



Germination and anaerobic metabolism of seeds of *Tabebuia cassinoides* (Lam.) DC subjected to flooding and anoxia

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ABSTRACT

Tabebuia cassinoides (Lam.) DC (Bignoniaceae) is an arboreal species common in seasonally or permanently waterlogged areas of the “restinga” forest (a type of forest that occurs on the sandbanks of the coastal plains of southeastern Brazil). The objectives of the present study were to establish seed germination responses of this species to flooding and anoxia and investigate the end products of the anaerobic metabolism of seeds subjected to these conditions, with the goal of understanding the adaptive strategies that enable this species to dominate flood prone areas of “restinga”, as well as determine reserves stored in their seeds. Seeds of *T. cassinoides* did not germinate under anoxia or complete submergence, but remained viable under these conditions for 15 and 20 days, respectively. Due to their membranaceous wings, the seeds float very well and reached 100% germination in this condition, an important adaptation to overcome the initial stages of development in flooded habitats. In relation to anaerobic metabolism, ethanol is the most important end product, while lactate is produced in lower concentrations. Seeds of *T. cassinoides* have very little endosperm and the reserves, mainly glycoproteins, lipids and free sugars, accumulate in the cotyledons. Free sugars may provide the substrate for the initial metabolism of seed germination, but the level of these reserves was not enough to allow germination under oxygen deprivation. Possibly, carbohydrate reserves were enough only to maintain seed viability for a relative short period under this condition.

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Introduction

Seeds of most higher plants have an absolute oxygen requirement for germination. However, anoxic germination has been reported among monocots in *Oryza sativa* (Alpi and Beevers, 1983; Davies, 1980) and *Echinochloa crus-galli* (Kennedy et al., 1980) and among the dicots, in *Chorisia speciosa* (Joly and Crawford, 1983) and *Erythrina caffra* (Small et al., 1989). In all cases, it was observed that, for subsequent seedling development, oxygen was necessary.

Under hypoxia, submerged seeds of gallery forest species of southeastern Brazil as *Sesbania virgata* (Okamoto, 1995) and *Inga affinis* (Lieberg and Joly, 1993) as well as seeds of Amazonian *Himatanthus sucuuba* (Ferreira et al., 2007) are able to germinate and to produce seedlings, while *Parkia discolor* (Coutinho and Struffaldi, 1971) and *Parkia pendula* (Scarano and Crawford, 1992) are unable to germinate in this condition. Nevertheless the seeds of these two legumes from the Amazonian Forests remain viable under this condition for 6 and 7 months, respectively. Other

species avoid hypoxia dispersing seeds and fruits during the dry season, when well-drained microsites are available in seasonally flooded areas, as reported by Lobo and Joly (1996) for *Talauma ovata*. These are examples of different regeneration strategies that allow the establishment of plants in an environment that is restricted to the majority of competing arboreal species.

Anaerobic stress in plants results in a rapid change of gene expression pattern (Sachs et al., 1980). Under such a condition, induction of fermentative metabolism is considered an adaptation to maintain the capacity of ATP synthesis in the absence of aerobic respiration (Davies, 1980).

The main product of fermentation in plants is ethanol. Both enzymes associated with alcoholic fermentation, pyruvate decarboxylase and alcohol dehydrogenase, are induced by anoxic stress (Ricard et al., 1986). However, the occurrence of lactic fermentation was also observed in anaerobic roots (Rivoal and Hanson, 1993) and in germinating seeds (Aldosoro and Nicolas, 1980). Data obtained *in vitro* (Davies et al., 1974) and *in vivo* (Roberts et al., 1984) led to the hypothesis that lactate production in plants is a transient phenomenon that controls the transition from aerobic metabolism to ethanolic fermentation. Any failure in the regulation of this metabolic response may be responsible for acidification and cell death (Roberts et al., 1984, 1985).

