elongation followed by cell division. These events coincided with abundance and reorientation of microtubules, accumulation of β -tubulin, cell expansion and elongation. ABA inhibited the accumulation of β -tubulin, cell elongation and cell division, but not the initial expansion, and organellar and nuclear DNA replication. All these events are sequentially summarized in figure 8.

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Abstract

The objective of this study was to determine the timing and location of endo- β -mannanase and β -mannosidase activity in the different seed parts of germinating coffee (*Coffea arabica* cv. Rubi) seeds, as well as the isolation of cDNA clones encoding these enzymes. Endo- β -mannanase and β -mannosidase activities increased during germination mainly in the endosperm caps of the coffee seed. Low levels of activities were observed in the rest of the endosperm and in the embryo prior to radicle protrusion. Two partial length cDNA clones encoding endo- β -mannanase and β -mannosidase were isolated from coffee endosperm caps. The deduced amino acid sequences of endo- β -mannanase and β -mannosidase exhibited high homology with those of other endo- β -mannanases and β -mannosidases from plants.

Keywords: coffee seed, *Coffea arabica*, endo- β -mannanase, β -mannosidase

Introduction

The coffee (*Coffea arabica* cv. Rubi) embryo is surrounded by endosperm tissue (Krug and Carvalho, 1939; Mendes, 1941). The endosperm cells possess thick cell walls, which are largely composed of mannan-rich polymers with 2% of galactose (Wolfrom, *et al.* 1961; Bewley and Black, 1994). Three enzymes have been suggested to be involved in the hydrolysis of the cell wall mannans: (1 \rightarrow 4)- β -mannan endohydrolase (EC 3.2.1.78; referred to here as endo- β -mannanase), β -mannosidase (EC 3.2.1.25) and α -galactosidase (EC 3.2.1.22).

The endosperm cap of the coffee seed, opposing the radicle tip, is weakened during germination (Chapter 3) and endo- β -mannanase activity increases before the first seed of the batch shows radicle protrusion (Chapter 3). Tissue printing showed occurrence of endo- β -mannanase activity, initially in the endosperm cap surrounding the radicle tip and only later, during germination, in the rest of the endosperm (Chapter 3). Three different isoforms of this enzyme were found in the endosperm cap during germination and another isoform was detected in the rest of the endosperm later during germination (Chapter 3). Marraccini *et al.* (2001) have demonstrated that whole coffee seeds contain 8 different isoforms of endo- β -mannanase, following germination. The same authors isolated only two different cDNA clones of endo- β -mannanase from germinated coffee seeds (*ManA* and *ManB*), which suggests that these different isoforms are at least partly caused by post-transcriptional modifications. It is not known whether there is endo- β -mannanase activity in the embryo prior to radicle protrusion. It is also not known what the role is of endo- β -mannanase in the rest of the endosperm (Chapter 3), and if the gene encoding for endo- β -mannanase is specific to the endosperm cap or not.

So far, β -mannosidase activity has not yet been monitored in the different seed parts of coffee seed during germination. This enzyme hydrolyzes the oligomeric mannose products resulting from endo- β -mannanase activity (Bewley and Black, 1994) and has been found and characterized in several seed species (McCleary and Mathenson, 1975; McCleary, 1982). It is likely to be present during coffee seed germination. Therefore, this study includes the timing and location of endo- β -mannanase and β -mannosidase activities in the different seed parts during germination of coffee seed and partial cDNA cloning of endo- β -mannanase and β -mannosidase cDNAs; also their deduced amino acids sequences are presented.

Material and Methods

Seed Source. Coffee seed (*Coffea arabica* L. cv. Rubi) was harvested in 2000 in Lavras-MG-Brazil, depulped mechanically, wet fermented for 24 hours, dried to 12% moisture content and shipped to The Netherlands and Canada where it was stored at 10 °C until use.

Germination conditions. The surrounding seed coat was removed by hand before the seed was sterilized in 1% sodium hypochlorite for 2 minutes, rinsed in water and imbibed in 10 ml demineralized water. The seeds were placed in Petri dishes on two layers of Whatman (No. 1) paper. During imbibition the seeds were kept at $30 \pm 1^\circ \text{C}$ in the dark (Huxley, 1965; Valio, 1976; Chapter 2).

Diffusion assay for endo- β -mannanase activity. Extracts were made from endosperm caps, embryos and rest of the endosperm isolated from water-imbibed seeds. Endo- β -mannanase was extracted in McIlvaine buffer (0.05 M citrate/0.1 M Na_2HPO_4 , pH5.0) with 0.5M NaCl and assayed in a gel (0,5 mm thick) containing 0.5% (w/v) locust bean gum (Sigma) in McIlvaine buffer (pH 5.0) and 0.8% type III-A agarose (Sigma) on Gelbond film (Pharmacia). Samples (2 μl) were applied to holes punched in the gel with a 2-mm paper punch. Gels were incubated for 21 h at 25° C, and then washed in McIlvaine buffer (pH 5.0) for 30 min, stained with 0.5% (w/v) Congo Red (Sigma) for 30 min, washed with 80% ethanol for 10 min, and destained in 1M NaCl for 5 h. Commercial endo- β -mannanase from *Aspergillus niger* (Megazyme, Cork, Eire) was used to generate a standard curve. The gel was scanned and printed out for calculation of enzyme activity according to Downie et al. (1994).

β -Mannosidase extraction and assay. Ten endosperm caps (0.1g), 10 embryos (0.026g) or 10 rest-of-endosperms (lateral endosperms) (2.1 g) were dissected and ground in a mortar with liquid nitrogen. Enzyme was extracted from the seed parts in McIlvaine buffer pH 5,0 with 0,5 M NaCl; the extracts were centrifuged for 20 minutes at 21000 g at 4°C. The supernatant was assayed using 75 μl MacIlvaine buffer pH 5,0, 15 μl 10 mM p-nitrophenyl- β -D-mannopyranoside (Boehringer, Mannheim) dissolved in MacIlvaine buffer, pH 5.0 and 60 μl enzyme extract. After incubation for 2 h at 37 °C the reaction was stopped by adding 75 μl 0.2M Na_2CO_3 . The yellow color produced was measured at OD₄₀₅ in a microtiter plate reader. The enzyme activity was expressed as p-nitrophenol released (nmol/min/gfw).

