

# pH effects on nodulation and biological nitrogen fixation in *Calopogonium mucunoides*

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**Abstract** *Calopogonium mucunoides* Desv. is able to form nodules, root organs in which biological nitrogen fixation takes place, after a symbiotic interaction with soil bacteria known as rhizobia. Such distinct advantage of some legume species faces different environmental abiotic factors such as acid and alkaline pH conditions of soil. Nodulation and symbiotic nitrogen fixation response in different pH ranges were determined under greenhouse conditions. Plants were cultivated in vermiculite and treated with nutritive solution adjusted to pH 4.0, 5.5, and 7.0, and after three months, the following variables were measured: nodule number, nitrogen fixation, tissue protein, amino acids, total ureides, allantoin, and allantoate. The number of nodules and nitrogen fixation were enhanced under acidic conditions, but nitrogenase activity was drastically decreased at pH 7.0. Acidic conditions decreased the amount of protein, amino acids, total ureides, allantoate, and allantoin in leaves and nodules, but at pH 5.5, only protein content was decreased. Symbiosis with *C. mucunoides* and biological nitrogen fixation were kept under acidic conditions, but it was negatively affected under conditions that are near to neutral. It is

reasonable to conclude that the association between *C. mucunoides* and nitrogen-fixing bacteria from “Cerrado” evolved in this context, which is supported by the difficulties faced to keep interaction under pH near neutrality.

**Keywords** Acid soils · “Cerrado” · Leguminous

## Introduction

Soil acidity has long been known to decrease symbiotic nitrogen fixation in legumes, negatively affecting growth and yield, especially in plants depending exclusively on symbiosis to acquire nitrogen (Mohammadi et al. 2012; Bekere et al. 2013). Nearly 30 % of earth’s land surface present acidic soil (pH < 5.5) including 40 % of arable land, affecting nutrient availability and root growth, and additionally increasing Al<sup>3+</sup> toxicity, which ultimately lead to losses in crop yield (Lin et al. 2012). Under acidic conditions, cell-membranes permeability are altered by the excess of H<sup>+</sup> inducing cation efflux, impairing plant nutritional status and growth. To solve negative influence of acidic soil on plant growth, liming is required to neutralize undesired effect of high H<sup>+</sup> in the soil (Guo et al. 2009). Hence, symbiotic plants are frequently exposed to a range of soil pH, acidic, neutral, and alkaline conditions. The association between plant and bacteria drives nitrogen fixation and can be positively or negatively affected by soil conditions, including soil pH.

Symbiotic plants have been used for recovering of degraded areas or other purposes since they play an important role for improving soil quality, apparently by providing N to soil (Leblanc et al. 2005; Nichols and Carpenter 2006). Leguminous plants have three alternative sources of inorganic N on environment: nitrate, ammonium, and N<sub>2</sub> (Pal’ove-Balang and Mistrik 2007) relative to the former,

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because these plants have the capability to incorporate N from atmospheric N<sub>2</sub> by association with rhizobium which promotes biological nitrogen fixation (BNF). In this way, it contributes to N input to ecosystem and consequently to improving soil fertility. However, nodulation and biological nitrogen fixation are sensitive to environmental stressful conditions, which may cause inhibition of initial steps of bacterial infection in roots (Bouhmouch et al. 2005). Nitrate reduction involves the enzyme nitrate reductase (NR), producing nitrite which is reduced to ammonia, in plastids, by the nitrite reductase enzyme (NiR); afterward, the NH<sub>4</sub><sup>+</sup> formed is incorporated into organic compounds by glutamine synthetase and glutamine 2-oxoglutarate amino transferase, or simply GS/GOGAT (Temple et al. 1998).