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Fermentation processes are absolutely dependent on the availability of carbohydrates (Drew, 1997). Lipid respiration consumes more oxygen than carbohydrate catabolism, and in seeds that are either flooded or otherwise deprived of oxygen, metabolism will be subjected to a greater hindrance from a lack of oxygen with a consequent loss in viability (Crawford, 2003). It is therefore not surprising that oil-rich seeds are more susceptible to oxygen deprivation than starchy seeds during the early stages of germination (Al-Ani et al., 1985; Raymond et al., 1985).

Tabebuia cassinoides (Lam.) DC is an arboreal Bignoniaceae, very common in the waterlogged areas of Restinga Forest that occupy geologically recent, Quaternary terrains between the Atlantic Rain Forest and the sea in southeastern Brazil. In some areas, where soil drainage has been altered by roads and/or other anthropogenic changes, becoming permanently waterlogged, *T. cassinoides* may form monospecific stands. This tree is an economically important species, because among the Brazilian taxa, it provides the best wood for pencil production, mainly for export. On a worldwide level, it is of the highest quality and is only exceeded by the American cedar *Libocedrus decurrens* (Kuniyoshi, 1993).

The winged seeds of *T. cassinoides* are wind dispersed in the rainy season, when the water table may be up to 50 cm above the soil surface. Therefore, to understand the regeneration process of these areas of Restinga Forest it was necessary to study the germination and anaerobic metabolism of *T. cassinoides* seeds under normoxia, flooding, and anoxia. In addition, seed reserves were investigated because they are key factors in enabling seeds to germinate or to maintain their viability under oxygen deficiency.

Materials and methods

Plants of *T. cassinoides*, in reproductive stage, present trumpet flowers, linear-oblong fruits and bialate seeds with membranaceous wings (Fig. 1). Fruits of this species were collected from several trees at the Núcleo Picinguaba (23°21'S and 44°51'W), Serra do Mar State Park, Ubatuba, State of São Paulo, Brazil. Completely developed fruits were collected before their total dehiscence to avoid seed dispersal by wind.

Seed germination

Immediately after harvesting, the fruits were taken to the laboratory where the seeds were separated from the pods. Before starting each experiment the seeds were surface sterilised by submersion for 3 min in 100 ml of a 1000 units ml⁻¹ solution of Micostatin (Bristol-Myers Squibb do Brazil S.A., São Paulo, Brazil), a powerful fungicide, and then washed in running distilled water for 5 min.

Normoxia conditions were obtained by placing the seeds in a plastic box lined with filter paper saturated with distilled water. Flooding conditions were obtained by disposing the seeds in 250 ml of distilled water. Partial submergence was imposed by allowing the winged seeds to float on water surface, while total submergence was attained by forcing the seeds to stand under the water surface. Anoxia treatments were carried out by placing the seeds in anaerobic jars (Oxoid Ltd., Basingstoke, Hampshire, England). In all treatments five plastic boxes or Petri dishes with 20 seeds each were used. Recovery experiments were carried out by placing three Petri dishes, containing ten flooded and anoxia treated seeds, under normoxic conditions.

All treatments (normoxia, flooding and anoxia) were applied in germinators (Fanem, São Paulo, Brazil) at 25 ± 2 °C with a 12 h white light photoperiod, and radicle protrusion was considered as



Fig. 1. Drawing of a reproductive branch and of the seed of *T. cassinoides*, showing the compact arrangement of seeds within the pod. Bar = 3 cm.

the indicator of germination. Seed photoblastism was tested only for normoxic conditions, and dark conditions were obtained by enclosing the plastic boxes in three black polyethylene bags, as described by Joly and Felipe (1979). In the dark experiment, seed germination was followed in a darkroom with a green security light (Joly and Felipe, 1979).

The plastic boxes were examined every day, and germinated or dead (putrefied) seeds were removed. The experiments were terminated when 100% of the seeds had germinated or were dead. In the case of the anoxic treatment daily examination of the seeds was possible without opening the jars, since they were transparent. Nevertheless, after 1, 2, 3, 5 and 15 days the jars were opened and the seeds examined more carefully for radicle protrusion.

