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IN VIVO METABOLIC REGULATION OF PLANT RESPIRATION UNDER SALT AND NUTRIENT STRESS. INTERACTION WITH MYCORRHIZA

Néstor Fernández Del Saz

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CERTIFICAMOS:

Que el presente trabajo titulado *"IN VIVO METABOLIC REGULATION OF PLANT RESPIRATION UNDER SALT AND NUTRIENT STRESS. INTERACTION WITH MYCORRHIZA"* presentado por Néstor Fernández Del Saz para optar al TÍTULO universitario oficial de DOCTOR por la *Universitat de les Illes Balears* dentro del programa de doctorado de Biología de las Plantas, se ha realizado bajo nuestra dirección.

Revisado el presente trabajo, autorizamos su presentación para que pueda ser juzgada por el tribunal correspondiente.

Palma de Mallorca, 22 de Mayo del 2017

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“*What helps you persevere is your resilience and commitment*.”

— Roy T. Bennett

SYMBOLS AND ABBREVIATIONS LIST

AM: arbuscular mycorrhiza

A_N: net photosynthesis

AOX: alternative oxidase

AOP: alternative pathway

ATP: adenosine triphosphate

COX: cytochrome oxidase

COP: cytochrome pathway

Δ_a: oxygen isotope fractionation by the alternative pathway

Δ_c: oxygen isotope fractionation by the cytochrome pathway

Δ_n: oxygen isotope fractionation in the absence of inhibitors

g_s: stomatal conductance

KCN: potassium cyanide

mETC: mitochondrial electron transport chain

v_{alt}: alternative pathway activity

v_{cyt}: cytochrome pathway activity

P: phosphorus

RGR: relative growth rate

SHAM: salicylhydroxamic acid

TCA: tricarboxilic acid

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SUMMARY

The regulation of the alternative respiration in plants under nutrient deficiency and osmotic stresses is not fully understood. Both stresses have important repercussions on plant growth, and they have shown to increase the activity of the alternative oxidase (AOX) in different situations, although not always. Such a disparity may reside in duration, stress severity and plant stress tolerance. Under stress, increases of AOX activity are considered as an adaptation of the respiratory metabolism when the activity of the cytochrome oxidase (COX) is restricted. This phenomenon was observed only in roots of plants grown under long-term P limitation; AOX activity allowed the synthesis of respiratory metabolites such as citrate, a tricarboxylic organic acid (TCA) that is exuded into the rhizosphere to increase the availability of P. It is also known that plants increase the synthesis of several metabolites in response to salinity, but their relation with AOX activity has not been tested. Thus, the metabolic regulation of AOX activity under both P-limitation and salinity has been poorly studied.

For a deeper understanding of the regulation of the respiratory metabolism under both stresses, I studied the effect of different long and short-term salt and nutrient stress. I also tested this regulation by using arbuscular mycorrhizal (AM) fungus colonization, reputed to benefit P uptake, to decrease the exudation of rhizosphere exudates, and to confer tolerance in plants. On the top of that, the role of AOX activity in AM plants is unknown, which could provide further information about the effect of AM colonization on plant respiration, a controversial issue in literature.

Tests were performed by using the oxygen isotope technique to measure the *in vivo* activities of COX and AOX. My results showed that AOX activity allowed an acclimation of respiration favoring the synthesis of TCA metabolites and others synthesized from TCA cycle under P limitation and salinity. Moreover, I observed different responses of AOX activity in AM organs with influence on plant growth. In AM leaves, increases of AOX activity contributed to synthesize ATP allowing faster rates of shoot growth under long-term salt stress. In AM roots, slower rates of AOX activity under long-term P limitation allowed an accumulation of carbon due to the lack of a demand for the synthesis of rhizosphere exudates. Finally, I noticed that changes on plant P status regulates P uptake by modulating AOX activity, which allows the synthesis of citrate.

RESUMEN

La regulación de la respiración alternativa en plantas bajo estrés nutricional y salino no es del todo comprendida. Ambos estreses tienen importantes repercusiones en el crecimiento vegetal, y además han incrementado la actividad de la oxidasa alternativa (AOX) en algunos casos. Tal disparidad puede residir en la duración, severidad del estrés, y la tolerancia de la planta frente a éstos estreses. Bajo estrés, incrementos de actividad son considerados adaptaciones del metabolismo respiratorio cuando la actividad de la citocromo oxidasa (COX) es restringida. Tal efecto se observó sólo en raíces de plantas crecidas bajo en deficiencia de fósforo (P); donde la actividad AOX permitió la síntesis de metabolitos respiratorios como citrato, un ácido tricarboxílico (TCA) que es exudado a la rizosfera para incrementar la disponibilidad de P. También se sabe que las plantas incrementan la síntesis de ciertos metabolitos en respuesta a la salinidad, pero su conexión con la actividad AOX no se ha comprobado. Por lo tanto, la regulación de la actividad AOX bajo déficit de fósforo y salinidad han sido pobremente estudiados.

He estudiado el efecto de diferentes duraciones de estrés nutricional y salino para una mejor comprensión de la regulación del metabolismo respiratorio bajo ambos estreses. Además, estudié esta regulación usando micorrizas, reputadas por incrementar la adquisición de P, disminuir la exudación de carboxilatos, y conferir tolerancia en plantas. Además, el papel de AOX en plantas micorrizadas es desconocido, y podría arrojar importante información sobre el efecto de la colonización de hongos formadores de micorrizas arbusculares, lo cual es un tema controvertido en la literatura.

La principal técnica empleada en esta Tesis fue la del fracionamiento isotópico, que permite medir las actividades COX y AOX. Mis resultados mostraron que la actividad AOX permitió, bajo estrés salino y nutricional, una aclimatación de la respiración favoreciendo la síntesis de metabolitos TCA y otros sintetizados a partir de este ciclo. Además, observé diferentes respuestas de la actividad AOX en diferentes órganos de plantas micorrizadas, associadas a efectos en el crecimiento vegetal. En hojas, incrementos de actividad AOX favorecieron la síntesis de ATP permitiendo rápidas tasas de crecimiento bajo ambos estreses. En raíces, tasas lentas de actividad AOX permitieron una acumulación de carbono debido a la ausencia de una demanda de carboxilatos. Finalmente, presencié cómo el contenido de P regula la adquisición de P a través de su efecto sobre la actividad AOX, la cual regula la síntesis de citrato.

RESUM

La regulació de la respiració alternativa en plantes sota estrès nutricional i salí no és del tot compresa. Ambdós estressos tenen importants repercussions en el creixement vegetal, i a més han incrementat l'activitat de l'oxidasa alternativa (AOX) en alguns casos. Tal disparitat pot residir en la durada, severitat de l'estrès, i la tolerància de la planta enfront d'aquests estressos. Sota estrès, increments d'activitat són considerats adaptacions del metabolisme respiratori quan l'activitat de la citocrom oxidasa (COX) és restringida. Aquest efecte es va observar només en arrels de plantes crescudes amb deficiència de fòsfor (P); on l'activitat AOX va permetre la síntesi de metabòlits respiratoris com citrat, un àcid tricarboxílic (TCA) que és exsudat a la rizosfera per incrementar la disponibilitat de P. També se sap que les plantes incrementen la síntesi de certs metabòlits en resposta a la salinitat, però la seva connexió amb l'activitat AOX no s'ha comprovat. Per tant, la regulació de l'activitat AOX sota dèficit de fòsfor i salinitat han estat pobrament estudiats. He estudiat l'efecte de diferents durades d'estrès nutricional i salí per a una millor comprensió de la regulació del metabolisme respiratori baix tots dos estressos. A més, vaig estudiar aquesta regulació emprant micorizes, reputades per incrementar l'adquisició de P, disminuir l'exsudació de carboxilats, i conferir tolerància en plantes. A més, el paper d'AOX en plantes micorizades és desconegut, i podria llançar important informació sobre l'efecte de la colonització de fongs formadors de micorizes arbusculars, la qual cosa és un tema controvertit en la literatura.

La principal tècnica emprada en aquesta Tesi va ser la del fraccionament isotòpic, que permet mesurar les activitats COX i AOX. Els meus resultats van mostrar que l'activitat AOX va permetre, sota estrès salí i nutricional, una aclimatació de la respiració afavorint la síntesi de metabòlits TCA i altres sintetitzats a partir d'aquest cicle.

A més, vaig observar diferents respostes de l'activitat AOX en diferents òrgans de plantes micorizades, associades a l'efecte en el creixement vegetal. En fulles, increments d'activitat AOX van afavorir la síntesi d'ATP permetent ràpides taxes de creixement sota ambdós estressos. En arrels, taxes lentes d'activitat AOX van permetre una acumulació de carboni a causa de l'absència d'una demanda de carboxilats. Finalment, vaig presenciar com el contingut de P regula l'adquisició de P a través del seu efecte sobre l'activitat AOX, la qual regula la síntesi de citrat.

BACKGROUND AND OBJECTIVES

The regulation of the alternative oxidase (AOX) activity under a large range of abiotic and biotic stresses has been extensively studied in plants (see Introduction). Among them, nutrient deficiency and osmotic stresses have important repercussions on crop productivity (Kasuga et al., 1999; Gamuyao et al., 2012).

Both stresses have also shown to increase the activity of AOX whilst respiration via cytochrome oxidase (COX) pathway was restricted (Gonzalez-Meler et al., 2001; Ribas-Carbo et al., 2005). Increases of AOX activity under COX restriction were first suggested (Lambers et al., 2005) and later demonstrated (Florez-Saraza et al., 2014) to constitute an adaptation of respiratory metabolism, especially under phosphorus (P) limitation. Florez-Sarasa et al., (2014) observed that growth under P limitation increased the activity of AOX in cluster roots of *Lupinus albus* allowing the synthesis of respiratory metabolites such as citrate, a root carboxylate that is exudated into the rhizosphere to increase the availability of P from soils (Lambers and Plaxton, 2015). It was stated that AOX contributes to dissipate the excess of NADH produced during the synthesis of this TCA metabolite when COX activity is restricted (Florez-Saraza et al., 2014). Nevertheless, in leaves, the response of AOX activity under P limitation has shown a disparity without any reasonable explanation, and it has not been related to any respiratory metabolite in this organ (Gonzalez-Meler et al., 2001).

Regarding salt stress, its effect on AOX respiration in roots has not been studied, while in leaves no response was observed in *Pisum sativum* plants under long-term stress (Marti et al., 2011). Nevertheless, it is known that short-term severe osmotic stress induces important metabolic changes in plants (Wormit et al., 2012). However, the regulation of AOX activity by TCA metabolites (or derivates) under salt stress has not been tested. Similar to what was observed under P limitation, one may think that an increase of AOX activity should occur only under conditions where the synthesis of TCA metabolites is also induced by osmotic stress.

Clearly, more evidences are needed to state that AOX activity permits a metabolic flexibility that helps overcoming the negative impact of both stresses on plant respiration. The application of both long-term and short-term stresses may contribute to clarify the implication of AOX activity in allowing an acclimation of metabolism, which may lead to enhanced plant tolerance to both salinity and P limitation in different plant organs.

Another useful scenario to test the effect of both salt and P limitation stresses on AOX activity is provided by using mycorrhiza, reputed to benefit plant growth and P uptake (Smith et al., 2008), as well as to confer plant tolerance to both stresses (Ruiz-Lozano 2003; Cantrell and Linderman 2001; Evelin et al. 2009). Arbuscular mycorrhizal (AM) symbioses play an important role in ecosystems increasing soil carbon storage (Fitter et al., 2000). By promoting P uptake and increasing carbon fixation in plants, they can also contribute to decrease anthropogenic [CO_2] from the atmosphere (Fitter et al., 2000), which is of interest in a future context of climate change also characterized by a severe nutrient deficiency and elevated levels of salts in soils (Munns and Tester, 2008; Gilbert 2009).

There is no consensus about the effect of AM colonization on plant respiration because it has been shown to be dependent of several factors, such as plant and fungal species (Pang and Paul 1980; Silsbury et al. 1983; Peng et al. 1993; Nielsen et al. 1998; Hughes et al. 2008; Rewald et al. 2015; Otgonsuren et al. 2016). The respiratory response of AM colonized plants under nutrient and salt stresses could be important to clarify whether AM symbioses would keep acting as a carbon sink in the future. Undoubtedly, the regulation of respiratory metabolism in AM colonized plants is a good starting point to understand how these abiotic stresses may affect their carbon metabolism. Heretofore, it is known that AM colonization decreases the amount of rhizosphere carboxylates such as citrate, as observed in several plant species (Ryan et al. 2012, Nazeri et al. 2013), and that it reduces root respiration also coinciding with a decrease in root metabolism (Romero-Munar et al., 2017). A further relation between the synthesis of carboxylates and the alternative respiration in AM colonized plants has not been tested and clearly it may provide more information not only about the regulation of AOX under P limitation, but also about the effect of AM colonization on plant respiration. Moreover, by studying this symbiotic interaction we could reveal the trigger of such a coordinated response between AOX activity and the synthesis of TCA metabolites, which may reside in the P concentration in plants, as was also demonstrated for other P starvation responses (Liu et al. 1998, Lambers et al. 2006, Chiou and Lin 2011).

Thus, the present Thesis is based on the idea that the use of mycorrhiza will provide important information about the regulation of AOX activity under salinity and P limitation. Moreover, I hypothesized that: (1) AOX activity confers tolerance in plants under salinity; (2) AOX activity allows the continuity of TCA cycle under salt stress; (3) AOX activity

contributes to increase growth in AM colonized plants under P limitation and salinity; (4) AOX activity contributes to decrease respiration in AM colonized roots due to the reduced synthesis of rhizosphere carboxylates; and (5) AOX activity is regulated by changes on plant P concentration.

The present hypotheses were partially based on the observations of a research paper not included as part of the present Thesis (Romero-Munar A, Del-Saz NF, Ribas-Carbo M, Flexas J, Baraza E, Florez-Sarasa I, Fernie AR, Gulías J (2017) Arbuscular mycorrhizal symbiosis with *Arundo donax* decreases root respiration and increases both photosynthesis and plant biomass accumulation. Plant, Cell and Environment, doi: 10.1111/pce.12902"). They were fully developed considering all the observations about AOX activity described in the **INTRODUCTION**, which has been submitted for publication:

> "Del-Saz NF, Ribas-Carbo M, McDonald A, Lambers H, Fernie AR and Florez-Sarasa I (2017) Closing in on the enigmatic role of the alternative oxidase pathway in plants (Submitted to Trends in Plant Science)"

All the experiments were performed using the isotope fractionation technique. Therefore, this is the only technique described in the **MATERIAL AND METHODS**, a section that has also been published as a book chapter:

> "Del-Saz NF, Ribas-Carbo M, Martorell G, Fernie AR, Florez-Sarasa I (2017) Measurements of electron partitioning between cytochrome and alternative oxidase pathways in plant tissues (MiMB, Plant respiration and internal oxygen: Methods and protocols. In press)"

All other methodologies used are described in each of the Chapters of the RESULTS section, which is organized as a series of five manuscripts published, either submitted, which address all the presented hypotheses, with the following five specific questions:

- **Chapter 1. Does AOX activity confer tolerance in plants under salinity?**

  "Del-Saz NF, Florez-Sarasa I, Clemente-Moreno MJ, Mhadhbi H, Flexas J, Fernie AR, Ribas-Carbo M (2016) Salinity tolerance is related to cyanide-resistant alternative respiration in *Medicago truncatula* under sudden severe stress. Plant, Cell and Environment 39: 2361–2369

- **Chapter 2: Does AOX activity really allow the continuity of TCA cycle under salt stress?**

  "Del-Saz NF, Alonso D, López-Gómez M, Palma F, Clemente-Moreno MJ, Florez-Sarasa I, Fernie AR and Ribas-Carbo M (2016). Sudden severe drought and salt stress in *Arabidopsis thaliana* increases the rate of both polyamine synthesis and leaf respiration via alternative oxidase (Submitted to Journal Experimental Botany)"

- **Chapter 3. Does AOX activity contribute to increase growth in AM colonized plants under P limitation and salinity?**

  "Del-Saz NF, Romero-Munar A, Alonso D, Aroca R, Baraza E and Ribas-Carbo M (2016) Respiratory ATP cost and benefit of arbuscular mycorrhizal symbiosis with *Nicotiana tabacum* at different growth stages and under salinity (Submitted to Enviromental and Experimental Botany)"

- **Chapter 4. Is root respiration of AM plants affected by the exudation of rhizosphere carboxylates?**

  "Del-Saz NF, Romero-Munar A, Cawthray GR, Aroca R, Baraza E, Flexas J, Lambers H, Ribas-Carbo M (2017) Arbuscular mycorrhizal fungus colonization in *Nicotiana tabacum* decreases the rate of both carboxylate exudation and root respiration and increases plant growth under phosphorus limitation. Plant and Soil doi:10.1007/s11104-017-3188-y"

- **Chapter 5. Is AOX activity regulated by plant Phosphorus status?**

  "Del-Saz NF, Romero-Munar A, Cawthray G, Palma F, Aroca R, Baraza E, Florez-Sarasa I, Lambers H and Ribas-Carbo M (2017) Phosphorus concentration coordinates respiratory bypasses, synthesis and exudation of citrate, and the expression of high-affinity phosphorus transporters in *Solanum lycopersicum* plants (Submitted to Plant, Cell and Environment)"

Finally, a general **DISCUSSION** is shown, preceding the **CONCLUSIONS** of the present Thesis.

INTRODUCTION

Closing in on the enigmatic role of the alternative oxidase pathway in plants

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ABSTRACT

After more than a century of research, the role of the alternative oxidase (AOX) pathway is still enigmatic. Despite intense research on the *in vitro* characterization of regulatory factors modulating AOX, the regulation of its activity *in vivo* is still not fully understood. Here, advances concerning *in vitro* and *in vivo* regulation of AOX are reviewed and regulatory factors that merit future research are highlighted. In addition, we review and discuss the main biological functions assigned to the AOX and suggest future experiments in order to unravel its enigmatic role.

1. The alternative oxidase: is there a paradigm?

Respiration in plants involves redox reactions that consume oxygen (O_2) and reducing equivalents (NAD(P)H and $FADH_2$), and produce carbon skeletons, carbon dioxide (CO_2) and energy (ATP). ATP synthesis in mitochondria is coupled to O_2 consumption in the mitochondrial electron transport system, mainly through the cytochrome oxidase pathway (COP). In the plant kingdom, there is a strongly conserved alternative non-phosphorylating electron transport pathway [1,2], that also reduces O_2 to H_2O. This is achieved by a terminal alternative oxidase (AOX) located in the inner mitochondrial membrane, and is hence termed the AOX pathway (AOP) [1].

The ubiquinone (UQ) pool is the branch point of electron flow between the two respiratory pathways [3]. In contrast to the COP, the transfer of electrons from reduced UQ to O_2 via the AOP is not coupled to the extrusion of protons from the matrix to the intermembrane space, and energy is hence lost as heat [3]. The AOX is insensitive to a number of respiratory inhibitors that affect the other components of the respiratory chain. In contrast, AOX, is sensitive to inhibition by salicylhydroxamic acid (SHAM) and *n*-propyl gallate [4].

For many years, electron flux to the AOX was assumed to occur only at a high reduction state of the UQ pool, when COP was at, or near, saturation [5,6]. This led to the paradigm that AOP acts as an energy overflow when COP operates at full capacity [7], and to the widespread use of specific inhibitors to ascertain the activity of the AOX. However, further studies on isolated mitochondria showed that AOX can be active at much lower UQ reduction levels, when COX is not saturated [8].

AOX displays a greater discrimination against the heavy isotope $^{18}O_2$ than does COX [9] which allowed the development of a non-invasive mass spectrometry technique to measure the electron partitioning between the two pathways in the absence of inhibitors [9]. Using this technique, it was demonstrated that AOP competes with the COP for electrons of the UQ pool [9,10], and that changes in respiration rates after SHAM addition can underestimate the actual activity of the AOP. Thereafter, the use of inhibitors was considered inadequate to determine the activities of the COP and AOP [11], and it is now widely accepted that the actual AOX activity can only be determined by the oxygen-isotope fractionation technique, whereas the use of inhibitors is valid only to determine the capacities (maximum electron fluxes) of COP and AOP [12,13].

A widely accepted function of the AOP is that of heat generation in thermogenic plants [14]. Oxygen-isotope fractionation measurements showed that a dramatic increase in AOX activity in the floral receptacles is associated with a high thermogenic activity in *Nelumbo nucifera*

[15]. However, AOX is abundant and active at considerable levels in many non-thermogenic tissues, but despite its extensive characterization at the biochemical and molecular levels, its *in vivo* role remains poorly defined. Many functions have been ascribed to the AOX, including preventing over-reduction of the UQ pool, thus reducing formation of reactive oxygen species (ROS) [12].

Despite reducing energetic efficiency, induction of AOX activity is required in several physiological situations in order to confer metabolic flexibility allowing plants to produce heat [15], tolerate stress [13] and maintain growth in balance with resource availability [16,17]. Under stress conditions, partitioning of electrons to the AOP (τ_a, the proportion of AOX to total respiration), is usually increased (Table 1). However, the *in vivo* activity of the AOX in the absence of stress accounts for 10 to 50% of total respiration [18], and this proportion is maintained at a range of temperatures normally encountered during plant growth [19]. These observations led to questioning the role of the non-phosphorylating AOP under non-stress conditions. Notably, τ_a increases also occur during development in floral tissues [15,20], roots [21,22] and leaves [23,24] and during cotyledon greening [25]. Therefore, the function of AOX would appear to extend beyond its role under stress. That said, it is likely that AOX plays a central role in cell reprogramming under stress, mainly due to its ability to control the production of signaling molecules such as ROS and reactive nitrogen species (RNS) that elicit mitochondrial retrograde signaling [26-29]. Recently, previously suggested roles for AOX and two newly proposed general ones including the maintenance of metabolic and signaling homeostasis, particularly under stress, have been compiled [13]. The use of the term ‘homeostasis’, which has slipped into the recent plant literature, is unfortunate, as the term implies stability, such as with blood sugar levels or body temperature [30]. Nevertheless, there may well be a role for AOX in modulating the production of signaling molecules, which affect gene regulatory networks with consequences for mitochondrial and other cell components involved in stress responses [13,28,31]. However, the production of signaling molecules such as ROS and RNS is likely associated with only transient changes in AOX activity that are not currently technically detectable with the O_2 isotope-fractionation method [13,32] and as such lie beyond the scope of this article.

Table 1. Literature on plant tissue electron partitioning to AOP (τ_a) response to different stresses across plant species. Data on wild-type plants was only used in the studies involving experiments with mutant or transgenic plants.

Species	Tissue	Stress	τ_a	AOX activity response	Stress duration	Refs
Glycine max	Cotyledons	Allelochemicals	Variable	No change	Short-term	[106]
Zea mays	Leaves	Chilling	Increase	Increase	Short-term	[101]
Medicago truncatula	Leaves	Salt stress	Variable	Variable	Short-term	[111]
Arabidopsis thaliana	Leaves	Temperature	Variable	Variable	Short-term	[99]
Nicotiana tabacum	Leaves	Bacterial infection elicitor	Increase	Increase	Short-term	[53]
Nicotiana tabacum	Leaves	Water stress	Increase	Increase	Short-term	[51]
Glycine max	Roots	Herbicides	Variable	Variable	Short-term	[73]
Medicago truncatula	Roots	Salt stress	Decrease	Decrease	Short-term	[111]
Arabidopsis thaliana	Leaves	High light	No change	Increase	Short-term	[61]
Arabidopsis thaliana	Leaves	High light	No change	Increase	Short-term	[89]
Arabidopsis thaliana	Leaves	High light	No change	Increase	Short-term	[18]
Cucumis sativus	Leaves	High light	No change	Increase	Short-term	[18]
Glycine max	Leaves	High light	No change	No change	Short-term	[18]
Nicotiana silvestris	Leaves	High light	No change	Increase	Short-term	[18]
Pisum sativum	Leaves	High light	No change	No change	Short-term	[18]
Cucurbita pepo	Leaves	Temperature	No change	Increase	Short-term	[19]
Nicotiana sativa	Leaves	Temperature	No change	Increase	Short-term	[19]
Vicia faba	Leaves	Temperature	No change	Increase	Short-term	[19]
Nicotiana tabacum	Leaves	Virus	No change	No change	Long-term	[122]
Glycine max	Leaves	Water stress	Increase	Increase	Long-term	[52]
Nicotiana silvestris	Leaves	Water stress	Increase	No change	Long-term	[55]
Beta vulgaris	Leaves	Zn toxicity	Decrease	Increase	Long-term	[123]
Glycine max	Cotyledons	High Temperature	Decrease	Increase	Long-term	[98]
Spinacia oleracea	Leaves	High light	Increase	Increase	Long-term	[94]

Alocasia odora	Leaves	High light	Increase	No change	Long-term	[94]
Cucumis sativus	Leaves	High light	No change	Increase	Long-term	[95]
Arabidopsis thaliana	Leaves	High light	No change	Increase	Long-term	[61]
Arabidopsis thaliana	Leaves	High light	No change	Increase	Long-term	[89]
Populus canandensis	Leaves	High Temperature	Increase	not shown	Long-term	[102]
Chionochloa rubra	Leaves	High Temperature	Variable	not shown	Long-term	[58]
Chionochloa pallens	Leaves	High Temperature	Variable	not shown	Long-term	[58]
Phaseolus vulgaris	Leaves	P deficiency	Increase	Increase	Long-term	[104]
Nicotiana tabacum	Leaves	P deficiency	Decrease	Decrease	Long-term	[104]
Gliricidia sepium	Leaves	P deficiency	Increase	Increase	Long-term	[104]
Pisum sativum	Leaves	Salt stress	Increase	No change	Long-term	[56]
Spartina densiflora	Roots	Cu toxicity	No change	Decrease	Long-term	[108]
Agrostis stolonifera	Roots	High Temperature	Decrease	Decrease	Long-term	[103]
Agrostis scabra	Roots	High Temperature	No change	Decrease	Long-term	[103]
Metrosideros umbellata	Leaves	N/P deficiency	Decrease	Decrease	Long-term	[59]
Weinmannia racemosa	Leaves	N/P deficiency	Decrease	Decrease	Long-term	[59]
Arabidopsis thaliana	Leaves	High CO_2	Increase	Increase	Long-term	[124]
Opuntia ficus-indica	Leaves	High CO_2	Increase	Increase	Long-term	[115]
Salsola divaricata	Leaves	Low temperature	Increase	Increase	Long-term	[100]
Poa annua	Roots	Low light	Increase	Increase	Long-term	[81]
Betula nana	Leaves	Low light/nutrient stress	No change	not shown	Long-term	[119]
Eriophorum vaginatum	Leaves	Low light/nutrient stress	No change	not shown	Long-term	[119]
Rubus chamaemorus	Leaves	Low light/nutrient stress	No change	not shown	Long-term	[119]
Nicotiana tabacum	Roots	P deficiency	Increase	Increase	Long-term	[105]

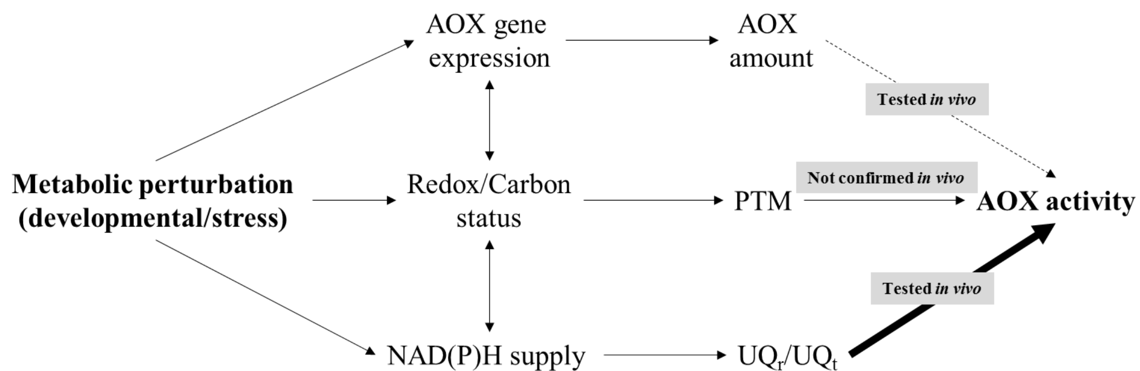
Box 1. Structural, genetic and evolutionary features of the AOX

AOX is a single subunit UQ oxidase in the inner mitochondrial membrane which facilitates the four-electron reduction of O_2 to H_2O using reduced UQ [2,33-36]. AOX comprises a conserved four-helix bundle that co-ordinates the binuclear di-iron center responsible for the binding and activation of O_2 [2,37,38].

AOX has been hypothesized to be lost in those organisms with sophisticated mechanisms to control the O_2 delivery to their cells, thus providing an explanation for the distribution of AOX across the different domains of life [39]. It was also suggested that O_2 scavenging is an ancestral relic of the transition to an oxygenic atmosphere [16]. Despite the fact that control of ROS production has been one of the main proposed roles of AOX in plants, the rates of ROS production and AOX respiration have not yet been quantitatively assessed in parallel**.**

In eukaryotes, AOX is a nuclear-encoded gene in various species of fungi, protists, and animals and found in all plants, often as a multigene family [40]. Recent protein phylogenies based on AOX gene family sequences analyses are refining the evolutionary history and taxonomic distribution of AOX from algae to angiosperm plants [41-43]. Two types of AOX are present in basal monocot and dicot plants (*AOX1* and *AOX2*), while in other monocots there is only one AOX [43-45]. *AOX* gene expression is influenced through the process of mitochondrial retrograde regulation (MRR), whereby changes in nuclear gene expression are directed by the mitochondrion [46]. The transcription factor abscisic acid-insensitive 4 (ABI4) plays a central role in mediating mitochondrial retrograde signals to induce *AOX1* expression [47]. This provides a molecular link between *AOX1* expression and signaling by abscisic acid which may explain why *AOX1* expression has been postulated to play an important role in the response to many stresses [13]. In addition, compounds associated with oxidative stress also influence *AOX1* transcript levels, as well as several systemic signaling molecules such as salicylic acid, methyl salicylate, ethylene and jasmonic acid [13]. Furthermore, intermediates of the TCA cycle such as citrate, malate and 2-oxoglutarate and metabolites such as acetate and cysteine can activate *AOX1* expression [13]. Inhibition of the TCA cycle, as well as, dysfunction or inhibition of complexes I, III, IV or V also induces *AOX1* expression [13]. By contrast, the specific expression of *AOX2* genes in different tissues and at different developmental stages suggests that these genes are rather involved in 'housekeeping' functions in. *e.g.*, reproductive tissues [48,49].

Box 2: Relevance of AOX protein levels on its activity *in vivo*



Metabolic perturbations associated with different developmental processes and/or environmental stresses induce changes in gene expression, redox/carbon status and NAD(P)H supply, potentially affecting AOX activity *in vivo*. Transcript abundance is frequently not a good predictor of protein abundance, which in turn may not be a good predictor of enzyme activity [50]. Furthermore, the *in vivo* activity of any given enzyme is often much lower than its capacity (maximum activity), because of limitations of substrates, allosteric activators or incomplete post-translational activation of the enzyme [50]. The study of AOX represents a rare case in which its *in vivo* activity can be measured by a non-invasive technique. In the last decade, several studies have combined AOX transcript, protein and *in vivo* activity determinations after stress treatments, thus confirming that AOX activity *in vivo* is mostly tuned at the post-translational level [18,51-60]. While stress application is useful to assess the impact of AOX protein or capacity changes on its activity *in vivo*, measurements of *in vivo* activity in AOX-genetically-modified plants are the best approach to assess the relevance of the post-translational regulation in the absence of other stress-related factors [51,61]. The AOX protein level does not regulate its activity *per se*; however, it can limit the extent to which the activity can increase. For instance, *in vivo* AOX activity is not induced in AOX anti-sense plants after high-light treatment, because of its protein/capacity limitation [61]. Indeed, an AOX overcapacity (i.e. higher AOX capacity than activity) has been observed in several species, albeit to a different extent [59,62]. Such overcapacity allows a rapid increase in AOX activity by the activation of the existing protein, circumventing the need for *de novo* synthesis. These observations support the concept that post-translational regulation confers flexibility and adaptability on respiratory metabolism thus enabling rapid adjustment to the prevailing environmental conditions [32,50,63]. In this respect, increased levels of α-ketoacids and redox state in the mitochondrial matrix can activate AOX through post-translational modifications (PTM), but this is not confirmed *in vivo* (see section 2). Finally,

increased levels of NAD(P)H in both the cytosol and mitochondrial matrix lead to increases in the reduction state of the UQ pool (see section 2) which ultimately affects AOX activity *in vivo*.

2. Regulation of AOX activity: from *in vitro* to *in vivo*

Biochemical characterizations of the AOX protein and its activity in isolated mitochondria highlighted regulatory factors modulating AOX activity including protein amount, activation state and substrate availability (Box 2). The extent to which these regulatory features affect the AOX activity *in vivo* was reviewed [13]. Since then, studies reporting AOX *in vivo* data have clarified some issues, while others remain unresolved. Post-translational regulation of AOX is crucial (Box 2), but to what extent does post-translational regulation, as characterized *in vitro*, actually regulate activity *in vivo*?

The reversible formation of dimers via oxidation/reduction of conserved Cys residues is the first post-translational modification described for AOX [64]. This mechanism clearly affects AOX activity *in vitro*, but AOX is generally in its reduced-active form in whole tissue extracts, thus questioning the significance of this mode of regulation *in vivo* [12]. However, some authors reported redox changes in AOX in leaves of the shade species, *Alocasia odora*, when plants were transferred from very-low to high-light (HL) conditions [65]. Notably, post-translational regulation of leaf AOX underlies the response of AOX *in vivo* activity in different species after HL treatment [18]. Additionally, the oxidized form of AOX occurs in tobacco leaf extracts, and its ratio to the reduced form increases at an advanced stage of programmed cell death (PCD) when AOX *in vivo* activity declines [53]. Thioredoxin is a candidate involved in redox activation of AOX [66]. While there is evidence for thioredoxin modulating the AOX reduction state and stimulating its capacity in isolated mitochondria from poplar [66], pea [67] and *Arabidopsis* leaves [68], there is as yet no evidence that AOX is regulated by the thioredoxin system *in vivo*. Given that the use of mitochondrial thioredoxin mutants has made it possible to unravel the redox regulation of mitochondrial TCA cycle enzymes *in vivo* [69,70], it follows that taking a similar approach to examine *in vivo* electron partitioning to AOX in these mutants should allow unambiguously resolution whether thioredoxin regulates AOX *in vivo*.

Once in its reduced state, AOX interacts with α-keto-acids via two conserved Cys residues [71], causing conformational changes in the protein and activity increases [72]. Pyruvate is one of the most effective activators at lower UQ reduction levels [8]. However, the relevance of pyruvate stimulation *in vivo* was questioned [12], e.g., because changes in AOX *in vivo*

activity during soybean root development do not correlate with changes in pyruvate levels [21]. Considering the low concentration of pyruvate needed for full activation of AOX and the likely intra-mitochondrial pyruvate concentration, pyruvate levels in tissues should be sufficient to maintain a fully-active AOX [12]. While other studies have reported a relation between pyruvate levels and the induction of AOX [73-75], changes in its activity *in vivo* have not been confirmed to be caused by changes in mitochondrial pyruvate levels. There are also reports of AOX isoforms that are regulated by succinate, due to a mutation of the first conserved cysteine residue to a serine [76]. Measurements of intra-mitochondrial levels of metabolites by the subcellular non-aqueous fractionation technique [77], in combination with *in vivo* AOX measurements are required to resolve the controversy.

When AOX is fully activated, its activity depends on the availability of substrates, i.e. the reduced fraction of the UQ pool (UQr/UQt) and oxygen. The kinetic properties of AOX have been characterized in isolated mitochondria [78,79]. A recent hypothesis postulates that AOX regulates the oxygen concentration inside mitochondria, thus limiting ROS production, without competing with COX for O_2 due to its lower affinity (Box 1). However, there is currently no information concerning electron partitioning between the two pathways under hypoxia, because determination of *in vivo* AOX activity under such conditions requires a refinement of the O_2-isotope fractionation technique. With regard to the other substrate, UQ, few studies have addressed the *in vivo* relevance of UQ reduction levels on the AOX activity. Previous studies analyzed UQr/UQt by HPLC measurements in *Poa anua* roots and after KCN and SHAM titrations [80]. UQr/UQt increases in KCN-treated roots after SHAM inhibition provided evidence for the role of AOX in stabilizing UQr/UQt when COX is inhibited, but *in vivo* AOX activities as a function of UQr/UQt have not yet been determined. Intriguingly, decreases in total root respiration during development [21] and after low-light transition [81] were not directly associated with changes in either UQ reduction levels or *in vivo* AOX activity. However, an interesting conclusion can be drawn from two recent studies on *AOX1a*-modified plants conducted using a very similar experimental design [61,82]. After a short-term HL treatment, a lack of induction of *in vivo* AOX activity [61] and higher leaf UQ reduction levels [82] were observed in *AOX1a*-suppressed plants than in WT plants. Importantly, respiration in *AOX1a*-antisense plants was not different from WT plants, due to an increased *in vivo* COX activity [61]. Taken together, these results suggest that the lack of HL-induced *in vivo* AOX activity in *AOX1a*-modified plants increased the UQ reduction level when COX was not saturated.

