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## Age Mediated Effects on the Alternative Respiratory Pathway in Leaves of *Rhodiola rosea* and *Ajuga reptans*

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**Abstract:** The influence of aging processes on respiration and the ratio of respiratory pathways in the leaves of *Rhodiola rosea* L. and *Ajuga reptans* L. under natural conditions was investigated. Young leaves had a higher respiration rate and higher activity of the alternative respiratory pathway than did mature leaves. The capacity of the alternative respiratory pathway did not show significant differences between the young and mature leaves of *R. rosea* and increased in the mature leaves of *A. reptans* (from 60% up to 88%). This fact may be explained by the higher requirements of growing tissues for metabolites. In this case, the alternative pathway could be responsible for the continuous activity of the citric acid cycle and glycolysis, and, therefore, deliver additional metabolites. The decline in activity of the alternative pathway with aging could be explained by the lower demand of the older leaves in metabolites. The high capacity of the alternative respiratory pathway in the mature leaves may be considered evidence of a high respiratory potential. We believe that this phenomenon may be explained by the fact that leaves are frequently confronted with numerous stress events during their life and, as a consequence, increase the respiratory capacity as a tool of stress resistance.

**Key Words:** alternative respiratory pathway, respiration, alternative oxidase, leaves, age, *Rhodiola rosea*, *Ajuga reptans*

### Introduction

There are two possible pathways of respiration in higher plants – a cytochrome pathway and an alternative pathway. The cytochrome pathway is the main source of ATP in plants, while the terminal cytochrome oxidase (COX) is the key enzyme of the electron transport chain. The COX is highly sensitive to cyanide. One of the striking features of the alternative oxidase (AOX), and, therefore, the alternative pathway, is its resistance to cyanide. The alternative pathway is considered to be nonphosphorylating (for an excellent review see Vanlerberghe & McIntosh, 1997).

However, both the role of AOX and how spread AOX is in nature, is still a subject of debate (Henry & Nyns, 1975; Ordentlich et al., 1991; Day et al., 1995). Better knowledge of different aspects of the alternative pathway is of great scientific value (Maxwell et al., 1999). Development state is one factor known to affect the activity of AOX (Vanlerberghe & McIntosh, 1997). However, more experimental data to reveal the influence of the age of leaves on the activity of AOX are urgently needed (Kumar and Knowles, 1996).

*A. reptans* L. and *R. rosea* L. are common members of natural assemblages at middle and higher latitudes, respectively. They differ in their prevalence for light conditions; *A. reptans* is shade-enduring while *R. rosea* has considerably higher light requirements. The aim of the present study was to reveal the effects of age on the activity of the respiratory alternative pathway in leaves of *Ajuga reptans* and *Rhodiola rosea*.

### Materials and Methods

Both *A. reptans* and *R. rosea* were studied under natural conditions with sufficient light supply. All experiments were carried out at 62° 52' N near Syktyvkar, north-east Russia. Young (end of May – beginning of June) and mature (middle of July) leaves were taken into consideration. The leaves were continuously sampled from 15 plants at 9:00 a.m. in order to avoid any possible artefacts caused by photosynthesis metabolites.

Small pieces (0.0028 dm<sup>2</sup>) were cut from the leaves sampled (three pieces per leaf). The pieces were placed in a cuvette and respiration was estimated by oxygen

consumption ( $\text{mmol O}_2 \text{ g}^{-1} \text{FW h}^{-1}$ ) with the aid of a Clark-type electrode at 2°C. The activity and capacity of the respiratory pathways were studied using the method of specific inhibitors (Bahr & Bonner, 1973; Theologis & Laties, 1978). Benzhydroxamic acid (BHAM, 25.0 mM) and KCN (5.0 mM) were used as inhibitors of AOX and COX, respectively. 2,4-dinitrophenol (DNP, 0.5 mM) was used as the uncoupler. The inhibitors were used both separately and together. All experiments were carried out in triplicate.

