Effect of water availability on changes in root amino acids and associated rhizosphere on root exudation of amino acids in Pisum sativum L.

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Abstract

Root exudation is considered to regulate the abundance of the microbial community. It may vary both qualitatively and quantitatively in response to the environment in which the plant is growing. A part of exuded N derives from amino acids (AAs). This, in turn, may help plants to cope with abiotic stresses by favouring positive interactions with the rhizosphere environment, thus playing a potential role in maintaining healthy plants. In this respect, an under-investigated area is the effect of stress due to water deficit (WD). It is proposed that the AA profile in the rhizosphere may be altered by WD, reflecting a modulation of root AA exudation linked to a physiological response of the plant to water stress. To investigate this, *Pisum sativum* L. plants, grown in unsterilised *Rhizobium leguminosarum*-enriched soil, were stem-labelled with ^{15}N-urea for 96 h, and then subjected/not subjected to 72 h of WD. The concentrations and abundance of ^{15}N-labelling in individual AAs were determined in both roots and the associated rhizosphere at 24, 48 and 72 h after stress application. It was found that both AAs metabolism in the pea root and AAs exudation were strongly modified in WD conditions. After 24 h of WD, the concentrations of all measured AAs increased in the roots, accompanied by a dramatic stress-related increase in the ^{15}N-labelling of some AAs. Furthermore, after 48-72 h of WD, the concentrations of Pro, Ala and Glu increased significantly within the rhizosphere, notably with a concomitant increase in ^{15}N-enrichment in Pro, Ser, Asn, Asp, Thr and Ile. These results support the concept that, in response to WD, substantial amounts of recently assimilated N are rapidly translocated from the shoots to the roots, a portion of which is exuded as AAs. This leads to the rhizosphere being relatively augmented by specific AAs (notably HSer, Pro and Ala) in WD conditions, with a potential impact on soil water retention.

Keywords*:* *Pisum sativum* L.; Leguminoseae; Amino acids; Water stress; Rhizosphere; CG-MS; ^{15}N-labelling

Abbreviations: AA, amino acid; DAS, days after sowing; DW, dry weight; GABA, γ-amino-butyric acid; HSer, homoserine; MWHC, maximum water holding capacity; PGPR, plant growth-promoting rhizobacteria; WD, water deficit.

1. Introduction

It is well established that intensive agricultural practices are involved in promoting climate change, notably because of pollution linked to mineral nitrogen (N) fertilisers. In this context, attention is being focused on the development of sustainable agriculture (Group, 2012), particularly the use of N-fixing legume (Fabaceae) crops in agrosystems in an effort to reduce the use of mineral N fertilisers (Garg and Geetanjali, 2007) by exploiting the capacity of these plants to assimilate atmospheric N_2 by establishing a symbiotic relationship with endophytic rhizobacteria (Hirsch et al., 2001). This N is converted into organic forms – mainly amino acids (AAs) – and can be transferred to the soil *via* rhizodeposition, during which a large range of organic compounds is released from actively growing roots at the soil-root interface (Badri and Vivanco, 2009; Farrar et al., 2003; Fustec et al., 2010).

With ongoing climate change, the severity, frequency, and duration of drought are expected to increase in legume-producing areas. In the rain-fed cropping systems of Northern Europe, legumes are particularly sensitive to drought stress, with a negative impact on biological N fixation, yields, and N rhizodeposition (Gutschick and BassiriRad, 2003). It has been shown that drought stress may be stimulated by a short-term exposure to water deficit (WD) (Gargallo-Garriga et al., 2014) but the impact this might have on the rhizosphere environment has been inadequately investigated.

Rhizodeposition consists of the release from actively growing roots of a large range of organic and inorganic compounds at the soil-root interface, including root debris, decomposed root material, cell lysates, mucilage and exudates (Lynch and Whipps, 1990; Uren, 2001). It is generally accepted that plants may modulate rhizodeposition patterns in order to facilitate positive interactions with their biotic and abiotic environment (van Dam and Bouwmeester, 2016). For instance, the exudation of mucilage plays a major role in the maintenance of rhizosphere humidity and root-soil contact under dehydrating conditions (Carminati et al., 2010; Walker et al., 2003). In addition, a number of studies focusing on exudates have demonstrated their important role on the regulation of microbial communities at the root-soil interface, with a possible associated positive effect on plant health by enhancing nutrient acquisition or helping plants tolerate abiotic stress (Albareda et al., 2006; Bais et al., 2006; Houlden et al., 2008; Moe, 2013; Zancarini et al., 2013). Thus, positive interactions with rhizosphere microorganisms, such as plant growth-promoting rhizobacteria (PGPR), may help plants tolerate stress due to WD (Ngumbi and Kloepper, 2016). For instance, treating maize plants subjected to drought with PGPR resulted in the accumulation of compounds that help in osmotic adjustment and in maintaining cellular turgor i.e. free AAs (e.g. Pro) and soluble sugars (e.g. mannitol) (Bano et al., 2013; Ngumbi and Kloepper, 2016; Vardharajula et al., 2011).

Among exuded compounds, AAs are strong candidates for components of the signalling events controlling relationships between the plant and the rhizosphere microbial growth (Moe, 2013): they are the major pool of organic N released into the rhizosphere (Farrar et al., 2003; Yu et al., 2002) and act as a C and N supply for soil microorganisms (Grayston et al., 1998; Kuzyakov, 2002; Read and Perez-Moreno, 2003). Nonetheless, relationships between plant physiological responses to WD and the exudation of AAs remain poorly documented. We hypothesised that WD may alter AA exudation both quantitatively and qualitatively and that exuded AAs may have a role in the physiological response of legumes to WD stress. To explore this relationship, we compared the dynamics of AAs in root tissues and in the rhizosphere in pea plants (*Pisum sativum* L., Leguminoseae) grown under controlled conditions and subjected/or not to WD. The modified dynamics of AA metabolism and exudation were probed by short-term ^{15}N-labelling from stem-fed (cotton wick) ^{15}N-urea prior to WD stress being applied, enabling the allocation and exudation of recently-assimilated ^{15}N under WD, to be recovered in AAs of the rhizosphere. AAs were investigated in the roots and in the rhizosphere before visible morphological adaptations, as the local response to water stress appears soon after the application of WD (Marino et al., 2007).

2. Results

2.1. Influence of WD on plant dry weight

In plants maintained at 65% maximum water holding capacity (65MWHC, see Materials and Methods for the definition), i.e. without WD stress, shoot dry weight (DW) increased with time from 24 to 72 h ($P<0.01$, Fig. 1, 'Control'), whereas it remained unchanged ($P>0.05$) in plants under a WD stress of 25% MWHC (25MWHC). Thus, after 72 h of WD, the shoot dry weight of the stressed plants was less than that of the unstressed ones ($P< 0.05$). In both kinds of treatments (with or without WD), root dry weight did not change over time ($P>0.05$).

2.2. Bacterial biomass in the soil in the presence and absence of a plant

The impact of 72h-WD (25MWHC) was to increase the bacterial biomass in the soil without plant (P^--soil, $P<0.05$), whereas no significant change was seen in the rhizosphere when a plant was present ($P+$-soil) ($P>0.05$). Without WD (65MWHC), the bacterial biomass (determined as $\mu g\ g^{-1}$ soil DW) was nearly two-fold in the rhizosphere soil (P^+-soil) than in the soil without plant (P^--soil) ($P<0.01$, Table 1).

2.3. Total N, NO_3^- and ammonium NH_4^+ concentrations, and $\%^{15}N$ in the roots and in the soil in the presence and absence of a plant

Total soil N was not altered by either the soil water content or the presence/absence of a plant (P>0.05, Table 1). The composition of the inorganic N pool of the soil was changed in the presence of a plant: in unstressed plants (65MWHC), the [NO_3^-] in the rhizosphere (P^+-soil) was significantly lower than that in the soil without plant (P^--soil) (P<0.05, Table 1), whereas the opposite trend was observed for NH_4^+, with [NH_4^+] being significantly higher in the rhizosphere (P^+-soil) than in the soil without plant (P^--soil, P<0.01, Table 1). These results were modified by a 72 h-WD, with a lower [NO_3^-] and [NH_4^+] in 25MWHC conditions than in 65MWHC for both P^+- and P^+-soils. Notably, the [NH_4^+] was decreased nearly two-fold in the rhizosphere (P^+-soil) of the stressed plants compared to the unstressed ones (P<0.001, Table 1).

