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Alternative respiration in seven *Quercus* Spp. of SW Spain

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Abstract

The terminal part of the mitochondrial electron pathway, consists of two terminal oxidases: the cytochrome oxidase (sensitive to cyanide) and the alternative oxidase (both resistant to cyanide and sensitive to salicylhidroxamic acid). This study assesses the presence and importance of these two respiratory branches in the roots of seven Mediterranean *Quercus* species (4 evergreens and 3 deciduous) typical of the South Spain landscapes. The experiments were carried out with seedlings growing under controlled conditions. Determinations were performed using specific inhibitors of both respiration systems (potassium cyanide and salicylhydroxamic acid). The results showed that, under the experimental growth conditions, root respiration of all species was sensitive to salicylhydroxamic acid, indicating that the alternative oxidase was engaged.

Under the experimental growth conditions, the seven woody species studied have a range of cyanide-resistant respiration (37.7 % - 60.9 %) which is in the line of the value published for *Q. suber*, but lower than those published for herbaceous species. This suggests that higher cyanide-resistant respiration might be adaptive in herbaceous species because of their generally higher growth and respiration rates. In the same line, the deciduous species considered in the present study showed a salicylhydroxamic acid sensitive respiration (32 % ± 2.2 %) that is significantly higher than that of the evergreen ones (20.7 % ± 0.8 %), which have generally lower growth and respiration rates. Besides, this suggests that the Mediterranean evergreen species are more efficient in the use of energy than deciduous ones, which is in accordance with the putative low fertility of their habitats.

Even not conclusive, the analysis of results shows a general pattern relating faster growth rates, more active tissues, higher respiration rates and higher alternative respiration rates; all which is in according with the expected as regards of the recent studies on the nematode *Caenorhabditis elegans*.

Introduction

The alternative oxidase system is an electron transport branch parallel to the cytochrome pathway from which it diverges at the ubiquinone pool level. Unlike the cytochrome, this branch is resistant to cyanide and sensitive to salicilhidroxamic acid (SHAM). It does not provide energy to the plant's metabolism, since the oxidative phosphorylation is only engaged to the electron flux through the cytochrome pathway (Lambers, 1982; Siedow and Umbach, 1995). Thus, the energy conservation is less than maximal if a part of the respiration proceeds via this nonphosphorylating pathway.

Diverse functions for the alternative oxidase have been postulated (see Millenaar and Lambers, 2003), although its only demonstrated function so far is related with the thermogenesis during the anthesis of *Arum* spadices (Meeuse, 1975). Until 1995, the most extended hypothesis on the main role of the alternative oxidase is to constitute an escape valve for the metabolic energy (Lambers, 1982). More recent studies suggest that alternative oxidase would prevent the formation of oxygen free radicals (Purvis, 1997) by transferring electrons directly to O_2 (Millenaar et al., 1998; Millenaar and Lambers, 2003).

In the roots of herbaceous plants, the cyanide resistant respiration can reach 70% of total respiration (Lambers

et al., 1979), which represents a very high fraction of the total respiration. Thus, from a strictly energetic point of view, this component of respiration should results in a low efficiency use of energy, which could in turn result in a significant decrease of potential growth rate. Besides, a low energy use efficiency (that is, to waste carbon) in water limited areas would result in higher leaf conductances, and so in a waste of water. Therefore, a relatively lower alternative component of respiration (that is, a higher both energy and water use efficiency) in the species typical of less fertile (nutrients and/or water limited) environments should be expected in comparison to those of more fertile habitats.

In the Mediterranean-climate type areas, evergreen *Quercus* species are associated to less fertile and, in general, more stressed habitats in contrast to the deciduous species (Kikuzawa, 1991). Therefore, a comparatively lower alternative respiration pathway should be expected in the evergreen species.

In the present study, we evaluated the relative importance of both cytochrome and alternative oxidases in the root respiration of seven *Quercus* species typical of Mediterranean South Spanish landscapes. Also we use published data on root growth rates of the same species and individuals as those considered in the present study (Martínez et al., 2002) to analyze the effect of alternative respiration on growth.