Germination was examined in order to calculate the Germination Speed Index (GSI) of Maguire (1962) and the percentage of germination. Statistical significance was determined by one-way ANOVA followed by Tukey's test, with the minimum level of significance set at $P < 0.05$.

Ethanol and lactate determination

In the experiments carried out to determine the level of ethanol and lactate in the germinating seeds kept under normoxia, flooding and anoxia for 0, 0.5, 1, 2 and 3 days, the seeds (± 1 g) were placed in liquid nitrogen after each period of treatment, and then macerated in 12 ml of chilled perchloric acid (6%). The homogenate was centrifuged at 3500 rpm for 20 min in a chilled centrifuge (0 °C). After centrifugation, the supernatant was removed and neutralised with 5 M K₂CO₃. Potassium perchlorate was removed by centrifugation (Joly and Brändle, 1995).

The enzymic determination of ethanol and lactate was carried out using Boehringer kits as described by Joly and Brändle (1995). The loss of metabolites during extraction was estimated by adding

known amounts of ethanol and lactate to an additional sample of seeds. The average recovery figures were as follows: ethanol 84% and lactate 93%. In all cases six replicates per treatment were used and the results analysed by ANOVA. To compare how much of the overall interspecific variance of a trait (ethanol, lactate) can be attributed to differences among time, treatment or to the interaction between time and treatment, we calculated the r^2 values for each trait. These were calculated from repeated ANOVAS, following Rosenthal and Rosnow (1985), where $r^2_x = SS_x/SS_{total}$; SS_x and SS_{total} are the sum of squares for factor x and the total sum of squares, respectively. The premises of normality, homoscedasticity and sphericity were checked and the data for ethanol were log-transformed.

Ethanol was also measured in the water where seeds were totally or partially submerged for 0.5 and 1 day.

Light microscopy

Samples of quiescent seeds were fixed in FAA 70 for 48 h, dehydrated in ethanol series and embedded in paraffin. Sections 8 μ m thick were obtained with a rotary microtome and stained in Toluidine Blue pH 4.0 (Silva et al., 1997) for acid sugars, mainly cell-wall pectins, and Xylidine Ponceau pH 2.5 (Cortelazzo and Vidal, 1991) for total proteins. For neutral polysaccharide detection, sections were stained in Periodic acid-Schiff (PAS); for a control the pre-treatment with the periodic acid was omitted (Cortelazzo, 1992). Identity of starch grains was confirmed by polarization microscopy in sections stained in Toluidine Blue. For lipid observation, freehand cross sections of fresh material were stained in Sudan Black B (Jensen, 1962).

Analysis of carbohydrate reserves

Cotyledons of quiescent seeds were dried at 80 °C for 48 h before they were ground to a powder using a mill.

Free sugars were determined from three replicates of 25 mg powder each. The fine powder was extracted with 80% ethanol at 80 °C for 15 min. After centrifugation, the pellets were re-extracted three times more as above. The soluble sugars were quantified colorimetrically in the pooled supernatants (Dubois et al., 1956).

Starch was evaluated in three samples of 50 mg powder each. The powder was first extracted with methanol:chloroform:water (12:5:3, v/v/v) for elimination of lipids and free sugars. After centrifugation, the pellet was incubated for 2 h in 30% HClO₄. After spinning, the pellet was extracted again as above. The pooled perchloric supernatants were used for starch determinations (Scott and Melvin, 1953). The values obtained were then multiplied by 0.9 to allow for conversion of hexose to starch (McCready et al., 1950).