In plants, a few elements are present in large amounts, such as nitrogen, which is extremely important for molecules like amino acids and nucleic acids (Williams and Miller Williams and Miller 2001). *Calopogonium mucunoides* Desv., a typical tropical legume from “Cerrado”, is used as green manure as well as a soil cover crop. In addition, *C. mucunoides* has high capacity to fix N<sub>2</sub> and can be cultivated in soils with low pH and low fertility (Izaguirre-Mayoral 1996). There are a few studies concerning nitrogen metabolism in this specie, but it has already been demonstrated that this plant specie has high relative abundance of ureides, a class xylem N-transporting compound in plants using nitrogen through nodule activity (Seiffert et al. 1985; Costa 1995). Preliminary studies from our research group demonstrated that symbiotic N<sub>2</sub>-fixing system established between rhizobium and *C. mucunoides* is tolerant to nitrate exposition (Camargos 2007), which is an interesting feature since nitrate is known to inhibit nodulation as well as N<sub>2</sub>-fixation. Nodulated *C. mucunoides* growing in “Cerrado” faces soil acidity, but preference for acidic, neutral, or alkaline soils by symbiotic system of *C. mucunoides* was not investigated, although certain species of *Lupins* and *Mimosa* isolated from Brazilian “Cerrado” and “Caatinga” biomes exhibit some tolerance to acidic conditions (Sprent 2009; dos Reis et al. 2010).

Since symbiotic nitrogen fixation is crucial to successful establishment of *C. mucunoides* and continuing productivity of plant. We aimed to assess the effects of different pH solutions on nodulation, N-fixing, and nitrogenous compounds in different organs in order to understand how this species would respond to different conditions of Brazilian soil pH.

## Materials and methods

### Plant material

Seeds of *C. mucunoides* were commercially obtained from Pirai Sementes (Piracicaba, Brazil) and were germinated in two 4-L pots with sand and vermiculite. Seedlings were transferred to

4-L pots filled with sand and vermiculite (1:1), two plants per pot, and inoculated with a suspension of nodules taken from the same species growing in its natural environment.

Plants were grown in a greenhouse located at Ilha Solteira-SP (20°25'58" S, 51°20'34" W; 335 m) with controlled temperature (min. av. 18.2—winter; max. av. 34—summer). Plants were watered with 100 mL of Hoagland and Arnon (1950) complete nutritive solution adjusted to pH 4.0; 5.5 and 7.0, twice a week. After 3 months, plants were harvested and tissues were separated into nodules, roots and leaves and stored in freezer for further analysis.

Nitrogenase activity of nodulated root system was determined by the in vivo assay in intact plants.

### Extraction and quantification of N compounds

Nitrogenous compounds were extracted from tissues 1:10 (w/v) by using a solution of methanol, chloroform and water (MCW—12/5/3 v/v/v, respectively) according to Bielecky and Turner (1966). After homogenization, the materials were centrifuged during 15 min at 1560×g and the supernatant was collected. For each 1 mL of supernatant recovered, it was added 0.25 mL of chloroform and 0.67 mL of distilled water was mixed, and then this mixture was vortexed and allowed to rest for 24 h at 4° C for phase separation. The upper phase was then collected for quantitation of N-compounds. Total soluble proteins were extracted from tissues 1:10 (w/v) using 0.1 M NaOH solution.

Proteins, amino acids, allantoin, and allantoate were determined according to Bradford (1976), Yemm and Cocking (1955), and Vogels and Van Der Drift (1970), respectively.

### In vivo N-fixation assay

The in vivo analysis of nitrogenase activity was evaluated by quantitation of H<sub>2</sub> released during nitrogenase activity, using the N-fixation system Package (Qubit Systems, Canada). The nodules were counted and weighed.

### Statistical analysis

Data were subjected to analysis of variance (ANOVA), and means were compared by Tukey's test at 5 % of probability using the software SISVAR<sup>®</sup>.

## Results

### pH effect on nodules' nitrogenase activity in *Calopogonium mucunoides*

The symbiosis and biological nitrogen fixation responded differently in each treatment. The higher nodulation rate

was observed at pH 4.0, and nodule number was similarly decreased at pH 5.5 and 7.0 (Table 1). Nitrogenase activity was higher at pH 4.0, and lower at pH 7.0, while at pH 5.5, we found intermediate nitrogenase activity values (Table 1).

### pH effect on organic nitrogenous compounds in - *Calopogonium mucunoides*

In leaves, we observed lower contents of amino acids, total ureides, allantoin, and allantoate at pH 4.0 in comparison to the other treatments (Table 2). In root tissues, we observed that protein content was influenced by pH in which the lowest protein concentrations were observed at pH 5.5 (Table 2), while the other nitrogenous compounds were not influenced by the treatments. In nodules, we also found that at pH 5.5 and 7.0, the proteins, amino acids, total ureides, allantoin, and allantoate contents were higher in comparison to the treatment of pH 4.0 (Table 2).