Although the total root N at 72 h was not affected by WD, the treatment was seen to impact the $\%^{15}N$ content, with the stressed roots (25MWHC) being about 2.4% richer in ^{15}N than the unstressed roots (65MWHC) (P<0.01, Table 1). Nonetheless, no difference was found in the $\%^{15}N$ in the rhizosphere (P^+-soil, P>0.05, Table 1).

2.4. AA concentration and ^{15}N-AA (%) in the roots and in the soil in the presence and absence of a plant

2.4.1. Range of AAs detected

In all, 22 AAs could be detected by GC-MS. However, for the minor components, the variation in the quantification was too great to be useful: therefore, only the 14 AAs composing the majority (>95%) of the total are presented (Table 2). These 14 target AAs could be quantified in all the samples and replicates, enabling a comparison of the fingerprints of root and rhizosphere AAs under WD/unstressed conditions at the three sampling times (24, 48, and 72 h).

2.4.2. Comparison of AAs between roots and rhizosphere

The non-protein AA HSer was by far the most abundant AA in both roots and the rhizosphere under all conditions and was consistently more abundant in the rhizosphere (between 1.2- and 1.7-fold, P<0.01, Table 2). Most of the AAs showed distinct differences in abundance between the roots and the rhizosphere, with the exceptions of Gly, Val, Ile and Leu (Table 2). The next most abundant AA in the roots was Asn (28.7%); otherwise only GABA (6.8%) and Ala (4.9%) composed ≥5% of the total. However, in the control rhizosphere, Thr (5.1%), Ser (4.4%) and Glu (4.1%) were more prevalent than in the roots, whereas Asn (6.3%), Asp (2.6%), Pro (1.8%), GABA (1.5%) and Phe (0.8%) were all less present. The differences in Asn (4.5-fold decrease), GABA (4.5-fold decrease) and Thr (1.8-fold increase) were particularly notable.

2.4.3. Impact of WD/unstressed status on AA concentrations

In unstressed controls (65MWHC), preliminary experiments showed no significant differences between samples at 24, 48 and 72 h (P>0.05, supplementary data). Therefore, only the data for root and

rhizosphere samples collected at 72 h are presented (Fig. 2 and Fig. 3). Following the application of WD stress (25MWHC), the [total AA] increased in roots such that after 48 h it was 2-fold higher than that in the roots in unstressed conditions ($P<0.01$, Fig. 2a). Thereafter, the concentration diminished, such that by 72 h the values were similar to those of unstressed control roots ($P>0.05$, Fig. 2a). In contrast, throughout the exposure to WD stress (25MWHC), the [total AA] in both (P^--soil) ($P>0.05$) and the rhizosphere (P^+-soil) ($P>0.05$; Fig. 2b) did not change significantly.

A negligible increase in [total AA] was seen in roots after 24 h of WD at 25MWHC compared to unstressed plants (Fig. 2a). After 48 h of WD at 25MWHC, significant increases in the concentration of most AAs, compared to unstressed plants, were observed ($P<0.05$), with the exceptions of Gly, Pro, Thr, Glu, Asn and Gln ($P>0.05$; Fig. 3a). The increases at 48 h of WD in Asp and Ser were significant ($P<0.05$), but the most marked increases were in GABA, Pro and HSer ($P<0.01$), with the [Pro] increasing nearly 4-fold.

Although the [Total AA] in the rhizosphere (P^+-soil) did not change (Fig. 2b), the profile of AAs present was considerably modified with the WD (Fig. 3b). Within 48 h of WD at 25MWHC, significant increases were found for Ala and Glu ($P<0.05$; Fig. 3b). Most marked, however, was the effect on Pro (Fig. 3b), which by 48 h from the beginning of WD in the rhizosphere (P^+-soil) had increased 8-fold relative to the unstressed control ($P<0.01$; Fig. 3b), thus becoming the second major AA of the total pool of AAs (Table 2). Furthermore, Pro increased significantly in the soil without plant (P^--soil) after 72 h of drought exposure ($P<0.05$; Fig. 4). In striking contrast to roots, [Ala] and [GABA] did not significantly alter in the stressed rhizosphere ($P>0.05$; Fig. 3b), nor did Ser or Asp.

By 72 h of WD, the contents of most of the AAs that had increased in either roots or the rhizosphere (or both) after 48 h of WD decreased so that by 72 h of WD at 25MWHC, the levels were not significantly different to the control (65MWHC). The marked exception was Pro, which remained at a high concentration in the roots ($P<0.05$; Fig. 3a), but not in the rhizosphere (Fig. 3b).

2.4.4. Impact of 65MWHC (unstressed) versus 25MWHC (stressed) conditions on ^{15}N content of individual AAs in the roots and rhizosphere

^{15}N-enrichment was found in all observed AAs in both roots and the rhizosphere within 24 h (data highly variable between individual plants and therefore excluded from the discussion) and remained present after 72 h in both unstressed (65MWHC) and stressed conditions (25MWHC) (Fig. 5). At 72 h of 25MWHC conditions, the majority of AAs in both compartments showed greater incorporation relative to the unstressed plants (65MWHC), notably for Pro and Ser ($P<0.05$ in the roots and rhizosphere). The

degree of incorporation ranged between 10 and 40% (Fig. 5) with a higher enrichment in the roots than in the rhizosphere, indicating that equilibrium between the compartments had not been reached.

In general, the degree of enrichment in both root and rhizospheric extracts showed a similar pattern, with HSer and Pro being the most enriched in both stressed and unstressed conditions (Fig. 5). Incorporation of ^{15}N into Pro was particularly marked, doubling in the first 48 h of WD in roots and tripling in the rhizosphere (data not shown), then further increasing substantially 3-fold and 6-fold the control values, respectively, by 48/72 h of WD ($P<0.05$; Fig. 5). Other AAs showing a significant increase in ^{15}N-AAs in the roots and in the rhizosphere by 48 h of WD were Leu, Ser and Asn-1/Asn-2 (all $P<0.05$; Fig. 5) and incorporation of ^{15}N remained higher in Ser in both compartment by 48 h of WD ($P<0.05$). The degree of enrichment in Thr increased by 72 h of WD, but only in the rhizosphere ($P<0.05$).

3. Discussion

3.1. ^{15}N labelling in root AAs and in AAs exuded into rhizospheric soil

3.1.1. Incorporation of ^{15}N into AAs from ^{15}N-urea

Assessing the flux of AAs from plants into the soil is very difficult due to microbial degradation (Owen and Jones, 2001) and sorption to mineral particles (Dashman and Stotzky, 1982). In the present study, we used stem-labelling *via* a cotton wick with ^{15}N-urea (Mahieu et al., 2009a) to label *de novo* synthesis of AAs *in planta* in order to follow their concentration and profile in roots and their rhizosphere after exudation. This method has no measurable effect on plant development and can be successfully used for labelling plants with ^{15}N (Wichern et al., 2010). Labelling with ^{15}N makes the identification of exuded AAs reliable even over short exudation periods. Urea was used as the ^{15}N carrier because it is rapidly assimilated by the action of the ubiquitous urease (Hertenberger and Wanek, 2004). Uptake of solution was complete for all plants. However, it must be noted that there may be several drawbacks to ^{15}N-labelling, such as a heterogeneous distribution of ^{15}N in the plant (Wichern et al., 2011) and the dilution effect of ^{14}N present in both the plant and the rhizosphere, although heterogeneity should be minimised by taking the entire sample of each compartment (aerial, roots, rhizosphere). Nonetheless, different rates of absorption/transport could add noise to the system. This is evident from the data collected for individual AAs after 24 h of WD treatment, which were too variable to be used to draw meaningful conclusions.

3.1.2. Composition of the root AA pool and incorporation of ^{15}N from ^{15}N-urea

The whole root biomass was treated as a single pool. Stem-labelling with ^{15}N-urea for 4 days resulted in the control roots (65MWHC) of all plants being ^{15}N-enriched. The degree of incorporation into different AAs was not homogeneous and by 72 h from the start of treatment it varied between 7 and 29% (Fig. 5). HSer, by far the most abundant AA (37% of total AAs recovered; Fig. 3), also had one of the highest ^{15}N-enrichments (26%). In contrast, although Asn was the second most abundant AA (29%), its degree of ^{15}N labelling (M+1 and M+2) was much lower (7%) than that of HSer. *P. sativum* is known to accumulate massively HSer and Asn during early growth and these two AAs are the major N compounds found in the phloem (Kuzmicheva et al., 2014; Rochat and Boutin, 1991; Urquhart and Joy, 1981). They are both thought to play an important role in long-distance N transport and storage (Bauer et al., 1977). Two possible reasons can be put forward to explain this difference in the extent of incorporation. First, Asn is mainly synthesised in nodules while ^{15}N provided by the cotton-wick technique could be preferentially directed to other organs, notably the leaves. Second, it could reflect a different rate of turnover for these two metabolically-related AAs. HSer and Asn are formed from Asp, by reduction and amination respectively: this may explain why they show an inverse abundance in relation to Asp. The results presented here indicate that in the leaves, urea-derived ^{15}N-Asp was more competitively used for chloroplastic Asp-derived AA biosynthesis than that by the cytosolic Asn synthetase.

