

Free amino acids, polyamines, soluble sugars and proteins during seed germination and early seedling growth of *Cedrela fissilis* Vellozo (Meliaceae), an endangered hardwood species from the Atlantic Forest in Brazil

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Abstract Germination, one of the earliest events in the plant life cycle, is a complex process in which the seed must physically recover from maturation drying, resume a sustained metabolic intensity, complete essential cellular events that allow for embryo emergence and prepare for subsequent seedling growth. Among the biochemical changes, compounds such as amino acids, proteins, soluble sugars and polyamines (PAs) presented relevant roles during seed development and germination. The aim of this work was to study the alterations in the content of free amino acids, PAs, soluble sugars and proteins during germination of *Cedrela fissilis* Vellozo (Meliaceae). The content of amino acids, soluble sugars and PAs were determined by high-performance liquid chromatography, and the

soluble protein in ethanol content was quantified using a 2-D Quant Kit. A triphasic pattern of germination was observed, and germination was completed with radicle protrusion on day seven of incubation. A significant decrease in the soluble proteins in ethanol and increase in the total free amino acid content during germination (5–7 days) suggests that amino acids might be provided by the mobilization of stored proteins in mature seeds. Among soluble sugars, sucrose presented the highest content in mature seeds decreasing significantly during germination, whereas glucose and fructose were only detected in seedlings, suggesting that the degradation of sucrose to these monosaccharides is important for seedling growth. Endogenous free spermidine (Spd) and spermine (Spm) are significantly mobilized during germination, while the PA ratio [Put/(Spd + Spm)] significantly increased in seedlings, due to the significant increase in putrescine (Put) contents, which is involved with cell division for seedling growth. Our results revealed changes in the contents and forms of the studied compounds, suggesting the involvement of these biomolecules in *C. fissilis* seed germination and early seedling growth. The results provide additional data on the biochemical and physiological changes that occur during seed germination, particularly in endangered hardwood species from Brazilian Atlantic Forest.

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Abbreviations

ABA	Abscisic acid
ANOVA	Analysis of variance
DAO	Diamine oxidase
DM	Dry matter
FM	Fresh matter
GABA	γ -Aminobutyric acid
HPLC	High performance liquid chromatography
OPA	o-Phthaldialdehyde
PA	Polyamine
Put	Putrescine
Spd	Spermidine
Spm	Spermine
WC	Water content

1 Introduction

Seed germination is one of the earliest events in the plant life cycle, and the transition from seed to seedling is one of the most drastic developmental processes in plants. Plants change from heterotrophic to autotrophic and from quiescent to active, and although they are initially protected within a seed coat, they are then exposed to ambient conditions (Donohue et al. 2010). Germination is a complex process in which the seed is physically capable to recover from drying, take up a sustained metabolic intensity, complete essential cellular events for the embryo emergence and, overall, adjust the whole metabolism for subsequent seedling growth (Obroucheva et al. 2006; Nonogaki et al. 2010).

To achieve germination, the seed employs three main phases: imbibition (phase I), germination sensu stricto (true germination process, phase II), and seedling growth (phase III) (Bewley 1997). Germination is considered complete when the radicle protrudes from the covering structures. Water uptake results in the resumption of respiratory activity and protein synthesis using extant mRNAs (phase I). Phase II is the most important phase, and it is associated with several cellular and biochemical events, including DNA repair and protein synthesis, the latter of which depends on the translation of de novo-synthesized RNA, as well as changes in the soluble sugar content (Bewley 1997).

Following the imbibition, in the dry seed, the reinitiation of metabolism and reestablishment of

chemicals and cellular structural integrity require the integration of events involved in synthesis and protection. Protein synthesis and respiration initially involve components stored within the mature dry seed, although transcription and translation commence early in imbibition (Nonogaki et al. 2010). Alterations or modifications of hormones, especially abscisic acid (ABA) and gibberellin, play an important role during germination (Nonogaki et al. 2010). In woody species, compounds such as amino acids, proteins, carbohydrates and polyamines (PAs) are also important during seed development and germination (Astarita et al. 2003; Santa-Catarina et al. 2006; Dias et al. 2009; Balbuena et al. 2009; Pieruzzi et al. 2011).

Proteins that were synthesized and stored during seed maturation are degraded into free amino acids for biosynthesis and energy generation (Onomo et al. 2010; Alhadi et al. 2012; Tan-Wilson and Wilson 2012). Seed storage proteins can be divided into four classes based on their solubility. Albumins are water soluble, globulins are salt soluble, prolamines are soluble in ethanol solutions, and glutelins are soluble in alkali or acid (Shotwell and Larkins 1989; Krishnan and White 1995). Prolamines are stored in the form of polymers or aggregates, referred to as protein bodies (Mitsukawa et al. 1999). In *Oryza sativa*, these proteins are accumulated during endosperm development and further utilized as a source of nitrogen, carbon, and sulfur for the young developing seedling (Kim and Okita 1988). In woody species, these proteins have been mobilized during the germinative process (Dantas et al. 2008). The mobilization of seed storage proteins following seed imbibition (phase I) and germination (phase II) is crucial for the establishment of seedlings (phase III). In certain species, such as *Ocotea catharinensis*, alterations in the amino acid content during seed development and germination have been observed (Santa-Catarina et al. 2006; Dias et al. 2009). These results suggest the involvement of amino acids in the synthesis of proteins such as late embryogenesis abundant (LEA) proteins, which are important for desiccation tolerance (Santa-Catarina et al. 2006).

