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How do phosphorus, potassium and sulphur affect plant growth and biological nitrogen fixation in crop and pasture legumes? A meta-analysis

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ABSTRACT

Worldwide, legumes are grown on approximately 250 M ha and fix about 90 Tg of N_2 per year. Plants involved in biological nitrogen fixation (BNF) are particularly sensitive to deficiencies of phosphorus (P), potassium (K) and sulphur (S). These nutrients can affect BNF directly; this is modulating growth of rhizobia, nodule formation and functioning, or indirectly by affecting the growth of the host plant. However, several process and mechanisms remain unclear. We compiled a data set (63 studies) on the effects of P, K, and S deficiency on shoot mass, nodule mass and number, nitrogenase activity (estimated by the acetylene reduction activity test, ARA) and the concentration of N, P, K and S in shoots and nodules. Our aims were (1) to compare the relative sensitivity of these traits to nutrient deficiency and (2) to probe for nutrient-specific patterns in trait responses.

Our quantitative analysis confirms that nodule growth and number are more sensitive than shoot mass in response to deficiency of P, K and S. In addition, nodule activity decreases more than both shoot and nodule mass, which indicates a reduction in nodule productivity; this is likely related with direct effects of these nutrients on physiological and metabolic processes of nodules. The conserved shoot N concentration, in comparison to concentration of P, K and S indicates a relatively greater accumulation of N that matches the proposed N-feedback mechanism down-regulating BNF in nutrient-deficient systems. Despite some nutrient-specific differences, i.e. smaller nodules and higher N/K ratio with shortage of P and K, respectively, the patterns of growth, nodule activity and nutrient concentration were similar for all three nutrients P, K and S. This indicates that a unique mechanism could be depressing BNF (N-feedback) in conjunction with direct effects of the nutrients on nodule activity.

Scarcity of data related to N, K and S concentration in nodules is a major constrain for deep analysis of the deficiencies of the nutrients. Critical concentrations of P, K and S in plant and nodule tissues are also a major gap. Models are needed that integrate the direct effect of the nutrients on nodule growth and activity with the N-feedback mechanism.

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1. Introduction

Worldwide, legumes are grown on approximately 250 M ha and fix about 90 Tg of N_2 per year (Kinzig and Socolow, 1994). Biological nitrogen fixation (BNF), offers a series of advantages over nitrogen (N) fertilizer, including higher efficiency in the utilization of N by the plant, the minimization of N leaching, and the reduction of soil and water contamination (Peoples et al., 1995). Moreover, BNF has beneficial effects for subsequent crops in rotations and for non-legumes in mixed stands, hence its importance for maintaining productivity in many agricultural systems (Graham and Vance, 2000). However, BNF is particularly sensitive to environmental stress like nutrient deficiency.

Phosphorus (P) is, after N, the most limiting nutrient for crops (Vance et al., 2000). Crop yield is limited by P availability in about 40% of the world's arable land, so studies on the response of legumes and rhizobia to P fertilization have received considerable attention (Almeida et al., 2000; Høgh-Jensen et al., 2002; Olivera et al., 2004; Schulze, 2006). To a lesser extent, research has focused on legume-rhizobia responses to potassium (K), which is also deficient in many soils (Römheld and Kirkby, 2010). The effect of sulphur (S) supply on BNF has received less attention, as deficits of this nutrient are less frequent in agricultural soils. Owing to S depletion in some soils,

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however, crop S deficiencies have been reported with increasing frequency (Scherer, 2001).

Legumes that acquire N by BNF generally have a higher requirement of P, K and S than those which only relay on soil N (Israel, 1987; Sulieman et al., 2013). These nutrients can affect BNF directly; this is by modulating nodule growth, nodule formation and functioning (Duke et al., 1980; Vadez et al., 1996; Pacyna et al., 2006), or indirectly by affecting the growth of the host plant (Duke et al., 1980; Almeida et al., 2000; Varin et al., 2010). Owing to the high ATP requirements for nitrogenase function, P availability is critical for nodule activity (Ribet and Drevon, 1995; Al-Niemi et al., 1997); and it also plays a role in signal transduction, membrane biosynthesis, and nodule development and function (Al-Niemi et al., 1997). Duke et al. (1980) demonstrated a direct effect of K status on BNF mediated by its influence in nodule growth and function, activity of enzymes involved in ammonia assimilation, amino acid interconversions, carbon supply and energy transduction. Varin et al. (2010) showed a close relationship between S supply and nitrogenase and leghaemoglobin content in nodules. Scherer et al. (2008) determined that S deficiency reduced BNF in pea (*Pisum sativum* L.) and lucerne (*Medicago sativa* L.) as a consequence of decreased ferredoxin and leghemoglobin concentrations as well as reduced ATP supply. Besides the direct impact of P, K and S in these aspects of carbon and N metabolism, it is generally accepted that their main effect on BNF is mediated by responses of host plant growth (Almeida et al., 2000; Høgh-Jensen, 2003; Varin et al., 2010). In this sense, when nutrient deficit reduces plant growth, an N-feedback is triggered that down-regulates nodule development and activity. This mechanism seems also responsible for the regulation of BNF when other stresses, such as drought, salt, toxic metals and pathogen attack, are involved (Lea et al., 2007).

The effects of P, K and S availability on plant legume growth and BNF are therefore biologically interesting and agronomically relevant, and a range of direct and indirect effects have been identified. However, several processes remain unclear and no attempts have been made to quantitatively consolidate the fragmented information on the effects of nutrients on BNF related traits. We compiled a data set on the effects of nutrient deficiency on shoot mass, nodule mass and number, nitrogenase activity and the concentration of N, P, K and S in shoots and nodules. Our aims were (1) to compare the relative sensitivity of these traits to nutrient deficiency and (2) to probe for nutrient-specific patterns in trait responses.

2. Method

2.1. Data sources

We compiled papers searching the Web of Knowledge^{SM} (Thomson Reuters, 2013) database for "nitrogen fixation + phosphorus/potassium/sulphur" with alternative formats. Some of the papers were also obtained searching in the reference lists of those found in the Web of Knowledge^{SM} database. We included both field and controlled environment experiments. We excluded studies with non-cultivated species, where plants were not inoculated with the specific symbiotic rhizobium, where nutrient supply was confounded with other sources of experimental variation, where N rate or source could distort the interpretation of the results, or where P, K or S rate was reported as toxic. Only experiments which established P, K and S treatments at early plant growth stages were considered; experiments with an abrupt withdraw of nutrients in late stages or where plants received differential rates of nutrients to relief deficiencies were excluded. We retrieved 124 papers, and the final database that satisfied our inclusion criteria consisted of 36 experiments on P, 15 experiments on K and 12 experiments on S (Table 1). We focused on traits that are relevant to plant and rhizobia growth and function, and are often reported in the literature, including shoot mass, nodule mass and number, nitrogenase activity (estimated by the acetylene reduction activity test, ARA) and the concentration of N, P, K and S in shoots, roots and nodules. The estimation of BNF through ARA was selected because it was the most abundant in the retrieved papers. Some authors noticed some problems of this assay related to an acetylene-induced decline in nitrogenase activity over the time (Michin et al., 1983). To account for this, we only considered determinations done with less than 60 min incubations. In general, nutrient stress was higher in controlled conditions due to a better control of nutrient provision. This was the main difference we detected between growing conditions; no attempt was made to use this as a criterion to data classification in the analysis.