Regardless of UQr/UQt, the total amount of UQ can also be a limiting factor for AOX activity [79]. Accordingly, both reduced and oxidized UQ levels were higher in leaves of HL-grown *Arabidopsis* plants than in low-light (LL) grown plants, whereas the UQ reduction level was similar [82]. These differences in UQ level can explain why the *in vivo* activities of both COX and AOX were greater in leaves of HL- than LL-grown plants [61]. Important recent advances in our understanding of the plant UQ synthesis pathway, combined with the availability of mutants of enzymes in this pathway [83] offer new opportunities to test the relevance of UQ levels for the activity of both respiratory pathways *in vivo*.

3. The physiological functions of the AOX pathway

Below, we describe putative functions of the AOX pathway based on three general physiological/metabolic situations (Figure 2), and review studies reporting *in vivo* AOX activities and/or using AOX-modified plants under different scenarios.

3.1. The alternative oxidase under energy and carbon excess

This case was originally termed the 'energy overflow hypothesis', but must be revisited, because the model was based on results using AOX inhibitors [3].

Stressful situations induce cellular changes, which may drastically affect the levels of metabolites, some of which are required for the stress response [84]. Such metabolic changes can lead to an imbalance in respiratory substrate availability and demand, potentially affecting the redox state of the electron transport systems of both mitochondria and chloroplasts. For instance, an excess of reductant and carbon occurs under HL conditions. The energy bypass systems of the mitochondrial electron transport system may dissipate excess carbon and reductant under HL conditions [85-87]. In this way, mitochondrial metabolism would play a role in ameliorating photoinhibition [87,88]. A combination of photosynthetic characterization, metabolomics, and *in vivo* AOX analysis has yielded important insights into the metabolic role of AOX under fluctuating light [18,60,61,89,90]. The *in vivo* activation of the AOX pathway in leaves under HL has been linked to changes in key metabolites involved in non-cyclic flux modes of the TCA cycle, possibly supporting both photosynthetic (by dissipation of energy at PSII) and stress-related amino acid metabolism (Figure 1A). Interestingly, the plastoquinone reduction levels becomes greater in *AOX1a*-altered plants than in WT plants after HL treatment [82], and ultimately causes an increase in chronic photoinhibition, which is very likely due to lower *in vivo* AOX activity (ν_{alt}) [61].

Experiments with AOX genetically-modified plants further support a role of AOX in the energy balance of chloroplasts thus affecting photosynthesis [91], and also photorespiration [92,93]. However, measurements of the *in vivo* activities of both AOX and COX pathways in plants are required to determine the extent to which each respiratory pathway is linked to photorespiration.

Previous studies showed that ν_{alt} was not correlated to light-induced changes in sugars levels in *Poa anua* roots and soybean cotyledons [25,81]. On the other hand, diurnal changes in sugar levels were linked to ν_{alt} changes in leaves of spinach and bean [94]. More recently, changes in the levels of amino and organic acids in leaves of *Arabidopsis* at different growth light and day/night conditions were strongly correlated with changes in both ν_{cyt} and ν_{alt}, while sugar levels displayed poor correlations [89]. More experimental evidence is needed including a broader coverage of time points and metabolites under different photoperiods and natural light conditions to definitively resolve this issue. Notably, metabolic changes associated with ν_{alt} after growth light changes [89,95] are different from those after short-term light changes [60,61,95], thus denoting the different metabolic roles of AOX depending on the duration and intensity of the light perturbation.

3.2. The alternative oxidase under COX restriction

Factors such as low temperature, nutrient limitation, allelochemicals, metal toxicity and nitric oxide (NO) can inhibit the activity of COX. Inhibition of the electron flow to COP can cause an over-reduction of the mitochondrial electron transport system, thus favoring the formation of ROS [96,97]. In this sense, the AOP, acting as an electron bypass for the COP, can potentially avoid the production of ROS and, at the same time, allowing respiration to continue under COX restriction.

It has been hypothesized that AOX activity allows acclimation of respiration to changes in temperature under COX restriction. Several authors examined the effect of short- and long-term changes in temperature on ν_{alt}. A greater contribution of the AOP to total respiration was observed in leaves of *Vigna radiata* grown at 19 °C, and in cotyledons of soybean grown at 28 °C, when measured at 15 °C [98]. In leaves of *Arabidopsis*, a peak of ν_{alt} was reported at day 4 of cold treatment which was reverted by day 10, coinciding with an enhanced ν_{cyt} [99]. These observations suggest that ν_{alt} may balance the rate of respiration at early stages of cold treatments until COP is fully functional again. More recently, increases in AOP measured at 25 and 35 °C in leaves of *Salsola divaricata* grown at 15 °C have been suggested to maintain

COP and photosynthetic functionality under sudden and severe changes of temperature [100]. Furthermore, the increase in ν_{alt} was associated with a higher stress level after recovering from chilling, probably lowering the production of ROS under COP restriction [101].

The effect of long-term high temperatures on ν_{alt} has also been focus of interest. The ν_{alt} was increased in *Nicotiana tabacum, Populus canadensis* and *Quercus rubra* at measurement temperatures above the growth temperature [51,90,102]. Similarly to what has been found under low temperatures [98,99], the foliar response of ν_{alt} to high temperatures appears to compensate for a decrease of ν_{cyt} until its recovery. In roots, ν_{cyt} increases in response to increasing temperatures in *Agrostis scabra* and *A. stolonifera*, and AOP displays high activity only in *A. scabra*, possibly compensating its lower COP response compared with that in *A. stolonifera* [103]. By contrast, short-term variation in temperature did not affect the electron partitioning in hypocotyls of *Vigna radiata* and leaves of *Cucurbita pepo*, *Nicotiana sativa, Vicia faba, Chionochloa rubra* and *C. pallens* of warm-grown plants and at different measuring temperatures [19,90,98]. In conclusion, AOP response appears to be coupled with COP restriction in most cases, but the AOP response to temperature depends on tissue, species and stress duration.

There is a restriction of COX under nutrient stress. Phosphorus (P) limitation can limit the activity of COX due to an increase in adenylate control. The AOP contribution to total respiration increased in leaves of *Phaseolus vulgaris* and *Gliricidia sepium* grown under P limitation [104]. The ν_{alt} in roots of *N. tabacum* plants grown under P limitation also increases [105], while it decreases at the leaf level in the same species [104]. Thus the AOP response to P limitation seems complex and varies among tissues and species.

Some studies reported decreases in ν_{cyt} and growth after exposure to allelochemicals [106] or herbicides [73], and exposure to long-term drought and salinity [52,55,56,107]. In these cases the contribution of AOX to total respiration increased mainly due to ν_{cyt} decrease while ν_{alt} was maintained or increased, possibly allowing TCA cycle reactions under low energy demand (i.e. high ATP/ADP ratios) for growth (Figure 2). Finally, ν_{cyt} can be inhibited by heavy metals [108] and NO. The mitochondrial synthesis of NO is affected in AOX-suppressed plants [109] due to an increased electron pressure at Complex III [29]. These observations provide a very interesting framework to further understand how NO is affecting the electron partitioning between COP and AOP *in vivo*.

3.3. The alternative oxidase and carbon demands for growth and maintenance

Given its non-phosphorylating nature, the AOX can re-oxidize NAD(P)H produced in the mitochondria and/or the cytosol allowing a continuation of the respiratory metabolism when the cellular ATP demand is low. In this way, the AOX can mediate the production of several carbon compounds essential for growth and cellular functioning under both stress and non-stress conditions.

Importantly, a lack of ν_{alt} has never been confirmed in any plant tissue, not even in *AOX1*-suppressed [51,62] or knockout plants (Del-Saz et al. unpublished). Indeed, ν_{alt} in AOX antisense plants is very similar to that in AOX over-expressers and wild-type plants grown at both low-light and HL intensities [61]. Moreover, ν_{alt} and its capacity were very similar in *AOX1a* antisense plants (i.e. AOP was fully engaged) at both growth light intensities [61]. Taken together, these results suggest that a 'crucial' threshold of ν_{alt} is always maintained in *AOX1a*-suppressed plants, possibly by compensation/ action of other AOX isoforms. Both stress- and non-stress-related isoforms of AOX are expressed at different stages of development in *Arabidopsis* leaves which suggests that AOX has a general role in balancing metabolism [110]. In this respect, ν_{alt} under non-stress conditions is fairly constant along leaf and root development, while the ratio AOX/COX increases [21,23,24]. It therefore becomes apparent that AOP contributes to respiratory metabolism at advanced stages of development, most probably facilitating the continued operation of the TCA cycle under conditions when the ATP demand is low due to reduced growth (Figure 2).

There is considerable evidence suggesting the importance of AOP in the production of several carbon compounds under stress conditions. At the leaf level, metabolites involved in osmoregulation are related to an increase in ν_{alt} in *Medicago truncatula* exposed to sudden and severe salt stress [111] (Figure 1A). In roots, different studies related the AOX response with the synthesis and exudation of carbon compounds into the rhizosphere. For instance, the faster rates of AOP were suggested to allow exudation of chemicals related to communication between *Pisum sativum* plants [112]. Moreover, an increase in ν_{alt} is closely associated with citrate synthesis and exudation in cluster roots of *Lupinus albus* [113] and roots of *N. tabacum* [105] grown under P limitation (Figure 1B). Together with other metabolic evidence on P acquisition under limiting conditions [114], these studies support a role of AOX in mediating root citrate synthesis and exudation (Figure 1B and 2).

A lower AOP contribution in leaves of *Alocasia odora*, a shade species, has been suggested to increase the energetic efficiency of respiration, thus sustaining growth under limiting light conditions [94]. Along these lines, the lower ν_{alt} in plants acclimated to low CO_2 concentrations suggest that reductions in the AOP can improve both leaf carbon balance and respiratory efficiency under carbon-limiting conditions [98,115]. In fact, increased carbon-use efficiency was observed under nutrient stress in AOX-suppressed cells of *N. tabacum* [116]. However, studies at the whole tissue level reflect a more complex scenario [13]. Indeed, the higher ν_{alt} observed in roots of fast- versus slow-growing species was interpreted as a role of AOP in reducing the over-reduction of UQ-pool and ROS production, thus allowing fast growth. The faster growth rates observed in *AOX1a*-overexpressing lines growing at low temperatures or under osmotic stress also support this view [117,118]. However, these observations could also be interpreted as another beneficial role of AOX - that of conferring metabolic flexibility.

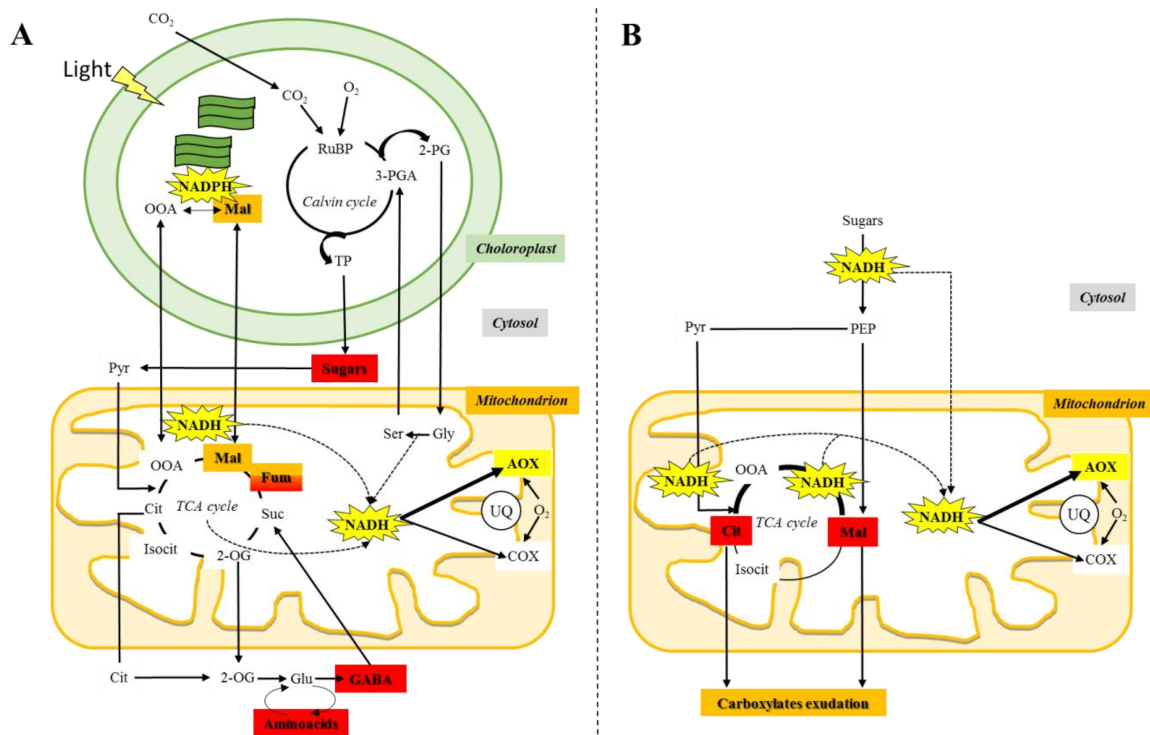


Figure 1. Schematic representation of the main metabolic pathways linked to AOX activity *in vivo* in leaves (A) and roots (B). Interactions between the different organelles and cellular compartments are shown, highlighting the key metabolites significantly correlating with AOX activity *in vivo* under different stress conditions. Changes in the levels of the highlighted metabolites are linked to the production/consumption of reducing equivalents, thus affecting AOP *in vivo* activity. In (A), red boxes indicate sugars (maltose, xylose), organic acids (fumarate), amino acids (asparagine, β-Alanine) and γ-

aminobutyrate (GABA) significantly correlate with AOX activity *in vivo* in leaves of different species after short-term exposure to high-light conditions [18]; orange boxes indicate organic acids (malate, fumarate) that are significantly correlated with AOX activity *in vivo* changes in leaves of *Medicago truncatula* after exposure to short-term and severe salt treatment [111]. In (B), red boxes indicate organic acids (malate and citrate) significantly correlating with AOX activity *in vivo* changes in roots of *Lupinus albus* and *Nicotiana tabacum* growing under long-term phosphorus-limitation and with rapid rates of carboxylates exudation [105,113]. 2-OG, 2-oxoglutarate; 3-PGA, 3-phosphoglyceric acid; 2-PG, 2-phosphoglycolate; Cit, citrate; Isocit, isocitrate; Glu, glutamate; Gly, glycine; Mal, malate; OAA, oxalacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; ribulose-1,5-diphosphate (RuBP); TP, triose phosphates; Ser, serine; Suc, succinate; UQ, ubiquinone.

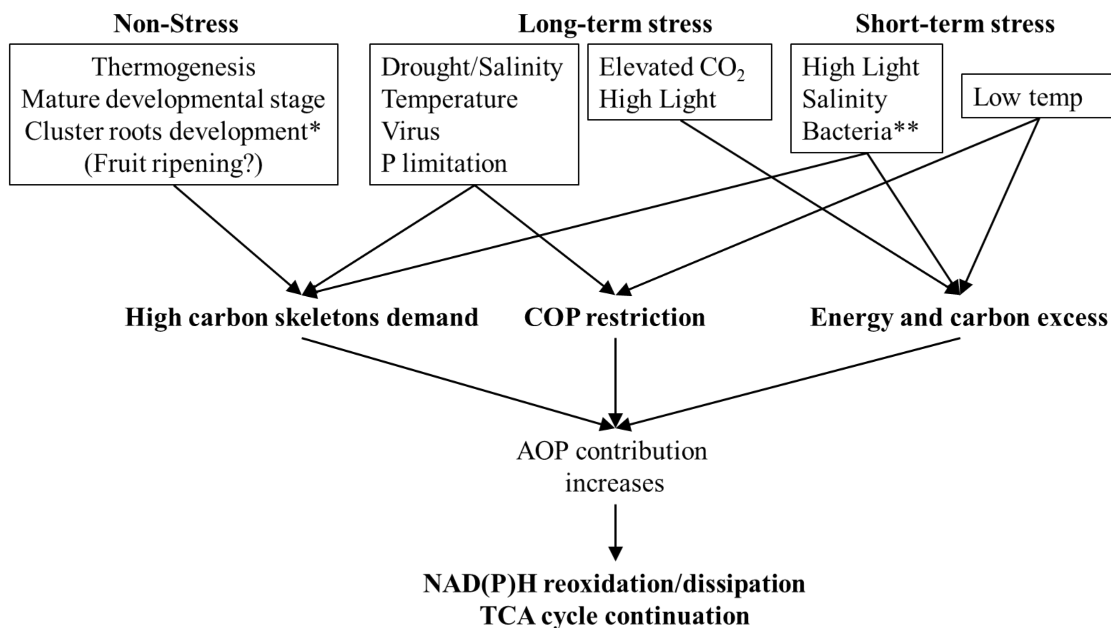


Figure 2. Schematic representation of factors that induce changes in AOP contribution *in vivo* as well as putative AOX roles under stress and non-stress conditions. Evidence on the regulation of the AOP activity *in vivo* (see Box 2) provides key clues to unravel the physiological/metabolic situations under which the AOX will be induced and therefore accomplish its function. The important contribution of the UQ reduction level denotes that activation of AOX will mainly occur under two situations: (i) under energy or carbon excess and/or (ii) under COP restriction. An altered cellular redox state and an accumulation of organic acids are expected under both situations, which will favor the activation of the AOX (although relevance of this mechanisms is still under discussion, see Box 2). As another possibility (iii) the AOX can mediate NAD(P)H re-oxidation in order to satisfy the demand for the synthesis of carbon skeletons under low ATP demand.*It is induced by stress, phosphorus (P)

limitation. **This refers to a study of programmed cell death induced by a bacterial elicitor, which is not a case of energy and carbon excess, but there is a high demand for both.

4. Concluding remarks and future directions

The *in vivo* regulation of the electron partitioning between COP and AOP mainly depends on the cellular demand for ATP and carbon skeletons, which depend on development and environment. COP is generally more dynamically regulated than AOP which reflects its function as the main ATP-producing pathway. However, AOP is involved in the supply of carbon skeletons for primary carbon and nitrogen metabolism. In the short-term, AOP facilitates the synthesis of metabolites involved in stress tolerance and dissipates excess carbon and reductant generated in photosynthesis (and possibly photorespiration) (Figures 1 and 2). These observations are in line with the previously suggested role of the AOX as a first line of defense against metabolic perturbations [32] and ROS production [97]. In the long term, a higher AOP/COP ratio allows the continuation of respiration when COP is restricted by stress-induced growth reductions (Figure 2). The ecophysiological function of the AOP in adding flexibility to plant stress responses should be further tested in plants naturally adapted to fast-changing environmental conditions which would require a further implementation and development of off-line systems for field measurements of the AOX activity *in vivo* [59,119]. Thus, plants with higher AOP should present greater acclimation to environmental conditions. In addition, the combination of AOX sequence, expression and *in vivo* activity analysis in poorly studied non-angiosperm plants [41] will be required to establish the importance of AOX during plant evolution, especially during the transition to land.

Under non-stress conditions, there is an important contribution of AOP to total respiration. New approaches involving complete knock out of the AOX expression under the control of tissue-specific and/or inducible promoters should be implemented to decrease the AOX *in vivo* activity under non-stress conditions in different tissues and/or developmental stages. Furthermore, technological improvements of new measuring systems (i.e. new membrane-inlet systems [120] to allow faster ('real-time') determinations of AOX *in vivo* activity combined with recently-developed cell-type-specific metabolic flux analysis [121] will be key to unravel the precise roles of AOX in plants.

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MATERIAL AND METHODS

Measurements of electron partitioning between cytochrome and alternative oxidase pathways in plant tissues

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ABSTRACT

Plant respiration is characterized by the existence of the alternative oxidase pathway (AOP) that competes with cytochrome oxidase pathway (COP) for the electrons of the ubiquinone pool of the mitochondrial electron transport chain, thus reducing ATP synthesis. The oxygen (O_2) isotope fractionation technique is the only available to determine the electron partitioning between two pathways and their *in vivo* activities in plant tissues. In this chapter, the basis of the O_2 isotope fractionation technique and its derived calculations are explained together with a detailed description of the dual-inlet isotope ratio mass spectrometry (DI-IRMS) system and protocol developed at the University of Balearic Islands. The key advantages of the DI-IRMS over other systems are highlighted as well as the main potential problems of this technique. Among these problems, those associated to leakage, diffusion and inhibitor treatments are noted and solutions to prevent, detect and repair these problems are detailed.

1. INTRODUCTION

Plant respiration is the combination of metabolic reactions where reduced carbon compounds are oxidized to CO_2 and H_2O and the energy released is used for the synthesis of reducing equivalents and ATP. The synthesis of ATP is linked to mitochondrial electron transport from respiratory substrates to terminal oxidases which reduced oxygen to water. Therefore, while respiration can be measured as CO_2 production when the focus of interest is on carbon budgets, measuring O_2 consumption allows a more direct assessment of the energetic efficiency of respiration.

Plants have a specialized mitochondrial electron transport chain (mETC) with several proteins that allow alternative electron transport routes thus modifying the energetic efficiency of respiration (reviewed in van Dongen et al., 2011; Gupta et al., 2015). Among these energy bypass systems, the alternative oxidase (AOX) couples the oxidation of the reduced ubiquinone (UQ) to the reduction of oxygen to water without proton translocation, thus dissipating energy as heat (Moore & Siedow, 1991). In addition, electron transport to AOX bypasses two of the three proton pumping sites of the cytochrome oxidase pathway (COP) thus considerably reducing ATP synthesis. Therefore, it is very important to know to what extent the AOX is engaged and how is regulated because of its major impact on the energetic efficiency of plant respiration.

Past investigations on regulatory properties of AOX such as redox (Umbach and Siedow, 1993) and allosteric activation by organic acids (Millar et al., 1993) indicated that the AOX pathway (AOP) could operate under conditions when the cytochrome oxidase pathway (COP) is not saturated. Therefore, when the AOP is in its fully active state it can compete with the COP for electrons of the UQ pool (Hoefnagel et al., 1995). Competition between the COP and the AOP was demonstrated in isolated mitochondria with measurements of the individual activities of both pathways by using the oxygen isotope fractionation technique (Ribas-Carbo et al., 1995). These lines of experimental evidence supposed that approaches based on respiratory inhibitor titrations were no longer appropriate to measure the *in vivo* activities of the COP and the AOP (Day et al., 1996). The basis of the oxygen isotope fractionation technique resides in the differential oxygen isotope fractionation by the cytochrome and the alternative oxidases (Guy et al., 1989). Both oxidases react preferentially with the $^{32}O_2$ rather than with the $^{34}O_2$ because molecules which contain heavier isotopes have chemical bonds that are more stable, and so take a greater amount of energy to break (Dawson and Brooks, 2001). However, the two oxidases use different mechanisms to break the O_2 molecule and this generates a different isotope fractionation (Ribas-Carbo et al., 2005), being higher in AOX

(24-31‰) than in COX (18-20‰) (see Table in Ribas-Carbo et al., 2005). Thus, the oxygen isotope fractionation during respiration, i.e.- by examining the isotope fractionation of the substrate oxygen as it is consumed in a closed and leak-tight cuvette, can be used to calculate the partitioning of electrons between the two respiratory pathways (τ) in the absence of inhibitors (Ribas-Carbo et al., 2005; and section 3.4 below).

Since the first oxygen isotope fractionation system (Guy et al., 1989), different measurement systems have been developed for determining τ. Ribas-Carbo et al. (2005) reviewed the history of this technique as well as the design advances. Since 2005, only two systems have been newly developed including an off-line gas phase system (Kornfeld et al., 2012) and on-line gas and liquid phase systems (Cheah et al., 2014). While technical problems on these new systems are overcome after leakage and/or fractionation corrections (for details see Kornfeld et al., 2012 and Cheah et al., 2014), the precision and sensitivity of the dual-inlet isotope ratio mass spectrometry (DI-IRMS) system is still higher than any system developed (for detailed explanations see section 3.2). In addition, DI-IRMS has been the most frequently used system for determining mitochondrial electron partitioning between COP and AOP in different tissues and species over the last 10 years (Del-Saz et al., 2016 and Florez-Sarasa et al., 2016 and references therein). For all these reasons and due to space limitation, only the gas-phase DI-IRMS system developed at the University of Balearic Islands (UIB) is detailed in this chapter with our focus being on *in vivo* determinations of AOP and COP in plant tissues. Note that liquid-phase systems allow for *in vitro* studies in isolated mitochondria or enzymes while off-line systems allow for *in vivo* field measurements. Readers are referred to Ribas-Carbo et al. (2005) for the description of previously developed systems (some of them still recently used by Miller et al., 2011 and Meier et al., 2013), and to Kornfeld et al., 2012 and Cheah et al., 2014 for the newly developed off-line and on-line systems, respectively.

2. MATERIALS

2.1 Sample collection system coupled to the dual-inlet isotope ratio mass spectrometer

The air-sample collection system allows the sequential withdrawn of air samples from the cuvette and its flux into the sample bellow of the dual-inlet isotope ratio mass spectrometer (DI-IRMS). The system consists of a 3-mL and 10 cm^2 stainless steel closed cuvette maintained at a constant temperature using a copper plate and a serpentine around the cuvette with a temperature-controlled water bath (Fig. 1). Typically up to three leaf discs of 9.6 cm^2 or 0.5 mg of fresh tissue can be fed into the cuvette; overfeeding of the cuvette can cause O_2 diffusion problems with its consequent impact on fractionation (see note 2). The respiration

cuvette is equipped with two inlets: one connected to the mass spectrometer sample bellow through a 1-m long capillary tube (0.127 mm inside diameter), and the other connected to a 1-mL air-tight syringe (Cromlab S.L., Barcelona, Spain) (Fig. 1). Throughout the experiment, the syringe is used to both mix the air in the cuvette and to maintain the cuvette at constant pressure. There is a pneumatically controlled on-off micro-needle valve at the capillary tube connecting the cuvette to the mass spectrometer (Fig. 1). Finally, there is a liquid N_2 trap removing the H_2O and CO_2 from the cuvette-sampled air before is fed into the mass spectrometer (Fig. 1). The removal of the H_2O must be ensured because is crucial for a correct functioning of the mass spectrometer (see preparatory checks in section 3.1). Also note that the advantage of this system is its simplicity which diminishes the possibilities for leaks, although they must be monitored conscientiously (see note 1).

2.2 Dual-inlet isotope ratio mass spectrometer

The development of a system based on dual-inlet mass spectrometry (DI-IRMS) improved the accuracy and sensitivity of the technique over previous on-line continuous flow systems (Ribas-Carbo *et al.*, 2005). The DI-IRMS is generally considered to be the most precise method of measuring the isotope ratios (for detail explanations read points d, e and g of the section 3.2). However, the technique requires greater preparation time and larger sample size than is required for the continuous flow mode

The DI-IRMS presents the following four independent systems (Fig. 1):

A. System of bellows and pneumatic valves: this module is used to collect the sample air- i.e. from the collection system described above, and to allow simultaneous and automated measurements of sample and reference gases (Fig. 1). Two inlets allow the entry of air into two bellows with a capacity of 30 mL. One of them is used as reference and other as sample, and both are able to be automatically compressed/decompressed; the possibility to compress the gas captured into the bellows allows to dynamically increase/decrease the amount of gas introduced into the ion source with the consequent increase/decrease on the signal intensity detected for each generated ion (i.e. the low signal intensity from a low amount of gas sampled can be increased for about 10 times after below compression thus allowing a more precise measurement). There are 12 individual valves that can be opened/closed in order to fill up sample air from the cuvette into the bellow and also to empty the bellow by connecting to the rotatory vacuum pump once measurement is finished and the system should be prepared for the next sampling. Moreover, the bellows are connected, via a

capillary, to a crimp which controls whether the sample or reference air goes either into the mass spectrometer or to a waste line (into the turbo pump in fig. 1) via a "change-over valve block", consisting of four valves. During the measurements, two valves are always open and two closed to ensure one bellow has an open path to the mass spectrometer, and the other to the waste line.

B. Gas ionization system: the ionization chamber consists of an ion source that ionizes the gas molecules throughout an electron beam, a positively charged ion repeller from the electron beam, a magnetic field (disposed in parallel to the electron flux) that allows the ions focusing, and an ion accelerator that confers velocity to the ions by applying an electric potential pulse before withdrawing the ionization chamber. The obtained velocity of the ions will depend on the mass-to-charge ratio.
C. System to separate ions on the basis of the mass-to-charge ratio: this consists of a flight tube by which the ions beam is directed by a magnetic field (or magnet) and separated into multiple beams depending of their masses (determined by their different isotopes composition), and Faraday cups which are collectors for that receive the impact of the ions. On each impact, the ions are neutralized as well as the electric potential, which reduction is proportional to the intensity of the ions beam. The DI-IRMS system of the UIB has eight Faraday cups thus allowing simultaneous detection of $^{14}N_2$, $^{32}O_2$ and $^{34}O_2$ among other gas molecules.
D. Data collection/monitoring system: it consists of a voltage/frequency converter coupled to a software able to monitor the ratio of the m/z 34/32 ($^{18}O_2/^{16}O_2$) and m/z 32/28 (O_2/N_2) ratios of the sample gas. Isodat 3.0 (Thermo Fisher Scientific, Massachusetts, USA) is the software for system control, data acquisition and data evaluation.

2.3 Chemicals for end-point determinations

For many years, it was thought that electrons were only available to the alternative oxidase pathway (AOP) when the cytochrome oxidase pathway (COP) was either saturated or inhibited, and the electron partitioning between the two respiratory pathways (τ) were studied only by using specific inhibitors of the two pathways, being the most used the potassium cyanide (KCN) for the COP, and salicylhydroxamic acid (SHAM) for the AOP. While the O_2 isotope fractionation technique can be used to determine τ in the absence of inhibitors by measuring O_2 isotope fractionation during tissue respiration (Δ_n), the end-point fractionation values for each pathway (Δ_c, COP discrimination; and Δ_a, AOP discrimination) are still

required for τ calculation (see section 3.4). These end-point values are obtained in independent experiments to those for obtaining Δ_n and are fairly constant in each specific tissue of the same species (see note 6); though once Δ_c and Δ_a are obtained in the tissue of interest, such values can be used for τ calculation in different experiments (but see note 6 for special considerations). Typical concentrations used are 10 mM KCN and 25 mM SHAM (for more details see section 3.3 and note 4).

There are other inhibitors that can be used as alternative to KCN or SHAM, although they have not been extensively used for the calculation of the respiratory end-points (Δ_c and Δ_a). Antimycin A is one of the first known and most potent inhibitors of the mitochondrial respiratory chain, and it is able to inhibit the flow of electrons through COP (Slater 1973; Huang *et al*., 2005). On the other hand, *n*-propyl gallate has routinely been used to document the presence of a cyanide-resistant respiratory pathway in a large number of tissues (Janes and Wiest, 1982). Recently, ascofuranone, an ubiquinone analog isolated from the pathogenic fungus *Ascochyta viciae*, has shown to be a potent inhibitor of trypanosomal alternative oxidase (Moore *et al*., 2013). Further studies using the oxygen isotope fractionation technique are needed in order to test the effectiveness and the appropriate concentration of these alternative inhibitors.

3. METHOD

3.1 Preparatory checks

Before starting an experiment different checks on the DI-IRMS system are recommended for its correct functioning. As for most mass spectrometer systems, the vacuum pressure in the whole system should be checked and kept at the levels indicated by manufacturer's recommendations; in case vacuum level is not achieved, vacuum pumps may need repairing or replacing but also some leak may be present and may require for technical assistance of the manufacturer's.

After checking correct vacuum levels on emptied system, the background contaminants should also be checked to be under the levels recommended by the manufacturers; these levels can vary from lab to lab though adequate levels of contaminant for correct functioning should be set and checked regularly). High water levels is one of the main and typical problems and probably the most difficult to solve (i.e. several days of vacuum-dry of the system may be required to remove water contamination); water condensed throughout the capillaries and ion source may react with the gas samples thus causing undesired isotope fractionations; in the system here described, special care must be taken when injecting gas samples across the

liquid N_2 trap though (Fig. 1) ensuring complete water removal (i.e. before starting the experiments, air sample tests can be introduced and m/z 18 can be monitored).

Once the system is ready to run samples, daily tests of machine stability are strongly recommended before starting an experiment. For this purpose, the two bellows can be filled up (for the procedure to injecting the air samples into the bellows see next section 3.2) with the same standard gas (i.e. normal atmospheric air can be used; this technique does not need a special standard gas due to the type of data generated and calculations used, see section 3.4). Then, several (at least 3 are recommended) runs of six cycle measurements (i.e. as for the 'real' experiments, see next section 3.2) can be started and two parameters should be checked: i) the isotope ratios m/z 34/32 ($^{18}O_2/^{16}O_2$) and m/z 32/28 (O_2/N_2) should be constant, i.e. standard deviations of the 6 replicate cycles should be less than 0.1 and 0.05, respectively; ii) the calculated isotope composition or delta (δ, calculation commonly used in stable isotopes studies which is usually given by the software and indicates isotope ratio of the sample in relation to that of the standard; for basic information on stable isotope analyses see Dawson and Brooks, 2001) should be constant and around 0 because the same air is using as reference and sample, thus indicating no fractionation due to technical problems.

3.2 Protocol for measuring oxygen isotope fractionation in the absence of inhibitors

a. Firstly, pre-evacuate the sample bellow (Fig. 1) and expand to its maximum volume (≈30 mL).
b. Close the vacuum line by closing valves nº2 and nº5, and open the valves nº 1, nº 3 and nº 4 that connect the sample bellow and ion source with the external capillary of the collection system (Fig. 1). No signal increase (i.e. m/z 32, O_2) should be detected, otherwise a leak is present which must be repaired (see note 1).
c. Open the pneumatically controlled micro-needle valve placed next to the cuvette (Fig. 1) to let the air from the cuvette enter into the bellow through the 'sample' inlet of the DI-IRMS. Keep this valve open until pressure in the sample bellow reaches aprox 2.5 mbars (a manometer is present in each bellow) and then close it. With this pressure in the 30 mL bellow volume, approximately 250 µL air sample is fed into the sample bellow. Note that before the air is introduced into the bellow, the sample air passes through a liquid N_2 trap to remove both H_2O and CO_2.
d. Once the m/z 32 signal is stable, close valve nº 3 to retain the air sample inside the bellow and compress it to increase signal intensity. This is a great advantage of the DI-IRMS because it facilitates recording of a high signal (see point A in section 2.2) of a

small amount of sampled air (i.e. 250 μL) thus allowing not consuming more than 30-50% of the total O_2 in the cuvette at the end of the experiment. While signal (i.e. m/z 32) is stabilizing, open valve nº2 to connect the external capillary to the rotatory vacuum pump in order to remove the remaining sample so that the system can be prepared for the next sample collection.

e. Once the m/z 32 signal is stable after bellow compression, the mass spectrometer simultaneously measures the m/z 34/32 ($^{18}O_2/^{16}O_2$) and m/z 32/28 (O_2/N_2) ratios of the sample gas. The sample gas is analysed against standard air contained in the other bellow (Fig, 1, reference bellow), previously filled with standard air (see section 3.1). Both sample and reference air can then enter ionization chamber under nearly identical conditions since the adjustable volume of the bellows allows the equilibration of the air pressures in both bellows in order to obtain the same signal intensity for the isotope measurements (typically m/z 32 is adjusted and this can be done automatically by the mass-spec software Isodat 3.0). This signal adjustment avoids signal linearity problems that occur when very different signals are compared.

f. Once the signals of the two bellows are adjusted, a run of six replicate cycles of each gas sample (sample and reference air) is initiated (i.e. by starting a programed sequence of six cycles) which takes approximately 10 minutes. The average values of the isotope ratios of the six replicate cycles are taken as one data single point in the plot for discrimination calculation (see Figure 2 and next section 3.4).

g. After the run of six replicate cycles is finished, empty the bellow by opening valve nº 5 connecting the bellow volume to the waste line of the vacuum rotatory pump. After evacuation, expand the bellow to its maximum volume and start from point 'a' in this section. Note that a complete respiration experiment usually consists of six data points with six cycles per point thus requiring around 90 min. In case of a high respiration rate, data points can be reduced to a minimum of five when the R^2 of the discrimination plot is higher than 0.995 (see figure 2 and section 3.4). Also, depending on the respiration rate of the tissue under study replicate cycles can be increased thus augmenting the sensitivity as needed and up to the limit of the machine. This possibility of measuring the sample and reference air as many times as necessary is one of the most important advantages of the DI-IRMS system because it allows high precision and sensitive measurements and thus experiments to be performed using plant material with very low respiration rates and/or with low amount of tissue.