3.1.3. Composition of the rhizosphere AA pool and incorporation of ^{15}N from ^{15}N-urea

It has been shown that roots release substantial amounts of AAs during vegetative growth (Bobille et al., 2016; Fischer et al., 2007; Nguyen, 2003). Consistent with this, in our study the total [AA]s recovered in the rhizosphere of plants (P^+-soil) were considerably greater than those recovered in the P^--soil, indicative of the release of these compounds by roots. Nonetheless, the concentration of all AAs in the rhizosphere was only around 1% that in the roots (Fig. 3). Even so, the patterns for both concentration and ^{15}N-incorporation in these two compartments were similar, with HSer being the major component in the rhizosphere (61.3%), and Asn, Ala, Thr, Ser and Glu all being more prevalent (between 4 and 6%), while Asp, Pro, GABA, Gln, Gly and Val were at low levels (Table 2, Fig. 3b). This finding, that those AAs abundant in the roots were also the most represented in the rhizosphere concurs with our previous study of *Medicago truncatula* (Bobille et al., 2016).

HSer has previously been reported as a major component of pea root exudates and as an important substrate for rhizosphere colonisation by *Rhizobium leguminosarum* (van Egeraat, 1975). Vanderlinde *et al*. (2014) showed that in *R. leguminosarum* bv. *vicae* 3841, the gene cluster pRL8JI for HSer catabolism is induced by pea root exudates. HSer utilisation by *R. leguminosarum* is correlated with the prevalence of

particular strains in pea nodules, unlike with other *R. leguminosarum* hosts (Hynes and McGregor 1990) and it is therefore not surprising that in this study, HSer was found to be the most abundant AA in the rhizosphere. This result suggests that the preferential exudation of HSer may be a means by which pea plants select their symbiotic bacteria, with an influence even at the level of the bacterial strain.

Investigating the flux of AAs in the rhizosphere in unsterilised soil is complex because *i)* the measured concentration represents the balance between the rate of exudation and the rate of utilisation by the soil microflora (Moe, 2013) and *ii)* the soil solution also comprises AAs released by microbes. C- and N-rich small molecular weight compounds can be quickly degraded by microorganisms, with an estimated half-life of the order of minutes to hours (Farrell et al., 2014). In the present study, it was found that bacterial biomass was higher in the control rhizosphere (P^+-soil) than in the P^--soil (Table 1), suggesting that their proliferation may be stimulated either by individual components of root exudate – e.g. HSer – or by a general greater availability of AAs and/or other organic compounds in the P^+-soil.

Notwithstanding a probable impact on the amount of assimilation of AAs by the soil microflora, following stem labelling with ^{15}N-urea for 4 days, substantial amounts of ^{15}N-AAs were found in the rhizosphere (Table 1, Fig. 5). Marked labelling in the P^+-soil was recorded in HSer, Ala, Glu+Gln, Thr, Pro, and Asp+Asn, demonstrating that the incorporation of ^{15}N into the exudate compartment occurred within a short period of time. However, with the notable exceptions of HSer and Asn, the level of enrichment was equivalent to or less than that in the roots. The relatively elevated ^{15}N incorporation into these AAs can be taken to indicate a high level of turnover in the rhizosphere pool. The results of AA labelling in the rhizosphere strongly suggest continual sequestration and metabolism of AAs by microorganisms, which might also explain the differences in the % composition: some AAs are more rapidly assimilated by the microflora than others.

However, it should be kept in mind that during their span life, microorganisms also exude a wide range of compounds including amino acids, the extent of this depending on their population dynamics and their community structure (Phillips et al., 2004). In our experiment, a part of the labelled amino acids recovered in the rhizosphere could therefore result from inorganic and organic N exudated by the plant before being transformed by microbial activity and released into the soil. With the presence of a plant, bacterial exudation can be expected to increase, as the growth of their populations is stimulated. The WD can also be anticipated to impact both qualitatively and quantitatively on the AAs exudated by microbial communities. Our results suggest that the contribution of this microbial exudation to the amount of labelled amino acids in the rhizosphere is negligible compared to the contribution of the plant, but it remains difficult to assess its impact on their profile. Moreover, during their growth, plants are also able

to absorb a part of the AAs released by their roots or exudated by the surrounding microbes, and this influx has been reported to depend on biotic and abiotic conditions. It must be considered that, our measurements represent a footprint of the plant rhizosphere, resulting from the complex processes of interactions, regulation and trade-offs existing between the plant and biotic and abiotic factors in the rhizosphere.

3.2. Effect of water deficit on N content, AA composition and %^{15}N in the roots

Under WD, the N content of roots (mg g^{-1} dry weight) did not change significantly (Table 1), even though the root dry mass increased with time whereas the increase in the shoot dry mass was insignificant. This finding is consistent with the well-established fact that WD limits photosynthesis and alters the partitioning of C and N compounds at the whole plant level, thus causing changes in the shoot:root biomass ratio (Chaves et al., 2002).

As AAs are the favoured form for the transport of organic N in plants, their transport and metabolism are likely be considerably affected by WD (Justino and Sodek, 2013; Serraj et al., 1999; Sulieman and Tran, 2013; Tsai et al., 2003). It has been consistently observed that, under WD, newly formed AAs (^{15}N-AAs) and the AA concentration increase in roots. In the present study, however, the responses of individual AAs were quite variable. Many AAs showed no WD-related effect on $[AA]_{root}$ (Gly, Val, Ile, Leu, Ser, Thr, Phe, Gln, Asn; Fig. 3), although there were some effects on their proportion of the total AA pool (Table 2). A smaller group, including Ala, Pro, Glu, HSer, GABA and Asp, showed an increase in $[AA]_{root}$. These can be related to specific roles. Under drought conditions, the conversion of ammonia into non-toxic forms appears critical for maintaining normal cellular functions (Oliver et al., 2011; Rabara et al., 2015). Hence, it might be expected that ^{15}N incorporation into Glu might be elevated in WD. This indeed is what was seen: ^{15}N incorporation into both Glu and Asp was 2-fold the control (65MWHC) after the first 48 h. Furthermore, this was transmitted to the principal AA constituting the major form for N transport and storage in pea, HSer, which was 1.6-fold enriched over the 72-h stress treatment relative to the control. Asn, although less enriched in the control (7.4%), showed a 2.4-fold mean increase in ^{15}N incorporation under WD. Whether this increased incorporation is due to enhanced assimilation via the GS-GOGAT pathway, or due to stress-induced GDH activity cannot be ascertained.

Although many AAs showed no change in $[AA]_{root}$, they all showed incorporation of ^{15}N during the labelling period (Fig. 5a). While in a few AAs this did not respond to WD (Val, Leu, GABA, Asp), most AAs showed an enhanced incorporation in response to WD (Gly, Pro, Ser, Thr, Glu, Asn, HSer). Specific AAs have an identified role in the enhancement of stress tolerance in plants, acting as osmoprotectants, precursors of

important metabolites, response and signalling molecules or in the detoxification of reactive oxygen species (Larrainzar et al., 2009; Okumoto et al., 2016). Two of the most common osmolytes accumulated by plant cells in response to osmotic stress are GABA and Pro, both derived from Glu (Okumoto et al., 2016; Vendruscolo et al., 2007). The accumulation of Pro helps for maintaining the root turgor pressure and improves the water uptake of roots during WD (Leport et al., 2006). Pro and GABA have both been found in substantial concentration in the phloem sap of the legume *Medicago sativa* grown under WD (Girousse et al., 1996). Similarly, in *P. sativum*, large increases in the $[GABA]_{root}$ and $[Pro]_{root}$ were found in the 25MWHC plants, 7-fold greater than in the unstressed roots at 72 h. High incorporation of ^{15}N into both these compounds within 48 h indicated extensive *de novo* synthesis (Fig. 5a). As chloroplasts are the primary site of Pro biosynthesis in pea leaves (Rayapati et al., 1989), this implies increased transport from shoot to roots through the phloem, as previously reported (Lee et al., 2009). GABA plays a similar role but is also important for maintaining the C:N balance under a stress affecting N metabolism through the conversion of GABA to Glu to succinate in the TCA cycle. Protection against oxidative stress is enhanced by the generation of NADH and succinate (Tripathi et al., 2016). This may explain the observed drop in $[GABA]_{root}$ between 48 and 72 h, a change not seen in $[Pro]_{root}$.