The activity of the alternative pathway ( $v_{\text{alt}}$ ) was calculated as the difference between total respiration (in the absence of inhibitors) and respiration in the presence of 25.0 mM BHAM. Residual respiration ( $v_{\text{res}}$ ) was measured in the presence of inhibitors of both the AOX and COX (25.0 mM BHAM + 5.0 mM KCN). Activity of the cytochrome pathway ( $v_{\text{cyt}}$ ) was calculated as the difference between respiration in the presence of the 25.0 mM BHAM and residual oxygen uptake. Capacity of the alternative pathway ( $v_{\text{alt}}$ ) was calculated as the difference between respiration in the presence of 5.0 mM KCN and residual respiration. Capacity of the cytochrome pathway ( $v_{\text{cyt}}$ ) was calculated as the difference between respiration in the presence of the 25.0 mM BHAM + 0.5 mM 2,4-DNP and residual oxygen uptake. The engagement of respiratory pathways ( $p_{\text{alt}}$  and  $p_{\text{cyt}}$ ) was estimated as the relation between respective activity and capacity.

Statistical analyses including the t-test were performed in the computer package Minitab 13.0.

## Results and Discussion

Results showing the influence of inhibitors on respiration in leaves of different age are presented in Tables 1 and 2. The young actively growing leaves had considerably higher total respiration compared to the older leaves. This fact is not surprising due to the higher requirements of younger leaves for energy and metabolites, while respiration in older leaves declines mainly due to a decrease in growth rate (Larcher, 1995). The young leaves were more sensitive to the inhibitor of AOX (BHAM). BHAM caused a decline by 30% (*R. rosea*) and 41% (*A. reptans*) in the young leaves and by 17% (*R. rosea*) and 18% (*A. reptans*) in the mature leaves (Figures 1 and 2). Cyanide led to a decrease in respiration by nearly 50% in both younger and older leaves of *R. rosea*. However, the differences in this case were not statistically significant. On the other hand, the cyanide-resistant respiration of leaves of *A. reptans* increased significantly with aging. In this case, cyanide inhibited the respiration of younger leaves by 30% but its effect on the mature leaves was not found to be statistically significant. 2,4-DNP stimulated the respiration of both young and mature leaves in *R. rosea* as well as in *A. reptans*.

Results on the activity and capacity of respiratory pathways are shown in Tables 3 and 4. Activity of the alternative respiratory pathway in the young leaves significantly exceeded that in the mature leaves of both *R. rosea* and *A. reptans*. The capacity of the alternative respiratory pathway did not significantly change in *R. rosea* with aging of the leaves. However, the capacity of the alternative respiratory pathway significantly increased

Table 1. Influence of inhibitors on respiration ( $\mu\text{mol O}_2 \text{ g}^{-1} \text{FW h}^{-1}$ ) in leaves of *R. rosea*. \*- the difference is statistically significant ( $P \leq 0.05$ ) compared to the control. The values of  $\pm$ SE are shown.

Inhibitors	Young leaves	Mature leaves
Control	13.3 $\pm$ 0.5	7.2 $\pm$ 0.2
BHAM (25.0 mM)	9.3 $\pm$ 0.5*	6.0 $\pm$ 0.4*
KCN (5.0 mM)	7.6 $\pm$ 0.8*	3.6 $\pm$ 0.4*
BHAM (25.0 mM) + KCN (5.0 mM)	3.0 $\pm$ 0.2*	1.3 $\pm$ 0.1*
2,4-DNP (0.5 mM)	20.5 $\pm$ 1.3*	8.4 $\pm$ 0.7
2,4-DNP (0.5 mM) + BHAM (25.0 mM)	15.0 $\pm$ 0.9	7.4 $\pm$ 0.6

Table 2. Influence of inhibitors on respiration ( $\mu\text{mol O}_2 \text{ g}^{-1} \text{FW h}^{-1}$ ) in leaves of *A. reptans*. \*- the difference is statistically significant ( $P \leq 0.05$ ) compared to the control. The values of  $\pm$ SE are shown.

