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Alternative oxidase respiration in the mycorrhizal fungus *Laccaria bicolor*

Respiration via alternativt oxidas i mykorrhizasvampen Laccaria bicolor

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Swedish University of Agricultural Sciences, Department of Forest Ecology and Management
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I denna rapport redovisas ett examensarbete utfört vid Institutionen för skogens ekologi och skötsel, Skogsvetenskapliga fakulteten, SLU. Arbetet har handletts och granskats av handledaren, och godkänts av examinator. För rapportens slutliga innehåll är dock författaren ensam ansvarig.

This report presents an MSc/BSc thesis at the Department of Forest Ecology and Management, Faculty of Forest Sciences, SLU. The work has been supervised and reviewed by the supervisor, and been approved by the examiner. However, the author is the sole responsible for the content.

Abstract

The temperature on Earth is rising, and one of the main drivers is anthropogenic greenhouse gases such as carbon dioxide (CO_2). The world's forests act as carbon sinks, binding carbon into their biomass. The net carbon assimilation is determined by the uptake and release of CO_2 through the processes of photosynthesis and respiration. Respiration in plants and most fungi can proceed via two pathways. The most frequently used pathway ends with the terminal complex Cytochrome C oxidase (COX), but it can also follow a less efficient alternative pathway, which ends with Alternative oxidase (AOX). The rate by which plants and other organisms use the alternative pathway affects their carbon use efficiency.

Both enzymes use atmospheric oxygen as their substrate, but they discriminate differently against the isotope ^{18}O. In this study, the presence of AOX in the mycorrhizal fungus *Laccaria bicolor* was proven, using isotope ratio mass spectrometry (IRMS). Based on the ^{18}O discrimination of the mycelium in the presence of pathway specific inhibitors, the electron partitioning to each pathway in untreated mycelium (i.e. without inhibitors) was estimated.

As found in previous studies on plants, the discrimination was found to be affected by the water content of the sample. Since this effect was probably derived from diffusion limitation, all discrimination values were normalized to correspond to the mean water content, 94.4%. The ^{18}O discrimination of *L. bicolor* was found to be 18.8±0.9, which is comparable to COX values previously found in plants and to discrimination values of baker's yeast, *Saccaromyces cerevisiae*, which lacks AOX. This indicated that the use of AOX in young mycelium of *L. bicolor* was negligible. However, a correlation was discovered between AOX contribution and age, suggesting that AOX plays an increasingly important role in ageing mycelium.

The oxygen isotope discrimination method is currently the only reliable way of measuring AOX/COX partitioning during respiration. However, despite numerous studies in various species, this is the first time it has been applied to fungal mycelium, and as such it represents an important step towards a greater understanding of fungal respiration. Further studies of fungal AOX under natural conditions, in combination with estimations of fungal biomass, has a great potential to improve the accuracy of carbon sequestration models.

Keywords: L. bicolor, mycelium, ectomychorriza, respiration, AOX, alternative oxidase, cytochrome oxidase, salicylhydroxamic acid, n-propyl gallate, cyanide

Sammanfattning

Temperaturen på Jorden stiger, och en av de främsta orsakerna är antropogena växthusgaser som koldioxid (CO_2). Världens skogar fungerar som kolsänkor, då de binder in kol i sin biomassa. Den totala mängd kol som lagras in bestäms av upptag och avgång av CO_2 under fotosyntes och respiration. Respiration i växter och de flesta svampar kan ske via två olika vägar. Den mest använda avslutas med enzymet cytokrom C (COX), medan den alternativa och mindre effektiva vägen slutar med enzymet alternativt oxidas (AOX). Användandet av AOX påverkar hur effektivt organismen använder kolet den tar upp.

Båda enzymerna använder sig av syre från atmosfären som substrat, men de diskriminerar olika starkt mot syreisotopen ^{18}O. I den här studien visas att enzymet AOX finns i mykorrizasvampen *Laccaria bicolor*, och dess användning mäts med hjälp av masspektrometri av isotopiska förhållanden (IRMS). Diskrimineringen av syreisotopen ^{18}O uppskattades för varje enzym med hjälp av inhibitorer och användes sedan för att räkna ut elektrondelningen mellan de olika respirationsvägarna i obehandlat mycel, d.v.s. utan tillsatta inhibitorer.

Liksom i tidigare studier visade sig diskrimineringen vara påverkad av provets vatteninnehåll. Eftersom denna effekt troligtvis uppkommit på grund av diffusionsbegränsning av substratet normaliserades alla diskrimineringsvärden till det som motsvarade det genomsnittliga vatteninnehållet, 94,4%. Diskrimineringen av ^{18}O i *L. bicolor* var 18,8±0,9, vilket är jämförbart med de värden som presenterats för växter och jästsvampen *Saccaromyces cerevisiae*, vilken saknar AOX. Detta indikerade att användandet av AOX är försumbart i ungt mycel. Dock kunde ett samband påvisas mellan AOX-användning och ålder, vilket antyder att AOX spelar en allt viktigare roll allt eftersom mycelet åldras.

IRMS är den enda tillförlitliga metoden för mätning av elektronfördelning mellan AOX och COX. Ändå, trots otaliga studier i olika arter, är detta första gången som denna metod används i svampmycel. Arbetet som sådant utgör därför ett viktigt steg mot en större förståelse av svampars respiration.

Vidare studier av AOX i svamp under naturliga förhållanden, i kombination med uppskattningar av svampars biomassa, har stor potential att ytterligare förbättra säkerheten i kolinlagringsmodeller.

Nyckelord: L. bicolor, mycel, ektomykorrhiza, respiration, AOX, alternativt oxidas, cytokrom C, SHAM, PG, KCN, cyanid

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Abbreviations

ETC	Electron transport chain
AOX	Alternative oxidase
AOP	Alternative oxidase pathway
COX	Cytochrome C oxidase
COP	Cytochrome C oxidase pathway
KOH	Potassium hydroxide
SHAM	Salicylhydroxamic acid
KCN	Potassium cyanide
mCLAM	m-Chlorobenzhydroxamic acid
n-PG	n-Propyl gallate
DMSO	Dimethyl sulfoxide
IRMS	Isotope ratio mass spectrometry
ROS	Reactive oxygen species

1 Introduction

1.1 Background

The past three decades on Earth have all been warmer than any decade since 1850 (Pachauri and Mayer, 2014). The most likely cause of the increasing temperature is anthropogenic greenhouse gases, where carbon dioxide (CO_2) is the most important one (Pachauri and Reisinger, 2007). The world's forests are important sinks in the global carbon cycle, with an estimated net uptake of 1.1 ± 0.8 Pg C $year^{-1}$ and a total carbon stock of 861±66 Pg (Pan *et al.*, 2011).

