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Fermentation and adenylate metabolism of *Hedychium coronarium* J. G. Koenig (Zingiberaceae) and *Acorus calamus* L. (Araceae) under hypoxia and anoxia

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Summary

1. Rhizomes of wetland plants are subjected to periods of hypoxia and/or anoxia by the seasonal or permanent waterlogging of their growing sites. *Hedychium coronarium,* the White Ginger, and *Acorus calamus,* the Sweet Flag, have their origin in India and were introduced into Latin America and Europe, respectively, more than three centuries ago. The White Ginger grows in humus-rich, shaded or semi-shaded areas subjected to waterlogging but it is never totally submersed, while the Sweet Flag grows at lake margins and is totally submersed during winter.

2. Winter rhizomes of both species were cultivated in water culture in a greenhouse. The end products of fermentation (ethanol, lactic acid, malic acid), overall rhizome pH, the adenylate pool of nucleotides, the energy charge and their capacity to resume growth, were measured after periods of 1, 2, 4, 8 and 16 days of anoxia and hypoxia. In all cases metabolic responses were also determined in rhizomes allowed to recover for 24 h in air.

3. Ethanol was the main fermentation end product in both species, reaching higher concentrations in the anoxia-treated rhizomes. In *H. coronarium*, there was also a significant increase in the levels of lactic acid, with a considerable drop in overall rhizome pH.

4. Anoxia and hypoxia induced, in both species, a significant drop in the energy charge values. Control plant rhizomes and rhizomes allowed to recover in air for 24 h had energy charge values of around 0·8. In rhizomes subjected to stress these values were lower, around 0·50 in *A. calamus* and as low as 0·3 in *H. coronarium.*

5. Although in both species there is also a decrease in the amount of total nucleotides, it was much more drastic in the case of anoxia treated rhizomes of *H. coronarium.* The pH drop was most probably the underlying cause of the metabolic disarray that lead to a depletion of the adenylate pool and, finally, failure to regenerate after 16 days of anoxia.

6. The results also show that energy charge values without measurements of the total adenylate pool may give a misleading impression of fitness. Thus, the anaerobic metabolism of *H. coronarium* is less efficient and more harmful than that of *A. calamus* and, although considerably tolerant to hypoxia, it does not tolerate strict anoxia as the latter species does.

Key-words: Adenylate energy charge, anaerobic metabolism, waterlogging

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Introduction

The study of the different aspects of anoxia and hypoxia tolerance in rhizomes (perennial organs with ample energy reserves and buds for regeneration) may result in a better understanding of the ecophysiology of wetland species (Brändle & Crawford 1987).

Hedychium coronarium J.G. Koenig (Zingiberaceae), the white ginger from Eastern India (Dahlgren, Clifford & Yeo 1985), was introduced into Brazil by the Portuguese some 300 years ago. Nowadays it is widespread in the Neotropical region, always growing in humus-rich shaded or semi-shaded

areas subjected to seasonal or perennial waterlogging. The sympodial rhizomes are horizontal and creeping at the soil surface, and are rich in starch grains. Plant stands are usually 2 m high and, therefore, even during the highest floods, the aerial part is never totally submerged. The large white flowers resemble a butterfly and have a strong aroma when opening at dusk.

The very aggressive growth of this species is a hazard for the regeneration of natural gallery forest trees, because the seedlings have no chance to compete with the aggressive propagation of the rhizomes. The objective of the present study was to understand the anoxia and/or hypoxia tolerance mechanism of the white ginger. A better understanding of this mechanism could be a useful tool for the control of the expansion of this species in the rehabilitation plots of gallery forest in the interior of the State of São Paulo (Joly 1994a).

The study was carried out comparing the behaviour of *H. coronarium* rhizomes and those of *Acorus calamus* L. (Araceae). Also originally from India, the sweet flag was introduced into Europe at least three centuries ago. It is an amphibious plant that colonizes the littoral zone of lakes, swamps or slow running water (Dykyjová 1980), as an emergent macrophyte during spring and summer, but totally submersed during winter. Its ability to survive long periods of anoxia is well documented (Brändle 1991; Henzi & Brändle 1993).