3.3. Effect of water deficit on organic N composition and $\%^{15}N$ in the rhizosphere

Under WD, the Total N content and $\%^{15}N$ within the rhizosphere did not significantly change. This is consistent with the study of Mahieu *et al.* (2009b) reporting that in pea the Total N derived from rhizodeposition is not modified even after 16 days of exposure to WD (24% of the MWHC of the soil). However, it is known that abiotic factors greatly affect the release of N compounds quantitatively and qualitatively (Canarini et al., 2016; Weston et al., 2012). Notably, soil water potential and temperature affect the release of AAs into the rhizosphere (Farrell et al., 2014; Jones et al., 2009). In the present study, it was found that [Pro] and [Glu] all increased within the rhizosphere in response to WD (Fig. 3b). Furthermore, through ^{15}N-labelling from ^{15}N-urea, it was shown that this increase contained considerable quantities of newly formed AAs (Fig. 5b). By 48 to 72 h of 25MWHC conditions, label incorporation into all AAs measured was increased, with the exception of Phe, for which the noise was too great to draw any conclusions. In some AAs, notably Ala, Ile, and Leu, a transient effect was seen, with a maximum at 48 h followed by a decrease by 72 h (Fig. 5b). The strongest labelling was into Ala, Pro, Asp/Asn and HSer, indicating that these AAs may have a specific role to play in the exudates.

The question remains as to whether the increase in [AAs] in the rhizosphere is due to active exudation, passive diffusion through a concentration gradient along the root (Paynel et al., 2001), or drought damage

to the plasma membrane, resulting in the "leakage" of soluble compounds out of root cells. Several lines of argument can be put forward in favour of active exudation. First, [Ala] and [Glu] increased in the rhizosphere after 48 h of WD, while they did not change in the root, indicating that a concentration gradient is not involved. Secondly, a very differential increase in ^{15}N-enrichment into several AAs was seen in the rhizosphere after 48/72 h of WD compared to the same AAs in the roots of control plants. This would count against an overall "leakage". Of particular note are the relative ^{15}N-incorporations into GABA, Asp and HSer. In these 3 AAs, WD (25MWHC) was associated with greater labelling in the root pool, whereas in the 65MWHC plants, the inverse was found. Thirdly, rhizospheric [$^{15}N_1$]-Asp was richer than root [$^{15}N_1$]-Asp in contrast to the other AAs. Bearing in mind that the synthesis of metabolites that affect osmoregulation to acquire drought stress tolerance represents an energy cost, these data can be taken to indicate that the release of AAs into the rhizosphere may be a regulated active phenomenon in response to drought.

WD has been shown to decrease the conductivity of soil and to create depletion zones around the roots that limit water uptake (Carminati et al., 2010). Some authors have recently highlighted the role of some kinds of rhizodeposits, such as mucilage or polygalacturonic acid, in improving water uptake by creating a built-in water potential gradient (Carminati et al., 2010; Ghezzehei and Albalasmeh, 2015). Accumulation zones of chemicals might be formed by the exudation of ions, especially protons, by plant roots (Hinsinger et al., 2003).

3.4. Effect of water deficit on the plant-bacterial relationship in the rhizosphere

The impact of WD on plant growth clearly modifies the allocation of photosynthates to the roots, exudation to the rhizosphere and, consequently, plant-microorganism relationships (Compant et al., 2010; Guenet et al., 2012). NH_4^+ content decreased with time in both the rhizospheric P^+- and P^--soils, indicating an increased microbial demand for N under WD (Table 1). This may result in the initiation of specific kinds of biotic interactions aimed at protecting the plant (Bertin et al., 2003). During WD, bacterial biomass increased in the P^--soil, unlike in the P^+-soil containing roots. This result suggests a competition for soil N components between the plant and microbial communities in the rhizosphere (Lipson and Monson, 1998). Many AAs exuded by the roots, particularly those that are proteogenic, can easily be used as a N supply by microorganisms, including plant growth-promoting rhizobacteria (PGPR) and mycorrhizal fungi (Belimov et al., 2015; Moe, 2013). In addition, many root-associated bacteria, particularly *Pseudomonas*, may stimulate root exudation of AAs, thereby changing the concentration of these compounds in the rhizosphere (Phillips et al., 2004). For instance, chemotaxis to AAs present in root exudates of tomato was

demonstrated for various PGPRs, and this trait was suggested as determining root colonisation by the bacteria (Oku et al., 2012). In addition, Ala can be used as the sole source of C and N in batch culture for *Pseudomonas oryzihabitans* Ep4 and inoculation with this PGPR improved potato yield in field experiments when plants were grown under WD (Belimov et al., 2015). Bacteria use Ala catabolically in the citric acid cycle and anabolically, to produce sugars or fatty acids as an energy supply (Apostel et al., 2013).

Microorganisms can also provide large quantities of compatible solutes, notably Pro and Gln, to cope with WD (Warren, 2014; Weston et al., 2012). Consistently, we found that Pro increased in $25P^{+}$-soil (Fig. 4), as did the bacterial biomass (data not shown), suggesting that the microflora may release Pro in response to drought stress. Moreover, Azcón *et al.* (2013) showed that three indigenous bacterial strains (*Pseudomonas pudita, Pseudomonas* sp. and *Bacillus megaterium*) isolated from soil under WD are able to improve plant tolerance against WD, with all three showing an increase in internal Pro content.

4. Conclusions

As AAs are the main N form exuded into the rhizosphere, their availability can be expected to have a major impact on the rhizospheric environment of the plant. We hypothesised that modified AAs released into the rhizosphere may constitute a response to WD and that understanding the impact of this stress should help in understanding N metabolism changes in plant–rhizosphere interactions. Taken as a whole, the present study shows that under WD, the amount of ^{15}N increases markedly in AAs of both roots and rhizosphere, indicating that the transport of recently-assimilated N from the shoots to the roots has been stimulated, and that a part of this is exuded. Furthermore, the differences in composition between the profile of exuded AAs and that of the roots suggest that some AAs are preferentially excreted. Despite their sequestration by microorganisms in the soil, we observed a significant accumulation of specific AAs that may play a role in increasing the water-holding capacity around the root and favour the establishment of positive plant–microorganism interactions at early stages of drought. A study aimed at establishing a better understanding of the relationship between microorganisms and AA release under WD is now necessary.

5. Materials and methods

5.1. Biological material and experimental design

Soil removed from a field (62.3% sand, 24.1% silt, 11.5% clay, 1.4% organic matter, pH_{KCl} 6.4) was inoculated with a fresh solution of *Rhizobium leguminosarum* bv. *vicae* (strain P221) previously grown on Bergersen medium (Bergersen, 1961). All soil used for experimentation was deliberately retained

unsterilised, so as to contain a natural microflora, but was inoculated with a high density of *Rhizobium leguminosarum* bv. *vicae* (strain P221) to ensure a reproducible and high level of nodulation. Experimental conditions used 100 g of this soil as the growth substrate in small pots (5 cm height × 5.5 cm diameter), giving a cell density of *R. leguminosarum* of 10^9 cells/per pot. Preliminary experiments indicated that this pot size was suitable to ensure a rapid colonisation of the soil by the roots, such that, at harvest, the whole soil was colonised by roots. Thirty-two pots were prepared.

Seeds of *Pisum sativum* L. var. Frisson (Leguminoseae) were sterilised (50% H_2SO_4, 10 min), rinsed with sterilised demineralised water, and germinated on moist filter paper with sterilised demineralised water (in darkness, 21°C, 65 h). Twenty-four pots of pre-inoculated soil were each planted with one germinated seed (hereafter called 'P^+-soil') and plants were developed in a growth chamber (25/16°C; 16/8 h photoperiod, 300 µmol photons m^{-2} s^{-1}, 70% relative humidity). The remaining eight pots of pre-inoculated soil were also placed in the growth chamber but without any plant (hereafter called 'P^--soil'). Separating P^+-soil and P^--soil into different pots enabled the bacterial populations of soil with and without rhizodeposition to be compared.

