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Plant low-K responses are partly due to Ca and the low-K biomarker putrescine does not protect from Ca side effects but acts as a metabolic regulator

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Abstract

Effects of K deficiency have been investigated for several decades and recently, progress has been made in identifying metabolomics signatures thereby offering potential to monitor the K status of crops in the field. However, effects of low K conditions could also be due to the antagonism with other nutrients like calcium (Ca) and the well-known biomarker of K deficiency, putrescine, could be a response to Ca/K imbalance rather than K deficiency. We carried out experiments in sunflower grown at either low or high K, at high or low Ca, with or without putrescine added to the nutrient solution. Using metabolomics and proteomics analysis, we show that a significant part of the low-K response such as lower photosynthesis and N assimilation, is due to calcium and can be suppressed by low Ca conditions. Putrescine addition tends to restore photosynthesis and N assimilation but but aggravates the impact of low-K conditions on catabolism. We conclude that (i) effects of K deficiency can be partly alleviated by the use of low Ca and not only by K fertilization, and (ii) in addition to its role as a metabolite, putrescine participates in the regulation of the content in enzymes involved in carbon primary metabolism.

Introduction

Potassium (K) is an essential nutrient (up to 5% of plant dry weight) involved in crucial physiological processes such as electrochemical homeostasis, stomatal aperture and enzyme catalysis (Wang & Wu, 2013; Anschütz *et al.* , 2014). K deficiency inhibits plant growth and primary production and therefore, intense efforts are being devoted to improve K acquisition by plants (Shin, 2014; Rawat *et al.* , 2016). K-deficient soils are relatively common in countries of the intertropical region, such as Brazil, Equatorial Africa, China and South-Eastern Asia (like Indonesia), and Australia (*www.fao.org*) and thus important crops like oil palm, sunflower, cotton, or rice are concerned. In addition, K-deficient areas have variable geological substratum and soil total base content (in particular exchangeable calcium), and as a result background nutrient (ionic) conditions that accompany K deficiency are highly variable. For example, in Sumatra (Indonesia) where oil palm is cultivated, both Ca-poor (histosol) and Ca-rich (cambisol) soils can be found, and such a difference in Ca has an important impact on K fertilization decisions.

Symptoms of K deficiency in plants have been studied for more than fifty years and include metabolic effects such as an inhibition of glycolysis due to a decrease in pyruvate kinase activity, as well as accumulation of typical metabolites such as putrescine (Jones, 1961; Jones, 1966; Okamoto, 1966; Freeman, 1967; Okamoto, 1967; Okamoto, 1968; Besford & Maw, 1976; Armengaud *et al.* , 2009; Hussain *et al.* , 2011; Sung *et al.*

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, 2015). Metabolomics analyses of K deficiency in*Arabidopsis* showed a reconfiguration of amino acid and organic acid synthesis, consistent with a change in nitrogen assimilation (in favour of neutral amino acids), and an inhibition of the most K-sensitive enzyme, pyruvate kinase, thereby leading to altered glycolytic metabolism (Armengaud *et al.* , 2009). Recently, metabolic changes caused by low K availability have been described in details in sunflower, and it has been shown that in addition to the well-known build-up of putrescine, low K induced a strong increase in respiratory CO_2 efflux and modified the flux through the C_5-branched acid pathway; furthermore, the natural$^{15}N/^{14}N$ isotope composition ($\delta^{15}N$) in leaf compounds showed that there was a change in nitrate circulation, with less nitrate influx to leaves under low K (Cui *et al.* , 2019a).

