UNIVERSIDADE FEDERAL DE LAVRAS

TATIANA ARANTES AFONSO VAZ

MORPHOLOGY AND ECOPHYSIOLOGY OF *Swartzia langsdorffii* Raddi. SEEDS

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2016

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Engenharia Florestal, área de concentração em Silvicultura e Genética Florestal, para a obtenção do título de Doutor.

Orientador
Dr. Antonio Claudio Davide

Coorientador
Dr. Peter Edwin Toorop

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Àquela a quem devo tudo o que sou,

Mãe, com o maior amor deste mundo,

Te dedico.

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"I have great faith in a seed. Convince me that you have a seed there, and I am prepared to expect wonders."

Henry D. Thoreau

RESUMO GERAL

Swartzia langsdorffii Raddi. é uma espécie arbórea nativa da Mata Atlântica e florestas semidecíduas distribuída na região sudeste do Brasil. Esta espécie possui frutos alaranjados, grandes, lignificados, com secreção abundante de resinas, alto conteúdo de água e com deiscência tardia. Cada fruto contém em média três sementes grandes amarronzadas envoltas em arilo amarelo, arredondadas, e tanto semente quanto arilo também possui alto conteúdo de água após a dispersão. De acordo com estas características as sementes de *S. langsdorffii* poderiam ser enquadradas no grupo das sementes recalcitrantes. Supostamente recalcitrantes, as sementes desta espécie são dispersas ainda na estação seca, em agosto, mês com mais baixa precipitação do ano na região de Lavras, MG. Assim, os objetivos neste trabalho foram caracterizar o comportamento fisiológico de sementes de *S. langsdorffii* de acordo com sua tolerância à dessecação e comportamento no armazenamento, e avaliar as características morfofisiológicas que permitam a manutenção da viabilidade das sementes após a dispersão (Artigo 1). Além disso, objetivou-se identificar a real contribuição do diásporo de *S. langsdorffii* na viabilidade e germinação das sementes, e identificar características morfoanatômicas que permitam que estas sementes sensíveis à dessecação mantenham sua viabilidade após a dispersão até que ocorra a germinação e estabelecimento da plântula (Artigo 2). Com os resultados obtidos no Artigo 1, tornou-se possível classificar as sementes de *S. langsdorffii* como recalcitrantes. Poros foram encontrados dispersos por todo o tegumento e também um grande número de estômatos na protoderme do eixo hipocótilo-radícula, podendo estas estruturas estarem relacionadas às trocas gasosas entre a semente e as estruturas que a circundam. Além disso, foi observada a presença de compostos fenólicos no eixo hipocótilo-radícula, que de acordo com alguns autores podem ser responsáveis por proteger as sementes contra decompositores e estresses abióticos. As sementes se mantiveram viáveis no solo por até sete meses após a dispersão sem perda significativa de umidade, apesar da baixa precipitação e umidade do solo antes do início do período chuvoso, provavelmente devido à proteção conferida pelo pericarpo e arilo. Já no Artigo 2 foi possível verificar que as sementes não perdem água mesmo quando não estão protegidas pelo pericarpo e arilo, provavelmente devido à sua forma, tamanho, umidade relativa da serapilheira e à um espessamento de cutina encontrado na parede celular da protoderme. Já as sementes que se encontravam dentro do pericarpo ou apenas envoltas pelo arilo, apresentaram maior conteúdo de água e porcentagem de germinação, mostrando que estas estruturas não eram responsáveis por evitar a perda de água pelas sementes, mas sim por permitir que estas sementes apresentem um conteúdo de água maior do que aquele no momento da dispersão.

Palavras-chave: Recalcitrantes. Poros. Estômatos. Sobrevivência.

ABSTRACT

Swartzia langsdorffii Raddi. is a native tree from Atlantic Rain Forest and semi deciduous forest in southeast region of Brazil. This species has large orange fruits, lignified, with abundant resin secretion, high water content and late dehiscence. Each fruit has approximately three large, rounded and brownish seeds, surrounded by a yellow aril, and both of them present high water content after dispersal. According to these characteristics *S. langsdorffii* seeds could be placed in the recalcitrant group. Although recalcitrant, seeds are dispersed in the dry season, August, the month with the lowest rainfall of the year in Lavras, MG. Thus, the aims of this work were to assess the physiological behaviour of *S. langsdorffii* seeds regarding its response to desiccation and storage, and evaluate the morphophysiological traits that allow seed viability maintenance after dispersal (Article 1). Additionally, it is aimed to identify the true contribution of *S. langsdorffii* diaspore to seed survival and germination, and identify mophoanatomical traits that allow these desiccation sensitive seeds to remain viable after dispersal (Article 2). The results found in the Article 1 show that *S. langsdorffii* seeds are safely classified as recalcitrant. Pores were found distributed in the seed coat and also a great amount of stomata in the hypocotyl-axis protoderm. These structures are probably related to gas exchange between seed and surrounding structures. Phenolic compounds were also observed in this area, and according some authors these substances are responsible for seed protection against decomposers and abiotic stresses. Seeds remain viable in the soil for up to seven months without significant reduction in water content, even despite the low rainfall and relative humidity of soil before the beginning of the rainy season. That occurs probably due to pericarp and aril protection. In the Article 2 was possible to verify that seeds do not lose water even when not protected by pericarp and aril, probably due to its shape, size, litter relative humidity and to cutin thickening in the protoderm cell walls. Seeds inside pericarp or surrounded by the aril presented higher water content and germination percentage, evidencing that these structures are not responsible for restriction of water loss but to allow higher seed water content and being the main responsible for *S. langsforffii* seeds survival and germination after dispersal.

Key words: Recalcitrant. Pores. Estomata. Survival.

SUMARY

1 INTRODUCTION

1.1 The studied species: Swartzia langsdorffii Raddi.

Swartzia Schreb. is a neotropical genus that belongs to Fabaceae family, Faboideae subfamily (COWAN, 1981), has about 140 species and considerable number of species occurring in Amazon and Southeast regions of Brazil (MARQUI et al., 2008). *Swartzia langsdorffii* Raddi. can reach 10 to 20 m in height and it has light brown stem with slits (MANSANO; LIMA, 2007). This species produces large fruits (6 a 9 cm), lignified, with abundant secretion of resins composed mainly of tannins, saponins and lipids, containing 2 to 4 arillated seeds (COLPAS; OLIVEIRA, 2003) and the dispersal starts in the late winter (VAZ et al., 2016).

Studying pericarps of *S. langsdorffii*, Colpas and Oliveira (2003) found several compounds related to seed protection. One example are the phenolic compounds, which could aid protection against fungi and bacterial decomposers (CONSTABEL et al., 2000), also acting as germination inhibitors (MACIEL et al., 1992; COLPAS et al., 2003) and have high antioxidant activity protecting cells from abiotic stresses (SWIGONSKA *et al*., 2014). *Swartzia langsdorffii* seeds are differentiated by their large size, moist aril, thin seed coat and big embryo, features of the group of recalcitrant seeds. However, until now, no studies were conducted to assess the desiccation tolerance/sensitivity or the seed anatomy, valuable information to understand the ecophysiology of this seed, especially in relation to the maintenance of viability in their natural habitat after the dispersal.

1.2 Seed storage behaviour

The seed ability to survive with low water contents during storage has fascinated people along the years and several researchers have developed methodologies aiming to improve seed storage. For this be possible was necessary to understand the seed storage behaviour and develop methods to evaluate this trait correctly.

Ewart (1908 apud HONG et al., 1998) segregate the species according to the life time during the storage, Elliot (1912 apud HONG et al., 1998) classify them according the desiccation sensitivity (seeds that tolerated full, partial and minimal drying). Other classifications were determined over time, e.g., Tillotson (1921 apud HONG et al., 1998) and Holmes and Buszewicz (1958 apud HONG et al., 1998), however none of them is currently used. A new classification was developed by Roberts (1973) not considering only the seed longevity but also their physiological response during storage under specific moisture and temperature conditions. This author introduced the terms orthodox and recalcitrant, wherein orthodox seeds are those capable to survive to drying until low moisture contents (2-5%) without seed damage; and the recalcitrant seed does not survive to desiccation, losing their viability when reaching about 12-31% moisture content. Roberts' classification is well accepted and used, however, as the seed behaviour of new species were being discovered, adaptations in this classification to better allocate species with different behaviours were needed.

Farrant et al. (1988) suggested that recalcitrant seeds should be divided into three groups: minimally, moderately and highly recalcitrant. However, these groups were not well divided, showing the existence of a *continuum* of recalcitrance in seeds, depending of their natural habitat. Bonner (1990) also suggested a complex classification adapted from Roberts, adding the sub-groups: truly orthodox seeds, capable to survive long periods at sub–zero temperatures; sub-orthodox seeds, capable to survive during storage at similar conditions of the truly orthodox seeds, but, for no longer time; temperate recalcitrant seeds, unable to survive drying but can be stored at low temperatures; and lastly, tropical recalcitrant seeds, unable to survive drying or storage without significant physiological damage. To simplify seed classification, Ellis et al. (1990), kept both classes suggested by Roberts (orthodox and recalcitrant) and added another, the intermediate seeds, based on the *continuum* concept created by Farrant et al. (1988). These seeds present intermediate behaviour between orthodoxy and recalcitrance, making important the study of individual species in order to know the specific needs of each one. After the establishment of this former classification, the development of a standard methodology in order to classify seeds correctly was necessary, then, Hong and Ellis (1996) created a protocol to determine the seed storage behaviour considering the seed ability to survive at different drying rate and storage methods.

1.3 Morphological and ecophysiological traits of recalcitrant seeds

Commonly, desiccation-sensitive seeds present large volumes and rounded shape, large embryos, thin seed coat, and are dispersed with high water contents, besides being enveloped by moist and permeable tissues (TOMPSETT, 1992; HONG; ELLIS, 1995; BERJAK; PAMMENTER, 2000; FARNSWORTH, 2000; TWEDDLE et al., 2003; DAWS et al., 2006; JAYASURIYA et al., 2012; HAMILTON et al., 2013). Pammenter and Berjak (2000) affirmed that do not exist a strong relationship between seed size and desiccation sensitivity, also reported by Hill et al. (2012), but Daws et al. (2005) in a study in a tropical forest from Panama, showed that species classified as recalcitrant presented larger seeds than those classified as orthodox. The size of recalcitrant seeds seem to be an ecological advantage for seed survival, since larger and voluminous seeds present less contact surface with the atmosphere decreasing the loss of viability due to desiccation damage (FARNSWORTH, 2000; PAMMENTER; BERJAK, 2000; TWEDDLE et al., 2003; HAMILTON et al., 2013).

In general, recalcitrant seeds have no or little endosperm and this lack of endosperm is correlated with the increase in embryo size, trait considered an evolutionary advance that enables the reserve deposition in the embryo itself and favouring the immediate reserve use for radicle and plumule expansion. It results in faster germination and improving the competitive skills of this group of species (TAKHTAJAN, 1991; VIVRETTE, 1993). A great number of recalcitrant seeds have starch as main storage reserve (VAN WYK; BOTHA, 1984). According to Farrant et al. (1992, 1993), these seeds have a competitive advantage compared with other species due this kind of reserve be immediately available for use, making the germination process faster and uniform. Another advantage is the high water content in desiccation-sensitive seeds and consequent active metabolism at the moment of dispersal, speeding germination (BERJAK et al., 1984). Daws et al. (2005) stated that faster germination allows the quick water access in the soil, minimizing the risk of death by desiccation when the rainy season is too short or is pointed by short dry periods. In case of exposure to water restriction, the seed metabolism is accelerated to facilitate the water imbibition, as exemplified by Berjak et al. (1984) with *Avicennia marina* seeds. Daws et al. (2005) also stated that desiccation-sensitive seeds germinate faster in the shadow than in the light because high light incidence could result in the water loss and associated death by desiccation damage (PAMMENTER; BERJAK, 2000). Molofsky and Augspurger (1992) showed that when seeds were exposed to forest gaps the germination was higher when seeds were covered by the litter. The advantages of litter covering were observed for *Quercus rubra* (GARCIA et al., 2002) and *Quercus ilex* (JOET et al., 2013) seeds.

Recalcitrant seeds are dispersed surrounded by soft and moist tissues (TOMPSETT, 1994; HONG; ELLIS, 1998; FARNSWORTH, 2000; PAMMENTER; BERJAK, 2000; TWEDDLE et al., 2003), which can help with the maintenance of the seed water content before germination. Bustamante et al. (1996) found that *Cryptocarya alba* pericarp decrease the rate of water loss in seeds after dispersal, mainly in the dry season. Studying *S. langsdorffii* pericarp, Colpas and Oliveira (2003) found several compounds related to protection against infection by fungi and bacteria (decomposers), and herbivory and predation. Further, phenolic compounds were found in the pericarp which could act against fungi and bacterial decomposers (CONSTABEL et al., 2000), also acting as germination inhibitors (MACIEL et al., 1992; COLPAS et al., 2003) and have high antioxidant activity protecting cells from abiotic stresses (SWIGONSKA *et al*., 2014). Then, *S. langsdorffii* pericarp and aril could not only lay a role in protection of desiccation, but also protect seeds from decomposition and predation, and preventing that germination occurs in unfavourable period for seedling establishment. These reports make studies needed to better understand these desiccation-sensitive seed traits.