3.3 Protocol for measuring end-point oxygen isotope fractionation

The same protocol described in the previous section 3.2 can be applied, except that a previous inhibitor treatment is needed to obtain end-point oxygen isotope fractionation values (see section 3.4 for the need of end-points values). In most organs, oxygen isotope fractionation by the AOP (Δ_a) is easy to obtain, since KCN penetrates tissues fairly easily; it can be applied by sandwiching the tissue between medical wipes soaked in concentration of KCN ranging 1-16 mM, depending of the plant species and incubation or inhibition periods (Ribas-Carbo *et al.*, 2005). In many cases, roots must be submerged in solutions of KCN to obtain a complete inhibition of COP. In recent years, full inhibition of the COP has been obtained in leaves and roots of several species after incubation with 10 mM KCN solutions (Florez-Sasara *et al.*, 2007; 2011, 2014, 2016; Del-Saz *et al.*, 2016). The alternative oxidase consistently gives Δ_a values between 24‰ and 27‰ in roots, while in leaves is less variable, with values ranging from 30-32‰ (Ribas-Carbo *et al.*, 2005).

In many tissues the application of SHAM in order to obtain oxygen isotope fractionation by the COP (Δ_c) is more problematic. In some cases addition of this inhibitor leads to soaking of the tissues, which can cause diffusion problems with subsequent fractionation measurements. Successful Δ_c measurements were however obtained by incubation of tissue slices in solutions of 25 mM SHAM diluted in either in DMSO or in warmed water (Martí *et al.*, 2011; Florez-Sarasa *et al.*, 2007; 2012; Del-Saz *et al.*, 2016). In addition, the effectiveness of this inhibitor can be tested using a Clark-type oxygen electrode (Rank Brothers, Cambridge, England) in a solution containing 30 mM MES (pH 6.2) and 0.2 mM $CaCl_2$, and by monitoring the oxygen consumption of plant tissues that have previously been treated with KCN (see note 4) . In this sense, the application of increasing concentrations of SHAM will decrease the cyanide-resistant respiration to some extent in which the remaining respiratory rate is non-SHAM sensitive, being defined as residual respiration (see note 5). The obtained values of Δ_c range between 16 and 20.8‰ in roots, and 19.6 and 20.2‰ in leaves (Ribas-Carbo *et al.*, 2005).

3.4 Calculations of the oxygen isotope fractionation and the electron partitioning

Oxygen isotope fractionation is calculated using the fractionation factor (D), which is derived from the slope of a linear regression through the origin of a plot of ($\ln R/R_0$) x 1000 versus $-\ln f$ (Guy et al., 1989):

$$D\ (‰) = \ln (R/R_0) / -\ln f$$

where R is the $^{18}O/^{16}O$ ratio of the sample, R_0 is the initial $^{18}O/^{16}O$ ratio and f is the fraction of remaining O_2. With the dual-inlet isotope ratio mass spectrometer (DI-IRMS) system, masses

34 ($^{18}O^{16}O$) and 32 ($^{16}O^{16}O$) are used to measure the oxygen-isotope $^{18}O/^{16}O$ ratios. On the other hand, masses 32 ($^{16}O_2$) and 28 ($^{14}N_2$) are used to calculate f and therefore total oxygen uptake (V_t); note that mass 32 ($^{16}O_2$) is used as a good approximation of total oxygen amount since natural abundance levels of ^{18}O is only 0.4% of the total oxygen, and N_2 is an inert non-reacting gas for the plant tissue. The values of m/z 34/32 ($^{18}O_2/^{16}O_2$) and m/z 32/28 ($^{16}O2/^{28}N2$) are obtained from a standard and the sample air with DI-IMRS analysis with six replicate cycles (see previous section 3.2) for each of the six data points regularly obtained for a complete plot to calculate D (Fig. 2). The r^2 values of all unconstrained linear regressions between –ln f and $\ln(R/R_0)$, with a minimum of five data points, must be at least 0.995, considered minimally acceptable (Ribas-Carbo et al. 1997). Note that D refers to fractionation of the substrate of the reaction but then it is transformed to the more common notation of 'Δ' using the formula (Guy et al., 1989):

$$\Delta = D/\ 1\text{-}(D/1000)$$

Once the Δ values are obtained, then the electron partitioning to the AOP (τ_a) can be calculated as follows (Guy et al., 1989):

$$\tau_a = \Delta_n - \ \Delta_c\ /\ \Delta_a - \Delta_c$$

where Δ_n, is the isotope fractionation in the absence of inhibitors, and Δ_c and Δ_a are the so called end-points corresponding to the fractionation by the cytochrome and alternative oxidase pathways, respectively (Fig. 2). These end-points are determined for each experimental system using inhibitors of the COP and AOP (see section 3.3 for detailed explanations).

Finally, the individual *in vivo* activities of the COP (ν_{cyt}) and AOP (ν_{alt}) are obtained by multiplying the total oxygen uptake rate (V_t) and the electron partitioning to the AOP as follows:

$$\nu_{cyt} = V_t\ x\ (1\text{-}\ \tau_a)$$

$$\nu_{alt} = V_t\ x\ \tau_a$$

4. NOTES

1- ***Leakage detection:*** The main problem of any isotope method of this type is the possibility of contamination from outside air in the form of leaks, which increases with every additional connection. The connections of the system such as the syringe, tight

connectors of capillaries and O-rings from inlet connectors (Fig. 1) are the main points of air leaks. This contamination affects Δ_n values, which can be lower than expected. In this situation, it is recommended to check/replace these connectors. If available, flushing argon around the connections allows testing for leaks by monitoring the m/z 40 signal. Also, cuvette can be filled with He or N_2, tightly closed and a full experiment can be performed (i.e. like a blank test); O_2 (m/z 32) signals should not be observed over the course of such experiment. Alternatively, in order to detect whether the leakage problem is affecting discrimination values, it is recommended to measure Δ_a value (i.e. tissue after KCN incubation) as it has shown to be consistent in each tissue type (check note 6 below); also note that any leakage will decrease the fractionation value obtained, thus an unusual (i.e. lower than expected) fractionation value is easier to detect in high (i.e. Δ_a) rather than in low (i.e. Δ_n or Δ_c) fractionation values.

2- ***Tissue diffusion problems:*** Poor diffusion through tissues is another limitation to the fractionation method. If diffusion limits the supply of oxygen to the terminal oxidases, then the discrimination values will be lower than those associated with respiration, and will vary depending on the rate of respiration. This is a problem with dense tissues such as stem and thick roots (discussed in Angert and Luz 2001) that recently, in another dense tissue such florets of *Philodendron bipinnatifidum*, was overcome by increasing the O_2 partial pressure (Miller et al., 2011). In leaves, diffusion is less problematic, although in some cases it is recommended cutting the leaf into slices to facilitate the oxygen diffusion. Diffusions problems in leaves can be also observed when there is excess tissue inside the cuvette, or in highly moisturized tissues such as fine roots. The latter case can be observed in roots that are washed to remove the excess of substrate after being removed from the pot, or after the incubation period using inhibitors. In both cases, it is recommended to left to air dry before placing the sample inside the cuvette. Note that KCN is highly volatile and its effect can be reverted (check note 4 recommendations).

3- ***Unusual O_2 consumption rates:*** the length of a complete respiration experiment depends on the respiration rate of the tissue being studied and the experimental conditions. Samples with fast respiration rates can be measured by shortening the air sampling time between experimental points. Nevertheless, faster respiratory rates can

run out the available oxygen inside the cuvette and the syringe (3+1 ml), which can result in unusual Δ_n values and respiratory rates. This problem can be solved increasing the initial volume of oxygen to be respired by pulling the plunger of the syringe. Another alternative is decreasing the amount of plant material that is placed in the cuvette and thus, reducing the consumption of available oxygen to be respired. On the other hand, in the event of samples with lower respiratory rates, the air sampling time between experimental points must be extended as a minimum consume of oxygen is needed to obtain the minimum acceptable R^2 values ≥ 0.995 of the linear regressions plots between ln (R / R_0) and -ln f (see section 3.4). In this situation, the sample amount can be increased, however, care must be taken in order not to cause diffusion problems (see note 2), especially in leaves. In the measuring system presented here, approximately up to three leaf discs of 9.6 cm^2 and or 0.5 mg of fresh tissue can be fed into the cuvette without causing diffusion problems.

4- ***Inhibitor effectiveness (partial inhibition and overexposure problems):*** Poor infiltration of the inhibitors can cause difficulties in determining the two end-points, especially in dense or waxy leaves. In such cases, it is recommended cutting the leaf into two to four slices before the incubation to facilitate the infiltration of the inhibitor. In KCN treatment experiments, a piece of paper/wipe soaked in the KCN solution can be placed inside the cuvette during the experiment to avoid decrease inhibitor concentration by cyanide evaporation. Vacuum infiltration/soaking of the inhibitor solutions are not recommended because infiltration by itself can cause diffusion problems with consequent discrimination effects. On the other hand, excessive periods of incubation or concentration of the inhibitors can also cause problems in determining the end-points (Moller et al., 1998). Such problems can be detected when very unusual fractionation value (usually lower than expected) is observed together with highly changed respiration rate after inhibitor treatment. In these situations, it is recommended to test the effectiveness of the inhibitors using a Clark-type oxygen electrode by monitoring the oxygen consumption in order to establish the appropriate concentration of inhibitor.

5- ***Residual respiration:*** The remained oxygen uptake that is observed in the presence of inhibitors of both the cytochrome (KCN) and alternative (SHAM) pathways is defined as residual respiration. It has been reported to have an isotopic fractionation between

19.6‰ and 21.0‰, much lower than Δ_a (Guy et al., 1989; Ribas-Carbo et al., 1997). Therefore, any significant residual respiration present in the tissue would decrease Δ_a value, compared to isolated mitochondria, since the latter do not present residual respiration. Ribas-Carbo et al. (1997) showed that Δ_a was similar in isolated mitochondria and intact tissues (30.9‰ and 31.5‰ respectively) of green soybean (*Glycine max*) cotyledons. A similar result was also observed in etiolated soybean cotyledons. These results suggest that residual respiration maybe an artifact which only occurs in tissues in the presence of both inhibitors and that does not interfere with the oxygen isotope fractionation measurements (Ribas-Carbo et al., 1997).

6- ***Consistency of Δ_a and Δ_c among treatments, tissues, genotypes and specie***s: the respiratory end-points are roughly constant in tissues of different species and treatments, but not between tissues or between green and nongreen tissues, especially Δ_a. Ribas-Carbo et al. (1997) observed that Δ_a in intact green cotyledons of soybean was high (31‰), and in roots and etiolated cotyledons was low (25-26‰). However, Δ_c was demonstrated to be constant in these tissues (19.5-21‰). These findings were confirmed in mitochondria isolated from each of these tissue types suggesting that the differential oxygen isotope fractionation can be due the existence of distinct AOX isozymes which appear to be differentially expressed in various plant tissues (Whelan et al., 1996); for instance AOX2 and 3, later renamed as AOX2a and 2b (Considine et al., 2002), were suggested to be responsible for the high and low Δ_a values, respectively (Ribas-Carbo et al., 2000). On the other hand, the consistency of the end-points under a wide range of conditions (e.g. reduction status of the ubiquinone pool, addition of pyruvate and DTT), using different mitochondrial preparations confirmed that these values can be used as standard values for each pathway in subsequent experiments under different treatments (Ribas-Carbo et al., 1995). In others words, the determination of both end-points is generally not necessary for every experimental treatment that can or cannot affect the respiratory rate and/or Δ_n value. Nevertheless, Δ_a changes were observed in AOX mutated proteins (Umbach et al., 2002) which could indicate that dramatic changes on expression of AOX different isoforms may have an impact on Δ_a (discussed in Umbach et al., 2002); however, similar Δ_a values were obtained in plants with acute changes of wild-type AOX1a expression (Florez-Sarasa et al., 2011) and also in mutated AOX1a overexpressors (I Florez-Sarasa personal communication). Finally, recent genetic analysis of AOX sequences revealed

a high variability on the main AOX isoform present in different plant species (Costa et al., 2014). Therefore, it is recommended to measure Δ_a and Δ_c values of species in which O_2 fractionation is measured for the first time, despite the observed consistency of Δ_a and specially of Δ_c observed among studied species (Ribas-Carbo et al., 2005; Florez-Sarasa et al., 2016) and among different AOX-alteredexpression genotypes (Florez-Sarasa et al., 2011; Del-Saz et al., 2016).

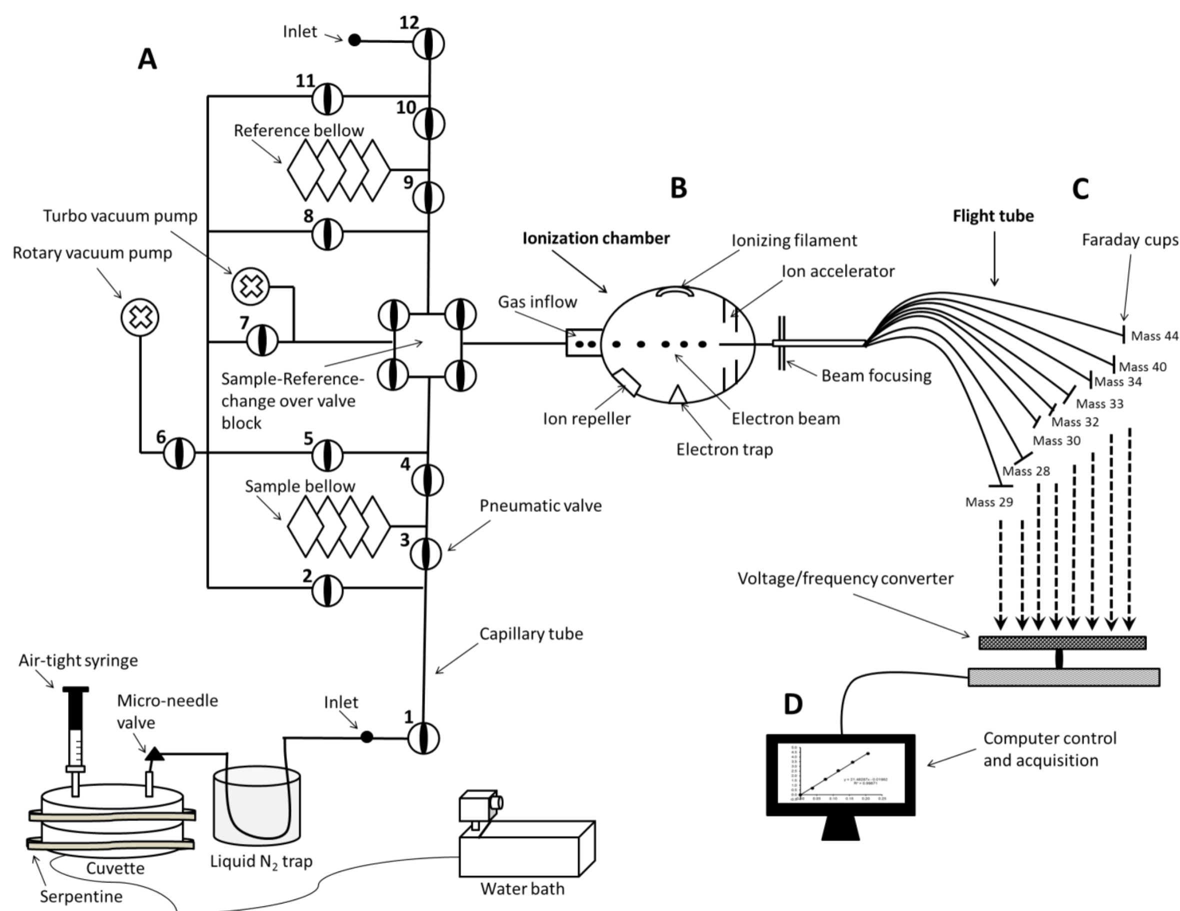

Figure 1. Diagram of the gas-phase collection system coupled to dual-inlet isotope ratio mass spectrometer (Delta XPlus, Thermo LCC, Bremen, Germany) developed at the Biology Department of the University of the Balearic Islands.

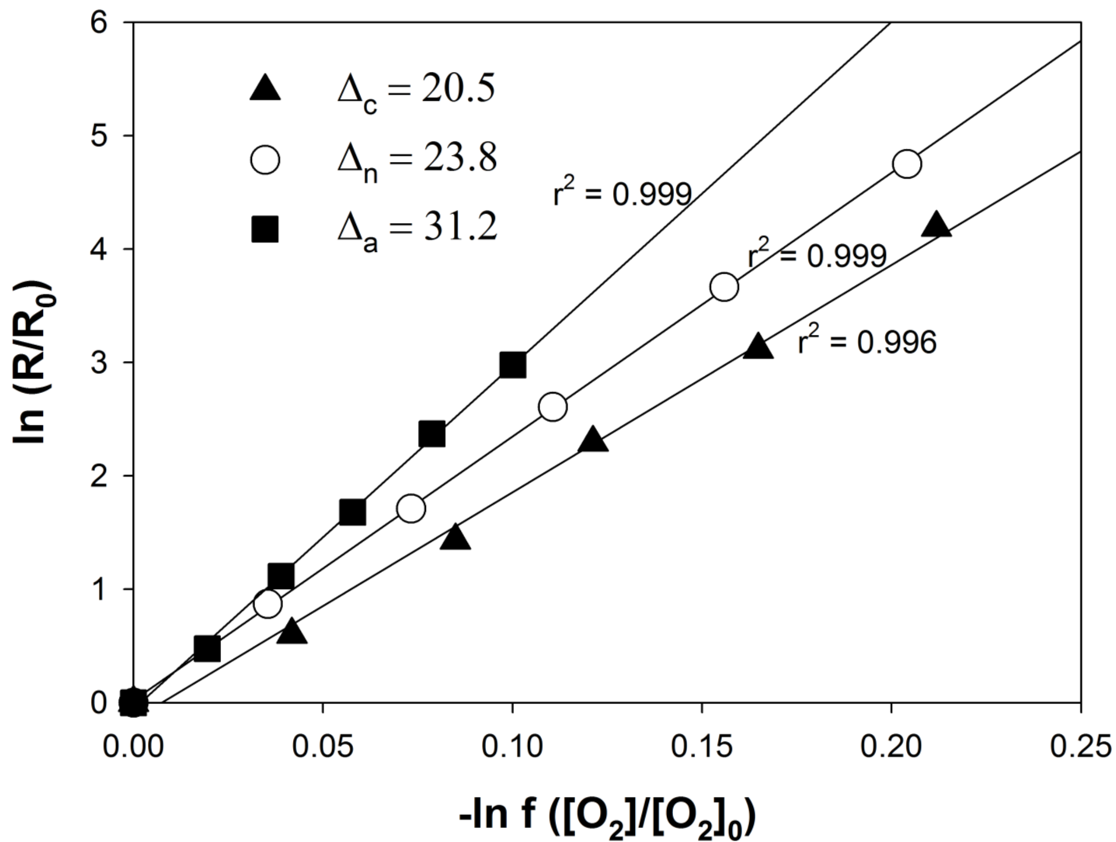

Figure 2. Representative plots of the oxygen isotope fractionation during respiration in the absence of inhibitors (Δ_n), in the presence of 25mM SHAM (Δ_c) and in the presence of 10mM KCN (Δ_a). The three plots were obtained in three separate respiration experiments in Florez-Sarasa et al. (2007). The fractionation factor (D) was obtained from the slopes of the linear regressions between –ln f and ln(R/R_0) and then transformed to Δ values (see section 3.4). The Δ values shown in the figure legend are expressed in per mil units (‰). The r^2 values of all unconstrained linear regressions were higher than 0.995.

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RESULTS

CHAPTER 1.

Salinity tolerance is related to cyanide-resistant alternative respiration in *Medicago truncatula* under sudden severe stress

Salinity tolerance is related to cyanide-resistant alternative respiration in *Medicago truncatula* under sudden severe stress

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ABSTRACT

Salt respiration is defined as the increase of respiration under early salt stress. However, the response of respiration varies depending on the degree of salt tolerance and salt stress.

It has been hypothesized that the activity of the alternative pathway may increase preventing over-reduction of the ubiquinone pool in response to salinity, which in turn can increase respiration. Three genotypes of *Medicago truncatula* are reputed as differently responsive to salinity: TN1.11, A17 and TN6.18. We used the oxygen-isotope fractionation technique to study the *in vivo* respiratory activities of the cytochrome oxidase pathway (COP) and the alternative oxidase pathway (AOP) in leaves and roots of these genotypes treated with severe salt stress (300 mM) during 1 and 3 days. In parallel, AOX capacity, gas exchange measurements, relative water content and metabolomics were determined in control and treated plants. Our study shows for first time that *salt respiration* is induced by the triggered AOP in response to salinity. Moreover, this phenomenon coincides with increased levels of metabolites such as amino and organic acids, and is shown to be related with higher photosynthetic rate and water content in TN6.18.

INTRODUCTION

In the 1930´s, Henrik Lundegårdh performed a series of experiments that raised a theory to describe a simultaneous absorption of salt with an increased respiratory rate (Lundegårdh & Burstrom 1933; Lundegårdh 1937, 1938). Lundegårdh (1939) described an increase in the respiratory rate shortly after salt addition in wheat which was termed as *anion or salt respiration*. Cyanide inhibited this respiratory increase, and a fix rate of respiration remained which was termed as *fundamental respiration* (Lundegårdh 1939). Later it was described that plant mitochondria possess a variable proportion of respiration that is not inhibited by potassium cyanide (KCN), suggesting the existence of an alternative oxidase (AOX) that consumes oxygen (Bonner, 1961). Currently, it is well known that AOX is the only protein of the cyanide-insensitive respiratory pathway (AOP), alternative to the cyanide-sensitive cytochrome oxidase pathway (COP) which draws electrons from ubiquinol to reduce oxygen to water, and is not linked to ATP synthesis (Guy *et al.*, 1989; Moore & Siedow 1991; Ribas-Carbó *et al.*, 1995, 1997).

The original concept of *salt respiration* endures over time and it is presently recognized that salinity can increase respiration (Eynard & Wiebe, 2005; Bothe 2012; Kosová *et al.*, 2013; Rewald *et al.*, 2013). However, the effect of salinity has been shown to depend on its severity and duration and also on the degree of the plant tolerance to salt, with increasing respiration being a distinctive feature (Jacoby *et al.*, 2011). Such a variable response of respiration has been previously observed, i.e. in *Nothofagus* species with different drought tolerance (Sanhueza *et al.*, 2013) and can be due to the different metabolic adaptations that have been observed between sensitive and tolerant closely related species (Sanchez *et al.*, 2011; Jacoby *et al.,* 2013), cultivars (Widodo *et al.*, 2009) or hybrids (Richter *et al.,* 2015).

Moreover, it has been hypothesized that AOP respiration may prevent over-reduction of the ubiquinone pool, avoiding the formation of reactive oxygen species (ROS) that result of the disruption of cellular homeostasis induced by salinity (Maxwell *et al.*, 1999; Møller, 2001). In fact, the induction of AOX capacity by salt stress observed in various studies suggests that AOP activity could have a role in salt stress tolerance or avoidance (Kreps *et al.*, 2002; Seki *et al.*, 2002; Martí *et al.*, 2011). However, no direct correlation between AOX protein abundance and its activity has been observed (Guy & Vanlerberghe 2005; Ribas-Carbó *et al.*, 2005; Vidal *et al.*, 2007; Grant *et al.*, 2008; Florez-Sarasa *et al.*, 2011, 2014).

In the present study, the effect of sudden severe salinity has been studied in three *M. truncatula* genotypes (TN1.11, A17 and TN6.18) that in previous studies showed different response to salinity based on the induction and sustained expression of antioxidant mechanisms (Mhadhbi *et al*., 2011; 2013). Leaf gas exchange and water content parameters were determined as physiological indicators of the salinity stress level, and *in vivo* leaf and root respiration has been measured using the oxygen isotope fractionation technique, which is the most reliable technique for studying the *in vivo* activities of COP and AOP (Day *et al*., 1996). In addition, leaf and root metabolites were determined by gas chromatography coupled to mass spectrometry (GC-MS) metabolite profiling. As previously suggested, an integration of molecular and physiological approaches will allow a more comprehensive understanding of the role of mitochondrial respiration during salinity stress (Jacoby *et al.,* 2011; 2013). The hypothesis behind this research is that increased respiration, especially through the AOP, will facilitate physiological and metabolic adaptations that should improve the response to sudden severe salt stress.

MATERIAL AND METHODS

Plant material and growing conditions

Three *M. truncatula* genotypes: TN1.11, Jemalong (A17) and TN6.18 have been studied. After scarification, seeds were placed at 4ºC for 24h in Petri dishes containing 0.8% agar medium for germination. Seedlings were transferred to pots containing sterile perlite-vermiculite (3:1) mix. Pots were placed in three plastic trays used for subirrigation. Plants were grown in a growth chamber for 40 days at 12-h/12-h photoperiod, 350 µmol quanta $m^{-2}s^{-1}$ light intensity, 25ºC/20ºC day/night temperature and relative humidity around 50%. Plants were watered twice a week with half-strength Hoagland's solution (Epstein 1972). On day 40, half of the plants were watered normally (control 2 Litres) while the rest were watered with 2 L half-strength Hoagland's solution containing 300 mM of sodium chloride (NaCl-treated plants). Physiological analyses of control and treated plants were performed 1 and 3 days after salt treatment.

Respiration and oxygen isotope fractionation measurements

For respiratory measurements, plants were placed for 30 min in the dark to avoid light-enhanced dark respiration. Leaves were harvested and placed in a 3-ml stainless-steel closed cuvette maintained at a constant temperature of 25ºC (Gastón *et al*., 2003). Air samples of 300 µL were sequentially withdrawn from the cuvette and fed into the mass spectrometer.

Changes in the $^{18}O/^{16}O$ ratios and oxygen concentration were obtained to calculate the oxygen-isotope fractionation and respiration rates as described in Ribas-Carbó *et al.* (2005). The electron partitioning to the alternative pathway (τ_a) was calculated as follows:

$$\tau_a = (\Delta_n - \Delta_c) / (\Delta_a - \Delta_c)$$

Where Δ_c, Δ_a are the oxygen isotope fractionation of the cytochrome (+SHAM) and alternative (+KCN) pathway, respectively and Δ_n, is the oxygen isotope fractionation of the respiration in the absence of inhibitors. In leaves, Δ_a was obtained by sandwiching leaves between medical wipes soaked with a solution of 10 mM KCN for 30 min. Values of Δ_a were 32.6±0.4‰, 31.8±0.3‰ and 30.9±0.2‰ for TN1.11, A17, and TN6.18 (three replicates per genotype), respectively. For the calculation of Δ_c, leaves from three replicates per genotype were submerged in different solutions of 25 mM SHAM for 30 min. The average value of Δ_c was 19.8‰ (19.7±0.1‰, 19.9±0.2‰ and 19.8±0.1‰ for TN1.11, A17, and TN6.18 , respectively).

The individual activities of the COP (v_{cyt}) and AOP (v_{alt}) were obtained by multiplying the total oxygen uptake rate (V_t) and the partitioning to each pathway as follows:

$$v_{cyt} = V_t \times (1-\tau_a)$$

$$v_{alt} = V_t \times \tau_a$$

Values presented are the mean ± SE of four measurements.

In vivo root respiration analysis was performed in the same plant as leaf. For Δ_a measurements, three roots per genotype were submerged in different solutions of 10 mM KCN for 30 min. The average value of Δ_a was 27.0‰ (26.9±0.1‰, 27.0±0.2‰ and 27.1±0.2‰ for TN1.11, A17, and TN6.18, respectively. For the calculation of Δ_c, three roots per genotype were submerged in different solutions of 25 mM SHAM for 30 min. The average value of Δ_c was 17.0‰ (17.1 ± 0.1‰, 17.1± 0.2‰ and 16.9 ± 0.5‰ for TN1.11, A17, and TN6.18, respectively).

Measurements of the AOP capacity

Leaves and roots were weighted and incubated in a solution with 10 mM KCN for 30 min. Oxygen uptake rates were measured in darkness using a liquid-phase Clark-type oxygen electrode (Rank Brothers) at a constant temperature of 25ºC in a solution containing 30 mM MES pH 6.2, 0.2 mM $CaCl_2$. Fresh solutions were prepared daily. Four replicates were performed per treatment.

Leaf gas exchange and water status measurements

Net photosynthesis (A_N) and stomatal conductance (g_s) were measured after steady state (after c. 20 min) with an open infrared gas-exchange analyser system (Li-6400; Li-Cor Inc., Lincoln, NE, USA) on the youngest fully expanded leaves of TN1.11, A17 and TN6.18 under light-saturating PPFD of 1500 µmol m^{-2} s^{-1}, 400 µmol CO_2 mol^{-1} air, 25 ºC and a relative humidity of the incoming air around 50%.

Leaf relative water content (RWC) was analyzed in four leaves as follows:

$$RWC = [(FW - DW) / (TW - DW)] \times 100$$

where fresh (FW), turgid (TW), and dry (DW) weight of leaves were measured. FW was determined immediately after sampling; TW was obtained after incubating leaf discs in distilled water for 48 h in the dark at 4 ºC (to minimize respiration losses) and DW was determined after drying for 72 h in an oven at 70 ºC.

Metabolite profiling

Leaves and roots were sampled after 30 min in darkness. Metabolite extractions, derivatization and gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) analyses were carried out as previously described (Lisec *et al*., 2006). The GC-TOF-MS system was composed of a CTC CombiPAL autosampler, an Agilent 6890N gas chromatograph and a LECO Pegasus III time-of-flight mass spectrometer running in EI+ mode. Metabolites were identified by comparison with database entries of standards (Kopka *et al*., 2005; Schauer *et al*., 2005). Data were normalized with respect to control plants (at days 1 and 3). Values presented are the mean of four to six measurements.

Statistical analyses

Five experiments separate in time were performed with different groups of plants grown under similar conditions. Statistical analysis was performed using the JMP®, Version 12.1.0 (SAS Institute Inc., Cary, NC, 1989-2007). A one-way analysis of variance (ANOVA) with a level of significance of P-value < 0.05 was performed followed by Student's t-test for all analysis. Pairwise Pearson correlation coefficients between metabolite levels and the alternative pathway activity (v_{alt}) in leaves were also performed in all three genotypes.

RESULTS

Photosynthesis and stomatal conductance

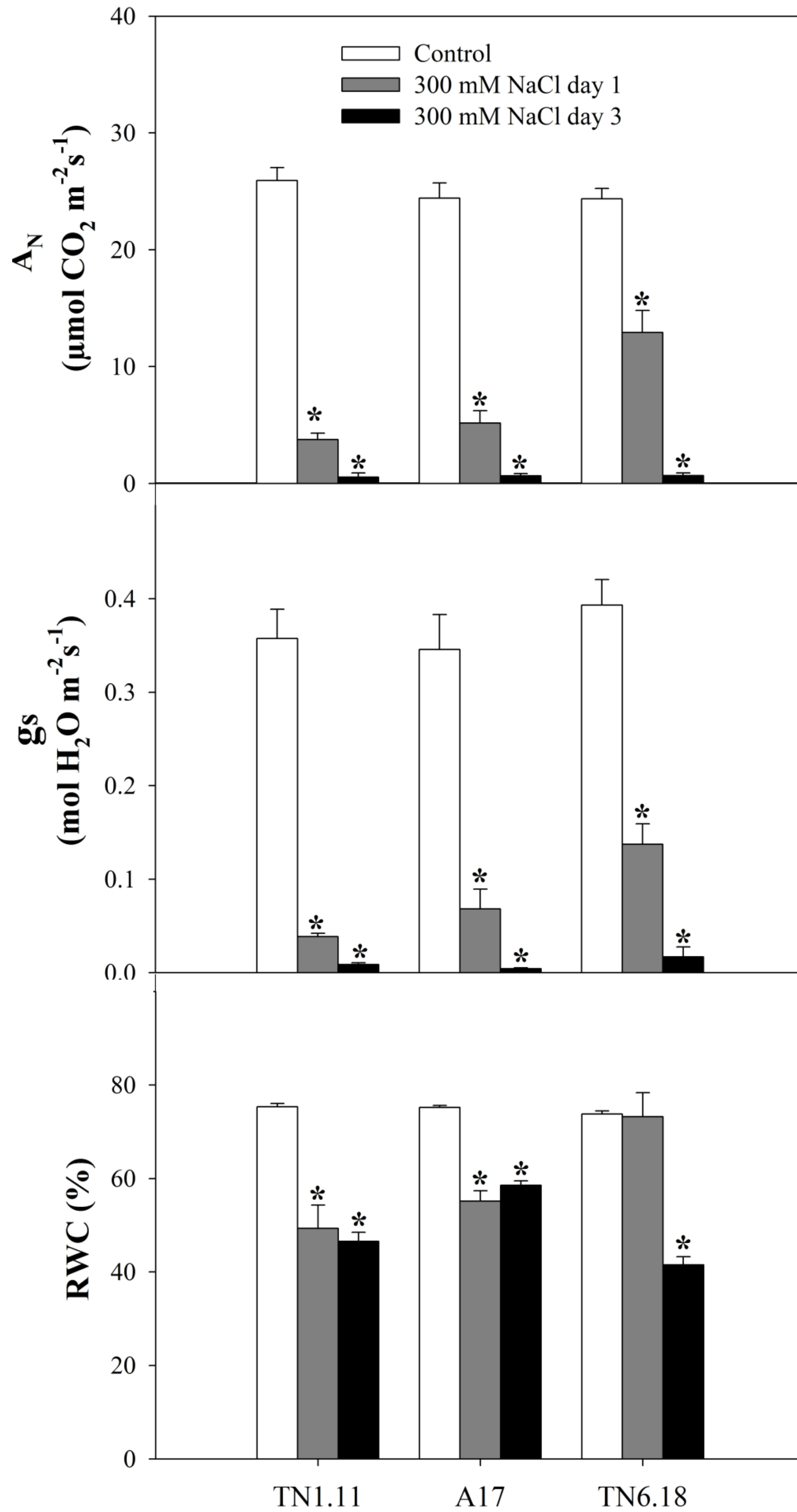
Net photosynthesis (A_N) was 25.9±1.1, 24.4±1.3 and 24.4±0.9 μmol CO_2 m^{-2} s^{-1} in control plants of TN1.11, A17 and TN6.18, respectively. After 1 day of salt treatment, A_N was reduced significantly by 86%, 78% and 48% in TN1.11, A17 and TN6.18, respectively. After 3 days A_N was reduced by more than 90% in all genotypes (Figure 1).

Stomatal conductance (g_s) was 0.357±0.031, 0.346±0.037 and 0.393±0.027 mol H_2O m^{-2} s^{-1} in control plants of TN1.11, A17 and TN6.18, respectively. After 1 day of salt treatment, g_s was reduced significantly by 89%, 81% and 68% in TN1.11, A17 and TN6.18, respectively. After 3 days g_s was reduced by more than 95% in all genotypes (Figure 1). TN6.18 genotype shows the lowest decrease in both A_N and g_s on the first day of salinity.

Relative water content (RWC)

RWC was around 75% in leaves of control plants of all three genotypes (Figure 1). After 1 day of salt stress, RWC decreased to 49% and 56% in TN1.11 and A17, respectively while remaining at 75% in TN6.18 (Fig. 1). After 3 days of salt, RWC was around 50% in all three genotypes (Figure 1).

Figure 1. The effect of salt treatment in leaves of *Medicago truncatula* genotypes TN1.11, A17 and TN6.18 under 0 mM NaCl (white bars), and after 1 (grey bars) and 3 days (black bars) of salt treatment (300 mM) on net photosynthesis (A_N), stomatal conductance (g_s) and relative water content (RWC). No significant differences were found in control plants between the two different days of analysis and consequently, their results were averaged as shown in the graph. * Means significant differences between control and its respective salt time level at p<0,05. Data are means ± SE of 4 replicates.


Control
300 mM NaCl day 1
300 mM NaCl day 3
A_N (µmol CO_2 $m^{-2}s^{-1}$)
gs (mol H_2O $m^{-2}s^{-1}$)
RWC (%)
TN1.11
A17
TN6.18

Respiration and electron partitioning to the AOX pathway

In leaves, total oxygen uptake (V_t) was 34.67±2.91, 37.50±1.74 and 35.41±1.27 nmol O_2 $g^{-1}DW$ s^{-1} in control plants of TN1.11, A17 and TN6.18, respectively. After 1 day of salt treatment, V_t was unchanged in TN1.11 and A17 and increased by 17% in TN6.18 (Figure 2). After 3 days of salt treatment, V_t was unchanged in A17 and decreased by 52% and 42% in TN1.11 and TN6.18, respectively (Figure 2).