Material and Methods

For the study, seven *Quercus* species (*Quercus canariensis* Willd., *Quercus coccifera* L., *Quercus faginea* Lam., *Quercus fruticosa* Brot., *Quercus pyrenaica* Willd., *Quercus rotundifolia* Lam. and *Quercus suber* L.) were considered. The set includes different leaf life-spans (evergreens and deciduous) and life forms (trees and shrubs).

For the roots of each species, cyanide sensitive respiration, SHAM sensitive respiration, cyanide resistant respiration, and residual respiration, were estimated. The first one represents an estimation of the respiration rate associated to cytochrome oxidase system. The second one represents the actual respiration rate associated to the alternative oxidase system, while the third one represents its maximum potential rate. The last one represents an estimation of the respiration rate associated to minor respiratory pathways other than either cytochrome or alternative ones.

Acorns of each species were sampled at several locations in SW Spain. In the sampling, different individuals growing in natural conditions were considered. Acorns of approximately the same weight were placed in a growth chamber, and kept on humid sand to induce the emergence of the radicle, at 450 mmol m^{-2} s^{-1} PAR, 14-hour photoperiod and 18ºC / 25ºC night and day temperatures. When the radicles were 6 cm long, the seedlings were transferred to a hydroponic culture with nutrient solution (Epstein, 1972). For more details see Martinez et al. (2002).

The total respiration was estimated quantifying the O_2 consumption of root fragments. A Clark type electrode (Hansatech Co. Ltd., Norlfolk, England) in a 2 ml cell volume was used for the determinations. To quantify the respiratory components, we used specific inhibitors: potassium cyanide (KCN) to inhibit the cytochrome oxidase and salicylhydroxamic acid (SHAM) to inhibit the alternative oxidase (Møller et al., 1988). Roots of seedlings 10 to 30 days old were cut with a sharp blade in 5 mm long fragments under nutrient solution. In previous experiments, the required time after the cut to reach stable respiration rates was determined. The stabilization time always ranged between 7 and 12 minutes. So, once the roots were cut, they were left to rest in nutrient solution for 12 minutes before the respiration determinations (Bloom, 1989; Aslam et al., 1996).

All the determinations were made in darkness, at 20ºC. Hamilton syringes were used to inject the inhibitors in the electrode cell, which was filled with nutrient solution. For the determinations, nutrient solution was buffered with a HEPES 10 mM buffer (pH = 6.5) in stead of EDTA-Fe in order to avoid SHAM precipitate formation in the cell electrode. At the start of each experiment, the electrode was calibrated at 0 and 100 by bubbling nitrogen and air respectively in the nutrient solution container. The duration of each experiment was always less than 30 minutes, so that oxygen consumption was always less than 60 % of the oxygen present in the electrode cell. At the end of the experiment, the roots fragments were washed using distilled water, and dried at 80 ºC to constant weigh.

To estimate residual respiration a two steeps titration was performed. The first one with KCN until reaching a stable respiration rate, and then a second one with SHAM until reaching a new stable respiration rate. This last respiration rate was considered the residual respiration. Inhibitors concentrations ranged from 0 to 4 mM KCN, and 0 to 14 mM SHAM; final concentrations depending on the particular species considered.

For each species, a titration with KCN (0 to 4 mM) was performed until reaching a stable respiration rate. Titrations were repeated with four different root samples and average rate calculated. This average rate, corrected for residual respiration yields the cyanide-resistant respiration.

To estimate SHAM-sensitive respiration, a titration with this inhibitor was performed. Only inhibitor concentrations lower than 1 mM were considered to avoid problems associated to SHAM (Møller et al., 1988; Millar et al., 1995). The titration was repeated with four different root samples until reaching stable respiration rates. The average rate yields the SHAM sensitive respiration.

For each species, cyanide sensitive respiration was obtained by subtracting its SHAM sensitive respiration from its total respiration corrected for residual respiration.