2.2. Statistical analysis

To account for differences between growing conditions (e.g. field, pots, etc.), species and other sources of variation, all traits were normalized as the ratio between the actual value for each trait and the maximum in the experiment. If secondary sources of variation were included in the experiment (e.g. soil moisture), the ratio was calculated for each level of the secondary variable. The ratios were ln-transformed before analysis (Hedges et al., 1999) but untransformed data are presented for easier interpretation. The normality and residual distributions were tested.

To compare the relative sensitivity of these traits to nutrient deficiency, we carried out pair-wise comparisons of traits for their responses to P, K and S deficiency; hence, data points where traits in both x and y axis were equal to 1, often treatments with no nutrient deficiency, were excluded from statistical analyses. The null hypothesis was that both traits in a pair (e.g. nodule mass vs. shoot mass) were equally responsive to nutrient deficit. Graphically, the null hypothesis is represented by scatter plots aligned with the $y = x$ line. Deviations of data from the $y = x$ line were calculated. Statistically, a t-test ($P < 0.05$) returning no significant deviations from $y = x$ supports the null hypothesis, significant positive deviations indicated that trait "y" is less responsive than trait "x", and significant negative deviations indicate that trait "y" is more responsive than trait "x". To probe for nutrient-specific patterns in trait responses, we used an F-test to compare traits deviations in response to P, K and S deficiency. When F-test was significant, Least Significant Difference (LSD) at the 0.05 level was calculated. Normality, t- and F-tests were all performed with the R program (R Development Core Team, 2009); SigmaPlot (version 11.0, Systat Software Inc., San Jose, CA, USA) was used to draw box plots.

3. Results and discussion

3.1. Shoot and nodule growth

Fig. 1 shows that nodule mass is more sensitive to nutrient deficit than shoot mass as most data points are in the right area below the $y = x$ line. The patterns for P, K and S were similar. For P and S, we hypothesize that this differential sensitivity is related to the greater concentration of nutrients in nodules compared with that in shoots or roots (Table 2) which may indicate a greater nutrient need to maintain high nodule growth rates. Qiao et al. (2007) provides direct evidence to support this hypothesis, as the critical P concentration for peak nodule growth of 5 mg P g DM^{-1} compares with the critical concentration of 1.5 mg P g DM^{-1} for maximum shoot growth in soybean (*Glycine max* (L.) *Merr.*). Similar thresholds were proposed for white lupinus (*Lupinus albus* L.), which lie between 3.1 and 1.1 mg P g DM^{-1} for shoots and 6.5-3.3 mg P g DM^{-1} for nodules (Schulze, 2006). Tang et al. (2001) reported

Table 1
Summary of experiments used in the analysis of plant-rhizobia responses to nutrient deficit.

Nutrient[a]	*Legume*	Culture	Date of measurements[b]	Other sources of variation	References
P (4)	*Glycine max* (L.) Merr.	Field	Physiological maturity	K rate	Abbasi et al. (2012)
P (4)	*Cajanus cajan* (L) Millsp.	Field	78 DAP	Genotypes	Adu-Gyamfi et al. (1989)
P (2)	*Vigna unguiculata* (L.) Walp.,	Hydroaeroponic and rhizotrons	45 DAP	Genotypes	Alkama et al. (2009)
P (4)	*Trifolium repens* L.	Pots w/quartz sand	55 DAP	Atmospheric CO_2 level	Almeida et al. (2000)
P (2)	*Vigna unguiculata* (L) Walp.	Pots w/soil and sand	96 DAP	Genotypes and inoculation (nc[c])	Ankomah et al. (1996)
P (2)	*Phaseolus vulgaris* L	Pots w/soil	Flowering	Genotypes	Araújo et al. (1997)
P (3)	*Phaseolus vulgaris* L.	Field	Harvest	Genotypes	Attar et al. (2012)
P (4)	*Glycine max* (L.) Merrr.	Field	Mid flowering	Inoculation methods	Bekere and Hailemariam (2012)
P (3)	*Phaseolus vulgaris* L	Pots w/soil	30 and 65 DAP	Soil moisture	Bonetti et al. (1984)
P (2)	*Vigna aconitifolia* (Jacq.) Marechal, *Vigna radiata* (L.) *R.*Wilczek and *Glycine max* (L.) Merr.	Hydroponic	56 DAP	No	Chaudhary et al. (2008)
P (2)	*Medicago sativa* L.	Field	First flower and one-tenth bloom	K rate (nc) and S rate (nc)	Collins, et al. (1986)
P (2)	*Pachyrhizus erosus (L.) Urb*	Pots w/soil	84 DAP	Bradyrhizobium strains, P source (nc) and N rate (nc)	Cruz et al. (1997)
P (2)	*Glycine max* (L.) Merr. and *Medicago sativa* L.	Pots w/silica sand	42 DAP	No	Drevon and Hartwig (1997)
P (5)	*Stylosanthes humilis* H.B.K	Pots w/soil	17, 23 and 26 DAP	S rate (nc)	Gates (1974)
P (2)	*Glycine max* (L.) Merr.	Hydroponic	28 DAT	No	Georgiev and Tsvetkova (2011)
P (8)	*Phaseolus vulgaris* L.	Field	42 DAP	No	Graham and Rosas (1979)
P (3)	*Trifolium repens* L.	Hydroponic	66 DAP	Time of P-deprivation (nc)	Høgh-Jensen, et al. (2002)
P (5)	*Cicer arietinum* L.	Field	Pre flowering stage	No	Idris et al. (1989)
P (6)	*Glycine max* (L.) Merr.	Pots w/perlite	40 DAT	No	Israel (1987)
P (5)	*Pisum sativum* L.	Pots w/soil and sand	19 and 24 DAE	Time of P-deprivation (nc) and N rate (nc)	Jakobsen (1985)
P (2)	*Phaseolus vulgaris* L.	Hydroponic	42 DAP	Genotypes	Jebara et al. (2005)
P (2)	*Phaseolus vulgaris L.*	Hydroponic	42 DAP	Genotypes	Kouas et al. (2009)
P (3)	*Phaseolus vulgaris* L	Pots w/perlite and vermiculite	21 DAP	N rate (nc)	Leidi and Rodríguez-Navarro (2000)
P (2)	*Trifolium repens* L.	Pots w/soil	47 DAP	Lime (nc) and mycorrhiza	Newbould and Rangeley (1984)
P (4)	*Cicer arietinum* L.	Field	15, 30 and 45 DAP	No	Nosheen and Shafique (2006)
P (6)	*Phaseolus vulgaris* L	Hydroponic	28 DAP	No	Olivera et al. (2004)
P (7)	*Phaseolus vulgaris* L.	Pots w/sand	33 DAP	Genotypes and N rate (nc)	Pereira and Bliss (1987)
P (2)	*Phaseolus vulgaris* L.	Pots w/sand	33 DAP	Genotypes (nc)	Pereira and Bliss (1989)
P (4)	*Glycine max* (L.) Merr.	Hydroponic	32, 40, 47 and 54 DAE	N rate (nc)	Qiao et al. (2007)
P (2)	*Glycine max* (L.) Merr.	Hydroponic	45 DAP	No	Ribet and Drevon (1995)
P (3)	Glycine max (L.) Merr.	Pots w/silica sand	42 DAP	Fe (nc)	Rotaru and Sinclair (2009)
P (2)	*Glycine max* (L.) Merr.	Pots w/perlite	30, 36, 44, 51 and 58 DAT	No	Sa and Israel (1991)
P (2)	*Lupinus albus* L.	Pots w/silica sand	21 and 37 DAT	No	Schulze (2006)
P (4)	*Glycine max* (L.) Merr.	Pots w/soil	33 DAP	Bradyrhizobium strains	Singleton et al. (1985)
P (5)	*Medicago truncatula* L.	Hydroponic	75 DAP	Symbiotic bacteria	Sulieman et al. (2013)
P (4)	*Medicago truncatula* L.	Hydroponic	35 DAT	No	Tang et al. (2001)
P (4)	*Vigna unguiculata* (L). Walp	Pots w/sand	21, 42, 63 and 84 DAP	No	Wan Othman et al. (1991)
K (3)	*Glycine max* (L.) Merr.	Field	Physiological maturity	P rate	Abbasi et al. (2012)
K (2)	*Glycine max* (L.) Merr.	Pots w/soil	72 DAP	Soil moisture and genotype	Abdelhamid et al. (2011)
K (3)	*Pisum arvense* L.	Hydroponic	37 DAP (seed filling stage)	No	Abdel-Wahab (1985)
K (3)	*Vicia faba* L.	Pots w/soil	46 DAP	Soil moisture	Abdel-Wahab and Abd-Alla (1995)
K (8)	*Macroptilium lathyroides* (L.) Urb, *M. atropurpureum* (Moc. & Sessé ex DC.) Urban, *Desmodium intortum* (Mill.) Urb, *D. uncinatum* (Jacq.) DC., *Stylosanthes humilis* Kunth, *Lotononis bainesii* Baker, *Centrosema pubescens* Benth, *Medicago sativa* L., *M. truncatula* Gaertn, *Trifolium repens* L.,*T. fragiferum* L..	Pots w/soil	Pre-flowering	Soil type	Andrew and Robins (1969a) and Andrew and Robins (1969b)

