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Research Paper

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 production during oxidative phosphorylation in leaves. Under stress, ATP is needed to maintain the accumulated biomass. ATP production mostly proceeds from the cytochrome oxidase pathway (COP), while respiration *via* the 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, fungal colonization 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 while decreasing them 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 suggest that AM symbiosis represents an ATP cost detrimental for shoot growth at early stages, whilst it represents a benefit on ATP allowing for faster rates of growth at mature stages, even under salinity conditions.

1. 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 impact the problem of low productivity by also decreasing cell turgor (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, 2006). Symbiosis with AM fungi may improve growth and confer tolerance to plants growing under P limitation and exposed to salt stress (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 the fungal species (Stribley et al., 1980) as well as the stage of colonization (Smith et al., 2009). At matures stages, symbiosis may improve P acquisition in P-deficient soils (Siddiqui and Pichtel, 2008; Smith and Read, 2008). Symbiosis imposes a carbon cost to the plant for its functionality (Pearson and Jakobsen, 1993; Jakobsen et al., 2002). For the plant, only when the benefits of AM symbiosis exceeds its costs, 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 leaf growth. 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).

Abbreviations: AM, arbuscular mycorrhiza; AOP, alternative oxidase pathway; AOX, alternative oxidase; COP, cytochrome oxidase pathway; COX, cytochrome oxidase; mETC, mitochondrial electron transport chain; UQ, ubiquinone pool

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Whenever the specific association plant-AMF is positive, one may think that if AM fungus colonization allows plants to grow quicker under P-limiting and saline soils, the ATP investment in growth should be higher in AM than in NM plants.

While AM symbiosis improves biomass accumulation at 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), given that fungal structures require plant carbon for their entire colonization (Graham and Eissenstat, 1994). Bearing this in mind, 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 the ubiquinone (UQ) to oxygen *via* the AOP bypasses complexes III and IV without protons transport and thus, decreasing the energetic efficiency of respiration (Robinson et al., 1995; Lambers et al., 2005). It is known that abiotic stress can restrict the activity of COP which may lead to an over-reduction of UQ. Under this situation, AOP may increase its activity to prevent it (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 (Gonzàlez-Meler et al., 2001; Del-Saz et al., 2016). The *in vivo* activities of the AOX and COX can only be measured by ($^{18}O/^{16}O$) oxygen isotope fractionation (Guy et al., 1989; Robinson et al., 1995; Ribas-Carbo et al., 2005) allowing the modelling of 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; Gonzàlez-Meler et al., 2001; Guy and Vanlerberghe, 2005; Vidal et al., 2007).

In the present study, 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 both AM and NM plants to produce the same amount of ATP at mature stage of colonization?

To answer these questions, *N. tabacum* plants were inoculated with *Rhizophagus irregularis,* a model AM fungi species for salinity experiments (Estrada et al., 2013; Aroca et al., 2013), that in previous studies was described to decrease root respiration in tobacco plants grown under P limitation (Del-Saz et al., 2017). In the present study, plants were grown for 49 days, and leaves respiration was measured using the oxygen-isotope-fractionation technique to study ATP production and respiratory components at different stages of plant growth; (i) in plantlets prior the inoculation (day 14), (ii) in AM and NM plants prior salt addition (day 28), and (iii) in salt and non-salt treated AM and NM plants (day 49).

2. Material and methods

2.1. Plant and fungal material

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

After the first inoculation (at day 14), plants were grown during 35 days in a growth chamber under controlled conditions of 27/20 °C 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 $(NH_4)_2HPO_4$, and 0.225 mM NH_4Cl to supply the total requirements of NH_4^+, as described in Del-Saz et al. (2017).

Four weeks-old NM and AM plants were harvested for respiratory measurements and biomass analysis (n = 4). Simultaneously, some lateral roots were used for analysis of AM fungus colonization. Moreover, on day 28, NM and AM plants were treated with NaCl (n = 4). The treatment was progressively applied 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-old NM and AM plants either treated or non-treated with salt were harvested for respiration measurements, biomass analysis, AM fungus colonization and phosphorus analysis (n = 4). Respiration measurements were performed from 10:00 a.m. to 18:00 p.m.

2.2. AM fungus root length colonization

Root samples (approximately 0.200 g FW) were thoroughly washed and clean 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 and Robson, 1984), where the frequency of colonization represents the ratio between the fragments of colonized root and the total number of root fragments examined.

2.3. Biomass measurements and phosphorus analyses

Tobacco plants were harvested on days 14, 28, and 49. Shoots were separated into stems and leaves. Dry weight (DW) of shoot components was 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 by Poorter (1989).

Dry leaf samples of seven week-old NM and AM 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).

2.4. Respiration and oxygen-isotope fractionation measurements

Leaves were incubated in the dark for 30 min to avoid light-enhanced dark respiration. Later, leaf disks were placed in a 3 mL stainless-steel closed cuvette maintained at a constant temperature of 25 °C 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