Importantly, as pointed out recently (Cui *et al.* , 2020), K deficiency is not associated with a general decrease, but actually leads to a significant increase in cation load, which comes from the considerable increase in Ca^{2+} (and occasionally in Mg^{2+}) in tissues. In fact, there is an antagonism between K^+ and Ca^{2+} (Dibb & Thompson, 1985; Jakobsen, 1993; Daliparthy *et al.* , 1994), and the K *x* Ca interference in fertilization has been documented for nearly 50 years in many crops (such as poplar, sugarcane, sunflower, rapeseed, tobacco, oil palm, soybean, castor bean or wheat). Also, if K-deficiency occurs under low Ca conditions, typical K symptoms such as putrescine accumulation are partly suppressed (Richards & Coleman, 1952; Coleman & Richards, 1956). Therefore, despite the role of Ca^{2+} in signaling to remobilize vacuolar K^+ ions under low K conditions (Amtmann & Armengaud, 2007; Pandey *et al.* , 2007; Tang *et al.* , 2020), some of the commonly observed K deficiency symptoms probably reflect the response to a disequilibrium in external (soil) cation composition, in which Ca^{2+} is over-represented. To our knowledge, this question has never been tackled directly. In a recent meta-analysis of papers dealing with the effect of nutrient antagonism on yield, the antagonism between K and Ca appears to be understudied as compared to K*x* Mg antagonism (Rietra *et al.* , 2017). Also, the detailed metabolic responses to K availability when Ca is varied have not been documented. In this context, putrescine is an interesting biomolecule, since its role under K deficiency has been suggested to be to both mitigate reactive oxygen species (ROS) production –and thus the down-regulation of mitochondrial damage– and participate in cellular Ca^{2+} homeostasis (Cui *et al.* , 2020).

To clarify these aspects, we cultivated sunflower plants under different K availability conditions (low, 0.2 mM, and high, 4 mM), under low or high Ca conditions, with or without addition of putrescine (experimental design detailed in Fig. S1). We carried out physiological, ionomics, metabolomics and proteomics analyses and also used^{15}N-labelling to assess nitrate absorption. Our objectives were to assess whether *(i)* some of the typical metabolic symptoms of K deficiency could actually be suppressed by the use of low Ca conditions, and *(ii)* putrescine addition alleviates some metabolic effects of K deficiency or on the contrary, triggers a low-K response. Our results show a considerable effect of Ca on metabolism, low Ca conditions compensating for some of low-K symptoms. Putrescine addition is found to improve N nutrition but exaggerates glycolysis inhibition, suggesting that it plays a dual role under K deficiency.

Material and methods

Plant material and photosynthesis

Sunflower (*Helianthus annuus* L.) var. XRQ was sown directly in sand (washed with distilled water) in the greenhouse, using 7-L pots. Growth conditions were: 12/12 h photoperiod, 25/18°C air temperature, 70/60% relative humidity day/night. Photosynthetic parameters were measured using a portable open system Li-Cor 6400 XT. Net assimilation (A) and conductance reported in Fig. 2 was obtained under saturating light (1500 µmol m^{-2} s^{-1}PAR) at 400 µmol mol^{-1} CO_2 and 21% O_2. Night respiration (R_n) was measured after photosynthesis measurements on dark-adapted leaves (30 min) at 400 µmol mol^{-1} CO_2, 21% O_2 and 25°C.

Nutrient conditions and waterlogging

The nutrient solution was after (Cui *et al.* , 2019a), where the amount of K^+ was varied by changing the amount of KCl. Two K availability conditions were used here: "low K" (0.2 mM) and "high K" (4 mM). The amount of nitrate and phosphate (in mM) in the nutrient solution was kept constant throughout experiments

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(nutrient solution composition in Table S1). Nitrate concentration of the nutrient solution was 15 mM to cover plant N needs and avoid any N-limitation effects. The amount of Ca^{2+} (low [0.4 mM], and high [4 mM]) was adjusted my changing the balance between Ca-nitrate and Na-nitrate (Table S1). Putrescine was added at a concentration of 0.4 mM. Taken as a whole, there were six nutrient conditions: low K and high Ca (LK-HCa), high K and high Ca (HK-HCa), low K and low Ca (LK-LCa), high K and low Ca (HK-LCa), low K and high Ca with putrescine addition (LK-HCa+P), and high K and high Ca with putrescine addition (HK-HCa+P). It should be noted that here, the condition low Ca + putrescine (under either low or high K) has not been used because the objective was to assess whether putrescine addition can compensate for the effects of high Ca. In figures, high Ca, low Ca and high Ca + putrescine conditions are shown with blue, green and pink shades, respectively.

