Germination and some aspects of the metabolism of *Chorisia speciosa* St. Hil. seeds under anoxia.

CARLOS A. JOLY AND ROBERT M.M. CRAWFORD

Department of Botany, The University, St. Andrews KY16 9AL, Scotland

ABSTRACT – (Germination and some aspects of the metabolism of *Chorisia speciosa* St. Hil. seeds under anoxia). Seeds of the tropical dry forest tree *Chorisia speciosa* St. Hil. (Bombacaceae) become covered in a muscilagenous gel three to four days after the start of imbibition suggesting that gas exchanges could be severely limiting in the early stages of germination. Germination, seed metabolism and seedling development were therefore compared under air and under conditions of strict anoxia in an atmosphere of 85% N_2, 10% H_2 and 5% CO_2. Germination as far as radicle protusion proceeded identically under air and anoxia. When anaerobic incubation was continued after radicle emergence further seedling development was inhibited. During the initial stages of germination before radicle emergence seed respiration rates, both aerobic and anaerobic, were very low with minimal accumulations of ethanol. After the protusion of the radicle there was a marked increase in both aerobic and anaerobic respiration with a rapid rise in ethanol accumulation in anaerobically incubated seedlings.

RESUMO – (Germinação e alguns aspectos do metabolismo de sementes de *Chorisia speciosa* St. Hil. mantidas em condições anóxicas). Três a quatro dias após o início do período de embebição as sementes de *Chorisia speciosa* (Bombacaceae) estão completamente cobertas por um gel mucilaginoso, o que sugere que na fase inicial da germinação as trocas gasosas podem ser um fator limitante. Por esta razão decidiu-se comparar a germinação, o metabolismo e o desenvolvimento das plântulas oriundas de sementes postas para germinar em um ambiente aeróbico e de sementes postas para germinar numa atmosfera com a seguinte composição: 85% N_2, 10% H_2 e 5% CO_2. A protusão da radícula, germinação, ocorre de forma idêntica em ambos tratamentos. Entretanto se após a germinação as plântulas são mantidas na câmara anaeróbica o desenvolvimento é completamente inibido. Em ambos os tratamentos, durante a fase inicial do processo de germinação, a taxa respiratória é baixa com um pequeno acúmulo de etanol. Após a protusão da radícula há um sensível aumento da taxa respiratória, com um grande acúmulo de etanol nas plântulas incubadas na câmara anaeróbica.

Key words: anoxia, germination, *Chorisia speciosa*.

Introduction

During imbibition, before the rupture of the testa, germinating seeds are naturally exposed to anoxia (Aldasoro & Nicolas 1980, Crawford 1977). Any prolongation of this natural period of anoxia can result in death (Crawford 1977, Rumpho & Kennedy 1981). *Oryza sativa* (Opik 1973, Vartapetian *et al.* 1978), *Zizania aquatica* (Campiranon & Koukkari 1977) and *Echinochloa crus-galli* (Kennedy *et al.* 1980) are exceptions as they are able to germinate and grow under anaerobic conditions. These species are all able to grow in waterlogged soils and it is not surprising that they are able to germinate and start seedling development in anaerobic conditions. The natural period of anaerobiosis that occurs during germination differs from that of flooding in roots because for seeds there is no possibility of the stress being alleviated by oxygen diffusing to the anaerobic region from the aerial shoot.

In a number of species the seeds during germination produce large amounts of muscilaginous gel (Mayer & Poljakoff-Mayber 1982). Apart from the role of the gel in seed dispersal (Fahn & Werker 1972, Van Der Pijl 1972) little is known of the biological significance of these gels and there is no evidence of any effect they may have on gaseous exchange in the germinating seeds. However, if the gel is impermeable to gases it is possible that seeds which have been covered with a muscilagenous coat while imbibing will be naturally exposed to anoxia or hypoxia for a longer period than seeds which do not produce such a coat. Seeds which

Present address of C.A.J.: *Departamento de Morfologia e Sistemática Vegetais, Instituto de Biologia, Universidade Estadual de Campinas, Caixa Postal 6109, 13100 Campinas, São Paulo, Brasil.*

produce such a gel might be expected to be able to germinate in low concentrations of oxygen and thus provide another example of a higher plant organ which can function anaerobically without injury.