The carbon balance of an ecosystem is determined by uptake and release of CO_2 through the processes of photosynthesis and respiration (Valentini *et al.*, 2000). Whereas only plants, algae and certain bacteria fix atmospheric carbon via photosynthesis, all living organisms contribute to the return of carbon to the atmosphere through respiration (Whitmarsh and Govindjee, 1999). Because of its important role in determining the net carbon assimilation of an ecosystem, respiration is an important input in carbon sequestration models (DeLucia *et al.*, 2007). Despite this, the regulating factors of respiration are poorly understood.

Respiration is a process in which ATP, carbon skeletons and CO_2 are produced (Smith, 2009). Carbon metabolism begins with glycolysis in the cytosol, where some ATP is synthesized. However, the major part of the ATP synthesis occurs in the mitochondria via oxidative phosphorylation. In the mitochondrial membrane, electrons are transported through a series of complexes to create a proton motive force and reduce oxygen to water (figure 1). This is called the terminal electron transport chain (ETC) and the proton motive force is used by ATP synthase (complex V) to combine ADP and a phosphate molecule, creating the energy carrying ATP molecule. This commonly occurs through the Cytochrome oxidase pathway, where cytochrome C oxidase (COX) catalyse the reduction of oxygen to water (Guy *et al.*, 1989, Joseph-Horne *et al.*, 2001, McDonald and Vanlerberghe, 2006). However, in many organisms (all plants, and many fungi) there is an alternative electron transport pathway (McDonald and Vanlerberghe, 2006). This pathway branches of from ubiquinone and consists of one single enzyme, the alternative oxidase (AOX) (Lambers and Ribas-Carbo, 2005, Smith, 2009).

The alternative oxidase pathway bypasses complex III and IV (COX). It is therefore less efficient in producing ATP than the cytochrome oxidase pathway, and thus have a lower carbon use efficiency (Sieger *et al.*, 2005, Smith, 2009). Just like COX, AOX use electrons to reduce oxygen to water, but instead of further increasing the proton gradient, some of the reducing power is dissipated as heat. This is used by thermogenic species to elevate the temperature of their tissues, possibly to attract pollinators (Wagner *et al.*, 2008). However, most plants are non-thermogenic and use AOX for other purposes (Vanlerberghe, 2013).

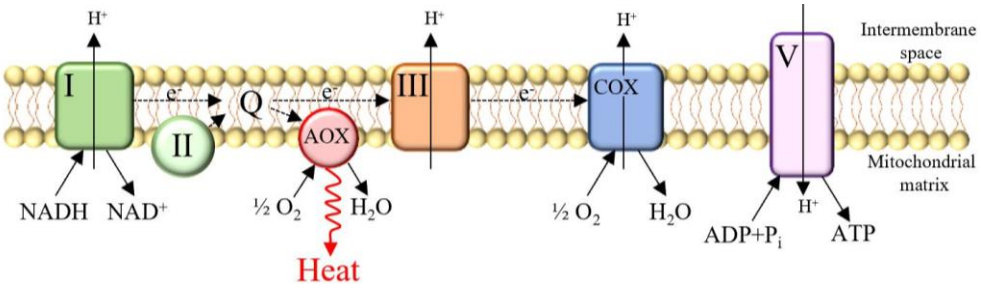

Figure 1. Mitochondrial electron transport chain. Electrons from NADH is transported through the complexes. The final electron acceptor is either of the two terminal oxidases, AOX and COX, which uses the electron to reduce oxygen to water. Protons are transferred from the mitochondrial matrix to the intermembrane space by complex I, III and COX. The transfer creates a proton gradient used by complex V to form ATP. Figure adapted from Vanlerberghe (2013).

In the pathogenic fungus *Ustilago maydis*, two main functions of AOX have been proposed (Juarez *et al.*, 2006).
First, it acts as a mechanism to reduce oxidative stress. Reactive oxygen species (ROS) can be formed when molecular oxygen is reduced by electron transport proteins (Smith, 2009, Xie *et al.*, 2019). In plants, this mainly occurs at complex I and complex III and as a response to stresses such as drought, extreme temperature or pathogen infection (Møller, 2001, Xie *et al.*, 2019). Formation of ROS can be prevented by the bypass of complex III by AOX.
Secondly, the alternative oxidase pathway (AOP) acts as a back-up system (Del-Saz *et al.*, 2018). In conditions where the cytochrome oxidase pathway (COP) is impaired, such as low temperature, metal toxicity or nutrient deficiency, AOP allows respiration to proceed.

Since respiration is one of the main components in the carbon budget of an ecosystem, understanding of its mechanisms is of great importance for any carbon sequestration model or estimation (Cannell and Thornley, 2000). Despite this, the impact of the AOX on respiration and carbon sequestration remains unknown (Del-Saz *et al.*, 2018).

1.2 Studying alternative oxidase

Until 1989, the common method to estimate AOX activity was titration with an AOX inhibitor, like salicylhydroxamic acid (SHAM), with and without the COX inhibitor potassium cyanide (KCN) (Guy *et al.*, 1989). The activity of AOX was determined with extrapolation. This method has one major drawback in that it does

not consider the fact that the pathways can partly compensate for each other if the activity of one of them is lowered (Day *et al.*, 1996).

In 1989, Guy *et al*. (1989) developed a non-invasive method, with which the activity of AOX can be measured in the absence of inhibitors (Guy *et al.*, 1989, Day *et al.*, 1996). This method uses isotope discrimination in combination with inhibitors to calculate AOX activity through the oxygen isotope fractionation technique (Guy *et al.*, 1989).

Isotopes of an element are atoms differing from each other in the number of neutrons found in their nucleus (Fry, 2008). Because neutrons do not carry any charge, their presence or absence does not affect the atomic properties of the element. Different isotopes of the same element take part in the same reactions, but the heavier isotopes react slower and require more energy to react. Usually in biological systems, there is a preference for the lighter isotopes over the heavy isotopes of the same element, referred to as *discrimination* (Fry, 2008, Martin and Hine, 2008).

