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Do metabolic changes underpin physiological responses to water limitation in alfalfa (*Medicago sativa*) plants during a regrowth period?

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ABSTRACT

Drought is one of the most limiting factors on crop productivity under Mediterranean conditions, where the leguminous species alfalfa (*Medicago sativa* L.) is extensively cultivated. Whereas the effect of drought on plant performance has been widely described at leaf and nodule levels, less attention has been given to plant-nodule interactions and their implication on metabolites exchange during a regrowth period, when water is limiting. For this purpose, physiological characterization and metabolite profiles in different plant organs and nodules were undertaken under water deficit, including regrowth after removal of aerial parts. In order to study in more detail how nitrogen (N) metabolism was affected by water stress, plants were labelled with N-enriched isotopic air ($^{15}N_2$) using especially designed chambers. Water stress affected negatively water status and photosynthetic machinery. Metabolite profile and isotopic composition analyses revealed that, water deficit induced major changes in the accumulation of amino acids (proline, asparagine, histidine, lysine and cysteine), carbohydrates (sucrose, xylose and pinitol) and organic acids (fumarate, succinate and maleic acid) in the nodules in comparison with other organs. The lower ^{15}N-labeling observed in serine, compared with other amino acids, was related with its high turnover rate, which in turn, indicates its potential implication in photorespiration. Isotopic analysis of amino acids also revealed that proline synthesis in the nodule was a local response to water stress and not associated with a feedback inhibition from the leaves. Water deficit induced extensive reprogramming of whole-plant C and N metabolism, including when the aerial part was removedto trigger regrowth.

1. Introduction

Alfalfa (*Medicago sativa* L.) is one of the forage crops most extensively cultivated in the Mediterranean region (Annicchiarico et al., 2011, 2015). Alfalfa is a temperate forage frequently exposed to abiotic stresses such as low water availability and high temperature conditions (Walsh, 1995). It is estimated that approximately 70% of yield reduction worldwide is the direct result of environmental stresses (Acquaah,

Abbreviations: A, Photosynthetic assimilation; Arg, arginine; AS, apical shoots; Asn, asparagine; BNF, biological nitrogen fixation; C, carbon; C_a, ambient CO_2 concentration; C_i, intercellular CO_2 concentration; DW, dry weigh; E, leaf transpiration rate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; GABA, gamma-aminobutyric acid; GC-C-IRMS, gas chromatography combustion isotope ratio mass spectrometry; GC-TOF–MS, gas chromatography with time-of-flight mass spectrometry; Gln, glutamine; g_s, leaf stomatal conductance; Glu, glutamate; Gly, glycine; J_{max}, maximum electron transport rate contributing to RuBP regeneration; HPLC, high performance liquid chromatography; l, stomatal limitation; Lys, lysine; Met, methionine; MEV, TIGR multi experiment viewer; MSTFA, *N*-methyl- *N* (trimethylsilyl)trifluoroacetamide; N, nitrogen; Nod, nodule; OPA, *o*-pthaldialdehyde; Orn, ornithine; Pro, proline; PPFD, photosynthetic photon flux density; PR, primary roots; RI, retention index; RWC, relative water content; Ser, serine; T0, first harvest; T8, second harvest; TFA, Trifluoracetic acid; Thr, threonine; TOM, total organic matter; Trp, tryptophan; V_{cmax}, carboxylation velocity of Rubisco; VSP, vegetative storage proteins; WW, well-watered; WS, water stress; Ψ_s, osmotic potential

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2012), where drought is considered the main environmental stress in agriculture (Cattivelli et al., 2008). In legumes, water limitation can reduce global N_2 fixation by up to 17 Gt N $year^{-1}$ (Burns and Hardy, 1975). Under drought conditions, alfalfa has a strategy of avoidance by stopping its vegetative growth and accessing water through its deep root system but in general has poor drought resistance and is rapidly affected by water shortage (Sheaffer et al., 1988) resulting in a decrease in yield depending on the severity and duration of drought stress. Alfalfa, similarly other forages, is frequently subjected to above ground organs cutting for animal feeding. Such cutting causes important modifications in carbon (C) and nitrogen (N) metabolism (Aranjuelo et al., 2014a) at the different organ levels. During this period, shoot removal requires the mobilization of C and N reserves from roots to shoots (Avice et al., 2003; Aranjuelo et al., 2014a), which means an inversion of source and sink organs due to the disappearance of aerial source organs and the formation of new sinks with developing shoots. Abiotic conditions that limit water availability after shoot removal can have significant effects on the dynamics of regrowth (Erice et al., 2007).

Alfalfa is a forage legume that stablishes a plant-rhizobia interaction in which plant photosynthesis supplies C to nodules, where it is used by the nitrogenase enzyme in the bacteroid as a source of energy and reducing power to fix nitrogen gas (N_2) (Streeter, 1987). On the other hand, the products of N_2 fixation, either amides or ureids, are exported to the plant via the xylem (Schubert et al., 1995) where they are used for the synthesis of proteins, secondary products and compounds involved in osmotic adjustment under stressful conditions (Fougère et al., 1991).