Results

The results in Fig. 2 show that the seeds are not photoblastic, although the presence of light increased significantly the speed of germination ($GSI = 6.68 \pm 0.07$) when compared with seeds germinated in dark condition ($GSI = 5.20 \pm 0.12$). Seeds of *T. cassinoides* were not able to germinate under total submergence or under anoxia and, after 20 and 15 days, respectively, they lost their viability (Fig. 3). In contrast, hypoxic seeds, floating in water, reached 100% of germination (Fig. 2), although a little slower ($GSI = 5.99 \pm 0.18$) than seeds kept under normoxia ($GSI = 6.68 \pm 0.07$). If kept floating for up to 30 days, these germinated seeds developed their radicles in water and their emerging

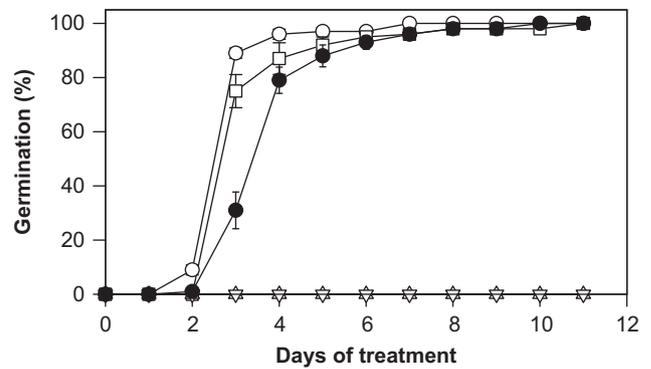


Fig. 2. Effect of light and of submergence (hypoxia) and anoxia on seed germination of *T. cassinoides*. Values are given as mean \pm s.e. of five replicates each with twenty seeds: (○) normoxia light, (●) normoxia dark, (□) Partial submergence, (△) total submergence, (▽) anoxia.

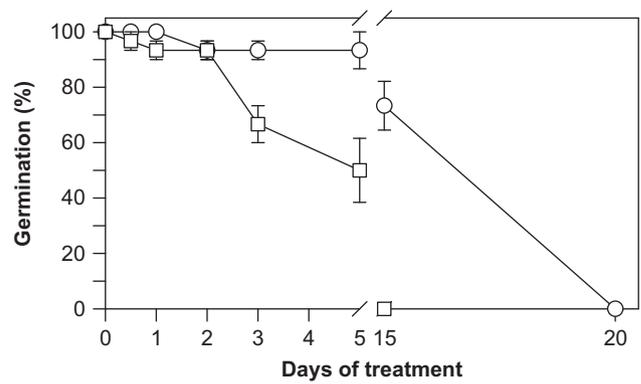


Fig. 3. Loss of viability of *T. cassinoides* seeds after different periods of total submergence and of anoxia. Values are given as mean \pm s.e. of three replicates each with ten seeds: (○) total submergence, (□) anoxia.

Table 1

Significance values and fraction of total interspecific variance (r^2) that is explained by time, by treatment and by the interaction of both on metabolite contents in seeds of *T. cassinoides*.

Trait	<i>p</i>	r^2
Ethanol		
Time	0.000	0.33
Treatments	0.010	0.07
Time \times treatments	0.030	0.12
Lactate		
Time	0.000	0.36
Treatments	0.026	0.06
Time \times treatments	0.358	0.07

cotyledons became green, apparently able to become active in terms of photosynthesis.

Time, treatments and the time \times treatments interaction had significant effects on ethanol production ($p < 0.05$). For lactate, only time and treatments were significant (Table 1). However, from the factors analysed, the time was the most important one (higher values of r^2), that explains the variations observed on metabolite contents in *T. cassinoides* seeds (Table 1, Fig. 4).