RNA isolation. Total RNA was extracted using the modified hot borate method (Wan and Wilkins, 1994). Endosperm caps were dissected from fifty coffee seeds after 5 days of imbibition. They were first frozen in liquid nitrogen and ground to a powder. The powder was again ground in a micro-dismembrator U (B. Braun Biotech International, Germany) at 2000 rpm for 3 minutes.

The powder was suspended in 700 μ l hot (80 °C) borate buffer (0.2 M sodium tetraborate decahydrate, 30 mM EGTA, 1% SDS and 1% sodium deoxycholate, pH 9,0) with 1.1 mg DTT, 14 mg PVP, 14 mg ascorbic acid and 1 mg proteinase K per sample. The sample was then transferred to a 2.2 mL Eppendorf tube and incubated in a water bath for 1h and 30 min at 42 °C. RNA was precipitated with 55 μ L 2 M KCl on ice for 1 hour. Samples were centrifuged for 20 minutes at 5 °C. The supernatant was transferred to a 15 mL tube and 270 μ L 8 M LiCl was added after which the samples were incubated overnight on ice to precipitate RNA. Samples were then centrifuged at 12,000 g for 20 minutes at 5 °C to pellet the RNA. The pellet was resuspended in 1 mL ice cold 2 M LiCl and subsequently centrifuged at 10,000 g for 10 min at 4 °C (the latter 2 steps were performed twice). The pellet was resuspended in 400 μ L 10 mM Tris-HCl and remaining debris was spun down at 12,000 g for 10 min at 4 °C. The supernatant containing the RNA was transferred to a 15 mL tube and 40 μ L 2 M KAc pH 5,5 was added and samples were centrifuged at 12,000g for 10 minutes at 5 °C. Cold ethanol (1.3 mL) was added and RNA was precipitated for 24 hours at -20 °C. The sample was centrifuged at 11,000g for 30 minutes at 5 °C to pellet the RNA. The pellet was washed with 70% cold ethanol, dried in a speed vacuum rotor and resuspended in 40 μ L RNase free water.

cDNA synthesis. Two and half μ g of total RNA isolated from coffee endosperm caps was used for cDNA synthesis using a SuperScript™ Preamplication System for First Strand cDNA Synthesis (Life Technologies) with oligo-p (dT) used as a primer and 1 μ l of SuperScript II Reverse Transcriptase (RT) (Life Technologies) according to the manufacturer's protocol. cDNA was used as a template for PCR reactions. Gene-specific primers were designed using the cDNA sequence for endo- β -mannanase from coffee seed (Marracini et al., 2001) and tomato seed (Nonogaki et al., 2000) and used in a PCR reaction that had 94 °C for 10 sec, 54 °C for 45 sec and 72 °C for 3 min. PCR products were separated in a 2% agarose TAE gel. The 412 bp DNA band was excised from the gel, purified, re-amplified under the same PCR conditions, cloned into pGEM-T Easy vector (Promega-USA) and sequenced.

Degenerate primers were designed using the cDNA sequence for β -mannosidase from tomato seed (Mo and Bewley, personal communication) and an Arabidopsis sequence of a putative β -mannosidase in a PCR reaction at 94 °C for 45 sec, 45 °C for 45 sec and 72 °C for 1 min. The PCR products were separated in a 2% agarose TAE gel. The 263 bp DNA band was excised from the gel, purified, re-amplified under the same PCR conditions, cloned into pGEM-T Easy vector and sequenced.

DNA Sequence Analysis. The nucleotide sequence was determined using a DNA sequencer (model ABI 373, PE-Applied Biosystems). Homology search within the databases was done using the BLAST program of the DNA databank (<http://www.ncbi.nlm.nih.gov/blast>). Alignments of the amino acid sequences were performed using the Clustal W program <http://www.ebi.ac.uk>

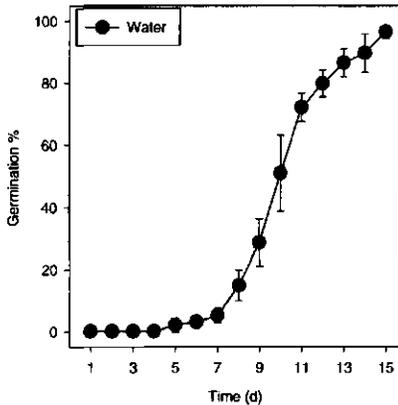


Figure 1 Germination of coffee seeds in water. Data points are averages of 4 replications of 25 seeds each; error bars indicate standard deviation.

Results

Germination. The first seeds showed radicle protrusion at 5 days from the start of imbibition and 50% of the seed population had their radicles protruded at 10 days. Germination of the whole population was completed at day 15 of imbibition (Fig. 1), confirming previous observations that coffee seed exhibits relatively slow and variable radicle protrusion.

Endo- β -mannanase activity. Previous work has shown endo- β -mannanase activity to be present in the endosperm of coffee seed during germination (Chapter 3). Here we observed endo- β -mannanase activity

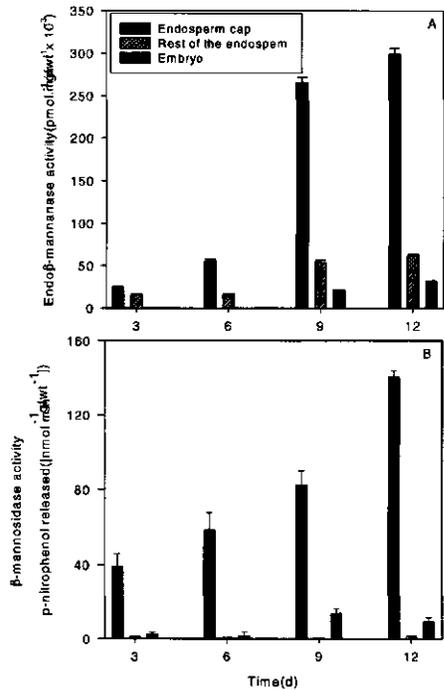


Figure 2 Endo- β -mannanase activity (A) and β -mannosidase activity (B) in seed parts from water-imbibed seeds. Data represent 3 replications of extracts from 10 endosperm caps, rest-of-endosperms and embryos; error bars indicate standard deviation.

in endosperm cap, rest of the endosperm and in the embryo prior to radicle protrusion. The increase in activity in the endosperm cap started immediately, whereas the activity in the rest of the endosperm increased from day 6 of imbibition onwards. In the embryo the activity appeared later during imbibition. However, the majority of the activity was located in the endosperm cap during germination with an almost fourfold increase near radicle protrusion as compared with the rest of the endosperm (Fig. 2A).