The electron partitioning to the alternative pathway (τ_a) was 0.20±0.01, 0.19±0.01 and 0.21±0.01 in control plants of TN1.11, A17 and TN6.18, respectively. After 1 day of salt treatment, τ_a unchanged in TN1.11, A17 and increased significantly to 0.31±0.04 in TN6.18 plants (Figure 2). After 3 days of salt treatment, τ_a remained unchanged in A17 and increased to 0.36±0.03 and 0.34±0.01 in plants of TN1.11 and TN6.18, respectively (Figure 2). These changes in V_t and τ_a were due to changes in the activity of the cytochrome (v_{cyt}) and alternative (v_{alt}) pathways.

Thus, v_{cyt} was 27.43±2.38, 30.57±1.54 and 28.55±1.14 nmol O_2 $g^{-1}DW$ s^{-1} in control plants of TN1.11, A17 and TN6.18, respectively. After 1 day of salt treatment, v_{cyt} was not significantly changed in any of the genotypes. After 3 days of salt treatment, v_{cyt} decreased significantly in TN1.11 by 62% and TN6.18 by 50% while remaining unchanged in A17 (Figure 2).

On the other hand, v_{alt} was 6.92±0.46, 7.09±0.54 and 7.79±0.58 nmol O_2 $g^{-1}DW$ s^{-1} in control plants of TN1.11, A17 and TN6.18, respectively. After 1 day of salt treatment, v_{alt} was not significantly changed in TN1.11, A17 while it increased by 80% in TN6.18 (Figure 2). After 3 days of salt treatment, v_{alt} was not significantly changed to control in any of the genotypes (Figure 2).

The capacity of the alternative pathway (V_{alt}) was 32.33±0.89, 30.57±0.49 and 39.20±3.03 nmol O_2 $g^{-1}DW$ s^{-1} in control plants of TN1.11, A17 and TN6.18, respectively. After 1 day of salt treatment, V_{alt} was not significantly changed in TN1.11 and TN6.18 genotypes while it was increased in A17 by 13% (Figure 2). After 3 days of salt treatment, V_{alt} decreased significantly by 62%, 18% and 69% in TN1.11, A17 and TN6.18, respectively (Figure 2).

In roots, total oxygen uptake (V_t) was 58.77±3.45, 49.45±4.84 and 48.95±2.96 nmol O_2 $g^{-1}DW$ s^{-1} in control plants of TN1.11, A17 and TN6.18, respectively. After 1 day of salt treatment, V_t was decreased in TN1.11 by 46% and unchanged in A17 and TN6.18 (Figure 3).

After 3 days of salt treatment, V_t was decreased by 45%, 59% and 57% in TN1.11, A17 and TN6.18 respectively (Figure 3).

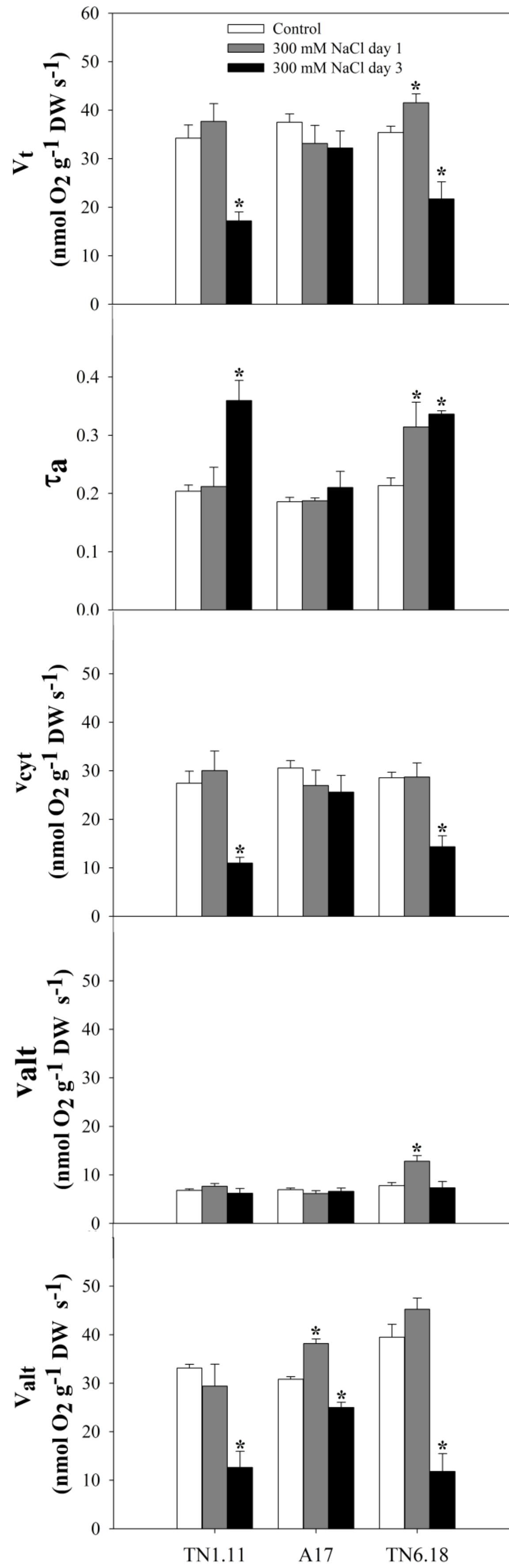
The electron partitioning to the alternative pathway (τ_a) was 0.16±0.01, 0.25±0.02 and 0.18±0.01 in control plants of TN1.11, A17 and TN6.18, respectively. After 1 day of salt treatment, τ_a was decreased by 75%, 80% and 72% in TN1.11, A17 and TN6.18 respectively (Figure 3). After 3 days of salt treatment, τ_a was not significantly changed to control in any of the genotypes (Figure 3). These changes in V_t and τ_a were due to changes in the activity of the cytochrome (v_{cyt}) and alternative (v_{alt}) pathways.

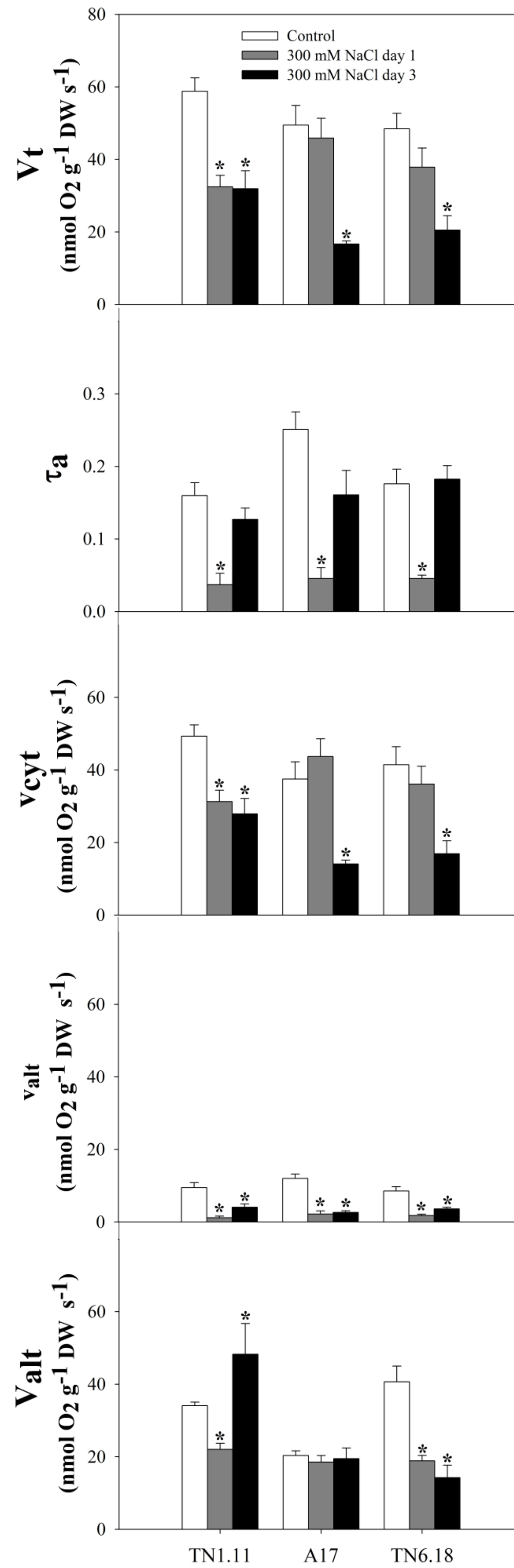
Thus, v_{cyt} was 49.31±2.58, 37.46±3.38 and 41.23±3.27 nmol O_2 g^{-1}DW s^{-1} in control plants of TN1.11, A17 and TN6.18, respectively. After 1 day of salt treatment, v_{cyt} was decreased only in TN1.11 by 40%. After 3 days of salt treatment, v_{cyt} decreased significantly by 40%, 66% and 67% in TN1.11, A17 and TN6.18 respectively (Figure 3).

On the other hand, v_{alt} was 9.46±1.28, 11.83±1.07 and 8.77±0.97 nmol O_2 g^{-1}DW s^{-1} in control plants of TN1.11, A17 and TN6.18, respectively. After 1 and 3 days of salt treatment, v_{alt} was significantly decreased by 65% in all genotypes (Figure 3).

The capacity of the alternative pathway (V_{alt}) was 34.16±0.71, 20.30±1.41 and 40.76±5.23 nmol O_2 g^{-1}DW s^{-1} in control plants of TN1.11, A17 and TN6.18, respectively. After 1 day of salt treatment, V_{alt} was significantly decreased by 32% and 50% in TN1.11 and TN6.18 respectively (Figure 4). After 3 days of salt treatment, V_{alt} increased significantly by 40% in TN1.11 and decreased significantly by 67% in TN6.18 (Figure 3).

Figure 2. Total respiration (V_t), electron partitioning to the alternative pathway (τ_a), cytochrome pathway activity (v_{cyt}), alternative pathway activity (v_{alt}) and alternative pathway capacity (V_{alt}) in leaves of *Medicago truncatula* genotypes TN1.11, A17 and TN6.18 under 0 mM NaCl (white bars), and after 1 (grey bars) and 3 days (black bars) of salt treatment (300 mM). No significant differences were found in control plants between the two different days of analysis and consequently, their results were averaged as shown in the graph. * Means significant differences between control and its respective salt time level at $p<0,05$. Data are means ± SE of 4 replicates.


Control
300 mM NaCl day 1
300 mM NaCl day 3
Vt (nmol O2 g-1 DW s-1)
τa
vcyt (nmol O2 g-1 DW s-1)
valt (nmol O2 g-1 DW s-1)
Valt (nmol O2 g-1 DW s-1)
TN1.11
A17
TN6.18



Control
300 mM NaCl day 1
300 mM NaCl day 3
Vt
(nmol O2 g-1 DW s-1)
τa
vcyt
(nmol O2 g-1 DW s-1)
valt
(nmol O2 g-1 DW s-1)
Valt
(nmol O2 g-1 DW s-1)
TN1.11
A17
TN6.18

Figure 3. Total respiration (V_t), electron partitioning to the alternative pathway (τ_a), cytochrome pathway activity (v_{cyt}), alternative pathway activity (v_{alt}) and alternative pathway capacity (V_{alt}) in roots of *Medicago truncatula* genotypes TN1.11, A17 and TN6.18 under 0 mM NaCl (white bars), and after 1 (grey bars) and 3 days (black bars) of salt treatment (300 mM). No significant differences were found in control plants between the two different days of analysis and consequently, their results were averaged as shown in the graph. * Means significant differences between control and its respective salt time level at p<0,05. Data are means ± SE of 4 replicates.

Metabolite profiling

The effects of sudden severe salt stress on the metabolite profiles of leaves was assessed by the detection of 33 metabolites comprising 12 amino acids, 5 sugars, 12 organic acids 4 other metabolites (Table 1).

After 1 day of salt stress significant changes were observed in 9 (2 up), 4 (1 up) and 11 (11 up) amino acids; in 4 (3 up), 2 (2 up) and 1 (1 up) sugars and in 10 (1 up), 8 (1 up) and 4 (4 up) organic acids for TN1.11, A17 and TN6.18, respectively. After 3 days of salt stress significant changes were observed in 11 (11 up), 10 (10 up) and 12 (12 up) amino acids; in 4 (2 up), 1 (0 up) and 4 (4 up) sugars and in 10 (0 up), 7 (0 up) and 8 (1 up) organic acids for TN1.11, A17 and TN6.18, respectively. Among the organic acids, fumarate decreased after 1 day of salt treatment in TN1.11, A17 while it was unchanged in TN6.18. After 3 days, fumarate decreased in all genotypes. Malate decreased after 1 day of salt treatment only in A17 while it was unchanged in TN1.11 and TN6.18. After 3 days, malate decreased in all genotypes (Table 1).

In addition, Pearson correlations were performed between log10 transformed-fold changes of *in vivo* respiratory activities and metabolite levels in order to get further insights into the influence of AOP and COP in primary metabolism and/or vice-versa. Among all the metabolites analysed, only malic and erythronic acids were significantly correlated with all respiratory activities (i.e. V_t, v_{cyt} and v_{alt}), while fumaric acid was also significantly correlated with v_{alt} (Figure 4).

Table 1. Metabolite profiling in leaves of *Medicago truncatula* genotypes TN1.11, A17 and TN6.18 treated with 300 mM NaCl. Relative values are expressed as fold changes after 1 and 3 days under salt treatment normalized to their respective control. Red and blue boxes denote significant increase and decrease as assessed by the Students *t*-test, at p<0,05, respectively. Data are means of 4 to 6 replicates.

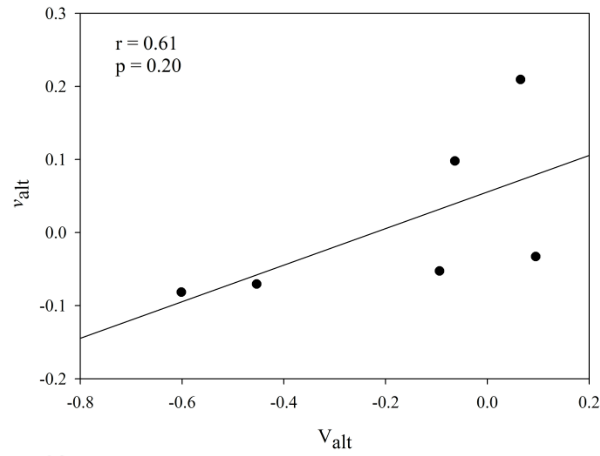
	300 mM NaCl vs C (day 1)			300 mM NaCl vs C (day 3)		
	TN 1.11	A17	TN 6.18	TN 1.11	A17	TN 6.18
Amino acids						
Alanine	0.37	1.14	1.85	3.85	4.17	3.87
Alanine, beta	0.26	0.82	1.92	2.70	2.74	5.60
Asparagine	0.16	0.57	1.24	0.76	0.95	4.01
Glycine	3.17	1.23	7.07	26.55	1.06	12.71
Isoleucine	0.51	0.45	9.02	7.18	5.65	3.83
Methionine	1.04	1.16	1.69	11.44	3.32	7.42
Phenylalanine	0.93	0.73	10.74	11.41	8.69	7.30
Proline	9.84	0.83	12.21	41.35	41.35	58.68
Serine	0.96	3.46	1.83	5.26	6.68	3.43
Threonine	0.25	0.41	2.08	6.65	2.51	2.88
Tyrosine	0.30	0.82	2.82	4.85	3.71	4.26
Valine	0.45	0.58	5.03	10.53	5.50	4.72
Organic acids						
Aspartic acid	0.28	0.36	1.95	0.04	0.12	0.25
Benzoic acid	0.26	0.41	1.05	1.02	0.85	1.11
Citric acid	0.66	0.84	0.96	0.50	0.40	0.61
Dehydroascorbic acid dimer	0.20	5.37	1.82	0.39	0.39	0.28
Erythronic acid	0.50	0.58	0.84	0.20	0.20	0.21
Fumaric acid	0.36	0.35	1.10	0.13	0.13	0.18
Glutamic acid	0.47	0.42	1.06	0.38	0.64	0.60
Glutaric acid, 2-oxo-	3.82	0.74	1.95	0.16	0.92	2.18
Lactic acid	0.33	1.36	1.35	0.96	1.10	1.12
Malic acid	0.93	0.37	1.08	0.18	0.17	0.20
Nicotinic acid	0.68	0.87	1.52	0.32	1.00	1.06
Pyruvic acid	0.70	0.56	1.26	0.42	0.90	1.44
Sugars						
Fructose	10.58	0.84	1.05	3.97	0.32	2.16
Glucose	6.39	1.14	1.38	20.17	1.08	7.11
Glucose, 1,6-anhydro, beta	0.40	1.94	1.57	0.17	0.76	1.49
Maltose	0.87	0.84	1.17	1.14	1.27	2.21
Sucrose	2.27	3.72	0.95	0.38	1.50	3.01
Others metabolites						
Cinnamic acid, 4-hydroxy-, trans-	0.47	1.01	0.77	0.38	0.56	0.76
Erythritol	1.65	0.97	1.17	0.84	1.17	4.04
Glycerol	0.45	1.21	4.53	3.27	1.81	7.15
Inositol, myo	0.43	0.69	1.04	0.42	0.84	0.98

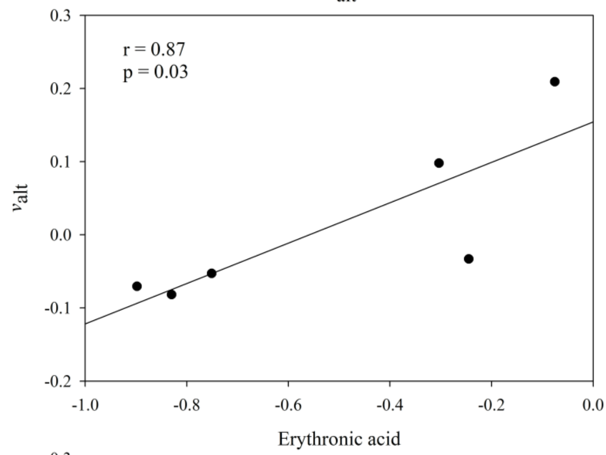
In roots, the effects of sudden severe salt stress on the metabolite profile was assessed by the detection of 26 metabolites comprising 9 amino acids, 3 sugars, 12 organic acids and two other metabolites (Table S1).

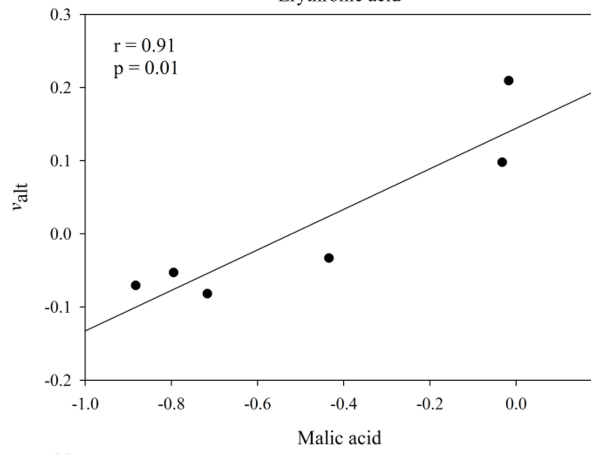
After 1 day of salt stress significant changes were observed in 4 (1 up), 5 (0 up) and 1 (0 up) amino acids; in 0, 0 and 1 (0 up) sugars and in 6 (0 up), 1 (0 up) and 4 (3 up) organic acids for TN1.11, A17 and TN6.18, respectively. After 3 days of salt stress significant changes were observed in 9 (8 up), 2 (1 up) and 6 (0 up) amino acids; in 0, 1 (0 up) and 3 (0 up) sugars and in 8 (6 up), 1 (0 up) and 8 (0 up) organic acids for TN1.11, A17 and TN6.18, respectively.

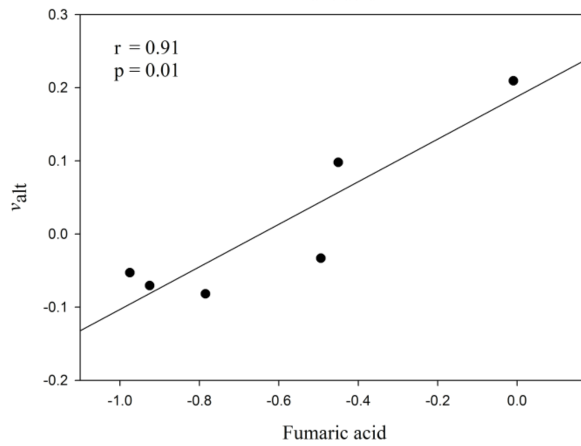
As for the leaves, Pearson correlations were performed between log10 transformed-fold changes of *in vivo* respiratory activities and metabolite levels. No significant correlations were observed at the root level.

Figure 4. Linear correlation plots between AOP activity (v_{alt}) and AOP capacity (a), erythronic acid (b), malic acid (c) and fumaric acid (d) in leaves of TN1.11, A17 and TN6.18 *M. truncatula* genotypes after 1 and 3 days of 300 mM NaCl are represented. Pearson correlation coefficients (r) and P-values (P) are indicated. Values are expressed as fold change (log10 transformed).


r = 0.61
p = 0.20
v_{alt}
V_{alt}


r = 0.87
p = 0.03
v_{alt}
Erythronic acid


r = 0.91
p = 0.01
v_{alt}
Malic acid


r = 0.91
p = 0.01
v_{alt}
Fumaric acid

DISCUSSION

The present research is based on the hypothesis that tolerance to sudden salinity stress is related to the capacity of the leaf tissue to respire (Jacoby *et al*., 2011) with more specific relevance of the respiration through the cyanide-resistant alternative pathway as it has been related to oxidative stress (Maxwell *et al*., 1999; Møller, 2001). Generally, salt stress causes a strong decrease in physiological leaf parameters like RWC, stomatal conductance (g_s) and consequently photosynthesis (A_N), and these parameters are related to the degree of salinity tolerance or sensitivity (Flexas *et al*., 2004; Sudhir & Murthy, 2004; Lopez-Climent *et al*., 2008). Thus, of the three genotypes of *M. truncatula* with different salinity responsiveness studied, TN6.18 clearly maintains the highest levels of these physiological traits after 1 day of sudden addition of 300 mM NaCl (Figure 1), being the only genotype to show an increased rate of respiration (i.e. *salt respiration*), which is not related to cytochrome oxidase but due to an increase in the alternative oxidase pathway activity *in vivo* (Figure 2). The observed increase in total respiration (V_t) after 1 day of severe salt treatment is in agreement with Jacoby *et al*. (2011). However, after a longer period of exposure to severe salt treatment (3 days), all genotypes presented a strong decay of physiological parameters (A_N, g_s and RWC) as seen in Figure 2.

The improved response to 1 day of salt stress in TN6.18 coincides with increased levels of metabolites such as amino, organic acids and other metabolites such as glycerol (Table 1) in agreement with previous observations in salt tolerant barley cultivar (Widodo *et al*., 2009), or in *Catharanthus roseus* under 250 mM NaCl (Chang *et al*., 2014). These metabolites have been considered as osmoprotectors (Sairam & Tiagy, 2004; Amini & Ehsanpour, 2005; Abhilash *et al*., 2014; Rybka & Nita, 2015), and they have also shown to be genotype dependent, taking part of osmotic adjustment to tolerate salinity (Marschner, 1995; Shabala 2013; Munns 2015; Flowers 2015). The increased level of osmoregulatory intermediates observed in TN6.18 after 1 day of sudden severe salt treatment (Table 1) may be a consequence of a higher TCA cycle flux which would be favoured by the increased rate of mitochondrial electron transport observed in TN6.18 (Figure 2). Moreover, the higher rate of AOP observed in TN6.18 after 1 day of salt stress would reduce the redox status of the ETC caused by the increased TCA cycle flux, thus improving the response to salt stress. The correlation observed between AOP activity and malate and fumarate (Figure 4c and d) reinforces the role of AOP on allowing a higher rate of TCA cycle as both are products of the mETC complex II or succinate dehydrogenase (Vanlerberghe 2013).

In addition, metabolite-AOP correlations can also reflect the important role suggested for AOP on improving photosynthetic performance (Vanlerberghe 2013). The TCA cycle regulation of leaf fumarate/malate levels has been shown to directly affect stomatal conductance in tomato plants (Araujo *et al.*, 2011) which can, at least partly, explain the improved g_s and A_N observed in TN6.18 under salinity (Figure 1). On the other hand, AOP has been shown to act as an electron sink under stress conditions allowing the oxidation of excess reducing power from the chloroplast, i.e. transported through the malate valve (Noguchi & Yoshida 2008, Florez-Sarasa *et al.*, 2011) which would also be in the line of the malate correlations observed (Figure 4). Finally, erythronic acid, which is a degradation product of ascorbate (Green & Fry, 2005), significantly correlated with the respiration activities. This may indicate a contribution of the mitochondrial electron transport chain and AOP to ascorbate synthesis, as previously reported (Millar *et al.*, 2003; Bartoli *et al.*, 2006).

In roots, it is well known that respiration decreases under severe salt stress (Richardson & McCree, 1985; Hwang *et al.*, 1994; Moud & Maghsoudi, 2008). In agreement with previous observations, root respiration of *Medicago truncatula* genotypes treated with 300 mM NaCl decreased after 3 days of treatment (Figure 3). The increased capacity of AOP observed in TN1.11 after 3 days of salt treatment may be explained by the increase of AOX1a expression observed in this genotype by Mhadhbi *et al.*, (2013). Nevertheless, neither a correlation between root respiration nor the activity of the AOP with the responsiveness to severe salt stress has been observed. In addition, the lack of correlations observed between metabolites and respiratory activities in roots also suggests that, in our experimental conditions, AOP was not involved in the metabolic adaptation of the roots to salinity.

Increase of *salt respiration* combined with the *in vivo* activity of AOP in leaves has been correlated to AOP-driven metabolic adaptations on organic and amino acid metabolism. Such a respiratory response could be related with salinity tolerance as it allowed sustaining higher photosynthetic rates and water content under short-term severe salt stress in *Medicago truncatula* TN6.18 genotype. Nevertheless, long-term experiments are needed to ascertain the tolerance/sensitivity degree of these genotypes.

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Supplementary table S1

Table S1. Metabolite profiling in roots of *Medicago truncatula* genotypes TN1.11, A17 and TN6.18 treated with 300 mM NaCl. Relative values are expressed as fold changes after 1 and 3 days under salt treatment normalized to their respective control. Red and blue boxes denote significant increase and decrease as assessed by the Students *t*-test, at p<0,05, respectively. Data are means of 4 to 6 replicates.

	300 mM NaCl vs C (day 1)			300 mM NaCl vs C (day 3)		
	TN 1.11	A17	TN 6.18	TN 1.11	A17	TN 6.18
Amino acids						
Alanine	0.69	0.32	0.72	0.46	0.28	0.13
Alanine, beta	0.60	0.37	0.67	2.58	0.80	0.16
Asparagine	0.04	0.95	0.96	7.40	1.26	0.57
Glycine	1.36	0.41	0.78	2.44	1.28	0.51
Isoleucine	0.95	1.19	0.74	19.97	2.01	0.89
Phenylalanine	0.62	0.40	0.67	7.11	1.61	0.80
Serine	0.85	1.13	0.83	3.72	0.94	0.39
Threonine	0.52	0.54	0.68	5.64	1.29	0.64
Valine	0.69	0.90	0.46	11.48	2.19	0.97
Organic acids						
Aspartic acid	0.17	0.62	0.73	6.26	0.98	0.78
Butyric acid, 4-amino-	1.08	0.76	0.87	1.34	0.71	0.34
Citric acid	0.17	0.73	3.75	0.78	0.25	0.32
Fumaric acid	0.94	0.99	4.11	1.20	2.04	0.54
Glutamic acid	0.51	0.95	1.11	2.12	0.92	0.59
Glutaric acid, 2-oxo-	1.08	0.96	1.37	0.55	1.67	0.40
Lactic acid	1.37	0.71	0.35	1.88	1.23	1.13
Malic acid	0.71	1.18	3.28	0.35	0.75	0.33
Nicotinic acid	0.57	1.89	0.84	4.02	0.92	0.42
Phosphoric acid	0.48	1.29	1.02	1.00	1.63	0.54
Pyroglutamic acid	0.87	0.25	1.03	4.29	0.87	0.90
Pyruvic acid	0.56	0.87	0.83	1.53	0.98	0.97
Sugars						
Fructose	1.14	1.01	1.48	0.97	0.84	0.17
Glucose	1.23	1.34	1.21	1.13	1.14	0.37
Sucrose	0.83	0.91	0.87	0.89	0.62	0.82
Other metabolites						
Glycerol	1.51	0.98	0.96	1.54	1.35	0.28
Inositol, myo	0.74	0.51	0.42	0.98	1.01	0.26

CHAPTER 2.

Sudden severe drought and salt stress in *Arabidopsis thaliana* increases the rate of both polyamine synthesis and leaf respiration via alternative oxidase

Sudden severe drought and salt stress in *Arabidopsis thaliana* increases the rate of both polyamine synthesis and leaf respiration via alternative oxidase

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ABSTRACT

Salt and drought stress induce plants to accumulate free polyamines (PAs) putrescine, spermidine and spermine. Their metabolism is linked to tricarboxylic acids (TCAs) such as citrate, malate and fumarate, which in turn, are involved in respiration. Under drought and salt stress, respiration can be restricted at the level of the ubiquinone pool, however, the activity of the alternative oxidase pathway (AOP) is thought to prevent this from occurring. The present research is based on the hypothesis that the accumulation of TCAs and PAs under drought and salt stress will depend on the response of the leaf AOP activity. To test this, the oxygen-isotope-fractionation technique was used to study the *in vivo* respiratory activities of the cytochrome and the alternative oxidase pathways (COP and AOP) under sudden severe drought and salt conditions (1 day, 300 mM of mannitol or salt) in leaves of wild-type *Arabidopsis thaliana* plants and in *aox1a* T-DNA mutants. In addition, the levels of free folial PAs and TCAs were determined. Our results demonstrate that the lack of *AOX1a* coincided with lower accumulation of TCAs and PAs and lower rates of AOP respiration, while in the wild-type AOP aids the synthesis of PAs by allowing the acceleration of the TCA cycle needed for their synthesis.

INTRODUCTION

Plants acclimate to adverse environmental conditions, partially, via the accumulation of metabolites that play protective roles. Well-known examples of metabolites that are involved in stress responses are the low-molecular-weight aliphatic polyamines (PAs) (Andronis *et al.*, 2014; Liu *et al.*, 2015). The three common free PAs in plants are spermidine (Spd), spermine (Spm), and their obligate precursor putrescine (Put) (Gill and Tuteja, 2010; Minocha *et al.*, 2014). Polyamines modulate the defense response of plants to abiotic stresses, most remarkably on salt and drought stress (Gill and Tuteja, 2010; Gupta *et al.*, 2013; Shu *et al.*, 2015).

The metabolism of PAs requires a continuous production of tricarboxylic acid (TCA) cycle intermediates to sustain their synthesis (Minocha and Minocha, 2006). In fact, it has been recently described that there is an expanded use of the TCA cycle to provide biosynthetic precursors for the synthesis of metabolites involved in stress acclimation (O´Leary and Plaxton, 2016). In line with this, Matto *et al.* (2006) observed an accumulation of distinct TCAs such as citrate, fumarate and malate as well as PAs in transgenic tomatoes engineered to accumulate high amounts of Spd and Spm. Moreover, the synthesis of TCA cycle intermediates is associated to the generation of reducing equivalents (NADH and $FADH_2$) that are oxidized in the mitochondrial electron transport chain (mETC). A distinctive feature of the plant mETC is the presence of two terminal oxidases. In addition to the cyanide sensitive cytochrome oxidase (COX), the presence of a cyanide-resistant alternative oxidase (AOX) that couples the oxidation of ubiquinol with the reduction of O_2 to H_2O, confers to mETC the capacity to sustain O_2 consumption under COX restriction. Electron transport via the AOX pathway (AOP) is not coupled with energy conservation (Ribas-Carbo *et al.*, 2005a; Vanlerberghe, 2013). Nonetheless, the AOP provides metabolic flexibility that helps plants acclimate to stress under conditions where the cytochrome oxidase pathway (COP) is restricted thus allowing the oxidation of reducing equivalents coupled to TCA cycle reactions (Lambers *el al.*, 2005). In fact, the accumulation of TCAs and PAs observed by Matto *et al.* (2006) also coincided with higher respiratory rates, suggesting a more active mitochondrial metabolism. Nevertheless, the influence of the AOX activity in modulating the synthesis of PAs is currently unknown.

AOX protein is encoded by a multigene family which exhibits tissue-specific expression (Saisho *et al.* 1997). *AOX1* isoforms generally show the highest expression levels, being expressed in most tissues and induced by stress treatments in several species (Vanlerberghe and McIntosh, 1992; Simons *et al.*, 1999; Djajanegara *et al.*, 2002; Clifton *et al.*, 2005; Sieger

et al., 2005; Vanlerberghe, 2013). Over the last few years, reverse genetics has been used to decrease *AOX1* gene expression in order to test hypotheses regarding the role of AOP. Following the suppression of *AOX1* gene expression, the amount of the AOX protein has been found to be decreased concomitantly with a decrease in its capacity (the maximum possible flux of electrons to AOX) (Guy and Vanlerberghe, 2005; Umbach *et al*., 2005; Watanabe *et al*., 2010; Florez-Sarasa *et al*., 2011). In parallel, a large number of studies have observed an increase in AOX protein or capacity under stress in wild-type plants (Vanlerberghe *et al*., 2013). In fact, it is well known that a regulated overcapacity of AOX is required to ensure adequate activity under conditions which induce metabolic fluctuations (Rasmusson *et al*., 2009; Florez-Sarasa *et al*., 2011). By contrast to AOX capacity, AOX activity can be directly quantified *in vivo* in the absence of added inhibitors by assessing the differential fractionation of ^{16}O and ^{18}O isotopes (Day *et al*., 1996; Ribas-Carbo *et al*., 2005a; Vanlerberghe, 2013).

In previous studies, the i*n vivo* AOX activity was reported to increase in response to severe drought and salt stress (Ribas-Carbo *et al*., 2005b; Del-Saz *et al*., 2016). On the other hand, it is well known that the synthesis of PAs is induced under both stresses (Gupta *et al*., 2013). Thus, the present study was conducted in wild type *A. thaliana* plants and in *aox1a* T-DNA insertion mutants that display a reduced levels of AOX protein and capacity (Watanabe *et al*., 2010; Yoshida *et al*., 2011). In an effort to gain insight into the role of the AOX in the synthesis of PAs, the two genotypes were treated with high concentrations (1 day, 300 mM) of mannitol (drought) and salt. The *in vivo* activities of COP and AOP in leaves were measured using the oxygen-isotope-fractionation technique and in parallel, leaves were harvested for determination of the levels of free PAs and TCAs. We hypothesize that the reduction of AOX will be associated with lower leaf rates of AOP respiration and less accumulation of PAs under severe water and salt stress.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds from *A. thaliana* Columbia 0 (Col 0) and *aox1a* T-DNA insertion line (SALK_ 084897) were sown in pre-wetted pots with a mixture of peat-perlite (3:1). The T-DNA insertion within the *AOX1a* gene was confirmed by genomic PCR with T-DNA-specific primers and seeds from a homozygous line were used for the experiments. Pots were placed in two plastic trays used for sub-irrigation. Plants were grown in a growth chamber for 35 days at 12-h/12-h photoperiod, 150 µmol quanta m^{-2} s^{-1} light intensity, 25 °C/20 °C day/night temperature and relative humidity above 50%. Plants were watered twice a week with half-strength

Hoagland's solution (Epstein 1972). On day 35, half of the plants were watered normally (control plants) while the rest were irrigated with half-strength Hoagland's solution containing 300 mM of sodium chloride (NaCl treated plants) or mannitol (Mannitol-treated plants). The analyses of control and treated plants were performed one day after the irrigation.

Respiration and oxygen-isotope fractionation measurements

Leaves were placed in the dark for 30 min to avoid light-enhanced dark respiration. Respiration and oxygen isotope fractionation measurements were performed as described in Del-Saz *et al*. (2016).

Respiratory partitioning between the two respiratory pathways was calculated from the oxygen isotope fractionation by the alternative oxidase (Δ_a) and the cytochrome oxidase (Δ_c). Value of Δ_a, determined in the presence of 10 mM of KCN. The Δ_a values were not significantly different between the genotypes and thus, the Δ_a mean value of 30.8 ± 0.445‰ (six replicates) was used for all the partitioning calculations. The oxygen isotope fractionation value for the cytochrome pathway (Δ_c), determined in presence of 25 mM of SHAM, was 20.5‰ (four replicates). Dry weights were determined after drying leaves for 2 days at 60 °C. Results were expressed as nmol O_2 g^{-1} dry weight s^{-1}. Results were expressed as nmol O_2 g^{-1} dry weight s^{-1} as the mean ± SE of six or eight biological replicates.