Materials and methods

PLANT MATERIAL AND GENERAL CONDITIONS

Rhizomes of the white ginger *H. coronarium* collected in Campinas, State of São Paulo, Brazil, in July/August, and of the sweet flag *A. calamus* collected in the lake of Moosseedorf, Bern, Switzerland in October/November, were cultivated in hydrocultures in an ordinary glasshouse at 16–20 °C. The aerial part and the roots of the collected rhizomes were cut and the rhizomes were surface sterilized for 10 min with 2% sodium hypochlorite and then rinsed for another 10 min in tap water, before planting. The development of these plants was considered as control.

After some months in the glasshouse the rhizomes selected for experimental purpose underwent the same procedure again. Roots and aerial parts were removed and the rhizome was surface sterilized.

Anoxia treatments were carried out in an anaerobic workbench (Forma Scientific, Marietta, OH, USA). The hypoxia treatments were carried out in Perspex boxes with an inner coverage of aluminium foil, flushed continuously with a gas mixture with 1% oxygen. Oxygen concentration, in these incubation boxes, was monitored with an Oxygen Analyser (LF 700D, Toray, Lippke, Neuwied, Rhein, Germany). All experiments (anoxia and hypoxia) were carried out in the dark at room temperature (20 °C) and with rhizomes covered with a moist filter paper to avoid dessication during the incubation period. During the 24 h recovery period the rhizomes, still covered with moist filter paper, were incubated in Perspex boxes flushed continuously with air.

In all the experiments, at the end of the incubation period, some rhizomes were replanted in water culture in the greenhouse to verify their ability to restore growth; i.e. the survival capacity of the species under anoxia and hypoxia. Results were analysed, and compared with the control plants, 30 days after re-planting.

EXTRACTIONS AND MEASUREMENTS

At the end of the incubation period the rhizomes were dropped into liquid nitrogen to stop immediately any metabolic changes. The epidermis of the frozen tissue was then removed and the frozen tissue was pulverized in a dismembrator (Mikro II, B. Braun, Melsungen, Germany) for 1 min. The frozen powder was sieved and placed in plastic vessels kept in liquid nitrogen. Half a gram of this powder was homogenized and de-proteinized in 9 ml of perchloric acid (6%) with a polytron (Kinematica, Luzern, Switzerland), and subsequently centrifuged for 10 min at 24 000 × g at 0 °C. The supernatant was neutralized with ice cold 5 M K_2CO_3 solution, in an ice bath. Potassium perchlorate was removed by a second centrifugation. Aliquots of the extract, kept in liquid nitrogen, were used for the determination of fermentation products and adenosine nucleotides. Aliquots of the same powder were extracted in 0·2 M sodium-phosphate buffer pH 8·0 before protein determination with a Bio Rad test kit (Bio-Rad, Richmond, CA, USA).

In order to measure the pH of anoxia incubated rhizomes, 1 g of fresh material was extracted in 10 ml bidistilled water with a polytron (Kinematica, Luzern, Switzerland). Measurements of five replicates were carried out immediately after extraction.

Spectrophotometric determination of ethanol, lactic acid and malic acid concentrations were carried out at 365 nm, using test kits (Boehringer, Mannheim, Germany). The determinations of adenosine nucleotides were performed with the luciferin-luciferase system (Lumac/3M, Schaasberg, The Netherlands) according to Sieber & Brändle (1991).

STATISTICS

Statistical data analyses were carried out using analyses of variance, considering only the points with at least four replicates available. Significance was defined at the 95% level.

Table 1. Growth responses of rhizomes of *Hedychium coronarium* and *Acorus calamus* subjected to hypoxia and anoxia after 1 month of recovery in the greenhouse

	1 day	2 days	4 days	8 days	16 days
H. coronarium					
Control	++	++	++	++	++
Hypoxia	++	++	++	++	+
Anoxia	++	++	++	+	–
A. calamus					
Control	++	++	++	++	++
Hypoxia	++	++	++	++	+*
Anoxia	++	++	++	++	++

++, Vigorous growth; +, growth; – no growth.
*Lateral bud growth because apical buds were dead.