β -Mannosidase activity. β -mannosidase activity increased mainly in the endosperm cap before radicle protrusion. Only very low activities were observed in the embryo and in the rest of the endosperm prior to radicle protrusion (Fig 2B).



Figure 3 Amino acid sequence alignment of predicted gene product based on putative cDNA clone for endo- β -mannanase from coffee (*Coffea arabica* cv. Rubi) seed endosperm caps (1), compared with the corresponding parts of *ManA* (2) and *ManB* (3), also from coffee seeds (Marracini *et al.* 2001), and *LeMan2* (4) from tomato seeds (Nonogaki *et al.*, 2000). Amino acids, identical to those of *ManA* are shaded in black.

Cloning of endo- β -mannanase and β -mannosidase cDNA. Partial cDNA clones for endo- β -mannanase (412 bp) and for β -mannosidase (263 bp) were obtained from endosperm caps of coffee seeds. The predicted gene product encoding for endo- β -mannanase revealed high homology with endo- β -mannanase from germinated coffee seed (99%) (*ManA*) (Marracini, *et al.* 2001) (GEN Bank nr. AJ 293305) while homology with the second endo- β -mannanase from germinated coffee (*ManB*) (Marracini *et al.*, 2001) was low (40%) (Fig. 3). The homology with



Figure 4 Amino acid sequence alignment of predicted gene product based on a partial cDNA clone for β -mannosidase from coffee seed endosperm caps (*Coffea arabica* cv. Rubi) (1) compared with the corresponding part of β -mannosidase from tomato (*Lycopersicon esculentum*) seeds (Mo and Bewley, personal communication) (2). Amino acids, identical to those of β -mannosidase are shaded in black.

the endo- β -mannanase from tomato seed (*LeMAN2*) (Nonogaki *et al.* 2000) was 64 %, (GEN Bank nr AF 184238). For β -mannosidase the predicted gene product revealed high homology (86%) with tomato β -mannosidase (Fig. 4; Mo and Bewley personal communication)(GEN Bank nr AF403444).

Discussion

Coffee endosperm cell walls are composed mainly of mannans with 2% of galactose present in the side chain (Wolfrom *et al.*, 1961; Bewley and Black, 1994). The cells in the coffee endosperm cap have thinner cell walls than the cells of the rest of the endosperm (Chapter 2), which indicates that the region in the endosperm where the radicle will protrude is predestined. However, it does not exclude the requirement of endosperm cap weakening to facilitate radicle protrusion (Chapter 3). Endo- β -mannanase, an enzyme involved in the degradation of the mannan backbone (Bewley, 1997), increased mainly in the endosperm cap before completion of germination of the first seed, with low amounts observed in the rest of the endosperm and embryo. In addition, tissue printing has shown that endo- β -mannanase activity appeared first in the endosperm cap and only later in the rest of the endosperm (Chapter 3). β -Mannosidase, an enzyme that hydrolyses the oligomeric mannose products resulting from endo- β -mannanase activity, increased in activity mainly in the endosperm cap prior to radicle protrusion, following the same trend as endo- β -mannanase (Fig. 3). The low levels of endo- β -mannanase activity in the rest of the endosperm that were observed during germination might be required to supply energy to the growing coffee embryo while the embryo is still enveloped by the endosperm. This activity may contribute to lowering the osmotic potential of the coffee embryo during germination thereby allowing more influx of water to the embryo cells and, hence, embryo growth, as proposed in Chapter 3. Obviously, the activity in the rest of the endosperm will increase after radicle protrusion and in this case endo- β -mannanase seems to have again considerable importance in degrading the thick cells of the rest of the endosperm and in supplying energy to the growing

seedling. The presence of endo- β -mannanase activity in the embryo later during germination in the embryo, coincided with embryo growth by cell elongation and the time of the appearance of a protuberance in the endosperm cap (Chapter 3, 5), which indicates that endo- β -mannanase activity in the embryo is probably necessary to facilitate embryo cell elongation, although conclusive evidence is lacking.

Thus, endo- β -mannanase and β -mannosidase activities prior to radicle protrusion in the endosperm cap appeared to be involved in the germination process *i.e.* weakening of the endosperm cap. Evidently, endo- β -mannanase is the first enzyme in the hydrolysis of the mannan backbone and is likely to be the main enzyme involved in endosperm cap weakening in coffee whereas β -mannosidase hydrolyzes the oligomeric mannose products resulting from endo- β -mannanase activity (Bewley and Black, 1994). Enzyme activities in the rest of the endosperm of coffee appear to be required during seedling establishment after radicle protrusion.

The predicted gene product encoded by the endo- β -mannanase cDNA revealed high homology to post-germinative *ManA* from coffee seed (Marracini *et al.* 2001) (Fig. 3). However, the homology with another endo- β -mannanase from coffee seed (*ManB*) was only 40% (Marracini, *et al.* 2001). This result shows that there are different genes encoding for endo- β -mannanase in coffee seed. In addition, different isoforms have been separated by isoelectric focussing (Chapter 3; Marracini *et al.* 2001). This implies that some of the isoforms observed are probably the result of post-transcriptional modifications, generated during and following germination of the coffee seed and that they may originate from at least 2 gene products. The high homology displayed by endo- β -mannanase to post-germinative *ManA* shows that this gene product was not only expressed in the endosperm of the coffee seed after radicle protrusion but also prior to radicle protrusion. We propose that *ManA* and the endo- β -mannanase cloned here, that represents 25% of the full length cDNA from *ManA*, are the same and that this gene is expressed in the endosperm cap region during coffee seed germination and thus encodes an endo- β -mannanase that plays a role in germination. Furthermore, homology with another endo- β -mannanase from the endosperm cap of tomato seeds (*LeMAN2*) was also observed (Fig. 3).

β -Mannosidase from the endosperm cap of coffee seed cloned here representing 15% of the full length cDNA from β -mannosidase from tomato seed (Mo and Bewley, personal communication), showed high homology (86%). These endo- β -mannanase and β -mannosidase genes play an important role in coffee seed germination.

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CHAPTER 7

General discussion

Coffee (*Coffea arabica* cv. Rubi) seed germination: mechanism and regulation

E. A. Amaral da Silva

This thesis describes a number of processes that contribute to the mechanism and regulation of coffee seed germination. They are summarized in Figure 1 and put on the time scale of germination, starting with the dry seed (day 0) and ending with radicle protrusion at day 10.