Table 1 (*Continued*)

Nutrient[a]	*Legume*	Culture	Date of measurements[b]	Other sources of variation	References
K (3)	*Medicago sativa* L.	Pots w/soil	First flower stage	K source (nc)	Collins and Duke (1981)
K (3)	*Medicago sativa* L.	Field	First flower and one-tenth Bloom stage	Soil type, P rate (nc) and S rate (nc)	Collins et al. (1986)
K (3)	*Medicago sativa* L.	Field	Third cut (4 years old pasture)	K source (nc)	Duke et al. (1980)
K (2)	*Trifolium repens* L.	Hydroponic	71 DAP	withdrawal of K supply (nc)	Høgh-Jensen (2003)
K (5)	Trifoliunz oesiculosum Sav.	Pots w/soil	3 years	P rate	Lynd et al. (1984)
K (2 Exp. 1 and 3 Exp. 2)	*Vicia faba* L.	Hydroponic	28 (Exp. 1)and 42 DAP (Exp. 2)	No	Mengel et al. (1974)
K (4)	*Glycine max* (L.) Merr.	Hydroponic	52 DAP	No	Premaratne and Oertli (1994)
K (3)	*Vicia faba minor* L. and *Phaseolus vulgaris* L.	Pots w/silica	R1 stage	Temperature	Sangakkara et al. (1996a)
K (3)	*Vicia faba minor* L. and *Phaseolus vulgaris* L.	Pots w/silica	R1 stage	Soil moisture	Sangakkara et al. (1996b)
K (4)	*Lablab purpureus* L.	Pots w/soil	70 DAP	Soil moisture	Younis (2010)
S (8)	*Macroptilium lathyroides* (L.) Urb, *M. atropurpureum* (Moc. & Sessé ex DC.) Urban, *Desmodium intortum* (Mill.) Urb, *D. uncinatum* (Jacq.) DC., *Stylosanthes humilis* Kunth, *Lotononis bainesii* Baker, *Glycine wightii* (Wight & Arn.) Verdc., *Medicago sativa* L.,*M. truncatula* Gaertn, *Trifolium repens* L., T. semipilosum Fresen	Pots w/soil	Pre-flowering	Soil type	Andrew (1977)
S (3)	*Lupinus albus* L.	Field	Physiological maturity	No	Cazzato et al. (2012a,b)
S (3)	*Vicia faba* L.	Field	Physiological maturity	No	Cazzato et al. (2012a,b)
S (3)	*Glycine max* (L.) Merr.	Field	Physiological maturity	Inoculation (nc)	Hussain et al. (2011)
S (3)	*Cicer arietinum* L.	Field	Physiological maturity	Genotypes and S source	Islam et al. (2012)
S (2)	*Vicia faba minor* L.	Pots w/perlite	49, 56, 63, 70 and 77 DAP	No	Pacina et al. (2006)
S (2)	*Medicago sativa* L., Trifolium pratense, *Pisum sativum* L. and *Vicia faba minor* L.	Pot w/perlite	57 DAP	N rate	Scherer and Lange (1996)
S (2)	*Pisum sativum* L.	Pot w/perlite	46, 53 and 60 DAS	No	Scherer et al. (2006)
S (2)	*Pisum sativum* L. and *Medicago sativa* L.	Pots w/perlite	63 and 70 DAP (*Pisum sativum* L.) and 70, 77 and 84 DAP (*Medicago sativa* L.)	No	Scherer et al. (2008)

[a] Number in parentheses represents levels of nutrient.

[b] DAP: Days after planting; DAT: days after transplanting; DAE: Days after seedling emergence.

[c] nc: not considered in the analysis of the data.

a comparable concentration, 4–5 mg P g DM^{-1}, for optimum nodule growth in *Medicago truncatula* L.. Critical concentrations have not been proposed for K and S. Of interest, the concentration of K seems to be lower in nodules than in shoots (Table 2), hence the differential responsiveness to K deficiency in nodule and shoot mass could not be explained by the same mechanism as for S and P.