Ethanol was the main end product of the anaerobic metabolism, while lactate was produced in lower amounts (Fig. 4). Seeds of *T. cassinoides* had a high concentration of ethanol, even before the start of the experiments, and under 1 day of normoxia, after the imbibition process started, there was a 50% increase in ethanol

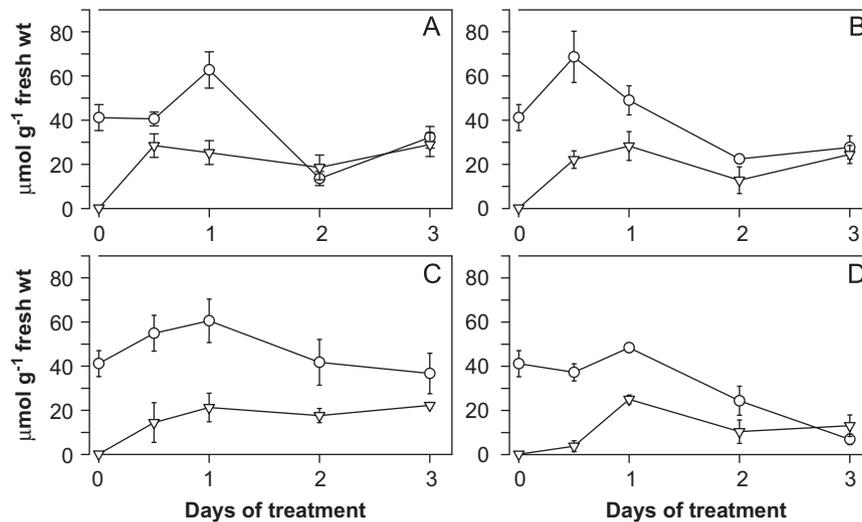


Fig. 4. Effect of time of normoxia (A), partial (B) and total submergence (C) and anoxia (D) on metabolite contents in seeds of *T. cassinoides*. Values are given as mean \pm s.e. of six replicates: (○) ethanol, (▽) lactate.

concentration, increasing from 41.2 ± 5.9 to 62.8 ± 8.3 $\mu\text{mol g}^{-1}$ fresh weight (Fig. 4A). This increase occurred 12 h earlier, in seeds subjected to total or partial submergence (Fig. 4B and C). Anoxia treated seeds did not present such a high increase in ethanol levels (Fig. 4D). Ethanol concentrations after 3 days of normoxia or partial submergence or anoxia were lower. This decrease was associated with radicle protrusion under normoxia and partial submergence (Fig. 2) and with partial loss of seed viability under anoxia (Fig. 3).

Ethanol was also found in the water where the seeds were totally or partially submerged (around 50% of the ethanol produced by the seeds was in the medium). Lactate, which was not present in seeds before their imbibition, increased to approximately $20 \mu\text{mol g}^{-1}$ fresh weight in all treatments (Fig. 4).

The endosperm is small (few layers) in *T. cassinoides* seeds and their reserves are mainly accumulated in the cotyledons, like in other Bignoniaceae seeds (see Gabrielli and Castro, 1995, and references therein). The thin cell walls of the cotyledons, as shown by staining with Toluidine Blue (Fig. 5A), do not play a role as a site of reserve deposition. Inside cells, small starch grains were visualized by polarization (Fig. 5A, detail). The strong staining with Xylidine Ponceau (Fig. 5B) and the weak staining with PAS (Fig. 5C) suggested that glycoproteins are the main reserve of these seeds. Another important component seems to be lipids, shown by Sudan Black B staining (Fig. 5D).

We decided to measure only those reserves that provide substrate for anaerobic metabolism of seed germination. Seeds of *T. cassinoides* have a very low percentage of starch (0.7%) and a considerably higher level of free sugars (17.3%, in relation to cotyledons dry mass).

Discussion

T. cassinoides seeds are dispersed in the rainy season, when the soil is flooded. The capacity of these seeds to germinate while fluctuating on water surface (see also Lopez, 2001; Wittmann et al., 2007), and the development of their radicles under water, might allow the establishment of this species wherever radicles can anchor to a substrate. Scarano et al. (1997) observed *T. cassinoides* seedlings growing on litter trapped by tanks of understory bromeliads. The same authors suggested that these litter-traps provide a “suspended soil” which might serve as a substrate for seed germination and seedling growth. Germina-

tion while floating was also observed in seeds of *Carapa guianensis*, a tree from flood-prone forests of the Amazon (Scarano et al., 2003).