Coffee seed germination: sequence of events and their interrelationships

Two phases of germination

Our results make clear that coffee seed germination, resulting in radicle protrusion, is the net result of embryo growth and endosperm weakening prior to radicle protrusion. From the germination events that we have monitored in this study it becomes apparent that the subsequent steps in embryo growth and endosperm weakening coincide to a large extent and are interdependent.

Two phases can be recognized in the overall germination process. Phase 1 occurs between 0 and 5 days of imbibition and includes the full hydration of the seed between day 0 and 3. Phase 2 stretches from day 5 to day 10 and ends with protrusion of the radicle. Note that these two phases do not coincide with any of the 3 phases of water uptake that are common in seeds (Bewley and Black, 1994; Chapter 3, fig. 2).

In the embryo phase 1 is characterized by isodiametrical growth (including swelling) and a build-up of turgor pressure. Concomitantly, nuclear and organellar DNA synthesis take place, presumably as part of the DNA repair process. During this phase β -tubulin accumulates and the microtubular cytoskeleton becomes apparent, predominantly in a random orientation. Evidently, the cells in the embryonic axis are preparing for expansion growth: the increase in turgor pressure indicates that water is taken up by the cells but that relaxation of the walls is largely absent. This may be caused by a limitation of the cell wall extensibility at that time or by the fact that expansion is hindered by the opposed mechanical restraint of the surrounding endosperm. Only by the end of phase 1 relaxation of the cell walls takes place and, hence, cells expand and their turgor decreases. The endosperm may have enabled this expansion because hydrolytic degradation of endosperm cell walls is operational during phase 1; this includes increased activities of endo- β -mannanase and cellulase and results in a decrease of the required puncture force. In this phase also porosity in the cell walls closest to the embryo is observed. Endosperm cell wall degradation may create space for the embryo to expand, or it may increase the plasticity of the endosperm cell walls. The occurrence of the latter possibility is supported by the appearance of a protuberance at the end of phase 1 in which the growing radicle is surrounded by a still intact endosperm cap.

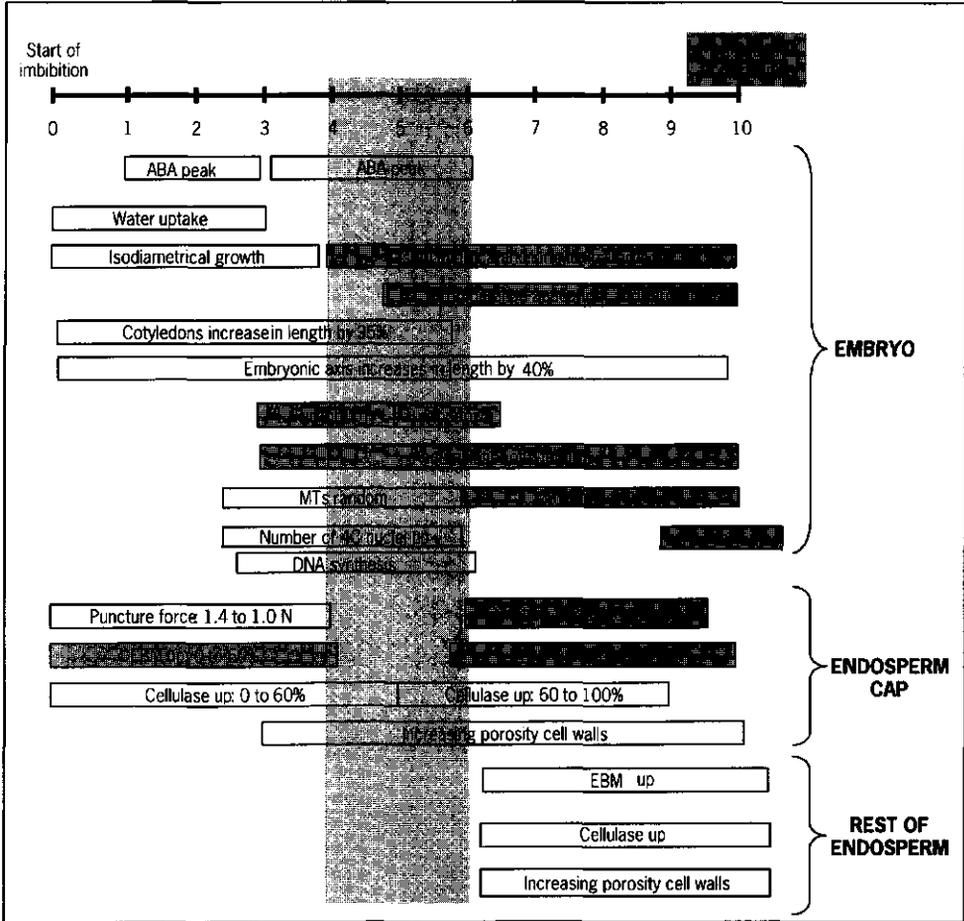


Figure 1. Schematic overview of events occurring in the embryo and endosperm during the germination of coffee seeds between the start of imbibition and radicle protrusion. Shaded bars indicate that the event is affected by ABA. MT: microtubules; EBM: endo- β -mannanase. % in EBM and cellulase bars indicates activity relative to maximum. "up": indicates increase. Shaded column between 4 and 6 days of imbibition indicates transition between phase 1 and phase 2 of germination.

The transition from phase 1 to phase 2 (4-6 days) is marked by a decrease in turgor pressure of the embryo and the occurrence of plateau phases in endo- β -mannanase and cellulase activities, required puncture force, and in the duplication of nuclear DNA.

From day 6 onwards embryo growth becomes predominantly longitudinal and the protuberance becomes more prominent. In parallel, the number of cells with transversally orientated microtubules increases and β -tubulin accumulation continues. In the endosperm cap both endo- β -mannanase and cellulase activities increase again, the required puncture force displays a second decrease and the porosity of cell walls in the endosperm cap becomes more intense and spreads out further to the periphery of the endosperm cap. It is clear that in phase 2 the endosperm cap 'gives way' to the growing embryo. At this stage the cells in the endosperm cap appear compressed, reinforcing the notion that embryo 'thrust' is increasing. It is possible that the second step in endosperm weakening is partly caused by the growing embryo. Shortly before radicle protrusion there is a marked increase in the number of 4C nuclei and mitotic figures can be observed. Also in phase 2 degradation of the rest of the endosperm commences and this may be regarded as the beginning of reserve mobilisation to accommodate the growing embryo with nutrients for energy metabolism.