Experimental design and sampling

The experimental design is schematized in Fig. S1a. After emergence (1 week after sowing), plants were cultivated for 2 weeks under specific nutrient conditions (described above). Three weeks after emergence, plants had one true leaf pair, and after four weeks, there were two leaf pairs. These two pairs are referred to as "young" and "old" (Fig. S1b). There were two sampling campaigns: sampling 1 two weeks after emergence (3 weeks old plants), and sampling 2 three weeks after emergence (4 weeks old plants). Two weeks and four days after emergence, half of the plants were labelled with ^{15}N-nitrate (nutrient solution of exactly the same composition, with just nitrate replaced to its ^{15}N form) while the other half was kept at natural abundance. The day before sampling, photosynthesis and dark respiration (reported in Fig. 2) were measured on the same plants. Upon sampling, plants were measured for size, fresh weight, leaf surface, and dissected and kept in liquid nitrogen for analyses. Roots were sampled after having removed sand and washed with water. In practice, roots were washed, rapidly dried with absorbing paper and quenched in liquid nitrogen within 2-3 min. Extractions for metabolomics and proteomics analyses were performed on fresh material. Isotope and ionomics analyses were performed on freeze-dried material.

Omics

Ionomics and metabolomics were performed ICP-OES and by GC-MS, respectively, as in (Cui *et al.* , 2019a). Proteomics analysis was carried out as in (Cui *et al.* , 2019b) on total protein obtained by trichloroacetate-acetone extraction. Peptide identification and quantitation was done using the same workflow, except that protein abundance reported here are from extracted chromatograms (XIC). Details are provided in Supplementary methods S1.

Isotope analysis and %N

Freeze-dried samples (powdered total organic matter) were weighed in tin capsules, and δ^{15}N values and %N were measured using an elemental analyser (Carlo-Erba) coupled to isotope ratio mass spectrometer (Isochrom, Elementar) run in continuous flow. All sample batches included standards (glycine, +0.66previously calibrated against IAEA standards glutamic acid USGS-40 and caffeine IAEA-600) each ten samples.

Statistics

Five replicates were done for all conditions for ionomics, isotopes, metabolomics and four replicates were done for proteomics. Supervised multivariate analysis of omics data was carried out by orthogonal projection on latent structure (OPLS) (Bylesjö *et al.* , 2007) with Simca (Umetrics), using K, Ca and putrescine as predicted Y variables and metabolites (or proteins) as predicting X variables. The absence of statistical outliers was first checked using a principal component analysis (PCA) to verify that no data point was outside the 99% confidence Hotelling region. The goodness of the OPLS model was appreciated using the determination coefficient R^2 and the predictive power was quantified by the cross-validated determination coefficient, Q^2. The significance of the statistical OPLS model was tested using a χ^2 comparison with a random model (average ± random error), and the associated P -value ($P_{\text{CV-ANOVA}}$) is reported (Eriksson *et al.* , 2008). Best discriminating features were identified using volcano plots whereby the logarithm of the P -value obtained in univariate analysis (two-way ANOVA; factors used: K x Ca or K x putrescine) was plotted

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against the rescaled loading (p_{corr}) obtained in the OPLS. In such a representation, best biomarkers have both maximal –log(P) and p_{corr} values. Univariate analysis of statistical classes in bar plots was performed using a two-way ANOVA (Fisher statistics), with a threshold of P as indicated in figure legends. When all conditions were compared at once, a one-way ANOVA was conducted.

Results

Elemental composition

The effect of nutrient conditions was first checked on leaf and root elemental composition analyzed by ICP-OES (Fig. 1). As expected, there was a clear and substantial effect of K conditions on tissue K content in all organs. K conditions impacted on tissue Ca, Na, Mg demonstrating the antagonism between major cations (Fig. 1a-c). But conversely, there was no effect at all of Ca (and putrescine) on K tissue content. Low Ca conditions were associated with lower Ca content in all tissues and this was compensated for by higher Na content. There was little effect of K on micronutrients, except for Fe which appeared to be more abundant at low K in roots (other micronutrients are not shown). Taken as a whole, when all cations contents were used to calculate the total positive charge (cation charge balance), there was no effect of K and Ca conditions in roots. By contrast, there was a very clear effect in leaves which exhibited a much higher total cation charge balance at low K (Fig. 1d).