The respiration rates were expressed as nmol O_2 $s^{-1}g^{-1}$, and as a percentage of the total respiration measured in absence of inhibitors. For more details on methods see Møller et al. (1988).

Comparisons between evergreen and deciduous species were performed using a one-way ANOVA and grouping the variables considered as either evergreen or deciduous.

In order to analyse the effect of alternative oxidase respiration on growth, we proceeded as follows. Since the use of inhibitors only yields proximate estimations of the flux of electrons throughout both respiratory oxidases, we considered only the global trends of growth and respiration relationships by establishing the regression of average growth rates published by Martinez et al (2002) for the same species and individuals as those considered in

the present study, on both their total respiration (which includes cytochrome and alternative respiration) and their cyanide-sensitive respiration (which includes the cytochrome respiration mainly).

The regression of growth rate on total respiration represents an estimation of the rate of growth attained by respiring at different rates, while the regression on cyanide sensitive respiration represents an estimation of the growth rate that could be attained in the absence of alternative respiration; that is, in the case that all the electrons were flowing throughout the phosphorylating (cytochrome) pathway. Consequently, the difference between slopes of both regression lines represents an estimation of the global effect of alternative respiration on growth rate.

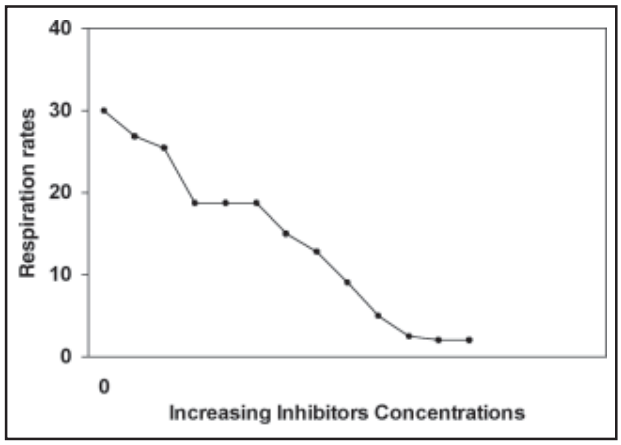

Figure 1. Evolution of the respiration rate (nmol O_2 s^{-1} g^{-1}) in response to increasing concentrations of KCN and SHAM in the case of a representative species (Q. coccifera). Black and white arrows indicates the starting of the titration with each inhibitor. The roots of all the seven species displayed a similar pattern in the titrations.

Results

Total respiration rates of the seven *Quercus* species range from 22.5 ± 1.3 to 58 ± 2.7 nmol O_2 s^{-1} g^{-1} for *Q. fruticosa* and *Q. faginea* respectively, with an average value of 39.5 ± 14.0 nmol O_2 s^{-1} g^{-1} (Table1).

The respiration of all species was sensitive to both KCN and SHAM (Figure 1), indicating that the cytochrome and the alternative respiration were engaged. Respiration was gradually inhibited at increasing KCN concentrations until reaching stable rates at concentrations in the range from 1.5 to 3.5 mM KCN depending on the species (Figure 1). Subsequent increases of SHAM concentration gradually inhibited root respiration until reaching stable rates at SHAM concentrations between 10 to 14 mM (Figure 1). These final rates represent the residual respiration of each species, and ranged from 6.7 % (*Q. fruticosa*) to 11.3 % (*Q. pyrenaica*) of the total respiration (Table 1). Inverting the titration sequence (that is: starting with SHAM and finishing with KCN), yielded stable rates (data not presented) similar to the previous ones; suggesting that the residual respiration values are consistent.