As yet, we can not explain the occurrence of two phases in coffee seed germination. It is tempting to speculate that the presence of ABA during imbibition plays a role in this. The occurrence of the second transient rise in ABA content prior to phase 2 supports this hypothesis. Also, the targets of exogenous ABA are mainly in phase 2, implying that the processes occurring during phase 1 are relatively insensitive to ABA. Only endo- β -mannanase activity of phase 1 is inhibited by ABA.

Hormonal regulation: ABA and GA

Exogenous ABA inhibits germination of coffee seeds as in many other species. Here we have shown that the targets of ABA are located both in the embryo and endosperm. In the embryo all the growth related processes are inhibited, except water uptake and swelling, and nuclear and organellar DNA synthesis. It is possible that the number of targets of ABA in the embryo is limited because inhibition of one of the growth related processes may lead to inhibition of subsequent events. It can be argued that inhibition of β -tubulin accumulation prevents the assembly of microtubules, the formation of microtubular arrays and, hence, modifications of the cell walls (including increase in extensibility) and cell expansion and division.

On the side of the endosperm only the second phase of the weakening process is inhibited. Endo- β -mannanase activity is inhibited by ABA throughout germination whereas cellulase activity is not. From this we may conclude that endo- β -mannanase is not involved in the first step of endosperm weakening. Furthermore, as porosity in phase 2 is still observed in the presence of ABA, endo- β -mannanase is not involved in the development of porosity. Finally, the inhibition of the second step of endosperm weakening may also indirectly be caused, at least partly, by the absence of embryonic 'thrust' in the presence of ABA.

GA displays a two-fold effect on coffee seed germination. Firstly, GA is absolutely required for germination. This is true for many other species (Bewley and Black, 1994). Inhibitors of GA-biosynthesis effectively inhibited germination. These inhibitors both prevented embryo growth and the second step of endosperm weakening in a similar fashion as ABA: no increase in turgor around day 5, no protuberance occurring and no increase in endo- β -mannanase activity. Secondly, higher concentrations of GA inhibit coffee seed germination. However, embryo growth at high GA concentration was normal, as in water imbibed seeds. In the endosperm cap, however, activities of endo- β -mannanase and β -mannosidase were substantially higher than in the control, both in phase 1 and in phase 2. In phase 1, endo- β -mannanase was up to 10-fold higher and in phase 2 up to 3 fold. The combination of normal embryo growth and substantially enhanced endosperm degradation causes death of the embryo just prior to radicle protrusion. This lethal event is not caused by increased concentrations of degradation products from endosperm cell wall hydrolysis. We hypothesize that the endosperm is normally subject to programmed cell death but that the high concentration of GA speeds up this process whereby factors that are involved in programmed cell death may reach the embryo before it has protruded (and harmful components may leach out). Apparently, this is lethal to the embryo.

Coffee and tomato seed germination: a comparison

The coffee embryo grows inside the endosperm prior to radicle protrusion by an increase in the embryo turgor pressure and cell wall extensibility. In contrast, tomato embryos do not show any changes in their osmotic potential ψ_x (more negative values) or a build-up of pressure potential (turgor) (ψ_p) before radicle protrusion (Haigh and Barlow, 1987). In coffee embryos ABA inhibited the increase in the embryo growth potential and, thus, inhibited cell wall loosening, whereas in tomato embryos ABA displays a minor effect on embryo growth potential. Regarding GAs, similar results were observed during germination in coffee and in tomato embryos: in both species GAs are required to induce embryo cell elongation.

However, an inhibiting effect of high GA-concentrations has not been reported for tomato seed germination.

Both in coffee and tomato seeds the endosperm cap weakens, following a biphasic pattern during germination (Fig. 1 of Chapter 1; Chapter 3; Toorop *et al.*, 2000). In the coffee seed the first step correlates with cellulase activity and the second step with endo- β -mannanase activity. ABA inhibits at least 2 isoforms of endo- β -mannanase in the endosperm cap. In tomato seed the inhibition of germination by ABA is through the endosperm cap only, but endo- β -mannanase activity is not inhibited by ABA (Fig. 1 of Chapter 1; Toorop *et al.*, 2000). This leads to the suggestion that another unknown enzyme that is inhibited by ABA regulates tomato seed germination. In coffee seeds GA has no effect on the first step of endosperm cap weakening but it appears to control the second step. GAs, possibly emanated from the embryo, are required to induce endo- β -mannanase in the endosperm cap and, thus, endosperm cap weakening. In tomato seeds GAs appear to control the second step of the endosperm cap weakening, as in coffee seeds.

In conclusion, radicle protrusion in coffee seeds is controlled by both embryo growth and weakening of the endosperm cap, more precisely in the second phase of germination. In tomato seed radicle protrusion seems to be exclusively controlled by endosperm cap weakening.

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Summary

Coffee (*Coffea arabica* cv. Rubi) seed germination: mechanism and regulation

Summary

Coffee seeds display slow and variable germination which severely hampers the production of seedlings for planting in the following growth season. Little work has been done with the aim to understand the behavior of coffee seeds during germination and there is a lack of information concerning the regulation of the germination process. This thesis addresses questions concerning the mechanism and regulation of coffee seed germination.

Initial experiments showed that radicle protrusion in the dark at 30 °C was initiated at around day 5 of imbibition. At day 10, 50% of the seed population displayed radicle protrusion and at day 15 most of the seeds had completed germination. The water uptake by the coffee seeds followed a common triphasic pattern as described for many other species (Chapter 2 and 3). During imbibition the coffee embryo grew inside the endosperm. The cotyledons increased in length by 35% and the axis by 40%, resulting in the appearance of a protuberance in the endosperm cap region. There was an increase in the embryo pressure potential up to day 5 of imbibition followed by a release of turgor thereafter, indicating relaxation of embryonic cell walls (Chapter 3). Light microscopy demonstrated that the cells of the embryonic axis displayed isodiametric growth (swelling) at the beginning of the imbibition process followed by both isodiametric and longitudinal growth later during imbibition. The isodiametric growth coincided with a random orientation of the microtubules whereas longitudinal growth was accompanied by a transversal orientation. Accumulation of β -tubulin, an increase in the number of 4C nuclei and DNA replication were evident during imbibition. These cell cycle events coincided with the growth of the embryo and the appearance of cell division prior to radicle protrusion. However, cell division was not a prerequisite for radicle protrusion in coffee seeds (Chapter 5).