All pots were watered daily with a sterilised N-free nutrient solution (modified from Murashige and Skoog, 1962) (mM): 1 $CaCl_2$, 1 $MgSO_4$, 2 KH_2PO_4, 2 K_2HPO_4, 1 NH_4NO_3, 4 KNO_3, 0.1 $MnSO_4$, 0.03 $ZnSO_4$, 1.10^{-3} Na_2Mo_4, 0.1 H_3BO_3, 5.10^{-3} KCl, 1.10^{-4} $CoCl_2$, 25.10^{-3} $FeSO_4$, 25.10^{-3} Na_2EDTA (pH 5.8), to enhance biological nitrogen fixation and to maintain the soil relative humidity at 65% of the maximum water holding capacity (MWHC, 100%). (This was determined on four pots filled with fresh soil which were weighed, watered in excess, and reweighed after being left to drain overnight.)

5.2. Labelling with ^{15}N-urea

Plants were labelled with the cotton-wick method (Mahieu et al., 2009a) after 3 leaves had developed (11 days after sowing (DAS)), corresponding to the phenological growth stage 13 (see the BBCH-identification keys of pea: Meier, 2001; Weber and Bleiholder, 1990). A cotton wick was inserted through a 0.5-mm hole made in the internode below the first true leaf. The cotton wick was protected by two silicon tubes (to prevent evaporation of the solution) and the two ends were immersed in 1 mL of 80 mM ^{15}N-urea solution (99 atom% ^{15}N) contained in a reservoir (2-mL microtube). Absorption of the labelling solution was complete by 15 DAS.

5.3. Imposition of water deficit conditions

At 15 DAS (growth stage 14), 12 pots of P^+-soil and 4 pots of P^--soil were subjected to a water deficit (WD) regime by lowering the soil moisture to 25% MWHC and were maintained at this level to the end of the experiment. These pots are defined as $25P^+$-soil and $25P^-$-soil, respectively. The other 12 pots of P^+-soil and 4 pots of P^--soil were maintained at 65% MWHC to the end of the experiment. These pots are defined as $65P^+$-soil and $65P^-$-soil, respectively. All pots were thereafter watered with only sterilised demineralised water to avoid differences in nutrient concentrations.

5.4. Harvesting and sampling

Four replicates of each modality and of P^--soil were harvested and sorted after 24 h of WD (16 DAS), 48 h of WD (17 DAS) and 72 h of WD (18 DAS). In modalities with a plant, the small pots were entirely colonised by the roots, therefore the whole soil (including adherent soil was considered as rhizosphere. Plants were cut at the stem base and soil (adherent and not adherent) and roots were carefully sorted by hand. Shoots were dried at 70°C for 48 h for dry mass, N (%) and ^{15}N:^{14}N ratio measurements (Nu Instruments Ltd horizon stable isotope ratio mass spectrometer coupled with a EuroVector elemental analyser). Roots were shaken for 10 s in 50 mL of 2 mM formic acid (pH 2.5) before being rinsed with demineralised water, and lyophilised for 72 h for dry mass and GC-MS analysis. The fresh weight of recovered soil was recorded. 40 g of soil was then transferred to 250-mL polypropylene bottles containing 150 mL of 2 mM formic acid (pH 2.5). To this was added the 50 mL of formic acid used to rinse the recovered roots. For the control pots without plant, 40 g of soil was directly transferred into 200 mL of 2 mM formic acid. The suspension was shaken (1 h, 200 rpm, 20°C), centrifuged (5 min, 8000 rpm), filtered (0.22 µm) and stored at -20°C. Amino acids were extracted as previously described (Bobille et al., 2016). For each pot, ~20 g of fresh soil was dried at 105°C for 24 h to measure the water content (% MWHC), N content and for ^{15}N:^{14}N measurements. The remaining soil was stored at -80°C for bacterial biomass, nitrate and ammonium analyses.

5.5. GC-MS analysis of AAs

The AAs present in extracted samples of soil (n=4) and roots (n=3) were analysed by GC-MS for identification and quantitation. A standard (2 µL of 2.5-mM α-aminobutyric acid in deionised water) was added as the quantitation standard to a known volume of the AA extract and the mixture was reduced to dryness in vacuum in microtubes. Freshly prepared reagent mix (50 µL, acetonitrile/*N*-methyl-*N*-*tert*-butyldimethylsilyl-trifluoroacetamide/trimethylamine, 20:20:1) was added to the dried extract (Godber and Parson, 1999) and the microtubes were thoroughly vortexed, centrifuged and incubated in a heated

block (95°C, 30 min). Following cooling, 25 µL of the derivatised extracts was transferred to a GC vial containing an insert for GC-MS analysis. Injection (0.25 µL) was performed in the split mode-4. The GC-MS system used was composed of a BR5MS column (Bruker; 5% diphenyl/95% dimethylpolysiloxane, 30 m × 0.25 mm i.d., 0.25 µm film thickness) mounted in a 436-GC coupled to a simple Quadruple (SQ) SCION MS (Bruker). The GC was operated in constant pressure mode with helium as carrier gas (initial flow, 1 mL min^{-1}). The injector, MS transfer line and source temperatures were 280°C, 290°C and 220°C, respectively. The chromatogram was developed with a thermal gradient of: 60°C for 1 min, linear gradient of 30°C min^{-1} to 120°C, linear gradient of 8°C min^{-1} to 300°C, 300°C for 5 min, resulting in a total run of 39 min. The mass spectrometer was programmed in single ion monitoring (SIM) mode, with the mass range *m/z* of 50 – 650 Da, electron impact (EI) ionisation at 70 eV and monitoring the mass (M) of the largest significant component, mass plus 1 (M+1), and mass plus 2 (M+2) ions, for each AA. The separation of 22 AAs was achieved, and quantification was by reference to calibration curves.

5.6. Concentration of mineral N in the soil

15 g of fresh soil (previously stored at -80°C) was extracted in 75 mL of 1 M KCl, mixed by repeatedly turning (1 h, 40 rpm) and decanted (1 h, 18°C). Nitrate (NO_3^-) and ammonium (NH_4^+) were determined by continuous flow analysis on a Skalar San Plus Auto Analyser (Skalar Inc., Breda, The Netherlands) using a spectrophotometric detector.

5.7. Concentration of bacterial biomass in the soil

Bacterial biomass in the soil was determined by real time PCR with fluorescence monitoring using an external standard curve generated from serial dilutions of a pure culture of *Escherichia coli*. For each pot, 2 × 500 mg of soil were extracted separately using a NucleoSpin® Kit (MACHEREY-NAGEL GmbH and Co. KG) as described by the manufacturer. The two DNA extracts were pooled, re-suspended in 50-µL ES buffer (5 mM Tris/HCl, pH 8.5) and quantified using a Thermo Scientific NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, Wilmington, MA, USA). Amplification was performed with 1 µL of a dilution of first DNA strands (20 ng $µL^{-1}$), 10 µL of Supermix Sso FastTM Eva Green® (©Biotium, Inc.), 1 µL 10 mM of each primer in a total volume of 25 µL in water. The primers used for the quantitative PCR were a reverse primer (5'-GGACTACCAGGGTATCTAATCCTGTT-3') and a forward primer (3'-TCCTACGGGAGGCAGCAGT-5') from Nadkarni *et al.* (2002). Each measurement was carried out using triplicate PCR reactions to determinate C_t values and the mean value of the quantification was calculated. A standard curve provided a full concentration range of *E. coli,* extending from 10 to 10^{-4} ng $µl^{-1}$ and the

PCR efficiency was established with a minimum of 0.99 (99%). The cycling programme consisted of denaturation at 98°C for 2 min and 40 cycles at 98°C for 30 s and at 50°C for 3 s.

5.8. Calculations and statistical analysis

The concentration of each AA in a given sample is expressed in µmol g^{-1} dry matter (soil or roots). The abundance of a single AA, denoted here as %*x*, was calculated from equation (1):

$$\%x = \frac{x}{total\ \mathrm{AAs}} \times 100 \quad (1)$$

where *x* is the concentration of the given AA *X* in the sample and *'total AAs'* is the sum of the concentrations of all AAs.