Our analysis using pooled data from many studies strongly supports a larger sensitivity of nodule mass compared to shoot mass in response to P, K and S deficiency (Fig. 1), but reports on the effect of these nutrients on nodule number are controversial, especially for P. It has been reported that P deficiency may increase (Schulze, 2006), decrease (Pereira and Bliss, 1989) or have no effect (Drevon and Hartwig, 1997) on nodule number per unit shoot mass. But

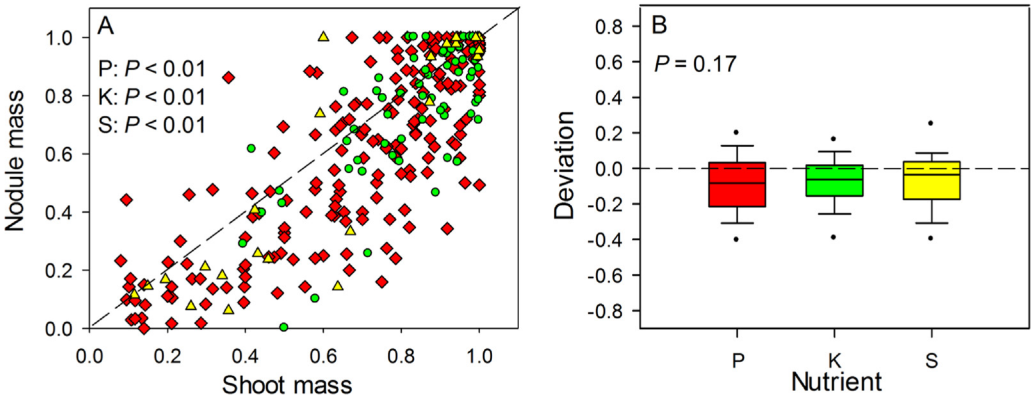

Fig. 1. (A) Comparison of relative nodule mass with relative shoot mass for legumes grown with different P (diamonds; $n = 264$), K (circles; $n = 86$) and S (triangles; $n = 38$) rates. Dotted line is $y = x$. (B) Frequency distribution of deviations from $y = x$ in response to P, K and S deficiency. Box lines are percentile 0.25, 0.50 and 0.75; whiskers are 0.10 and 0.90, and dots are 0.05 and 0.95 percentile. P indicates (A) significance of deviations from $y = x$ for each nutrient and (B) significance of differences among P, K and S.

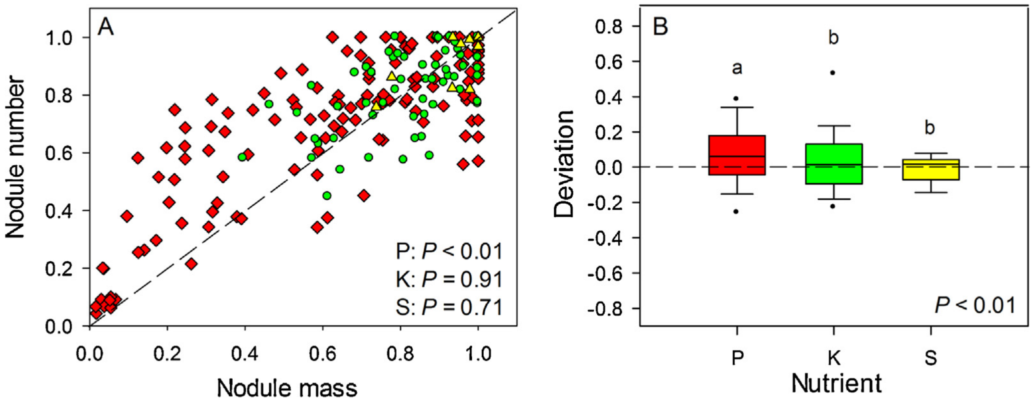

Fig. 2. (A) Comparison of relative nodule mass with relative nodule number for legumes grown with different P (diamonds; $n = 163$), K (circles; $n = 82$) and S (triangles; $n = 12$) rates. Dotted line is $y = x$. (B) Frequency distribution of deviations from $y = x$ in response to P, K and S deficiency. Box lines are percentile 0.25, 0.50 and 0.75. Whiskers are 0.10 and 0.90 and dots 0.05 and 0.95 percentile. P indicates (A) significance of deviations from $y = x$ for each nutrient and (B) significance of differences among P, K and S. In (B) different letters indicate differences between nutrients at $P < 0.05$ using Fisher protected LSD.

it is accepted that P-deficient plants tend to have smaller nodules. Ribet and Drevon (1995) proposed that this strategy increases the nodule surface/volume ratio, thereby facilitating oxygen diffusion into the nodule, which is critical for effective BNF (Layzell et al., 1990). Fig. 2 supports this proposition, as nodule number decreases less than nodule mass under P deficiency. Working with *Lupinus albus* L., Schulze (2006) showed that not only the number of nodules increases with P deficiency but also that nodule location shifts, as the smaller nodules were located in the vicinity of cluster root zones where P uptake is presumably highest. In contrast to these responses to P, nodule number and nodule mass are equally affected by deficit of K and S. The conclusion for K is robust, as it is based on $n = 82$, but the conclusion for S needs to be considered cautiously as it is based on few observations ($n = 12$).

Special consideration should be given to sampling date on trait responses. This is illustrated in Fig. 3, which shows that differences between shoot mass and nodule mass reduction in response to P deficiency decreases with plant age. Qiao et al. (2007) determined that 2 weeks after transplanting, early nodule formation was not affected by external P supply in soybean. Indeed, they proposed that P from seed reserves could support rhizobial infection and nodule initiation. In the period from 3 to 4 weeks after sowing, P deficit markedly decreased nodule formation but did not affect the growth of host plant and after 5 weeks, both nodule formation and plant growth were depressed, although the effect was greater on nodule formation. No consistent explanations have been proposed to account for this pattern.

The interaction between plant age and nodule type might further bias the conclusions regarding nutrient effect on BNF and related traits. Nodules can be determinate or indeterminate, depending on the host plant species (Hirsch, 1992). Indeterminate nodules continuously produce new cells from a persistent meristem to replace older senescent cells. They are found in species such as clover, lucerne and pea. By contrast,in determinate nodules found in most monocarpic legumes, the end of the nodule's lifecycle coincides with pod filling (Puppo et al., 2005). Thus, the relations between plant growth and nodule growth and activity in response to P, K and S deficiencies could be influenced by the interaction between plant age and nodule type. In our analysis, sampling dates were similar for P, K and S (Table 1); no attempt was made to account for plant age or nodule type, but these factors should be considered in more detailed analyses.

Table 2
Summary statistics of nutrient concentration (%) in shoots, roots and nodules in legume-rhizobium systems in response to P, K and S deficit.