Physiological parameters

Total biomass, leaf photosynthesis (under standard conditions) and respiration were also measured to appreciate the general impact of nutrient conditions on carbon assimilation (Fig. 2). After 3 weeks under high Ca, shoot and root biomass was significantly affected by K conditions. Conversely, the effect of Ca and putrescine was modest under high K. After 4 weeks, the effect of K conditions was very clear, with no effect at all of Ca and putrescine in shoots. In roots, low Ca and putrescine increased biomass very slightly (Fig. 2a). Net CO_2 assimilation was significantly lower at low K under high Ca, and this effect disappeared at low Ca in young leaves (Fig. 2b). Putrescine was also beneficial to photosynthesis and suppressed the effect of low K conditions in young leaves. In old leaves, K availability always had an effect on photosynthesis, and putrescine increased photosynthesis only slightly under low K. In old leaves, changes in photosynthesis were mostly caused by the effect of nutrient conditions on conductance, the variations of which paralleled that of photosynthesis (Fig. 2c). In young leaves, the effect of nutrient conditions (K in particular) on conductance was not mirrored by photosynthesis showing that photosynthetic capacity was also affected. As expected, low K was associated with higher respiration rates (Fig. 2d). Surprisingly, low Ca conditions further increased respiration rates in all leaves.

Nitrogen assimilation

Nitrogen assimilation was examined using elemental N content analysis and ^{15}N-nitrate labelling to measure nitrate absorption and distribution in shoots and roots (Fig. 3). In all tissues, N elemental content was significantly lower at low K, showing the general inhibition of N assimilation under low K availability (Fig. 3a). Low Ca conditions resulted in slightly lower N content in old leaves under high K. Putrescine was beneficial to the N content in young leaves under low K. N absorption measured via^{15}N-labelling was much lower under low K, and this was alleviated to some extent by putrescine. Interestingly, low Ca was beneficial to N absorption at low K but detrimental at high K, suggesting that the balance between cation species was essential for nitrate absorption by root cells.

Metabolic signature of nutrient conditions

The metabolic impact of nutrient conditions was analyzed using GC-MS profiling. 178 (leaves) and 187 (roots) analytes were identified and quantified. The multivariate statistical analysis by OPLS allowed facile discrimination of samples in both leaves (Fig. 4a) and roots (Fig. 5a). The multivariate statistical model was good and robust as shown by high R^2 and Q^2 values (0.94 and 0.87, respectively, in both leaves and roots), and highly significant ($P_{\text{CV-ANOVA}}<10^{-30}$ (K), $< 10^{-22}$ (Ca) and $<10^{-11}$(putrescine)). Axis 1 was driven by K conditions, while Ca and putrescine conditions were driven by both axes 2 and 3, showing some interaction

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in their metabolic effect. A specific analysis of putrescine content is provided in Fig. S2. When all samples were pooled together (old and new leaves), putrescine addition was not found to cause an important increase in tissue putrescine pool in leaves (on average, × 2) and roots (on average, × 1.25). In roots, putrescine was 2-fold lower under low Ca.

In leaves, low K conditions were associated with many highly significant changes, typically sugar (arabinose, ribose), putrescine, citrate and ascorbate accumulation and a decrease in glucose 6-phosphate, inorganic phosphate (Pi), threonate, amino acids (glutamate, aspartate) and their derivatives (cystathionine, β-alanine) (Fig. 4b). Ca conditions were not associated with many significant metabolites and only one metabolite increased by high Ca was above the Bonferroni threshold, tartarate (reduced form of oxaloacetate) (Fig. 4c). Low Ca was associated with an accumulation of organic acids of the tricarboxylic acid pathway (TCAP), such as citrate, isocitrate, fumarate and aconitate. Under low Ca, there was also more Pi and glutamine, suggesting a beneficial effect of low Ca on N and P nutrition. Very few metabolites were affected above the Bonferroni threshold by putrescine addition (Fig. 4d): putrescine homoserine and serine (increased) and cellobiose and gulonate (decreased). Interestingly, with a P -value lower than 0.001, tartarate appeared to be decreased by putrescine (thus counteracting the effect of high Ca), while sucrose increased.

Like in leaves, many metabolites were significantly affected by low K conditions in roots, including sugars (maltose, xylose, allose, gentiobiose), putrescine and ascorbate (increased), and aspartate, threonate, cystathionine, β-alanine (decreased) (Fig. 5b). Also, low Ca led to an accumulation in organic acids (glutarate, isocitrate, succinate, aconitate, citrate, citramalate) (Fig. 5c). Interestingly, spermidine (derivative of putrescine) was found to increase at high Ca. Very few metabolites were affected by putrescine addition: putrescine, glutamine, asparagine and methylaspartate (increased), and glutamine, fumarate, xylulose, glucuronate (decreased) (Fig. 5d).