Most of the recalcitrant species occurs in sites with great water availability and capable to offer suitable conditions to germination and seedling establishment (PAMMENTER; BERJAK, 2000). Seeds of these species, in general, are non-dormant, with prompt germination after dispersion and do not form persistent seed banks (VÁZQUES-YANES; OROZCO-SEGOVIA, 1993). Tweddle et al. (2003) used 886 species to report that desiccation-sensitive seeds occur more commonly in tropical rain forest (47% of all studied species) and less frequently in dry sites (12%), and in these drier environments species can decrease the desiccation risk dispersing their seeds in the month with higher precipitation (PRITCHARD et al., 2004). However, several studies report desiccation-sensitive seed dispersal in seasonal environments to the month with higher rainy (BERJAK; PAMMENTER, 2000; FARNSWORTH, 2000; TWEDDLE et al., 2003; PRITCHARD et al., 2004), but *Swartzia langsdorffii* seeds, even desiccation-sensitive, are dispersed in the dryer month of the year in the region of study. This was also observed for other recalcitrant species (CARVALHO et al., 1998; DUSSERT et al., 2000; PRITCHARD et al., 2004; DAWS et al., 2005; YU et al., 2008; HILL et al., 2010) but was not investigated how these species keep their seeds viable in the soil until the beginning of the rainy season. Then, the aims of this work were to characterize *Swartzia langsdorffii* seed storage behaviour and evaluate which anatomical, morphological and physiological traits are responsible to allow seed survival and germination after dispersal in the late winter.

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3 ARTICLE 1: *Swartzia langsdorffii* Raddi: morphophysiological traits of a recalcitrant seed dispersed during the dry season

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Swartzia langsdorffii Raddi: morphophysiological traits of a recalcitrant seed dispersed during the dry season

Short title: Morphophysiology of *Swartzia langsdorffii* seeds

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Abstract

Swartzia langsdorffii seeds have recalcitrant characteristics. Nonetheless, dispersal begins in the month with the lowest precipitation in the studied region, which could lead to seed death by desiccation. Therefore, the aims of this study were to (1) characterise the physiological behaviour of *S. langsdorffii* seeds related to their desiccation sensitivity/tolerance and (2) assess the morphophysiological characteristics that enable the seeds to remain viable after dispersal. Fruits and seeds were subjected to biometric evaluation and the anatomical and ultrastructural characterisation of the seeds was determined. Field assessments were performed to determine the capacity of the seeds to maintain viability and to verify the relation between seed viability, pericarp water content and environmental variables. These seeds are recalcitrant, have pores that are distributed throughout the seed coat and contain a large number of stomata that are found in the hypocotyl-radicle axis epidermis. Moreover, phenolic compounds were found throughout the radicle region. Seeds remain viable in the soil for up to seven months after dispersal without a significant decrease in water content, despite the low precipitation and soil water content. Radicle protrusion begins five months after dispersal and coincides with partial fruit decomposition at the beginning of the rainy season. Then, the possible microclimate created by the pericarp composed by the moisture of the aril and the soil, the presence of the structures in the axis such as the pores and stomata, the chemical composition and the morphology of *S. langsdorffii* seeds could favour their viability maintenance until the beginning of the rainy season.

Introduction

Recalcitrant seeds lose viability when dried below 12-31% water content and do not tolerate sub-zero storage (Roberts, 1973). They are usually spherical, voluminous, large (larger than 4 cm or 4 g) and surrounded by moist and permeable tissues during dispersal. They also usually have large embryos and little or no endosperm (Chin *et al*., 1989; Farnsworth, 2000; Pammenter and Berjak, 1999; Tweddle *et al*., 2003; Daws *et al*., 2005; Hamilton *et al*., 2013). In addition, these seeds have the following characteristics: lack of post-maturation drying, leading to high water content during dispersal (> 35%); rapid and uniform germination; limited storability and no formation of persistent soil seed banks. It is thought that morphophysiological characteristics work to minimise water loss and, consequently, seed death by desiccation damages (Berjak *et al*., 1989; Ellis *et al*. 1990; Hong *et al.,* 1998; Pammenter and Berjak, 1999, 2000; Kermode and Finch-Savage, 2002; Berjak and Pammenter, 2008). Studies have reported that drying causes structural damage in recalcitrant seeds. However, seed drying does not represent a problem for the natural regeneration of species as recalcitrant seeds germinate shortly after dispersal and form a seedling bank in the forest understory (Tweddle *et al*., 2003). Furthermore, these seeds are more common in non-seasonal and constantly moistened environments and less frequently found in dry and seasonal environments, where their dispersal is usually concentrated on a period just before or during the months with greater precipitation (Roberts and King 1980; Dickie *et al.* 1992; Tompsett, 1992; Hong *et al.,* 1998; Tweddle *et al*., 2003; Pritchard *et al*., 2004; Daws *et al*., 2005).

Swartzia langsdorffii Raddi. [(Fabaceae – Faboideae (Swartzieae)] is a perennial woody species that reaches up to 20 m in height (Paiva *et al*., 2004) and is distributed throughout the southeastern region of Brazil, associated with the Atlantic Rainforest and seasonal forest formations (Santos, 1979; Marangon *et al*., 2003). The fruits of *S. langsdorffii* are follicle type, large (6 - 9 cm) and lignified, have abundant secretion of resins mainly composed of tannins, saponins and lipids, and contain two to four large seeds surrounded by an aril (Colpas and Oliveira, 2003). The fruits are dehiscent but only open after dispersal during the dry season (austral winter) and the fruits tissues remain intact, covering seeds for many months while in the soil.

Although the seeds of *S. langsdorffii* are large, rounded, surrounded by fleshy tissues and have high water content – typical characteristics of recalcitrant seeds – their dispersal begins in August, the month with the lowest precipitation of the year. Thus, the objectives of this study were to (1) characterise the physiological behaviour of *S. langsdorffii* seeds related to their desiccation sensitivity/tolerance and (2) assess the morphophysiological characteristics that enable the seeds to remain viable even after the diaspores (fruits containing seeds) have been dispersed.

Material and methods

Diaspores collection and processing

S. langsdorffii diaspores were collected from five trees in the municipality of Lavras, Minas Gerais, Brazil (21°14'S; 45°00'W, 918 m a.s.l.) in August 2010 and August 2011, after the beginning of natural dispersal, when they have a dark orange colour (mature diaspores). Diaspores were opened with a knife to remove the seeds, and when necessary, the seed aril was manually removed to perform the laboratory experiments. Subsequently, the seeds were washed up in tap water, blotted dry and maintained in a perforated plastic bag under laboratory conditions for up to 10 days.

Diaspores biometry and seed viability assessment

The number of seeds per diaspore and biometric characteristics of the pericarps and seeds were obtained from 135 fruits and 60 seeds. The water of the different parts of the diaspores was determined separately for the pericarp, aril and seed, using the oven method at 103°C for 17 hours (ISTA, 2004), with four replicates of 20 g each. The structures were cut in small parts when fresh weighed to facilitate water loss, and the results were expressed on a fresh weight basis. The seed viability, with and without the aril, was tested through germination tests in plastic trays (40 x 25 x 8.5 cm) with a 6 cm layer of washed, autoclaved and pre-moistened sand. Seeds were sown 1 cm deep, and incubated in a germination incubator (Mangelsdorf) at 25°C under constant light. The experiments were designed with four replicates of 15 seeds each, and the emergence of the shoots was assessed weekly until all seeds germinated or had died (rotted).

Structural characterisation of the seeds

Five samples containing seed coat, embryonic axis and cotyledons were fixed separately in modified Karnovsky solution (2.5% glutaraldehyde; 2.5% formaldehyde and 0.05 M sodium cacodylate buffer, pH 7.2; and 0.001 M $CaCl_2$) (Karnovsky, 1965) for at least 24 hours. After fixation, the samples were transferred to a 30% glycerol solution for 30 minutes. Subsequently, the samples were cryofractured in liquid nitrogen depending on the tissue area to be observed and washed in distilled water. After sectioning, the samples were dehydrated in a graded acetone series for 10 minutes each in concentrations of 25, 50, 75 and 100% and with three repetitions for the 100% concentration. After dehydration, the samples were transferred to a critical point device (Bal-Tec, São Paulo, Brazil), mounted on stubs, sputtercoated with gold (Bal-Tec, São Paulo, Brazil) and examined under a scanning electron microscope (SEM) LEO EVO 40 XVP (São Paulo, Brazil).

For the light microscopy analysis, seeds were fixed in formalin-acetic acid-alcohol (FAA 50) and conserved in 70% ethanol. The embryonic axis was isolated from the cotyledons for the inclusion procedures. The samples were dehydrated in graded alcohol series and embedded in hydroxyethyl methacrylate (Leica®, São Paulo, Brazil) according to the manufacturer's instructions. The resulting material was sectioned longitudinally in a rotating microtome to obtain approximately 5 µm thick sections. The sections were stained with 0.05% toluidine blue O (O'Brien and McCully, 1981), and the resulting slides were mounted with Permount® synthetic resin. The toluidine blue stains cells with primary wall in blue purple, cells with primary and secondary lignified wall in light green and the phenolic compounds in dark green. The images were recorded in a trinocular microscope (Primo Star Carl Zeiss®, São Paulo, Brazil) connected to a digital camera (Axiocam Erc 5S, São Paulo, Brazil).

Seed drying and storage

The diaspores collected in 2010 and 2011 were processed (seeds taken out as described above), and the seeds were placed in plastic trays (40 × 25 × 8.5 cm) in a single layer and subjected to the drying process in a climate-controlled room [20 ± 2°C and 50 ± 2% relative humidity (RH)]. The target weights (Cromarty *et al.*, 1985) corresponding to 40, 35, 30, 25 and 20% water content were calculated to monitor seed drying.

Seed samples of each water content target were used to determine drying velocity and to assess the seedling emergence, as described above.

For the storage experiment carried out in 2010, seeds were removed immediately after diaspore collection and placed in semipermeable perforated plastic bags. The bags containing seeds were stored in the dark in a cold chamber at a constant temperature of 5°C and 40% RH. Each month for five months a sample was collected to assess seed water content and seedling emergence, as described above.

Seed germination in field conditions

These experiments began in August 2010 and August 2011 and were carried out in the understorey of a seasonal semideciduous montane forest with an emergent canopy (Veloso *et. al*, 1991) located in the Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil. The Köppen climate classification for Lavras is Cwa with Cwb characteristics, and there are two well-defined seasons: a dry season from April to September and a rainy season from October to March (Köppen, 1936).

In August 2010, four blocks with 200 diaspores each were randomly distributed in a single layer directly above the litter in the forest understory. From August 2010 to January 2011, samples containing 10 diaspores per block were collected every 15 days to assess the seed water content and germination. Approximately 20 seeds were obtained from the 10 diaspores sampled; five of these seeds were used for determine the seed water content and 15 were used to count the number of germinated, dead and firm seeds. The criterion used to assess germination was the protrusion of the radicle ≥ 1mm. Seeds were considered dead if they were rotten, and firm seeds were those with an intact and apparently healthy structure but no protrusion of primary root.

A second experiment was implemented from August to December 2011 to assess the relationship between environmental variables and water content of the fruits, arils and seeds. A total of 600 diaspores per block were distributed over the litter in a single layer, and the pericarp, aril and seed water content were assessed monthly (as described above) along with the soil moisture, which was measured using four replicates with 20 g of soil each (EMBRAPA, 1997). The environmental data – mean, maximum and minimum air temperature and rain precipitation - were collected in the climatic station situated near the forest, at UFLA.

Statistical analysis

All collected data were submitted to normality and homoscedasticity tests, if data are normal and homoscedastic ($p \geq 0.05$) data were submitted to an analysis of variance (ANOVA) at 5% probability. When statistically significant differences were found ($p \leq 0.05$), qualitative means were compared using Tukey test at 5% probability (R Development Core Team, 2011) and quantitative data were analysed using regression (SigmaPlot® software - Systat Software Inc., San José, California, USA). When the data distribution were not normal and/or homoscedastic, data were submitted to Generalized Linear Models (GLM) tests (R Development Core Team, 2011), and the means analysed as described above. Furthermore, biometric data were subjected to a box-plot analysis using SigmaPlot® software (Systat Software Inc., San José, California, USA). A Pearson correlation analysis was conducted using R 2.12.0 software (R Development Core Team, 2011) with data of moisture content of fruits, arils, seeds and soil, and the environmental data collected in the Climate Station of Engineering Department of UFLA. Only the means obtained before the rainy season were included in this latter analysis to avoid the influence of rain on the moisture values.