The seeds of *Chorisia speciosa*, a tropical tree typical of the forests that occur in mesotrophic soils of Central and South-East Brazil, are completely convered by a gel 3 to 4 days after being placed in moist conditions. The formation of the gel causes a complete rupture of the seed coat, and the gel becomes the only barrier between the developing embryo and the atmosphere. The seeds of this species germinate readily after imbibition making this species suitable for testing whether or not such gel-covered seeds are capable of germinating in low concentrations of oxygen.

Material and methods

Effect of anoxia upon seed germination – Seeds of *Chorisia speciosa* St. Hil. (Bombacaceae), collected in the state of São Paulo, Brazil, were surface sterilized by mechanically shaking for 15min. in 100ml of 6% sodium hypochlorite followed by rinsing four times with sterile distilled water. After sterilization the seeds were placed in Petri dishes with a double layer of Whatman No. 3 paper. Five Petri dishes, with ten seeds each, were placed in an incubator at 25°C in the dark, and another five placed in an anaerobic workbench (Forma Scientific) with an atmosphere of 85% N_2, 10% H_2 and 5% CO_2, also at 25°C in the dark. The Petri dishes containing the seeds were introduced into the anaerobic workbench via a pretreatment chamber. In this chamber, the seeds were submited to three cycles of vacuum removal of any oxygen that might be trapped in the seeds. The sterile distilled water used to wet the filter paper in these Petri dishes was bubled with O_2-free nitrogen during 30min., and was also submited to the three cycles of vacuum removal of oxygen and equilibration with the gas used in the anaerobic workbench. The maintenance of anaerobic contitions throughout the experiments was shown by a methylene-blue indicator placed in the anaerobic workbench. The Petri dishes were examined every day and radicle protusion was considered as germination.

Effect of anoxia upon the metabolism of the germinating seed – Seeds were surface sterilized and placed in Petri dishes in an incubator or in the anaerobic workbench as described above. After 3, 6 and 9 days of incubation the aerobic and anaerobic respiration rates were determined with a Warburg respirometer. The seeds were placed in Warburg flasks containing 2ml of sterile 0.05M citrate-0,1M phosphate buffer pH 5.4. The flasks had been previously sterilized with boiling distilled water. All preparations were carried out in a sterile room. After the flasks had been sealed they were transferred to a water bath at 25 ± 1°C. The flasks used to determine anaerobic respiration rates were flushed with O_2-free nitrogen for 15min. (Crawford 1966). The flasks were allowed to equilibrate for 30min. before readings were started. Oxygen uptake, CO_2 evolution under air and under nitrogen were then measured for 2h. with readings each 15min. For each treatment five flasks with two seeds each were used.

Simultaneously after 3, 6 and 9 days of incubation seeds of both treatments were extracted as described by Joly & Crawford (1982) for enzymatic determination of ethanol (Bernt & Gutmann 1974), lactate (Gutmann & Wahlefeld 1974a) and malate (Gutmann & Wahlefeld 1974b). Two seeds per replicate, five replicates per treatment were used.

Ethanol, lactate and malate were also determined in seedlings kept in the anaerobic workbench for 1, 2 and 3 days after germination. Here too there were five replicates of two seeds each.

All data are expressed in seed dry weight that was determined from 200 seeds weighed in aliquots of ten seeds. The significance of change in the level of the metabolites was determined by a *t* test. The loss of metabolites during extraction was estimated by adding known amounts of ethanol, lactate and malate to an additional sample of two seeds. The average (n = 5) recovery figures were as follows: ethanol 82%, lactate 91% and malate 93%.