Despite the similarities in the response to K, Ca or putrescine, metabolites associated with a significant K x putrescine effect were different in leaves and roots. Typically, under low K in leaves, putrescine addition led to a decrease in several sugars, polyols and shikimate, and an increase in glycolate (and, of course, putrescine) (Fig. 4e). In roots, putrescine addition decreased isoprenoids (phytol, tocopherol), sugars and sugar derivatives (2-oxogluconate, rhamnose, xylulose) (Fig. 5e).

Proteome changes caused by nutrient conditions

Proteomics analyses were carried out, and the full list of significant proteins in tabulated in Table S2. A summary showing proteins involved in primary CNS metabolism is provided in Table 1. As expected, in leaves, many proteins involved in photosynthesis and photorespiration (Rubisco, Rubisco activase, phosphoribulokinase, serine glyoxylate aminotransferase, etc.) were significantly affected by K conditions, showing the effect of K availability on the development of the photosynthetic machinery. Importantly, key enzymes of catabolism were more abundant under low K (isocitrate dehydrogenase, aconitase, malic enzyme, pyruvate dehydrogenase, etc.) showing the up-regulation of respiration. Also, low K was associated with an increase in carbamoyl phosphate synthase (required for arginine and polyamine synthesis), and a decrease in pyruvate kinase. The decrease in pyruvate kinase content reflects the down-regulation of this K-dependent metabolic step when K is limiting. In roots, a small number of proteins were significant, and included also pyruvate kinase and proteins directly involved in N assimilation (nitrate transporters, glutamine synthetase), all decreased under low K.

High Ca impacted negatively on pyruvate kinase and several enzymes of photosynthesis in leaves. In roots, only two enzymes had aP -value above the Bonferroni threshold and these included pyruvate kinase (decreased at high Ca). Putrescine addition caused an increase in the content of enzyme involved in photosynthesis and glucose oxidation (glucose/ribitol dehydrogenase and glucose 6-phosphate dehydrogenase) in leaves. In roots, putrescine addition caused a decline in proteins involved in nitrogen assimilation: nitrate transporters, aspartate aminotransferase and glutamine synthetase.

Effect of nutrient conditions on selected proteins

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A focus on enzymes involved in pyruvate metabolism is provided in Fig. 6. As mentioned above and shown in Table 1, pyruvate kinase was down-regulated under low K and this effect could be alleviated by low Ca conditions in both roots and leaves. Putrescine addition tended to increase the content in pyruvate kinase under low K but decreased it at high K. For other enzymes (NADP-dependent malic enzyme, aconitase, phospho*enol* pyruvate carboxylase), both low Ca and putrescine tended to attenuate the increase in protein content observed under low K in leaves. In roots, low Ca has little effect on these enzymes while putrescine increased their content at high K.

Transporters and channels that were both detected and associated with significant changes are shown in Figs. S3 and S4. As expected, a high affinity K transporter was induced at low K in roots, and in both leaves and roots, the subunit β2 of the voltage dependent aldo/keto reductase-potassium channel was downregulated at low K + high Ca and upregulated at low K + low Ca. In leaves, low K caused a decline in two ABC cassette containing proteins (TAP1 and cABCI6) and an ammonium/urea transporter, suggesting changes in nitrogen/protein homeostasis. In roots, nitrate transporters were upregulated at low K + high Ca, high K + low Ca and were clearly downregulated by putrescine.

The two-way ANOVAs carried out to look at significant proteins were used to visualize proteins associated with specific interactions effects (Fig. S5). In the group of roots proteins upregulated under high K + low Ca, proteins involved in nitrogen assimilation appeared clearly (group 1, Fig. S5a). In leaves, some enzymes of catabolism (isocitrate dehydrogenase, aconitase) were specifically enhanced at low K + high Ca (group 1, Fig 5b). Interestingly, many proteins of the photosynthetic machinery (including magnesium chelatase, Rubisco and proteins of the chloroplastic electron transfer chain) were downregulated in this specific condition, suggesting that the effect of low K was mostly driven by high Ca (group 2, Fig. 5b). K x putrescine interaction analysis clearly showed that, as found above, the downregulation of pyruvate kinase was strong under low K + high Ca, and that putrescine addition minimized differences between low K and high K for key catabolic enzymes (Figs. 5c-d).