In a closed system, this preference results in a gradual enrichment of the heavier isotope of the reaction's substrate, termed isotopic *fractionation* (Hoefs, 2009).
The most commonly used elements for fractionation studies in biological systems are carbon, oxygen, nitrogen, hydrogen and sulphur (Fry, 2008). These are all abundant in organic compounds and have stable isotopes. Their light atomic mass allows a single neutron to significantly affect the overall mass of the atom, enabling reliable measurements of the isotopic fractionation (Hoefs, 2009).

Most of the atmospheric oxygen has the atomic mass of 16g/mol, ^{16}O, but there is also a small fraction (~0.2%) of a slightly heavier isotope, ^{18}O (Michener and Lajtha, 2007). Both of the terminal oxidases in the respiratory pathway, AOX and COX, consume oxygen from the atmosphere, but they differ in their discrimination against ^{18}O (Guy *et al.*, 1989). By determining their respective discrimination factors, and the discrimination of total respiration, the electron partitioning to each enzyme can be calculated using linear interpolation.

The isotopic discrimination of COX and AOX respectively can be determined by specific inhibition of one or the other (Guy *et al.*, 1989). COX can be inhibited by incubation with KCN and AOX can be inhibited using for example SHAM. Using isotope ratio mass spectroscopy (IRMS) it is possible to measure the $^{18}O/^{16}O$ isotopic ratio of air, and by monitoring the enrichment of ^{18}O in the residual air of a sealed reaction vial, the discrimination of the enzyme of interest can be determined (Del-Saz *et al.*, 2017).

1.3 This study

This study investigates the use of the AOX pathway in the fungus *Laccaria bicolor*, an ectomycorrhizal fungus that is naturally occurring in boreal forests. The involvement of AOX is estimated using IRMS, providing new information about the properties of respiration in fungi. Additionally, the respiration of young and old mycelium is studied, revealing important metabolic patterns. This is the first time this

method is applied to fungal mycelium, despite the abundance and important functions of fungi in terrestrial ecosystems (Wallander *et al*., 2001). The lack of isotopic measurements on fungal AOX/COX partitioning represents an important gap in the scientific understanding of carbon cycling in natural systems. In the long term, such information could improve carbon budgets and growth models.

2 Materials & Methods

2.1 In summary

The fungus *L. bicolor* was grown on top of cellophane sheets placed on P20-medium. After 3 weeks, the cultures were harvested by scraping the mycelium off the cellophane. The samples were placed in vials that were sealed with airtight caps. The air in the vials was replaced with outdoor air, to ensure equal conditions. Thereafter, an IRMS autosampler was used to measure the rate of respiration and the isotopic discrimination against ^{18}O in each vial separately. SHAM, n-PG, mCLAM and KCN were used as inhibitors for measurements of the isotopic discrimination of each enzyme separately (Figure 2).

2.2 Detailed description

2.2.1 Culturing of fungi

A protocol from the Judith Felten research group at Umeå Plant Science Centre (UPSC), Umeå, was used for the culturing of fungal material (Felten, 2018).

2.2.2 Inoculation and growth (figure 2.1)

Existing cultures of *L. bicolor* grown on P20-media were used to grow material for this study (Judith Felten research group, UPSC, Umeå). Petri dishes with P20-media were prepared with 4 cellophane sheets and inoculated with 4 plugs of mycelium, one on each sheet.
In order to produce a measurable isotopic enrichment of the substrate, the sample must consume a sufficient amount of oxygen during the incubation. A test-run performed on pre-grown fungal cultures indicated that each vial should contain 4 cultures of fungus, to ensure sufficient respiration. Given the size of the autosampler, respiration could be measured on 8 samples a day. Therefore, 8 new plates were started every day for two weeks (10 days in total). The inhibited samples were predicted to have a lower respiration rate. Therefore, 12 plates were started every day for the next 2 weeks (10 days), to be used during the inhibitor studies. The samples were grown at room temperature, in a dark, ventilated locker for 3 weeks before harvest.

2.2.3 Sample preparation and inhibitory treatments

The protocol from Henriksson *et al.* (2019) was adapted for the KCN treatment and the initial SHAM treatment. Necessary adaptations were made to accommodate the mycelial tissue. These adaptations, as well as further inhibitor trials, are described in detail in the following section.

2.2.3.1 Controls (figure 2.2)

The inoculation plug was removed from each sample, and the mycelium scraped off the cellophane. The samples were placed in numbered 22mL vials, 4 colonies in each vial.

2.2.3.2 Young/old mycelium (figure 2.3)

A subset of the untreated colonies was divided into young and old mycelium. The mycelial colonies grow radially from the central inoculation plug. To divide the colony into young and old, a circular border was cut at half the colony radius.
The mycelium was thicker close to the centre of the colony, therefore this division gave samples of similar weight. The mycelium within the border was classified as old and the mycelium outside of the border was classified as young. They were separated using a scalpel and placed in separate vials. The inoculation plug was removed.

2.2.3.3 KCN treatment (figure 2.4)

The samples were placed in vials before KCN-treatment, in the same manner as for the controls. 8 colonies were used in each vial. Two cotton swabs dipped in 1M KCN solution were placed in each vial, and they were sealed with lids. After 30 minutes of incubation the swabs were removed, half a KOH pellet in a plastic cup was added, and new lids were attached. All handling of KCN was made with great caution in a fume hood.

2.2.3.4 SHAM treatment and additional treatments (figure 2.5)

Petri dishes were prepared with a sheet of absorbing tissue (Versi-Dry Lab-soakers, Nalgene) and a solution of 30mM SHAM in 2% DMSO was added until saturation. The cellophane sheets with fungal cultures were placed on the soaked tissues for 1 hour. The cellophane was permeable and allowed uptake from the soaked tissue. After this, the samples were scraped off the cellophane and placed in vials. Six colonies were used in each vial. Controls for this treatment were prepared in the same way, but with H_2O instead of SHAM solution.

The SHAM concentration used in Henriksson *et al.* (2019) was not appropriate for the mycelium in this study (details in the results section below), and a re-evaluation of the inhibitor protocol was required. A range of SHAM concentrations

were tested, as well as the alternative AOX inhibitors n-PG and mCLAM, and effects of the solvent itself, using the same application method as described above (Wedding *et al.*, 1973). For a complete compilation of the test results, see table 1. To test the effect of DMSO on the SHAM treatment an additional SHAM solution was prepared, without DMSO. In order to dissolve SHAM in water without addition of solvent, an ultrasound bath was used.