## Discussion

The present study shows the importance of understanding both nodulation and biological nitrogen fixation to understand the symbiosis in *C. mucunoides* under different conditions of pH. Limitation of crop productivity in arable soils worldwide is due to acidic soil, which account for around 50 % of world's potential usable land, and the pH of these soils are 5.5 or lower (Kochian et al. 2004). The importance to study the response of non-crop plants to low pH involves, additionally, aluminum toxicity, and *C. mucunoides* response may suggest different adaptive mechanisms to cope with this kind of stress. This species is a soil cover crop used as a green manure in crop rotation and presents high capacity to fix nitrogen (biological nitrogen fixation–BNF) (Camargos and Sodek 2010). Rhizobia present in the soil in this experiment provided effective nodulation, but only in lower pH conditions, especially pH 4.0 (Table 1). Aluminum toxicity and pH effect on nodulation and biological nitrogen fixation are solved by raising pH to a more suitable level, and for this

purpose, liming is used as a common technique for raising soil pH (Helyar and Anderson 1974). *Calopogonium mucunoides* did not nodulate and fix nitrogen efficiently when pH was equal or higher than 5.5 (Table 1). Therefore, liming can reduce nodulation and nitrogen fixation in *C. mucunoides*, and additionally, over-liming, however, can reduce crop yield (Calder et al. 1965). Apparently, according to our results, both *C. mucunoides* and rhizobia were shown to be tolerant to acidic soils, and they are able to keep fixing molecular nitrogen under this condition.

According to Bordeleau and Prevost (1994), soil pH may influence nodulation and/or nitrogen fixation; the first case is when the bacteria is sensitive to low pH, and the second case, after nodule establishment, when it has its functioning impaired by acidic conditions; in this way, the identification of acid-tolerant rhizobia has brought good results (Meghvansi et al. 2005). Our data are in accordance with such statement, since at pH 4.0 we observed the highest nodulation and nitrogenase activity (Table 1) which means that the rhizobium that interacted with *C. mucunoides* in this work is tolerant to soil acidity as well as its functioning, which is not so common based on the results from Lin et al. (2012) that found that nodulation is severely affected under low pH conditions in soybeans. Curiously, at pH 7.0, we observed that there was low nitrogenase activity, although it has the same nodulation rate of plants from treatment of pH 5.5; in this case, we can also conclude that the response of *C. mucunoides* to pH is affected, thereby affecting nitrogen fixation in nodules (Bordeleau and Prevost 1994). It was reported that decreases in BNF occurred in both tropical and temperate species grown in acidic soils (Munns 1986; Thomas et al. 1997). Interestingly, nodulation was decreased to around 90 % and nodule dry weight to about 50 % in species such as soybean, pea, cowpea, *Medicago*, and lucerne under low pH conditions (Lie 1969; Mohebbi and Mahler 1989; Vargas and Graham 1989; Alva et al. 1990). In common bean, under low soil pH conditions, both ultrastructure of nodules and nitrogenase activity were affected negatively (Vassileva et al. 1997). According to Hungria and Stacey (1997), low soil pH disrupts the signal exchange between rhizobia and the host plant. However, our experimental results showed that in this plant, a native from acidic soil, the mechanism of symbiotic association needs to be deeply investigated since nodulation and BNF work well even under pH 4.0. Apparently, rhizobia do not get attached to root when pH is low and root colonization can be decreased (Caetano-Anollés et al. 1989), but we did not observe similar situation in our experiment since nodule formation was higher under lower pH conditions. It is noteworthy that the precise mechanism involved in the decrease of rhizobia root-hair infection is unclear (Lie 1969).