Discussion

Response mechanisms to K deficiency have been extensively studied and include changes in metabolism (such as putrescine build-up), gene expression and physiological parameters such as photosynthesis, chlorophyll synthesis and growth. Our study is broadly consistent with documented effects of low K conditions on metabolism (Ashley *et al.* , 2006; Armengaud *et al.* , 2009; Wang & Wu, 2013). Typically, there were *(i)* a general decline in photosynthesis due to lower stomatal conductance and a down-regulation of the biosynthesis of the photosynthetic machinery; *(ii)* an inhibition of nitrate absorption and assimilation; and *(iii)* changes in respiratory metabolism, with higher respiration rates and higher content in several catabolic enzymes (such as malic enzyme) despite an inhibition of glycolysis due to lower pyruvate kinase activity. The typical inhibition of pyruvate kinase by low K conditions comes from the fact that this enzyme uses K^+ as a cofactor (Evans, 1963; Nowak & Mildvan, 1972) but also, as found here, a lower enzyme abundance as also found in tomato (Besford & Maw, 1976). Here, we have carried out a dissection of metabolic responses to K availability when it interacts with calcium and putrescine to better understand specific pathways affected by K itself, and effects of Ca/K balance or putrescine accumulation.

The response to low K is partly driven by Ca

A common drawback in manipulating nutrient composition is the potential change in other cations due to electro-neutrality, making overall mineral conditions not comparable (Cramer *et al.* , 1986). Here, we have used low and high Ca conditions that were compensated for by sodium (Na) for the chemical form of nitrate. However, low Ca conditions were not associated with a drastic augmentation of the Na content in tissues and thus an onset of salt stress: in fact, (*i*) Na always remained a minor component in leaves; and *(ii)* in roots, Na was increased by about 20% only under low Ca. Therefore, the effects we found here under low Ca conditions were not caused by salinity.

Our results clearly demonstrate that amongst effects observed under low K conditions, the decline in photosynthesis was in reality partly due to Ca. In fact, in young leaves, low Ca conditions could restore some

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photosynthetic activity (while it was not so in old leaves) (Fig. 2). In addition, the decline in the content of many proteins involved in photosynthesis could be compensated for by low Ca conditions (Fig. S5b). Surprisingly, low Ca aggravated the increase in leaf dark respiration triggered by low K conditions (Fig. 2), in agreement with TCAP intermediates being higher under both low K and low Ca (Fig. 4), whereas some proteins of respiratory metabolism were more abundant under low K + high Ca, not low K + low Ca (such as aconitase, Fig. 6). This contradiction is perhaps explained by *(i)* changes in post-translational modifications, *(ii)* the action of effectors on enzymes and/or *(iii)* a lower ATP/O_2 efficiency of mitochondrial metabolism. In particular, we note that low Ca conditions led to a decline in Mg leaf content, and in principle, this must have affected mitochondrial-cytosolic ADP/ATP exchange and interconversion (Bligny & Gout, 2017).

It is worth noting that unlike photosynthesis, low Ca conditions did not compensate for the alteration of N metabolism at low K. In fact, low Ca aggravated the decline in the content of root proteins involved in N metabolism (Fig. S5a) and increased the content in non-aminated precursors (2-oxoglutarate) (Fig. 5c). The reverse was true at high K, where low Ca upregulated the content in proteins involved in root N metabolism, including nitrate transporters. However, nitrate assimilation measured using ^{15}N-nitrate was increased by low Ca under low K conditions, and decreased by low Ca at high K (Fig. 3). This surprising result likely reflects the fact that under K-deficient conditions, the general effect of low K impacts not only on root development and protein synthesis but also on root cation balance (increase in Ca^{2+} but low content in unicharged species) and this leads to a decline in nitrate capture by root cells. In fact, Ca^{2+} addition has been found to be detrimental to nitrate influx in root cells (Kafkafi *et al.* , 1992; Aslam *et al.* , 1995). Accordingly, here^{15}N-nitrate absorption was found to be low. When low Ca conditions are used, Na^+ can partly substitute for K^+ (Fig. 1) and this restores nitrate absorption, thus downregulating the synthesis of high affinity nitrate transporters.