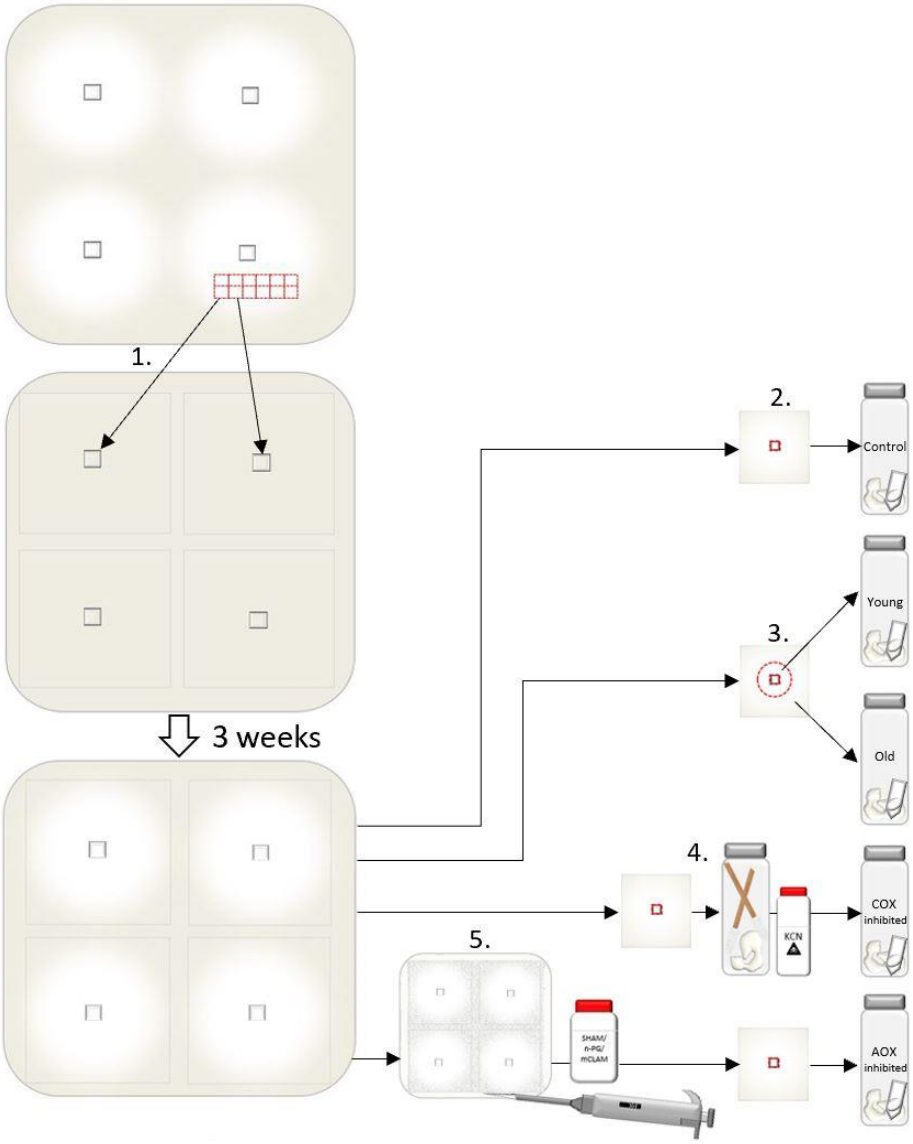

*Figure 2***.** Sample preparation. 1. Mycelium inoculation on cellophane sheets placed on P20 medium. Mycelium was grown for 3 weeks. 2. Preparation of control samples. 3. Preparation of young/old mycelium samples. 4.Preparation of COX inhibited samples: 2 cotton swabs were dipped in a 1M KCN solution and placed in the vial. A lid was put on and the sample was incubated for 30 minutes. The swabs were then removed, and a new lid was put on. 5. Preparation of AOX inhibited samples: Inhibitor solution was pipetted onto an absorbing tissue placed in a petri dish. The cellophane sheets with mycelium were placed on the tissue and incubated for 1 hour. After incubation the samples were placed in vials.

2.2.3.5 Common treatment for all samples

The vials were weighed before and after the mycelium was added, for retroactive dry weight calculations. Half a potassium hydroxide (KOH) pellet in a small plastic cup was placed in each vial, to prevent build-up of CO_2 during incubation, which could impede respiration. An additional vial was prepared simultaneously, but without mycelium, to be used as a standard. The empty standard vial represented the air at time zero and was used for calculations of the gradual isotopic enrichment in the mycelium vials. The vials were sealed with airtight caps with rubber septa. A gas exchange system was used to replace the air in the vials with identical outdoor air. The vials were emptied and refilled three times, to make sure the air was properly replaced. This ensured identical starting conditions for all vials and the standard. The time at the end of the last air replacement marks time zero for the IRMS measurement.

2.2.4 Incubation and gas sampling

The instruments and protocols for this study have been set up at SSIL (SLU stable isotope laboratory) and is described in detail in Henriksson *et al.* (2019).
The vials were placed in an IRMS autosampler equipped with a syringe, that penetrated the septa of the vials and injected helium (He) gas into one vial at a time (Henriksson *et al.*, 2019). This created an over-pressure that forced a small amount of the air in the vial to move through a pipe into the IRMS instrument. At each sampling, 10 µl of air was collected, and a total of 0.5ml of He was injected into each vial. One sample was taken every 15 minutes, so that a total of 135 minutes had passed before it returned to the first vial. The sampling proceeded for approximately 24 hours, after which each vial had been sampled 12 times. After analysis, all vials were dried in an oven for ≥48h and then weighed to calculate moisture content.

2.2.5 Data filtering and analysis

The output data received from the IRMS analysis contained data on the remaining proportion of initial O_2 and the isotopic composition of the oxygen in the vial. Each vial was sampled 10-12 times, depending on if the analysis had to be interrupted before it had finished (this did not negatively affect the resulting data). The discrimination value for each sampling occasion was calculated by a script using equations 1-3 as in previous studies (Guy *et al.*, 1989, Henriksson *et al.*, 2019).