Carbohydrates are involved in cross-talk with hormone signaling networks to modulate critical growth processes, such as embryo establishment, seed germination and seedling growth (see Eveland and Jackson 2012). During these processes, carbohydrates act as substrates for intermediary metabolism,

supplying the energy necessary for initial growth and seedling development (Peterbauer and Richter 2001; Tahir et al. 2011), or they act as signaling molecules (Smeekens et al. 2010). The mobilization of carbohydrates during germination is highly dynamic (Ferreira et al. 2009; Carrijo et al. 2010), and alterations in the profiles of these compounds have been observed in mature seeds and during germination (Borek et al. 2006).

In plants, the PAs putrescine (Put), spermidine (Spd), and spermine (Spm) act as regulatory molecules in several cellular processes, including cell division, cell differentiation and proliferation, cell death, DNA and protein synthesis and gene expression (Boucher-eau et al. 1999; Kusano et al. 2008; Álcazar et al. 2010; Cai et al. 2015). These PAs have been implicated in abiotic and biotic plant stress responses as well as many physiological processes (Baron and Stasolla 2008; Kusano et al. 2008; Álcazar et al. 2010), including seed development and germination (Astarita et al. 2003; Santa-Catarina et al. 2006; Dias et al. 2009; Balbuena et al. 2009; Pieruzzi et al. 2011; Krasuska et al. 2014).

Cedrela fissilis Vellozo is a native woody species in the Atlantic Forest. Because of its economic importance, especially for wood production, this species has been heavily exploited and is currently included in the endangered category of the Red List of Threatened Species published by the International Union for Conservation of Nature (2014). *C. fissilis* seeds are considered orthodox (Carvalho et al. 2006), although their reduced viability during storage has been problematic for seed conservation and reforestation program development (Carvalho 2003). Therefore, studies on the biochemical aspects of seed development and germination could contribute to the conservation of this endangered species.

In this present work, the contents of amino acids, soluble proteins in ethanol, soluble sugars and PAs during germination and initial growth seedling in *C. fissilis* were analyzed, and the relationship between these compounds during germination was studied. The results illustrate the physiological changes that occur during the germination process in this species, and the data provided here will be useful for monitoring seed germination during storage and improve research on the somatic embryogenesis of woody species for use in conservation programs.

2 Materials and methods

2.1 Seed germination

Mature seeds of *C. fissilis* Vellozo were obtained in October 2011 from the Sementes Caiçara Nursery, which is located in Brejo Alegre, Sao Paulo State, Brazil (21°10'S and 50°10'W). Prior to imbibition, the seeds were surface-disinfected according to the method of Santa-Catarina et al. (2001). The seeds were washed with 250 ml distilled water and then immersed in 70 % (v/v) ethanol for 1 min and commercial bleach Qboa® (Anhembi SA, Osasco, Brazil) containing 2.0–2.5 % active chlorine supplemented with the fungicide Derosal® (Bayer, São Paulo, Brazil) (200 µl Derosal® per 1000 ml solution) for 30 min. The seeds were subsequently washed five times with distilled and autoclaved water and then placed in Petri dishes containing two layers of germibox paper with 10 ml distilled and autoclaved water containing Derosal® (200 µl Derosal®/1000 ml solution). The seeds were incubated in a growth chamber with a photoperiod of 16 h, light intensity of 22 µmol m² s⁻¹, and temperature of 25 ± 2 °C. The percentage of germination was obtained after 20 days from seven replicates with 10 seeds per replicate.

The germination phase establishment and sampling times were defined in previous assays. Germination process was monitored daily over 7 days and growth seedling observed until the 20th day. For the biochemical analyses, triplicate seed samples were collected prior (mature seed—time 0) and at 2, 5, 7, 10 and 17 days of imbibition (Fig. 1a). Each triplicate sample was obtained from a bulk sample of at least 100 seeds. The seed tegument was removed, and samples consisting of 200 mg fresh mass (FM) were collected for amino acid, protein and PA analyses, and samples consisting of 300 mg FM were collected for carbohydrate analyses. The samples were stored at -20 °C until further biochemical analysis.

The water content (WC) during germination was analyzed by evaluating the FM and dry mass (DM) of the seeds prior (mature seed) and at 2, 5, 7, 10 and 17 days after imbibition. For DM analyses, the seeds were weighed and maintained at 105 °C for 24 h. The WC was determined using the following formula: WC = FM - DM. Seven replicates with 10 seeds per replicate were used for each analysis.