The enrichment of each AA with ^{15}N (derived from the labelling), denoted here as ^{15}*N-AA,* is calculated from equation (2):

$$^{15}N\text{-}AA = \frac{(M+n)_{\mathrm{obs}} - (M+n)_{\mathrm{nat}}}{[(\mathrm{M})+(\mathrm{M+n})]\mathrm{obs} \ -(M+n)_{\mathrm{nat}}} \times 100 \quad (2)$$

where M and M+*n* (n=1 or 2) are the respective areas of the ^{14}N and ^{15}N ion fragment peaks from the most significant fragments for an AA derivative in the labelled sample (obs) or in an unlabelled sample analysed on the same GC-MS run (nat).

All data are presented as the mean with standard deviation (mean±SD) of values from at least three independent repetitions.

Statistical analysis was carried out with R software version 3.1.2 (R foundation for Statistical Computing). The normality of data (assessed with the Shapiro Wilk test, P>0.05) and the homoscedasticity of the variances (assessed with the Bartlett test, P>0.05) were tested. The significance was tested at P<0.05 with a one-factor analysis of variance (ANOVA) test accompanied by Tukey's HSD test for multiple comparisons. In the case of non-normality (P<0.05), the significance was tested with the Kruskal-Wallis test.

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Figure captions

Figure 1. Shoot (a) and root (b) dry weight (mg) of *Pisum sativum* plants subjected to a water deficit (WD) stress at 25% MWHC (25MWHC) and without stress at 65% MWHC (65MWHC), monitored at 24, 48 and 72 h after WD was applied. Data represent the mean±SD of at least three biological replicates. Asterisks indicate statistically significant differences for one organ (shoot or roots) between the 65MWHC and 25MWHC plant at a given time point (ANOVA test, *P<0.05).

Figure 2. Total amino acid ($[AA]_{total}$) content in (a) the roots (µmol g^{-1} dry matter) and (b) the rhizosphere (µmol g^{-1} dry soil) of *Pisum sativum* cultures after 24 h, 48 h and 72 h of water deficit stress at 25% MWHC (designated (25)) and after 72 h without stress (65% MWHC, designated (65)). (In a preliminary experiment, no significant difference was found in 65MWHC plants at 24, 48 and 72 h, therefore only the 72 h data are illustrated.) Values are mean±SD of at least three biological replicates. The letters indicate significant differences between control pots (65MWHC) and WD pots (25MWHC) at 24 h, 48 h and 72 h of WD (Tukey HSD test, P<0.05). Note scale difference between (a) and (b).

Figure 3. Concentration (µmol g^{-1}) of amino acids in (a) the roots (µmol g^{-1} dried matter) and (b) the rhizosphere (µmol g^{-1} DW soil) of *Pisum sativum* cultures after 24 h, 48 h and 72 h of water deficit stress at 25% MWHC (25MWHC) and after 72 h without stress (65% MWHC, 65MWHC). (In a preliminary experiment, no significant difference was found in 'Control' plants at 24, 48 and 72 h, therefore only the 72 h data are illustrated.) Data are mean±SD of at least three biological replicates. Letters indicate significant differences for a single amino acid between control pots and pots at 24 h, 48 h and 72 h of WD (Tukey HSD test, P<0.05). Non-standard abbreviations: GABA, γ-amino-butyric acid; HSer, homoserine. Note scale difference between (a) and (b).

Figure 4. Concentration (µmol g^{-1} DW soil) of proline in P^--soil and in the rhizosphere (P^+-soil) of *Pisum sativum* culture subjected to a water deficit (WD) stress at 25% MWHC (25MWHC) and without stress (65% MWHC, 65MWHC), monitored at 72 h after WD was applied. Data are mean±SD of at least three biological replicates. Asterisks indicate statistically significant differences between the control and WD pots (Kruskal-Wallis test, *P<0.05, **P<0.01).

Figure 5. Impact of water deficit (WD) on incorporation of ^{15}N (%) from stem-fed ^{15}N-urea into AAs in the roots and in the rhizosphere of *Pisum sativum* plants. a) Label incorporation in AAs extracted from roots; b) Label incorporation in AAs extracted from the rhizospheric soil; c) Differences in ^{15}N incorporation between roots and rhizosphere. All plants were exposed to ^{15}N-urea from 11 to 15 DAS, then were maintained at 65% MWHC (65MWHC) or exposed to a WD at 25% MWHC (25MWHC). Data are shown for 25% MWHC plants monitored at 48 and 72 h after WD was applied and for 65% MWHC plants at 72 h. (In a preliminary experiment, no significant difference was found in 65MWHC plants at 48 and 72 h). Values represent the mean±SD of at least three biological replicates. The letters indicate significant differences for a single AA between control pots (72 hat 65MWHC) and pots exposed to WD stress (25MWHC) after 48 h and 72 h (Tukey HSD test, $P<0.05$). Values for an individual amino acid that are not significantly different from each other ($P>0.05$) are indicated by the same letter while values within an individual amino acid with a dissimilar letter differ significantly ($P<0.05$). No comparison is indicated either between individual AAs or between different compartments (Roots, Rhizosphere). Abbreviations: nd, not detected; GABA, γ-amino-butyric acid; HSer, homoserine; Asn-1/Asn-2 and Gln-1/Gln-2, the M+1/M+2 mass isotopomers of Asn and Gln into which one or two ^{15}N heavy isotopes have been incorporated..

Figure 1. Shoot (a) and root (b) dry weight (mg) of *Pisum sativum* plants subjected to a water deficit (WD) stress at 25% MWHC (25MWHC) and without stress at 65% MWHC (65MWHC), monitored at 24, 48 and 72 h after WD was applied. Data represent the mean±SD of at least three biological replicates. Asterisks indicate statistically significant differences for one organ (shoot or roots) between the 65MWHC and 25MWHC plant at a given time point (ANOVA test, *P<0.05).

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Figure 1.

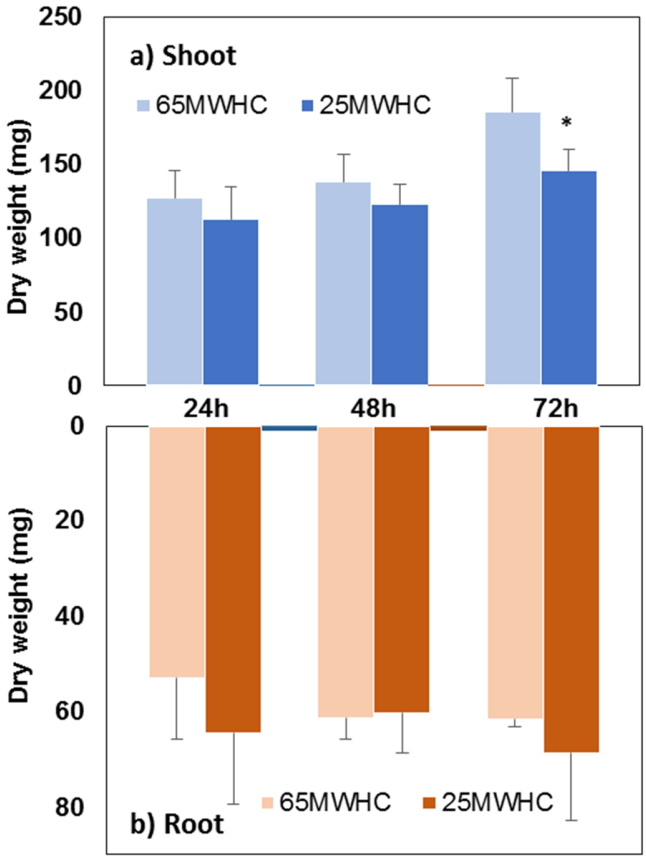
a) Shoot
65MWHC
25MWHC
Dry weight (mg)
250
200
150
100
50
0
*
24h
48h
72h
0
20
40
60
80
Dry weight (mg)
65MWHC
25MWHC
b) Root

Figure 2.