The proportion of remaining O_2 was calculated according to equation 1.

$$f^* = \frac{^{32}O^*}{^{32}O_0^*} = \frac{^{32}O_2\ /^{28}N_2}{(^{32}O_2\ /^{28}N_2\ )_0} \quad (1)$$

Where
f is the proportion of remaining oxygen after incubation as compared to time zero (0).*

The ^{18}O enrichment was calculated by comparing the $^{18}O/^{16}O$ ratio after incubation to that of time zero as described in equation 2.

$$\delta^{18}O(‰) \approx \delta^{34}O_2(‰) = (\frac{^{34}R}{^{34}R_0} - 1) \times 1000 \quad (2)$$

Where
***δ*^{18}*O(‰)** is the ^{18}O enrichment;*
***^{34}R** is the $^{34}O_2/^{32}O_2$ ratio; and*
***^{34}R$_0$** is the initial ratio (represented by the standard vial).*

The discrimination (D) was calculated as the slope of a linear regression fitted to the obtained values from each vial over time (equation 3). Because each vial is sampled repeatedly, this regression accurately describes the gradual ^{18}O enrichment of the substrate air.

$$1000 \times \ln\left(\frac{^{34}R}{^{34}R_0}\right) = a - D\ lnf^* \quad (3)$$

Where
***^{34}R** is the $^{34}O_2/^{32}O_2$ ratio;*
***^{34}R$_0$** is the initial ratio (represented by the standard vial);*
f is the proportion of remaining oxygen after incubation as compared to time zero (0); and*
***a** is the intercept.*

A prerequisite for equation 3 is that the isotopic discrimination remains constant throughout the incubation. In this type of experiment, where the substrate (O_2) pool is finite and the reaction proceeds one-way without being affected by build-up of the product the isotopic discrimination is expected to follow a Rayleigh distillation curve (equation 4) (Kendall and Caldwell, 1998, Henriksson *et al.*, 2019). If the partitioning of electrons between AOX and COX were to shift during the course of the incubation, the discrimination values would deviate from the curve. If the obtained ^{18}O enrichment values fit the Rayleigh equation, it means that the discrimination is constant over time. In order to test this criterium, the ^{18}O enrichment data from each vial was subjected to a rigorous test.
The test consisted of a stepwise fitting of the data to the Rayleigh distillation curve. The IRMS data points were continuously added to the curve and cut if the R^2 of the fit dropped below 0.995 for three points in a row. If the last data points gave an R^2 below 0.995, they were removed. The minimum requirement for the data to be used was 5 points. The first point from each analysis was removed due to an instrumental artefact.

$$\frac{R}{R_0} = (\frac{X}{X_0})^{\alpha-1} \quad (4)$$

where
***R** is the $^{18}O/^{16}O$ ratio;*
***R₀** is the initial $^{18}O/^{16}O$ ratio;*
***X** is the oxygen concentration in residual air;*
***X₀** is the initial oxygen concentration; and*
***α** is the isotopic discrimination factor.*

2.2.6 Water content normalization

Diffusion of O_2 across liquid water does not fractionate against ^{18}O, but can lead to substrate limitation, which in turn reduces discrimination of the consuming reaction (Guy *et al.*, 1989). Mycelium water content was found to affect respiratory discrimination. This effect was removed by fitting a linear regression to the obtained discrimination values and normalizing all values to correspond to the mean water content, 94.4%.

2.2.7 Estimation of AOX contribution

The estimated mean discrimination values for COX and AOX were used as endpoints in a linear regression. The discrimination value of COX represented 0% AOX contribution and the discrimination value of AOX represented 100% AOX contribution. The obtained function was used to place all measured discrimination values along the slope, obtaining sample specific estimates of AOX contribution.

3 Results

The discrimination of control samples decreased significantly (p=0.046, ANCOVA) with increased water content (figure 3). Samples with a water content below 90% and above 95.8% were excluded due to extreme influence on the slope. Approximately 5% of the samples were excluded at this step. For the remaining samples, the discrimination values were adjusted along the slope of the regression to the mean water content, 94.4%, before further analyses. The O_2 uptake rate displayed no such correlation and did not have to be adjusted.

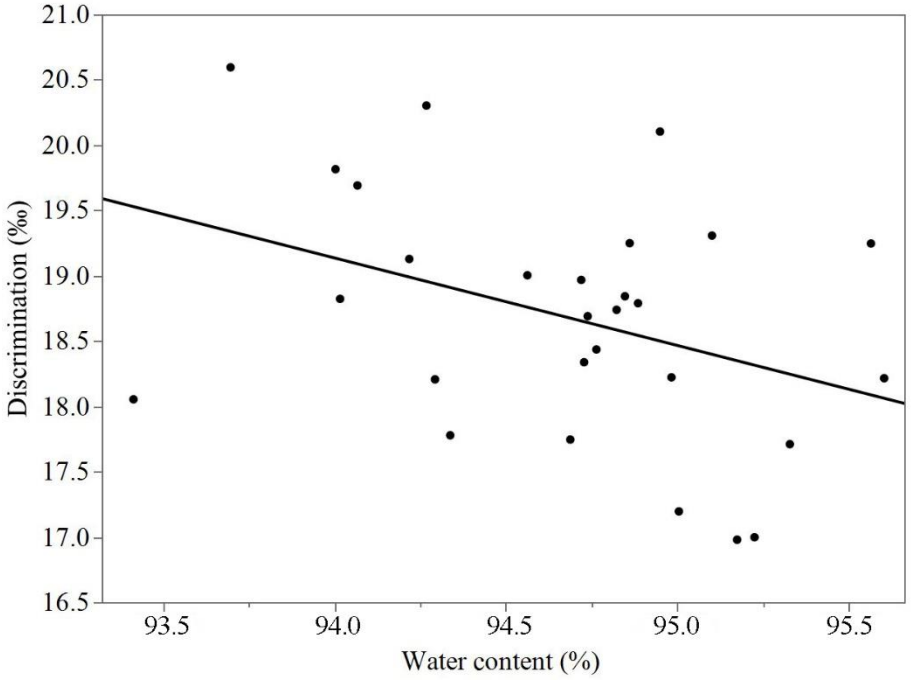

Figure 3. Correlation between discrimination and water content of control samples (p=0.046).

The old mycelium (outer 50% of the colony radius) had a mean discrimination of 19.5±0.4‰, which was significantly higher than the discrimination of young mycelium (inner 50% of the colony radius), 18.8±1.0‰ (p=0.042, Tukey's range test; figure 4a). The lowest discrimination was found within the young mycelium samples. The O_2 uptake rate was significantly lower for old mycelium than for young mycelium (165.4±15.6 µmol g^{-1} min^{-1} and 239.9±31.9 µmol g^{-1} min^{-1}, respectively, $p<0.0001$, Tukey's range test; figure 4b). The discrimination and O_2 uptake rate of the control group was different from that of the old mycelium (p=0.045 and

p<0.0001, respectively, Tukey's range test). The new mycelium was not significantly differing from the controls in any of the analyses.