**Table 1** Number of nodules and nitrogenase activity ( $\mu\text{mol H}_2 \text{ g}^{-1} \text{ h}^{-1}$ ) in plants of *Calopogonium mucunoides* growing under different pH conditions

Treatment	Number of nodules	Nitrogenase activity
pH 4.0	57 a	4.78 a
pH 5.5	30 b	1.13 b
pH 7.0	32 b	0.07 b

Different letters in the same parameter indicate significant difference at 5 % by Tukey's test.  $n = 3$

**Table 2** Proteins ( $\text{mg g}^{-1}$  DW), soluble free amino acids ( $\mu\text{mol g}^{-1}$  DW), ureides ( $\mu\text{mol g}^{-1}$  DW), allantoin ( $\mu\text{mol g}^{-1}$  DW), and allantoate ( $\mu\text{mol g}^{-1}$  DW) concentrations in leaves, roots, and nodules of *Calopogonium mucunoides* growing under different pH conditions

Treatments	Protein	Amino acids	Ureides	Allantoin	Allantoate
Leaf tissue					
pH 4.0	2.06 a	0.63 b	1.84 ns	0.89 b	2.09 b
pH 5.5	0.52 b	1.47 a	2.68 ns	2.15 a	8.13 a
pH 7.0	1.37 a	1.61 a	2.26 ns	1.74 a	11.58 a
Root tissue					
pH 4.0	0.40 a	0.94 ns	2.04 ns	1.28 ns	0.75 ns
pH 5.5	0.08 b	1.06 ns	1.79 ns	1.04 ns	0.75 ns
pH 7.0	0.34 a	0.94 ns	1.87 ns	1.21 ns	0.66 ns
Nodules					
pH 4.0	0.13 ab	2.63 b	2.09 b	0.896 b	2.94 b
pH 5.5	0.226 a	3.89 ab	8.13 a	3.02 ab	5.11 a
pH 7.0	0.07 b	7.53 a	11.58 a	5.05 a	6.54 a

Different letters for the same parameter in the same tissue indicate significant differences at 5 % by Tukey's test.  $n = 3$

ns non-significant difference

The observation made above is of great importance to legumes because it may help us to select tolerant rhizobium strains and assess it with different crops in order to find a good interaction between plant and rhizobium. It would be of great importance because some crops, such as soybean, are non-dependent of N manure and associating this feature with a good low pH tolerant crop could mean a productivity increase. Miransari et al. (2013) suggested this approach in a way of afterward identification of tolerant bacteria, where the target should be on specific gene expression under acidic condition in order to improve plant-bacterial interaction.

In considering the pH influence on nodulation or biology of rhizobium, we have to consider its influence on nitrogen metabolism as a whole. In leguminous plants, N can enter biological system by fixation of atmospheric  $\text{N}_2$  due to nitrogenase activity in root nodules (Camargos and Sodek 2010).

By measuring nitrogenase activity, it is an excellent parameter to know nodule functioning. Different from the results found in the model crop soybean, nodulation was decreased at pH 4.0 (Lin et al. 2012), our observation that at the same pH, *C. mucunoides* presented the highest nodulation and nitrogenase activity (Table 1) brings a new scenario where  $\text{N}_2$  can be fixed even under low pH values.

Experiments to determine symbiotic efficiency of rhizobium to acidic pH conditions were carried out by Choudhury et al. (2010) using several legumes (*Cajanus cajan* Mill., *Lens culinaris* Medic., *Phaseolus aureus* Roxb., *Phaseolus mungo* Roxb., *Pisum sativum* L. and *Dlichos labab* L). They showed that some rhizobia are more sensitive than others, and it can be a key to isolate acid-tolerant rhizobia that may serve to produce specific bioinoculant for improving plant growth in acid soils. The

next step of our work is exactly to isolate and characterize some strains, directly from root nodules of *C. mucunoides*, and assess its interaction with crop plants such as promiscuous-nodulating genotypes of soybean and common beans—with the expectation that bacteria isolated from nodules of other species may be symbiotically effective (Bromfield and Barran 1990; Sanginga et al. 1997; Gwata et al. 2004) and thus favoring the use of N by the plants.

The nutrient N is one of the most important elements to plants because it integrates molecules of amino acids, proteins, and nucleic acids. Hence, any disturbance in its metabolism and/or uptake leads to severe consequences in plant growth and development (Fukushima and Kusano 2014). Amino acids are the primary molecules formed by N assimilation through GS/GOGAT pathway, the way in which every  $\text{NH}_4^+$  produced or uptaken gets into organic compounds (Masclaux-Daubresse et al. 2006). In nodules, N can be assimilated and transported as glutamine and asparagine (amides) or as allantoin and allantoate (ureides), these N-rich compounds are metabolized in leaves, so we analyzed their content to estimate if pH has influence on N metabolism.