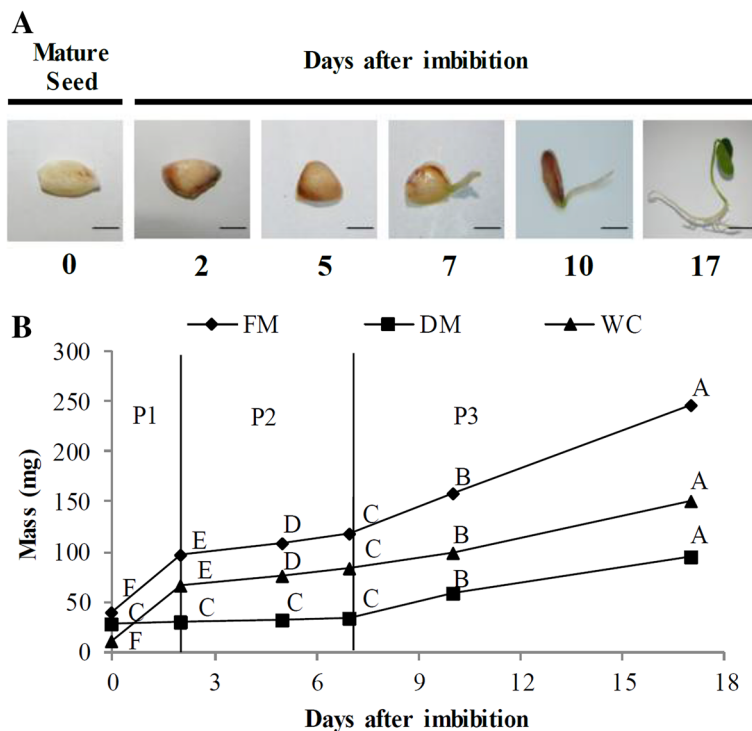


Fig. 1 Morphological aspects of *C. fissilis* seeds during germination and early seedling development (a) and imbibition curve after 2, 5, 7, 10 and 17 days of imbibition (b). P1 phase I, characterized by the rapid absorption of water and reactivation of metabolism; P2 phase II, characterized by slow water absorption and completed with radicle protrusion; P3 phase III (final phase), characterized by continuous water absorption and seedling

growth. DM dry mass, FM fresh mass, and WC water content. Capital letters denote significant differences in each analysis during the days of imbibition. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test ($n = 7$; CV FM = 4.6 %; CV DM = 8.5 %; CV WC = 7.0 %). CV coefficient of variation. Bars in a = 0.5 cm

2.2 Free amino acids and soluble proteins in ethanol determination

The amino acid content was analyzed according to the method of Santa-Catarina et al. (2006). Amino acid samples were prepared in biological triplicate and separated using high-performance liquid chromatography (HPLC) on a reversed-phase C18 column (Shim-pack CLC ODS, Shimadzu Corporation, Kyoto, Japan). The peak areas and retention times of each amino acid were evaluated through comparisons with known concentrations of aspartic acid, glutamic acid, asparagine, serine, glutamine, histidine, glycine, arginine, threonine, alanine, γ -aminobutyric acid (GABA), tyrosine, methionine, tryptophan, valine, phenylalanine, isoleucine, leucine, ornithine and lysine.

The contents of soluble proteins in ethanol were quantified using ethanolic extracts from the amino acid extractions and performed using the 2-D Quant

Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's recommendations.

2.3 Soluble sugars determination

The soluble sugars content was determined according to the method of Filson and Dawson-Andoh (2009). The samples (300 mg FM) were ground in 1 ml extraction solution containing 80 % (v/v) ethanol (Merck), 3 % (m/v) polyvinylpyrrolidone (Sigma-Aldrich) and 1 % (m/v) ascorbic acid (Sigma-Aldrich) at 4 °C. The extracts were then incubated at 70 °C for 90 min. Following centrifugation at $20,000 \times g$ for 10 min, the supernatant was removed, and the pellets were then re-extracted with 1 ml extraction solution and centrifuged again at $20,000 \times g$ for 10 min. The supernatants were combined and filtered through a 20 μ m membrane, and the carbohydrate content was identified using HPLC

(Shimadzu) with an evaporative light scattering detector (ELSD-LT II—Shimadzu) at a temperature of 40 °C, nitrogen gas pressure of 350 MPa, gain of 9 and a filter setting of 4. A Prevail Carbohydrate ES (Alltech Associates, Deerfield, IL, USA) 5 μm (250 \times 4.6 mm) HPLC column and Prevail Carbohydrate ES (Alltech Associates) 5 μm (7.5 \times 4.6 mm) pre-column were used. The gradient was achieved by mixing decreasing proportions of absolute acetonitrile (Merck) with water. The acetonitrile gradient was programmed as follows: 80 % during the first 16 min, 80–70 % between 16 and 23 min and 70 % from 23 to 30 min. The flow rate was 1 ml min⁻¹ at 25 °C. A 5 μl sample was injected, and the peak areas and retention times were measured by comparison with known quantities of carbohydrate standards containing sucrose, fructose, and glucose (Sigma-Aldrich).

2.4 Free polyamine (PA) determination

The free PA content was determined according to the method of Santa-Catarina et al. (2006). PA samples were prepared in biological triplicate. PAs were separated by HPLC using a reversed-phase C18 column (Shim-pack CLC ODS, Shimadzu Corporation, Kyoto, Japan), and the peak areas and retention times of each PA were evaluated by comparison with known concentrations of Put, Spd and Spm, which are the major PAs found in plants.

2.5 Statistical analyses

The data were analyzed by an analysis of variance (ANOVA) ($P < 0.05$) followed by the Student–Newman–Keuls (SNK) test (Sokal and Rohlf 1995) using the software program SAEG[®] v.9.1 (Fundação Arthur Bernardes, Viçosa, Brazil).