After correcting for residual respiration, cyanide resistant respiration ranged from 37.7 % to 60.9 % of total respiration for *Q. suber* and *Q. fruticosa* respectively, with an average value of 48.3 % for the seven species (Table 1). The cyanide sensitive respiration ranged from 66.5 % to 80.4 % of total respiration for *Q. faginea* and *Q. rotundifolia* respectively, with an average value of 73.1 % of total respiration (Table 1). The SHAM sensitive respiration ranged from 19.6 % to 34.5 % for *Q. rotundifolia* and *Q. faginea* respectively, with an average value for the seven species of 27.1 % of total respiration (Table 1). Cyanide-sensitive respiration was significantly bigger than SHAM-sensitive respiration (73.1 ± 6.2 % versus 27.1 ± 6.5 %; $p<0.001$, test LSD).

When species were grouped according to its deciduous or evergreen character, no significant differences in the average respiration rates of any of the analysed respiration components (total, residual, cyanide- resistant, cyanide- sensitive and SHAM-sensitive) were detected (Table 2). When relative rates (percentage of total respiration) were considered, cyanide sensitive respiration in evergreen species was higher than in the deciduous ones (79.3 ± 0.8 % versus 68.5 ± 1.8 %, respectively, $p<0.01$) (Table 2). Consequently, SHAM sensitive respiration was smaller in evergreens (20.7 ± 0.8 % versus 32.0 ± 2.2 %, $p< 0.01$) (Table 2).

Figure 2 presents the regression lines of growth rate on both, total and cyanide-sensitive respiration. There is a positive and significant relationship between respiration (both, total and cyanide-sensitive) and growth ($p<0.05$ and $p<0.01$ respectively). Also, species with higher growth

Table 1. Respiratory components (average and standard deviation) in the roots of the species considered in three present study. D: deciduous, E: evergreen. Values in nmol O_2 s^{-1} g^{-1} and as a percentage of total respiration corrected for residual respiration.

SPECIES	RESPIRATORY COMPONENTS								
	TOTAL	RESIDUAL		CYANIDE-RESISTANT		CYANIDE-SENSITIVE		SHAM-SENSITIVE	
	Rate	Rate	%	Rate	%	Rate	%	Rate	%
Q. pyrenaica D	57.3±4.2	6.5±0.1	11.3	27.8±1.8	54.7	36.3±4.0	71.5	14.5±4.0	28.5
Q. canariensis D	31.7±3.2	2.5±0.03	8.2	12.0±0.5	41.1	19.8±1.7	68.5	9.5±1.7	32.5
Q. faginea D	58.0±2.7	4.0±0.05	7.0	21.0±2.0	38.9	35.7±4.5	66.5	18.8±4.5	34.5
Q. fruticosa D	22.5±1.3	1.5±0.03	6.7	12.8±0.8	60.9	14.0±0.2	67.6	6.8±0.2	32.4
Q. suber E	44.3±1.8	4.0±0.05	9.0	15.2±0.8	37.7	31.3±2.0	78.4	8.7±2.0	21.6
Q. rotundifolia E	32.6±4.2	3.0±0.07	9.0	13.0±0.3	43.9	23.7±1.3	80.4	5.8±1.3	19.6
Q. coccifera E.	30.0±2.3	2.2±0.03	7.0	16.8±1.2	60.5	22.0±0.3	79.1	5.8±0.3	20.9
AVERAGE ± S.D.	39.5±14.0	3.4±1.6	8.3	16.9±5.7	48.3	26.1±8.5	73.1	10.0± 4.9	27.1

Table 2. Comparison of respiratory components (average and standard deviation) in roots of evergreen and deciduous species. Respiration rates in nmol O_2 s^{-1} g^{-1} and as a percentage of total respiration. **: p< 0.01 (ANOVA).