In this way, the observation that at pH 5.5 and 7.0, the contents of amino acid, allantoin, and allantoate were higher than at pH 4.0 (Table 2) suggests to us that very low acidic conditions affect N metabolism, although at pH 4.0, we observed the higher nitrogenase activity. A possible explanation for this scenario is that other steps, afterward nitrogenase, are more dramatically affected under these conditions. Our results are supported by the findings from Pal'ove-Balang and Mistrik (2011) who found that at pH 4.0, nitrogen assimilation through GS/GOGAT was drastically decreased, and special attention was given to the

decrease in Asn content, an important amino acid related to N transport from roots to shoot. In addition, Ouzounidou et al. (2015) also found that acidic pH negatively affected the accumulation of several metabolites, including proteins in *Salvia hispanica* L., thus showing the negative impact of low pH on plant metabolism as a whole. As observed for amino acids, the pattern of variation in the ureide content was the same, and we may conclude that N transport is affected under low pH 4.0.

The impacts of disturbance in N transport lead to negative consequences in growth and productivity; for this reason, the exploration of how native low-pH growing plants cope with acidity and its effects on N metabolism may serve as future improvement of productivity of important crops, such as soybean and common beans. Our plant model, *C. mucunoides*, presents different and important features such as nodulation and nitrogen fixation even under nitrate exposition (Camargos and Sodek 2010), besides our new report showing that it can nodulate and fix nitrogen under pH as low as 5.5. The consequences of understanding its behavior will help us to comprehend how this plant is adapted to acidity, and in future, it will serve as a tool for improving production and decrease the costs with liming and N-fertilizers.