Determination of the capacity of the alternative pathway (V_{alt}) was performed in five replicates of leaves, with an oxygen electrode as described in Del-Saz *et al*. (2016). Results were expressed as nmol O_2 g^{-1} dry weight s^{-1} as the mean ± SE of five biological replicates.

Determination of free polyamines in leaves

The analysis of free PAs was performed as described in Lopez-Gomez *et al*. (2016). Leaves extracts were prepared from 0.2 g of fresh tissue with 0.6 ml of 5% (v/v) cold perchloric acid (PCA) and incubated 24 h at 4 °C. The homogenate was centrifuged (3,000x*g*, 5 min, 4 °C) and 0.2 ml aliquots of the supernatant were dansylated as described below. Derivatization was performed by mixing 0.2 mL aliquots of the extracts prepared with 0.4 mL of dansyl chloride (prepared fresh in acetone, 10 mg/ml) and 0.2 mL of saturated sodium carbonate. After a brief vortexing, the mixture was incubated in darkness at room temperature overnight. Excess dansyl reagent was removed by reaction with 0.1 mL (100 mg/ml) of added proline, and incubation for 30 min. Dansylpolyamines were extracted in 0.5 ml toluene. The organic phase was collected and evaporated to dryness under a stream of nitrogen, and re dissolved in 0.1 mL acetonitrile.

The analysis of free PAs putrescine, spermidine and spermine was performed by HPLC using a Hewlett-Packard system equipped with a reverse phase column (4.6 x 250 mm C18). Column flow was 1.5 ml min^{-1} and the elution gradient was prepared with water and acetonitrile. The column was equilibrated with 70% acetonitrile and 30% water before injecting 0.01 mL samples. This was followed by a linear gradient ending with 100% acetonitrile after 9 min. The final step was held for 4 min before regenerating the column. Detection was done with a fluorometer using excitation and emission wavelengths of 415 and 510 nm, respectively, according to Flores and Galston (1982). A relative calibration procedure was used to determine the PAs in the samples, using 1,7- diaminoheptane (HTD) as internal standard and PAs standards amounts ranging from 0.3 to 1.5 nmol purchased. Results were expressed as nmol g^{-1} fresh weight. Values presented are the mean ± SE of five biological replicates.

Determination of organic acids in leaves

Leaf extracts were prepared from 0.3 g of fresh tissue with 1.6 mL of cold ethanol/chloroform/water (12/5/1, v/v/v) and the homogenate was centrifuged at 4°C and 12000×*g* for 15 min. The supernatant was separated into aqueous and chloroform phases by the addition of chloroform and water. The aqueous phase was evaporated under a flow of nitrogen. Dry residues were resolubilized in buffer (organic acids), centrifuged (10,000× *g* 15 min at 4°C) and the supernatant was suitably diluted.

Determination of citric, malic and fumaric acids were analyzed by HPLC using a Hewlett-Packard system equipped with a reverse phase column (4.6 x 250 mm C18). Solvent flow was 0.8 mL min^{-1} and the elution gradient was prepared with 0.01 mol L^{-1} KH_2PO_4 buffer solution, pH = 2.60 adjusted with o-phosphoric acid) and methanol. The linear gradient profile was applied as follow (t (min); % KH_2PO_4 buffer solution): (0; 93%), (8; 93%), (9; 85%), (13; 85%), (14; 93%). The final step was held for 2 min before regenerating the column. Detection was done with a diode array at 210 nm and the amount was determined from its peak area with a calibration curve. Results were expressed as μg g^{-1} fresh weight. Values presented are the mean ± SE of five biological replicates.

Statistical analyses

All data presented belong to three experiments with different groups of plants grown on separate dates in the same growth chamber and under the same conditions. One way analysis of variance (ANOVA) with a level of significance of P value <0.05 was performed. Student's

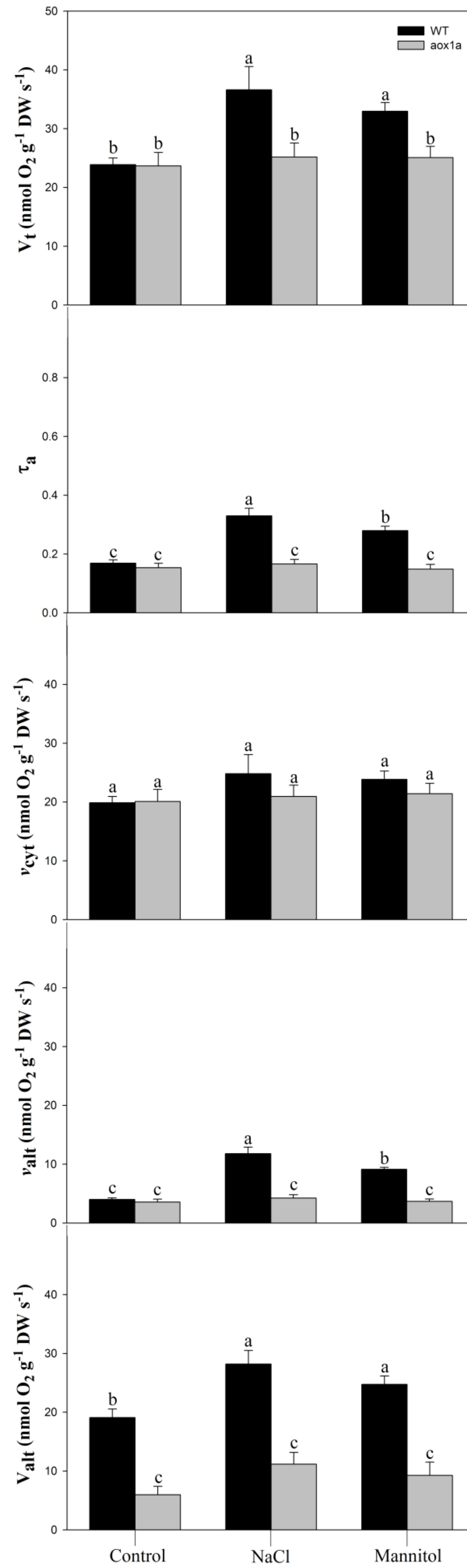
t-test was used when statistically significant differences were obtained. No transformation of the data was needed. Statistical analyses were performed using the JMP®, Version 12.1.0 (SAS Institute Inc., Cary, NC, USA, 1989-2007).

RESULTS

Sudden severe salt and mannitol stress increase respiration and electron partitioning in *Arabidopsis thaliana* wild type plants

Wild-type (WT) and *aox1a* mutant plants were watered with 2 L half strength Hoagland's solution containing 300 mM of sodium chloride (NaCl-treated plants) or mannitol (Mannitol-treated plants). Respiratory rates and oxygen isotope fractionation were determined one day after salt and mannitol treatments (Fig. 1). Total oxygen uptake (V_t) and electron partitioning to the alternative pathway (τ_a) were similar in WT and *aox1a* mutants under control conditions. Salt and mannitol treatments significantly ($P<0.05$) increased respiration only in WT plants from 23.9 to 36.6 and 32.9 nmol O_2 g^{-1} DW s^{-1}, respectively. Similarly, τ_a significantly increased under salt and mannitol treatments in WT plants from 0.169 to 0.329 and 0.279, respectively. On the other hand, no significant change in the τ_a was observed in *aox1a* mutants after salt and mannitol treatments. The activity of the alternative pathway (v_{alt}) was similar in both lines under control conditions and increased significantly by 194% and 127% in WT plants under salt and mannitol treatments respectively, whilst it was unaltered in *aox1a* mutants. The activity of the cytochrome pathway (v_{cyt}) was similar in the two lines in control and treated plants. Finally, the capacity of the alternative pathway (V_{alt}) was significantly lower in *aox1a* mutants than in WT plants (5.9 and 19.1 nmol O_2 g^{-1} DW s^{-1}, respectively) under control conditions and it was significantly increased only in WT after salt and mannitol treatments from 19.0 to 28.2 and 24.7 nmol O_2 g^{-1} DW s^{-1}, respectively.

Figure 1. Total respiration (V_t), electron partitioning to the alternative pathway (τ_a), cytochrome pathway activity (v_{cyt}), alternative pathway activity (v_{alt}) and alternative pathway capacity (V_{alt}) in leaves of Col 0 (WT) and *aox1a Arabidopsis thaliana* plants after 0 mM NaCl or Mannitol (Control), and after 1 day of severe NaCl or Mannitol treatments (300 mM). Values are means ± SE of 6-8 biological replicates. Significant differences ($P < 0.05$) are denoted by different letters.


WT
aox1a
V_t (nmol O_2 g^{-1} DW s^{-1})
τ_a
v_{cyt} (nmol O_2 g^{-1} DW s^{-1})
v_{alt} (nmol O_2 g^{-1} DW s^{-1})
V_{alt} (nmol O_2 g^{-1} DW s^{-1})
Control
NaCl
Mannitol

The organic acid and polyamine levels of *Arabidopsis thaliana* lines change differentially under sudden severe salt and drought stress

In parallel to the respiratory measurements, we determined the levels of TCAs malate, fumarate and citrate, as well as of free PAs putrescine, spermidine and spermine in leaves of *A. thaliana* WT and *aox1a* mutant treated for one day with NaCl or mannitol (300 mM) (Table 1).

Under control conditions, no differences between genotypes were found in the amount of fumarate and citrate, whilst the amount of malate was significantly lower (47%) in *aox1a* mutants. After 300 mM NaCl treatment, the amount of malate was unaltered in both genotypes, however, the amount of fumarate and citrate were significantly increased in WT plants only by 72% and 43%, respectively (Table 1). On the other hand, the amount of malate was significantly increased by 94% after mannitol treatment only in WT plants, whilst the amounts of fumarate and citrate were unaltered in both genotypes (Table 1).

Table 1. Concentrations of organic acids malate (Mal), fumarate (Fum) and citrate (Cit) and free polyamines (PAs) putrescine (Put), spermidine (Spd) and spermine (Spm) in leaves of Col 0 (WT) and *aox1a* (see Materials and Methods) *Arabidopsis thaliana* plants after 0 mM NaCl or Mannitol (Control), and after 1 day of severe NaCl or Mannitol treatments (300 mM). Values are means ± SE of five biological replicates. Significant differences ($P < 0.05$) are denoted by different letters.

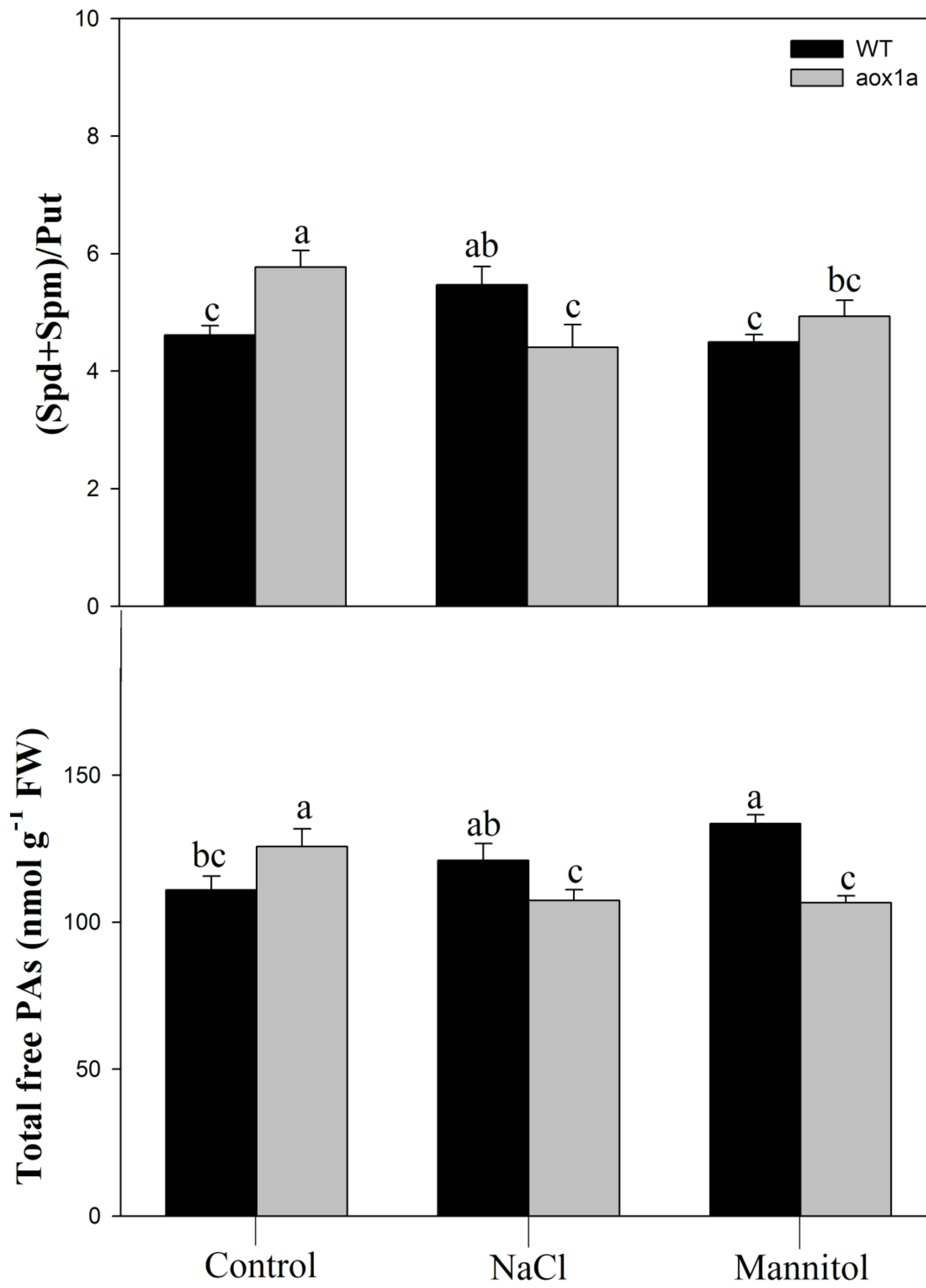
		Mal (µg g^{-1} FW)	Fum (µg g^{-1} FW)	Cit (µg g^{-1} FW)	Put (nmol g^{-1} FW)	Spd (nmol g^{-1} FW)	Spm (nmol g^{-1} FW)
Control	**WT**	311.9±52.2b	986.1±40.7b	69.7±4.22b	19.8±0.703b	62.9±3.44cd	28.2±0.943cd
	aox1a	166.6±15.9c	1455.7±296.1ab	73.4±4.77b	18.6±0.611b	73.3±4.39ab	33.9±1.49ab
NaCl	**WT**	238.6±24.9bc	1693.6±173.8a	99.8±9.77a	18.9±1.37b	67.8±3.79bc	34.3±1.26a
	aox1a	179.5±10.2c	1310.6±32.3ab	70.6±7.10b	20.3±1.73b	56.9±2.93d	30.1±1.15cd
Mannitol	**WT**	604.0±30.0a	1235.9±32.4ab	63.8±8.81b	24.3±0.235a	78.3±2.46a	30.9±0.874bc
	aox1a	229.4±52.5bc	1512.6±139.7a	65.8±8.94b	18.1±0.834b	61.3±1.78cd	27.2±1.09d

Regarding the levels of free PAs, putrescine content was not significantly different between genotypes in non-treated plants, while the contents of spermidine and spermine were significantly higher (17% and 20%, respectively) in *aox1a* mutants, and thus, the ratios of spermidine and spermine to putrescine (4.61 and 5.77 in WT and *aox1a* plants, respectively) as well as the level of total free PAs (110.9 and 125.8 in WT and *aox1a* plants respectively) were also significantly elevated in the mutant (Table 1, Fig. 2).

Following salt treatment, spermidine and spermine levels significantly decreased by 24% and 11%, respectively, in *aox1a* mutants, whilst spermine significantly increased by 22% in WT plants (Table 1). By contrast, putrescine levels were unaltered in both genotypes (Table 1). When taken together these results explain the opposite trends observed in *aox1a* mutant (decreased) and WT (increased) plants following salt treatment with regard of their ratios of spermidine and spermine to putrescine as well as of their total free PA pool size (Fig. 2).

Under 300 mM mannitol, the amount of putrescine and spermidine were increased significantly by 31% and 25% in WT plants. By contrast, the amount of putrescine was not significantly changed in *aox1a* mutants, whilst the spermidine and spermine levels were decreased significantly by 16% and 20%, respectively. Consequently, the ratio of spermidine and spermine to putrescine was decreased significantly from 5.77 to 4.93 in *aox1a* mutants after mannitol treatment. Finally, the total free PA level was reduced by 15% in *aox1a* mutants while it increased significantly by 21% in WT plants.

Figure 2. Ratio of spermidine and spermine to putrescine and concentration of total free polyamines (PAs) in leaves of Col 0 (WT) and *aox1a Arabidopsis thaliana* plants after 0 mM NaCl or Mannitol (Control), and after 1 day of severe NaCl or Mannitol treatments (300 mM). Values are means ± SE of five biological replicates. Significant differences ($P < 0.05$) are denoted by different letters.


WT
aox1a
(Spd+Spm)/Put
10
8
6
4
2
0
c
a
ab
c
c
bc
Total free PAs (nmol g-1 FW)
150
100
50
0
bc
a
ab
c
a
c
Control
NaCl
Mannitol

DISCUSSION

Plant responses to salt and drought stress include the accumulation of biogenic amines, such as putrescine, spermidine and spermine, of which in plants PAs are the most prevalent (Mattoo *et al*., 2006). Their synthesis requires continuous operation of the TCA cycle which is in turn sustained by respiration (Minocha and Minocha, 2006). However, respiration can be restricted at the level of the ubiquinone pool under abiotic stress (Vanlerberghe, 2013; Møller, 2001). Nonetheless, the activity of the AOP is thought to be able to circumvent the over-reduction of the ubiquinone pool which in turn can allow an increase in respiration under stress conditions (Lambers *et al*., 2005; Del-Saz *et al*., 2016). Based on previous evidence, we tested the hypothesis that sudden severe mannitol and salt treatments will increase leaf respiration via AOP and the amount of PAs in *A. thaliana* Col 0 (wild-type, WT) plants. In addition, we also tested whether the reduction of *aox1a* will be related to the absence of both AOP response and PAs accumulation under mannitol and salt treatments in *A. thaliana* mutant plants lacking *aox1a* expression.

As expected, the capacity of the AOP was significantly lower in *aox1a* mutant than in WT plants (Fig. 1) in agreement with the data of Watanabe *et al*. (2010). Despite the reduced AOP capacity, *aox1a* mutants displayed similar total respiration and electron partitioning to AOP as WT plants under control conditions (Fig. 1). These results are very similar to those obtained in *aox1a* anti-sense plants grown under different light intensities (Florez-Sarasa *et al*., 2011), thus confirming that *AOX1a* isoform is not essential for maintaining *in vivo* AOX activity under non-stress conditions. Nevertheless, in non-treated plants, *aox1a* mutants displayed lower levels of malate and higher Spd and Spm levels than the WT control (Table 1). Several studies in *aox1a* mutant or transgenic plants have shown a mild perturbation of metabolism under optimal growth conditions (Parsons *et al*., 1999; Giraud *et al*., 2008; Strodtkötter *et al*., 2009), including similar reductions in malate levels (Strodtkötter *et al*., 2009). However, metabolic changes have been demonstrated to be far more pronounced when the plants were under stress (Umbach *et al*., 2005; Giraud *et al*., 2008; Watanabe *et al*., 2008; Strodtkötter *et al*., 2009) as we also observed in the present study.

Under both salt and mannitol treatments, the lack of *AOX1a* was associated with the absence of respiratory response. Contrarily to *aox1a* mutants, WT plants increased their leaf respiratory rates under severe drought and salt stress (Fig. 1) as previously observed by Ribas-Carbo *et al*. (2005b) in soybean and by Del-Saz *et al*. (2016) in *Medicago truncatula* plants.

The increased respiration was mainly related to a higher AOP *in vivo* activity (v_{alt}) in close agreement with Del-Saz *et al*. (2016). On the other hand, the v_{alt} in *aox1a* mutants was limited by the reduced AOP capacity under salt and mannitol treatments, whilst in WT plants, an overcapacity rate, approximately 3 times higher than v_{alt}, was observed. This overcapacity has been previously reported in several plant species in response to high light conditions when a sufficient abundance of AOX protein was able to maintain a certain required level of v_{alt} (Florez-Sarasa *et al*., 2016). In our study, this overcapacity also allowed the v_{alt} to increase under salt and drought stress in WT plants (Fig. 1).

With regard to PA analysis, Spd and Spm were the most abundant Pas observed in our study. Several reports have emphasized the protective role for Spd and Spm against abiotic stress in plants (Yamaguchi *et al*., 2007; Kubiś, 2008). It has been described that the ability to accumulate high Spd and Spm levels is the main factor responsible for the stress response (Matto *et al*., 2010; Pál *et al*., 2015). Nevertheless, the PAs evaluated in this study showed different accumulation under both stresses in WT plants (Table 1). Such a phenomenon has been recently reviewed by Gupta *et al*. (2013) who reported that significance of PAs may assume distinct functions under both stresses. Our results show that salt treatment increased the accumulation of Spm and the ratio of Spd and Spm to Put as previously observed (Chattopadhayay *et al*., 2002; Maiale *et al*., 2004; Zapata *et al*., 2004; Roy *et al*., 2005; Mahajan *et al*., 2008; Roychoudhury *et al*., 2011), whilst mannitol treatment increased the accumulation of Put, Spd, and consequently, the total PA level in agreement with the data reported by Liu *et al*. (2007, 2011).

The metabolism of the PAs Put, Spd and Spm is intimately linked with the TCA cycle (Fig. 3), which provides the carbon skeletons required for their synthesis (Matto *et al*., 2010). The TCAs evaluated in this study also showed different accumulation under both stresses as was previously discussed in the review of Obata and Fernie (2012). In WT plants, an accumulation of citrate and fumarate was observed following salt treatment (Table 1) in agreement with Widodo *et al*. (2009) and Gong *et al*. (2005), whilst malate accumulated following mannitol treatment (Table 1), as was also previously described (Guicherd *et al*., 1997; Patonnier *et al*., 1999; Sairam and Tiagy, 2004). The observed accumulation of TCAs and PAs in WT plants under salt and mannitol treatments suggests that TCA cycle activity and as a consequence PAs synthesis are increased in WT plants (Fig. 3) in agreement with Matto *et al*. (2006, 2010)

observations. An increase in the TCA cycle activity is linked to the re-oxidation of reducing equivalents which would, in turn, explain the higher AOP activity (v_{alt}) observed in WT plants. Also, an increase in organic acid levels can allosterically activate the AOX protein (Millar *et al.*, 1996; Oliver *et al.*, 2008). Furthermore, the lack of v_{alt} response in *aox1a* mutants together with the lack of significant increase in the amount of TCAs and PAs after both stress treatments, strongly suggest an important role of AOP in the synthesis of PAs (Fig. 1-3).

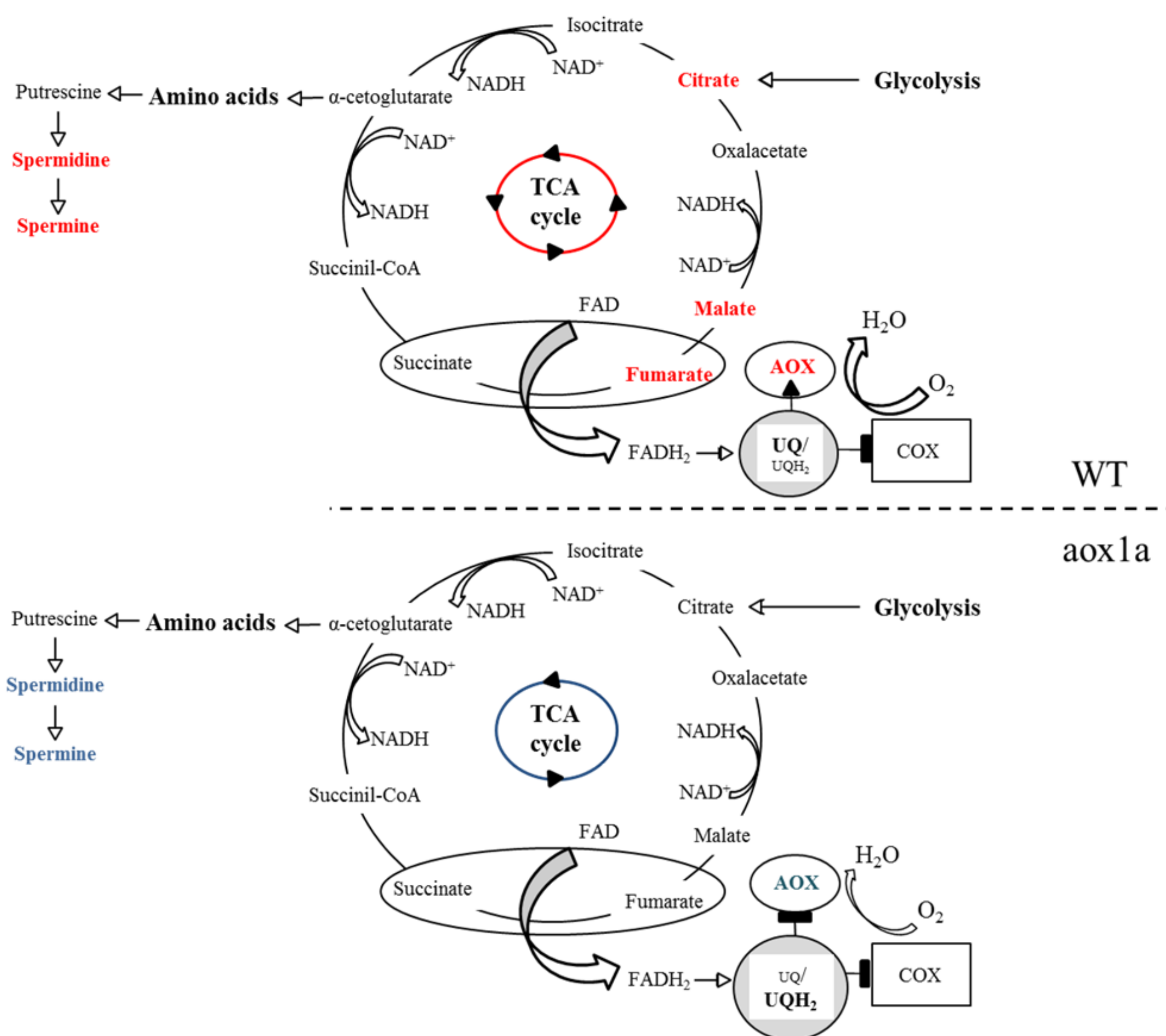


Figure 3. Biosynthesis pathways showing interconnections between glycolysis, TCA cycle, mETC, amino acids and PAs. Under severe drought and salt stress, the metabolism of PAs requires a continuous flux of TCA cycle intermediates to sustain their synthesis. The synthesis of several TCAs is associated to the generation of NADH in the mitochondrial matrix. Succinate is oxidized by the membrane-bound succinate dehydrogenase which is an enzyme shared by the TCA cycle and the mETC (complex II). Electrons from succinate reduce FAD to $FADH_2$ which enter the mETC trough

reduction of the UQ. In WT plants, the AOX couples the oxidation of UQ with the reduction of O_2 to H_2O when the COP is restricted, whilst the reduction of AOX in *aox1a* mutants is associated to an over-reduction of the UQ, which results in slower leaf respiration and deceleration of the TCA cycle. Consequently, the synthesis of the PAs spermidine and spermine is reduced. Red and blue colours denote increases and decreases (respectively) of TCA cycle, TCAs, AOX activity and PAs.

In summary, under salt and drought stress, the accumulation of PAs was associated with rapid respiratory rates through AOP and increased levels of TCAs citrate, malate and fumarate. Conversely, the reduction of AOX was associated with less accumulation of TCAs and PAs and slower leaf respiration rates through AOP. These results suggest that AOP aids the synthesis of PAs under salt and drought stress by allowing the acceleration of the TCA cycle needed for the PAs synthesis while the COP is unable to compensate for a lower of AOX.

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CHAPTER 3.

Respiratory ATP cost and benefit of arbuscular mycorrhizal symbiosis with *Nicotiana tabacum* at different growth stages and under salinity

Respiratory ATP cost and benefit of arbuscular mycorrhizal symbiosis with *Nicotiana tabacum* at different growth stages and under salinity

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ABSTRACT

Growth and maintenance partly depend on both respiration and ATP produced during oxidative phosphorylation in leaves. Under stress, ATP is needed to maintain the accumulated biomass. ATP production mostly proceeds via the cytochrome oxidase pathway (COP), although respiration via alternative oxidase pathway (AOP) may decrease the production of ATP per oxygen consumed, especially under phosphorus (P) limitation and salinity conditions. Symbiosis with arbuscular mycorrhizal (AM) fungi is reputed by their positive effect on plant growth under stress at mature stages of colonization, however, their construction may decrease plant growth at early stages. Thus, the present research is based on the hypothesis that AM fungus colonization will increase both foliar respiration and ATP production at mature stages of plant growth whilst it will decrease both at early stages. We used the oxygen-isotope-fractionation technique to study the *in vivo* respiratory activities and ATP production of the COP and AOP in AM and non-AM (NM) tobacco plants grown under P-limiting and saline conditions in sand at different growth stages (14, 28 and 49 days). Our results show that AM symbiosis represents an ATP cost detrimental for shoot growth at early stages, whilst it represents a ATP benefit that allowed faster rates of growth at mature stages, even under salinity conditions.

INTRODUCTION

Phosphorus (P) is a major mineral nutrient required by plants, and one of the most immobile and poor nutrients in soils (Vance et al. 2003). Phosphorus limitation may decrease both carbon assimilation and plant growth (Lambers et al. 2006). This is of particular concern in soils also affected by salinity because salt stress may further compound the problem of low productivity by decreasing cell turgor as well (Matar et al. 1992; Ford et al. 1993; Munns 2005).

Arbuscular mycorrhizal (AM) fungi widely exist in salt affected environments forming symbiotic associations in roots of most plant species (Juniper and Abbott 1993). Symbiosis with AM fungi may improve growth and confer tolerance to plants when they grow under P-limiting and saline conditions (Ruiz-Lozano 2003; Cantrell and Linderman 2001; Evelin et al. 2009). However, the effect of AM fungi on plant growth is variable as it depends on the host plant and fungal species (Stribley et al. 1980) and the stage of colonization (Smith et al. 2009). At matures stages, symbiosis may improve the P acquisition in soils deficient in this nutrient (Siddiqui and Pichtel 2008; Smith and Read 2008). The symbiosis imposes a carbon cost for the plant as it requires photosynthetic carbon for its functionality (Pearson and Jakobsen 1993; Jakobsen et al. 2002). Only when the benefit of the AM symbiosis for the plant exceeds its cost, more carbon will be available to be invested in plant growth and thus, the mycorrhizal association will be positive (Koide and Elliott 1989; Hughes et al. 2008).

Both biosynthesis of new plant constituents and maintenance of pre-existent structures are fuelled with ATP produced during respiration (Lambers and Poorter 1992). In this context, respiration in leaves is partitioned into two functional components termed growth and maintenance (Lambers et al. 2008). Under non-stress conditions, more ATP is invested in growth than in maintenance during the vegetative phase. Later, the investment turns into maintenance purposes (Amthor 1989; Bouma 2005). Nevertheless, when grown under stress, maintenance respiration may be as important as growth respiration (Lambers et al. 2002). Regarding AM plants, one may think that whether AM fungus colonization allows plants to accumulate more biomass in P-limiting and saline soils, the ATP investment in growth should be higher as well (relative to non-inoculated plants), whenever the specific association plant-AMF is positive.

Whilst AM symbiosis improves biomass accumulation during matures stages of colonization, it imposes an important cost for plant growth at early stages (Pearson and Jakobsen 1993; Li et al. 2005; Smith et al. 2009), since fungal structures require plant carbon for their entire

construction (Grahan and Eissenstat 1994). Following this reasoning, AM symbiosis should induce plants to invest less ATP in growth at early stages.

ATP synthesis depends on two electron pathways that consume oxygen in the mitochondrial electron transport chain (mETC); the cytochrome oxidase pathway (COP) and the alternative oxidase pathway (AOP). Respiration mostly proceeds via the COP (O´Leary and Plaxton 2016), which consists of complexes I, III, and IV operating in series to generate a proton gradient in the intermembrane space coupled to ATP synthesis by complex V. By contrast to the COP, the transfer of electrons from UQ to oxygen via the AOP bypass complex III and IV (with no extrusion of protons), and thus, decreasing the energetic efficiency of respiration (Robinson et al. 1995; Lambers et al. 2005). Abiotic stress can restrict the activity of COP which may lead to an over-reduction of UQ. In this situation, AOP may increase its activity to prevent such phenomenon to occur (Lambers et al. 2005). Moreover, the alternative respiration was shown to contribute to maintenance under non-stressful conditions at mature stages of growth (Millar et al. 1998; Florez-Sarasa et al. 2007; Priault et al. 2007) and to be sensitive to both P limitation and salinity (Gonzalez-Meler et al. 2001; Del-Saz et al. 2016). The *in vivo* activities of the AOX and COX only can be measured by ($^{18}O/^{16}O$) oxygen isotope fractionation (Guy et al. 1989; Robinson et al. 1995; Ribas-Carbo et al. 2005) allowing us to model the ATP production considering the non-phosphorylating nature of the alternative respiration (Vidal et al. 2007; Florez-Sarasa et al. 2007; Galle et al. 2010). This estimation is useful to analyse ATP costs and benefits of AM symbiosis at different stages of colonization and growth conditions, and to evaluate the potentiality of symbiosis in crop plants such as *Nicotiana tabacum*, a model species for oxygen fractionation studies (Lennon et al. 1997; Gonzalez-Meler et al. 2001; Guy and Vanlerberghe 2005; Vidal et al. 2007).

In the present study, the following three specific questions were addressed: (i) Is there any respiratory cost imposed by AM inoculation detrimental for plant growth at early stages of colonization? (ii) Is there any ATP retribution from AM fungi significant for growth at mature stages of colonization under P limitation? and (iii) does salinity lead inoculated and non-inoculated plants to produce the same amount of ATP at mature stage of colonization?

To test these hypotheses, *N.tabacum* plants were inoculated with *Rhizophagus intraradices,* a model AM fungi species for salinity experiments (Estrada et al. 2013; Aroca et al. 2013). Plants were grown during 49 days, leaves respiration was measured using the oxygen-isotope-fractionation technique in different stages of plant growth; (i) in plantlets prior the inoculation (day 14), (ii) at day 28 in AM and NM plants prior salt addition (150 mM), and (iii) at day 49 in salt and non-salt treated AM and NM plants.

MATERIAL AND METHODS

Plant and fungal material

Seeds of *N. tabacum* L. cv Petit Havanna were planted in trays of agricultural substrate containing autoclaved nutrient-rich black peat (KEKKILÄ DSM 1 W ©). At day 14, tobacco plantlets (n=4) were harvested for foliar respiratory measurements and shoot biomass analysis. Moreover, on this day, tobacco plantlets of a similar size were taken away from the trays and peat free roots were inoculated with 3 mL of commercial monoxenic inoculum of *Rhizophagus intraradices* containing 50 000 propagules mL^{-1} (MYCOVITRO S.L., Granada, Spain). Immediately after the inoculation (on the same day), peat free non-AM (NM, obtained by non-inoculation) and AM plants were transplanted to pots containing sand that was previously sterilized once per day during three consecutive days. A second inoculation (3 mL) was applied seven days later to ensure the infection according to the manufacturer's recommendation.

Following the first inoculation (at day 14), plants were grown during others 35 days in a growth chamber under controlled conditions of 27/20 ºC day/night temperature, above 50 % relative humidity and 12/12 h light/dark, and 800 µmol m^{-2} s^{-1} of photosynthetic photon flux density. Plants were grown under P-limiting conditions, irrigated two times a week with 0.5 L of a Hoagland nutrient solution (Epstein 1972) modified to contain 0.025 mM NH_4HPO_4, and 0.225 mM NH_4Cl to supply the total requirements of NH_4^+, similar to Del-Saz et al. (2017).

Four weeks NM and AM old plants were harvested to respiratory measurements and biomass analysis (n=4). Simultaneously, from the same plants used for respiration and biomass, some lateral roots were used for analysis of AM fungus colonization. Moreover, on this day (28), NM and AM plants were treated with NaCl (n=4). The treatment was applied on progressively by supplementing 50, 100, and 150 mM NaCl to Hoagland solution on days 28, 30 and 33. Plants were watered with 150 mM NaCl from day 35 to 49. Finally, seven weeks NM and AM old plants treated and non-treated with salt were harvested for respiration, biomass analysis, AM fungus colonization and phosphorus analysis (n=4).

AM fungus root length colonization

The root sample (approximately 0.200 g FW) was thoroughly washed and cleared in 10% (w/v) KOH, and stained with 0.05% (v/v) trypan blue in lactic acid, according to Phillips and Hayman (1970). AM fungus root length colonization was assessed using the magnified intersections method (Abbott et al. 1984), where the frequency of colonization represents the

ratio between the fragments of colonized root and the total number of root fragments examined.