3 Results

3.1 Germination

During *C. fissilis* seed imbibition, a triphasic development pattern was observed (Fig. 1b). The first phase (P I) began at the mature seed and lasted until the second day of imbibition when rapid water absorption occurred. The second phase (P II) began on day two and was characterized by a reduction and stabilization of water

absorption, and it was completed on day seven of incubation when radicle protrusion occurred. The third phase (P III) began after day seven and was characterized by continued water absorption and post-germinative events. During imbibition, the FM showed a pattern similar to that of the WC with a significant increase and higher values on the 17th day of seedling development (Fig. 1b). The DM value remained constant until day seven (Fig. 1b), and then a significant increase occurred. The germination percentage obtained in conditions of this experiment was 83 %.

3.2 Free amino acids and soluble proteins in ethanol

In *C. fissilis*, a significant increase in the total free amino acid content (Fig. 2) was observed on the 2nd day of imbibition (phase I) and similar values occurred during radicle protrusion (phase II) until the 10th day of imbibition. Moreover, a significant decrease in the total free amino acids occurred during seedling development in *C. fissilis* on the 17th day of imbibition.

Among the analyzed amino acids, threonine was predominant during *C. fissilis* seed germination and early seedling growth (Table 1), and the contents of citrulline, glycine, lysine and ornithine were higher in the mature seed than at the onset of imbibition (Table 1). However, certain amino acids, such as aspartic acid, glutamic acid,

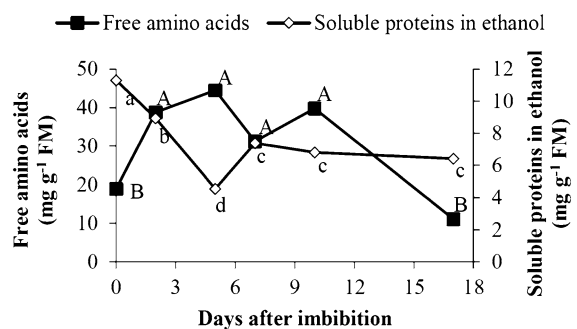


Fig. 2 Free amino acid contents (mg g⁻¹ FM) and soluble proteins in ethanol (mg g⁻¹ FM) in seeds of *C. fissilis* before (mature seed—time 0) and after 2, 5, 7, 10 and 17 days of imbibition. Lower-case letters denote significant differences in the soluble proteins in ethanol contents during the days of imbibition. Capital letters denote significant differences in the total free amino acid contents during the days of imbibition. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test ($n = 3$; CV total free amino acids = 20.2 %; CV soluble proteins in ethanol = 6.8 %, CV coefficient of variation)

Table 1 Free amino acid contents ($\mu\text{g g}^{-1}$ FM) in seeds of *C. fissilis* before (mature seed—time 0) and after 2, 5, 7, 10 and 17 days of imbibition

Amino acids	Days after imbibition						CV (%)
	0	2	5	7	10	17	
Aspartic acid	39.73CD ^a	163.33B	86.80C	5.13D	80.29C	464.32A	15.4
Glutamic acid	644.04B	502.13BC	399.84C	187.37D	597.90BC	2546.50A	12.0
Citrulline	5991.33A	2286.73B	2179.83B	113.23C	1967.38B	661.12C	21.7
Asparagine	142.70C	414.98B	110.72C	5.73D	112.33C	991.20A	11.9
Serine	41.87E	81.65D	148.53C	10.18E	219.85B	748.27A	9.1
Glutamine	14.46C	143.60C	153.84C	154.50C	407.70B	1841.41A	10.5
Histidine	18.15D	12.03D	29.48C	17.38CD	48.43B	228.58A	9.8
Glycine	2500.36A	169.04B	160.45B	53.26B	85.12B	152.80B	46.9
Arginine	308.42C	348.01BC	264.90C	77.11D	384.71B	897.87A	7.8
Threonine	8953.24B	34140.43A	40585.98A	30559.95A	35436.51A	1632.53B	23.9
Alanine	2.13D	10.40D	110.49B	3.70D	69.27C	197.08A	15.8
Gaba	26.20C	46.85C	67.76B	3.69C	94.35B	271.00A	27.8
Tyrosine	120.67B	244.22A	0C	0C	0C	0C	25.8
Tryptophan + methionine	9.11D	24.42C	18.18C	0.68E	34.93B	106.99A	12.6
Valine	4.26D	29.49C	44.04C	2.76D	67.27B	196.44A	15.2
Phenylalanine	8.81D	22.95C	26.86C	6.97D	39.60B	84.05A	10.9
Isoleucine	1.47E	9.42D	16.26C	3.09E	22.54B	59.05A	17.5
Leucine	2.41D	29.64C	48.89C	6.73D	74.66B	148.86A	20.7
Ornithine	65.21A	25.95C	24.62C	5.78D	20.62C	39.51B	16.5
Lysine	65.49A	32.73B	25.99B	36.78B	28.12B	45.14B	23.8

CV coefficient of variation

^a Capital letters denote significant differences for each amino acid throughout the days of imbibition. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test ($n = 3$)

glutamine, arginine, asparagine, serine, GABA, alanine, histidine, valine, leucine, phenylalanine and isoleucine, were significantly higher in the seedlings of *C. fissilis* (phase III) compared with the mature seed and phases I and II (Table 1). Among the amino acids, asparagine, aspartic acid, serine, GABA, valine, phenylalanine, leucine, isoleucine and alanine were observed in significant reductions on the 7th day during radicle protrusion followed by a significant increase during early seedling development on the 17th day (Table 1). The glutamine content did not change significantly during the imbibition phase (phase I), but an increase occurred after radicle protrusion, reaching the highest value on the 17th day of imbibition (Table 1).