Results

Effect of anoxia upon seed germination – There are no differences between the time course for germination of seeds for seeds kept in air and that of seeds kept in the anaerobic workbench (Figure 1). Both germination rate and the final percentage of germination are identical under air and in the anaerobic workbench.

Effect of anoxia upon seedling development – Radicle length, radicle weight and shoot + cotyledons weight were considered as growth parameters. The length of the radicle within the dry seed was measured with a Leitz magnifying glass adapted with a millimetric scale. Both radicle and shoot + cotyledons weights were determined to the nearest mg. These parameters have been measured in germinated seeds and seedlings from the following treatments: a) germinated and grown in air for 1, 2 and 3 days; b) germinated and kept in the anaerobic workbench for 0, 1, 2 and 3 days. The effects of anoxia on development were assessed from measurements made three days after the seedlings were transferred to air. The significance of the differences was tested with a *t* test; in all cases n = 25.

anaerobic workbench.

Effect of anoxia upon seedling development – Three-day old seedlings originated from seeds germinated in the anaerobic workbench and immediately transferred to air, cannot be distinguished from the controls (Table 1). However, if germinated seeds remain under anoxia, further growth is inhibited and after 3 days the seedlings are dead (Table 1). The radicle that emerges through the gel is ten times longer than that of the embryo within the dry seed (Table 2). After emergence further radicle extension and development is dependent on a supply of oxygen (Table 2).

Table 1. Effect of anoxia upon *Chorisia speciosa* seedling development expressed in fresh weight. Seedlings kept in air or in an anaerobic workbench with an atmosphere of 85% N_2, 10% H_2 and 5% CO_2 at 25 ± 1°C, in Petri dishes with two layers of moist Whatman No. 3 paper. 5 seedlings per Petri dish, 10 Petri dishes per treatment. Significance was tested in relation to the control by *t* test, *significant at the 5% level, **significant at the 1% level, ***significant at the 0,1% level.

Parameter / Treatment	Percentage of survival	Seedling weight (g)	Radicle weight (g)
3-day old aerobic (control)	100%	0.321 ± 0.004	0.078 ± 0.002
3-day old germinated under anoxia; immediately transferred to air	100%	0.343 ± 0.018	0.084 ± 0.006
1-days anerobic + 3-days aerobic	75%	0.299 ± 0.010	0.049 ± 0.004*
2-days anerobic + 3-days aerobic	28%**	0.288 ± 0.009*	0.016 ± 0.003***
3-days anaerobic + 3-days aerobic	0%	–	–

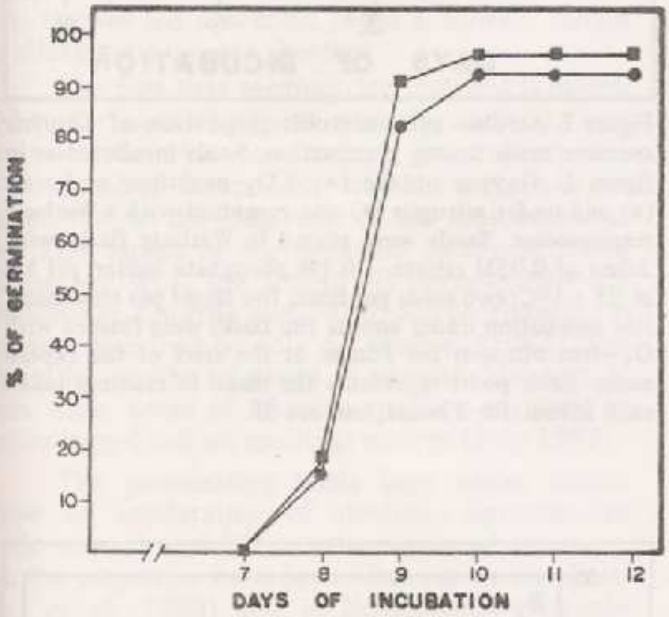


Figure 1. Effect of anoxia upon the germination of *Chorisia speciosa* seeds. Five Petri dishes containing 10 seeds each were placed in air (●) or in an anaerobic incubator (■) at 25°C in the dark. Seeds were surface sterilized with 6% sodium hypochlorite, washed and placed in the Petri disches.