Results

Diaspores biometry and seed viability assessment

The fruits had an average length of 93 ± 18.8 mm, width of 57 ± 8.1 mm and thickness of 37 ± 4.2 mm, and had 2.0 ± 1.0 seeds per fruit (Fig. 1A, B, C and D). The seeds had an average length of 32.5 ± 3.3 mm, width of 24.5 ± 2.9 mm and thickness of 19.6 ± 2.0 mm. The seed coat had an average thickness of 0.5 ± 0.1 mm (Fig. 1E, F, G and H), and the seeds, aril and fruit had an average water content of 47.7% (±0.46), 77.2% (±0.69) and 83.3% (±0.54), respectively, at dispersal.

A significant difference ($p \leq 0.0001$) was found between the percentage of shoot emergence in seeds with and without aril. Seeds with aril reached 32% germination with shoot emergence beginning on the 42nd day after sowing and stabilised on the 80th day. Seeds without aril had 100% germination with shoot emergence beginning on the 27th day after sowing and stabilising on the 49th day.

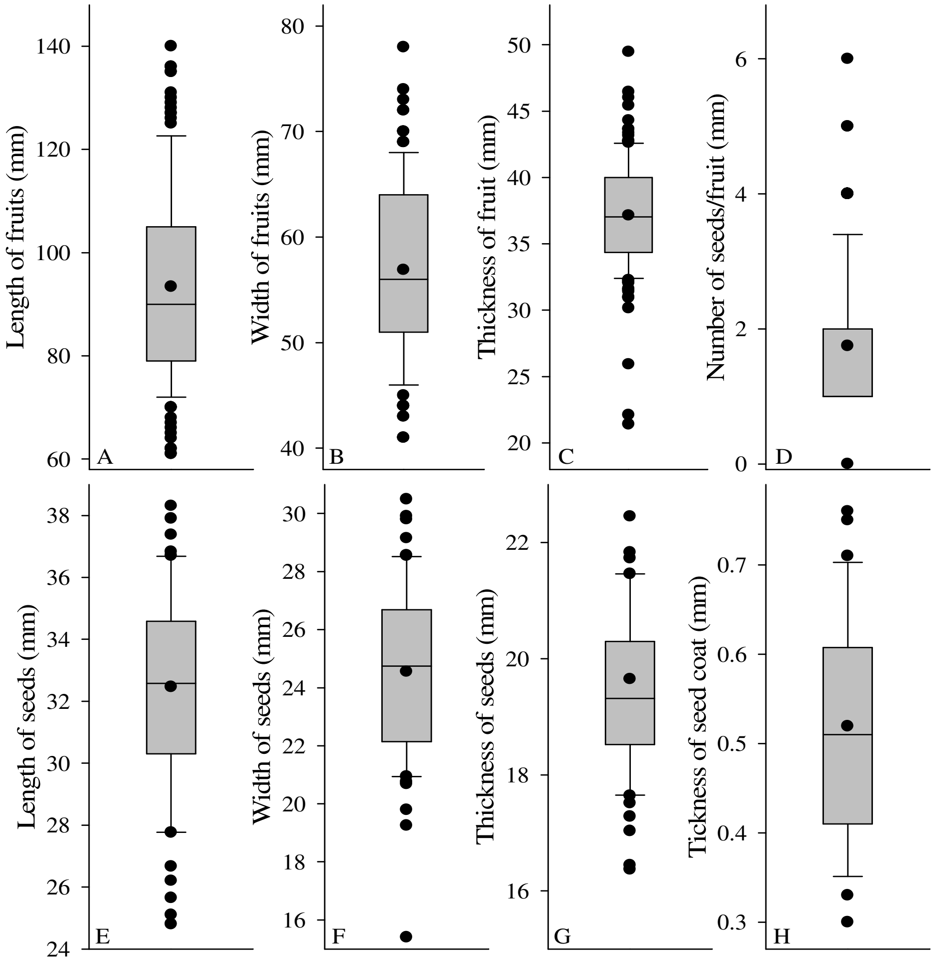

Figure 1. Length, width and thickness of the fruits and seeds, number of seeds per fruit and seed coat thickness for *Swartzia langsdorffii*. The circles inside the boxes indicate the mean, the circles outside the boxes indicate outliers present in the data and the lines inside the boxes indicate the median values.

Structural characterisation of the seeds

S. langsdorffii seeds have a seed coat with pores that are distributed evenly along its surface (Fig. 2A, B). These pores consist of regions where the seed coat cells are discontinuous and form gaps that may be observed by the naked eye as small whitish points. Those pores have subepidermal parenchymal tissue with large intercellular spaces (Fig. 2B), which can be observed along the entire extension of the thin seed coat in a cross section (Fig. 2C). The embryo is axial and containing two fleshy cotyledons. The embryonic axis is composed of a hypocotyl-radicle axis that has a conical shape and reduced size (Fig. 2D) compared to the cotyledons. A single small convex protuberance is observed in the middle region of the seed, opposite to the hilum, where the micropyle is located. Numerous stomata can be observed in the hypocotyl region closer to the cotyledon node (Fig. 2E). The plumule is located between the cotyledons and is not easily observed.

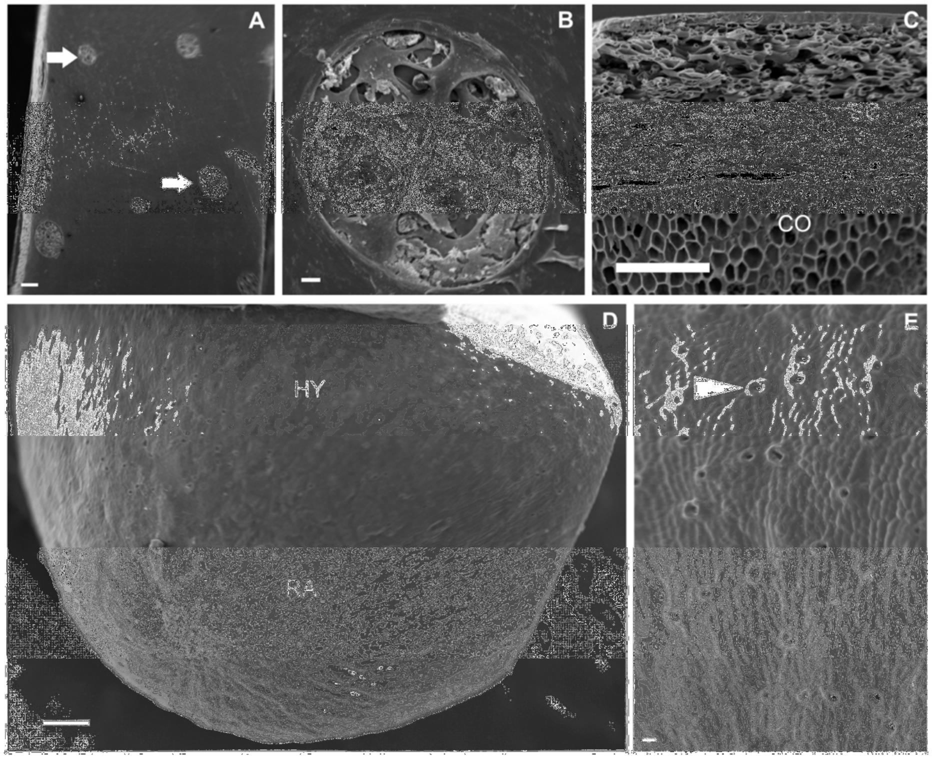

Figure 2. Ultrastructure of *Swartzia langsdorffii* seed parts. A) General view of the seed coat showing the presence of pores (arrows). B) Detail of the pore, showing loose subepidermal tissue and large intercellular spaces. C) Longitudinal section of the seed, with the thin seed coat showing intercellular spaces in the mesophyll; as well as some cotyledonary tissue. D) General aspect of the embryonic axis, with numerous stomata dispersed randomly in the hypocotyl region and few stomata in the radicle region. E) Detail of the stomata in the hypocotyl region (arrowhead). (CO = cotyledon; HY = hypocotyl; RA = radicle; SC = seed coat). Bars: 200 µm (A, C, D), 20 µm (B, E).

Anatomically, the plumule is rudimentary and has little relief above the cotyledon node and no leaf primordia (Fig. 3A, B). The protoderm has radially elongated cells, and the fundamental meristem has isodiametric cells with thin cell walls and a conspicuous nucleus (Figure 3B). The hypocotyl-radicle axis has an approximately conical shape with a pointed apical region and wider distal region as a result of the wide cotyledon node (Fig. 3A). Differentiated stomata were observed in the cotyledon protoderm close to the plumule (Fig. 3 B, C). Stomata were also observed in the protoderm of the distal region of the axis (hypocotyl) (Fig. 3D, E). The protoderm has cells with conspicuous phenolic content throughout the entire extension of the embryonic axis (Fig. 3D, F), the procambium has elongated cells, and the fundamental meristem is distributed irregularly in the cortex and medulla (Fig. 3A). In the extremity of the radicle, there is a group of cells with reduced size and irregular shape that form a primordial cap (Fig. 2D, Fig. 3A, F, G). Amyloplasts and granules that were stained with a strongly green colour were observed in this region and proximal to it, indicating the presence of phenolic compounds in the cells of this region (Fig. 3G).

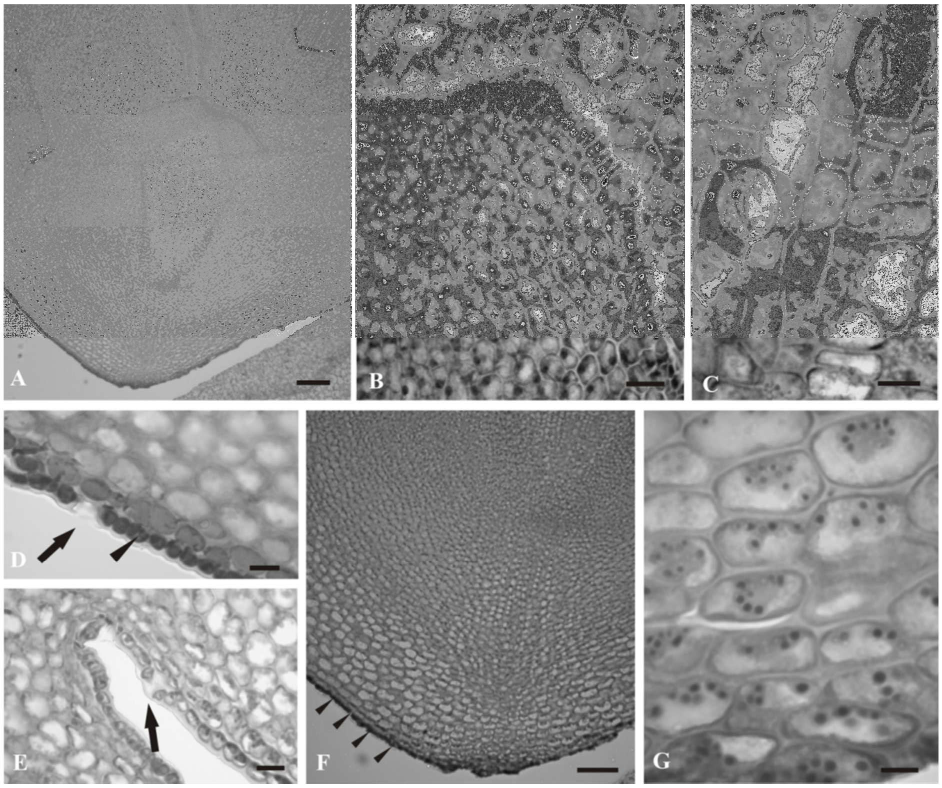

Figure 3. Anatomy of *Swartzia langsdorffii* embryo. A) General aspect of the embryonic axis. B) Detail of the undifferentiated plumule. C) Detail of the stomata on the cotyledons. D and E) Detail of the stomata on the embryonic axis, with arrows indicating the position of the stomata and arrowheads indicating phenolic compounds. F) Detail of the hypocotyl-radicle axis and presence of phenolic compounds (arrowheads). G) Detail of the apical cells of the primordial root cap with amyloplasts. Bars: 200 µm (A), 100 µm (F), 20 µm (B, C, D, E), 10 µm (G).

Seed drying and storage

The initial water content of seeds was 46% and reached 22% after 46 to 55 days of artificial drying (Fig. 4B). The critical point after which seeds lost viability (Pammenter and Berjak, 1999), measured as germination capacity, was reached at moisture levels between 33 and 38%, and the lethal point was reached at 22%, when all seeds had lost viability. The drying behaviour of the seeds collected in 2010 and 2011 was similar (Fig. 4A). Water loss followed an exponential decay occurring more rapidly until the 10th day, when the critical 38% moisture point was reached, while subsequently water loss was slower and the lethal point (22% moisture) was reached around the 50th day after the start of the drying process (Fig. 4B).