A significant reduction was observed in the content of soluble proteins in ethanol content until 5th day, prior to radicle protrusion, and an increase occurred on the 7th day during radicle protrusion, with similar values maintained during early seedling growth (Fig. 2).

3.3 Soluble sugars contents

The soluble sugars sucrose, fructose and glucose were analyzed during *C. fissilis* germination and early growth seedling (Fig. 3). The higher sucrose content (Fig. 3a) was observed in mature *C. fissilis* seeds before imbibition; this value decreased significantly during germination (phases I and II) and early seedling development (phase III). However, fructose and glucose were only detected in *C. fissilis* after germination, from 10 to 17 days, during early seedling development.

3.4 Free polyamines (PAs) contents

The free PAs Put, Spd and Spm were identified in germination and early growth seedling in *C. fissilis* (Fig. 4a–c). The Spd contents decreased significantly during imbibition (phase I), maintained a similar content until seed germination on the 7th day (phase

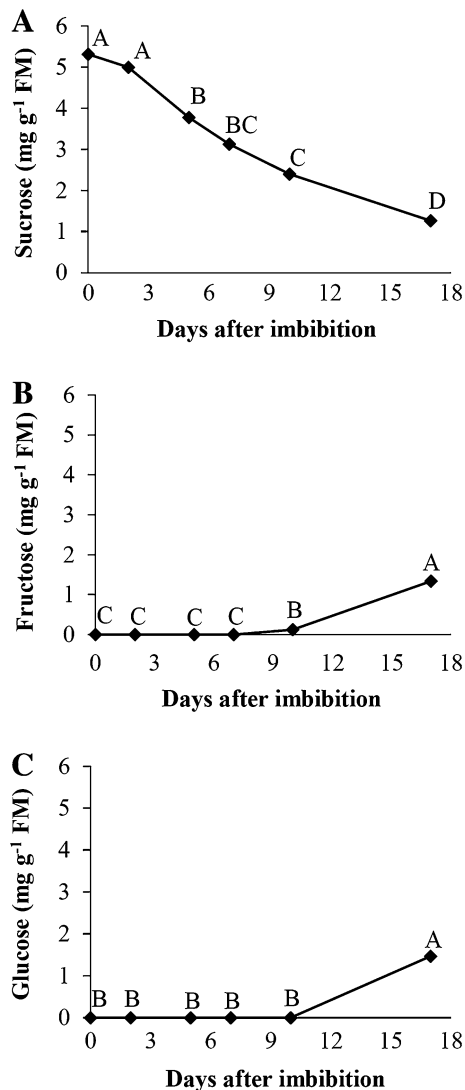


Fig. 3 Soluble sucrose (a), fructose (b) and glucose (c) contents (mg g^{-1} FM) in seeds of *C. fissilis* before (mature seed—time 0) and after 2, 5, 7, 10 and 17 days of imbibition. Capital letters denote significant differences in the content of each carbohydrate during the days of imbibition. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test ($n = 3$; CV sucrose = 8.5 %; CV fructose = 6.6 %; CV glucose = 17.1 %). CV coefficient of variation

II), and increased significantly during seedling development (Fig. 4b). Free Put (Fig. 4a) presented the second-highest content among the PAs identified during germination process (phases I and II), and the content increased significantly in early seedling growth (Fig. 4a). In contrast, the Spm contents decreased significantly between imbibition (phase I),

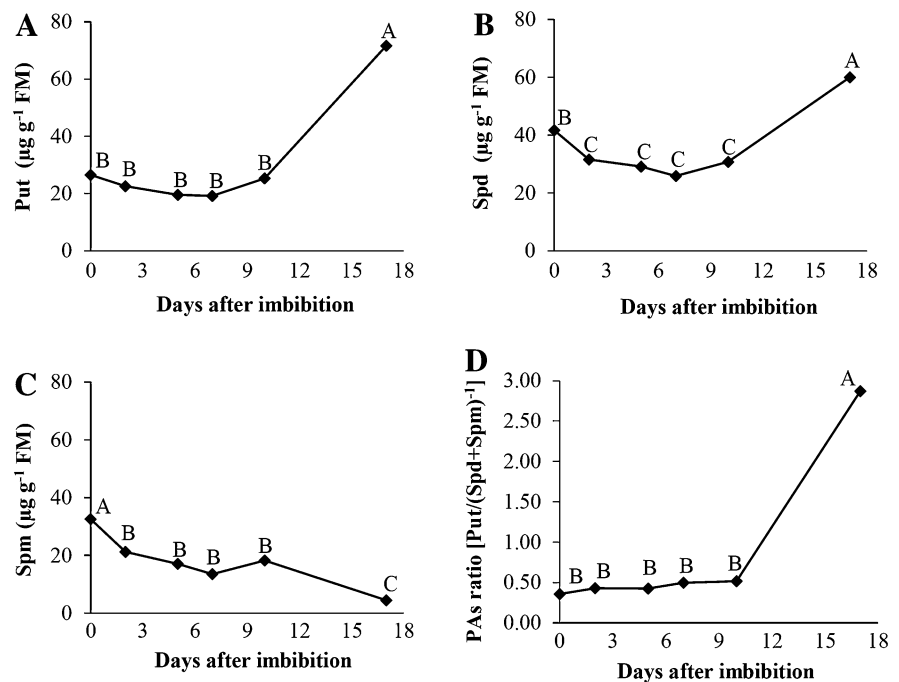
germination (phase II) and early seedling development (Fig. 4c). Among the free PAs, a higher content of Spd (Fig. 4b) occurred during germination than Put (Fig. 4a) and Spm (Fig. 4c).