Results

ANOXIA AND HYPOXIA SURVIVAL

Table 1 summarizes the results of the survival experiment. *Hedychium coronarium* rhizomes survived all hypoxia treatments well and also up to 8 days of anoxia. After 16 days of anoxia the rhizomes failed to regenerate. On the other hand, *A. calamus* plants survived to all anoxia and hypoxia treatments.

FERMENTATION END PRODUCTS

Ethanol is the main fermentation product of both species and increased almost linearly at the beginning of the incubation period (Fig. 1a,b). Later on the curve reached a maximum in the case of *H. coronarium* but *A. calamus* sustained a linear increase of ethanol levels under anoxia throughout the experimental period.

Compared with the hypoxia treatments the rate of accumulation was significantly higher under anoxia in both species. During the 24 h of recovery, both species showed a high capacity to reduce the ethanol concentration in the rhizomes. Ethanol levels reached values above 20 μmol $\times$ mg protein^{-1} in *H. coronarium* and above 10 μmol $\times$ mg protein^{-1} in *A. calamus*.

The accumulation of lactic acid (Fig. 1c,d) followed the same pattern but at a much lower scale, not reaching more than 2·5 μmol $\times$ mg protein^{-1} in the case of *H. coronarium* and 0·25 μmol $\times$ mg protein^{-1} in the case of *A. calamus*. In all the 24 h air-recovery treatments there was a drop in lactic acid concentrations, most obviously in the hypoxia treatments of *H. coronarium* (Fig. 1c).

Malic acid concentrations (Table 2) did not change in the rhizomes of *H. coronarium* subjected to hypoxia or to anoxia. Nevertheless, after 24 h of recovery in air, the values were slightly lower than in control and incubated plants. In *A. calamus,* both treatments induced a significant increase in the levels of malic acid. Levels obtained for anoxia treated plants were significantly higher than those of hypoxia treated plants but the recovery capacity was somewhat higher in the latter plants.

There was a significant acidification of the rhizomes extracts of *H. coronarium* (Table 2), with a decrease of one unit after 16 days of anoxia. The pH in *A. calamus* did not change significantly (Table 2).

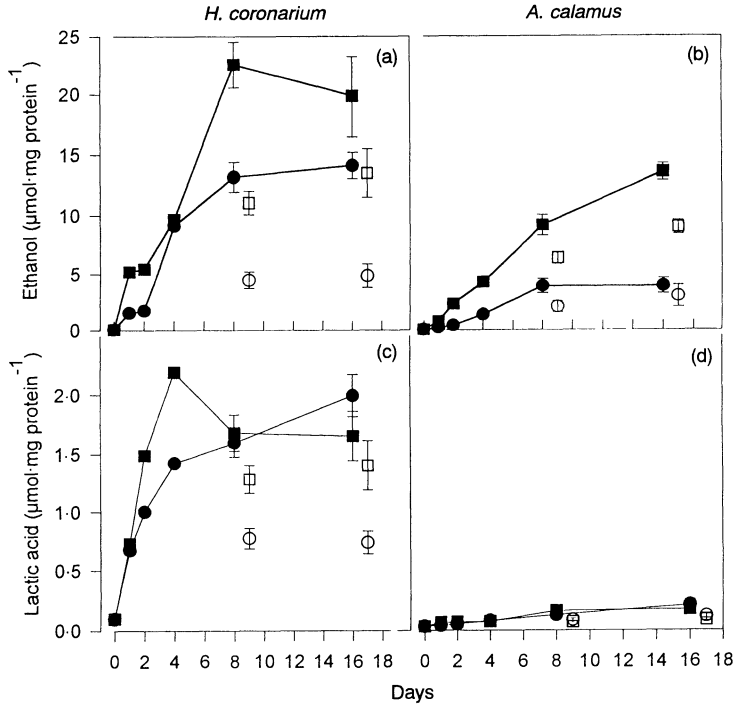

Fig. 1. *Hedychium coronarium* and *Acorus calamus* rhizome concentrations of ethanol (a & b) and lactic acid (c & d), under anoxia (black squares), hypoxia (black circles) and after 24 h air recovery (hollow symbols). Data in μmol $\times$ mg protein^{-1}. Bars area means ± SD.