Table 2. Effect of anoxia upon growth in lenght of radicle of *Chorisia speciosa*. Seed and/or seedling incubated in air or in an anaerobic workbench with an atmosphere of 85% N_2, 19% H_2 and 5% CO_2 at 25 ± 1°C; in Petri dishes with two layers of moist Whatman No. 3 paper. 5 seeds per Petri dish, 5 Petri dishes per treatment. Significance was tested in relation to the controls by *t* test, *significant at the 5% level, **significant at the 1% level, ***significant at the 0.1% level.

Treatment	Radicle length (cm)
Seed	0.058 ± 0.008 cm.
Germinated aerobically (control)	0.61 ± 0.019 cm.
Germinated anerobically	0.58 ± 0.012 cm.
2-day old aerobic (control)	1.67 ± 0.14 cm.
2-day old anaerobic	0.54 ± 0.021 cm.***
1-day anerobic 2-days aerobic	1.18 ± 0.040 cm.*
2-days anaerobic 2-days aerobic	0.46 ± 0.059 cm.***

Table 3. Content of ethanol, lactate and malate present in *Chorisia speciosa* seedlings germinated and kept in the anaerobic workbench for 1, 2 and 3 days. Seedlings incubated in an anaerobic workbench with an atmosphere of 85% N_2, 10% H_2 and 5% CO_2 at 25 ± 1°C. Five replicates containing 2 seedlings each. Values presented are mean ± SE, significance was tested by *t* test, *significant at the 5% level.

Days of incubation after germination	Metabolite		
	Ethanol	Lactate	Malate
1 day	30.08 ± 1.94	0.257 ± 0.018	0.123 ± 0.020
2 days	25.60 ± 2.69	0.116 ± 0.013*	0.098 ± 0.012
3 days	28.41 ± 2.11	0.229 ± 0.025	0.087 ± 0.010

Effect of anoxia upon the metabolism of the germinating seed – Both aerobic and anaerobic metabolic rates are very low before the emergence of the radicle (Figure 2). The gas exchange quotient (volume of CO_2 evolved/volume of O_2 consumed) is slightly above unity, which indicates that even in air the metabolism of the germinating seed is not fully aerobic. After the emergence of the radicle there is a marked increase in both aerobic and anaerobic respiration rates (Figure 2).

The production of malate shows an increasing trend until the sixth day of incubation in air, thereafter ethanol is the main product to accumulate in the germinating seeds (Figure 3A), which supports the conclusion that even in air the metabolism is not fully aerobic. The level of lactate did not change significantly throughout the germination period.

Ethanol was the main product to accumulate during the period of incubation in the anaerobic workbench. After six days of anaerobic incubation the amount accumulated under anoxia is significantly greater than that in air (Figure 3B). After nine days of incubation irrespective of whether it is aerobic or anaerobic the radicle emerges and at the same time there is a marked increase in both aerobic and anaerobic metabolism. As a result, the level of ethanol in those seedlings incubated under anoxia exceeds that of seedlings incubated in air to a much greater degree (sixteen-fold) than that observed in the earlier stages of germination up to six days (Figures 3A and B).

The results presented in table 3 suggest that also the metabolism is inhibited while the seedlings are kept in the anaerobic workbench, since there are no significant changes in the levels of ethanol and malate, and the fluctuation of the level of lactate has no biological significance.