The PA ratio [Put/(Spd + Spm)] did not change throughout the germination process (phases I and II); however, on day 17 of seedling development, a significant increase in this ratio was observed (Fig. 4d) due of the increased Put contents (Fig. 4a).

4 Discussion

Seed germination is a complex process that starts with water absorption by quiescent seed and ends with radicle protrusion (Bewley 1997), and all of the subsequent events are classified as post-germinative (Gallardo et al. 2002). Germination in *C. fissilis* ends with radicle protrusion on the 7th day of imbibition (Fig. 1b) and a triphasic development pattern is observed (Bewley and Black 1994). In *C. fissilis*, the first phase (phase I) began with the mature seed and lasted until the second day of imbibition when rapid and significant water absorption occurred (Fig. 1b). According to Bove et al. (2001), rapid imbibition in phase I results in the re-initiation of basal metabolism. During this phase, which is known as physical imbibition, a step-by-step activation of metabolic pathways is caused by a gradual increase in hydration. When the level of hydration exceeds 60 %, the rate of hydration slows (phase II) and new physiological mechanisms enable cell expansion along the embryonic axes, which culminates in the onset of cell elongation (Bove et al. 2001). This process most likely occurs in preparation for the cellular elongation that results in radicle protrusion (phase II) at day seven in *C. fissilis*. In this phase, osmotically active substances, such as sugars, amino acids and potassium ions, accumulate, and acidification of the cell wall leads to a loosening of the bonds between cell-wall polymers. These events coincide with the activation of H^+ -ATPases in the plasmalemma, which results in a further increase in water uptake that might coincide with the weakening of the surrounding tissues (endosperm) as the embryonic axes elongate and germination is completed (Bove et al. 2001). In addition, the imbibition curve in *C. fissilis* shows significant increases in WC and FM during all phases, although these effects are more pronounced in phases I and III

Fig. 4 Free PAs Put (a), Spd (b), Spm (c) contents ($\mu\text{g g}^{-1}$ FM) and PA [Put/(Spd + Spm) $^{-1}$] ratio (d) in seeds of *C. fissilis* before (mature seed—time 0) and after 2, 5, 7, 10 and 17 days of imbibition. Capital letters denote significant differences in each PA during the days of imbibition. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK test ($n = 3$; CV Put = 10.8 %; CV Spd = 6.4 %; CV Spm = 9.8 %; CV PA ratio = 13.6 %). CV coefficient of variation



and increases in DM in phase III (Fig. 1b), most likely because of radicle elongation and subsequent seedling growth.

Certain biomolecules synthesized and stored during seed development, such as proteins and amino acids, are mobilized during germination and early seedling growth (Gallardo et al. 2002; Penfield et al. 2005; Catusse et al. 2008). In *C. fissilis*, a significant decrease in soluble proteins in ethanol and increase in total amino acids during the first 5–7 days were observed (Fig. 2), suggesting that amino acids might be produced by the mobilization of storage proteins in mature seeds during germination in *C. fissilis*. Proteins that were synthesized and stored in the mature seed are broken down into free amino acids for biosynthesis and energy generation (Tan-Wilson and Wilson 2012). The soluble proteins in ethanol could be prolamines (Shotwell and Larkins 1989; Krishnan and White 1995), being described as the source of energy for the seedling development in *O. sativa* (Kim and Okita 1988). Similarly to *C. fissilis*, in *Caesalpinia pyramidalis*, a woody species, the contents of prolamines decreased after the start of imbibition and increased in phase II (Dantas et al. 2008). Furthermore, the increase in total free amino acid content observed in *C. fissilis* might be associated with the role of these compounds

as osmolytes, which are required to complete the germination process. According to Bove et al. (2001), physiological processes occur prior to root elongation to prepare the embryo for growth, and the level of osmotically active substances, such as amino acids, increases and may enhance the entry of water into the cell and contribute to root elongation.

In addition, storage nutrients in the cotyledon and/or endosperm, such as lipids, proteins or starch, begin to mobilize before germination ends and are used during post-germinative events to sustain the young plant in its early growth stages before it becomes autotrophic (Bove et al. 2001). Therefore, the significant decrease in the total free amino acid content in phase III from the 7th to 17th day (Fig. 2) might be associated with post-germinative events to support seedling growth in *C. fissilis*.