Variable	Percentile					
	n	5	25	50	75	95
Phosphorus						
N in shoots	165	1.19	1.85	2.87	2.87	4.64
N in roots	92	0.90	1.40	1.70	2.42	3.14
N in nodules	74	4.11	5.20	6.20	7.32	9.58
P in shoots	233	0.07	0.13	0.22	0.32	0.49
P in roots	85	0.06	0.12	0.17	1.00	1.61
P nodules	133	0.21	0.34	0.40	0.48	1.81
Potassium						
N in shoots	171	2.90	3.50	3.89	4.33	7.09
N in roots	5	–[a]	2.42	3.78	4.02	–
N in nodules	3	–	6.11	6.38	6.40	–
K in shoots	164	0.46	0.84	1.71	2.21	3.26
K in roots	9	–	0.78	1.96	3.80	–
K in nodules	3	–	0.49	1.12	1.48	–
Sulphur						
N in shoots	314	1.97	2.55	3.01	3.40	4.00
N in roots	11	–	1.95	2.31	2.80	–
N in nodules	9	–	3.63	5.03	5.67	–
S in shoots	309	0.08	0.11	0.16	0.22	0.29
S in roots	31	0.03	0.05	0.14	0.35	1.06
S in nodules	19	0.09	0.21	0.25	0.37	0.60

[a] Not calculated due to small sample size.

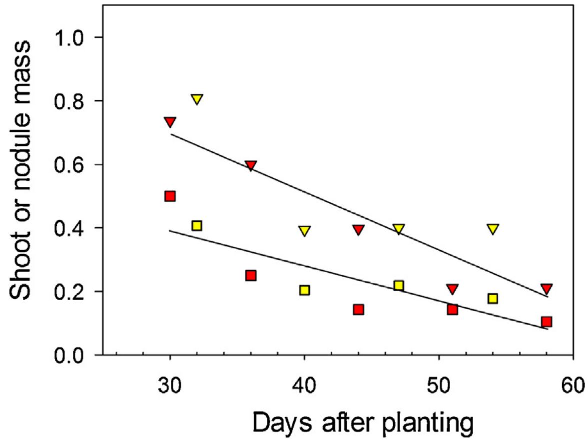

Fig. 3. Relative shoot (triangles) and nodule (squares) mass of P-deficient soybean plants as a function of days after planting. Data from Saa and Israel (1995) (red) and Qiao et al. (2007) (yellow). The slopes, $-0.019\,d^{-1}$ ($R^2 = 0.80$) for shoot and $-0.011\,d^{-1}$ ($R^2 = 0.73$) for nodules, are different at $P < 0.01$. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)

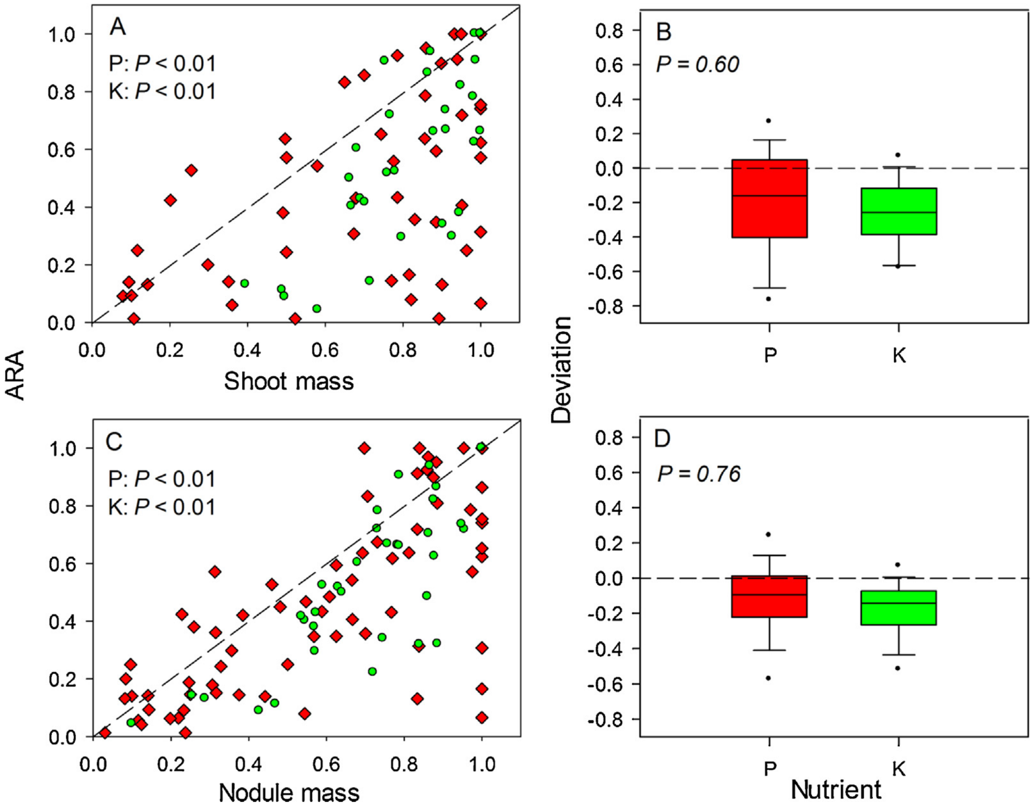

Fig. 4. Comparison of relative acetylene reduction activity (ARA) with (A) relative nodule mass and (C) relative nodule number for legumes grown with different P [diamonds; $n = 65$ (A) and 91 (C)] and K [circles; $n = 37$ (A) and 43 (C)]. Dotted line in AC is $y = x$. (B, D) Frequency distribution of deviations from $y = x$ in response to P, S and K deficiency. Box lines are percentile 0.25, 0.50 and 0.75; whiskers are 0.10 and 0.90, and dots are 0.05 and 0.95 percentile. P indicates (A, C) significance of deviations from $y = x$ for each nutrient and (B, D) significance of differences among P, K and S.

3.2. Biological nitrogen fixation

Nitrogen fixation seems more responsive to P and K deficit than shoot (Fig. 4A and B) and nodule mass (Fig. 4CD) as significantly larger proportions of data points are in the right area below the $y = x$ line. Shortage of data ($n = 3$) precluded the analysis for S. Reductions in BNF in response to P and K deficit may be mediated by reductions in nodulation, nodule productivity (i.e., moles of N_2 fixed per unit time per unit mass of nodule) or both (Duke and Collins, 1985). Few studies focused on the physiological and metabolic processes involved in these responses. Sa and Israel (1991) made a comprehensive analysis of the nodule energy status and functioning of soybean, and concluded that reduced nitrogenase activity under P starvation relates to the inhibition of ATP-dependent reactions in the plant cell fraction of nodules but not in nodules. They also fund no relation between ARA and bacteroid mass per unit nodule mass. Some authors have demonstrated a direct involvement of K in the activation of the enzyme nitrogenase (Duke et al., 1980; Barta, 1982; Lynd et al., 1981; Lynd and Ansman, 1989). Duke et al. (1980) determined that adequate K supply is required to sustain acetylene reduction rates and nodule productivity by directly activating the N assimilating enzymes, including those involved in ammonia assimilation, amino acid interconversions, carbon supply and energy transduction. Biochemical and immunological studies showed a close relationship between S supply and protein content in the nodules, particularly nitrogenase and leghaemoglobin (Varin et al., 2010). This is consistent with the high proportion of S-containing amino acids in both components of the nitrogenase complex, the Fe protein and the Fe–Mo protein (Curatti et al., 2006). As leghaemoglobin maintains a low free O_2 concentration within the nodule (Gordon et al., 2001) low concentrations of this protein may result in higher O_2 concentration. Moreover, Scherer et al. (2008) determined that under S starvation, BNF was reduced in pea and lucerne as a consequence of decreased ferredoxin and leghemoglobin concentrations as well as reduced ATP supply. In addition, the decreasing proportion of N derived from symbiosis in response to deficit of P (Cadisch et al., 1993; Almeida et al., 2000), K (Fajri, 2006) and S (Scherer and Lange, 1996), may also reflect a direct effect of these nutrients on BNF. This indicates that BNF is depressed to a greater extent than the plant N demand.