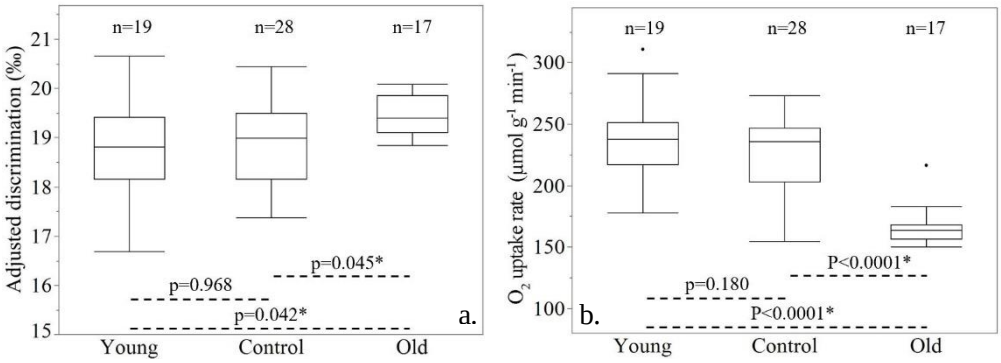

Figure 4. Discrimination adjusted for water content (a) and O_2 uptake rate (b) for controls, young and old mycelium. p-values from Tukey's range test is included for all pairs, as well as sample size.

The discrimination of the controls ranged from 17.5-20.4‰ and increased over time (p<0.0001, ANCOVA; figure 5a). During the first half of the sampling period, the mean discrimination was 18.5±0.7‰ (n=22) while for the second half it was 20.0±0.3‰ (n=5). Conversely the O_2 uptake rate decreased over time (p<0.0001, ANCOVA; figure 5b), with a mean of 238.1±21.4 µmol g^{-1} min^{-1} (n=22) for the first half of the sampling period, and 173.6±16.1 µmol g^{-1} min^{-1} for the second half (n=5; figure 5b).

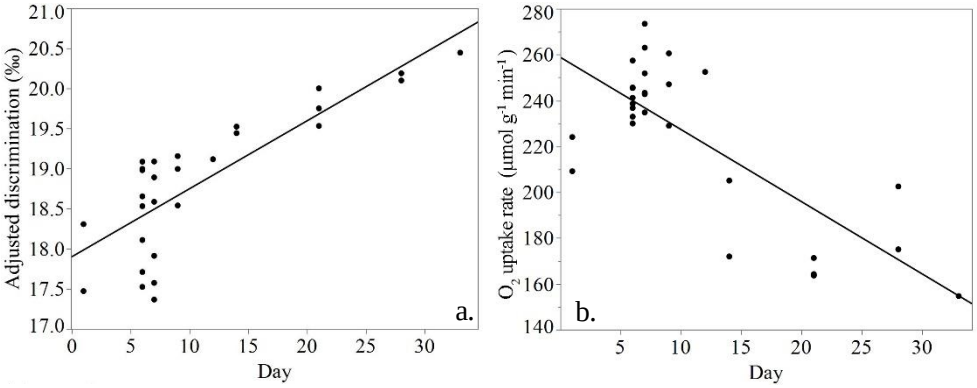

Figure 5. Discrimination adjusted for water content (a) and O_2 uptake rate (b) during the course of the trial.

The KCN treated samples displayed discrimination values well above the controls, 31.0±2.5‰ versus 18.8±0.9‰ (p<0.0001, Tukey's range test), and O_2 uptake rates well below, 35.8±9.9 µmol g^{-1} min^{-1} versus 224.0±34.4 µmol g^{-1} min^{-1} (p<0.0001, Tukey's range test).

None of the AOX inhibited samples displayed a lower discrimination value than the controls. The O_2 uptake rate was lower in the 5 and 10mM SHAM (DMSO)-treated samples than in the controls (p=0.006 and 0.001, Dunnett's test). Additionally, the samples treated with 10 mM n-PG displayed a lower O_2 uptake rate than

the controls ($p<0.0001$, Dunnett's test), however the sample size is too small to give a reliable result (n=3).

The SHAM-treated samples displayed a concentration dependant increase in discrimination and decrease in oxygen consumption ($p<0.0001$, ANCOVA; figure 6a,b).

The same correlation was seen in the n-PG-treated samples. The discrimination increase was significant ($p=0.001$, ANCOVA; figure 6a,b) while the decrease in O_2 uptake rate was not.

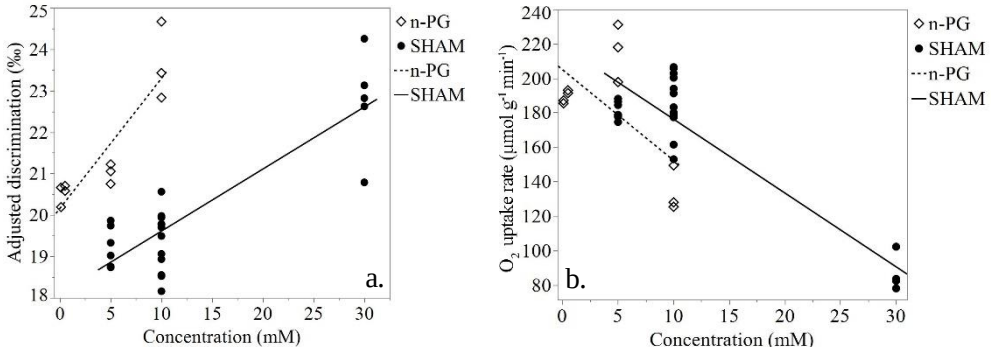

Figure 6. Dose dependant increase in discrimination (a) and decrease in O_2 uptake rate (b) for SHAM and n-PG treated samples.

The lowest discrimination value observed in this study was that of young mycelium (18.8±1.0‰). This is in the range of the discrimination values of COX reported for plants, and also similar to reported values for baker's yeast (*S. cerevisiae*), in which AOX is absent. The discrimination of AOX was approximately 12‰ higher than that of these samples. This is a relatively big difference, considering that the difference between COX and AOX discrimination within the same experiment is usually 8-9‰ (Henriksson *et al.*, 2019). Therefore, it was concluded that the AOX contribution was close to zero in this tissue. Based on this assumption, the AOX contribution could be estimated, using the discrimination value of young mycelium as a conservative lower endpoint for interpolation.