	RESPIRATORY COMPONENTS								
SPECIES	TOTAL	RESIDUAL		CYANIDE-RESISTANT		CYANIDE-SENSITIVE		SHAM-SENSITIVE	
	Rate	Rate	%	Rate	%	Rate	%	Rate	%
Deciduous	12.6 ± 4.3	42.4±18.5	8.3	18.4±7.5	48.9	26.4±11.3	68.5±1.8 **	12.4±5.3	32.0 ± 2.2 **
Evergreens	14.4 ± 1.7	35.6±7.6	8.3	15.0±1.9	47.4	25.7±5.2	79.3±0.8 **	6.8±1.6	20.7±0.8 **

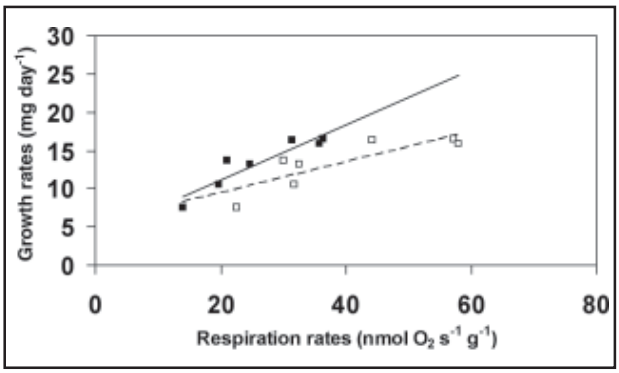

Figure 2. Relationship between root growth rate and the rates of both total respiration (open symbols) and cyanide- sensitive respiration (full symbols).

rates have higher both nitrogen concentration and maintenance respiration (Table 3).

The difference between slopes is also significant ($p<0.05$, Tukey test), showing that the average negative effect of estimated alternative respiration on growth rate (difference between growth rate observed minus predicted in the case that the full electron flow proceeds throughout the citochrome pathway only) increases with the increase in growth rate of the species (Figure 2).

Discussion

The total respiration rates in this study (between 22.5 and 58.0 nmol O_2 $s^{-1}g^{-1}$) are similar to those published for the roots of other tree species (Szaniawski, 1981; Mata et al., 1996).

Higher respiration rates are associated to higher growth rates (Figure 2), showing that comparatively more energy is required for growing faster. Species with higher growth rates probably requires of more active tissues as reflected by their positive correlation with both nitrogen concentration and maintenance respiration (Table 3). Consequently, the values of total respiration found in the present study for the roots of the seven *Quercus* species are lower than those published for the root systems of agricultural species, as soybean (between 70 and 100 nmol O_2 $s^{-1}g^{-1}$, Vessey and Layzell, 1987), or for a set of temperate herbaceous species (between 41.7 and 80 nmol O_2 $s^{-1}g^{-1}$, Poorter et al., 1991). Agricultural and herbaceous species -unlike woody ones- have generally been selected for faster growth rates, so explaining the observed differences in respiration rate.

Table 3. Significance of correlation coefficients (all positive) between nitrogen concentration, growth rate, maintenance respiration (data from Martinez et al., 2002) and total respiration (this study) in roots of the seven species studied. **: p<0.01. *:p<0.05. Test LSD.

	NITROGEN	MAINTENANCE COST	TOTAL RESPIRATION
GROWTH	**	**	*
NITROGEN		*	*
MAINTENANCE COST			**

The use of inhibitors for the estimation of respiratory components has generated controversy (see for example Atkin et al., 1995; Millar et al., 1995; Siedow and Umbach, 1995); since studies have demonstrated that alternative and cytochrome oxidases compete for electrons from ubiquinol (Ribas-Carbo et al., 1995; Day et al., 1996); so excluding the possibility of to precisely quantify cytochrome and the alternative respiration rates.

However, the use of specific inhibitors still remains adequate to discuss the global trends in the respiration components for comparative purposes, providing both the use of low concentrations of inhibitors (KCN and SHAM) and the reaching of successive stable respiration rates (Hoefnagel et al., 1995). In the present study total respiration rate decreased immediately in response to the addition of SHAM; even at very low concentrations (lower than 1 mM) of this inhibitor (see Results) and reached stable rates in the titrations with both inhibitors (Figure 1).