ENERGY CHARGE AND ADENYLATE NUCLEOTIDES

Anoxia and hypoxia induced, in both species, a significant drop in the energy charge values. Control plant rhizomes and rhizomes allowed to recover in air for 24 h had energy charge values of around 0·8. In rhizomes subjected to stress these values were lower, around 0·50 (Fig 2a,b). The very low value found in *H. coronarium* plants subjected to 16 days anoxic treatment, and the large standard deviations, suggest that these rhizomes were dying (Fig. 2a).

The amount of total nucleotides in the rhizomes of *H. coronarium* dropped drastically at the onset of the stress (Fig. 2c). In both treatments there seems to be a slight recovery of the levels of total nucleotides after 4 days of incubation. However, while in the hypoxia treatment the values are stable thereafter, in the anoxia-treated rhizomes the adenylate pool continues to decline. The recovery capacity of rhizomes kept in air for 24 h is not significant in the hypoxia-treated plants, however in the anoxia treated rhizomes it is high after 8 days of incubation decreasing thereafter (Fig. 2c). *Acorus calamus,* on the other hand, had a significant capacity to recover the level of total

nucleotides following hypoxia and anoxia (Fig. 2d). Moreover, here too both treatments induced a significant drop in the concentrations of total nucleotides, before stabilizing at intermediate values in the hypoxic treatment. The decrease following anoxia is slow in comparison with *H. coronarium* (Fig. 2d).

Discussion

ANOXIA AND HYPOXIA SURVIVAL AND FERMENTATION END PRODUCTS

The survival of plants under reduced oxygen availability has led to the proposal of contradictory theories, but the enhancement of the fermentative pathway (Crawford 1992), as also seen in the case of *H. coronarium* and *A. calamus*, is certainly the most widespread response.

The differences between the survival capacity of both species (Table 1) can be linked with differences in the final steps of their energy metabolism. The fermentative pathway induced by anoxia and hypoxia in rhizomes of the sweet flag led to a large accumulation of ethanol but not of lactate (Fig. 1). Nevertheless, the levels of ethanol present in this winter rhizome were considerably lower than those found in summer rhizomes (Sieber & Brändle 1991). Malic acid accumulation in the rhizomes of *A. calamus* probably was not directly linked with the energy metabolism and as a weak acid it did not affect overall rhizome pH (Table 2).

On the other hand, the significant change in overall rhizome pH (Table 2) observed in the white ginger was because of lactic fermentation (Fig. 1). Lactic acid levels were similar to those reported by Sieber & Brändle (1991) for anoxia non-tolerant potato tubers.

It is important to bear in mind that pH changes induced by malic acid accumulation are low and usually do not affect cell metabolism because largely malic acid is stored in the vacuoles (McManmon & Crawford 1968; Joly 1994b). However, lactic acid stays in the cell cytoplasm, leading to an overall change in metabolism that is pH regulated (Davies 1986).

In *H. coronarium* the large amount of lactic acid found in rhizomes subjected to 4 days of anoxia, and the lower capacity to reduce these levels under post-anoxia when compared with the hypoxia treated ones, may result in cytoplasmatic acidosis (Roberts *et al.* 1989). Prolonged stress periods (8 and 16 days) of anoxia, may have led to irreversible cell damage that resulted in the failure to resume growth of rhizomes under post-anoxia (Table 1). The slower increase in lactic acid levels in hypoxia treated rhizomes (Fig. 1) of *H. coronarium* may delay major damage to cell metabolism but even these rhizomes were showing signals of a gradual decline in their survival capacity when the same level of lactic acid was reached (Table 1).