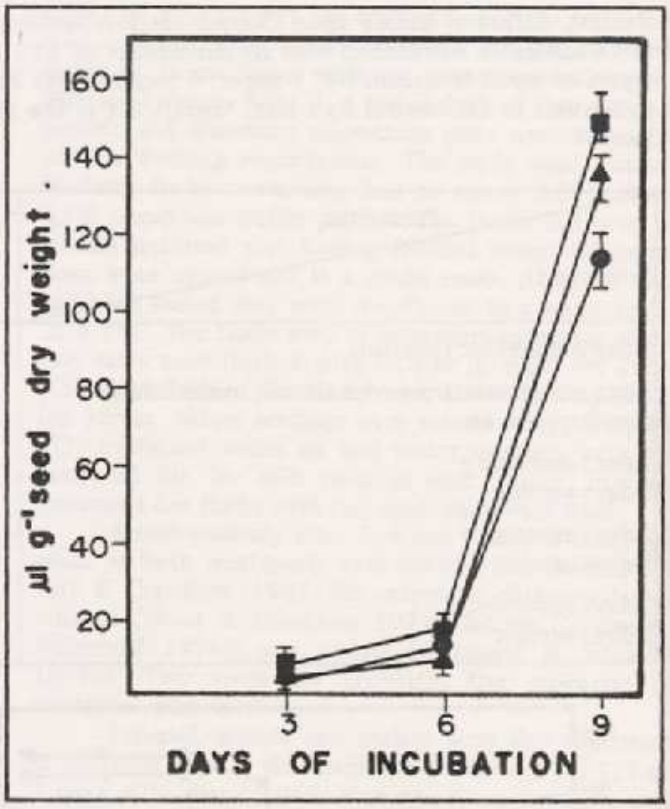
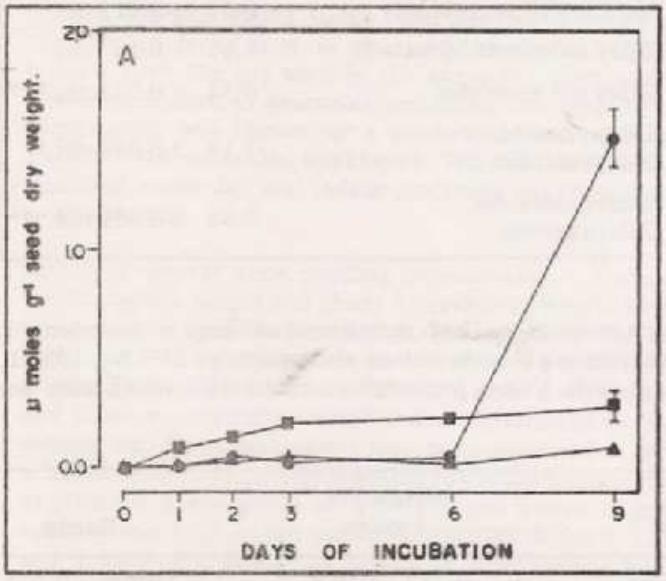


Figure 2. Aerobic and anaerobic respiration of *Chorisia speciosa* seeds during germination. Seeds incubated as in figure 1. Oxygen uptake (●), CO_2 evolution under air (■) and under nitrogen (▲) was measured with a Warburg respirometer. Seeds were placed in Warburg flasks with 2.0ml of 0.05M citrate – 0.1M phosphate buffer, pH 5.4 at 25 ± 1°C; two seeds per flask, five flasks per treatment. For incubation under anoxia the flasks were flushed with O_2–free nitrogen for 15min. at the start of the experiment. Each point represents the mean of readings taken each 15min. for 2 hours; bars are SE.