Biomass measurements and phosphorus analyses

Tobacco plants were harvested on days 14, 28, and 49. Shoots were partitioned into stems and leaves. Dry weights (DW) of shoot components were determined after drying for 48 h at 70 °C. In addition, relative growth rate at t_1 (from days 14 to 28) and t_2 (from days 28 to 49) in shoots (leaves plus stems) was calculated as described in Poorter (1989).

Dried leaves samples of seven weeks NM and AM old plants treated and non-treated with salt were ground into a fine powder with a mixer mill MM 200 (Restsch®, Haan, Germany). Leaf [P] was determined by ICP/OES (Varian 720-ES ICP Optical Emission Spectrometer, Münster, Germany).

Respiration and oxygen-isotope fractionation measurements

Leaves were incubated in the dark for 30 min to avoid light-enhanced dark respiration. Later, leaves disks were placed in a 3 mL stainless-steel closed cuvette maintained at a constant temperature of 25 ºC and measured as described in Del-Saz et al. (2016). For Δ_a measurements, leaves were submerged in a solution of 10 mM KCN for 30 min. A value of Δa of 30.2±0.1‰ (n=3) was obtained. On the other hand, an assumed value of 19.8‰ for the Δ_c was used for the electron partitioning calculations as this has been shown to be fairly constant in leaves and species examined up to date (Ribas-Carbo et al. 2005). Changes in the $^{18}O/^{16}O$ ratios and oxygen concentration were obtained to calculate the oxygen-isotope fractionation and respiration rates of COX and AOX as described in Ribas-Carbo et al. (2005).

Mitochondrial ATP production was modelled from the activities of the COP and AOP of each plant, assuming that electron flow through the AOP drives some synthesis of ATP via complex I contributing to proton extrusion. It is considered that 11/6 ATP are formed for each O_2 consumed by the AOP and 29/6 ATP through the COP (Amthor 1994; Gonzalez-Meler et al. 2001; Vidal et al. 2007).

Statistical analyses

Two experiments separated in time were carried out with different groups of plants grown under similar conditions. Data of stem biomass, total respiration (V_t) and ATP production were log-transformed in order to assume normality an homoscedasticity. One-way ANOVA

was performed to compare responses variables between NM and AM plants at day 28, and to compare between plants of the same treatment between different days (14 against 28, 28 against 49) in NM and AM plants. Two-way ANOVA, with salinity level and AM colonization as factors was performed at day 49. The post-hoc t-test was used when statistically significant differences (P-value < 0.05) were obtained. Statistical analyses were performed using the JMP®, Version 12.1.0 (SAS Institute Inc., Cary, NC, USA, 1989-2007).

RESULTS

Arbuscular mycorrhizal fungus root length colonization

The AM fungus colonization of *N. tabacum* roots was examined in inoculated plants at days 28 and 49. Colonization frequency was 23±4.4% (at day 28), and significantly higher ($p<0.05$; one way ANOVA) at day 49; 40±1.4% (with no salt addition), and 43±3.1% (with salt addition). Non-inoculated plants were examined to confirm the absence of AM fungus colonization at days 28 and 49.

Dry biomass and leaf phosphorus content

At day 28, shoot biomass of both inoculated and non-inoculated plants was significantly higher than at day 14 (Table 1). AM inoculation significantly ($p<0.05$; one way ANOVA) decreased shoot biomass by 19%, due to a lower leaf and stem biomass, by 34% and 51%, respectively (Table 1). Similarly, AM inoculation significantly ($p<0.05$; one way ANOVA) decreased RGR by 12%.

At day 49, biomass and RGR of shoots of both inoculated and non-inoculated plants was significantly higher than at day 28 (Table 1). Salt level significantly affected leaf ($p=0.044$; two-way ANOVA), stem ($p<0.0001$; two-way ANOVA) and shoot ($p<0.0001$; two-way ANOVA) dry biomass, together with RGR ($p<0.0001$; two-way ANOVA) and P concentration ($p=0.0045$; two-way ANOVA), whereas AM fungus colonization affected shoot dry biomass ($p=0.0031$; two-way ANOVA) and its RGR ($p<0.0001$; two-way ANOVA). In general, the effect of AM fungus colonization depended on salt level, but there was significant interaction between the two factors on stem ($p=0.0460$; two-way ANOVA), shoot biomass ($p=0.0028$; two-way ANOVA) and shoot RGR ($p=0.0039$; two-way ANOVA) (Table 1).

At day 49 with no salt addition, AM fungus colonization significantly increased shoot RGR by 34%, and shoot biomass by 19%, due to an increased leaf and stem biomass by 15% and 34%. With salt addition, AM fungus colonization did not affect any biomass parameter because salinity significantly decreased shoot biomass in both NM and AM plants, by 21%

and 33%, respectively, although RGR was increased by 27% in AM shoots. Stem biomass were decreased by 44% in NM plants, while leaf and stem biomass were decreased by 21% and 44% in AM plants under salinity (Table 1).

AM fungus colonization significantly increased leaf P concentration by 65% only with no salt addition, because salinity significantly increased leaf P concentration by 108% in NM plants, reaching similar values than that were observed in AM plants (Table 1).

Table 1. Effect of arbuscular mycorrhizal (AM) fungus colonization on shoot dry weight (DW) [determined by leaves and stem dry weight], relative shoot growth rates (RGR) and foliar phosphorus (P) concentration of tobacco plants at different growth stages (days 14, 28 and 49) and salt conditions from days 28 to 49. Values are means ± SE for 4 biological replicates. Different letters denote statistical significant differences between NM and AM plants at day 28 ($p < 0.05$ one-way ANOVA) and at day 49 ($p < 0.05$ post-hoc t-test). Asterisks denote significant biomass increments ($p < 0.05$ one-way ANOVA) between days 14 and 28, and between days 28 and 49 in NM and AM plants, separately.

		Leaf DW (g)	**Stem DW (g)**	**Shoot DW (g)**	**Shoot RGR ($mg\ g^{-1}\ d^{-1}$)**	**P concentration (mg/g)**
day 14	**Non-inoculated**	0.017±0.001	0.066±0.006	0.088±0.009	-	-
day 28	**NM**	2.85±0.311A*	0.356±0.087A*	3.21±0.398A*	257±9.04A	-
	AM	1.88±0.120B*	0.175±0.036B*	2.06±0.132B*	226±4.53B	-
day 49	**NM (0 mM NaCl)**	13.6±1.05ab*	4.98±0.81b*	18.7±0.606b*	84.9±1.52c*	0.571±0.031b
	AM (0 mM NaCl)	15.6±0.934a*	6.65±0.352a*	22.3±0.678a*	114±1.41a*	0.940±0.122a
	NM (150 mM NaCl)	11.9±0.271b*	2.8±0.147c*	14.7±0.358c*	73.6±1.15d*	1.19±0.102a
	AM(150 mM NaCl)	12.3±0.347b*	2.38±0.340c*	14.8±0.063c*	93.9±0.409b*	1.100±0.158a

Leaf respiration and electron partitioning to the AOX pathway

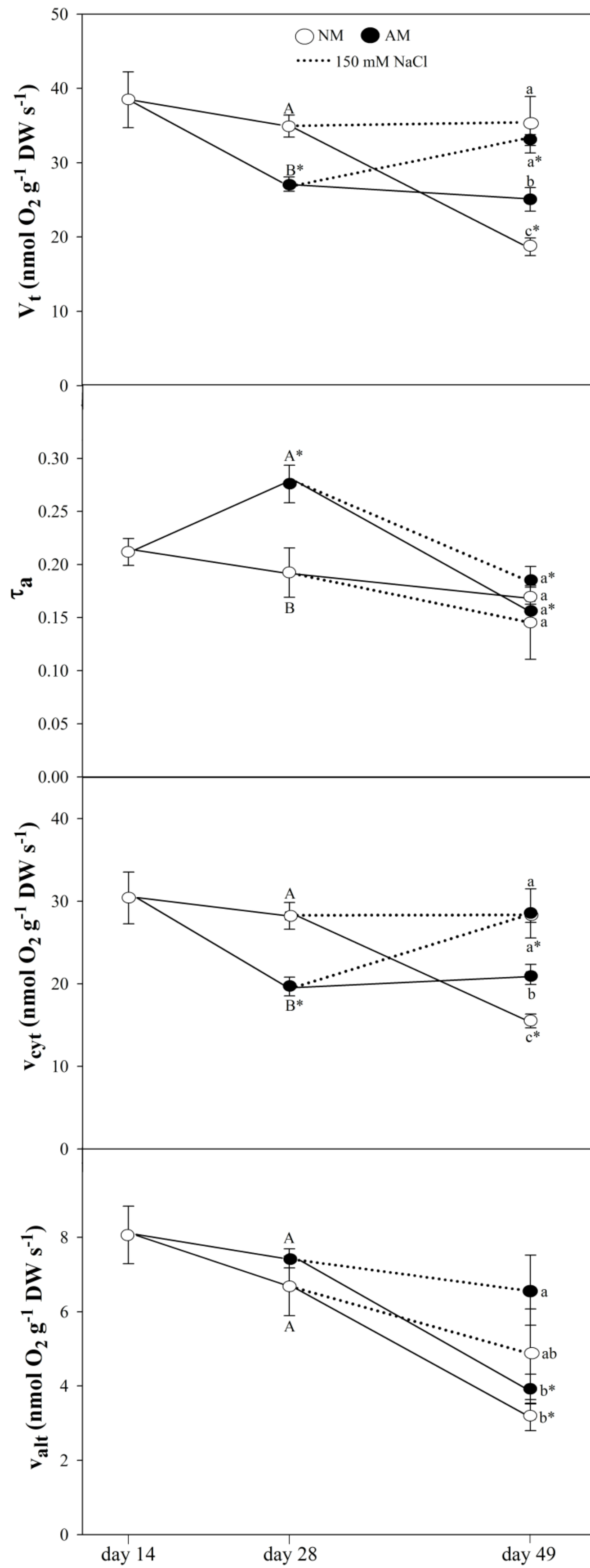
At day 28, AM inoculation significantly decreased total respiration (V_t) and the *in vivo* activity of COX (v_{cyt}) by 22% and 30%, respectively, also increasing significantly the electron partitioning to the AOP (τ_a) by 47%. The *in vivo* activity of AOX (v_{alt}) was not affected by AM inoculation (Fig. 1).

At day 49, in NM plants, V_t and v_{cyt} were significantly lower by 46% and 45% than at day 28 with no salt addition. τ_a was significantly lower in AM plants relative to 28 d-old plants with and without salt addition, and v_{alt} was significantly lower in both NM and AM plants without salt addition by 53% and 47%, respectively (Fig. 1). Salt level significantly affected V_t ($p<0.0001$; two-way ANOVA), v_{cyt} ($p<0.0001$; two-way ANOVA) and v_{alt} ($p=0.0208$; two-way ANOVA) whereas AM fungus colonization only affected V_t ($p=0.0377$; two-way ANOVA). There was no significant interaction between the two factors for any respiratory parameter (Fig. 1).

At day 49 with no salt addition, AM fungus colonization significantly increased V_t and v_{cyt} by 34% and 36%, respectively (Fig. 1). Under salt addition, none respiratory parameter was affected by AM fungus colonization because salinity increased V_t and v_{cyt} by 77% and 82% in NM plants, and by 40% and 35% in AM plants. Moreover, salinity significantly increased v_{alt} in AM plants by 68% (Fig. 1).

At day 28, AM inoculation significantly decreased ATP production by 27%. At day 49, in NM plants, ATP production was significantly lower by 46% than at day 28 with no salt addition (Fig. 2). ATP production was significantly affected by both salt level ($p<0.0001$; two-way ANOVA) and AM fungus colonization ($p=0.0395$; two-way ANOVA). At day 49 with no salt addition, AM fungus colonization significantly increased ATP production by 35%. Under salt addition, ATP production was not affected by AM fungus colonization because salinity increased ATP production by 80% and 37% in NM and AM plants, respectively (Fig. 2).

Figure 1. Effect of arbuscular mycorrhizal (AM) fungus colonization on total respiration (V_t), cytochrome pathway activity (v_{cyt}), electron partitioning to the AOP (τ_a) and alternative pathway activity (v_{alt}) in tobacco leaves at different growth stages (days 14, 28 and 49) and salt conditions from days 28 to 49. Values are means ± SE for 4 biological replicates. Different capital letters denote significant differences at day 28 ($p< 0.05$ one-way ANOVA) and small letters denote significant differences at day 49 ($p< 0.05$ post-hoc t-test). Asterisks denote differences between days 14 and 28, and between days 28 and 49 in NM and AM plants, separately ($p< 0.05$ one-way ANOVA).



NM
AM
150 mM NaCl
V_t (nmol O_2 g^{-1} DW s^{-1})
τ_a
v_{cyt} (nmol O_2 g^{-1} DW s^{-1})
v_{alt} (nmol O_2 g^{-1} DW s^{-1})
day 14
day 28
day 49

DISCUSSION

The present research is based on the hypothesis that both plant growth and maintenance of accumulated biomass are related to the capacity of leaf respiration to produce ATP. This capacity may be reduced when the contribution of AOP to overall respiration increases, especially under abiotic stresses such as P limitation and salinity (Gonzalez- Meler et al. 2001; Martí et al. 2011; Del-Saz et al. 2016). Under stress conditions, respiration may produce high amount of ATP to be invested in the maintenance of the biomass accumulated (Lambers et al. 2002). Symbiosis with AM fungi may increase the proportion of ATP produced to accumulate new biomass under P-limiting and saline conditions (Evelin et al. 2009; Ruiz-Lozano et al. 2012). On the other hand, it is recognized that AM symbiosis affects plant biomass accumulation distinctly at different stages of plant growth, with growth depressions often occurring at early stages, and the growth recovering occurring at mature stages (Smith et al. 2009). At early stages, the construction of the fungal structures may impose an ATP cost significant for plant growth by decreasing the availability of respiratory substrate (Smith et al. 2009). On the other hand, in mature associations, AM symbiosis may benefit ATP production if the symbiosis association is positive (Evelin et al. 2009). To evaluate the effects of AM fungus colonization on ATP production, accurate estimations of ATP production can be obtained measuring *in vivo* the activity of AOX (Vidal et al. 2007; Florez-Sarasa et al. 2007; Galle et al. 2010). Here, we tested the effects of AM symbiosis with *N. tabacum* on leaves respiration and ATP production, together biomass accumulation at different stages of plant growth (days 14, 28, and 49) and salt conditions (from day 28 to 49) in P limiting soils.

We observed in inoculated plants an incompletely formed AM symbiosis at day 28 (compared to day 49), together with lower RGR in shoots in agreement with Smith et al. (2009), also coinciding with lower rates of respiration (as compared to non-inoculated plants). At day 49, AM fungus colonization was increased. Moreover, both RGR and leaf respiration were higher in inoculated plants as compared to NM, with no salt addition. On the other hand, salinity decreased similarly shoot growth and induced both NM and AM leaves to respire the same, although AM fungus colonization allowed shoots to grow faster (as compared to early stages). At mature stages of plant growth (day 49), AM fungus colonization increased respiration via COP (Fig. 1). Therefore, the synthesis of ATP was also higher (Fig. 2), coinciding with higher biomass accumulation as compared to NM plants (Table 1). In NM plants, the decreased ATP production was related to a slower respiratory rate via COP, also coinciding with decreasing P concentration (relative to AM plants). Phosphorus limitation may restrict the overall respiratory rate in leaves through COP due to an increase in adenylate control (Theodorou and

Plaxton 1993; Gonzalez-Meler et al. 2001). This restriction would result in a reduction of oxidative phosphorylation leaving less ATP to be invested in biomass accumulation. It is recognized that restriction of the COP by P limitation can result in over-reduction of the UQ-pool. In this situation, the AOP can increase acting as a bypass for the COP accepting electrons from the UQ (Plaxton and Carswell 1999; Gonzalez-Meler et al. 2001; Lambers et al. 2005). Nevertheless, in our study, AOP did not contribute to sustain the respiratory rate in NM plants as previously was observed in *N. tababum* by Gonzalez-Meler et al. (2001), who concluded that such phenomenon is species dependent. Contrary to what observed in NM plants, AM fungus colonization improved P accumulation thus avoiding COP restriction and favoring both ATP production and shoot growth.

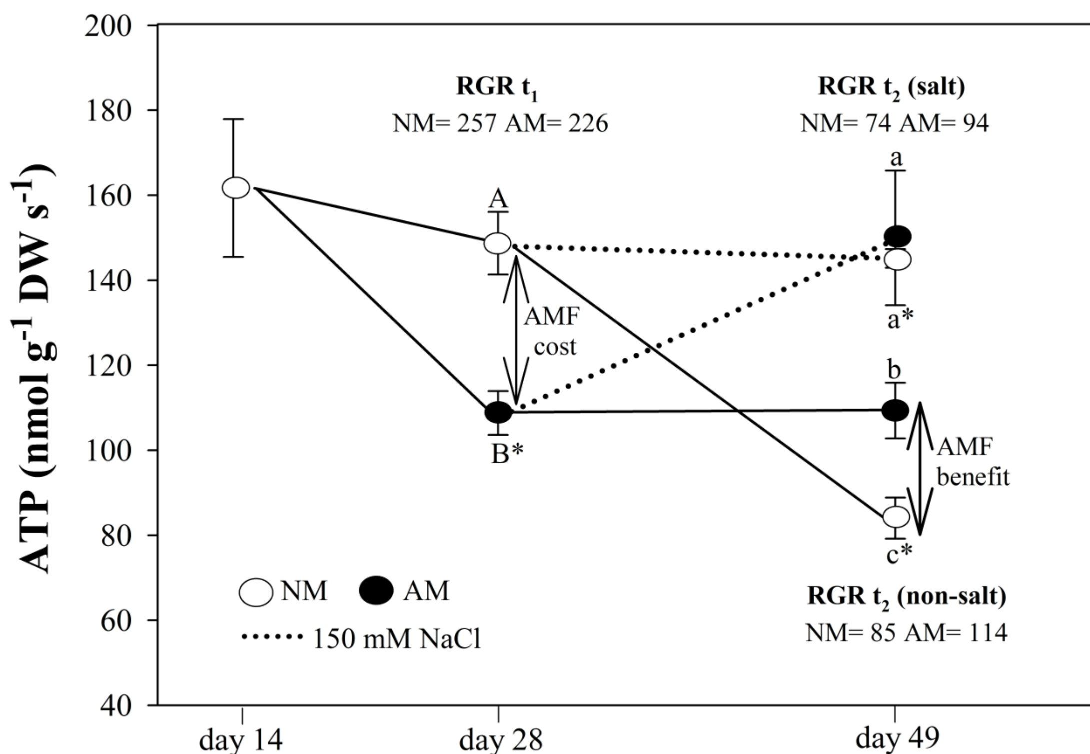
The influence of salinity on P accumulation and AM fungus colonization is variable and depends on the plant species and experimental conditions (Grattan and Grieve 1992; Ouziad et al. 2006; Porcel et al. 2012). Salinity may enhance P uptake (Grattan and Maas 1985; Roberts et al. 1984; Grattan and Grieve 1992; Sharpley et al. 1992) as was observed in NM leaves. In fact, most studies that observed that salinity increased P accumulation under P deficiency were conducted in sand (Grattan and Grieve 1992) as in our study. Besides, the accumulation of P in NM leaves under salinity reached similar levels than that were observed in AM plants. That would avoid any restriction of COP as we observed. In AM plants, salinity did not affect P accumulation, although the existing accumulation of P allowed both V_t and v_{cyt} to increase. Increased rates of respiration and ATP production under salinity are considered a distinctive feature of salt response (Jacoby et al. 2011; Del-Saz et al. 2016) to satisfy maintenance purposes such as to counteract the toxic effects of NaCl and to maintain the protein turnover (Jacoby et al. 2011). That means that less ATP would be available to be invested in the construction of new plant constituents in both NM and AM plants as we observed.

In general, the contribution of the AOP to overall respiration decreased at mature stages of growth and thus, increasing the energetic efficiency of respiration. Moreover, previous studies suggested that decreasing rates of AOP may be related to a better carbon use efficiency during stress (Noguchi et al. 2001; Gomez-Casanovas et al. 2007). In our study, salinity increased the activity of AOP only in AM plants, also coinciding with increased rates of respiration via COP. This increase of respiration may be associated to a higher electron flow in the mETC, which could led to an over-reduction of the UQ pool. To avoid this, AOX accepts electrons from the UQ helping COP to display fast rates as was observed in AM plants. Such a phenomenon was also observed under stress conditions in leaves of *Nicotiana sylvestris*

(Vidal et al. 2007), *Agrostis scabra* (Rachmilevitch et al. 2007) and *Beta vulgaris* (Sagardoy et al. 2009).

The growth component of respiration is clearly more important than that maintenance at early stages of growth (at day 28), as both NM and AM plants showed similar RGR to that fast growing species (Millenaar et al. 2001). At mature stages (day 49), maintenance became more important than growth considering the slow RGR observed in both NM and AM shoots. Inoculation at early stages (day 28) seems to decrease the growth component of respiration as both ATP production and biomass accumulation were slower (Fig. 2). This could be due to an uncomplete AM fungus colonization which construction requires plant carbon (Smith et al. 2009). In this sense, the ATP not synthesized in leaves of inoculated plants, that could have been invested in growth (as was observed in non-inoculated plants), represents the ATP cost of the symbiosis at early stages of colonization (Fig. 2). At mature stages (day 49), RGR in both AM and NM is similar to that slow growing species (Millenaar et al. 2001), although slightly slower in NM plants (compared to AM). In these plants, growth component of respiration seems to be reduced because ATP production was decreased as well. On the other hand, AM fungus colonization induced both ATP production and shoot growth (as compared to NM plants). The increased rate of ATP production that is invested in a faster growth in AM plants (relative to NM plants) represents the ATP benefit of the AM fungus colonization at mature stages of colonization (Fig. 2). On the other hand, salinity seems to increase the ATP demand for maintenance in both NM and AM plants as shoot RGR slowed down and ATP synthesis increased. Nonetheless, it seems that the proportion of ATP invested in growth was slightly higher in AM plants as they displayed faster RGR.

Figure 2. Modeling of foliar ATP production in NM and AM plants at different growth stages (days 14, 28 and 49) and salt conditions from days 28 to 49. At day 49, AM fungal (AMF) symbiosis imposes an cost of 40 nmol ATP g^{-1} DW s^{-1} when shoots show fast relative growth rates (RGR, mg g^{-1} d^{-1}), whilst AMF symbiosis represents a benefit of 25 nmol ATP g^{-1} DW s^{-1} when shoots show slow RGR with no salt addition. Values are means ± SE for 4 biological replicates. Different capital letters denote significant differences at day 28 ($p < 0.05$ one-way ANOVA) and small letters denote significant differences at day 49 ($p < 0.05$ post-hoc t-test). Asterisks denote differences between days 14 and 28, and between days 28 and 49 in NM and AM plants, separately ($p < 0.05$ one-way ANOVA).



In summary, we provide evidences that symbiosis with *Rhizophagus intrarradices* affects both respiration and ATP synthesis differently at different growth stages when plants grow in P deficient and saline soils. Symbiosis represented an ATP cost for the tobacco leaves detrimental for shoot growth at early stages. Later, this cost turned into an ATP benefit, which allowed a faster growth. Moreover, we observed with salt addition that AOP contributed, together with COP, in the acclimation of leaf respiration to adjust ATP production to the same level than that non-inoculated plants.

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CHAPTER 4.

Arbuscular mycorrhizal fungus colonization in *Nicotiana tabacum* decreases the rate of both carboxylate exudation and root respiration and increases plant growth under phosphorus limitation

Arbuscular mycorrhizal fungus colonization in *Nicotiana tabacum* decreases the rate of both carboxylate exudation and root respiration and increases plant growth under phosphorus limitation

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ABSTRACT

Background and aims Under phosphorus (P) limitation, plants tend to maximize their efficiency of P acquisition by increasing the exudation of root carboxylates, such as citrate, whose synthesis is mediated by respiration via the alternative oxidase (AOX) in cluster roots. However, high respiratory costs related to nutrient uptake are associated with slower plant growth, whereas arbuscular mycorrhizal (AM) fungus colonization increases plant growth and decreases the exudation of citrate and malate. Thus, the present research is based on the hypothesis that AM fungus colonization will decrease root respiration via the alternative pathway and the amount of carboxylates in the rhizosphere, and increase plant growth.

Methods We used the oxygen-isotope-fractionation technique to study the *in vivo* respiratory activities of the cytochrome and the alternative oxidase pathways (COP and AOP) in AM and nonmycorrhizal (NM) tobacco plants grown under P-sufficient (0.25 mM) and P-limiting (0.025 mM) conditions in sand. The amount of root exudates in the rhizosphere, total biomass and root P content were determined.

Results Under sufficient P, the amount of citrate and malate was higher in NM plants, while no differences were found in respiration between NM and AM plants. On the other hand, low P increases the exudation of citrate and respiration via AOP in NM plants, while it does not affect the amount of carboxylates, nor the respiratory rate in AM plants. Biomass production was reduced only in NM plants under low P.

Conclusions Our results highlight that AM fungus colonization decreases the rate of root respiration and the exudation of citrate and malate, whilst increasing plant growth. Conversely, respiration, via AOX, is associated with the exudation of citrate and less biomass production in NM roots.

INTRODUCTION

We live in an era in which global rock phosphate reserves are being depleted, with major repercussions for future crop production (Marschner 2012; Lambers and Plaxton 2015). In the past few years, there is an increasing awareness regarding the efficiency of the use of P reserves to produce crops in order to maintain or increase the current agricultural productivity (Cordell et al. 2009; Gilbert 2009).

Arbuscular mycorrhizal fungi (AMF) can enhance plant P acquisition and growth (Siddiqui and Pichtel 2008; Smith and Read 2008). The underlying mechanism of arbuscular mycorrhizal (AM) plants to efficiently acquire P from soils resides in their external AM hyphae, which extend from the root surface to the soil beyond the root P-depletion zone and have access to a greater volume of soil than the roots and root hairs alone (Smith and Read 2008). Moreover, the advantages of increased P uptake and growth, compared with that of nonmycorrhizal (NM) plants, increase with decreasing soil P availability (Nagy et al. 2009). However, the effect of AM on plant growth is variable and depends on the host plant and the fungal species (Bryla and Eissenstat 2005).

An important physiological process determining plant growth is respiration, especially in roots; root respiration increases under low P availability in NM plants (Baas et al. 1989; Nielsen et al. 1998). Moreover, Nielsen et al. (2001) observed in NM common bean plants faster root respiratory rates and less biomass production than AM plants. When plants are P limited to an extent that growth is reduced, respiratory costs of growth and nutrient uptake increase (Lambers et al. 2008; Nielsen et al. 2001). Under P limitation, NM plants increase the exudation by roots of low-molecular weight carboxylates such as citrate and malate (Veneklaas et al. 2003; Pearse et al. 2007; Suriyagoda et al. 2012), whose synthesis requires faster respiratory rates (Florez-Sarasa et al. 2014). These compounds contribute to the solubilization and acquisition of mineral P from the soil (Lambers et al. 2006, 2015). Interestingly, AM fungus colonization decreases root exudation under P limitation (Ryan et al. 2012; Nazeri et al. 2013).

In the mitochondrial electron transport chain, respiration mostly proceeds via the cytochrome oxidase pathway (COP), coupled to adenosine triphosphate (ATP) synthesis; however, under conditions where COP activity is restricted (i.e. by low ATP demand or P availability), the alternative oxidase pathway (AOP) may allow the oxidation of NADH and continuation of the tricarboxylic acid cycle (TCA) reactions to support various biosynthetic reactions such as the synthesis of carboxylic acids (Shane et al. 2004; Florez-Sarasa et al. 2014). In *Lupinus albus,* respiration through the AOP is increased *in vivo* in cluster roots, which is an adaptation to

severe P limitation. This coincides with an increased concentration of malate and citrate in cluster roots (Florez-Sarasa et al. 2014).

There is much less precise information regarding root respiration in AM plants. Whereas Silsbury et al. (1983), Rewald et al. (2015) and Otgonsuren et al. (2016) showed that colonization by AMF can decrease the rate of root respiration, there are several other studies that show the opposite (Pang and Paul 1980; Peng et al. 1993; Nielsen et al. 1998; Hughes et al. 2008). In addition to the specificity of the plant-fungus interaction, other factors such as the stage of AM fungus colonization might explain these contrasting results, as during early stages the respiratory costs are high as most of the new fugal tissue is being produced and a large amount of ATP is needed, but the costs decrease as the association matures (Bryla and Eissenstat 2005).

The effect of AM fungus colonization on alternative oxidase (AOX) activity is unknown, and there is only one study focused on AOX protein abundance, which found in cold acclimated *Plantago lanceolata* plants that protein is less abundant in AM roots relative to NM roots (Atkin et al. 2009). Moreover, there is no clear correlation between AOX protein abundance and AOX activity *in vivo* (Ribas-Carbó et al. 2005; Florez-Sarasa et al. 2011, 2014; Del-Saz et al. 2016). Consequently, studies about the effect of AM fungus colonization on the *in vivo* root COP and AOP activities are needed. They can be measured by mass spectrometry studying the oxygen-isotope fractionation to determine the *in vivo* activities of the two mitochondrial terminal oxidases, cytochrome oxidase (COX) and AOX (Day et al. 1996; Ribas-Carbó et al. 2005). We hypothesize that AM fungus colonization will decrease both alternative root respiration and the amount of carboxylates in the rhizosphere. We also hypothesize that this respiratory response will be associated with greater biomass production in AM plants.

In contrast to *Lupinus albus*, *Nicotiana tabacum* does not produce cluster roots (Lambers et al. 2006), although exudes organic acids for P uptake (Lü et al. 2011). Moreover, their symbiotic interaction with the fungus *Rhizophagus intraradices* is well studied (Shaul-Keinan et al. 2002; Porcel et al. 2005). Thus, the present study has been conducted using AM and NM tobacco plants inoculated or not inoculated with *Rhizophagus intraradices* and grown at a limiting or abundant P supply. *In vivo* root respiration was measured using the oxygen-isotope-fractionation technique. In addition, the amount and composition of root exudates in the rhizosphere were determined, as was root P content and total biomass. Altogether, the rate of root respiration in AM plants is expected to be slower than that in NM plants, especially

due to the important role of respiration through the AOP in the synthesis of carboxylic acids in NM plants.

MATERIAL AND METHODS

Plant and fungal material

Seeds of *Nicotiana tabacum* L. cv Petit Havanna plants were planted (one day per week during six consecutive weeks) in trays of agricultural substrate containing autoclaved nutrient-rich black peat (KEKKILÄ DSM 1 W ©). Two-week old plants were taken away from the trays and peat free roots were inoculated with 3 mL of commercial monoxenic inoculum of *Rhizophagus intraradices* containing 50,000 propagules mL^{-1} (MYCOVITRO S.L., Granada, Spain). The medium used to obtain the inoculum was the M medium described in Bécard and Fortin (1988). Immediately after the inoculation (on the same day), peat free NM (obtained by non-inoculation) and AM plants were transplanted to pots (2 L volume) containing approximately 4 Kg of sand (grain size distribution ≥ 0.4 mm and < 0.8 mm) that was previously sterilized by tyndallization. A second inoculation (3 mL) was applied seven days later to ensure the infection according the manufacturer's recommendation.

Following the first inoculation, plants were grown during 55 days in a growth chamber under controlled conditions of 25/20 ºC day/night temperature, above 40% relative humidity and 12 h photoperiod, and 350 µmol m^{-2} s^{-1} of photosynthetic photon flux density. Plants were irrigated three times a week with 0.5 L of a Hoagland nutrient solution (Epstein 1972) modified to contain one quarter strength with P supplied at 0.25 mM $(NH_4)_2HPO4$ (P-sufficient solution) or 0.025 mM (P-limiting solution) similar to previous studies (Israel 1987; Xing and Wu 2014). In order to supply the total requirements of NH_4^+, 0.225 mM NH_4Cl was added to the P-limiting solution.

There was one harvest day (in which one biological replicate per treatment was obtained) per week from 10 am to 18 pm (during six consecutive weeks alternating the order of harvest). Plants were harvested every two hours and used for all the different analysis described below. First, plants were carefully remove from the pots. Root systems from intact plants were firmly shaken to remove excess sand, with special attention to collect the roots that could separate from the plant. Simultaneously, from the same plant, some lateral roots were used for analysis of mycorrhizal colonization, others for respiratory measurements and others were used to sampling the carboxylates of the rhizosphere. Finally, the remaining plant material was

collected for biomass analysis. Phosphorus analysis was performed in the same plants used for biomass analysis, respiration and rhizosphere measurements.

Mycorrhizal colonization

The root sample (approximately 0.150 g FW) was thoroughly washed and cleared in 10% (w/v) KOH, and stained with 0.05% (v/v) trypan blue in lactic acid, according to Phillips and Hayman (1970). Arbuscular mycorrhizal fungal colonization was assessed using the magnified intersections method (Abbott et al. 1984), where the frequency of colonization represents the ratio between the fragments of colonized root and the total number of root fragments examined. An average of 300 root pieces per plant were examined. The colonization of *N. tabacum* roots was examined in six AM plants under P-sufficient or P-limiting conditions. Colonization frequency was 49±9.7% (P-sufficient) and 52±14% (P-limited), with no significant differences ($p>0.05$). Non-inoculated plants were examined to confirm the absence of AM colonization.

Rhizosphere measurements

NM and AM roots were harvested after 55 days of growth under P-sufficient and P-limiting conditions to analyze the amount of carboxylates in the rhizosphere. In order to do that, root systems were firmly shaken to remove excess sand, with the sand that remained attached to the roots defined as rhizosphere soil. The roots (1.09±0.162 g DW) and attached sand (80.6±4.99 g DW) were transferred to a beaker containing a measured volume (43.2±1.28 mL) of 0.2 mM $CaCl_2$ to allow repeated immersion of the root system during 15 minutes; the use of $CaCl_2$ ensured cell-membrane integrity was maintained. A 1 mL subsample of the rhizosphere extract was filtered through a 13-mm syringe filter with 0.2 µm nylon membrane into a high-performance liquid chromatography (HPLC) vial. Carboxylate concentrations in the extracts were determined by HPLC according to Cawthray (2003). Values presented are the mean ± SE of four biological replicates.

Biomass measurements and phosphorus analyses

Six NM and AM plants were harvested after 55 days of growth under P-sufficient and P-limiting conditions. Shoots were partitioned into stems, green leaves and senesced leaves. Dry weight (DW) of shoot components and remaining roots were determined after drying for 48 h at 70 °C.

Dried root samples were ground into a fine powder with a mixer mill MM 200 (Restsch®, Haan, Germany). Root [P] was determined by ICP/OES (Varian 720-ES ICP Optical Emission Spectrometer, Münster, Germany). Total root P content per plant was calculated by multiplying the tissue [P] and the tissue dry weight per plant.

Respiration and oxygen-isotope fractionation measurements

For respiratory measurements, roots (approximately 0.350 g FW) were harvested and immediately carefully rinsed using a soft water jet. Roots then were left to air dry for 15 minutes before being placed in a 3 mL stainless-steel closed cuvette maintained at a constant temperature of 25 ºC (Gastón et al. 2003). The respiration cuvette was equipped with two inlets: one connected to the mass spectrometer (Delta XPlus, Thermo LCC, Bremen, Germany), and the other connected to a 2-mL air-tight syringe (Cromlab S.L., Barcelona, Spain). Throughout the experiment the syringe is used to both mix the air (every 10 minutes) in the cuvette and to maintain the cuvette at constant pressure. Air samples of 300 µL were sequentially (every 20 minutes six times) withdrawn from the cuvette and fed into the mass spectrometer. Changes in the $^{18}O/^{16}O$ ratios and oxygen concentration were obtained to calculate the oxygen-isotope fractionation and respiration rates as described in Ribas-Carbó et al. (2005). The electron partitioning to the alternative pathway (τ_a) was calculated as follows:

$$\tau_a = (\Delta_n - \Delta_c) / (\Delta_a - \Delta_c)$$

Where Δ_c, Δ_a are the oxygen isotope fractionation of the cytochrome (+SHAM) and alternative (+KCN) pathway, respectively and Δ_n, is the oxygen-isotope fractionation of the respiration in the absence of inhibitors. For Δ_a measurements, roots were submerged in a solution of 10 mM KCN for 30 min. A value of Δ_a of 27.1±0.2‰ (n=3) was obtained. For the calculation of Δ_c, roots were submerged in fresh solutions of 25 mM SHAM for 30 min. A value of Δ_c of 16.8±0.1‰ (n=3) was obtained.