Although part of the coastal vegetation, environmental conditions of restingas are not as severe as those of estuarine areas, and *T. cassinoides* seeds are not subjected to anoxia as reported by Wetson et al. (2008) for *Suaeda maritima*. So, it was not surprising that *T. cassinoides* seeds were not able to germinate under this condition.

Flooding tolerance of seedlings of *T. cassinoides*, which are able to keep a growth rate slightly higher when subjected to flooding (Kolb and Joly, 2008), is another key factor for successful establishment in these permanently or seasonally waterlogged forest areas of the coastal region. In these naturally flood prone areas, flooding intensity and duration has such an important selective impact that it affects both inter- and intra-specific distribution of plant species along a gradient of waterlogging (Parelle et al., 2007).

During fruit sampling in the Restingas of Ubatuba we observed, in some cases, seeds starting to germinate while still inside the pods. The highly dense arrangement of seeds within pods (see Fig. 1) suggests that in this condition access to oxygen might be restricted, and may explain the high levels of ethanol in seeds of *T. cassinoides* at the start of the experiment. Similar results were reported by Okamoto and Joly (2000) for *I. sessilis* seeds.

Ethanol and/or lactate accumulation in seeds germinating under normoxia conditions, as observed here (Fig. 4), are common and have been previously reported for *E. caffra* (Small et al., 1989), *E. crus-galli* (Fukao et al., 2003; Rumpho and Kennedy, 1981), *I. sessilis* (Okamoto and Joly, 2000). Usually, with radicle protrusion or cotyledon swelling, fully aerobic metabolism becomes predominant (Côme et al., 1991; Rolletschek et al., 2002). In some species, any extension of the period of natural anaerobiosis, because the seed is submerged or buried in waterlogged soil, may result in death (Crawford, 1992).

There are increasing indications that ethanol is the main product of fermentation in plants (Crawford, 1992; Kolb et al., 2002). In *T. cassinoides* submerged seeds, ethanol easily diffused into the medium. Rumpho and Kennedy (1981) observed that 85% of the ethanol produced by *E. crus-galli* var. *oryzicola* seeds were eliminated into the medium and suggested that this is important to avoid the accumulation of this metabolite to toxic levels. Bertani et al. (1980) proposed that one of the most important mechanisms to tolerate anaerobiosis in rice seedlings is the