Among the analyzed amino acids, threonine, which is an essential amino acid and one of the products of the aspartic acid pathway, was predominant during *C. fissilis* seed germination process and early growth seedling (Table 1); this amino acid plays an important role during plant growth and development (Jander and Joshi 2010) and is involved in important metabolic pathways, including cellular energy production and photorespiration in plants (Joshi et al. 2006; Kang

et al. 2006). Other free amino acids are predominant during germination in various species, such as asparagine in *O. catharinensis* (Dias et al. 2009) and GABA in *Cola acuminata* and *Cola anomala* (Onomo et al. 2010), suggesting that the predominance of a particular amino acid might be species-dependent.

The contents of certain free amino acids, such as aspartic acid, glutamic acid, glutamine, arginine and asparagine (Table 1), were significantly higher in seedlings in phase III. These results suggest that the increased amino acid content during early seedling growth in *C. fissilis* could be associated with nitrogen availability to support growth. This hypothesis is based on these amino acids acting as nitrogen donors and amino acid transporters in plant growth and their involvement in the biosynthesis of other amino acids, such as lysine, threonine, isoleucine and methionine (Azevedo et al. 2006). In addition, a significant reduction in glutamic acid content during imbibition and radicle protrusion in *C. fissilis* (Table 1) suggests that this amino acid is involved in the interconversion of other amino acids during imbibition and germination (phases I and II) and may be a precursor for glutamine and proline synthesis, which are used as substrates for respiratory enzymes during imbibition (Rocha et al. 2010). Glutamine content increased after radicle protrusion and reached high values during early seedling development of *C. fissilis* (Table 1); because glutamine is a nitrogen and transporter amino acid in plants, this behavior suggests that it is necessary for seedling growth (Oliveira et al. 2001).

GABA is a non-protein amino acid that is synthesized through glutamic acid decarboxylation (Satya-Narayan and Nair 1990) and the PA pathway (Bouchereau et al. 1999). This amino acid is associated with several processes in plants, including cell proliferation and embryo differentiation in carrot (*Daucus carota*) cultures (Kamada and Harada 1984); cellular differentiation and embryogenic competence acquisition during somatic embryogenesis development in *Acca sellowiana* (Booz et al. 2009); zygotic embryo development in *O. catharinensis* (Santa-Catarina et al. 2006); nitrogen storage in pea (*Pisum sativum* var. Esla), bean (*Phaseolus vulgaris* var. La Granja) and lentil (*Lens culinaris* var. Castellana) seeds (Rodriguez et al. 2008); and germination in *C. acuminata*, *C. anomala* (Onomo et al. 2010) and *C. fissilis* (Table 1). GABA is synthesized de novo during early seedling growth, and its significant reduction

during phases I and II and significant increase in phase III (Table 1) suggests that this amino acid might play an important role in the events prior to radicle protrusion (germination) in *C. fissilis*. In addition these observations suggest an important role of GABA during seed development and germination, although its precise role remains poorly understood.

Tyrosine degradation is associated with alanine and glutamic acid production (Alhadi et al. 2012). A reduction in tyrosine content corresponded with an increase in alanine content on day five of imbibition in *C. fissilis*, which may have been caused by tyrosine degradation and alanine production. Furthermore, the increased tyrosine contents might have been associated with the inhibition of ABA synthesis, which is responsible for the suppression of germination (Ghelis et al. 2008). In addition, an increase in arginine content (Table 1) might be related to germination in *C. fissilis* as demonstrated by Alhadi et al. (2012). These authors showed that increased arginine contents promote the synthesis of gibberellins, which are germination promoters.

Carbohydrates are a source of cellular energy and act as osmolytes, which maintain the integrity of the plasma membrane, as well as signaling molecules in plants (Smeeckens et al. 2010). The higher sucrose content observed in mature *C. fissilis* seeds (Fig. 3a), might be associated with the accumulation of this carbohydrate during embryonic development (Focks and Benning 1998). Higher sucrose contents and lower monosaccharide contents were observed in the mature orthodox seeds of certain species (Obendorf 1997; Kuo et al. 1998). During imbibition (phase I) and germination (phase II), a significant decrease in sucrose contents occurred in the seeds of *C. fissilis* (Fig. 3a). High contents of sucrose are considered important for the maintenance of osmotic status in cells and may favor the process of imbibition (Pastorini et al. 2003). The aforementioned authors demonstrated that sucrose is stored in the final stages of embryo development in *Arabidopsis thaliana* and subsequently used during germination. Specific growth and metabolic responses might be activated and/or modulated based on the nature of the sugar signal. Sucrose, the primary transport sugar in plants, can be sensed as a direct signal (Chiou and Bush 1998).

Alternatively, a signal may be produced because of its hexose cleavage products, such as glucose or uridine diphosphate (UDP)-glucose and fructose

(Rolland et al. 2006; Li et al. 2011). The decrease in sucrose content observed in *C. fissilis* seedlings (phase III) might be associated with its degradation to fructose (Fig. 3b) and glucose (Fig. 3c), which were only detected after germination; however, these monosaccharides might be more relevant during seedling growth. Our results are consistent with those observed by Obroucheva et al. (2006) during germination in *Aesculus hippocastanum*. These authors demonstrated that sucrose content was higher in the beginning, whereas fructose and glucose increased at the end of germination (Obroucheva et al. 2006). Taken together, these results suggest a relationship between the contents of these soluble sugars and seed germination in certain species, including *C. fissilis*.