Whereas the general effects of drought on leaf gas-exchange in forages (Cornic, 2000; Lawlor, 2002; Aranjuelo et al., 2011) and on the sensitivity of plant-bacteria symbiosis have been extensively studied (Aranjuelo et al., 2014b; and references therein), relatively little is known about the effect of water availability in plant-nodule interactions and its implications in plant functioning and metabolites exchange during a regrowth period. Indeed, some authors reported that the effect of water deficit on plant performance is associated with the deleterious impact of drought on N_2 fixation rather than on photosynthesis itself (Serraj et al., 1999a; Thomas Robertson et al., 2004). Previous studies reveal that biological nitrogen fixation (BNF) under drought condition is affected by (1) C supply to nodules (Galvez et al., 2005; Larrainzar et al., 2009); (2) respiration decrease and the resulting lower oxygen (O_2) consumption may locally inhibit nitrogenase activity (Galvez et al., 2005; Aranjuelo et al., 2011) and (3) the accumulation in the nodule of N compounds can induce a feedback mechanism (Serraj et al., 1999b). Several molecules like glutamine (Gln) (Neo and Layzell, 1997), ureides (Serraj et al., 2001), and asparagine (Asn) have been suggested to be involved in such a mechanism (Bacanamwo and Harper, 1997).

In alfalfa plants, Asn, together with ammonia, is the major organic N compound transported to the plant from the nodule (Groat and Vance, 1981). Some amino acids can be further transported back to the nodule from the shoots as a systemic signal for BNF regulation under drought conditions (Bacanamwo and Harper, 1997; King and Purcell, 2005; Neo and Layzell, 1997; Serraj et al., 2001). However, studies under drought conditions in pea suggest a local signal in addition to the systemic signal involved in BNF activity (Marino et al., 2007). Another point of controversy concerns the different sources of C required for amino acid synthesis. Although organic acids (mainly maleic acid and succinate) represent an important pool of C skeletons in the bacteroid (Lodwig and Poole, 2003), other studies suggest that some amino acids, like glutamate (Glu), Gln, glycine (Gly), proline (Pro) and tryptophane (Trp), can also be remobilized and thus represent an alternative source of C and energy to nodules (Kohl et al., 1994; Udvardi and Day, 1997; Molero et al., 2011). However, Prell and Poole (2006) suggested that amino acid supply to the bacteroid appears to be related to the synthesis of alanine (Ala) and aspartate (Asp). Disparities amongst results highlight the current uncertainties on the role of amino acids in nodule metabolism and their partitioning through the plant, particularly under drought conditions (Lodwig and Poole, 2003; Lodwig et al., 2003). Some studies suggest that regrowth after shoot removal may be more dependent on the availability of N reserves rather than of C reserves (Avice et al., 1996; Kim et al., 1993; Ourry et al., 1994; Volenec et al., 1996).

Thus, understanding the exchange of C and N metabolites between plant and nodules is of prime importance, especially under water deficit conditions. Fluxomics (i.e. the study of the concentration and fluxes of metabolites in an organism) and isotopic tracing can provide insightful information about how different metabolites are exchanged and transferred in a biological system (Tardieu et al., 2017; Salon et al., 2017). The study of plant metabolites can therefore provide new insights on how specific processes involved in C and N metabolism may confer a better tolerance to water limitation in a context where the aerial part has been removed and, therefore, limiting the C supply to the nodule.

The objective of this study was to identify possible target specific compounds (soluble sugars, organic acids and amino acids) that may be involved in controlling plant performance during a regrowth period under drought conditions, by taking advantage of physiological and isotopic measurements. Here, we focused on the characterization of water availability effects in different organs (leaves, roots and nodules) and carried out metabolic analysis. N-enriched isotopic air ($^{15}N_2$) was used as labelling gas and enabled us to study N fixation in total organic matter (TOM) and individual amino acids and N exchange between different organs.

2. Material and methods

2.1. Experimental design and water status

The alfalfa (*Medicago sativa* L.) cultivar Demnat from Morocco, identified as well adaptated to frequent cuts under warm and irrigated conditions (Annicchiarico et al., 2013; Nanni et al., 2014), was selected for the study. Seeds were surface sterilized in 10% commercial bleach for 30 min., and rinsed three times with deionized water. Sterilized seeds were germinated on Petri dishes and planted on 7 L white plastic pots filled with sand. Plants were grown at 25/15 °C (day/night) with a photoperiod of 14 h in growth chambers (Conviron E15, Controlled Environments ltd., Winnipeg, Canada) equipped with fluorescent lamps (SylvaniaDECOR183, Professional-58 W, Germany) that provided a photosynthetic photon flux density (PPFD) of *ca.* 400 µmol m^{-2} s^{-1}. During the first month, plants were inoculated three times a week with 3 mL (per plant) of a sucrose solution at 2% containing *Sinorhizobium meliloti* strain 102F78 that was resuspended from agar media. Plants were watered twice a week with Hoagland N-free nutrient solution (Hoagland and Arnon, 1950) and once a week with deionized water to avoid salt accumulation in pots. As described below, when plants were 61 days old and the main root was totally developed we performed $^{15}N_2$ labelling during 5 days. This plant stage was chosen for labelling since at this stage, there is an important C and N remobilization from aboveground organs toward taproot that acts as the major storage organ (Avice et al., 1996). Immediately after the labelling, the first harvest was undertaken from a subset of four control and four labelled plants (T0). Once the harvest was finished, the aboveground part of the remaining plants was cut (to a 5 cm stem height) so to analyse plant regrowth capacity. Parallel with shoot cutting, waters stress treatment was imposed. Half plants were kept under optimal irrigation conditions (well-watered, WW), whereas in the other half water stress (WS) was imposed through water withholding. A second harvest was performed 8 days after cutting (T8), when plants were 74 days old. In each harvest, plants were separated into apical shoot, primary root and nodules. Four plants were collected per treatment and were immediately frozen in liquid N and stored in −80 °C freezer. A subsample of each organ was separated and dried in an oven during 48 h at 60 °C in order to determine dry weight. Metabolite measurements were conducted in only three replicates per organ and water regime.