ENERGY CHARGE AND ADENYLATE NUCLEOTIDES

Transfer of plants, or plant organs, to an oxygen-deprived atmosphere induces significant drops in the adenylate energy charge values (Pradet & Raymond 1983). In the winter rhizomes of *A. calamus* the maintenance of medium adenylate energy charges throughout the experimental period (Fig. 2), differs from the data reported by Sieber & Brändle (1991) for summer rhizomes, where energy charge recovery under anoxia occurred. The same seasonal-dependent behaviour has been shown for *Schoenoplectus lacustris* (Steinmann & Brändle 1984). Nevertheless, in all treatments, 24 h of recovery were sufficient for a

Table 2. Tissue concentration of malic acid in rhizomes of *Hedychium coronarium* and *Acorus calamus* subjected to anoxia and hypoxia as well as following a 24 h recovery period in air, and tissue extract pH of anoxia treated rhizomes. Values of malic acid are presented in $\mu mol \times mg\ protein^{-1}$ and are averages ± SD

	pH	anoxia	24 h recovery	hypoxia	24 h recovery
H.Coronarium					
0 days	6·2±0·14	1·12±0·10	(= control)		
8 days	5·6±0·16	1·11±0·14	0·70±0·09	0·99±0·07	0·79±0·08
16 days	5·2±0·20	0·95±0·17	0·48±0·09	0·96±0·13	0·79±0·09
A. Calamus					
0 days	6·1±0·07	0·52±0·06	(= control)		
8 days	5·8±0·14	1·56±0·14	1·26±0·08	1·19±0·13	0·44±0·06
16 days	6·0±0·10	1·92±0·08	1·53±0·08	1·20±0·10	0·54±0·04

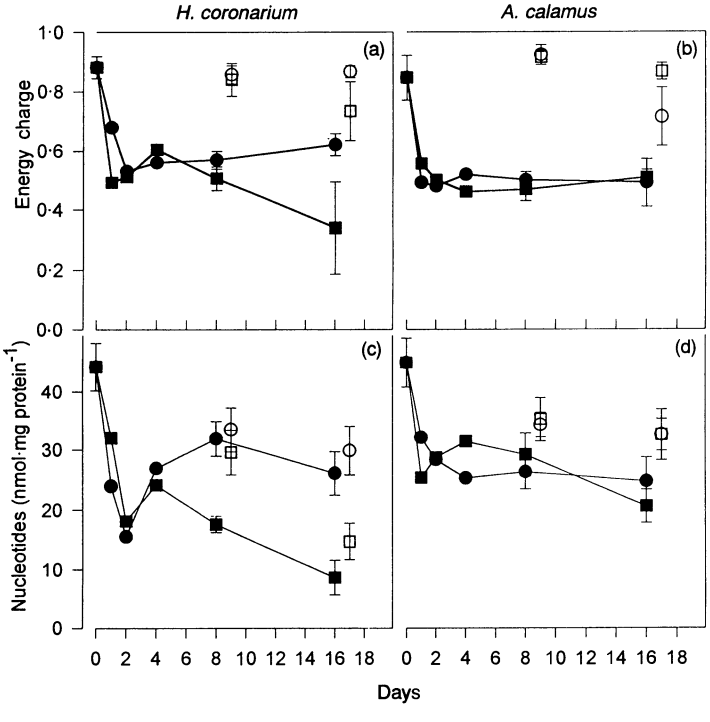

Fig. 2. *Hedychium coronarium* and *Acorus calamus* adenylate energy charge (a & b) and rhizome concentrations of the adenosine nucleotides ATP + ADP + AMP (c & d) under anoxia (black squares), hypoxia (black circles) and after 24 h air recovery (hollow sytmbols). Data in $nmol \times mg\ protein^{-1}$. Bars area means ± SD.

rapid re-establishment of adenylate energy charge values similar to that of control plants. Similar data were obtained with summer rhizomes of *H. coronarium* in a later experiment (R. Brändle, in preparation).