The values published by Mata et al. (1996) for cyanide resistant respiration of *Q. suber* roots (13 % of the total respiration) are lower than the estimated in the present study for the same species (37.7 % of total respiration). Since, on the contrary, the results of both studies are coincident in the magnitude of the cyanide sensitive respiration of this species (between 50 and 72% of the total respiration in the study of Mata et al., 1996; and 78.4 % in the present study), the low value of cyanide resistant respiration observed by Mata and co-workers might be a consequence of the high value of the residual respiration found by these authors, which gets as far as between 23% and 37% of the total respiration. It should be noted that residual respiration rates higher than 10% should be considered unrealistic (Møller et al., 1988).

It is important to point out that, at difference of the present study, Mata and co-workers used intact root systems, in stead of excised roots. So, considering the low diffusion capacity of SHAM in plant tissues, it could be concluded that in their experiments, the inhibition of SHAM sensitive respiration was incomplete, with the result of an unrealistic very high residual respiration. If so, recalculating the values by Mata et al. (1996) for *Q. suber* assuming a residual respiration of 10 %, yields a cyanide resistant respiration (between 26 % and 40 %) similar

to the estimated in the present study for that species (Table 1). All that suggest that the results fund in the present study are congruent with those published following the same method.

In the seven species studied average cyanide sensitive respiration is significantly higher than average SHAM sensitive respiration (73.1 % versus 27.1 %, $p<0.001$), suggesting that root respiration happens mainly through the cytochrome oxidase system, although a significant fraction of electrons flows through the alternative oxidase system. The effect of that energy loss is patent in Figure 2, which shows that alternative respiration increases with growth rate; with the result of a progressive loss in growth rate. The observed association of higher alternative respiration rates to faster growth rates could be considered adaptive.

In effect, recent studies on *Caenorhabditis elegans* (a nematode) have demonstrated that the production of free radicals is proportional to the respiration rate, because the electron leaking out from the respiratory chain increases with the magnitude of electron flux (Braeckman et al., 1999; Branicky et al., 2001; Asencio et al., 2003). In this context, alternative oxidase can drive a fraction of the electrons from the ubiquinone complex directly to the oxygen (Purvis, 1997; Karaffa et al., 2001), so decreasing the electron flux throughout the respiratory chain and thus minimizing the generation of free radicals. Thus, the association of higher alternative oxidase respiration (higher SHAM sensitive respiration) to faster growth rates found in the present study (Figure 2) could be mandatory to prevent massive electron leaking and thus, free radical generation, even at the cost of a significant loss of the growth rate. A such association has been described by Millenaar et al. (2001) in herbaceous plants.

In the same line, as compared with the deciduous species, the comparatively lower SHAM sensitive respiration in the evergreens (Table 2) is consistent with their generally lower both growth and respiration rates (Merino et al., 1982; Antúnez et al., 2001). Besides, this different distribution of the electrons among both alternative and cytochrome oxidases means that, at least under the experimental conditions of the present study, the evergreen species are more efficient in the use of the energy than the deciduous ones, since they synthesize more ATP per glucose respired. Roots more efficient in the use of energy (that is, with a lower SHAM sensitive respiration) may be very important for evergreen Mediterranean species which have to remain active during prolonged periods of the year in which the carbon balance of the individuals has to be very close to zero.

Finally, although the published data are scarce, the comparison of results available for woody species (Weger and Guy, 1991; Mata et al., 1996; present study), with those published for herbaceous ones (van der Plas et al., 1977; Lambers et al., 1979; Laties, 1982; Blacquière and de Visser, 1984; Gifford et al., 1985; Poorter et al., 1991; Atkin et al., 1995), suggests that roots of woody species possess a lower cyanide resistant respiration. The possible adaptive significance of this difference might be related to differences associated to both forms of life. Thus, as compared with woody species, herbaceous species have inherently faster growth rates usually associated to more active tissues and higher respiration rates (Poorter, 1990; Cornelissen et al., 1996; Reich, 1998), which would require the existence of an alternative oxidase system with a capacity higher enough to cope with the fraction of electrons able to generate free radicals. Interesting enough, *Q. fruticosa*, which can be considered of intermediate characteristics between herbaceous and arboreal species due to its shrub like morphology, presents the highest cyanide resistant respiration rate in this study, thus supporting the above considerations.

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