The endosperm of the coffee seeds possesses polygonal and rectangular cell types located in different parts of the endosperm. The endosperm cap cells have smaller and thinner cell walls than the rest of the endosperm, suggesting that the region where the radicle will protrude is predestined in coffee seed. Low temperature scanning microscopy revealed that during imbibition cells in the endosperm cap became compressed which was followed by a loss of cell integrity, appearance of a protuberance and occurrence of cell wall porosity (Chapter 3). As in many other species, the hemi-cellulose fraction of endosperm cell walls of coffee seeds consists mainly of mannans and galacto-mannans. These polysaccharides are commonly deposited in the cell walls as food reserve. Upon germination, these galacto-mannans are degraded through the action of hydrolytic enzymes, including endo- β -mannanase, β -mannosidase and α -galactosidase resulting in a weakening of the cell walls. The coffee endosperm cap weakens in two steps: cellulase activity correlated with the first

step and endo- β -mannanase activity with the second step. Endo- β -mannanase activity appeared first in the endosperm cap and only later in the rest of the endosperm, and coincided with a decrease in the required puncture force and appearance of cell wall porosity. Different isoforms of endo- β -mannanase were found in the endosperm cap and in the rest of the endosperm. The activity of β -mannosidase increased predominantly in the endosperm cap. However, low levels of endo- β -mannanase and β -mannosidase activities were also observed in the rest of the endosperm and in the embryo prior to radicle protrusion (Chapter 3 and 6). Two partial length cDNA clones encoding for endo- β -mannanase and β -mannosidase, respectively, were isolated from coffee endosperm caps. The deduced amino acid sequences exhibited high homology with those of other endo- β -mannanases and β -mannosidases from plants (Chapter 6).

Abscisic acid (ABA) inhibited germination of coffee seeds but not their water uptake, isodiametric growth, increase in 4C nuclei and DNA synthesis in the embryo cells. In the endosperm cap ABA inhibited the second step of endosperm cap weakening, presumably by inhibiting the activities of at least two endo- β -mannanase isoforms. However, ABA had no effect on endo- β -mannanase activity in the rest of the endosperm or on cellulase activity. Two peaks of endogenous ABA occurred in the embryo cells during germination. The first peak was observed at day 2 of imbibition and the second (smaller) peak at day 5 of imbibition. The occurrence of these ABA peaks coincided with the increase in the embryo growth potential and the second step of endosperm cap weakening, which makes these processes possible targets of ABA action (Chapter 3).

Exogenous gibberellin (GA_{4+7}) inhibited coffee seed germination. The response to GA_{4+7} showed two sensitivity thresholds: a lower one between 0 and 1 μM and a higher one between 10 and 100 μM . However, it was shown that radicle protrusion of coffee seeds depended on *de novo* synthesis of GAs. Endogenous GAs were required for embryo cell elongation and the second step of endosperm cap weakening. Incubation of seeds in exogenous GA_{4+7} resulted in a loss of embryo viability and the occurrence of dead cells, as observed by low temperature scanning microscopy. We suggest that the inhibition of germination by exogenous GAs is caused by factors that are released from the endosperm cap during or after its weakening. Exogenous GAs greatly accelerated the degradation of the endosperm cap. Factors that are involved in (normal) programmed cell death of the endosperm may reach the embryo during germination, causing cell death in the embryonic axis and, hence, inhibition of radicle protrusion. The results presented in this thesis show that coffee seed germination is controlled both by embryo growth and the second step of endosperm cap weakening (Chapter 4).

Finally, the sequence of events during coffee seed germination and their interrelationships are presented and discussed (Chapter 7). The events occurring in embryo and endosperm all followed a two-phase pattern. The first phase occurred during the first 5 days of imbibition and the second phase thereafter, until radicle protrusion. The results make clear that the germination processes are temporally and spatially coordinated and that disturbance of this coordination, as in the presence of GAs, may severely affect seed behaviour.

Samenvatting

Coffee (*Coffea arabica* cv. Rubi) seed germination: mechanism and regulation

Samenvatting

Zaden van koffie vertonen langzame en variabele kieming waardoor de productie van zaailingen voor planten in het volgende seizoen ernstig bemoeilijkt wordt. Er is tot dusver weinig onderzoek gedaan met als doel het gedrag van koffiezaad tijdens de kieming beter te begrijpen. Bovendien is er weinig informatie beschikbaar over de regulatie van het kiemingsproces. Dit proefschrift gaat in op vragen omtrent het mechanisme en de regulatie van de kieming van koffiezaad.

De eerste experimenten wezen uit dat bij 30 °C in het donker het kiemworteltje na ca. 5 dagen van imbibitie uit het eerste zaad tevoorschijn kwam. Na 10 dagen was dit voor ongeveer 50% van de zaden het geval en na 15 dagen waren de meeste zaden gekiemd. De wateropname door de zaden volgde het bekende drie-fasen patroon zoals beschreven voor vele andere soorten (Hoofdstuk 2 en 3).

Het koffie-embryo bleek al tijdens de imbibitie te groeien binnen het omhullende endospermweefsel. De cotylen namen zo'n 35% in lengte toe en de embryonale as ongeveer 40%. Dit resulteerde in de verschijning van een uitstulping in de omgeving van het endospermkapje. Er ontstond een toename van de drukpotentiaal van het embryo tot aan de vijfde dag van imbibitie. Dit werd gevolgd door een afname van de turgor wat wijst op een relaxatie van de celwanden in het embryo (Hoofdstuk 3). Met behulp van lichtmicroscopie werd aangetoond dat de cellen in de embryonale as aan het begin van het imbibitieproces uitsluitend isodiametrische groei (zwellings) vertoonden, gevolgd door zowel isodiametrische als longitudinale groei later tijdens de imbibitie. De isodiametrische groei viel samen met het voorkomen van microtubuli in een ongeordende oriëntatie terwijl de latere longitudinale groei gepaard ging met transversale oriëntatie van de microtubuli. Tijdens de imbibitie werd accumulatie van β -tubuline, een toename van het aantal 4C celkernen en DNA replicatie waargenomen. Deze onderdelen van de celcyclus verliepen gelijktijdig met de groei van het embryo en voorkomen van celdelingen vlak voor doorbraak van het kiemworteltje. Celdeling bleek echter geen voorwaarde voor wortel doorbraak in koffiezaad (Hoofdstuk 5).