Under high K conditions, such a role for Na^+ ions is unnecessary and essentially, nitrate absorption is accompanied by K^+ absorption. In cotton grown at 4 mM $[K^+]$, increasing $[Ca^{2+}]$ from 2 to 10 mM has only a small effect on nitrate (and K) absorption (Leidi*et al.* , 1991). Also in cotton grown at 2.5 mM $[K^+]$ in the presence of NaCl, low $[Ca^{2+}]$ (<0.25 mM) inhibits nitrate absorption (Gorham & Bridges, 1995). Similarly, nitrate absorption by wheat seedling is inhibited by Ca deficiency (Minotti *et al.* , 1968). K and nitrate absorption requires sufficient Ca^{2+} to maintain transmembrane electrochemical gradient and therefore, low Ca has a negative impact on nitrate absorption. It is also interesting that low Ca conditions at high K inhibited nitrate translocation to shoots (Fig. 3), probably due to similar ion imbalance in xylem sap loading and electroneutrality.

Can putrescine alleviate effects of K deficiency?

Putrescine addition partly alleviated the effects of low K on glycolysis (regulation of pyruvate kinase content under low K) (Figs. 6, S5c-d), photosynthesis (Fig. 2) and N assimilation (Fig. 3), and typically increased root glutamine and asparagine content (Fig. 5). However, putrescine did not suppress the increase in leaf respiration at low K (Fig. 2) despite the down-regulation of several enzymes involved in respiratory metabolism (Fig. S5d). Diverse mechanisms have been suggested to explain why putrescine is accumulated under low K, including the regulation of mitochondrial ion channels to avoid excessive oxidative damage and mitochondrial permeability transition (Cui *et al.* , 2020). Here, the addition of putrescine did not have a clear effect on mitochondrial proteins, except for alternative NAD(P)H dehydrogenase in roots (Table 1, Fig. S5).

The fact that putrescine (*i*) had rather contrasted effects (different effects in roots and leaves, e.g. Fig. 6), and *(ii)*caused a decline in pyruvate kinase content at high K, suggests that putrescine is not a molecule that only compensates for cellular K scarcity but rather, is involved (or one of its products) in low-K signaling, perhaps including root-to-shoot signaling. It has been proposed that putrescine regulates metabolism via an increase or a decrease in reactive oxygen species (ROS) depending on its concentration and the potential involvement of polyamine oxidase, which generates H_2O_2 (Verma & Mishra, 2005; Zepeda-Jazo*et al.* , 2011; Shelp *et al.* , 2012; Zhang *et al.* , 2014). It is worth noting that if putrescine is effectively oxidized, this can interact directly with Ca and K absorption since K efflux and Ca influx in root cells have been found to

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be regulated by ROS (Demidchik *et al.* , 2003). Here, we did not find any protein annotated as polyamine oxidase but found two peptides associated with copper amine oxidase (also referred to as diamine oxidase; CAO). Interestingly, putrescine addition caused an increase in CAO in roots but not in leaves (Fig. S6). It is plausible that putrescine addition thus led to an increased production of NH_4^+ and H_2O_2 in roots, thereby triggering ROS signaling and improving N nutrition (higher glutamine and asparagine content in roots). In leaves, the most significant metabolites under putrescine addition were serine and homoserine. Putrescine oxidative deamination produces γ-aminobutyrate semialdehyde further oxidized to GABA (Shelp *et al.* , 2012), which can be in turn incorporated into the TCAP via the GABA shunt and thus feed the synthesis of asparate (precursor of homoserine). That said, the simultaneous increase in serine and homoserine also likely reflects the regulation of sulfur (S) metabolism by putrescine (serine and homoserine are precursors of cysteine and methionine, respectively) (illustrated in Fig. S7).