S. langsdorffii seeds that were stored in a cold chamber with an initial water content of 47% had an initial viability of 92.5% and lost viability after four months of storage (Fig. 5). The seed water content remained constant during this period.

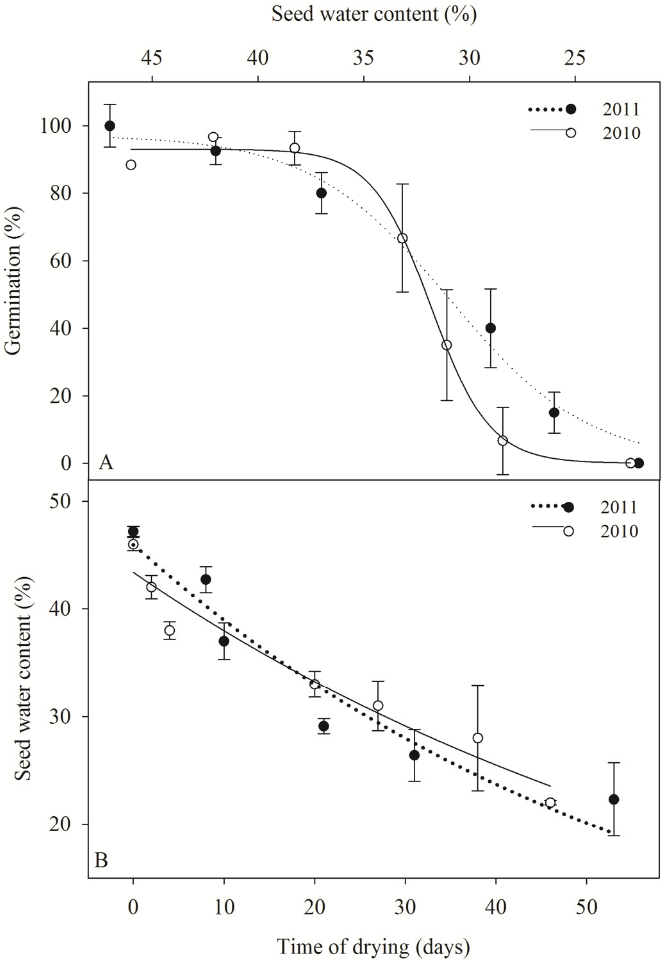

Figure 4. Effects of drying at 20 °C and 50% RH on germination and moisture of *Swartzia langsdorffii* seeds collected in 2010 and 2011. A) Germination percentage in relation to the seed water content (2011 $R^2 = 0.98$; 2010 $R^2 = 0.99$) expressed on a fresh weight basis. B) Water content of the seeds during drying (2011 $R^2 = 0.93$; 2010 $R^2 = 0.94$) expressed on a fresh weight basis.

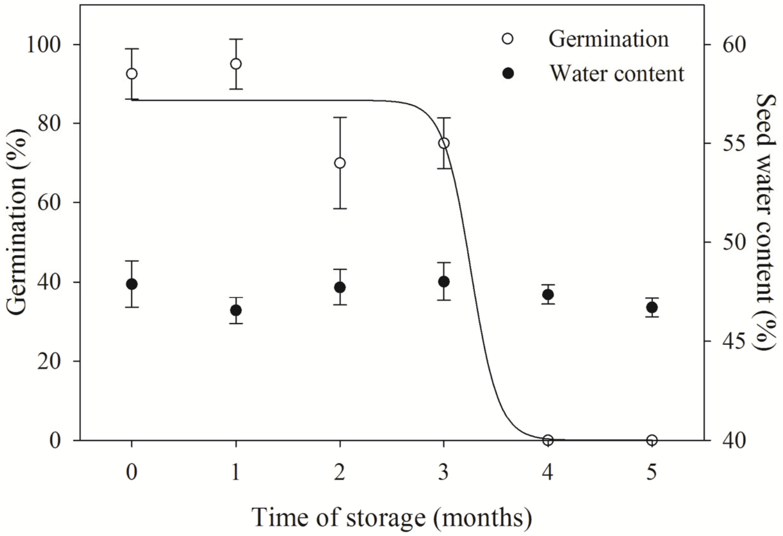

Figure 5. Germination percentage and water content (fresh weight) of *Swartzia langsdorffii* seeds during storage in a cold chamber (5ºC/40% RH); $R^2 = 0.96$.

Seed germination in field conditions

Significant variation occurred in the mean moisture values (p<0.0001) and germination percentage (p<0.0001), as well as in the number of dead (p<0.0001) and firm (p<0.0001) seeds during the experimental period. The seed water content ranged between 35 and 48%, remaining above the critical point (Fig. 6) that was determined in the laboratory experiment (Fig. 4A). Germination was observed after December 15th, five months after the dispersal of the diaspores, and reached 30% in January. The rainy period began after September 15th, and until the end of October there were sparse rainfall events followed by many days with no rain. Germination began 113 days after dispersal, when rain had already become abundant and regular (Fig. 6). The percentage of firm seeds remained stable until the 43rd day, after which it gradually decreased until reaching 43%. Dead seeds were first found on the 28th day and reached a maximum percentage of 27% at the end of the experiment (Fig. 6).

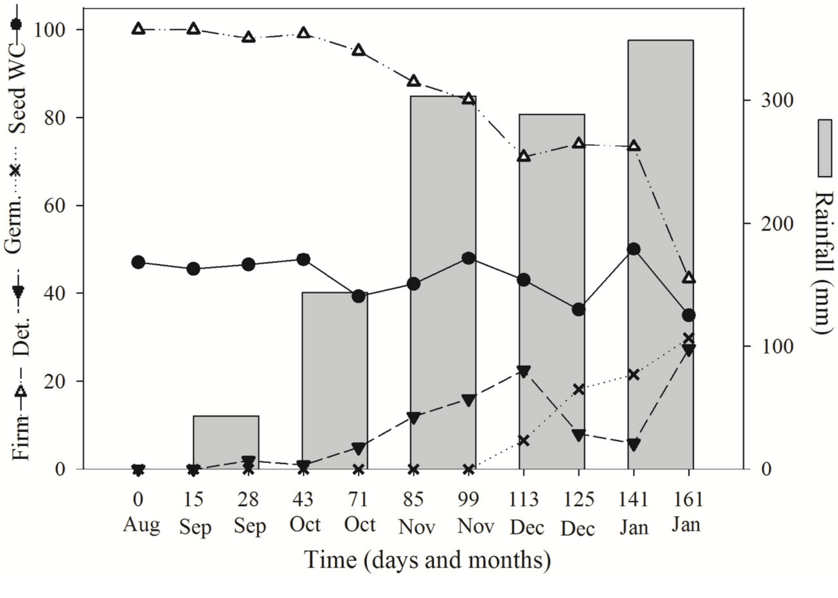

Figure 6. Percentage of germinated, dead and firm seeds and water content of *Swartzia langsdorffii* seeds placed in the field in 2010. Bars indicate cumulative monthly precipitation.

A wider difference between minimum and maximum air temperatures was observed in the first three months of the experiment, followed by a decrease in this range in October with the increase in the minimum air temperature, coinciding with the beginning of the rainy season. During the experiment, the maximum temperature ranged from 26.1 to 29.6 °C, mean temperature ranged from 19.0 to 22.1°C and minimum temperature ranged from 12.6 to 18.1 °C. The soil temperature remained stable at approximately 17.0 °C until the 28th day, after which it gradually increased to 19.1 °C at the end of the experiment (Fig. 7A).

There was significant variation in the water content of the fruits ($p<0.0001$), arils ($p<0.0001$), seeds ($p=0.0012$) and soil ($p<0.0001$) during the experimental period (Fig. 7B). The water content of the fruits varied between 27.9 and 83.3%, the water content of the arils varied between 71.7 and 78.3%, the water content of the seeds varied between 41.0 and 57.1%, and the water content of the soil varied between 20.4 and 26.9 %. Similar to the experiment conducted in the previous year, the water content of the seeds remained above the critical point for germination (Figs 6A and 7B).

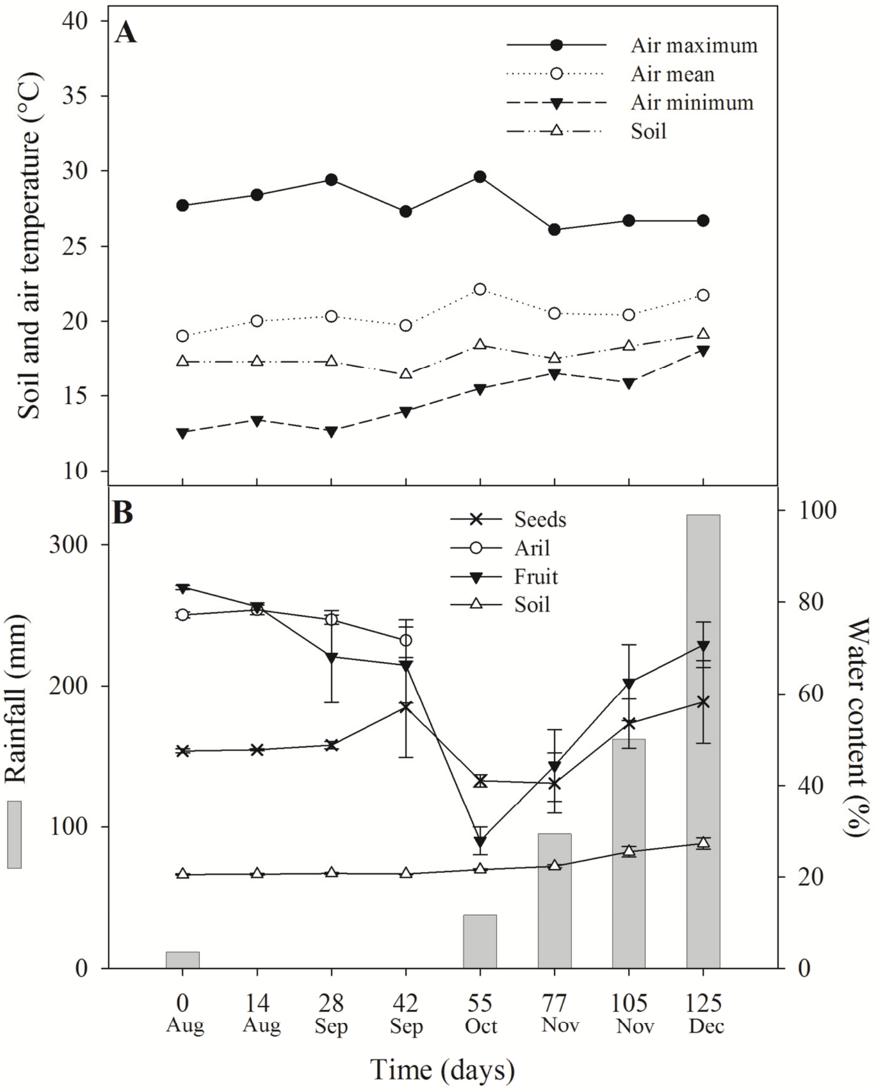

Figure 7. A) Maximum, mean and minimum air temperature and soil temperature in the experimental area. B) Water content of *S. langsdorffii* seeds, aril and fruit, soil moisture and precipitation from August to December 2011.

The seed moisture content was not correlated with either the fruit or aril moisture content, but showed a strong negative correlation (p=0.0026; R=-0.98) with the soil temperature. However, the fruit water content had strong negative correlation with the mean (p=0.0158; R=-0.94) and minimum (p=0.0347; R=-0.90) air temperatures and soil water content (p=0.0035; R=-0.98). After 55 days of the beginning of the experiment there was a decrease in the water content of the fruit, a significant increase in the mean and minimum air temperatures and an increase in the soil temperature. As described above, only the means collected before the rainy season were used to perform this analysis, since all the evaluated parameters showed increased water contents after the rainy period began.

Discussion

Swartzia langsdorffii seeds are desiccation-sensitive since they lose their viability when dried to 22% water content (Fig. 4). Desiccation-sensitive seeds usually have large volumes, round shapes, large embryos, thin seed coat and high water content during dispersal, and are usually dispersed inside the diaspores composed by water-permeable and moist structures (Tompsett, 1992; Farnsworth, 2000; Tweddle *et al.*, 2003;

Daws *et al*., 2005; Jayasuriya *et al*., 2012; Hamilton *et al*., 2013), as the morphological results showed in this work. Many researchers consider seed size of recalcitrant species an ecological advantage, once larger and generally rounder, seeds have a smaller surface area to volume ratio which slows water loss and delays desiccation-induced viability loss (Farnsworth, 2000; Pammenter and Berjak, 2000; Tweedle *et al*., 2003; Hamilton *et al*., 2013), and, as recalcitrant seeds tend to form seedling banks, it is expected these seeds to be large (Pammenter and Berjak, 2000). The correlation between seed size and desiccation sensitivity has been found in different studies conducted in tropical forests (Souza and Válio, 2001; Daws *et al*., 2005; Yu *et al*., 2008), and seeds classified as recalcitrant were larger than those classified as orthodox. Although *S. langsdorffii* has a large embryo that occupies the entire seed, most of the embryo consists of cotyledons.