Het endosperm van de koffiezaad bevat zowel polygonale als rechthoekige cel typen die in verschillende delen van het endosperm zijn gelokaliseerd. De cellen van het endospermkapje zijn kleiner en bevatten dunnere celwanden dan die in het overige endosperm. Dit doet vermoeden dat het deel van het endosperm waar de wortel zal doorbreken voorbereid is op deze gebeurtenis. Met behulp van lage temperatuur scanning electronenmicroscopie werd waargenomen dat tijdens de imbibitie de cellen in het endospermkapje werden samengedrukt gevolgd door een verlies van de celintegriteit, het ontstaan van de uitstulping en het voorkomen van celwandporositeit (Hoofdstuk 3). De hemi-cellulose fractie van de endospermcelwanden in koffiezaad bestaat, evenals in vele andere soorten, voornamelijk uit mannanen en galacto-mannanen. Deze polysacchariden worden veelal als reservevoedsel opgeslagen in de celwanden. Bij kieming worden de (galacto)mannanen afgebroken door hydrolytische enzymen als endo- β -mannanase, β -mannosidase en galactosidase, wat resulteert in een verzwakking van de celwanden. Het endospermkapje van koffiezaad verzwakt in twee stappen:

de eerste is geassocieerd met cellulase activiteit en de tweede met activiteit van het endo- β -mannanase. Endo- β -mannanase activiteit werd het eerst waargenomen in het endospermkapje en pas daarna in de rest van het endosperm. De enzymactiviteit ging gepaard met een afname in de kracht die nodig is om door het endosperm heen te breken, en met het verschijnen van porositeit in de celwanden. In het endospermkapje en in de rest van het endosperm werden verschillende iso-vormen aangetoond van het enzym endo- β -mannanase. Activiteit van β -mannosidase nam voornamelijk toe in het endospermkapje. Niettemin werden ook lage activiteiten van beide enzymen waargenomen in het overige endosperm en in het embryo, voorafgaand aan doorbreking van het kiemworteltje (Hoofdstuk 3). Er werden twee 'partial length clones' die coderen voor endo- β -mannanase en β -mannosidase geïsoleerd uit endospermkapjes. De afgeleide aminozuurvolgorden vertoonden een hoge homologie met die van andere endo- β -mannanases en β -mannosidases uit planten (Hoofdstuk 6).

Abscisinezuur (ABA) remde de kieming van koffieezaden maar niet hun wateropname, isodiametrische groei, toename in aantal 4C kernen en DNA-synthese. In het endospermkapje remde ABA de tweede stap van de endospermverzwakking, waarschijnlijk via de remming van minstens twee iso-vormen van het endo- β -mannanase. ABA had echter geen effect op de endo- β -mannanase activiteit in de rest van het endosperm en ook niet op de cellulase activiteit. Tijdens kieming werden twee pieken in het endogene ABA-gehalte gemeten in het embryo. De eerste piek verscheen rond 2 dagen van imbibitie en de tweede (kleinere) piek rond dag 5. Het voorkomen van deze ABA-pieken viel samen met de toename in de groeipotential van het embryo en de tweede stap van de endospermverzwakking waardoor dit twee mogelijke doelen van de ABA-werking kunnen zijn (Hoofdstuk 3).

Exogene gibberellinen (GA_{4+7}) remden de kieming van koffiezaad. De response op GA_{4+7} vertoonde twee gevoeligheidsdrempels: een lage tussen 0 en 1 μM en een hogere tussen 10 en 100 μM . Er werd echter ook aangetoond dat de kieming afhankelijk was van *de novo* synthese van GAs. Endogene GAs bleken nodig voor strekking van de embryonale cellen en de tweede stap van endospermverzwakking. Incubatie van zaden in oplossingen van GA_{4+7} resulteerde in verlies van levensvatbaarheid van het embryo en het afsterven van een deel van de cellen. We stellen ons voor dat de remming van de kieming door exogene GAs wordt veroorzaakt door factoren die vrijkomen uit het endospermkapje tijdens of na de verzwakking hiervan. Exogene GAs bleken de afbraak van het endospermkapje aanzienlijk te versnellen. Factoren die betrokken zijn bij het geprogrammeerd afsterven van cellen van het endosperm zouden tijdens de kieming het embryo kunnen bereiken alwaar afsterving van cellen wordt geïnduceerd en waardoor het kiemworteltje niet meer kan doorbreken. De resultaten in dit proefschrift laten zien dat de kieming van koffiezaad afhankelijk is van zowel embryogroei als de tweede stap van endospermverzwakking (Hoofdstuk 4).

Tenslotte wordt de volgorde van de bestudeerde kiemingsprocessen en hun onderlinge samenhang besproken (Hoofdstuk 7). Hieruit blijkt dat de kiemingsprocessen in embryo en endosperm

alle een twee-fasen patroon volgen. De eerste fase doet zich in de eerste 5 dagen van imbibitie voor en de tweede fase in de tijd daarna, tot aan de doorbraak van het kiemworteltje. Uit de resultaten blijkt duidelijk dat de kiemingsprocessen in tijd en ruimte zijn gecoördineerd en dat verstoring van deze coördinatie, zoals in aanwezigheid van GAs, het gedrag van het zaad ernstig kan verstoren.

Sumário

Coffee (*Coffea arabica* cv. Rubi) seed germination: mechanism and regulation

Sumário

As sementes do café têm germinação lenta e variável, o que dificulta severamente a produção de mudas para a estação chuvosa seguinte. Pouco trabalho tem sido feito com o objetivo de entender o comportamento das sementes de café durante a germinação, faltando informação a respeito da regulação do processo germinativo. Portanto, o mecanismo e regulação da germinação de sementes de café foram estudados nesta tese.