In effect, low K conditions reconfigured S metabolism. Under low K availability, the sulphur elemental content has been found to change significantly in sunflower (increase in leaves, decline in roots) (Cui*et al.* , 2019a) and in Arabidopsis, the tissue content in sulphate, cysteine and O -acetylserine decrease strongly (Forieri*et al.* , 2017). Here, we observed at low K an increase in cysteine metabolism (increased cysteine synthase, Table 1, lower cystathionine content, Fig 4) at the expense of methionine metabolism (decreased methionine synthase and SAM synthase, Table 1, lower methionine-to-cysteine ratio, Fig. S7). That is, cysteine was consumed to synthesize homocysteine (trans-sulfuration onto homoserine), which was then recycled back to cystathionine by cystathionine β-synthase (CBS), thereby consuming serine and avoiding methionine synthesis. Such an effect of low K on S metabolism is very likely a consequence of the inhibition of SAM synthase catalysis, which has been shown to be K^+-dependent (Takusagawa *et al.* , 1996). Parenthetically, the inhibition of SAM synthase further exaggerates putrescine accumulation since the conversion of putrescine to other polyamines requires SAM. It is also interesting to note that homocysteine production liberates pyruvate (Fig. S7). Therefore, homocysteine production and recycling to cystathionine represents an alternative pathway for pyruvate production (from serine). This is clearly advantageous under low K conditions where pyruvate kinase is inhibited.

Quite critically, the effect of low K on cysteine metabolism appeared to be modulated by Ca and putrescine: low Ca led to an increase in cystathionine β-synthase in leaves, and putrescine addition caused a decline in cysteine synthase in roots (Table 1). This suppression effect by putrescine was probably sufficiently strong to explain why both serine and homoserine increased. Also, putrescine impacted modestly on enzyme content in leaves (the effect of putrescine and K $x$putrescine on SAM synthase and CBS was associated with a P -value ($3.64\ 10^{-5}$ and $4.5\ 10^{-5}$, respectively) just above the Bonferroni threshold ($3.54\ 10^{-5}$), Table S2).

Perspectives

Taken as a whole, our results show that amongst typical symptoms of K deficiency, the alteration of photosynthesis can be in part compensated for by low Ca nutrition, via the upregulation of proteins involved in the photosynthetic machinery. Similarly, the inhibition by low K of pyruvate kinase could be partly alleviated by low Ca conditions. By contrast, low Ca could not compensate for changes in N assimilation, likely due to complicated interactions with other ions (Na^+) and transmembrane potential. Low Ca down-regulated the amount of putrescine in roots only. Putrescine itself was able to alleviate some effects of low K such as the reconfiguration of S metabolism, but exaggerated other effects (pyruvate kinase content) suggesting a role of putrescine in signaling.

Other consequences of K and Ca availability have not been extensively discussed here, such as the modulation of micronutrients: (i) iron (Fe) content and iron-dependent enzymes (2-oxoglutarate Fe^{2+}-dependent, ferrochelatase, ferritin) are affected by K and/or Ca level (Figs. 1, S5), and (ii) magnesium is affected by both K and Ca (magnesium content, Fig. 1; magnesium chelatase, Fig. S5). Of course, a detailed analysis of micronutrient absorption and allocation would require further analyses, including the use of isotopic tracers (^{54}Fe,^{25}Mg). Similarly, more work would be required to better understand the specific metabolic effects of putrescine, using isotopically labelled putrescine (^{15}N or^{13}C) in particular to identify key reactions and quantify putrescine catabolism to NH_4^+(via CAO) and CO_2 (via the TCAP).

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Our results also raise the question as to whether low Ca conditions or putrescine addition are viable solutions to alleviate some effects on K deficiency in crops. Although our results are limited to young sunflower plants under controlled conditions, it seems that none of these two possibilities is ideal. In fact, low Ca further increases respiration and thus the overall impact on carbon mass balance might not be beneficial. Putrescine addition is costly ([?] \$1 g^{-1}) and does not suppress the strong effect of low K on respiratory efflux (Fig. 2). Therefore, monitoring plant K status using new technologies such as metabolomics (Cui *et al.* , 2021), and sustainable K fertilization might be better options to ensure optimal K nutrition for crops in the field.

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Supplementary information

Methods S1. Proteomics analyses.

Table S1. Summary of nutrient conditions.

Table S2. List of significant proteins.

Fig. S1. Overview of the experimental design.

Fig. S2. Summary of putrescine content in leaves and roots.

Fig. S3. Significant transporters and channels in leaves.

Fig. S4. Significant transporters and channels in roots.

Fig. S5. Proteins associated with a significant K x Ca and Kx putrescine interaction.

Fig. S6. Relative content in copper amine oxidase.

Fig. S7. S assimilation pathway, and methionine and cysteine content.

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