Collectively, the analysis in Fig. 4AB helps to solve some controversies in the scientific literature. Almeida et al. (2000) found increases in specific BNF (g N_2 fixed g nodule^{-1}) of white clover (Trifolium repens L.) grown with low P supply. For the same species, Høgh-Jensen et al. (2002) reported that specific nitrogenase activity declined under P deprivation when expressed as N_2 fixation per unit root fresh weigh, but when expressed on a per unit nodule mass basis, P-deprived plants tended to maintain a higher levels than those with sufficient P supply. Similarly, experiments with different approaches to determine N_2 fixation concluded that K supply does affect the plant yield but not the nitrogenase activity (Mengel et al., 1974; Feigenbaum and Mengel, 1979; Duke et al., 1980; Collins and Duke, 1981; Barta, 1982; Collins and Lang, 1985). Sangakkara et al. (1996a) determined that *Vicia faba* L. and common bean (*Phaseolus vulgaris* L.) maintained the specific activity on nodules (mg N fixed g nodule^{-1}) when they were exposed to different K rates and concluded that K affects nodulation rather than nodule productivity. Moreover, Varin et al. (2010) reported a reduction of 81% in N fixed by white clover plants in S-free nutrient solution in comparison with those which adequate S supply. In this particular experiment, S-deficiency inhibited nodulation to a greater extent than BNF, which indicates increases in specific BNF. Our analysis

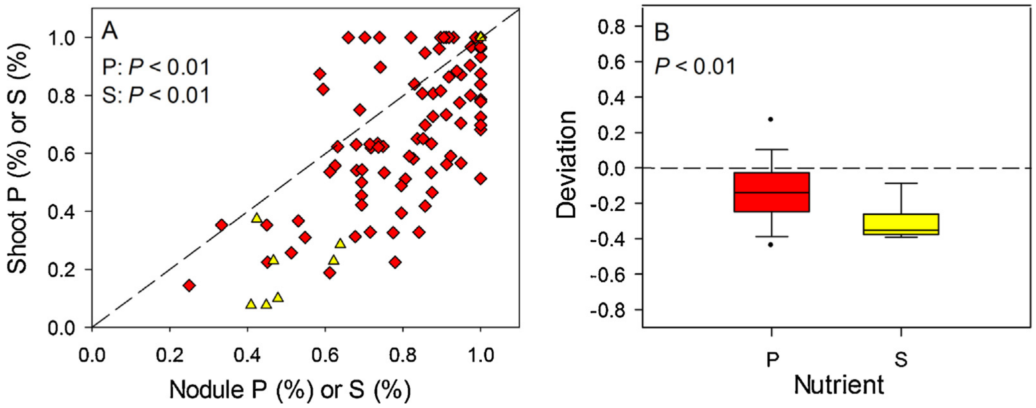

Fig. 5. (A) Comparison of relative P or S concentration in shoot mass with relative P or S concentration in nodules for legumes grown with different P (diamonds; $n = 112$) and S (triangles; $n = 7$) rates. Dotted line is $y = x$. (B) Frequency distribution of deviations from $y = x$ in response to P and S deficiency. Box lines are percentile 0.25, 0.50 and 0.75; whiskers are 0.10 and 0.90, and dots are 0.05 and 0.95 percentile. P indicates (A) significance of deviations from $y = x$ for each nutrient and (B) significance of differences between P and S.

supports that P, K and S starvation may directly impair nodule productivity. However, positive or neutral effects are also feasible, depending on the combination of plant species, the severity of the nutrient stress and the ratio between N demand and nodule mass. For example, Fig. 2 gives evidence that at early plant stages the difference between shoot (N demand) and nodule mass is greater than later. This may cause decreases in nodule productivity over time.

Soil water availability may condition the relative response of shoot growth and BNF to K dose (Abdel-Wahab and Abd-Alla, 1995). Sangakkara et al. (1996b) and Younis (2010) coincided that common bean and *Lablab purpureus* (L.) plants with adequate K supply shows lower diminutions in nodulation and BNF than K-stressed plants under water stress conditions. The scarcity of information in the retrieved papers precluded a consistent analysis of this issue, especially for P and S. Bonetti et al. (1984) determined that P addition increased shoot growth under water deficit but slightly modified ARA in common bean plants. This indicates that P does not improve BNF tolerance to water stress as occur with K.

3.3. Nutrient concentration in nodules

We compared the capacity of the plant and nodules to maintain P and S concentration as the provision of these nutrients changes. Fig. 5 shows that concentration of these nutrients in shoots is more responsive than their concentration in nodules. The pattern was different for both nutrients but this conclusion might be biased by the scarcity of data for S ($n = 7$). Only one paper presented the K concentration in nodules and shoots (Høgh-Jensen, 2003), thus precluding any analysis for this nutrient ($n = 3$).

Almeida et al. (2000) found that the concentration of P in nodules was much higher than in the host plant under severe P deficiency. Whether the difference is due to selective partitioning of P to nodules or P uptake by nodules is not clear, but Al-Niemi et al. (1998) suggest that nodules appear to take up P directly from nutrient solution. Israel (1993) reported that during recovery from P deficiency, soybean plants initially and preferentially imported this nutrient into nodules thus supporting the high strength of nodules as a sink for P. These mechanisms partially explain the relatively high concentration of P in nodules even when the provision changes. Almeida et al. (2000) concluded that it is unlikely that the concentration of P in the nodule directly limits nodule growth and functioning given its stability. In fact, they also argue that the increase on specific BNF determined in some cases under P deficiency indicates that nodules continued to function even when P supply to the growing plant is very low.