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## References

- Alva A, Asher C, Edwards D (1990) Effect of solution pH, external calcium concentration and aluminum activity on nodulation and early growth of cowpea. *Aust J Agric Res* 41:359–365. doi:10.1071/AR9900359
- Bekere W, Kebede T, Dawud J (2013) Growth and nodulation response of soybean (*Glycine max* L.) to lime, *Bradyrhizobium japonicum* and nitrogen fertilizer in acid soil at Melko, South Western Ethiopia. *Int J Soil Sci* 8:25–31. doi:10.3923/ijss.2013.25.31
- Bieski RL, Turner NA (1966) Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. *Anal Biochem* 17:278–293. doi:10.1016/0003-2697(66)90206-5
- Bordeleau LM, Prevost D (1994) Nodulation and nitrogen-fixation in extreme environments. *Plant Soil* 161:115–125. doi:10.1007/BF02183092
- Bouhmouch I, Souad-Mouhsine B, Brhada F, Aurag J (2005) Influence of host cultivars and Rhizobium species on the growth and symbiotic performance of *Phaseolus vulgaris* under salt stress. *J Plant Physiol* 162:1103–1113. doi:10.1016/j.jplph.2004.12.003
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254. doi:10.1006/abio.1976.9999
- Bromfield ESP, Barran LR (1990) Promiscuous nodulation of *Phaseolus vulgaris*, *Macroptilium atropurpureum*, and *Leucaena leucocephala* by indigenous rhizobium meliloti. *Can J Microbiol* 36:369–372
- Caetano-Anollés G, Lagares A, Favelukes G (1989) Adsorption of Rhizobium meliloti to alfalfa roots: dependence on divalent cations and pH. *Plant Soil* 117:67–74. doi:10.1007/BF02206258
- Calder F, Bishop R, MacLeod L, MacEachern C (1965) Effect of limestone on pasture sward renovation with and without cultivation. *Can J Soil Sci* 45:251–256
- Camargos LS (2007) Alteração no metabolismo de compostos nitrogenados em *Calopogonium mucunoides* em resposta a diferentes fontes de nitrogênio: Efeito na nodulação e na fixação. Thesis, Universidade Estadual de, Campinas
- Camargos LS, Sodek L (2010) Nodule growth and nitrogen fixation of *Calopogonium mucunoides* L. show low sensitivity to nitrate. *Symbiosis* 51:167–174. doi:10.1007/s13199-010-0063-5
- Choudhury B, Azad P, Kalita MC (2010) Variability in Symbiotic Effectiveness of Native Rhizobia in Acid Stress. *Curr Microbiol* 61:85–91. doi:10.1007/s00284-009-9579-4
- Costa NL (1995) Nitrogen fertilization and association of elephant-grass (*Pennisetum purpureum* cv. Cameroon) with tropical forage legumes. *Pesq Agrop Bras* 30:401–408
- dos Reis FB, Jr Simon MF, Gross E, Boddey RM, Elliott GN, Neto NE, Loureiro MD, de Queiroz LP, Scotti MR, Chen WM, Noren A, Rubio MC, de Faria SM, Bontemps C, Goi SR, Young JPW, Sprent JI, James EK (2010) Nodulation and nitrogen fixation by Mimoso spp. in the Cerrado and Caatinga biomes of Brazil. *New Phytol* 186:934–946. doi:10.1111/j.1469-8137.2010.03267.x
- Fukushima A, Kusano M (2014) A network perspective on nitrogen metabolism from model to crop plants using integrated 'omics' approaches. *J Exp Bot* 65:5619–5630. doi:10.1093/jxb/eru322
- Guo Y, Yu Y, Yuan L, Huang J (2009) Effects of liming and *Sinorhizobium* inoculation on growth, nodulation and nutrient concentrations of Lucerne in acid soils. *Trop Grasslands* 43:112–117
- Gwata ET, Wofford DS, Pfahler PL, Boote KJ (2004) Genetics of promiscuous nodulation in soybeans: nodule dry weight and leaf color score. *J Hered* 95:154–157. doi:10.1093/jhered/esh017
- Helyar KR, Anderson AJ (1974) Effects of calcium carbonate on the availability of nutrients in an acid soil. *Soil Sci Soc Am J* 38:341–346
- Hoagland DR, Arnon DI (1950) The water culture method for growing plants without soil. *Agri Exp Stn Bull*, California
- Hungria M, Stacey G (1997) Molecular signals exchanged between host plants and rhizobia: basic aspects and potential application in agriculture. *Soil Biol Biochem* 29:819–830. doi:10.1016/S0038-0717(96)00239-8
- Izaguirre-Mayoral ML, Vivas AI (1996) Symbiotic N<sub>2</sub>-fixation in tropical legume species growing at high geographic elevation. *Symbiosis* 21:49–60
- Kochian LV, Hoekenga OA, Pineros MA (2004) How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. *Annu Rev Plant Biol* 55:459–493. doi:10.1146/annurev.arplant.55.031903.141655
- Leblanc HA, McGraw RL, Nygren P, Le Roux C (2005) Neotropical legume tree *Inga edulis* forms N<sub>2</sub>-fixing symbiosis with fast-growing Bradyrhizobium strains. *Plant Soil* 275:123–133. doi:10.1007/s11104-005-0808-8
- Lie T (1969) Effect of low pH on different phases of nodule formation in pea plants. *Plant Soil* 31:391406. doi:10.1007/BF01373812
- Lin M, Gresshoff PM, Ferguson BJ (2012) Systemic Regulation of Soybean Nodulation by Acidic Growth Conditions. *Plant Physiol* 160:2028–2039. doi:10.1104/pp.112.204149
- Masclaux-Daubresse C, Reisdorf-Cren M, Pageau K, Lelandais M, Grandjean O, Kronenberger J, Valadier MH, Feraud M, Joulet T, Suzuki A (2006) Glutamine synthetase-glutamate synthase