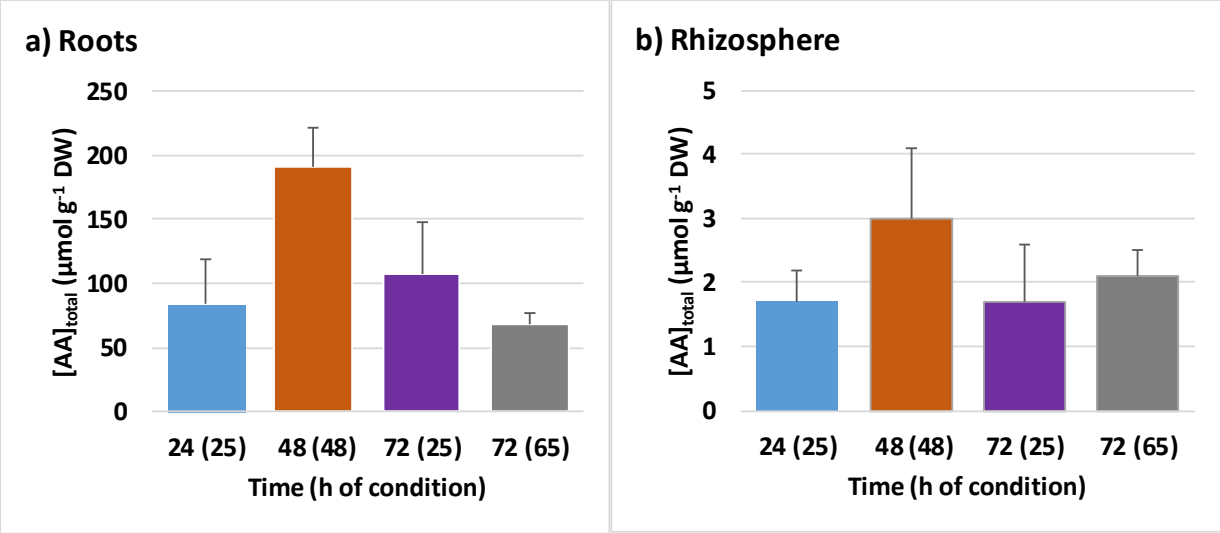
a) Roots
250
200
150
100
50
0
[AA]total (µmol g-1 DW)
24 (25)
48 (48)
72 (25)
72 (65)
Time (h of condition)
b) Rhizosphere
5
4
3
2
1
0
[AA]total (µmol g-1 DW)
24 (25)
48 (48)
72 (25)
72 (65)
Time (h of condition)

Figure 3 (colour in print)

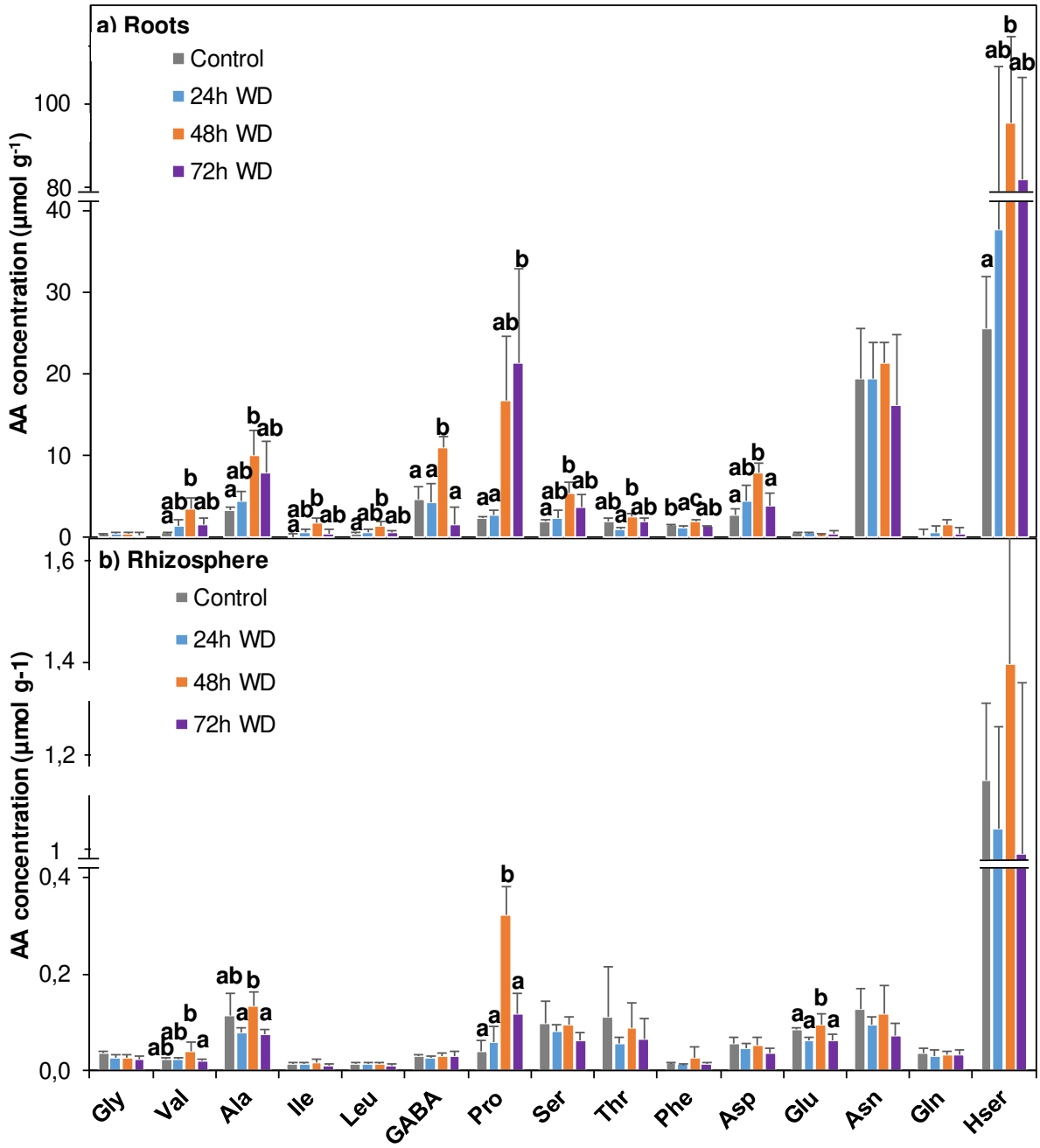

Figure 4.

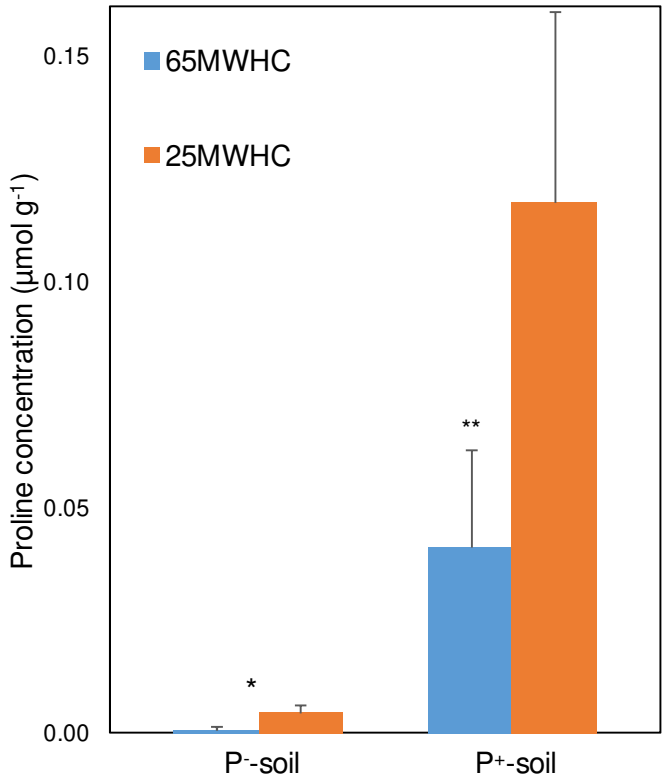
65MWHC
25MWHC
Proline concentration (µmol g-1)
0.15
0.10
0.05
0.00
*
**
P--soil
P+-soil