In plants, glucose has been highlighted as a signaling molecule in several processes associated with growth and development, such as germination, hypocotyl elongation, cotyledon expansion and leaf development (Rolland et al. 2006). In *C. fissilis*, glucose was only observed during early seedling development (Fig. 3c), suggesting that this sugar might not be required for germination process in this species. The exogenous addition of glucose has been shown to inhibit the germination and initial development of seedlings (Price et al. 2003; Gibson 2005; Hu et al. 2012). Studies have demonstrated that hexoses such as glucose and fructose show a greater signaling potential in the promotion of cell proliferation and organ growth. Thus, these sugars are observed during seedling growth, whereas sucrose is typically associated with differentiation and seed maturation (Borisyuk et al. 2004; Koch 2004).

In addition to soluble sugars, PAs are also important compounds that modulate several physiological and biochemical processes in plants (Kusano et al. 2008; Álcázar et al. 2010). Among the free PAs, Put, Spd and Spm were identified during *C. fissilis* germination process and early growth seedling (Fig. 4). The Spd and Spm contents were higher than Put in mature seeds before imbibition, suggesting that these PAs are accumulated in late seed development in this species. Similar results were observed in the seeds of *Araucaria angustifolia* (Astarita et al. 2003) and *O. catharinensis* (Santa-Catarina et al. 2006), and higher contents of Spd and Spm were observed in the final phase of seed development and might have been associated with cell elongation as well as desiccation tolerance (Astarita et al. 2003; Santa-Catarina et al.

2006). In *O. catharinensis*, a reduction in Put and increase in Spm might be important in the final stage of zygotic embryo development when cell elongation occurs (Santa-Catarina et al. 2006). In addition, *Zea mays* seeds showed high contents of Spd and Spm at the end of seed development, suggesting that these PAs are important during this phase (Cao et al. 2010). This pattern of PA content is similar in the seeds of several species, suggesting the role of these compounds in germination.

Free Spd contents (Fig. 4b) were higher throughout germination process (phases I and II) in *C. fissilis* than free Put (Fig. 4a) and Spm (Fig. 4c). These results suggest that the role of free Spd in germination of *C. fissilis* is similar to that observed in *A. thaliana*, where higher Spd contents were observed relative to other PAs (Puga-Hermida et al. 2006). Similar results were also observed in *Malus domestica* seeds (Sinska and Lewandowska 2006). In *Arabidopsis*, the addition of exogenous Put (but not Spd or Spm) resulted in the inhibition of germination (Mirza and Bagni 1991).

Furthermore, the significant increase in free Put (Fig. 4a) and decrease of Spm (Fig. 4c) contents in seedlings of *C. fissilis* on day 17 (Fig. 4a, c) might be associated with cell division processes during seedling growth. Matilla (1996) associated cellular differentiation with higher contents of Spd and Spm, whereas Put content appeared to be more closely associated with cell division. In addition, the activity of diamine oxidase (DAO), a Put-degradation enzyme, was significantly higher in the shoots relative to the other organs during seedling development in fava bean (*Vicia faba*) (Yang et al. 2011). Although DAO activity was not evaluated in this work, the reduction of Put contents (Fig. 4a) might be related to the degradation of Put and production of GABA (Table 1) during germination in *C. fissilis* since Put degradation by DAO leads to GABA biosynthesis (Bhatnagar et al. 2001).

The PA ratio [Put/(Spd + Spm)] could be used as a biochemical marker (Shoeb et al. 2001) of the different stages of embryo development (Santa-Catarina et al. 2006) and germination (Pieruzzi et al. 2011). A significant increase in this ratio (Fig. 4d) during early seedling development in *C. fissilis* occurred due of the higher Put content (Fig. 4a), indicating a high regeneration capacity of seedling. These results indicate that Put plays an important role in promoting the cell cycle and mitotic divisions necessary for seedling growth in *C. fissilis*, wherein

Put is essential for cellular division as suggested by Maki et al. (1991). These authors demonstrated that the inhibition of Put biosynthesis halted cellular division and inhibited G1/S phase progression in suspension cultures of *Catharanthus roseus*.

This work is the first to report an association between free amino acids, free PAs, soluble sugars, as well as soluble proteins in ethanol, during seed germination process and early growth seedling of *C. fissilis*. Soluble proteins are mobilized during germination (phases I and II), which is related with the significant increase of total free amino acids contents at the same time. In addition, a significant degradation of sucrose was observed during seed germination (phase I and II), possibly to supply fructose and glucose for seedling growth (phase III). Moreover, endogenous Spd and Spm are significantly mobilized during germination (phase I), while Put seems important for seedling growth (phase III), possible by the promotion of cell divisions necessary at this time. We conclude that changes in the contents of these biomolecules are associated with seed germination and early seedling growth in this specie. These results will be useful to further studies on seed storage and germination in orthodox tree species, as well as to establish a network of these compounds in the regulation of germination, increasing our knowledge about this process in native endangered species from Brazilian Atlantic Forest. In addition, further researches related to enzymes involved in amino acid, PA and carbohydrate metabolism might contribute to a better understanding of the metabolism of these compounds during the germination of *C. fissilis*.

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