Inhibitors	Young leaves	Mature leaves
Control	13.9 $\pm$ 0.8	8.7 $\pm$ 0.5
BHAM (25.0 mM)	8.2 $\pm$ 0.4*	7.1 $\pm$ 0.4*
KCN (5.0 mM)	9.7 $\pm$ 0.7*	9.2 $\pm$ 0.3
BHAM (25.0 mM) + KCN (5.0 mM)	1.4 $\pm$ 0.2*	1.6 $\pm$ 0.1*
2,4-DNP (0.5 mM)	20.7 $\pm$ 0.9*	13.4 $\pm$ 0.4*
2,4-DNP (0.5 mM) + BHAM (25.0 mM)	14.0 $\pm$ 0.4	12.5 $\pm$ 0.1*

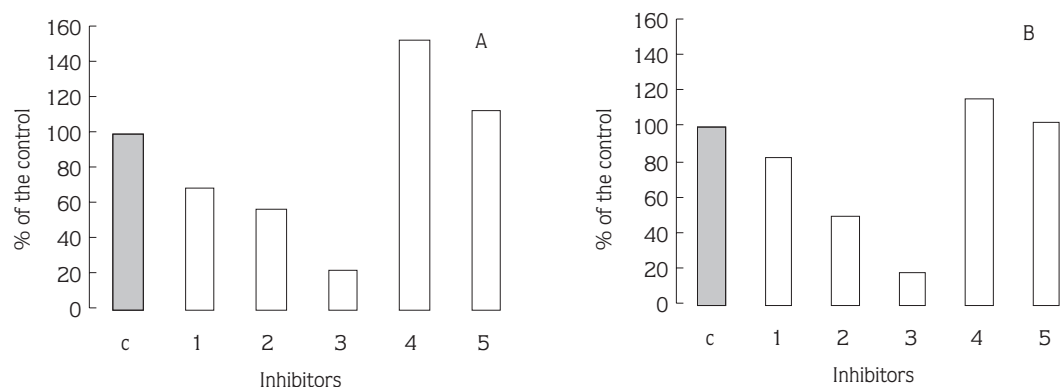


Figure 1. Influence of inhibitors on respiration (percentage compared to the control) in leaves of *R. rosea*. A – young leaves. B – mature leaves. C – control (no inhibitors), 1 - 25.0 mM BHAM, 2 - 5.0 mM KCN, 3 - 25.0 mM BHAM + 5.0 mM KCN, 4 - 0.5 mM 2,4-DNP, 5 - 0.5 mM 2,4-DNP + 25.0 mM BHAM.

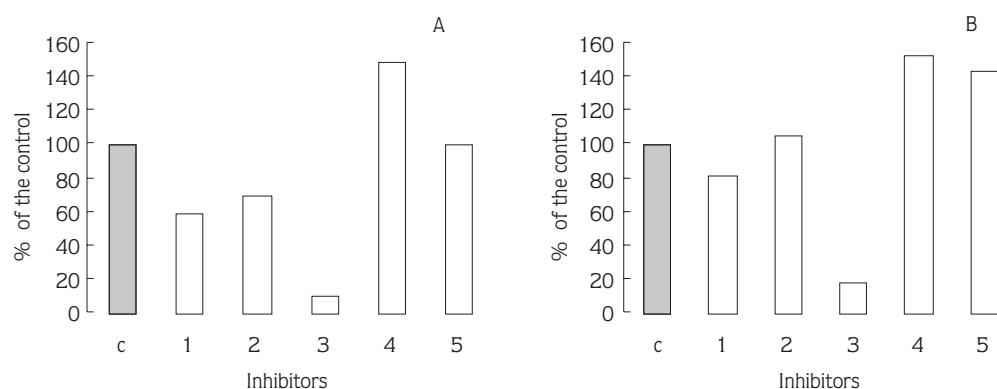


Figure 2. Influence of inhibitors on respiration (percentage compared to the control) in leaves of *A. reptans*. A – young leaves. B – mature leaves. C – control (no inhibitors), 1 - 25.0 mM BHAM, 2 - 5.0 mM KCN, 3 - 25.0 mM BHAM + 5.0 mM KCN, 4 - 0.5 mM 2,4-DNP, 5 - 0.5 mM 2,4-DNP + 25.0 mM BHAM.

Table 3. Parameters characterising respiratory pathways in leaves of *R. rosea*. AP – alternative pathway, CP – cytochrome pathway (percentages of the control). The values of  $\pm$ SE are shown.