In the case of the white ginger, an additional significant drop in the adenylate energy charge values (Fig. 2) of anoxia-treated rhizomes occurred between 4 and 8 days, when the fermentative metabolism (Fig. 1) also started to show signs of disarray. Dramatic changes in energy metabolism occur in plants (Roberts *et al.* 1989) as well as in animals (Fan *et al.* 1993) when lactic acid concentrations increase above the buffering capacity of the cells. In well-adapted plants, such as *A. calamus,* high levels of amides, and especially arginine (Weber & Brändle 1994), may increase this buffering capacity. This is a further explanation for the low changes in the pH of the extracts of anoxia incubated rhizomes despite malic acid concentrations increasing more than threefold (Table 2). In addition, the preservation of a regulated metabolism in *A. calamus* under anoxia is also highly dependent on membrane integrity and function (Henzi & Brändle 1993).

The most striking differences between both species, and between the hypoxia and the anoxia treatments in *H. coronarium,* lies in the adenylate pool (Fig. 2). Under anoxia, rhizomes of the white ginger show a steady drop in this pool. The depletion of the adenylate pool is irreversible after 16 days of anoxia. This is a consequence of the metabolic disarray and is most probably the underlying cause of the failure to resume growth (Table 1). This response is similar to that reported for potato tubers by Sieber & Brändle (1991), although in that case depletion and death already occurred after 2 or 3 days.

These results also show that isolated values of energy charge, without an estimation of the change of the total adenylate pool, may give a misleading impression of the conditions of rhizomes that are actually dying. The best example can be seen in Fig. 2, where after 24 h in air the rhizomes of *H. coronarium* presented energy charge values above 0·7 but were unable to resume growth.

Although typical of waterlogged areas *H. coronarium,* is rarely subjected to strict anoxia because the aerial part is always in an oxygen-rich atmosphere. Summer rhizomes of *H. coronarium* growing in a waterlogged area in Campinas (SE Brazil) contained ethanol ($0{\cdot}86 \pm 0{\cdot}11\ \mu mol \times mg\ protein^{-1}$) and lactic acid ($0{\cdot}24 \pm 0{\cdot}03\ \mu mol \times mg\ protein^{-1}$) concentrations similar to those found in rhizomes subjected to mild hypoxia.

Furthermore, using methylene blue in a 1% reduced agar medium (Armstrong & Armstrong 1988) it was possible to prove considerable oxygen diffusion from the aerial part to the rhizomes, roots and rhizosphere of *H. coronarium* rhizomes (data not shown). Aerenchyma formation in the roots of wetland species is genetically controlled and is not a response to external conditions (Konings & Lambers 1991). However, the tolerance of *H. coronarium* to short periods of anoxia is similar to that reported by Monk, Crawford & Brändle (1984) for *Iris germanica* and is much shorter than that of typical wetland plants.

The results presented reinforce the idea that the differences between anoxia and hypoxia tolerance is rather a question of metabolic regulation than the evolution of new pathways (Henzi & Brändle 1993). Winter rhizomes of *A. calamus* are as tolerant to anoxia as the summer rhizomes studied by Sieber & Brändle (1991) and they are also hypoxia tolerant. Winter rhizomes of *H. coronarium* are considerably hypoxia tolerant but not at all anoxia tolerant. Its anaerobic metabolism is less efficient and more harmful than that of the sweet flag.

Therefore one possible way of controlling the expansion of this alien species in native gallery forest areas could be similar to that proposed by Birch & Cooley (1983) for *Zizaniopsis miliacea.* In watersheds controlled by artificial reservoirs, the harvesting of the aerial part, followed by the maintenance of a high water table for a period up to 2 weeks, will kill a large proportion of rhizomes of *H. coronarium.* On the other hand, the flood tolerant seedlings of native trees would survive (Joly 1991) and thus the natural regeneration process of the gallery forest would be preserved and/or restored.

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