- pathway and glutamate dehydrogenase play distinct roles in the sink-source nitrogen cycle in tobacco. *Plant Physiol* 140:444–456. doi:[10.1104/pp.105.071910](https://doi.org/10.1104/pp.105.071910)
- Meghvansi MK, Prasad K, Mahna SK (2005) Identification of pH tolerant *Bradyrhizobium japonicum* strains and their symbiotic effectiveness in soybean *Glycine max* (L.) Merr. in low nutrient soil. *Afr J Biotechnol* 4:663–666
- Miransari M, Riahi H, Eftekhar F, Minaie A, Smith DL (2013) Improving soybean (*Glycine max* L.) N<sub>2</sub> fixation under stress. *J Plant Growth Regul* 32:909–921. doi:[10.1007/s00344-013-9335-7](https://doi.org/10.1007/s00344-013-9335-7)
- Mohammadi K, Sohrabi Y, Heidari G, Khalesro S, Majidi M (2012) Effective factors on biological nitrogen fixation. *Afr J Agric Res* 7:1782–1788
- Mohebbi S, Mahler R (1989) The effect of soil pH on wheat and lentils grown on an agriculturally acidified northern Idaho soil under greenhouse conditions. *Commun Soil Sci Plant Anal* 20:359–381. doi:[10.1080/00103628909368088](https://doi.org/10.1080/00103628909368088)
- Munns D (1986) Acid soil tolerance in legumes and rhizobia. *Adv Plant Nutr* 2:63–91
- Nichols JD, Carpenter FN (2006) Interplanting *Inga edulis* yields nitrogen benefits to *Terminalia* Amazonia. *Forest Ecol Manag* 233:344–351. doi:[10.1016/j.foreco.2006.05.031](https://doi.org/10.1016/j.foreco.2006.05.031)
- Ouzounidou G, Skiada V, Papadopoulou KK, Stamatis N, Kavvadias V, Eleftheriadis E, Gaitis F (2015) Effects of soil pH and arbuscular mycorrhiza (AM) inoculation on growth and chemical composition of chia (*Salvia hispanica* L.) leaves. *Braz J of Bot* 38:487–495
- Pal'ove-Balang P, Mistrik I (2007) Impact of low pH and aluminium on nitrogen uptake and metabolism in roots of *Lotus japonicus*. *Biologia* 62:715–719. doi:[10.2478/s11756-007-0133-1](https://doi.org/10.2478/s11756-007-0133-1)
- Pal'ove-Balang P, Mistrik I (2011) Effect of aluminium on nitrogen assimilation in roots of *Lotus japonicus*. *Plant Biosyst* 145:527–531. doi:[10.1080/11263504.2011.575608](https://doi.org/10.1080/11263504.2011.575608)
- Qubit Systems < disponível em: <http://qubitsystems.com/plant-and-soil/q-box-nf11p-nitrogen-fixation-package/>>
- Sanginga N, Dashiell K, Okogun JÁ, Thottappilly G (1997) Nitrogen fixation and N contributions by promiscuous nodulations soybeans in the southern Guinea savanna of Nigeria. *Plant Soil* 195:257–266
- Seiffert NF, Zimmer AH, Schunke RM, Behling-Miranda CH (1985) Nitrogen recycling in mixed pastures of *Calopogonium mucunoides* and *Brachiaria decumbens*. *Pesq Agrop Bras* 20:529–544
- Sprent JI (2009) Legume nodulation a global perspective. Wiley, Chichester
- Temple SJ, Vance CP, Stephen-Gantt J (1998) Glutamate synthase and nitrogen assimilation. *Trends Plant Sci* 3:51–56. doi:[10.1016/S1360-1385\(97\)01159-X](https://doi.org/10.1016/S1360-1385(97)01159-X)
- Thomas R, Asakawa N, Rondon M, Alarcon H (1997) Nitrogen fixation by three tropical forage legumes in an acid-soil savanna of Colombia. *Soil Biol Biochem* 29:801–808. doi:[10.1016/S0038-0717\(96\)00212-X](https://doi.org/10.1016/S0038-0717(96)00212-X)
- Vargas AAT, Graham PH (1989) Cultivar and pH effects on competition for nodule sites between isolates of *Rhizobium* in beans. *Plant Soil* 117:195–200. doi:[10.1007/BF02220712](https://doi.org/10.1007/BF02220712)
- Vassileva V, Milanov G, Ignatov G, Nikolov B (1997) Effect of low pH on nitrogen fixation of common bean grown at various calcium and nitrate levels. *J Plant Nutr* 20:279–294. doi:[10.1080/01904169709365250](https://doi.org/10.1080/01904169709365250)
- Vogels GD, Van der Drift C (1970) Differential analysis of glyoxilate derivatives. *Anal Biochem* 33:143–157. doi:[10.1016/0003-2697\(70\)90448-3](https://doi.org/10.1016/0003-2697(70)90448-3)
- Willians LE, Miller AJ (2001) Transporters responsible for the uptake and partitioning of nitrogenous solutes. *Annu Rev Plant Physiol Mol Biol* 52:659–688. doi:[10.1146/annurev.arplant.52.1.659](https://doi.org/10.1146/annurev.arplant.52.1.659)
- Yemm HE, Cocking EC (1955) The determination of amino acids with ninhydrin. *Analyst* 80:209–213. doi:[10.1039/an9558000209](https://doi.org/10.1039/an9558000209)