3.4. Are the P, K and S-deprived plants N-deficient?

One of the main questions regarding the effect of P, K and S is whether the diminution of nodulation and BNF is related to plant-N feedback. To answer this question, it is important to observe if plants starved of these nutrients are also N-deficient. Fig. 6A and B shows the concentration of N in shoots is conserved in relation to shoot growth in response to P, K and S deficiencies as most data points are in the left area up the $y = x$ line. This pattern is more pronounced for P than for K and S. The concentration of N in shoots decreased in a leaser proportion than the concentration of P, K and S in shoots (Fig. 6C and D). As a consequence, all three ratios, i.e. N/K, N/P and N/S, increased as nutrient deficiency was greater, but the N/K ratio increased more markedly. This is consistent with the accumulation of N compounds such as ammonia, urea, amino acids and amides (DeBoer and Duke, 1982). Høgh-Jensen et al. (2002) found higher concentration of asparagine in the phloem of P deficient *Trifolium repens* L. plants. This amide is involved in the N-feedback mechanism that restricts BNF (Schulze, 2004; Sulieman et al., 2010; Sulieman et al., 2013). A similar conclusion was reached by Almeida et al. (2000) who measured the asparagine concentration in P-deficient and non-deficient *Trifolium repens* L. plants grown with inorganic N supply. Varin et al. (2010) proposed a similar mechanism for S starved plants based on their determinations in *Trifolium repens* L. They postulated that the lower BNF of S-deficient plants may have resulted from an N-feedback mechanism down-regulating nodule development and nitrogenase and leghaemoglobin production, as indicated by the increased concentration of both N and asparagine observed in leaves and stolons. These authors also demonstrated that this N-feedback mechanism down-regulates the absorption of mineral N from the nutrient solution which further demonstrates that both pathways of N assimilation, soluble N uptake and N_2 fixation are reduced in S-deficient plants. Asparagine is mentioned as one of the main candidates for regulating the feedback inhibition of N assimilation but, owing to the variability in the BNF systems depending on amide and ureide transporters, different regulatory compounds (e.g. glutamine and ureide) may also be involved (Neo and Layzell, 1997; Parson, 1997; Schulze, 2004).

3.5. Effect of carbohydrates supply to nodules

Whereas carbohydrate supply was not the main focus of this review and the data analyzed do not allow for a direct test of their role, models of legume-rhizobium responses to nutrients would be incomplete without consideration of this aspect of BNF. Hence,

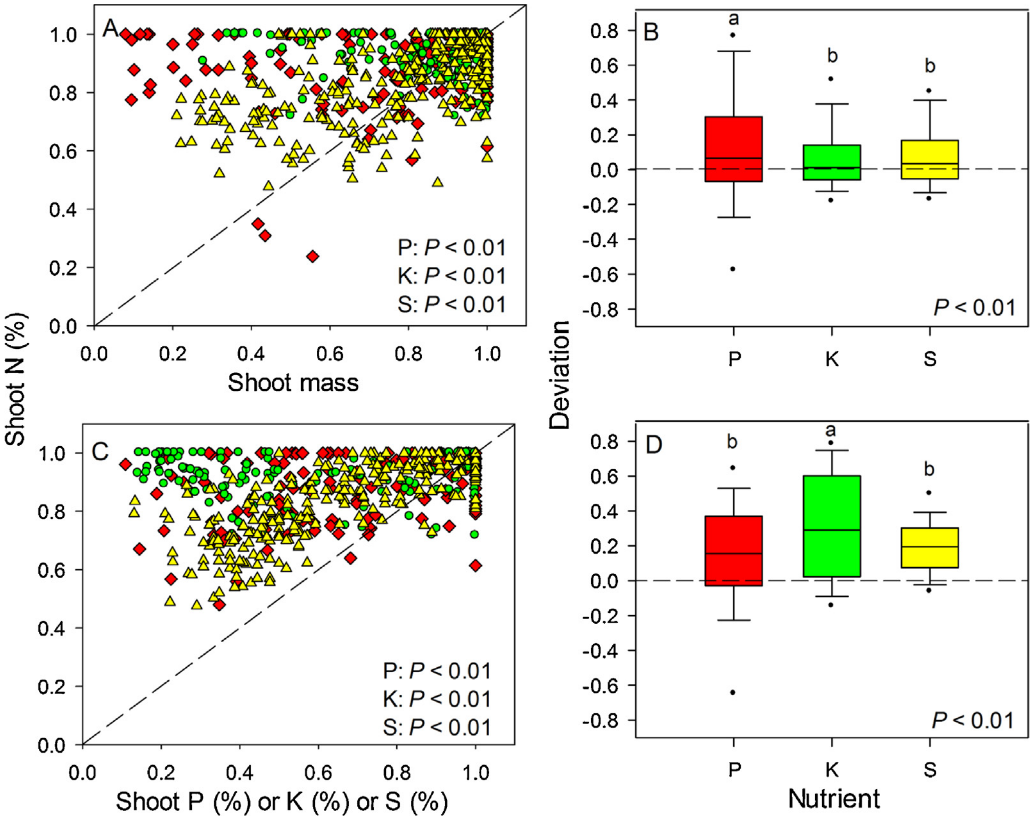

Fig. 6. Comparison of relative N concentration in shoot mass with (A) relative shoot mass and (C) P, K and S concentration in shoot mas for legumes grown with different P [diamonds; $n = 145$ (A) and 151 (C)], K [circles; $n = 156$ (A) and 155 (B)] and S [triangles; $n = 314$ (A) and 290 (CB)] rates. Dotted line is $y = x$. (B and D) Frequency distribution of deviations from $y = x$ in response to P, S and K deficiency. Box lines are percentile 0.25, 0.50 and 0.75; whiskers are 0.10 and 0.90, and dots are 0.05 and 0.95 percentile. P indicates (A, C) significance of deviations from $y = x$ for each nutrient and (B, D) significance of differences among P, K and S. In (B, D) different letters indicate differences between nutrients at $P < 0.05$ using Fisher protected LSD.