The individual activities of the COP (v_{cyt}) and AOP (v_{alt}) were obtained by multiplying the total oxygen uptake rate (V_t) and the partitioning to each pathway as follows:

$$v_{cyt} = V_t \times (1 - \tau_a)$$

$$v_{alt} = V_t \times \tau_a$$

Values presented are the mean ± SE of four biological replicates on a dry weight basis. Dry weights from this material was added to the total root biomass.

Measurements of the AOP capacity

Four roots per treatment were weighed (approximately 0.150 g) and incubated in a solution with 10 mM KCN for 30 min. Oxygen-uptake rates were measured in darkness using a liquid-phase Clark-type oxygen electrode (Rank Brothers, Cambridge, England) at a constant temperature of 25 ºC in a solution containing 30 mM MES pH 6.2 and 0.2 mM $CaCl_2$. Fresh solutions were prepared daily. The measurements were performed in parallel to oxygen-isotope fractionation measurements.

Statistical analyses

Six experiments (one biological replicate per experiment) separated in time (one per week during six consecutive weeks) were carried out with different groups of plants grown under similar conditions. Two-way ANOVA (P level, AM colonization) with a level of significance of P-value < 0.05 was performed. The post-hoc t-test was used when statistically significant differences were obtained. No transformation of the data was needed. Statistical analyses were performed using the JMP®, Version 12.1.0 (SAS Institute Inc., Cary, NC, USA, 1989-2007).

RESULTS

Dry biomass and root phosphorus content

Phosphorus level significantly affected total, shoot and root dry biomass, and P content, whereas AM fungus colonization did not affect any of these variables (Table 1). In general, the effect of mycorrhiza depended on P level (Table 2), but there was only a significant interaction between the two factors on shoot and total biomass (Table 1).

Under P-limiting conditions, AM fungus colonization significantly increased total biomass by 33%, due to an increased shoot biomass by 28%. Under P limitation, total biomass was significantly less in NM plants (64%) due to a lower shoot and root biomass (by 66% and 55%, respectively) (Table 2).

Total root P content was not affected by AM fungus colonization, whereas under P limitation it was significantly less, by 65%, in both NM and AM plants (Table 2).

Table 1. Significance of sources of variation after two-way ANOVA for each parameter. The sources of variance were phosphorus (P) level, arbuscular mycorrhizal (AM) fungus colonization and their interactions (P level x AM colonization). *p<0.05, **p<0.01, ***p<0.001, ns not significant effect.

	P level	AM colonization	P levelx AM colonization
Citrate	***	***	**
Malate	ns	**	ns
Lactate	ns	ns	ns
Fumarate	***	ns	ns
Oxalate	ns	ns	ns
Leaves DW	**	ns	ns
Stem DW	**	ns	ns
Shoot DW	***	ns	*
Root DW	**	ns	ns
Root/Shoot	ns	ns	ns
Total DW	***	ns	*
Root P content	***	ns	ns
$\mathbf{V_t}$	ns	**	*
v_{cyt}	ns	**	ns
v_{alt}	***	*	***
$\mathbf{V_{alt}}$	*	ns	ns

Table 2. Effect of arbuscular mycorrhizal (AM) fungus colonization on total dry weight (DW) [determined by shoot (leaves and stem dry weight) and root biomass] and phosphorus (P) content of tobacco plants grown under P-sufficient (Sufficient P, 0.25 mM) or P-limiting conditions (Low P, 0.025 mM). Values are means ± SE for 4-6 biological replicates. Different letters indicate significant differences with a P-value <0.05 determined by t-test.

		Leaf DW (g)	**Stem DW (g)**	**Shoot DW (g)**	**Root DW (g)**	**Root/Shoot DW**	**Total DW (g)**	**Root P content (mg plant^{-1})**
Sufficient P	**NM**	10.5±1.7a	5.6±0.7a	16.0±1.4a	2.2±0.4ab	0.14±0.03	18.2±1.3a	4.3±0.26a
	AM	9.1±2.0a	3.9±1.2ab	13.0±2.2ab	2.7±0.7a	0.20±0.02	15.8±2.8ab	3.4±0.83a
Low P	**NM**	4.1±0.3b	1.5±0.2b	5.5±0.5c	1.0±0.1b	0.19±0.02	6.5±0.5c	0.89±0.09b
	AM	7.0±0.7ab	3.2±0.6b	10.2±0.5b	1.7±0.2ab	0.17±0.02	11.9±0.6b	1.3±0.08b

Carboxylate concentrations in the rhizosphere

We determined the concentration of malate, citrate, lactate, fumarate and oxalate in the rhizosphere of NM and AM plants grown under P-sufficient or P-limiting conditions (Table 3). The amount of fumarate and oxalate was consistently lower than that of all other carboxylates measured in the rhizosphere. The amount of lactate and oxalate was not affected by either AM fungus colonization or P limitation. There was a significant effect of both P level and AM fungus colonization and their interaction on the amount of citrate, a significant effect of AM fungus colonization on the amount of malate, and a significant effect of P level on the amount of fumarate (Table 1).

Under P-sufficient conditions, AM fungus colonization significantly decreased the amount of malate and citrate in the rhizosphere, by 50% and 74% respectively, while the amount of fumarate was unaffected by inoculation with AM fungi (Table 3).

Under P-limiting conditions, AM fungus colonization significantly decreased the amount of malate and citrate, by 50% and 72%, respectively, relative to NM plants. The amount of citrate was significantly increased, by 157%, in NM plants, relative to P-sufficient conditions, while the amount of fumarate was very small, but significantly decreased in both NM and AM plants under P-limiting conditions (Table 3).

Table 3. Effect of arbuscular mycorrhizal (AM) fungus colonization on the amount of rhizosphere carboxylates [malate, citrate, lactate, fumarate and oxalate (μmol g^{-1} root DW)] of tobacco plants grown under phosphorus (P)-sufficient (Sufficient P, 0.25 mM) or P-limiting conditions (Low P, 0.025 mM). Values are means ± SE for four biological replicates. Different letters across treatments within the same organic acid indicate significant differences with a P-value <0.05 determined by t-test.

	Sufficient P		**Low P**	
	NM	**AM**	**NM**	**AM**
Malate	47±4.2a	24±1.6bc	34±3.2ab	14.5±5.7c
Citrate	14±1.3b	3.6±0.4c	36±2.9a	9.9±4.3bc
Lactate	4.7±1.4	7.9±1.4	16.8±5.8	13.8±6.5
Fumarate	0.62±0.09a	0.54±0.02a	0.13±0.09b	0.15±0.05b
Oxalate	0.71±0.04	0.62±0.06	1.08±0.07	2.6±1.9

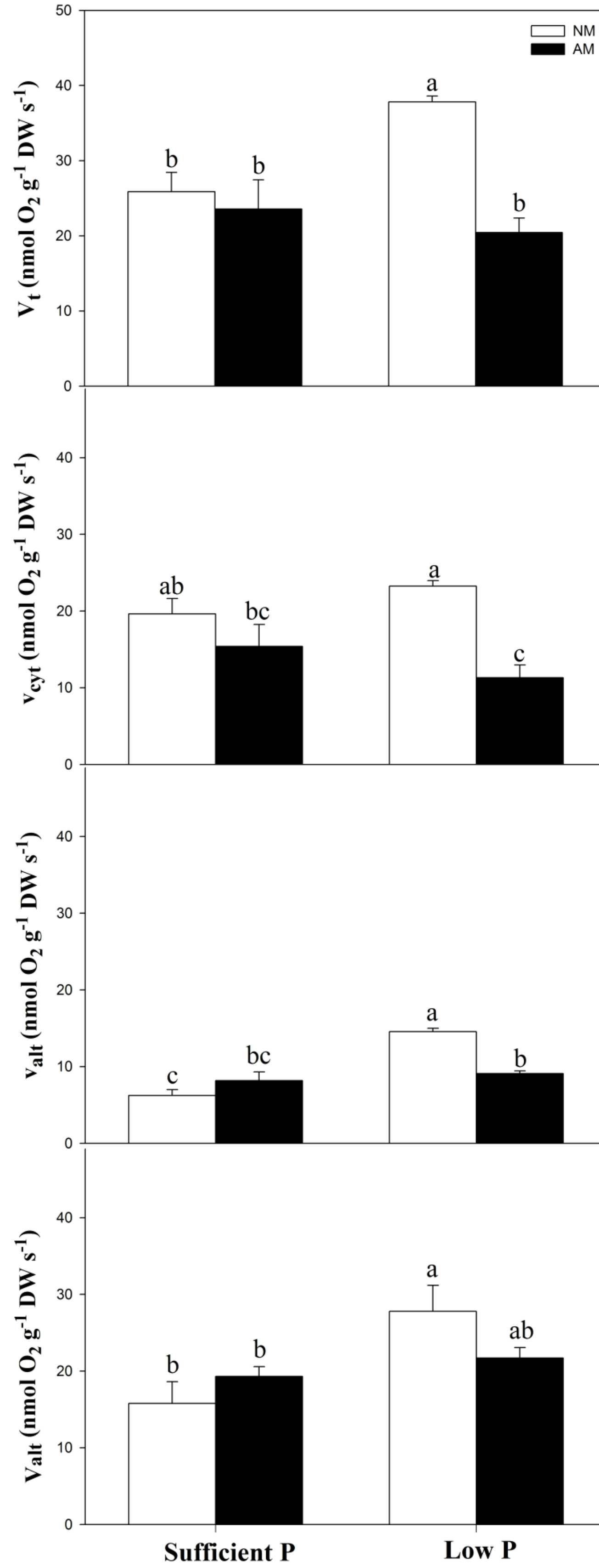
Root respiration and electron partitioning to the AOX pathway

There was a significant effect of both P level and AM fungus colonization and their interaction on v_{alt}, a significant effect of AM fungus colonization and the interaction of P level, and AM fungus colonization on V_t, a significant effect of AM fungus colonization on v_{cyt}, and a significant effect of P level on the capacity of the alternative pathway (V_{alt}) (Table 1).

Under P-sufficient conditions, total oxygen uptake (V_t), the *in vivo* activities of the cytochrome oxidase (v_{cyt}) and alternative oxidase (v_{alt}), and V_{alt} of tobacco plants were unaffected by AM fungus colonization (Fig. 1). Conversely, under P-limiting conditions, AM fungus colonization significantly decreased V_t by 46%, v_{cyt} by 51% and AOP by 37%, but V_{alt} was not significantly affected, relative to NM plants (Fig. 1).

Phosphorus limitation significantly increased V_t by 46%, AOP by 134% and V_{alt} by 76% in NM roots (Fig. 1), whereas there was no significant effect upon these parameters for mycorrhizal roots.

Figure 1. Effect of arbuscular mycorrhizal (AM) fungus colonization on total respiration (V_t), cytochrome pathway activity (v_{cyt}), alternative pathway activity (v_{alt}) and alternative pathway capacity (V_{alt}) in roots of plants grown under phosphorus (P)-sufficient or P-limiting conditions. Different letters indicate significant differences with a P-value <0.05 determined by t-test. Bars represent means ± SE of 4 replicates.


NM
AM
V_t (nmol O_2 g^{-1} DW s^{-1})
v_{cyt} (nmol O_2 g^{-1} DW s^{-1})
v_{alt} (nmol O_2 g^{-1} DW s^{-1})
V_{alt} (nmol O_2 g^{-1} DW s^{-1})
Sufficient P
Low P

DISCUSSION

Under P-limiting conditions, plants tend to maximize the efficiency of P-acquisition by increasing the exudation of root carboxylates, such as citrate and malate, which are major components of exudates released by roots (Lambers et al. 2006). Changes in internal concentrations of citrate and malate in cluster roots have previously been associated with increased partitioning of respiration via AOP, when COP activity was restricted by low ATP demand (Florez-Sarasa et al. 2014). However, high respiratory costs related to nutrient uptake may be associated with reduced plant growth when P is limiting (Nielsen et al. 2001; Lambers et al. 2008). Plant growth is often increased under AM fungus colonization (Smith et al. 2008), while the exudation of citrate and malate is reduced when plants establish a symbiosis with AM fungi (Ryan et al. 2012; Nazeri et al. 2013). Based on this, a slower respiratory rate and exudation of root carboxylates would be expected in AM plants. However, the effect of AM fungus colonization on the *in vivo* AOP and COP activities is unknown. Here, we tested the hypothesis that AM fungus colonization will decrease root respiration via AOP and the amount of carboxylates in the rhizosphere in *N. tabacum* plants.

Our results show that P-limitation decreased plant growth in NM plants, coinciding with a faster root respiratory rate in agreement with Baas et al. (1989) and Nielsen et al. (2001). The faster respiration was related to a higher alternative pathway activity in agreement with Florez-Sarasa et al. (2014), and more exudation of citrate. Citrate is generally more effective than malate or lactate in mobilizing P from soil (Veneklaas et al. 2003), and its exudation is often increased under P-limitation (López-Bucio et al. 2000; Lambers et al. 2002; Veneklaas et al. 2003; Pearse et al. 2007; Suriyagoda et al. 2012).

An adaptive response of respiratory metabolism and the mitochondrial electron transport chain to P limitation has been reported for NM roots (Theodorou et al. 1991, Rychter et al. 1992, Hoefnagel et al. 1994; Parsons et al. 1999). Any significant reduction in the concentration of adenylates or P may limit the activity of the COP, which is coupled to ATP synthesis (Rychter and Mikulska 1990; Juszczuk et al. 2001; Shane et al. 2004; Sieger et al. 2005). However, an increase of alternative respiration, which is not coupled to ATP production and responsible for cyanide-resistant respiration, could allow respiratory carbon flow avoiding any restriction caused by a limiting ADP availability. Increased AOX capacity has been observed under P limitation in several studies (Rychter et al. 1992; Parsons et al. 1999; Juszczuk et al. 2001; Sieger et al. 2005; Florez-Sarasa et al. 2014; Funayama-Noguchi et al. 2015). Moreover, citrate is a potent inducer of AOX protein synthesis (Vanlerberghe and McIntosh 1996; Gupta et al. 2012) which is consistent with increased AOX capacity in NM

roots under P-limiting conditions. The production of large amounts of citrate involves a high respiratory flux (Massonneau et al. 2001; Florez-Sarasa et al. 2014) as observed in NM roots, under conditions where ADP may limit COP activity, whilst the AOP would allow the oxidation of NADH and continuation of the TCA cycle to support the synthesis of citrate (Lambers et al. 2005; Florez-Sarasa et al. 2014).

Contrary to what we observed in NM plants, AM fungus colonization allowed sustained plant growth under P-limiting conditions, as previously observed (Fay et al. 1996; Paradi et al. 2003; Grace et al. 2008) and reduced the exudation of malate and citrate, especially under P-limitation, in agreement with previous studies (Ryan et al. 2012; Nazeri et al. 2013). Concomitantly, a slower rate of root respiration under AM fungus colonization relative to NM plants was observed at low P, as previously observed in AM plants (Silsbury et al. 1983). This reinforces a role of respiration in the exudation of carboxylates in NM roots, as suggested in previous studies (Kania et al. 2002; Shane et al. 2004; Florez-Sarasa et al. 2014). Moreover, the slower respiratory rate appears associated with a lower amount of malate and citrate in the rhizosphere which is indicative of a decreased metabolic activity in roots induced by AMF. In fact, several studies showed that AM fungus colonization affects root metabolism (Schliemann et al. 2008; Laparre et al. 2014; Rivero et al. 2015), and that AMF improve P acquisition through mechanisms other than the exudation of organics acids (Marschner and Dell 1994). In this situation, the decreased TCA cycle flux would decrease the rate of mitochondrial electron transport, mainly through COP. Moreover, the observed decrease of v_{alt} reinforces the suggested role of the alternative path when a large amount of NADH is generated in the synthesis of carboxylates (i.e. citrate), when COP is restricted (Lambers et al. 2005; Florez-Sarasa et al. 2014).

In summary, under P limitation, the exudation of citrate in NM roots was associated with rapid respiratory rates through AOP, whilst decreasing plant growth. Conversely, AM fungus colonization was associated with less exudation of citrate and malate and slower root respiration rates through both activities of COP and AOP, leaving more carbon for biomass production, as hypothesized.

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CHAPTER 5.

Phosphorus concentration coordinates respiratory bypasses, synthesis and exudation of citrate, and the expression of high-affinity phosphorus transporters in *Solanum lycopersicum*

Phosphorus concentration coordinates respiratory bypasses, synthesis and exudation of citrate, and the expression of high-affinity phosphorus transporters in *Solanum lycopersicum*

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ABSTRACT

Plants exhibit respiratory bypasses (*e.g.*, the alternative oxidase, AOX) and increase the synthesis of carboxylates in their organs (leaves and roots) in response to phosphorus (P) deficiency which increases P uptake capacity. They also show differential expression of high-affinity inorganic phosphorus (Pi) transporters, thus avoiding P toxicity at a high P availability.

The association between AOX and carboxylate synthesis was tested in *Solanum lycopersicum* plants grown at different soil P availability, by using plants grown under P-sufficient and P-limiting conditions, and by applying a short-term (24 h) P-sufficient pulse to plants grown under P limitation. Tests were also performed with plants colonized with arbuscular mycorrhizal (AM) fungi, which increased plant P concentration under reduced P availability. The *in vivo* activities of AOX and cytochrome oxidase (COX) were measured together with the concentration of carboxylates and the P concentration in plant organs. Gene transcription of Pi transporters (*LePT1* and *LePT2*) were also studied. A coordinated response between plant P concentration with these traits was observed, indicating that a sufficient P availability in soil led to a suppression of both AOX activity and synthesis of citrate and a down-regulation of the transcription of genes encoding high-affinity Pi transporters, presumably to avoid P toxicity.

INTRODUCTION

Phosphorus (P) is a key element for carbon and energy metabolism, playing an important role in the export of triose phosphate from the chloroplast and phosphorylation of ADP (Lambers *et al.*, 2008a). It is the least accessible macronutrient required by plants, representing about 0.2% of their dry weight, and when it is in short supply, it limits both glycolysis and mitochondrial electron transport, which ultimately leads to decreased plant growth (Schachtman *et al.*, 1998; Plaxton & Tran, 2011; Marschner, 2012). In response to P limitation, plants trigger a series of physiological, biochemical and molecular adjustments, including respiratory bypasses of adenylate reactions as well as the activation of other mechanisms like carboxylate exudation (Lambers *et al.*, 2006; Chiou & Lin, 2011; Plaxton & Tran, 2011).

Respiration is affected by inorganic phosphorus (Pi) availability in soils. The significant reductions in intracellular Pi during P deficiency may restrict both electron flow and oxygen consumption through the cytochrome oxidase pathway (COX), which is coupled to ATP synthesis. Consequently, the proton gradient increases, which may lead to decrease in the re-oxidation of NADH produced in the tricarboxylic acid (TCA) cycle (Lambers *et al.*, 2005; Plaxton & Tran, 2011). Accumulation of NADH may interrupt the synthesis of respiratory metabolites. To prevent that, respiration via the COX pathway is bypassed by an alternative path. The bypass occurs at the level of ubiquinone (UQ), from which electrons are transferred to the alternative oxidase (AOX) which consumes oxygen like COX but without proton translocation, thus decreasing the ATP yield of respiration (Gonzalez-Meler *et al.*, 2001; Vanlerberghe, 2013). Moreover, AOX activity may oxidize the excess of NADH resulting from restricted COX activity, allowing the continuation of TCA cycle reactions under COX restriction (Lambers *et al.*, 2005; Del-Saz *et al.*, 2016).

The alternative respiratory path responds to soil P availability, although showing different responses among species and organs. Gonzalez-Meler *et al.* (2001) observed increases of AOX activity in leaves of *Phaseolus vulgaris* and *Gliricidia sepium* plants grown under P limitation, but a decrease in *Nicotiana tabacum*. This contrasting result in leaves of different species has not been fully resolved. In roots, AOX activity is invariably increased under P limitation. Florez-Sarasa *et al.* (2014) and Del-Saz *et al.* (2017) reported higher AOX activity in cluster roots of *Lupinus albus* and roots of *N. tabacum* plants grown under P limitation. These studies also showed that in roots exposed to P limitation, AOX activity may be associated with the synthesis of metabolites such as citrate, which is exuded into the rhizosphere to displace P from insoluble complexes, thus increasing its availability for plant

uptake (Lopez-Bucio *et al*., 2000a; Veneklaas *et al*., 2003). Similarly, in leaves of plants grown under P limitation, an accumulation of carboxylates such as citrate, malate and fumarate has been reported (Lopez-Bucio *et al*., 2000b; Alexova *et al*., 2016). Carboxylates in leaves can be transported via the phloem and directed to roots for exudation (Lopez-Bucio *et al*., 2000b; Alexova *et al*., 2016). However, their synthesis in leaves has not been linked to AOX activity when plants are grown in P-poor soils. Such a link might lead to a better understanding of the regulation of AOX activity in leaves of plants exposed to P deficiency.

Uptake of Pi from soil and its translocation through roots require several transport processes across membranes. Pi uptake occurs at the outer root cells, followed by release from parenchyma cells into the root xylem (Bieleski, 1973). Its uptake occurs through high-affinity Pi transporters encoded by *PHT1* genes, predominantly expressed in the epidermal cells (Lambers & Plaxton, 2015). The transcripts encoding these transporters are highly conserved in plant species such as *Arabidopsis thaliana*, *Medicago truncatula* and *Solanum lycopersicum,* and their expression decreases when P is abundant (Bucher, 2007; Nussaume *et al*., 2011). In tomato, the expression of high-affinity Pi transporters, *LePT1* and *LePT2*, is strongly dependent on plant P status (Liu *et al*., 1998; Muchhal & Raghothama, 1999; Nagy *et al*., 2005, 2009). The downregulation of these genes is important to avoid P toxicity at a high P supply, rather than its upregulation to enhance the capacity of P uptake at limiting P availability, because the rate of Pi uptake is more dependent on soil characteristics than on the kinetic properties of Pi transporters at limiting P availability (Lambers & Plaxton, 2015).

Plant nutrient status, rather than its availability in soil, serves as a signal triggering nutrient starvation responses (Abel *et al*., 2002; Shane *et al*., 2003; Nagy *et al*., 2005; Chiou & Lin 2011; Roche *et al*., 2017). In relation to P nutrition, it is expected that a low P status in plant organs mediates a coordinated response in which induction of AOX activity occurs to allow the synthesis of citrate, and that contrarily, a high P status leads to a suppression of both AOX activity and synthesis of citrate, whilst the transcription of genes encoding high-affinity Pi transporters is repressed to avoid P toxicity. A close association of P status in plant organs with a coordinated response involving these traits has never been demonstrated. This association could be studied under different scenarios of soil P availability and plant P status. For instance, arbuscular mycorrhizas (AM) fungi, reputed to improve P status in colonized plants (Smith & Read, 2008), provide an extra scenario to study the effects of changing organ P status under similar soil P availability. Moreover, comparison of AM plants with non-colonized plants is of interest because colonization decreases both the exudation of root carboxylates and the AOX activity in plants grown under P limitation (Ryan *et al*., 2012;

Nazeri *et al*. 2013; Del-Saz *et al*., 2017). However, the effect of AM symbiosis on the synthesis of carboxylates in leaves is unknown.

Tomato is a model species for the study of P limitation and AM fungal colonization (Nagy *et al*., 2005, 2009). Growth, the exudation of carboxylates as well as the expression of high-affinity Pi transporters (*LePT1* and *LePT2*) have been well studied in this species (Daram *et al*., 1998; Liu *et al*., 1998; De Groot *et al*., 2001; Nagy *et al*., 2009). The present study was conducted using both AM and non-AM (NM) *Solanum lycopersicum* plants grown under P-sufficient and P-limiting conditions. In addition, plants grown under P limitation were treated with a short-term (24 h) P-sufficient pulse (P-pulse), which is expected to reestablish plant P status, possibly leading to the suppression of plant responses to P limitation. We hypothesize that AOX activity is involved in the synthesis of citrate in leaves. Moreover, we also expect a coordinated response between plant P status with a respiratory bypass, synthesis of carboxylates such as citrate, and expression of high-affinity Pi transporters. To test this hypothesis, the *in vivo* activities of AOX and COX in leaves and roots were measured using the oxygen-isotope-fractionation-technique (Ribas-Carbo *et al*., 2005). We also determined the amount of carboxylates in the rhizosphere and leaves together with P concentration and the expression of high-affinity Pi transporters, *LePT1* and *LePT2*.

Material and Methods

Plant and fungal material

Seeds of *Solanum lycopersicum* L. cv Moneymaker plants were planted in trays of a substrate containing nutrient-rich black peat (KEKKILÄ DSM 1 W ©). Two-week old plants were transplanted into pots (2 L volume) containing autoclaved sand whilst half were also inoculated with 3 mL of commercial monoxenic inoculum of *Rhizophagus intraradices* containing 50000 propagules mL^{-1} (MYCOVITRO S.L., Granada, Spain). A second inoculation was applied seven days later, according to manufacturer's instructions.

Following the first inoculation, plants were grown during 55 days in a growth chamber under controlled conditions of 25/20 ºC day/night temperature, above 40% relative humidity and 12 h photoperiod (350 µmol m^{-2} s^{-1} of photosynthetic photon flux density). Plants were irrigated three times a week with Hoagland nutrient solution (Epstein, 1972) supplemented with P (supplied as $NH_4H_2PO_4$) at 0.25 mM (P-sufficient solution) or 0.025 mM (P-limiting solution). In order to supply the total requirements of NH_4^+, 0.225 mM NH_4Cl was added to the P-limiting solution similar to previous studies (Del-Saz *et al*., 2017). At day 55, plants were harvested every four hours and used for all the different analyses described below. First,

leaf disks of each plant were harvested for measurements of respiration. Later, the same plant was carefully removed from the pot, and the root systems from intact plants were firmly shaken to remove excess sand. Some lateral roots were used for analysis of mycorrhizal colonization, measurements of respiration, rhizosphere carboxylates, and gene expression of *LePT1* and *LePT2*. Finally, the remaining plant material was collected for P analysis. In parallel, different 55-days old plants grown under P-limiting conditions were irrigated with a P-sufficient (0.25 mM) solution (P-pulse), and the same analyses were performed on these plants 1 day after its application.

Mycorrhizal colonization

The root sample (approximately 150 mg FW) was thoroughly washed and cleared in 10% (w/v) KOH, and staining with 0.05% (v/v) trypan blue in lactic acid, according to Phillips and Hayman (1970). Arbuscular mycorrhizal fungal colonization was assessed using the magnified intersections method (Abbott *et al*., 1984), where the frequency of colonization represents the ratio between the fragments of colonized root and the total number of root fragments examined. Values presented are the mean ± SE of five measurements.

Phosphorus analysis in plant organs

Dried samples of leaves and roots from four plants per treatment were ground into a fine powder with a mixer mill MM 200 (Restsch®, Haan, Germany). Leaf and root [P] were determined by ICP/OES spectrometry (Varian 720-ES ICP Optical Emission Spectrometer, Münster, Germany).

Determination of carboxylates in leaves

Leaves were ground in liquid nitrogen and homogenized with cold ethanol/chloroform/water (12/5/1, v/v/v) (1:5, w/v). The homogenate was centrifuged at 4°C and 12000×g for 15 min and the supernatant was separated into aqueous and chloroform phases by the addition of chloroform and water. The aqueous phase was evaporated under a flow of nitrogen. Finally, dry residues were resolubilized in milli-Q water, centrifuged (10000×g 15 min at 4 ºC), and the supernatant was filtered through 0.22 µm nylon filter.

Citrate, fumarate, and malate were separated and quantified by ion chromatography and suppressed conductivity detection, performed on a DionexICS-3000 chromatograph (Dionex Corp., Sunnyvale, CA, USA). A Dionex Ionpac AG11-HC guard column (50 mm×4 mm) and a Dionex Ionpac AS11-HC separation column (250 mm×4 mm) were used throughout. The

column temperature was 35 ºC and the working electric current was 223 mA. The ASRS 300 4mm suppressor was connected between the analytical column and the conductivity detector. Solvent flow was 1.5 mL min^{-1} and the elution gradient was prepared with eluent A (mili-Q water) and eluent B (NaOH 100 mM). The gradient profile was applied as follow (t (min);%A): (0; 99%), (15; 99%), (25; 85%), (35; 70%), (45; 40%), (46; 99%), (50; 99%). A relative calibration procedure was used to determine the carboxylate concentrations in the samples, using trifluoroacetic acid as the internal standard. Results were expressed as µg g^{-1} fresh weight (FW) of leaf. Values presented are the mean ± SE of four biological replicates.

Rhizosphere carboxylates measurements

Non-AM (NM) and AM roots were harvested to analyze the amount of carboxylates in the rhizosphere as described in Del-Saz *et al*. (2017). Carboxylate concentrations were determined by HPLC according to Cawthray (2003). Values presented are the means ± SE of four biological replicates.

Respiration and oxygen-isotope fractionation measurements

For respiratory measurements of leaves and roots, plants were placed for 30 min in the dark to avoid light-enhanced leaf dark respiration. Organs were harvested and placed in a 3 mL stainless-steel closed cuvette maintained at a constant temperature of 25 ºC (Gastón *et al*., 2003). Air samples of 300 µL were sequentially withdrawn from the cuvette and fed into the mass spectrometer. Changes in the $^{18}O/^{16}O$ ratios and oxygen concentration were obtained to calculate the oxygen-isotope fractionation and respiration rates as described in Ribas-Carbo *et al*. (2005). The electron partitioning to the alternative pathway (τ_a) was calculated as follows:

$\tau_a = (\Delta_n - \Delta_c) / (\Delta_a - \Delta_c)$

Where Δ_c, Δ_a are the oxygen isotope fractionation of the cytochrome (+SHAM) and alternative (+KCN) pathway, respectively and Δ_n, is the oxygen-isotope fractionation of the respiration in the absence of inhibitors. For the calculation of Δ_a, leaves and roots were treated with a solution of 10 mM KCN for 30 min. Values of Δ_a of 30.3±0.3‰ (n=3) and 26.1±0.1‰ (n=3) were obtained in leaves and roots, respectively. For the calculation of Δ_c, both organs were submerged in different solutions of 25 mM SHAM for 30 min. Value of Δ_c of 19.9±0.1‰ (n=3) and 17.6±0.2‰ (n=3) were obtained for leaves and roots, respectively. The individual activities of the COP (v_{cyt}) and AOP (v_{alt}) were obtained as described in Del-Saz *et al*., (2016). Values presented are the mean ± SE of five biological replicates.

Transcription of high-affinity Pi transporter genes *LePT1* and *LePT2*

Gene transcription of high-affinity Pi transporters *LePT1* and *LePT2* from roots of three tomato plants per treatment was quantified. RNA was extracted from roots by using the RNeasy Plant Mini kit (Qiagen, Germantown, USA), following the manufacturer's instructions. The cDNAs were synthesized using the High Capacity cDNA Reverse Transcription (RT) Kit (Applied Biosystem, ThermoFisher, USA), involving 1 × RT buffer, 1 × dNTP Mix, 1 × RT Random Primers, 1 μL of MultiScribe™ Reverse Transcriptase, 1 μL of RNase Inhibitor, 2 μg of RNA, and Nuclease-free H_2O to complete the reaction. The thermal cycler conditions were 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Semi-quantitative PCR was performed using the specific primers designed from the Lpt1 and Lpt2 sequence: Lpt1fw (5′-AAC-GAA-GGG-GAA-GAG-GAA-AC–3′) and *Lpt1*rv (5′-GCA-TTG-TAG-TGT-ATA-CTA-ACT-G-3′); *Lpt2* fw (5-GGA-AGC-ATC-ACA-AGA-AAC-TAT-A-3′) and *Lpt2* rv (5′-CTT-ACA-CAA-TAC-AAA-GAA-AAC-TG-3′). Tomato actin specific primers were used for DNA amplification control: *Tomact* fw (5′-TTC-CGT-TGC-CCA-GAG-GTC-CT-3′) and *Tomact* rv (5′-TCG-CCC-TTT-GAA-ATC-CAC-ATC-3′). PCR conditions were: 29 cycles of 95°C for 50 s, 60°C for 50 s, 72°C for 50 s, and 72°C for 5 min. The ratio of expression values of target gene to ubiquitin was used to standardize the expression data.

Statistical analyses

Five experiments (one biological replicate per respiratory experiment) separated in time (one per week during five consecutive weeks) were carried out with different groups of plants grown under similar conditions. Data of root P concentration were log-transformed in order to meet normality and homoscedasticity. Two-way ANOVA with P level (Sufficient P, Low P and P-pulse) and AM colonization as fixed factors was performed. The post-hoc t-test was used when statistically significant differences were obtained. No transformation of the data was needed. After logarithmic transformation, pairwise Pearson correlation coefficients between P concentration with respiratory parameters, carboxylates and the accumulation of *LePT1* and *LePT2* transcripts were also performed using the average value per treatment. Statistical analyses were performed using the JMP®, Version 12.1.0 (SAS Institute Inc., Cary,NC, USA, 1989–2007).

RESULTS

Arbuscular mycorrhizal colonization

Colonization was 49 ± 9.7% (P-sufficient), 52 ± 14% (P-limited), and 46 ± 11% (P-pulse), with no significant differences between the treatments ($p>0.05$ post hoc t-test). NM plants did not exhibit any fungal colonization.

Phosphorus concentration

Phosphorus availability significantly affected the P concentration of leaves and roots, whereas AM colonization only affected P concentration in roots (Table 1). Under P limitation, the leaf P concentration was significantly reduced (90% and 73% in NM and AM plants, respectively) and the root P concentration was also reduced (85% and 46% in NM and AM plants, respectively). There was a significant interaction effect of both factors on the P concentration of both organs (Table 1). The P-pulse significantly increased P concentration of leaves and roots, by 152% and 146%, respectively in NM plants, whilst in AM plants, no significant increase was detected (Figs 1a and 2a). Moreover, under P limitation, AM colonization significantly increased leaf and root P concentration, by 155% and 194%, respectively, while no significant effect was detected in the other P treatments (Figs 1a and 2a).

Table 1. Significance of sources of variation after two-way ANOVA analyses for each parameter. The sources of variance were phosphorus (P) availability (P level), arbuscular mycorrhizal (AM) colonization and their interactions (P level x AM colonization). *p<0.05, **p<0.01, ***p<0.001, ns not significant effect.

	P level	**AM colonization**	**P levelx AM colonization**
Leaf P concentration	***	ns	*
Root P concentration	***	**	***
Leaf citrate	**	ns	ns
Leaf fumarate	ns	ns	ns
Leaf malate	ns	ns	ns
Rhizosphere citrate	***	*	**
Rhizosphere lactate	**	ns	ns
Rhizosphere malate	*	ns	ns
Leaf V_t	***	ns	ns
Leaf v_{cyt}	***	ns	**
Leaf v_{alt}	***	**	***
Root V_t	ns	ns	ns
Root v_{cyt}	ns	**	ns
Root v_{alt}	ns	ns	***
***LePT1* expression level**	***	*	ns
***LePT2* expression level**	**	ns	ns

Determination of carboxylates in leaves and rhizosphere

Fumarate and malate were unaffected by any of the two factors, neither by their interaction (Table 1 and supplementary Table S1). Under P limitation, the amount of citrate was significantly and similarly increased in NM and AM plants by 200%, whilst a P-pulse did not affect it (Fig 1b).

In roots, there was a significant effect of both P availability and AM colonization and their interaction on the amount of citrate in the rhizosphere (Table 1). Phosphorus limitation significantly increased the amount of citrate only in the rhizosphere of NM plants (Fig 2b). Regarding other carboxylates, the amounts of lactate and malate were similar in both NM and AM plants under P-sufficient and P-limiting conditions. A P-pulse significantly increased the amount of lactate and malate, by 400% and 89%, respectively, in NM plants, while the amount of citrate was significantly decreased, by 47% in NM plants, and significantly increased in AM plants (by 350%). On the other hand, the amounts of lactate and malate were not affected by a P-pulse in AM plants (Fig 2b; Table S1).

Organ respiration and electron partitioning to the AOX pathway

In leaves, there was a significant effect of P availability on total oxygen uptake (V_t) and on the *in vivo* activities of the cytochrome (v_{cyt}) and the alternative oxidase (v_{alt}) (Table 1). In addition, there was a significant effect of the interaction of the two factors (P availability, AM colonization) on v_{alt} and v_{cyt} (Table 1). Phosphorus limitation significantly decreased V_t and v_{cyt}, by 25% and 36%, respectively, in NM plants, whilst in AM plants, both parameters were also reduced but to a lesser extent 17% (V_t) and 22% (v_{cyt}) respectively (Table 2, Fig 1c). The decrease of P availability significantly increased v_{alt} by 58% and by 30% in NM and AM plants, respectively. A P-pulse significantly increased V_t and v_{cyt} by 21% and 35%, and decreased v_{alt} by 22% in NM plants, while in contrast, in AM plants, significantly increased v_{alt}, by 48%, and decreased v_{cyt}, by 18% (Fig 1c-d; Table 2).