Experimentos iniciais mostraram que a protrusão da radícula, quando as sementes foram embebidas no escuro a 30° C, iniciou-se em torno do quinto dia de embebição. No décimo dia de embebição, 50% das sementes apresentaram protrusão radicular e no décimo quinto dia a maioria das sementes germinou. A embebição das sementes apresentou um padrão trifásico comum, como o descrito para muitas outras espécies (Capítulos 2 e 3). Durante a embebição da semente o embrião cresceu dentro do endosperma. Os cotilédones cresceram em comprimento por volta de 35% e o eixo embrionário em torno de 40%, levando a ocorrência de protuberância na região do endosperma "cap". Houve aumento no potencial de pressão do embrião até o quinto dia de embebição, seguido por uma liberação do turgor, indicando relaxamento das paredes celulares do embrião (Capítulo 3). Estudos de microscopia ótica mostraram que as células do eixo embrionário apresentaram crescimento isodiamétrico no início da embebição, seguido por crescimento isodiamétrico e longitudinal, durante a embebição. O crescimento isodiamétrico coincidiu com uma orientação aleatória dos microtúbulos e o crescimento longitudinal foi acompanhado por orientação transversal. Acumulação de β -tubulina, aumento no número dos núcleos 4C e de replicação do DNA foram também observados. Estes eventos do ciclo celular coincidiram com o crescimento do embrião e ocorrência de divisão celular antes da protrusão da radícula. Entretanto, divisão celular não foi pré-requisito para a protrusão da radícula (Capítulo 5).

O endosperma das sementes de café possui células do tipo poligonal e retangulares, situadas em diferentes regiões do endosperma. As células do endosperma "cap" são menores e têm paredes celulares menos espessas, enquanto que as paredes celulares do resto do endosperma são mais espessas, sugerindo que a região onde a radícula irá protrudir é pré-definida. Estudos usando microscopia de varredura mostraram que, durante a embebição, as células do endosperma "cap" tornaram-se comprimidas, houve perda da integridade celular, presença de protuberância e da ocorrência de porosidade nas paredes celulares do endosperma (Capítulo 3). Como em muitas outras espécies, a fração de hemi-celulose das paredes celulares do endosperma consiste principalmente de mananas e de galacto-mananas. Estes polissacarídeos são geralmente depositados como fonte de reservas da semente. No momento da germinação, as galacto-mananas são degradadas pela ação de enzimas, incluindo endo- β -

mananase, β -manosidase e galactosidase resultando no enfraquecimento das paredes celulares do endosperma. O enfraquecimento do endosperma "cap" ocorreu em duas fases, sendo que na primeira houve uma correlação com a atividade de celulase e, na segunda, com a atividade de endo- β -mananase. A atividade de endo- β -mananase ocorreu inicialmente no endosperma "cap" e apenas mais tarde no resto do endosperma, coincidindo com uma diminuição na força requerida para o embrião penetrar o endosperma e a ocorrência de porosidade na parede celular. Diferentes isoformas de endo- β -mananase foram observadas no endosperma "cap" e no resto do endosperma. A atividade de β -manosidase aumentou principalmente no endosperma "cap". Entretanto, níveis baixos de atividade de endo- β -mananase e de β -manosidase foram observados no resto do endosperma e no embrião, antes da protrusão da radícula (Capítulos 3 e 6). cDNAs parciais que codificam para endo- β -mananase e β -manosidase, respectivamente, foram isoladas do endosperma "cap". As seqüências deduzidas dos amino-ácidos exibiram alta homologia com outras endo- β -mananases e β -manosidases que ocorrem em plantas (Capítulo 6). Ácido abscísico (ABA) inibiu a germinação, mas não a embebição de água pela semente, o crescimento isodiamétrico, o aumento em núcleos 4C e a síntese do DNA nas células do embrião. No endosperma "cap", ABA inibiu a segunda fase do enfraquecimento do endosperma e a atividade de pelo menos duas isoformas de endo- β -mananase. Entretanto, o ABA não teve nenhum efeito na atividade de endo- β -mananase no resto do endosperma e na atividade de celulase. Dois picos de ABA endógeno foram observados nas células do embrião durante a germinação. O primeiro, no segundo dia de embebição e o segundo, menor, no quinto dia de embebição. A ocorrência dos picos de ABA coincidiu com o aumento no potencial de crescimento do embrião e com a segunda fase de enfraquecimento do endosperma "cap", sendo esses processos possíveis alvos da ação de ABA (Capítulo 3).

Giberelina exógena (GA_{4+7}) inibiu a germinação de sementes de café. Foram observados dois pontos de sensibilidade a GA_{4+7} : um entre 0 e 1 μ M e outro, mais elevado, entre 10 e 100 μ M. Entretanto, observou-se que a protrusão da radícula dependeu da síntese de GAs endógenas. GAs endógenas foram necessárias para promover a elongação da radícula e a segunda etapa do enfraquecimento do endosperma "cap". A incubação das sementes em GA_{4+7} exógena resultou em uma perda da viabilidade do embrião e da ocorrência de células mortas, observada por meio de microscopia de varredura. Sugere-se que a inibição da germinação por GA exógena é causada por fatores que são liberados do endosperma "cap" durante ou após o seu enfraquecimento. GA exógena acelerou extremamente a degradação do endosperma "cap". Fatores que normalmente estão envolvidos na morte programada das células do endosperma podem alcançar o embrião durante a germinação, causando a morte das células do eixo embrionário e assim, levando a inibição da protrusão radicular. Os

resultados apresentados nesta tese mostram que a germinação de sementes de café é controlada pelo crescimento do embrião e pela segunda etapa do enfraquecimento do endosperma.

Finalmente, a seqüência de eventos durante a germinação em sementes de café e suas inter-relações, são apresentadas e discutidas (Capítulo 7). Todos os eventos que ocorreram no embrião e no endosperma seguiram um padrão bifásico. A primeira fase ocorreu durante os primeiros 5 dias de embebição, seguida pela segunda fase, até a protrusão da radícula. Os resultados mostram claramente que os processos que ocorrem durante a germinação em sementes de café são temporariamente e espacialmente controlados e que distúrbios nessa coordenação, como a presença de GA, podem afetar severamente o comportamento da semente.

Curriculum vitae

Edvaldo Aparecido Amaral da Silva was born on February 26, 1967 in Avaré, State of São Paulo-Brazil. In January 1990 he started the Agronomy course at the Universidade Federal de Lavras (UFLA), graduating five years later. In 1995 he initiated his Master Degree in Seed Science at the Department of Crop Science at UFLA, receiving his Master Degree two years later.

In January 1998 he started his Ph.D. project on coffee seed germination: mechanism and regulation. His project involved a collaboration between the Laboratory of Plant Physiology and the Laboratory of Plant Cell Biology of Wageningen University and the Universidade Federal de Lavras (UFLA) in Brazil.

The work presented in this thesis was carried out within the Graduate School of Experimental Plant Sciences at Wageningen University, Laboratory of Plant Physiology and Laboratory of Plant Cell Biology, Wageningen.

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