Thin seed coats enable gas exchange with the environment (Berjak and Pammenter, 2000; De Souza and Marcos Filho, 2001; Hamilton *et al*., 2013), which could be helped by the presence of pores and intercellular spaces, also it does not prevent the expansion of the embryonic axis. Pores have been reported in soybean seed coat and are common in the Fabaceae family, with pore formation occurring in the latter development stages of the seed, concomitantly with the beginning of the maturation drying (De Souza and Marcos Filho, 2001). In this case, pores could help in viability maintenance allowing gas exchange between the seeds and their surrounding structures. Additionally, the presence of a large amount of stomata in the hypocotyl of *S. langsdorffii* embryonic axis may facilitate gas exchange and maintain an active metabolism even when the seeds are surrounded by pericarp and aril, considering that, according to Berjak *et al*. (1989), recalcitrant seeds have high requirements of oxygen due to their high metabolism during and after dispersal. Still, according the latter authors, this high metabolism is due to fact that the recalcitrant seeds continue to develop after shedding and it is not possible to detect when germination metabolism begins.

Although the water content of *S. langsdorffii* seeds remained unaltered during cold storage there was a complete loss of viability after the fourth month. Several authors reported on chilling sensitivity in tropical species, recommending storage at temperatures above 15 °C (Berjak and Pammenter, 2008; Dresch *et al*., 2014). In addition, seed storage with high water content is often hampered by fungal contamination in the seed coat (Calistru *et al*., 2000). A massive presence of fungi, that completely covered the seed coat, was observed starting in the third month of storage at 5 °C, when the seeds were opened to assess the presence of contamination inside the seeds; this confirmed that it only occurred on the outside of the seed coat.

Phenolic compounds act as protection against fungi and bacterial decomposers (Constabel *et al*., 2000), also acting as germination inhibitors (Maciel *et al*., 1992; Colpas *et al*., 2003) and have high antioxidant activity protecting cells from abiotic stresses (Swigonska *et al*., 2014). The massive presence of phenolic compounds in embryonic axis of *S. langsdorffii* seeds could indicate a possible strategy to avoid early germination and protect them against pathogens and possible environmental stresses, whereas seeds are dispersed during the dry season and take more than 100 days to start germination (Fig. 6). Colpas and Oliveira (2003) also noted these compounds in *S. langsdorffii* pericarp.

In relation to seed dispersal, considering morphological traits of diaspores such as colour, size and texture, Martins *et al*., (2014) supposed *S. langsdorffii* diaspores are dispersed by mammals, since the bright colour and odour of the pericarp and aril may act attracting these animals.

Nonetheless, *S. langsdorffii* diaspores are rich in secondary compounds such as tannins and saponins in the pericarp (Colpas and Oliveira, 2003) and phenolic compounds and saponins in the aril (data not published). These secondary metabolites are known by their strong role in seed defence against herbivory and decomposition (Cipollini and Levey, 1997) and, specifically for saponins, as a strong antinutrient (Francis *et al.*, 2002). A more detailed work should be developed aiming to understand the specific role of the diaspore on *S. langsdorffii* seed dispersal.

Numerous studies have correlated the dispersal of desiccation-sensitive seeds in seasonal habitats to the highest-precipitation periods of the year (Farnsworth, 2000; Pammenter and Berjak, 2000; Tweddle *et al.*, 2003; Pritchard *et al.*, 2004). However, *S. langsdorffii* seeds are desiccation-sensitive and dispersed in the month of the year with the lowest precipitation. The dispersal of other recalcitrant seeds has also been associated with the dry season, as reported by Dussert *et al.* (2000), Daws *et al.* (2005) and Yu *et al.* (2008); however, these authors reported these species are associated with swamp or riparian environments.

The pericarp of *S. langsdorffii* remained intact for approximately three months after the beginning of the experiments followed by drying in the tissues that were not in contact with the soil, but did not deteriorate; however, the tissues in contact with the soil did experience deterioration. These seeds maintain their high water content during a long period after dispersal and, besides have not been tested in this work; it is possible that the pericarp protects seeds from desiccation by creating a moist microclimate in the field. Chacón and Bustamante (2001) studied seeds of *Cryptocarya alba* and stated that the presence of pericarp decreases seed water loss after dispersal, especially during the dry season. The same role of the pericarp was found by Sobrino-Vesperinas and Viviani (2000) in seeds of *Quercus suber*. Then, the possible microclimate created by the pericarp composed by the moisture of the aril and the soil, the presence of the structures in the axis such as the pores and stomata, the chemical composition and the morphology of *S. langsdorffii* seeds could favour their viability maintenance until the beginning of the rainy season.

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Conflicts of interest

None.

4 ARTICLE 2: Influence of the surrounding structures on seed survival and germination of *Swartzia langsdorffii* Raddi.: the role beyond dispersal

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Preparing for submission

Original Article

Influence of the surrounding structures on seed survival and germination of *Swartzia langsdorffii* Raddi.: the role beyond dispersal

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Running title: Surrounding structures on seed survival and germination of *Swartzia langsdorffii*

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ABSTRACT

- **Background and Aims** Some studies hypothesize that diaspore structure plays a role in seed viability and/or germination but the ecological importance of this trait needs to be tested in the field. Then, the objectives of this work were (1) to identify the true *in situ* contribution of diaspore on seed viability and germination, and (2) to identify the morphoanatomical traits of *Swartzia langsdorffii* diaspore that aid its desiccation sensitive seed to remain viable after dispersal.

- **Methods** It was evaluated the role of pericarp and aril on seed survival and germination by placing the whole fruit [pericarp+aril+seed (treatment 1)], aril+seed (treatment 2) and bare seed (treatment 3) on the soil in the forest understory and monthly assessment of the number of germinated (radicle protrusion), dead, firm and predated seeds, and water content (WC) of all structures of the dispersal unit (pericarp, aril and seed); the environmental variables also were evaluated. In adition, the histochemical features of the diaspore were verified.
- **Key Results** The pericarp greatly reduced its WC over time, while the aril maintained its WC, covering the seeds for only one month, seeds do not lose water even without the presence of the rest of pericarp and aril. However, pericarp promoted seed viability and germination long after dispersal. The embryo presented a thickening in the external and internal periclinal cell wall covering

the embryonic axis and in the external periclinal cell wall around the cotyledons, in both cases consisting of cutin.

- **Conclusions** Pericarp and aril are not essential to prevent water loss in seeds, but the moisture in the pericarp and aril in the time of dispersal are likely to favour viability maintenance and promote germination more than two months later, during rainy season, when radicle protrusion becomes detectable. Morphoanatomical features of seeds are suggested as the main factor to prevent water loss. Survival of these desiccation sensitive seeds upon dispersal during the dry season appears to be facilitated by multiple diaspore features preventing water loss.

Key words: aril, cutin, desiccation sensitivity, diaspore, embryo cell wall, pericarp, seed viability, *Swartzia langsdorffii*, water loss restriction.

INTRODUCTION

Diaspore dispersal allows seeds, fruits or infructescences to be transported away from the mother plant via biotic or abiotic agents (Van Der Pijl, 1972). The diaspores adaptations such as colour, size, shape, odour and appendages act as a specific dispersal agents, and contributed to the so-called dispersal syndrome (Willson *et al.*, 1989) by attracting animals. Most of the has focused on the role of the pericarp in seed dispersal (Van Der Pijl, 1969; Rodriguez *et al.*, 2013; Martins *et al.*, 2014) or mechanical restriction to germination (Jayasuriya *et al.*, 2012; Lu *et al.*, 2015; Zhou *et al.*, 2015) but its importance on seed viability and promoting germination has been largely overlooked. Only some studies have shown the importance of pericarp in essential steps for seed germination and viability maintenance after dispersal. Xia *et al.* (2012), in a study with nine species of *Quercus*, correlated water loss and uptake with the pericarp anatomy, and the same role in the control of seed imbibition was found for *Glebionis coronaria* pericarp by Puglia *et al.* (2015). The pericarp preventing seed water loss has also been reported for other species (Sobrino-Vesperinas and Viviani, 2000; Chacón and Bustamante, 2001; Mamut *et al.*, 2014; Joët *et al.*, 2016). The control of water loss is an even more important factor for seeds that are desiccation sensitive, being a key issue for survival.

Swartzia langsdorffii [Fabaceae – Faboideae (Swartziae)] produce an orange and large pericarp, containing 1 to 6 brownish recalcitrant seeds with yellowish aril (Fig. 1), and all the diaspore structures have high water content at the time of dispersal (Vaz *et al.*, 2016). Considering these traits, Martins *et al*., (2014) suggested that *S. langsdorffii* diaspores are dispersed by mammals, since the bright colour and odour of the pericarp and aril may attract these animals. Nevertheless, Vaz *et al*. (2016) neither detected significant seed predation or removal during a field study with this species, nor observed any animal attempting to feed on the diaspores. Furthermore, *S. langsdorffii* diaspores are rich in secondary compounds such as tannins and saponins in the pericarp (Colpas and Oliveira, 2003), saponins in the aril (A. T. Nakamura, personal communication) and phenolic compounds in the seeds (Vaz *et al*., 2016). These secondary metabolites are known for their role in seed defence against herbivory and pathogens (Cipollini and Levey, 1997) and, specifically for saponins, as a strong antinutrient (Francis *et al*., 2002). Other interesting characteristics is as the pericarp retention a large amount of seed coat pores and stomata in the seed embryo aiding gas exchange with the surrounding structures (Vaz *et al*., 2016) suggest a suit of mechanisms involved in prolonged maintenance of seed viability. Therefore, these features lead to the hypothesis that *S. langsdorffii* pericarps have an important role in seed viability maintenance after dispersal, since the seeds are desiccation sensitive, dispersed in the dry season and do not lose viability until the rainy season begins (Vaz *et al*., 2016).

Some studies hypothesized that diaspore structure plays a role in seed viability and/or germination but the ecological importance of this trait remains to be tested in the field. Then, the objectives of this work were (1) to investigate the role of fruit tissues of *S. langsdorffii* on seed survival subsequent to dispersal, and (2) to identify any ecological relevant morphoanatomical traits of *Swartzia langsdorffii* diaspores that aid its desiccation sensitive seeds to maintain viability after dispersal.

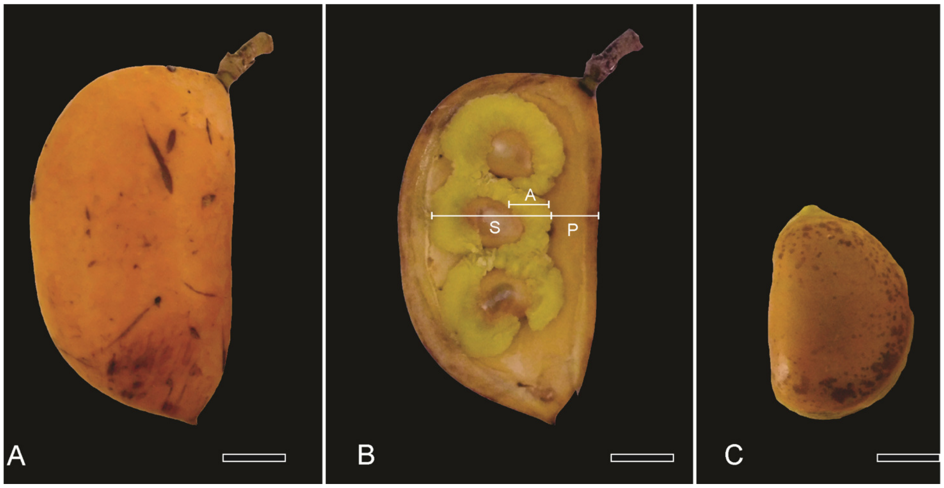

Fig. 1. *Swartzia langsdorffii* diaspore. Front (A) and transversal (B) view of the diaspore, showing arillated seeds. Front view of seed without aril (C). (P = pericarp, A = Aril, S = seed). Scale bars = 2 cm (A, B), 1 cm (C).

MATERIALS AND METHODS

Diaspore collection and processing

Swartzia langsdorffii diaspores were collected from trees in the municipality of Lavras (MG, Brazil; 21º 14' S, 45º 00' W, 918 m a.s.l.) in August 2013 and September 2014, after the beginning of natural dispersal when they presented a dark orange colour, indicative of seed maturation. When necessary, diaspores were opened with a knife to isolate the seeds and the aril was manually removed. Subsequently, the seeds were rinsed in tap water, their surfaces were blotted dry and seeds were placed in a perforated plastic bag under laboratory conditions until the start of all assays, one week later.