Figure 5

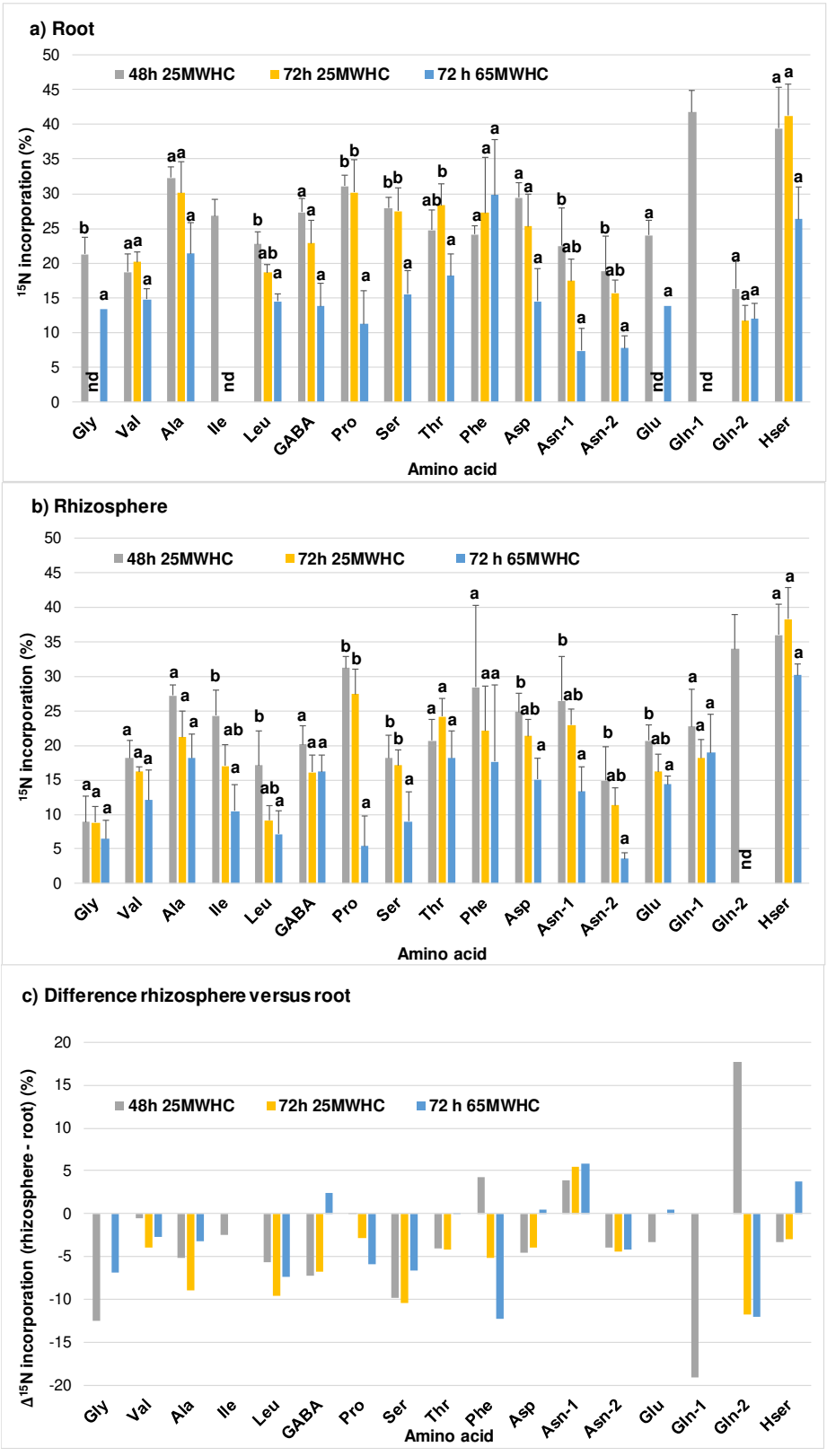
a) Root
48h 25MWHC
72h 25MWHC
72 h 65MWHC
15N incorporation (%)
Amino acid
Gly
Val
Ala
Ile
Leu
GABA
Pro
Ser
Thr
Phe
Asp
Asn-1
Asn-2
Glu
Gln-1
Gln-2
Hser
b) Rhizosphere
c) Difference rhizosphere versus root
Δ15N incorporation (rhizosphere - root) (%)

Table 1. Total nitrogen (N) content, $\%^{15}N$, nitrate (NO_3^-) and ammonium (NH_4^+) concentrations, and bacterial biomass in soil without a plant (P^--soil), in the roots of *Pisum sativum* plants (roots (P^+-soil), and associated rhizosphere (Rhizosphere (P^+-soil), following exposure to 72 h of either water deficit (WD) or not (No WD)). Values are mean±SD of at least three biological replicates.

	Compartment					
	P^--soil		Rhizosphere (P^+-soil)		Root (P+-soil)	
Conditions	No WD	WD	No WD	WD	No WD	WD
Exposure period (h)	72	72	72	72	72	72
Bacterial biomass (µg g^{-1} soil DW)	2.25±0.23 b	2.99±0.25 B*	4.30±0.36 a	3.86±0.27 A	-	-
Total N (mg g^{-1} DW)	0.95±0.13 a	1.01±0.06 A	0.78±0.04 a	0.79±0.02 A	28.53±1.68	24.64±2.49
NO_3^- (µg g^{-1} DW)	3.03±0.56 a	2.51±0.39 A	1.44±0.55 b	0.88±0.07 B	-	-
NH_4^+ (µg g^{-1} DW)	0.71±0.03 a	0.55±0.03 A**	0.84±0.04 a	0.47±0.07 A***	-	-
^{15}N (%)	0.37 a	0.37 A	0.48±0.02 a	0.50±0.05 A	8.47±0.44	10.85±0.30**

* indicates significant differences for one parameter betweenstressed and unstressed compartment of bulk soil, rhizosphere or roots (ANOVA's test, *P<0.05, **P<0.01, ***P<0.001). Letters a, b indicate significant differences between P^--soil and P^+-soil without WD, and A, B indicate significant differences between P--soil and P+-soil under WD. DW=dry weight.

Table 2. Partitioning of the 14 major amino acids as % of the total amino acid content (summed for 22 amino acids) in the roots and in the rhizosphere of *Pisum sativum* in control conditions and under water deficit (WD) for 24 to 72 h. Values are mean±SD of at least three biological replicates.

	Roots (P^+-soil)				Rhizosphere (P^+-soil)			
	Control	24 h WD	48 h WD	72 h WD	Control	24 h WD	48 h WD	72 h WD
Glycine	0.7±0.1	0.5±0.1	0.3±0.0	0.2±0.2	1.7±0.3	1.7±0.7	1.0±0.3	1.6±0.5
Valine	1.0±0.1	1.7±1.1	1.8±0.6	1.6±0.6	1.1±0.2	1.5±0.3	1.5±0.8	1.5±0.7
Alanine	4.9±0.4	6.0±2.3	5.2±1.3	8.1±3.8	5.6±2.8	4.7±1.2	4.9±1.3	5.4±1.9
Isoleucine	0.2±0.4	0.9±0.5	0.9±0.4	0.5±0.5	0.6±0.2	0.9±0.2	0.7±0.3	0.8±0.4
Leucine	0.8±0.1	0.9±0.5	0.8±0.3	0.6±0.2	0.7±0.2	0.9±0.2	0.6±0.2	0.8±0.4
GABA	6.8±1.8	6.2±5.0	5.8±1.3	1.2±1.4	1.5±0.4	1.7±0.5	1.1±0.4	2.0±0.6
Proline	3.5±0.3	3.9±1.8	8.6±3.1	19.9±5.2	1.8±0.9	4.2±2.9	11.1±3.2	8.0±3.7
Serine	2.8±0.3	3.0±1.1	2.8±0.6	3.7±1.6	4.4±1.4	4.9±0.8	3.4±0.8	4.2±0.9
Threonine	2.8±0.2	1.4±0.4	1.3±0.1	2.1±0.9	5.1±4.5	3.7±1.9	3.1±2.2	4.6±3.4
Phenylalanine	2.4±0.3	1.7±0.5	1.1±0.2	1.4±0.5	0.8±0.1	0.9±0.3	1.0±0.5	1.1±0.5
Aspartate	4.1±0.9	5.8±3.2	4.2±0.9	3.9±1.1	2.6±0.6	2.8±0.6	1.9±0.4	2.6±0.7
Glutamate	0.9±0.1	0.8±0.2	0.2±0.0	0.5±0.4	4.1±0.7	3.7±0.9	3.4±1.2	4.3±1.3
Asparagine	28.7±9.3	27.2±13.8	11.2±0.7	15.5±5.1	6.3±3.0	5.7±1.0	3.9±0.8	4.9±2.2
Glutamine	0.5±0.9	1.2±1.0	0.9±0.2	0.3±0.6	1.7±0.8	1.9±0.3	1.2±0.5	2.2±1.1
Homoserine	37.1±7.0	37.8±25.6	52.8±7.0	40.2±18.6	61.6±5.6	61.3±11.0	60.8±7.8	56.0±13.7
Total AA content (µmol g^{-1} DW)	68.7±8.7	83.5±35.6	191.6±29.7	106.5±41.0	2.1±0.4	1.7±0.5	3.0±1.1	1.7±0.9

Graphical abstract