The AOX contribution was increasing over time ($p<0.0001$, ANCOVA) and ranged from 0 to 13.7% (figure 7). The highest levels of AOX activity were found in the controls from the second half of the experiment, as well as the old mycelium.

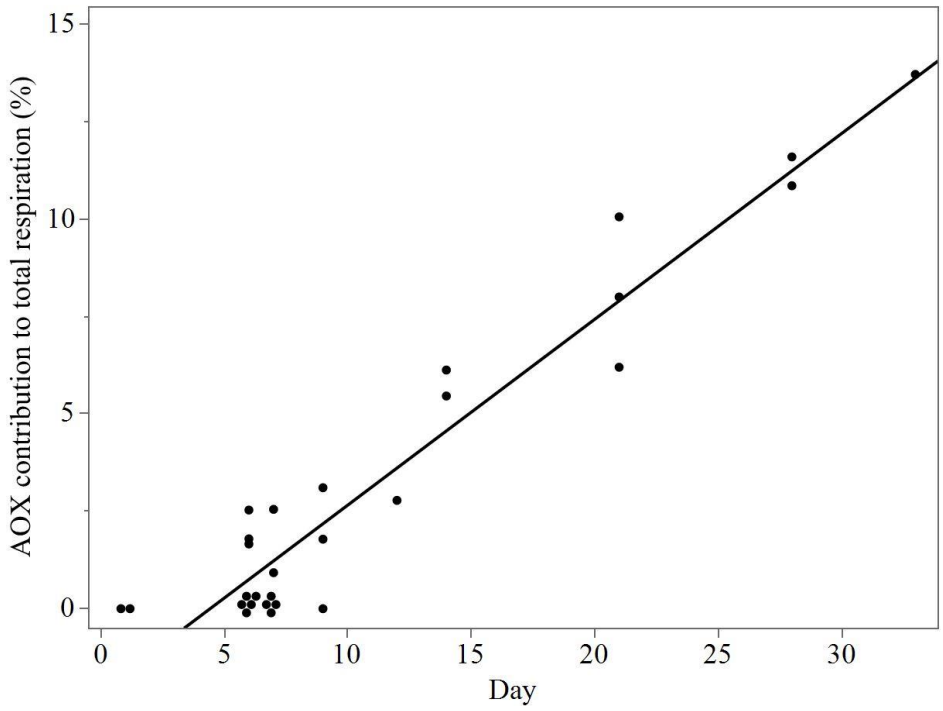

Figure 7. AOX contribution of control samples over time ($p<0.0001$).

The obtained discrimination and O_2 uptake rate values are summarized in table 1.

Table 1. *Discrimination and oxygen uptake rate for all treatments.*

Treatment	Solvent	n	O_2 uptake rate (µmol g^{-1} min^{-1})	SD	Discrimination (‰)	SD
Control		28	223 .97	34 .43	18 .84	0 .87
KCN		4	35 .78	9 .86	30 .96	2 .48
SHAM 5mM	DMSO	6	181 .75	5 .43	19 .25	0 .49
SHAM 10mM	DMSO	6	172 .44	12 .17	18 .97	0 .68
SHAM 10mM	H_2O	6	200 .18	6 .28	19 .79	0 .52
SHAM 30mM	DMSO	5	85 .79	9 .45	22 .73	1 .25
n-PG 0,1mM	EtOH	2	186 .36	1 .19	20 .43	0 .33
n-PG 0,5mM	EtOH	2	192 .29	1 .17	20 .65	0 .09
n-PG 5mM	EtOH	3	215 .73	16 .79	21 .01	0 .24
n-PG 10mM	EtOH	3	134 .33	13 .19	23 .65	0 .94
mCLAM 3.96mM	DMSO	4	225 .97	13 .35	19 .27	0 .35
Young		19	239 .86	31 .93	18 .78	0 .99
Old		17	165 .38	15 .58	19 .46	0 .41
1% EtOH		3	190 .35	12 .60	21 .04	0 .69
2% DMSO		8	177 .49	9 .22	19 .60	0 .25

4 Discussion

This is the very first time that the oxygen isotope discrimination method has been applied to measure AOX/COX partitioning in a fungal mycelium. As such, the current work represents a major step in advancing our understanding of carbon cycling in complex natural systems where both plants and fungi are present. The presented results reveal novel insights of the properties of AOX in mycorrhizal fungi and also highlight some of the difficulties with this method.

Before further analysis of the obtained data, external factors needed to be excluded. In the current study a linear correlation was found, showing that discrimination decreased as water content increased within the range of 90-95.8% water content ($p=0.046$, figure 3). The few samples with a water content below 90.0% displayed anomalous discrimination values, possibly caused by input errors. The samples with more than 95.8% water had an extreme influence on the slope and appeared to be affected by an additional factor (since the correlation was no longer linear above this value). Therefore, the samples with a water content below 90.0% and above 95.8% were excluded. Less than 5% of the data was discarded at this step. Additionally, all discrimination values were normalized to the mean water content, 94.4%, to exclude the influence of water content before further analyses.

The correlation discovered in this study was in line with that of a previous study performed by Henriksson *et al.* (2019), where the water content of *Pinus sylvestris* roots was significantly altering respiratory ^{18}O discrimination. A possible explanation for the interaction is limitations occurring during diffusion through liquid water. Usually, there is no isotopic fractionation during the diffusion step (Fry, 2008). However, when diffusion is limiting the transfer of substrate to the reaction site, the enzyme is more likely to react with the heavier isotope, thereby lowering the discrimination (Guy *et al.*, 1989, Miller *et al.*, 2011, Henriksson *et al.*, 2019).

The discrimination of the control samples ranged from 17.5-20.4‰, which is similar to the discrimination values previously reported for plants, 18.5-23.9‰ (Henriksson *et al.*, 2019).

During the 33 days of the experiment, the discrimination of non-inhibited control samples gradually increased. At the same time, their O_2 uptake rate decreased. The fact that both discrimination and O_2 uptake were observed to change over time, suggests that there is a metabolic difference among the prepared mycelial cultures.

The mycelium was grown to exclude as many external factors as possible, although there is one factor that might have caused this drift. During the culturing of fungal material, mycelium plugs were harvested from plates of pre-grown fungi. These cultures aged throughout the experiment and may have caused metabolic differences in the grown mycelium. However, it is important to note that the old plug of mycelium was removed from the sample before analysis.