Determination of water content

Pericarp, aril and seed water content (WC) were determined by oven drying at 103 °C for 17 hours, taking the fresh weight before and dry weight after drying, using four replicates of 20 g each. Due to the large structures, diaspore parts were cut in small pieces to facilitate the water loss. The results for WC were expressed on a fresh weight basis (ISTA, 2004).

Seed viability

The seed viability was assessed through germination tests using four replicates of 15 seeds each placed in plastic trays (40 x 25 x 8.5 cm) with a 6 cm layer of washed, autoclaved and pre-moistened sand. Seeds were sown 1 cm deep and incubated at 25 °C under constant light (cool white fluorescent tubes, approx. 40 µmol m^{-2} s^{-1}) and the radicle protrusion and shoot emergence was assessed weekly until all seeds had germinated or died, confirmed by cutting the seeds.

Influence of the pericarp and aril on seed survival and germination

To assess the role of pericarp and aril on seed survival and germination an experiment composed of three treatments was designed: 1) fresh intact diaspores (P+A+S); 2) seeds covered by aril upon pericarp removal (A+S); and 3) bare seeds upon removal of pericarp and aril (S). One hundred and twenty samples containing 60 dispersal units each (three treatments x four blocks x eight sampling times) were placed in permeable nylon bags and randomly distributed on the soil in the forest understory. Four bags of each treatment were taken monthly from the soil for 10 months to assess the number of germinated (radicle protrusion), dead and firm seeds, and WC (as described above) for all structures of the dispersal unit (pericarp, aril and seed). Firm seeds were placed in germination conditions in laboratory (as described above) to assess viability. Air temperature and humidity 5 cm above the soil were recorded continuously using a datalogger. The rainfall data of the experimental area were collected at the Universidade Federal de Lavras climatic station.

Histochemical characterisation of diaspore structures

Fresh pericarps, aril, seed coats, cotyledons and embryonic axis were hand sectioned using a razor blade, some sections of each structure were stained with safranin plus astra-blau [Safrablau (Bukatsch, 1972, modified by Kraus and Arduin, 1977)] and other sections of each structure were submitted to histochemistry tests according to Ventrella *et al*. (2013), using the solutions of Sudan IV for lipid localization; Lugol for starch localization; and Potassium Dichromate for phenolic compounds localization. Images were taken with a trinocular microscope (Primo Star, Carl Zeiss®) equipped with a digital camera (Axiocam Erc 5S).

Statistical analysis

The assays were plotted in a fully randomized design with missing plots, containing three treatments and four blocks. All the data were submitted to normality (Shapiro Wilk) and homoscedasticity (Bartlett) tests. Data for water content, germination, seedling formation, mortality and viability were submitted to Kruskal-Wallis test and a post-hoc comparison of pairs was performed using the Mann-Whitney test. For all the tests a 5% interval of confidence was applied. Additionally, a Spearman's correlation analysis was conducted using physiological (pericarp, aril and seed WC, germination, viability and mortality) and environmental parameters (temperature of litter and soil, soil WC, litter equilibrium RH and rainfall). Statistical analyses were conducted with the R 2.12.0 software (R Development Core Team, 2011).

RESULTS

Although *S. langsdorffii* is a recalcitrant seeded species dispersal occurs in the winter (August), which is a seasonally dry period in the region of the study. The rainy season starts in September becoming regular just in the second half of November, but even in the periods with less rainfall the relative humidity (RH) of the litter never fell bellow than 75%. Water content and temperature of the soil, and temperature of litter showed very little variation during the experimental period (Fig. 2).

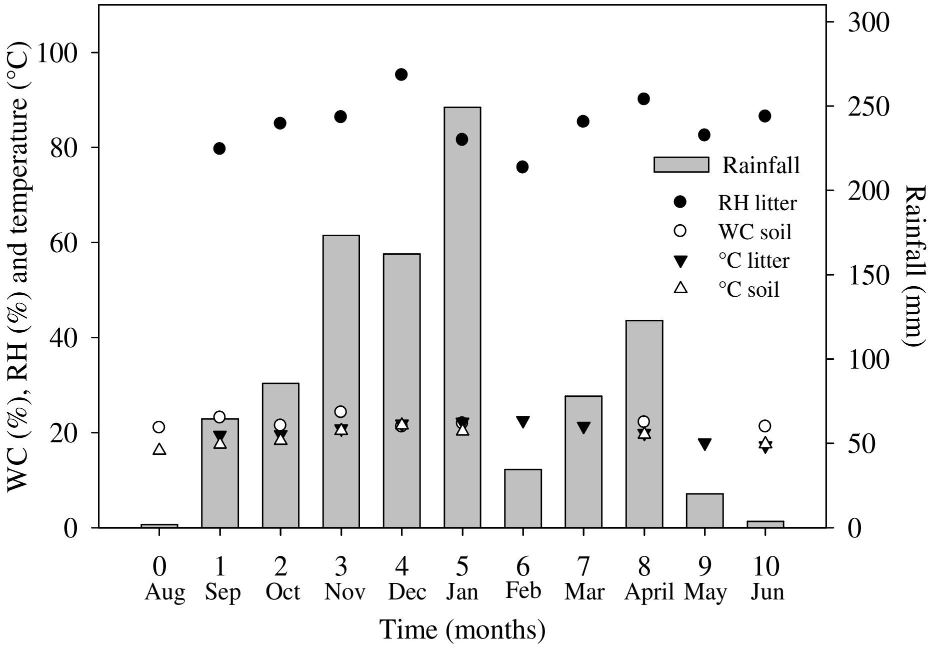

Fig. 2. Accumulated rainfall (mm), means of relative humidity of litter and soil (%), and temperature of litter and soil (°C) during the experimental period.

There were changes in the water content of different dispersal structures in time in the different treatments (Fig. 3A-C). The pericarp in treatment 1 (P+A+S) started to lose water after the beginning of the experiment, dropping from 83 to 38% at the end of the fifth month. The arils did not lose water but were totally consumed by small insects and microorganisms after the first month and the seed WC ranged from 47 to

56% during the experimental time without an obvious decrease (Fig. 3A). Arils from treatment 2 (A+S) were also consumed similar to treatment 1 (P+A+S), and the seed WC content ranged in a comparable way from 45 to 52%. Seeds from treatment 3 (S) presented a variation in the WC from 44 to 50% which was similar again. Considering only seed WC it was noticed that seeds of treatment 3 (S) lost a small amount of water from the second to the fourth month on the soil, but during the subsequent months the WC was equal to or higher than the WC at dispersal. Seeds from treatment 1 (P+A+S) and 2 (A+S) were WC equal to or higher than the WC at the time of seed dispersal. There was a WC increase only in seeds covered by at least one structure of the diaspore in the third month (Fig. 3D). It is worth to notice that in the three treatments the seed WC was never close to or below the critical point, that would result in a loss of 50% seed viability, as previously established by Vaz *et al*. (2016).

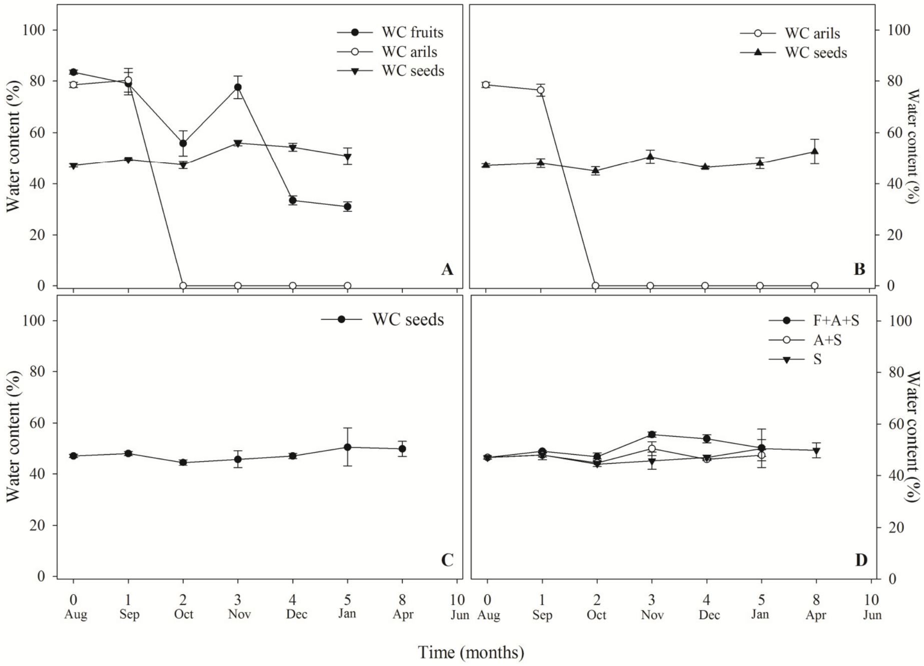

Fig. 3. Water content of *Swartzia langsdorffii* dispersal structures in each treatment along the time. A) Treatment 1 (P+A+S), B) Treatment 2 (A+S), C) Treatment 3 (S), and D) Seed water content in each treatment. (P = pericarp, A = aril, S = seed).

In all the three treatments, seed viability was not lost until the end of the rainy season. Regarding germination it was observed that seeds from treatment 1 (P+A+S) had higher germination percentages compared with treatments 2 (A+S) and 3 (S) until the fifth month. The same pattern was observed for the percentage of shoot emergence (Fig. 4). Although after the 5th month the mortality percentage had increased, seeds from treatment 2 (A+S) had higher germination and shoot emergence percentages than seeds from treatment 3 (S). In the tenth month, seeds from treatments 2 (A+S) and 3 (S) had all germinated or died, despite 1% still have remained viable (but without germinate in the field) in the treatment 3 (S) (Fig. 4B, C).

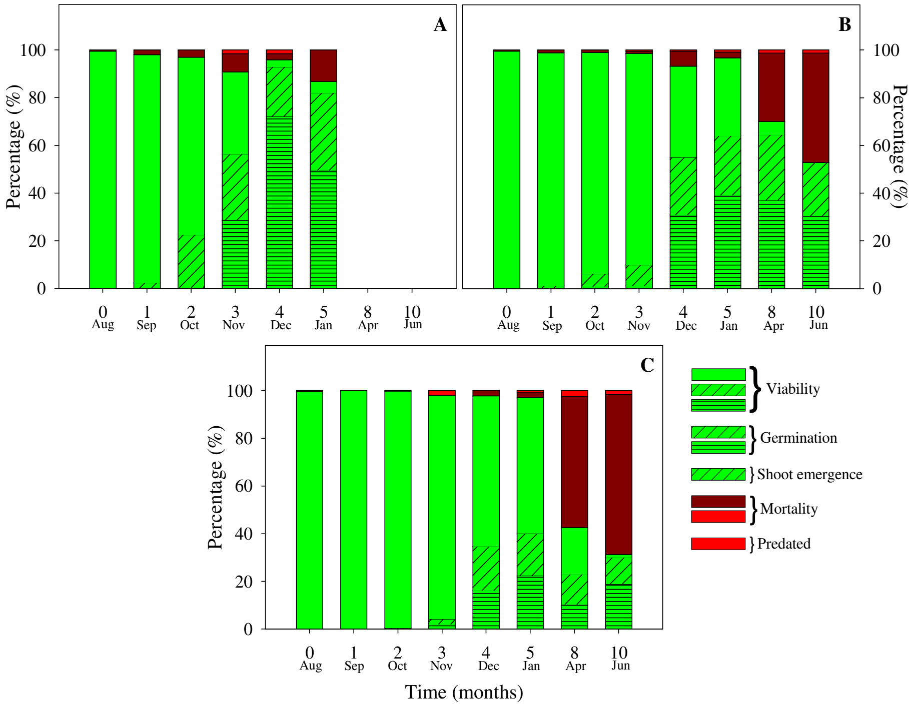

Fig. 4. Viability, germination, shoot percentage, mortality and predation of *Swartzia langsdorffii* seeds during the experimental period influenced by dispersal structures. A) Treatment 1 (P+A+S), B) Treatment 2 (A+S), and C) Treatment 3 (S). (A = aril, P = pericarp, S = seed).

Treatments 1 (P+A+S) and 2 (A+S) presented a higher germination percentage ($p<0.0229$) than treatment 3 (S) (Fig. 5A) none of the other evaluated parameters (i.e. shoot emergence, viability and mortality), differed statistically (Fig. 5B, C, D).