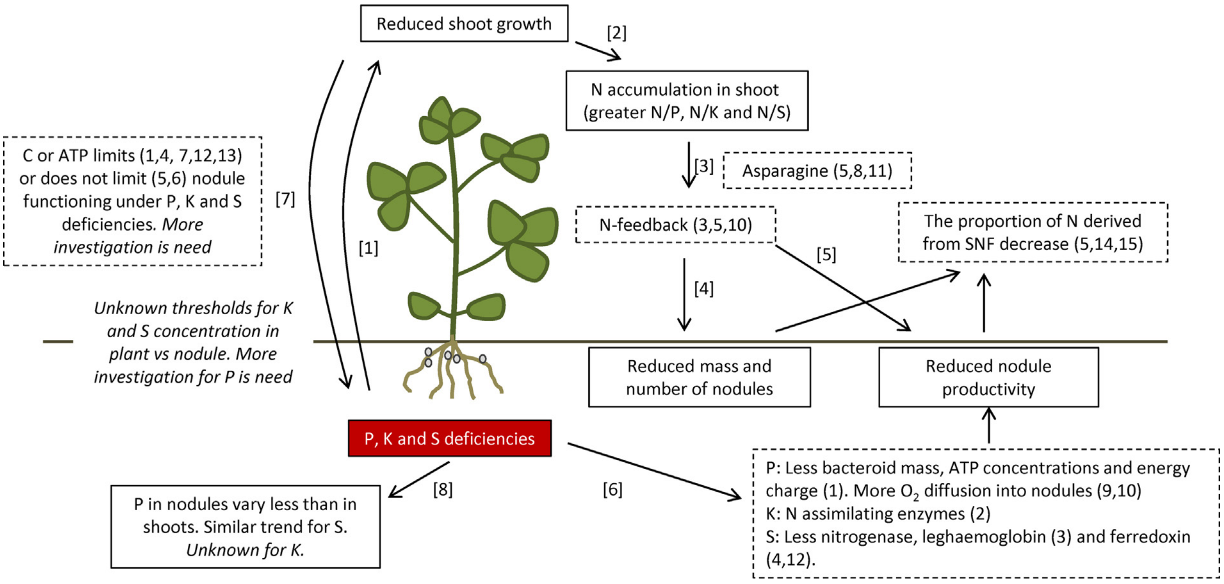

Fig. 7. Physiological mechanisms of legume-rhizobia responses to P, K and S deficiencies. Pathway [1] involves reduction in shoot growth in response to nutrient deficit. Pathway [2] involves a relative accumulation of N in shoot mass. Pathway [3] involves the N-feedback mechanism that down regulates biological N fixation (BNF). Asparagine is mentioned as a main regulator. Pathway [4] involves reduction in nodule mass and number and pathway [5] reduction in nodule productivity . Pathway [6] involves direct effects in nodule growth and functioning. Pathway [7] involves the effect of carbon limitation in nodule functioning. Pathway [8] involves maintenance of high nutrient concentration in nodules. This conceptual model is based on the original analysis in this paper (full line boxes) and supporting literature (dashed-line boxes) as follows: (1) Sa and Israel (1991); (2) Duke et al. (1980); (3) Varin et al. (2010); (4) Scherer et al. (2008); (5) Almeida et al. (2000); (6)Høgh-Jensen (2003); (7) Premaratne and Oertli (1994); (8) Sulieman et al. (2013); (9) Ribet and Drevon (1995); (10) Vadez et al. (1996); (11) Høgh-Jensen et al. (2002); (12) Pacyna et al. (2006); (13) Scherer et al. (2006); (14) Fajri (2006); (15) Scherer and Lange (1996). Text in italic indicates research gaps.

the aim of this section is not to exhaustively account for the role of carbohydrates but to use selected studies to emphasize their importance.

Schulze (2004) proposed three mechanisms that regulate BNF in legumes: (1) the carbon supply regulation, stating that BNF is regulated by either current nodule assimilate supply or through metabolism of assimilates into compounds that can be used by bacteroids, (2) the oxygen supply regulation, that assumes that the diffusion of this molecule into nodules acts as a regulation factor and (3) the N-feedback mechanism outlined above. The three mechanisms are not mutually exclusive, and the carbon and N based mechanisms could indeed be integrated in a carbon/N ratio mechanism; this ratio is relevant to many biological processes (Sterner and Elser, 2002).

The N-feedback mechanism is likely to be relevant in response to P, K and S, starvation, but several authors suggests that the decrease in the photosynthetic activity of the plant may lead to shortage of carbohydrates in nodules (Menguel et al., 1974; Jakobsen, 1985; Feigenbaum and Mengel, 1979; Barta, 1982; Sa and Israel, 1991, 1998; Scherer et al., 2006). Evidence for (Jakobsen, 1985; Sa and Israel, 1991; Gordon et al., 1997) and against (Almeida et al., 2000) the carbon-supply regulation hypothesis has been presented. In response to shortage of P, both Rubisco activity and rate of leaf photosynthesis diminish, but starch concentration in leaves increases consistent with sink-limited photosynthesis (Almeida et al., 2000). Higher concentrations of water soluble carbohydrate in roots than in nodules of P-deficient plants indicate that nodules are a weaker sink for these compounds and that they are not necessarily carbon limited (Almeida et al., 2000).

Several studies support the theory of carbon supply regulation in response to K deficit (Menguel et al., 1974; Feigenbaum and Mengel, 1979; Barta, 1982; Premaratne and Oertli, 1994). However, later research by Høgh-Jensen (2003) determined that the ratio of net CO_2 to N_2-fixation increases with lower K supply, and this author proposed that even mild K deficiency induced a feedback reduction of BNF. Scherer et al. (2006) determined that the level of glucose and sucrose in non-deficient soybean plants was up to five times higher than in S deficient. These observations suggest that available photosynthate could limit BNF as a consequence of a lower energy production or carbon skeletons for ammonia assimilation.

4. Concluding remarks

This review identified general patterns and nutrient-specific responses regarding the effect of P, K and S on traits related to BNF; research gaps were also highlighted. In seeking for patterns, a trade-off is involved where generality was achieved at the expense of specificities; i.e. we pooled data across species, nodule type (determinate vs. indeterminate), growing conditions, and plant developmental stage.

Given its importance for plant nutrition and its scarcity in many soils, significant efforts has been allocated to research the role of P supply in the rhizobia-legume symbiosis, whereas the effects of K and S have received less attention. Scarcity of measured N, K and S concentration in nodules impairs our understanding of the direct impact of nutrient deficiencies on nodule growth and function. Except for few reports on P, the lack of critical nutrient concentrations in plant and nodule tissues hampers the development of quantitative relationships between nutrition, plant growth and metabolic activity of rhizobia-legume systems.

Most studies converge to support a N-feedback mechanism triggered by a reduction in plant host growth and an accumulation of N compounds in shoots underlying the reduction of BNF under P, K and S deficit (Almeida et al., 2000; Vadez et al., 2000; Høgh-Jensen et al., 2002; Varin et al., 2010; Sulieman et al., 2013). However, there is also evidence for a direct effect of the nutrients on nodule growth and activity but few attempts have been made to integrate direct and indirect effects of nutrient deficit on the physiology of rhizobia-legume systems (Fig. 7). Some questions are advanced for further research:

(1) If nutrients control the BNF through the plant growth rate (N demand), why is BNF reduced proportionally more than N demand? Is this an indication of direct effect of nutrients?
(2) If P, K and S concentrations are more conserved in nodule than in shoot, why nodule productivity is more responsive than shoot growth to nutrient deficit?

In conclusion, our analysis confirms that deficiency of P, K and S reduces nodule growth and number to a greater extent than shoots mass, clarifying some controversies regarding the effect of P deficiencies on nodule number. In addition, nodule activity decreases more than both shoot and nodule mass, which indicates a reduction in nodule productivity. This conclusion is one of the main contributions of our analysis, given the discrepancies about this process in the literature. This effect would be related with direct effects of these nutrients on physiological and metabolic processes. Finally, the conserved shoot N concentration, in comparison to concentration of P, K and S indicates a relative N enrichment that matches the proposed N-feedback mechanism down–regulating BNF in nutrient deficient systems. Despite some nutrient-specific differences, i.e. smaller nodules with shortage of P and higher N/K ratio with shortage of K, the patterns of growth, nodule activity and nutrient concentration were similar for all three nutrients P, K and S. This highlights the central role of N-feedback in conjunction with individual direct effects of the nutrients on both nodule growth and activity.

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