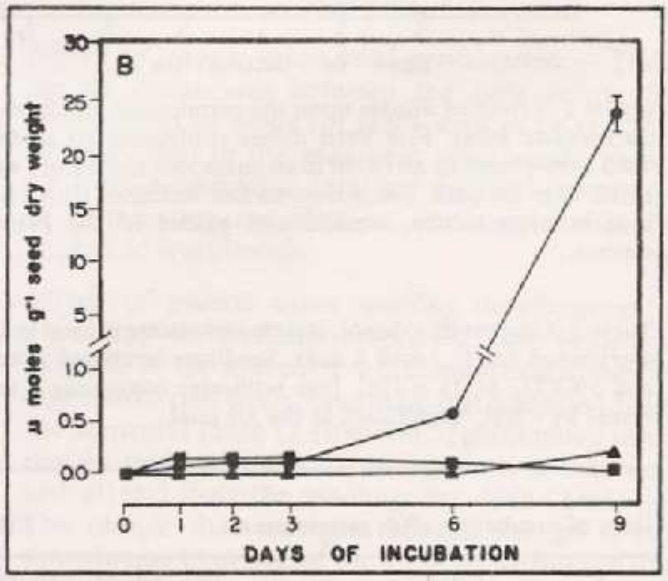


Figure 3. Content of ethanol (●), lactate (▲) and malate (■) in seeds of *Chorisia speciosa* during germination in air (A) and under anoxia (B). Seeds incubated as in figure 1. Each point is the mean of five replicates, with five seeds per replicate; bars are SE.

Discussion

Seed germination under anoxia has only been reported hitherto for the wetland species *Oryza sativa* (Öpik 1973, Vartapetian *et al.* 1978), *Zizania aquatica* (Campiranon & Koukkari 1977) and *Echinochloa crus-galli* (Kennedy *et al.* 1980). The present investigation shows that in a dry-land forest species, where gaseous exchange is apparently impeded by the production of a muscilagenous gel, oxygen is not necessary for germination to proceed successfully as far as radicle protusion. It was not possible to establish if radicle protusion was due to cell extension alone or whether cell division took place. Shoot extension under anoxia has been reported for a number of species (Vartapetian *et al.* 1978, Barclay & Crawford 1982) but claims for cell division under anoxia have only been made in respect of rice (Öpik 1973, Kordan 1976). The ecological advantage of radicle protusion in *Chorisia speciosa* under anoxia is directly comparable to the examples of shoot extension noted above in that it gives the anaerobic organ a 'snorkel' device to alleviate its oxygen shortage.

The fact that seedling development is inhibited under anoxia suggests that the ability to germinate in the absence of oxygen is a property that is restricted to overcoming only the oxygen deprivation due to the formation of the muscilagenous gel. This aspect of the tolerance to anoxia is not connected with the ability of three-month old seedlings of *Chorisia speciosa* to survive flooding as already described by Joly & Crawford (1982). The limitation to the tolerance of anoxia is also seen in that when seeds of *Chorisia speciosa* are sown in water-logged soil no seedlings emerge (Joly 1982).

The germinating seeds kept under anoxia show an acceleration of alcoholic fermentation with ethanol and CO_2 as the main end products. Similar responses have been observed in rice (Bertani *et al.* 1980) and in *Echinochloa crus-galli* (Rumpho & Kennedy 1981) but in these two species more than 80% of the ethanol produced is found in the imbibition solution. Although ethanol toxicity has recently been questioned by Jackson *et al.* (1982) there is a positive correlation between the increase in time of exposure to relatively high concentrations of ethanol and inhibition of seedling development which leads to the death of the germinated seeds after three days of incubation. The correlation between exposure to high concentrations of ethanol and loss of seed or seedling viability has also been observed in peas (Barclay & Crawford 1981) and in two tropical Leguminosae species necessary level, and/or vital metabolic routes could have been inhibited due to lack of oxygen.

Although naturally exposed to a period of anoxia *Chorisia speciosa* seeds do not present a diversification of end products of the anaerobic metabolism as reported for chick peas (Aldasoro & Nicolas 1980). An acceleration of glycolysis with the accumulation of large amounts of ethanol has also been observed during the natural period of anoxia in *Peltophorum dubium* and *Enterolobium contortisiliquum* (Joly 1982).

Considering these results and those of Harberd & Edwards (1982) it appears that during the initial stages of germination alcoholic fermentation is responsible for keeping the energy supply at the required level. Any prolongation of the natural period of anoxia into the phase when metabolism accelerates leads to the death of the seedling.

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(Joly 1982). Nevertheless, one can not overlook the fact that in all these cases seed or seedling death might have been caused by other factors. Alcoholic fermentation, although accelerated, might have been incapable of keeping the energy supply at the

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