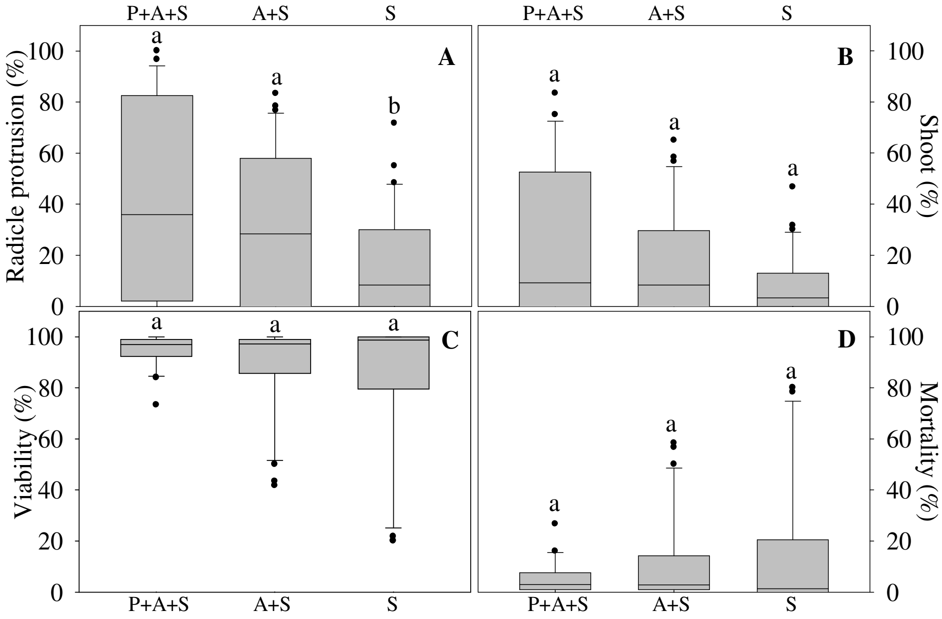

Fig. 5. Effect of different treatments on percentage of radicle protrusion (A), shoot emergence (B), viability (C), and mortality (D) of *Swartzia langsdorffii* seeds across sampling times. Boxes represent the interquartile range (IQR) between first and third quartiles and the solid line inside represents the median. (P = pericarp, A = aril, S = seed).

The months with highest germination and shoot emergence percentages were December and January (Fig. 6A, B) which are some of the months with higher rainfall in the year (Fig. 2), and after the ending of the rainy season seed viability decreased; with consequently a rise in seed mortality (Fig. 6C, D).

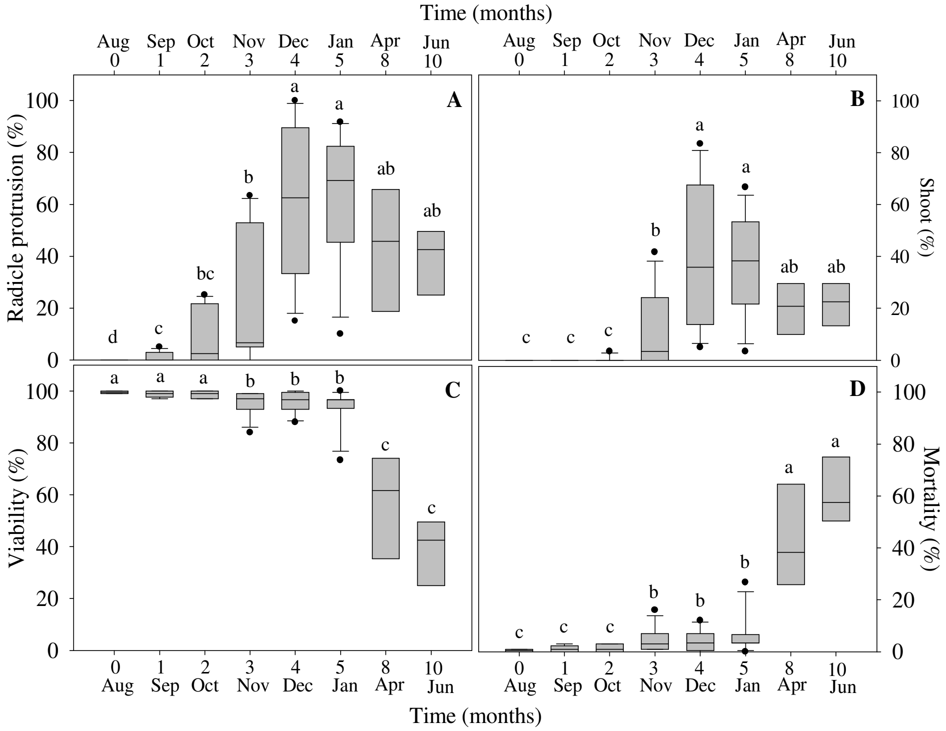

Fig. 6. Effect of time on percentage of radicle protrusion (A), shoot emergence (B), viability (C), and mortality (D) of *Swartzia langsdorffii* seeds across sampling times. Boxes represent the interquartile range (IQR) between first and third quartiles and the solid line inside represents the median.

Treatment 1 (P+A+S) had higher germination after two months (Oct) than treatment 3 (S), and a higher germination after three (Nov) and four (Dec) months than the both treatments; and treatment 2 (A+S) had higher germination after two and eigh months than the treatment 3 (S) (Fig. 7).

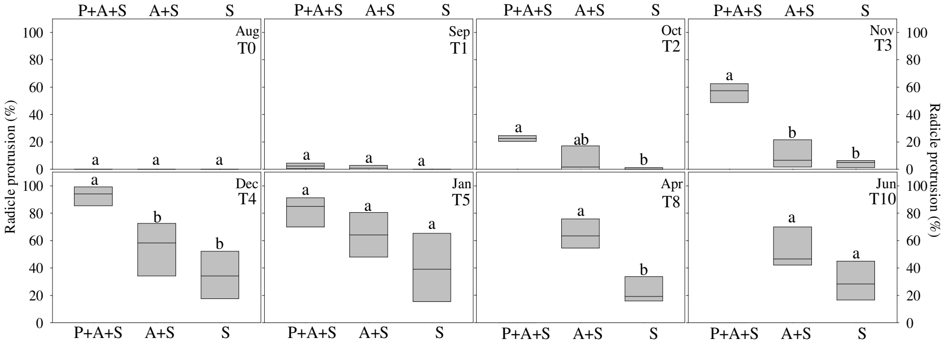

Fig. 7. Effect of the treatments in each month of field experiment on radicle protrusion of *Swartzia langsdorffii*. Boxes represent the interquartile range (IQR) between first and third quartiles and the solid line inside represents the median. (A = aril, P = pericarp, S = seed).

The correlation analysis conducted for the treatment 1 (P+A+S) (Table 1) found a positive correlation between seed WC and RH of litter (R=0.53), seed WC and soil WC (R=0.43), seed WC and soil temperature (R=0.72), seed WC and rainfall (R=0.63). Germination was strongly correlated with WC of pericarp (R=-0.85), with seed WC (R=0.62), with the temperature of the litter (R=0.90), with the UR of the litter (R=0.64), with soil WC (R=0.13), with the temperature of soil (R=0.89) and with the rainfall (R=0.83). Seed viability was correlated with the pericarp WC (R=0.56), with seed WC (R=-0.56), with temperature of the litter (R=-0.67), with soil WC (R=-0.50), with temperature of soil (R=-0.63) and with rainfall (R=-0.85). Seed mortality was correlated with pericarp WC

(R=-0.56), with seed WC (R=0.56), with temperature of the litter (R=0.67), with soil WC (R=0.50), with soil temperature (0.63) and with rainfall (R=0.85). Analyzing the data from treatment 2 (A+S), it was found correlation between seed WC and soil WC (R=0.42), germination and litter temperature (R=0.50), germination and soil temperature (R=45) and, germination and rainfall (R=0.48). For treatment 3 (S), it was found correlation between germination and seed WC (R=0.40), germination and litter temperature (R=0.45), germination and soil temperature (R=0.42) and germination and rainfall (R=0.45).

The thickening of cell walls of the ground meristem of cotyledons draws attention evidencing its pectic nature (data not shown) possible to observe using the Safrablau stain. The results for presence and absence of compounds based on the stains tested are found in Table 2.

Table 1. Spearman's correlation analysis among physiological features of *Swartzia langsdorffii* diaspore and environmental data along the field experimentation.

Treatment 1 – P+A+S								
	WC Pericarp	WC Aril	WC Seed	T °C litter	UR Litter	WC Soil	T °C Soil	Rainfall
WC Seed	R=-0.30 P=0.153	R=0.12 P=0.779	R=1.00 P=1.000	R=0.38 P=0.098	***R=0.53*** ***P=0.017***	***R=0.43*** ***P=0.034***	***R=0.72*** ***P<0.001***	***R=0.63*** ***P<0.001***
Germination	***R=-0.85*** ***P<0.001***	R=-0.25 P=0.558	***R=0.62*** ***P=0.001***	***R=0.90*** ***P<0.001***	***R=0.64*** ***P=0.002***	***R=0.13*** ***P<0.001***	***R=0.89*** ***P<0.001***	***R=0.83*** ***P<0.001***
Viability	***R=0.56*** ***P=0.004***	R=-0.62 P=0.103	***R=-0.56*** ***P=0.005***	***R=-0.67*** ***P=0.001***	R=-0.24 P=0.303	***R=-0.50*** ***P=0.012***	***R=-0.63*** ***P<0.001***	***R=-0.85*** ***P<0.001***
Mortality	***R=-0.56*** ***P=0.004***	R=0.62 P=0.103	***R=0.56*** ***P=0.005***	***R=0.67*** ***P=0.001***	R=0.24 P=0.303	***R=0.50*** ***P=0.012***	***R=0.63*** ***P<0.001***	***R=0.85*** ***P<0.001***
Treatment 2 – A+S								
	WC Pericarp	WC Aril	WC Seed	T °C litter	UR Litter	WC Soil	T °C Soil	Rainfall
WC Seed	-	R=0.29 P=0.493	R=1.00 P=1.000	R=0.09 P=0.682	R=-0.19 P=0.364	***R=0.42*** ***P=0.025***	R=0.09 P=0.663	R=0.25 P=0.192
Germination	-	R=-0.51 P=0.192	R=0.18 P=0.364	***R=0.50*** ***P=0.006***	R=0.01 P=0.973	R=-0.10 P=0.593	***R=0.45*** ***P=0.010***	***R=0.48*** ***P=0.006***
Viability	-	R=0.33 P=0.431	R=-0.21 P=0.273	R=0.02 P=0.937	R=-0.06 P=0.774	R=0.23 P=0.200	R=-0.08 P=0.646	R=-0.09 P=0.623
Mortality	-	R=-0.33 P=0.431	R=0.21 P=0.273	R=-0.02 P=0.937	R=0.06 P=0.774	R=-0.23 P=0.200	R=0.08 P=0.646	R=0.09 P=0.623
Treatment 3 – S								
	WC Pericarp	WC Aril	WC Seed	T °C litter	UR Litter	WC Soil	T °C Soil	Rainfall
WC Seed	-	-	R=1.00 P=1.000	R=0.26 P=0.212	R=-0.38 P=0.064	R=0.13 P=0.518	R=-0.02 P=0.936	R=0.08 P=0.679
Germination	-	-	***R=0.40*** ***P=0.033***	***R=0.45*** ***P=0.016***	R=0.12 P=0.536	R=-0.14 P=0.456	***R=0.42*** ***P=0.015***	***R=0.45*** ***P=0.010***
Viability	-	-	R=-0.27 P=0.159	R=-0.05 P=0.811	R=0.06 P=0.763	R=0.08 P=0.652	R=-0.04 P=0.833	R=-0.17 P=0.343
Mortality	-	-	R=0.27 P=0.159	R=0.05 P=0.811	R=-0.06 P=0.763	R=-0.08 P=0.652	R=0.04 P=0.833	R=0.17 P=0.343

Statistically significant (at $P < 0.05$) results are shown in bold type.

Table 2. Histochemical analysis of *Swartzia langsdorffii* pericarps, arils, cotyledons, embryonic axis and seed coats. The signs (+) mean the respective compound occur in the structure and (-) the compound does not occur in the structure.

	Pericarp	Aril	Seed coat	Cotyledons	Embryonic axis
Sudan IV	+*	+	-	+	+
Lugol	-*	-	-	+	+
Potassium dichromate	+*	-	+	-	+
Phloroglucinol-HCl	+*	-	+	+	+

*It was also tested by Colpas and Oliveira (2003).

It was evidenced by Sudan IV stain that a thick cuticle is present in the exocarp (external layer of the pericarp), and in all over the mesocarp lipid droplets are found dispersed in the cytoplasm (Fig. 8 A). The Potassium Dichromate stain revealed the presence of phenolic compounds stored in the phenolic idioblasts distributed throughout the mesocarp (Fig. 8 B). In the aril tissues this stain evidenced great amounts of lipid drops in the parenchyma cells cytoplasm (Fig. 8 C). A strong presence of phenolic compounds is found in the seed coat (Fig. 8 D) which has lignified cell walls. The cotyledons of *S. langsdorffii* are voluminous and present numerous starch grains stained by Lugol (Fig. 8 E). Starch is also observed in embryonic axis however in lower amount. In the embryonic axis was possible to observe a thin cuticle in the protoderm, however, a thickening in the cell was observed with accumulation of lipid compounds (possible cutin) in both the periclinal walls (Fig. 8 G). In the cotyledons this pattern was observed just in the external periclinal wall (Fig. 8 F). The positive reaction to Acid floroglucin was seen in the tracheal elements of xylem present in the pericarp, seed coat (in the vascular bundles of the hilum) and in the mesophyll wall, cotyledons and embryonic axis (data not shown).