Parameters	Young leaves	Mature leaves
Activity of AP ( $v_{alt}$ )	30 $\pm$ 2	17 $\pm$ 1
Capacity of AP ( $V_{alt}$ )	34 $\pm$ 2	32 $\pm$ 2
Engagement of AP ( $p_{alt}$ )	0.9	0.5
Activity of CP ( $v_{cyt}$ )	47 $\pm$ 1	65 $\pm$ 3
Capacity of CP ( $v_{cyt}$ )	95 $\pm$ 5	85 $\pm$ 4
Engagement of CP ( $p_{cyt}$ )	0.5	0.8
Residual respiration ( $v_{res}$ )	23 $\pm$ 2	18 $\pm$ 3

Table 4. Parameters characterising respiratory pathways in leaves of *A. reptans*. AP – alternative pathway, CP – cytochrome pathway (percentages of the control). The values of  $\pm$ SE are shown.

Parameters	Young leaves	Mature leaves
Activity of AP ( $v_{alt}$ )	41 $\pm$ 2	18 $\pm$ 3
Capacity of AP ( $V_{alt}$ )	60 $\pm$ 3	88 $\pm$ 4
Engagement of AP ( $p_{alt}$ )	0.7	0.2
Activity of CP ( $v_{cyt}$ )	49 $\pm$ 1	64 $\pm$ 2
Capacity of CP ( $v_{cyt}$ )	91 $\pm$ 4	126 $\pm$ 5
Engagement of CP ( $p_{cyt}$ )	0.5	0.5
Residual respiration ( $v_{res}$ )	10 $\pm$ 2	18 $\pm$ 1

in the mature leaves of *A. reptans* compared to the young leaves. The alternative pathway became nearly saturated in the young leaves and was far from saturation in the mature leaves of both species studied.

Activity of the cytochrome pathway was higher in the mature leaves than in the young leaves of both species investigated. Capacity of the cytochrome pathway did not change significantly in the leaves of different age in *R.*

*rosea*, but significantly increased with aging in *A. reptans*. The cytochrome pathway was near entire saturation in the mature leaves of *R. rosea*.

Residual respiration did not show statistically significant differences between the young and mature leaves in *R. rosea*, but increased significantly in the mature leaves of *A. reptans*.

Results published in the literature are often contradictory as to the influence of aging processes on the alternative respiratory pathway in plant tissues. Both increases and decrease in the activity on the alternative respiratory pathway with aging have been reported, sometimes on the same objects (e.g., McDonnell & Farrar, 1993; Day et al., 1994; Millar et al., 1998). Our results show that the activity of the alternative pathway was considerably higher in the young leaves. This fact may be explained by the higher requirements of growing tissues for metabolites. In this case, the alternative pathway could be responsible for continuous activity of the citric acid cycle and glycolysis, and, therefore, deliver additional metabolites. This explanation coincides well with Palmer's (1976) proposal concerning the role of the alternative pathway.

The capacity of the alternative pathway was considerable in the mature leaves of the species studied. However, the engagement of the alternative pathway considerably declined with aging. The decline in activity of the alternative pathway could be explained by the lower

demand of the older leaves in metabolites (Larcher, 1995). On the other hand, the high capacity in the mature leaves may be considered evidence of a high respiratory potential. We believe that this phenomenon can be explained by the fact that leaves are frequently confronted with numerous stress events during their life and, as a consequence, increase the respiratory capacity as a tool of stress resistance.

Bahr and Bonner (1973) proposed that the alternative respiratory pathway becomes active only when the cytochrome pathway reaches saturation ( $p_{\text{cyt}} = 1$ ). The inhibitory method of determination of AOX activity is based on the mentioned assumption. However, recently this model was subjected to criticism (e.g., Day et al., 1996). In some experiments with isolated mitochondria it was observed that AOX could also become active by the unsaturated cytochrome pathway ( $p_{\text{cyt}} < 1$ ) when the concentration of pyruvate was high (Millar et al., 1993; Umbach et al., 1994). It has been concluded that the inhibitory method can underestimate the real AOX activity because of the possibility of electron escape to the unsaturated cytochrome pathway. However, our experiments with the uncoupler (2,4-DNP) clearly indicated that respiration was strictly controlled by the energetic requirements of the cells (concentration of ATP). It may be concluded that no electron escape from the alternative respiratory pathway took place when using inhibitors of AOX.

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