Another fact that points towards an altered metabolism in the mycelium is that the same pattern was seen when fungal colonies were divided into old and young. They were significantly different in both discrimination and O_2 uptake rate.

Additionally, the discrimination and O_2 uptake rate of young mycelium (18.8±1.0‰ and 239.9±31.9 µmol g^{-1} min^{-1}) was similar to that of the controls from the first half of the sampling period (18.5±0.7‰ and 238.1±21.4 µmol g^{-1} min^{-1}), while the old mycelium was similar to the controls from the second half of the period (19.5±0.4‰, 165.4±15.6 µmol g^{-1} min^{-1} and 20.0±0.3‰, 173.6±16.1 µmol g^{-1} min^{-1}, respectively). The lower oxygen uptake combined with the higher discrimination in old mycelium indicate that the cytochrome pathway is less active, and possibly also that the alternative pathway is more active.

KCN treatment yielded discrimination values similar to those reported for AOX in plants (29.1-32.6‰) and somewhat higher than reported values for AOX in non-green tissue (25.6-25.7‰,)(Robinson *et al.*, 1995, Henriksson *et al.*, 2019). The elevated discrimination values in combination with an active, but reduced, O_2 consumption after treatment with KCN confirmed the presence of AOX in this species.

Conversely, the use of AOX-inhibitors to estimate the discrimination of COX turned out to be difficult. Several inhibitors and concentrations were tested with no satisfying result. The big variation within the control group, with discrimination values ranging from approximately 17.5‰ to 20.4‰, made comparisons and direct evaluation of the method problematic. However, as high concentrations of SHAM and n-PG resulted in significantly higher discrimination values than the control samples, it was clear that there were additional effects when using these inhibitors in mycelium, apart from their potential to block AOX activity. If no such effects occurred, the discrimination against ^{18}O for the AOX-inhibited samples would be equal to or below that of the controls.

There have been reported issues when using SHAM in intact tissues as opposed to when used in isolated mitochondria. The high discrimination values obtained with 30 mM SHAM could be caused by another oxygen consuming enzyme. For example, several studies have described extramitochondrial SHAM stimulated peroxidases (de Visser and Blacquière, 1984, Møller *et al.*, 1988, Diethelm *et al.*, 1990, Bingham and Stevenson, 1995). Diethelm *et al.* (1990) only found this effect in photosynthesising tissue, but others have found similar effects in various non-green tissues (Brouwer *et al.*, 1986, Bingham and Farrar, 1987, van der Plas *et al.*, 1987). These peroxidases are typically inhibited at higher concentrations of SHAM, but van der Plas *et al.* (1987) found that in potato tuber callus, 30mM SHAM did not fully inhibit the peroxidase. These studies were all performed on plants, so it is difficult to draw any conclusions regarding the possible effect of such a peroxidase in fungi. Another possible explanation is that the high concentrations of SHAM led to unspecific inhibition, meaning that not only AOX was inhibited (Bingham and Farrar, 1987, van der Plas *et al.*, 1987, Møller *et al.*, 1988).

The positive correlation between concentration and discrimination was also seen when using n-PG as the AOX-inhibitor. This is harder to explain, since n-PG is considered to be a more specific inhibitor than SHAM (Robinson *et al.*, 1995). There have been problems with uptake when using n-PG in intact tissues, but since the cells in the current study did react to the treatment this shouldn't be the case here. Since the same application method was used for all samples, it is not likely that the problem with the lower concentrations of SHAM and n-PG lies in uptake of the chemical.

As the current study focused on isotopic measurements of respiration, it is not possible to properly identify the cellular mechanisms underlying the unexpected values after addition of SHAM and n-PG. However, it is important to note that without isotopic measurements, these effects would not have been detected. Thus, if AOX/COX partitioning had been estimated merely from O_2 consumption measurements, the result would have been erroneous.

Although the AOX inhibitors did not give a satisfactory result, the contribution of AOX to total respiration could be estimated. Since the discrimination value of AOX was so much higher than that of the controls (31.0±2.5‰ versus 18.8±0.9‰), it was assumed that the *L. bicolor* mycelium in this study was not using AOX to a great extent. This is further supported by the reported value of ^{18}O discrimination in Baker's yeast (*S. cerevisiae*) which is within the same range (17.4±1.2‰) but represents COX respiration exclusively (Guy *et al.*, 1987).

The samples with the lowest discrimination values were the ones consisting of young mycelium (figure 4a). Following the reasoning above, it may be assumed that these samples had close to zero AOX activity and that the discrimination of these samples could be used as a conservatively estimated lower endpoint. Hence, a discrimination value of 18.8±1.0‰ was used as a conservative estimate of COX discrimination, similar to discrimination factors reported for COX in plants (15.7-21.1‰)(Henriksson *et al.*, 2019). Using the directly measured discrimination of AOX (in the presence of KCN) and the group with the lowest discrimination as a conservative estimate of COX discrimination, the AOX/COX partitioning was estimated.

The following conclusions could be drawn from the obtained data.
First, this study suggests that AOX does not play a large role in young mycelium of *L. bicolor* grown under lab conditions, but that this can increase significantly with age. Therefore, it cannot be ruled out that a significant portion of the fungal respiration could proceed via AOX under natural conditions, or that seasonal variation in AOX/COX partitioning may be considerable.

Second, a metabolic shift is suggested to occur as the mycelium ages. The shift discovered in this study was consistent and significant both when the cultured mycelial colonies were divided by age, as well as when whole such colonies grown from an ageing culture were compared.

Third, there are major issues when using existing protocols for inhibition of fungal AOX. In order to properly measure the discrimination of COX, a new protocol for inhibition must be developed. Several inhibitors and concentrations were tested in this study, but to successfully inhibit AOX, more time and effort is required. Future tests would benefit from immunoblots to determine the amount of AOX protein in the mycelium. This could also shed a light on the shifting AOX/COX patterns in ageing mycelium.

This novel study of AOX in mycorrhizal fungi reveals hitherto unobserved metabolic patterns in the mycorrhizal fungus *L. bicolor*. Further studies of fungal AOX under natural conditions, in combination with estimations of fungal biomass, has a great potential to improve our understanding of ecosystem carbon balances and increase the accuracy of carbon sequestration predictions.

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Tack!
Hilda Mikaelsson,
May 2019.

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