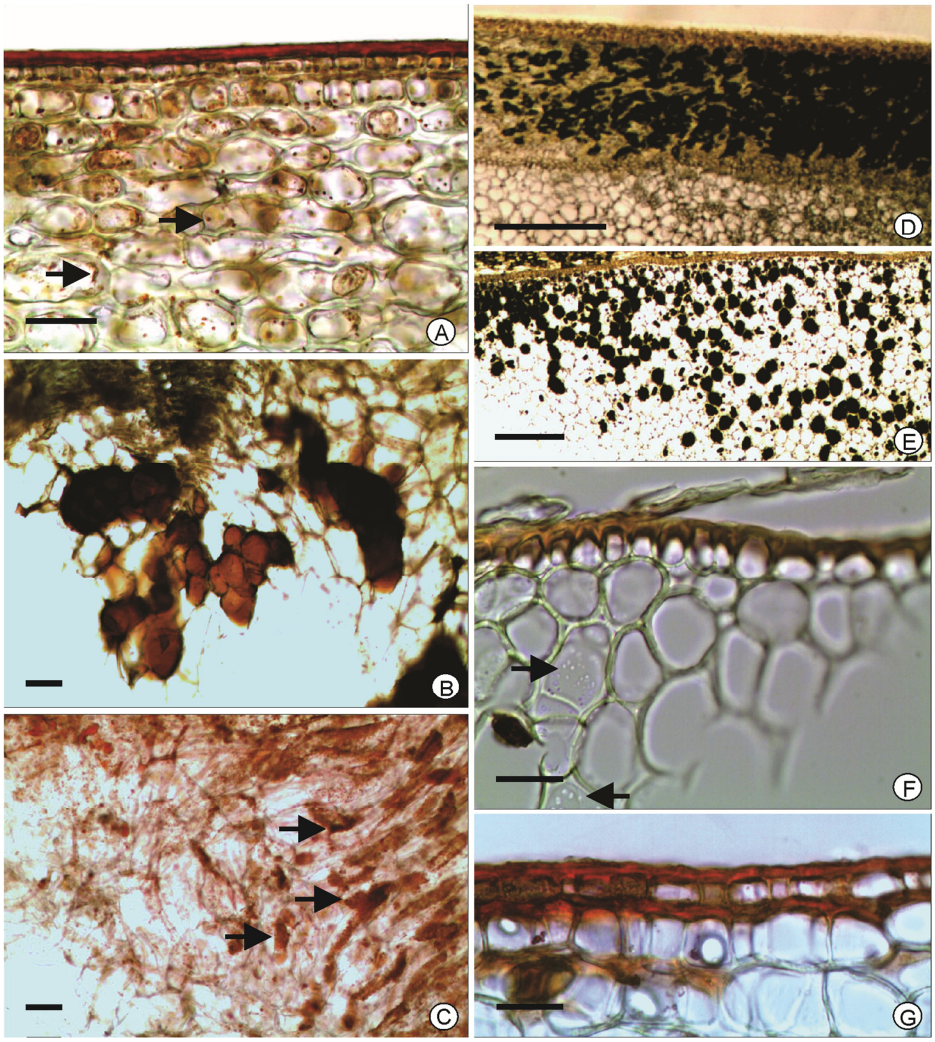

Fig. 8. Histochemical analysis of *Swartzia langsdorffii* pericarp, aril and seed. A) Exocarp presenting a thick cuticle layer and mesocarp fulfilled by lipid droplets (arrows). B) Phenolic idioblast fulfilled by resin. C) Aril presenting large amount of lipid drops (arrows). D) Phenolic compounds in the seed coat. E) Starch granules in the cotyledons. F) Cotyledons protoderm presenting a thickening in the external periclinal cell wall of cutin, with arrows indicating the primary pit fields. G) Thickening of cutin in the external and internal periclinal cell wall of embrionic axis.

DISCUSSION

Diaspore morphology and anatomy acting in seed water loss restriction

It was demonstrated that seeds surrounded by pericarp and/or aril do not lose water after dispersal (Fig. 3). One of the common traits of desiccation sensitive seeds is the presence of moist structures surrounding the seeds (Farnsworth, 2000), but despite this trait be present in *S. langsdorffii* diaspore, the seed itself is able to prevent water loss. An important feature which could aid to water loss restriction is the accumulation of lipid compounds in the thickened periclinal cell wall of seed protoderm, allegedly protecting it against desiccation. Exocarp also has an extra protection consisted by a very thick cuticle (Fig. 8) but when the dehiscence occur (after dispersal) the water loss cannot be avoided anymore.

Lipid layers are known by their function in the water loss restriction in plant leafs (Riederer and Schreiber, 2001; Taiz and Zeiger, 2013) and aiding in preventing water uptake in physically dormant seeds (Rangaswamy and Nandakumar, 1985; Egley, 1989; Kelly *et al.*, 1992; Zeng *et al.*, 2005). It is remarkable that bare seeds of *S. langsdorffii* do not lost significant amount of water during all the experimental time, probably owing to the lipid thickening in the cell wall of protoderm (Fig. 8), additionally to its large size and rounded shape, and the high RH near to the litter. Joët *et al*. (2013) stated that water loss before germination is the main problem for *Quercus ilex* establishment, unlike what happens in *S. langsdorffii*, which seems the main problem is the lack of additional water, since bare seeds do not lose water after dispersal but do not hold its viability as well.

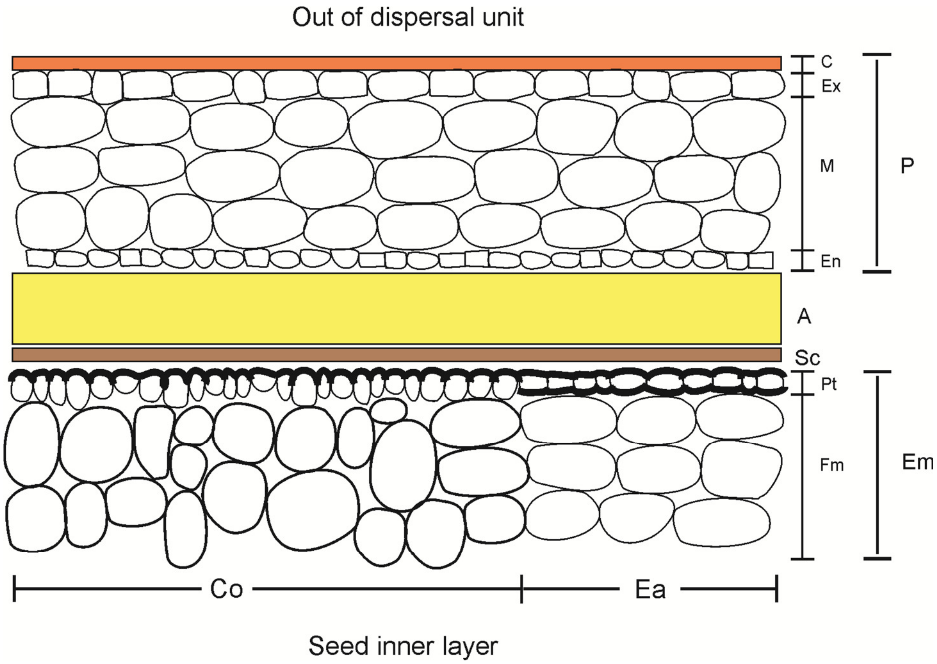

Fig. 9. Schematic diagram of transversal section of *Swartzia langsdorffii* diaspore. (A = aril, C = cuticle, Co = cotyledon, Ea = embryonic axis, Em = embryo, En = endocarp, Ex = exocarp, Fm = fundamental meristem, M = mesocarp, P = pericarp, Pt = protoderm, Sc = seedcoat).

Effect of the pericarp and aril on seed viability and germination

When seeds are still inside of the pericarp [treatment 1 (P+A+S)], or surrounded by the aril [treatment 2 (A+S)] its water content is always equal or higher than the bare seeds [treatment 3 (S)]. Further the water loss restriction, the pericarp and aril aid seeds to increase its water content during the months which they are germinating, even the aril surrounding the seeds only for one month (Fig. 3 D). It was seen in germination response, seed germination until the fourth month was significantly higher in treatment 1 (P+A+S) comparing to the other treatments (Figs 4 and 7). Regarding the aril actuation, the higher germination in the treatment 2 (A+S) at the eighth month (Fig. 7) could be related with a trait of aril (also the pericarp tissues when present), which even decomposed, could affect on soil water retention capacity with its residual structure. The seed role in soil water retention capacity has been proved to the myxospermous seeds of *Capsella bursa-pastoris,* wherein the seed coat mucilage can improve the soil water retention capacity especially in drier conditions (Deng *et al*., 2015). Probably, when the rainfall decrease in the months eighth and tenth (Fig. 2), this capacity of keep the soil moistened makes the difference among these treatments. Seed viability have not differed among the three treatments until the fifth month and also did not differ between treatment 2 (P+A+S) and 3 (S) in the eighth and tenth months, even then, seeds from treatment 2 have higher viability and germination, showing that pericarp and aril have a beneficial effect in the seeds after dispersal. As described by Vaz *et al*. (2016), *S. langsdorffii* pericarps remain intact in the soil for about three months after dispersal and after that, only the part in contact with soil experience deterioration and the arils are consumed by small animals from the first month (as also shown in Fig. 3). The role of the pericarp controling water loss is described in the literature (Sobrino-Vesperinas and Viviani, 2000; Chacón and Bustamante, 2001) as also controlling water uptake (Xia *et al*., 2012; Puglia *et al*., 2015), but in this study the pericarp and aril wholes go beyond that, acting as water improvement as well. In the *S. langsdorffii* case it is possible that the pericarp and aril tissues could incorporate in the soil after its deterioration as mentioned above, and the fibres and other compounds could increase soil water retention locally, just around the seeds, causing the higher water content and germination in seeds from treatment 1 (P+A+S) and, in a smaller scale, treatment 2 (A+S). As it was not noted before, the soil samples for water content measurement were taken from up to 5 cm depth, and was not possible to detect this possible variations in moisture caused by pericarps and arils.

Considering the correlation analysis it is very clear that the intact diaspore plays an important role on seed germination, being the responsible for seed response to the environmental conditions. In the treatment 1 (P+A+S) was found 17 significant correlation with the environmental parameters, while in treatment 2 (A+S) and 3 (S) was found just 4 and 3 significant correlations, respectively. Seed WC and the environmental parameters such as temperature and UR of litter, temperature and WC of soil, and the rainfall were essential for seed germination. All these factors were also reported important for *Quercus ilex* seed viability and germination after dispersal by Joët *et al.* (2015). Interestingly, germination had shown a strong negative correlation with pericarp WC (R=-0.85) maybe it is related to some germination inhibition compound found in the pericarps that need to be metabolized to allow germination, but it need to be better studied before any conclusion.

An interesting threshold was found for seed viability in the treatment 1 (P+A+S), despite the pericarp has a positive influence on this factor, the seed WC and viability have shown a negative correlation, also a negative correlation was found for the temperatures of litter and soil, WC of the soil and rainfall. Recalcitrant seeds do not tolerate water loss (Roberts, 1973) but it seems that too much moisture is not so beneficial for seed viability as well. It is important to highlight that without the pericarp, *S. langsdorffii* seeds loss its capability for respond or correlate with the environmental factors, showing weak correlations among seed WC and germination with the temperature of litter, WC and temperature of soil and rainfall.

What are the implications for S. langsdorffii seed dispersal?

Martins *et al*., (2014) stated that *S. langsdorffii* diaspores have characteristics for be dispersed by mammals, but as mentioned before, these diaspores are rich in secondary compounds (tannins and phenolic compounds) and saponins. Neither in the work of Vaz *et al*. (2016) nor this work was found significant seed predation (when it happened, seeds were partially bitten not all consumed) or, at least, diaspores removal from the experimental site. Perhaps the diaspore chemical composition is not suitable for animal consumption, since this could influence the physiological or metabolic cost for seed processing by animals (Cipollini and Levey, 1997). *S. langsdorffii* dispersal can feat to the

Attraction/Repulsion hypothesis, where the odour and colour attract dispersers but its composition repels them before consumption (Cipollini and Levey, 1997; Cipollini, 2000) or to the Extinct Megafauna, due diaspore morphology and the apparently absence of dispersers (Janzen and Martin, 1982; Guimarães *et al.*, 2008). It is strongly recommended the design of a work specifically for understand the possible dispersal syndrome of *S. langsdorffii* considering field observations and the species population structure and distribution. An important point is that, analysing the results of this work, whether an animal consume the pericarp or aril tissues it has no beneficial effect for seed establishment, since seeds removed from pericarps have lower germination percentages.

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