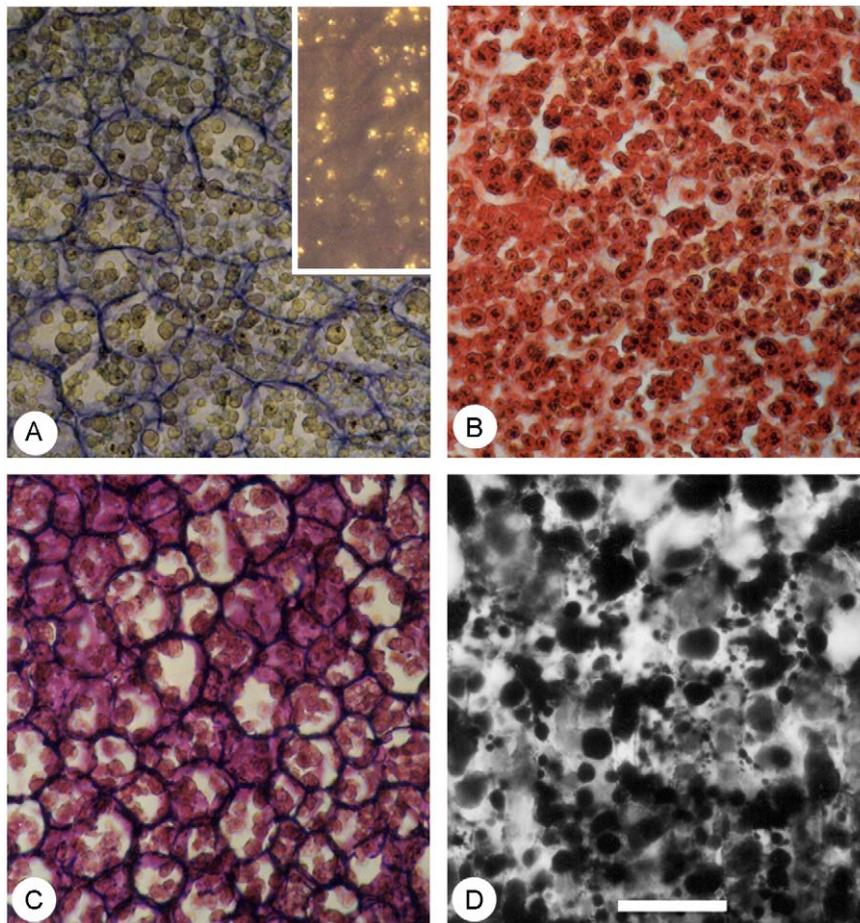


Fig. 5. Transverse sections of *T. cassinoides* cotyledons. (A) Toluidine Blue (polarizing microscope used in the detail), (B) Xylidine Ponceau, (C) PAS and (D) Sudan Black B. Bar = 50 μm .

presence of a powerful system of alcoholic fermentation connected to the capacity of diffusing the ethanol produced. This mechanism appears associated with the environment of the species, i.e., it is related to areas naturally subjected to flooding, as are the restinga forests of *T. cassinoides*.

The regulation of cytoplasmic pH is also considered a determinant factor of plant tissue survival under anoxia (Roberts et al., 1985). Cytosolic acidification has been related to lactate (Roberts et al., 1992). Probably this was not the case for *T. cassinoides* seeds because, regarding lactate, the time factor was more important than treatments or time \times treatments interaction (Table 1), and lactate was produced in similar amounts in germinated normoxic seeds and in non-germinated anaerobic seeds (Fig. 4).

Fermentation processes are absolutely dependent on the availability of free sugars (e.g., hexoses) (Drew, 1997). Fukao and Bailey-Serres (2004) proposed that a feedback mechanism, regulated by a G-protein, provides tolerance of oxygen deficiency through management of carbohydrate consumption and avoidance of oxidative stress. Al-Ani et al. (1985) and Raymond et al. (1985) reported that an adequate supply of fermentable sugars is important for germination and survival in hypoxic environments. Therefore, they related the germination capacity under low oxygen pressure with the presence of carbohydrate reserves in the seeds. Also, the tolerance of *Cyperus rotundus*, a troublesome sedge weed of rice, to flooding was attributed to a large carbohydrate content and the ability to maintain high levels of soluble sugars in the tubers during germination (Peña-Fronteras et al., 2008).

More oxygen is needed to degrade lipids compared with carbohydrates (Armstrong et al., 1994). This fact probably explains the differences observed by Al-Ani et al. (1985) and Raymond et al. (1985) in the hypoxic germination capacity between fatty and starchy seeds. *T. cassinoides* seeds are rich in glycoproteins, lipids, and free sugars, which are readily fermentable and may provide the substrate for the initial metabolism of seed germination. But these reserves are not enough to allow germination under total submergence and anoxia conditions, showing that in the initial stages both routes, aerobic and anaerobic, are essential. The loss of viability of seeds under these conditions was possibly due to exhaustion of respiratory substrates (see Manasse, 1990; Scarano et al., 2003) and/or from the toxic effect of the produced metabolites. Ethanol toxicity, however, is now known to be of less importance than previously thought (Jackson et al., 1982; Joly, 1994; Summers et al., 2000). However, some toxic effects of alcoholic fermentation have been attributed to acetaldehyde (Crawford and Brändle, 1996). Further experiments on the mobilization of seed reserves could highlight their use under oxygen deficiency conditions.

In conclusion, the capacity shown by *T. cassinoides* seeds to germinate, while fluctuating in the water that covers most of its native habitat when seeds are shed, helps us to understand why this tree is dominant in these restinga forests.

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