In roots, there was a significant effect of AM fungal colonization on v_{cyt} (Table 1). A significant effect of the interaction of the two factors (P availability, AM colonization) on v_{alt} was observed, whilst there was no effect of P availability on any respiratory parameter (Table 1). Phosphorus limitation significantly increased v_{alt} by 54% in NM plants, whilst no effect was observed in AM plants. A P-pulse significantly increased v_{cyt} by 48% and decreased v_{alt} by 44% in NM plants, while in AM plants, v_{alt} was significantly increased by 54%, and no effect was detected on v_{cyt} (Fig 2c-d; Table 2). No changes were observed in V_t among treatments.

Table 2. Effect of phosphorus (P) availability on the total respiration (V_t) in leaves and roots of NM and AM tomato plants grown under phosphorus (P)-sufficient (Sufficient P, 0.25 mM) or P-limiting conditions (Low P, 0.025 mM), and exposed to a sudden short-term (24 h) P sufficient-pulse (P-pulse). Values are means ± SE for five biological replicates. Different letters across treatments within the same organ indicate significant differences with a P-value <0.05 determined by post-hoc t-test.

		Leaf V_t (nmol O_2 g^{-1} DW s^{-1})	**Roots V_t (nmol O_2 g^{-1} DW s^{-1})**
	Sufficient P	28.2±1.98a	30.2±4.19
NM	**Low P**	20.6±1.39c	33.4±3.61
	P-pulse	26.3±1.00ab	35.4±2.65
	Sufficient P	29.2±0.978a	28.8±3.47
AM	**Low P**	24.5±0.324b	26.0±2.76
	P-pulse	24.2±0.461b	31.1±2.14

Differential gene transcription

There was a significant effect of P availability on the accumulation of both *LePT1* and *LePT2* transcripts (Fig. 2e-f), whilst there was a significant effect of AM colonization only on the expression of *LePT1* (Table 1). Phosphorus limitation significantly increased *LePT1* and *LePT2* transcription in both NM (3-fold and 14-fold, respectively) and AM (5-fold and 53-fold, respectively) roots. A P-pulse significantly increased the transcription of *LePT1* (2-fold) and significantly decreased the transcription of *LePT2* (by 67%) in AM roots, whilst no effect of a P-pulse was observed in NM roots on any high-affinity Pi transporter (Fig 2e-f).

Influence of phosphorus concentration on a respiratory bypass, synthesis and exudation of carboxylates, and accumulation of *LePT1* and *LePT2* transcripts

In order to gain insight into the influence of organ P concentration on the different acclimations to P limitation, Pearson correlations were performed between $\log_{10}$ transformed data of all parameters studied in leaves and roots of NM and AM plants grown under P-

sufficient and P-limiting conditions, and treated with a P-pulse (Table 3). Among all the parameters analysed, the following significant correlations were found between organs [P] and leaf V_t, leaf v_{cyt} and with rhizosphere citrate. Moreover, we observed a significant correlation between leaf [P] and *LePT2* expression level and the concentration of citrate in leaves with leaf v_{alt}. Other significant correlations (Table 3) showed an association between leaf [P] and root [P], the concentration of citrate in the rhizosphere with *LePT1* expression level, and leaf v_{alt} with both the concentration of citrate in leaves and leaf v_{cyt}. The interpretation of the significant correlations is represented in Figure 3.

Figure 1. Effect of P availability on (a) P concentration, (b) amount of citrate, and the *in vivo* activities of (c) COX (v_{cyt}) and (d) AOX (v_{alt}) in leaves of NM and AM tomato plants grown under phosphorus (P)-sufficient (Sufficient P, 0.25 mM) or P-limiting conditions (Low P, 0.025 mM), and exposed to a sudden short-term (24 h) P sufficient-pulse (P-pulse). Values are means ± SE for four-five biological replicates. Different letters indicate significant differences with a P-value <0.05 determined by post-hoc t-test.

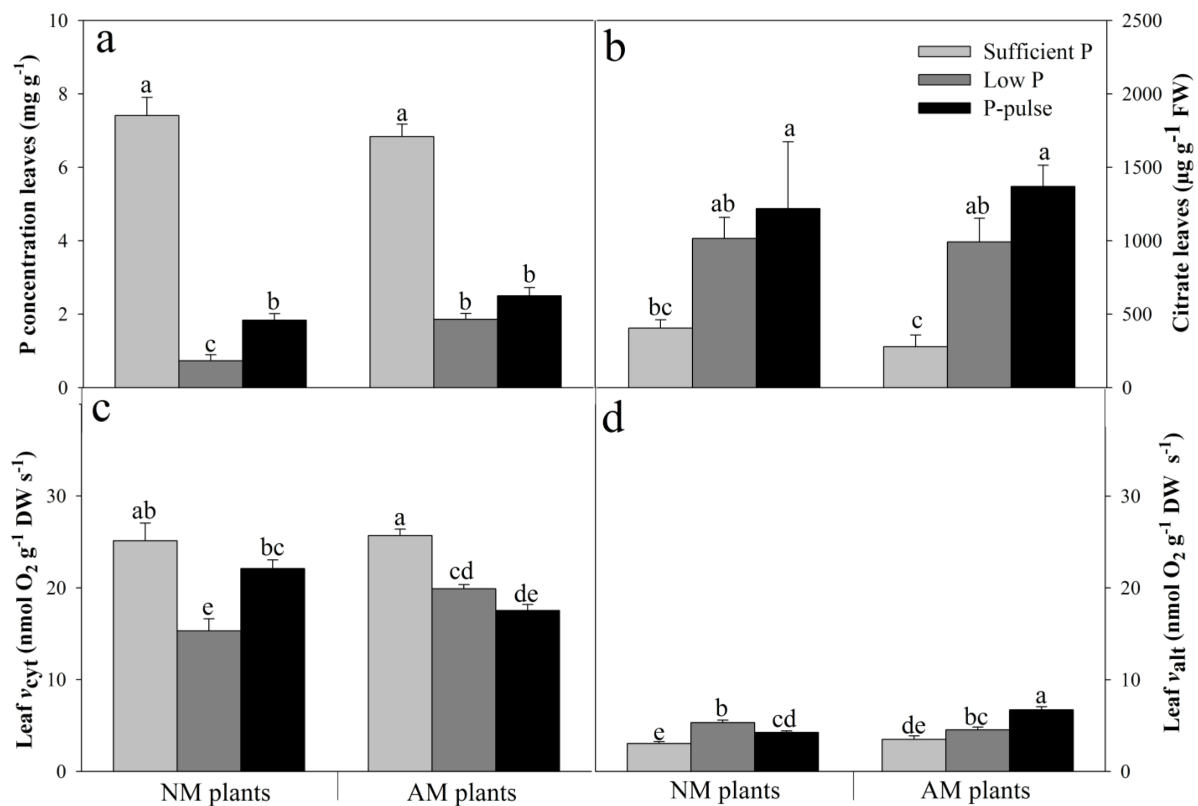


Figure 2. Effect of P availability on (a) P concentration, (b) amount of rhizosphere citrate, the *in vivo* activities of (c) COX (v_{cyt}) and (d) AOX (v_{alt}), and transcription of (e) *LePT1* and (f) *LePT2* genes in roots of NM and AM tomato plants grown under phosphorus (P)-sufficient (Sufficient P, 0.25 mM) or P-limiting conditions (Low P, 0.025 mM), and exposed to a sudden short-term (24 h) P sufficient-pulse (P-pulse). Values are means ± SE for four-five biological replicates. Different letters indicate significant differences with a P-value <0.05 determined by post-hoc t-test.

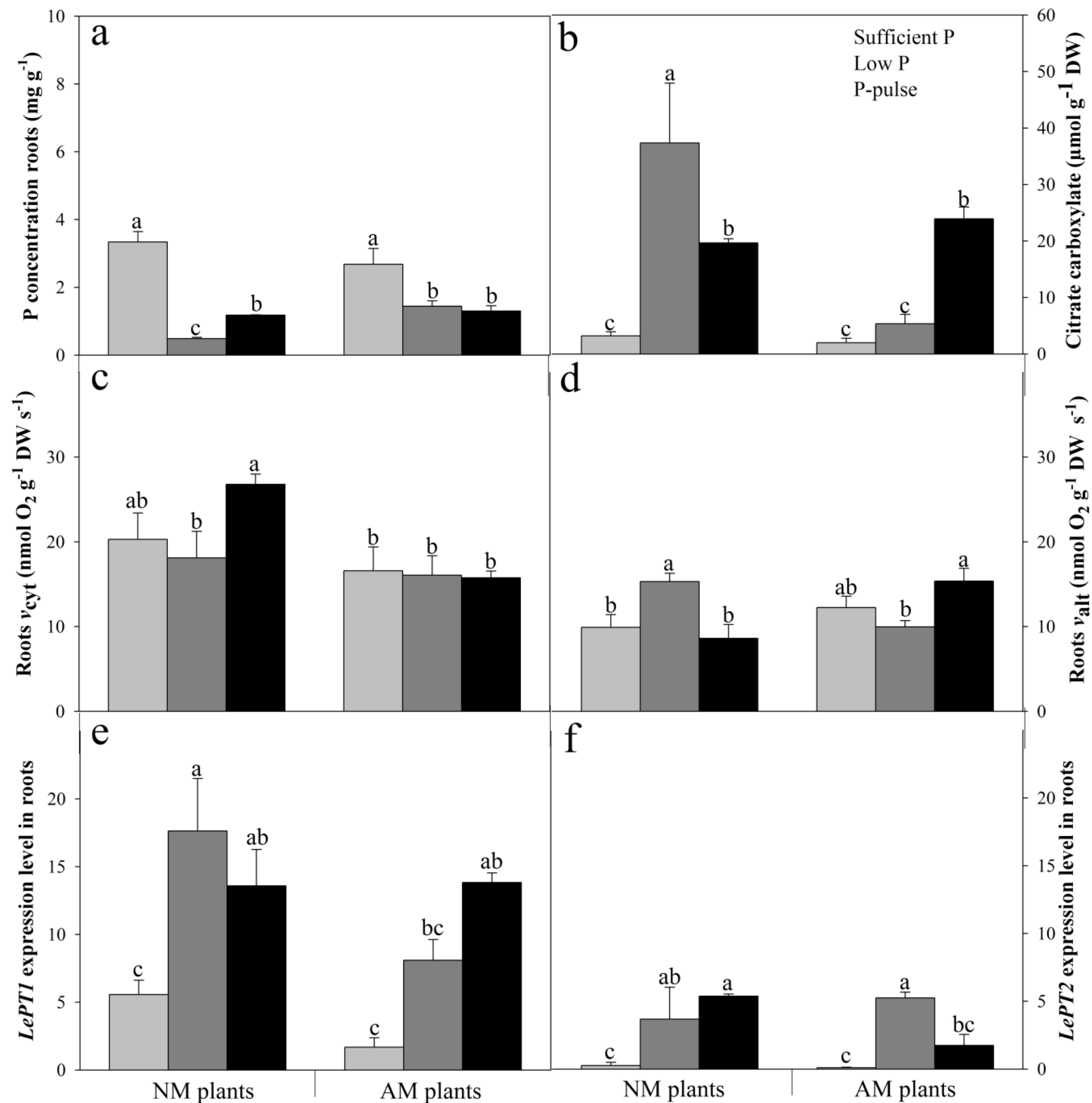


Table 3. Pearson correlation coefficients between organ P concentration [P] and respiratory parameters, carboxylates in leaves and rhizosphere, and transcription of *LePT1* and *LePT2* genes. P-values (p) are indicated as follow; *p<0.05, **p<0.01, ***p<0.001.

	[P] leaves	**[P] roots**
V_t leaf	0.96**	0.95**
v_{cyt} leaf	0.88*	0.91*
v_{alt} leaf	-0.69	-0.73
Citrate leaf	-0.77	-0.72
V_t roots	-0.43	-0.51
v_{cyt} roots	0.26	0.29
v_{alt} roots	-0.29	-0.43
Citrate rhizosphere	-0.84*	-0.86*
LePT1 expression level	-0.78	-0.73
LePT2 expression level	-0.82*	-0.72
Others correlations		
[P] leaf vs [P] roots		0.98***
Citrate rhizosphere vs *LePT1* expression level		0.91*
v_{alt} leaf vs Citrate leaf		0.86*
v_{alt} leaf vs v_{cyt} leaf		-0.86*

DISCUSSION

The present research was based on the hypothesis that plant P concentration modulates P responses to P availability, like the synthesis and exudation of carboxylates, the expression of high-affinity Pi transporters and a respiratory bypass. We especially focused on the relation between AOX activity in different plant organs and the amount of citrate in leaves and rhizosphere. Our hypothesis was tested in tomato plants grown under P sufficiency and P limitation, and also by exposing plants grown under P limitation to a P-pulse (24 h), in order to create different scenarios of P availability. An extra scenario of changing P concentration in plant organs was generated by using AM fungi, reputed for their effect on P acquisition (Smith & Read, 2008).

AOX activity and exudation of citrate are induced by P plant status

We observed several inductions and repressions of the AOX activity which correlated with the amount of citrate in leaves and roots under different scenarios of P availability (Figs 1-2). The AOX activity increased in leaves of both NM and AM plants grown under P limitation, coinciding with lower P concentrations and a greater amount of citrate (relative to P-sufficient plants). The same phenomenon was also observed in roots of tobacco NM plants (Del-Saz *et al*., 2017). On the other hand, P limitation affected neither AOX activity nor the amount of rhizosphere citrate in roots of AM plants in the present study, also in agreement with previous studies (Ryan *et al*., 2012; Nazeri *et al*., 2013; Del-Saz *et al*., 2017). Moreover, the highest AOX activities in tomato leaves were observed under COX restriction, likely due to P-deficiency-induced adenylate control as previously reported (Gonzalez-Meler *et al*., 2001). Therefore, our results suggest that the AOX activity *in vivo* allows the continuation of respiration under COX pathway restriction and also the synthesis of citrate under P limitation (Lambers *et al*., 2005; Florez-Sarasa *et al*., 2014; Del-Saz *et al*., 2017). Furthermore, after the application of a P-pulse, the concentration of P in leaves and roots of NM plants increased, which coincided with an increase of COX pathway (i.e. very likely due to a release of the P-deficiency–induced adenylate restriction), a decrease in AOX activity and a decrease in rhizosphere citrate. These results from P-pulse experiments further support for the role of AOX in citrate synthesis under COX restriction. A similar reversion of the AOX activity induction during recovery of COX pathway in NM plants was reported under temperature stress by Gonzalez-Meler *et al*. (1999) and Armstrong *et al*. (2008), who concluded that AOX activity tends to compensate a repressed activity of COX until its recovery. However, a different scenario was observed in AM plants after the application of a P-pulse, which displayed no changes in COX activity while AOX activity in both leaves and roots as well as the amount of rhizosphere citrate were increased. This suggests that AOX activity maintained its role in the synthesis of citrate after a P-pulse, independently of COX activity changes. Such a rapid AOX response would dissipate a sudden excess of NADH produced as a consequence of a rapid TCA cycle flux, as was also suggested to occur under sudden and severe salt and light treatments (Florez-Sarasa *et al*., 2016; Del-Saz *et al*., 2016).

The effect of P concentration on respiration, synthesis and exudation of carboxylates, and the expression of genes encoding high-affinity Pi transporters

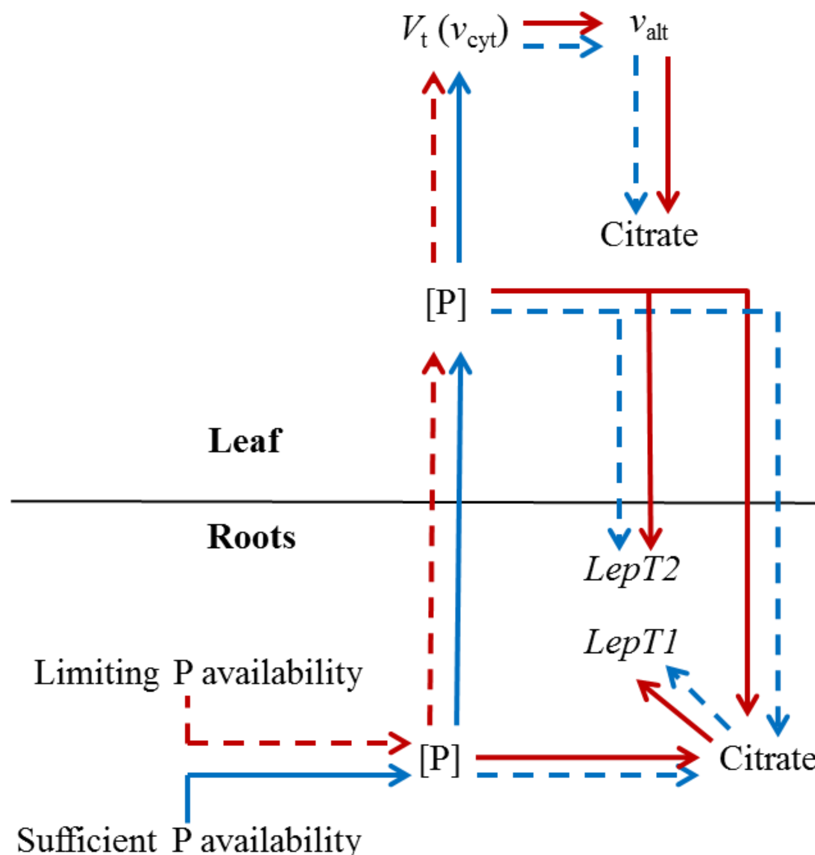
Plant P concentration was associated with different responses to P limitation analyzed in the present study on tomato plants (Table 3, Fig 3). As discussed above, a low P concentration led

to COX restriction and increased AOX activity, which allowed the synthesis of citrate. Indeed, P concentration was significantly and positively correlated with respiration via COX pathway in leaves, while a negative correlation was observed between COX and AOX activities. However, P concentration was not associated with total respiration or COX activity in roots.

Regarding high-affinity Pi transporters, the expression of *LePT1* and *LePT2* transcripts increased in both NM and AM plants under P limitation, as previously reported (Nagy *et al*., 2009). Conversely, the levels of *LePT1* and *LePT2* transcripts were kept high in NM roots after the P-pulse, whilst in AM roots, the transcript levels of *LePT2* were decreased. This is in the line with previous studies that reported an influence of AM fungal colonization on the expression of these high-affinity Pi transporters (Poulsen *et al*., 2005; Bucher, 2007). This is likely related to the existence of others Pi transporters in AM colonized roots that compose the mycorrhizal pathway for Pi uptake (Smith *et al*., 2004, 2011; Facelli *et al*., 2014). This pathway is thought to bypass the direct Pi-uptake pathway, consisting of *LePT1* and *LePT2* transporters, in a P availability-dependent manner (Karandashov & Bucher, 2005; Bucher, 2007; Watts-Williams *et al*., 2015; Sawers *et al*., 2017). Similarly, a downregulation of these high-affinity Pi transporters was observed in *Eucalyptus marginata* following a P-pulse only when it was colonized by AM fungi, which was interpreted as a mechanism to avoid P toxicity as this species occurs naturally in P-impoverished environments (Kariman *et al*., 2014). In our study, a similar phenomenon could be occurring in AM plants growing under P limitation bearing in mind the unaffected P concentration in AM plant organs following a P-pulse, and also considering that a sudden increase of P availability may lead to P toxicity (Shane *et al*., 2004a,b; Shane & Lambers, 2006). In line with this, the strong positive correlations between P concentration in both leaves and roots with the transcript levels of high-affinity Pi transporters suggests that tomato plants tend to avoid P toxicity under P sufficiency in soil. Moreover, P concentration in both leaves and roots was positively correlated with the amount of citrate in the rhizosphere which in turn correlated with the expression of *LePT1*. These observations are consistent with a coordinated response of citrate exudation (i.e. mediated by an increased AOX respiration) and the expression of high-affinity Pi transporters under low P availability in order to increase Pi remobilization for plant transport (Lopez-Bucio *et al*., 2000a; Veneklaas *et al*., 2003; Mitsukawa *et al*., 1997; Poulsen *et al*., 2005). An enhanced expression of these transporters is usually interpreted as an acclimation to P limitation in soil. However, the kinetic properties of high-affinity Pi transporters have very little effect on net P uptake (Lambers *et al*., 2006; Lambers & Plaxton,

2015, and references therein). In accordance, Rae *et al*. (2004) observed no effect on Pi uptake in transgenic *Hordeum vulgare* overexpressing high-affinity Pi transporters. This is because rates of P uptake from the soil solution are determined by the movement of P in soil, rather than by the kinetic properties of these transporters (Lambers & Plaxton, 2015).

Figure 3. Schematic representation of the events during P limitation (red lines) and P sufficiency (blue lines) based on the significant Pearson correlation coefficients of Table 3. Low P availability in soil decreases root and leaf [P]. A reduced leaf [P] decreases V_t in leaves via COX pathway (v_{cyt}). The reduction of v_{cyt} is associated with the occurrence of a respiratory bypass into AOX (v_{alt}), which allows the synthesis of citrate in leaves. Phosphorus concentration [P] in both organs triggers the transcription of *LePT2* genes and the exudation of citrate into the rhizosphere in order to maximize the capacity of P acquisition. Contrarily, sufficient P availability in soil increases root and leaf [P]. Sufficient leaf [P] allows to sustain faster leaf respiration via COX (v_{cyt}), which lead to decrease v_{alt} and consequently, the amount of citrate in leaves. An increase of P plant status suppresses the exudation of citrate into the rhizosphere and the transcription of *LePT1* and *LePT2* genes possibly to avoid P toxicity. Dashed lines indicate events that induce inhibitions, and continuous lines indicate events that induce activations.



Conclusions

We observed that in *Solanum lycopersicum* grown in symbiosis with the AM fungus *Rhizophagus intraradices* the P concentration in plant organs coordinated the exudation of citrate into the rhizosphere, expression of high-affinity Pi transporters and the occurrence of a respiratory bypass that allows the synthesis of citrate, especially in leaves. We surmise that the role of the AOX pathway is to overcome the adverse effects of Pi starvation on leaf respiration until the COX pathway is recovered. Moreover, AM plants under P limitation synthesized similar amounts of citrate in leaves as NM plants, but displayed greatly reduced levels of citrate in the rhizosphere concomitant with reduced AOX pathway activity. Interestingly, following a sudden P-pulse, we observed different responses in NM and AM plants; AM plants downregulated the expression of *LePT2* and increased both AOX activity and the synthesis of citrate, whilst the opposite was observed in NM plants. This different response might be due to the influence of the mycorrhizal Pi-uptake pathway as a protective mechanism to avoid P toxicity under sudden increases of soil P availability, as might happen after a rain event. Further research into the relation of rhizosphere citrate and Pi-acquisition pathways would be interesting to decipher the complexity of the regulation of P acquisition in AM plants.

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Table S1. Effect of P availability on the amount of carboxylates in leaf (malate and fumarate) and rhizosphere (malate and lactate) of NM and AM tomato plants grown under phosphorus (P)-sufficient (Sufficient P, 0.25 mM) or P-limiting conditions (Low P, 0.025 mM), and exposed to a sudden short-term (24 h) P sufficient-pulse (P-pulse). Values are means ± SE for four biological replicates. Different letters across treatments within the same carboxylate indicate significant differences with a P-value <0.05 determined by post-hoc t-test.

		Leaf carboxylates		Roots carboxylates	
		Malate ($\mu g\ g^{-1}$ FW)	**Fumarate ($\mu g\ g^{-1}$ FW)**	**Malate ($\mu mol\ g^{-1}$ DW)**	**Lactate ($\mu mol\ g^{-1}$ DW)**
NM	**Sufficient P**	1360±142	234±27.2	3.42±0.36b	7.60±0.84bc
	Low P	1107±145	220±32.3	3.80±1.27b	3.49±0.398c
	P-pulse	1419±237	300±56.3	7.20±0.824a	17.7±1.76a
AM	**Sufficient P**	1185±36.6	278±16.8	4.24±1.95b	14.9±2.38ab
	Low P	1466±233	260±36.6	3.76±0.79b	9.05±3.00abc
	P-pulse	1185±132	244±16.0	5.11±0.293ab	17.2±5.81a

GENERAL DISCUSSION

The present Thesis is based on the global hypothesys that AOX regulation will be dependent on metabolic and nutritional changes. This hypothesys was tested by the use of NM and AM colonized plants under different scenarios of changing concentrations of salt and P availability in soils. Several specific questions were addressed:

1- Does AOX activity confer tolerance in plants under salinity?

Differences in plant responses to salinity may occur among species and genotypes, as well as between NM and AM plants. Increasing respiratory rates were suggested to be a distinctive feature of salinity tolerance (Jacoby et al., 2011). On the other hand, AOX activity may increase under salt stress to dissipate an accumulation of NADH to prevent an over-reduction of UQ-pool, which in turn may increase respiration (Vanlerberghe, 2013). This phenomenon could be the consequence of an incremented synthesis of carbon compounds involved in the response to osmotic stress. I also tested if such a coordinated response between AOX activity and the synthesis of protective metabolites could also be associated to a better physiological status. To answer this question, I measured the *in vivo* activities of COX and AOX, gas exchange measurements, relative water content and metabolomics, in three genotypes of *Medicago truncatula*, reputed by their different response to salinity, and treated with sudden severe salt stress (300 mM) during 1 and 3 days. I observed that AOX activity increased coinciding with higher levels of osmoprotectans such as amino and organic acids, also showing a relation with a higher photosynthetic rate and water content. Therefore, I state that there is an association between AOX activity and TCA metabolites that may improve salt tolerance in leaves.

2- Does AOX activity really allow the continuity of TCA cycle flux under salt stress?

The incremented tolerance to salt stress observed in Chapter 1 suggested that AOX activity allows the continuity of TCA cycle reactions. Such a role for AOX needs to be further studied, for instance, by using mutants lacking AOX, under a situation that has been related to an accumulation of TCA metabolites and others carbon compounds synthesized from TCA cycle like PAs. In fact, an accumulation of organic acids and PAs in leaves has been observed under osmotic stress (Obata and Fernie, 2012; Andronis et al., 2014; Liu et al., 2015). To demonstrate the implication of AOX pathway in the synthesis of PAs, I tested the *in vivo* respiratory activities of the COP and AOP under sudden severe drought and salt conditions (1 day, 300 mM of mannitol or salt) in leaves of wild-type *Arabidopsis thaliana* plants and in aox1a T-DNA mutants. The results demonstrated that the application of

sudden and severe osmotic treatments increases the activity of AOX. Moreover, I observed that the lack of AOX1a coincided with lower accumulation of TCAs and PAs and lower rates of AOP respiration, while in the wild-type, AOP allowed a faster synthesis of PAs by allowing the acceleration of the TCA cycle needed for their synthesis. Therefore, I suggest that AOX activity may allow the continuity of TCA cycle reactions not only for the synthesis of TCA metabolites, but also for the synthesis of other metabolites which synthesis depends on TCA cycle reactions.

3- Does AOX activity contribute to increase growth in AM colonized plants under P limitation and salinity?

I have observed in chapter 1 that salinity increases the activity of AOX also coinciding with a better physiological status in leaves of plants exposed to severe and sudden salt stress. AM colonization is reputed for improving plant growth in P deficient and salty soils (Ruiz-Lozano 2003; Cantrell and Linderman 2001; Evelin et al. 2009). Nevertheless, the application of sudden and severe salt treatments is not useful to relate salt tolerance with plant growth due to its dramatic effect on both photosynthesis and respiration, the main physiological processes determining plant growth. Therefore, long-term salt treatments, in combination with long-term P limitation, seems to be a more realistic approach to test the effect on AM colonization on plant growth, and whether such effect is related to a differential AOX response. To test this, I studied the *in vivo* respiratory activities of the COP and AOP in AM and NM tobacco plants grown under P-limiting conditions and treated with a moderate salt stress during 21 days. The results showed that AOX activity is unaffected by AM colonization with no salt addition, whilst with salt addition, it was increased in AM plants allowing an increase of COX activity thus helping to increase both the rates of ATP production and shoot growth. Therefore, I state that AOX activity contributes to increase growth in AM colonized plants under both P limitation and salinity.

4- Is root respiration of AM plants affected by the exudation of rhizosphere carboxylates?

Similar to what I observed under salinity in Chapters 1 and 2, AOX activity may allow the continuity of TCA cycle reactions also under P limitation (Florez-Sarasa et al., 2014). It is known that roots exposed to P limitation increase the synthesis and exudation of rhizosphere citrate to increase the availability of P in soils (Lambers and Plaxton, 2015). On the other hand, it is known that AM plants release lower amounts of carboxylates like

citrate into the rhizosphere (Ryan et al. 2012, Nazeri et al. 2013). Bearing this in mind, together with what was observed in cluster roots of *Lupinus albus* (Florez-Sarasa et al., 2014), a diminished implication of AOX activity is expected in AM plants grown under P limitation. I observed that AM colonization not only decreased AOX activity together with the exudation of citrate, but also COX activity and thus total respiration. Moreover, this decrease on respiration contributed to increase biomass accumulation in AM plants, as was observed in previous studies (Romero-Munar et al., 2017). Therefore, I propose that root respiration of AM plants can be conditioned by the release of rhizosphere carboxylates, which in turn, may have an impact on plant growth.

5- Is AOX activity regulated by Phosphorus plant status?

The last question addressed in this Thesis is about what is the main trigger of the induction of AOX activity under P limitation. Is P availability in soil the regulator of AOX activity? Or is it plant P status? Recently, nitrogen plant status has been demonstrated to be responsible for the activation of nitrogen starvation responses (Roche et al. 2017). Regarding P, several studies related the induction of respiratory bypasses to P availability rather than plant P status (Gonzalez-Meler et al., 2001; Florez-Sarasa et al., 2014). I answered this question in *Solanum lycopersicum* plants under different scenarios of soil P availability and plant P status, by using plants grown at P-sufficient and limiting conditions, and by applying a sudden short-term (24 h) P-sufficient pulse in plants grown under P limitation. An extra scenario of changing P concentration in plant organs was generated by using AM fungi, reputed to improve P concentration in plants. Besides the *in vivo* activities of COX and AOX, the amounts of carboxylates in leaves and roots were measured, as well as gene transcription of Pi transporters (*LePT1* and *LePT2*), as a proxy for P starvation responses whose induction was related to plant P status (Abel et al. 2002, Shane et al. 2003, Nagy et al. 2005, Chiou and Lin 2011). I observed that changes on P concentration regulate these P starvation responses together with respiratory bypasses. Therefore, I conclude that changes on plant P status regulate AOX activity.

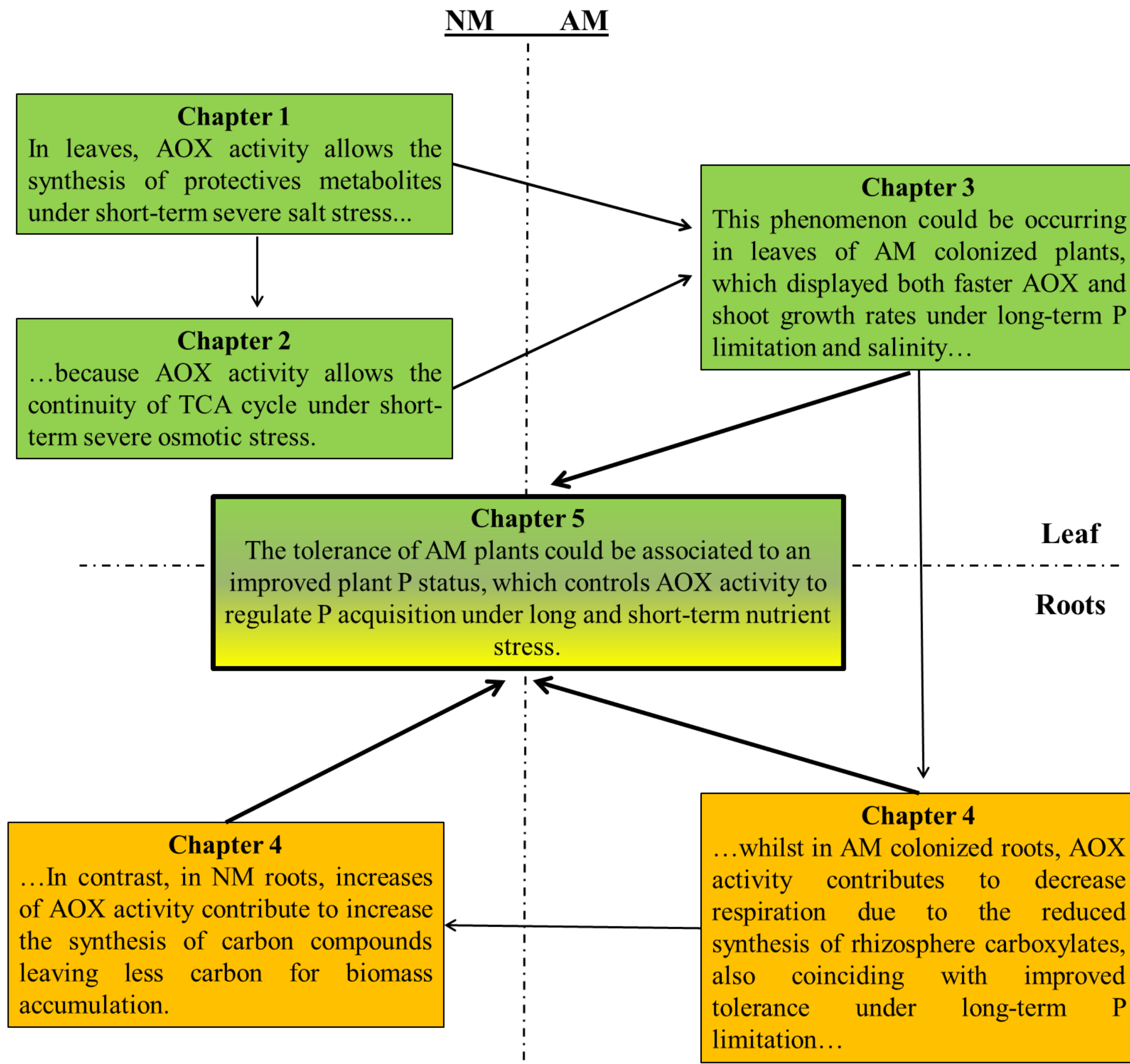

Figure 1. Schematic summary of the conclussions obtained in the five research chapters of the present Thesis.

In summary, I have demonstrated that AOX activity is increased in different plant organs in NM and AM colonized plants under osmotic stress and P limitation (Chapters 1-5). This phenomenon allows an acclimation of the respiratory metabolism under severe sudden osmotic stress that improves plant tolerance. Such acclimation response of respiration is observed only in leaves (Chapters 1-2). Regarding mycorrhiza, AM colonization has shown to improve tolerance under long-term salt and P limitation improving P acquisition also in coincidence with faster rates of AOX activity in leaves (chapter 3). Moreover, my observations suggest that the basis of this tolerance under P limitation also resides in the different mechanisms existing in plants to improve the capacity of P uptake; in NM plants, increases of AOX activity in roots are important to enhance the capacity of P uptake by the synthesis of citrate. However, the synthesis of rhizosphere exudates imposes an important carbon cost detrimental for plant growth (chapter 4). In this context, AM colonized plants display higher tolerance because they do not invest as much carbon as NM plants in such purpose, thus respiring less, and allowing an accumulation of carbon on them (chapter 4). Finally, I demonstrated by using mycorrhiza that the trigger of AOX activity resides in plant P concentration rather than its availability in soil (Chapter 5).

Therefore, the use of mycorrhiza has provided important information about the regulation of AOX activity under different scenarios of changing concentrations of salt and P availability in soils, which was dependent on metabolic and nutritional status, as originally hypothesized.

CONCLUSIONS

1) AOX activity allows the synthesis of protectives aminoacids and organic acids in a particular collection of *Medicago truncatula* genotypes under sudden severe salt stress. Increases of leaf respiration via AOX pathway are suggested to be distinctive feature of salt tolerance.

2) AOX activity allows the continuity of TCA cycle under sudden severe osmotic stress in *Arabidopsis thaliana*. The lack of *AOX1a* leads to a deacceleration of TCA cycle and to a decrease of the synthesis of PAs, which depend on the continuity of TCA cycle.

3) AOX activity contributes to increase shoot growth in *Nicotiana tabacum* colonized with *Rhizophagus intraradices* under long-term P limitation and salinity. This symbiotic association with AMF allows to increase the synthesis of ATP in leaves, which mostly proceeds via COX pathway.

4) AOX activity contributes to decrease respiration in AM colonized roots of *Nicotiana tabacum* due to the reduced synthesis of rhizosphere carboxylates, which synthesis was favoured by AOX activity in NM plants, leading to an increase of total respiration associated to lower biomass accumulation.

5) AOX activity is regulated by changes on phosphorus concentration in NM and AM colonized plants of *Solanum lycopersicum* grown at different P availability in soil. The sincronicity between plant P status and respiratory bypasses together with other P starvation responses suggests that AOX activity is involved in the regulation of plant P uptake.

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HAPPY END ☺