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GROWING THE ENDANGERED SPECIES *Astragalus nitidiflorus* IN THE NURSERY: FERTILIZATION RATE AFFECTS GROWTH, AND LEAF NUTRIENT AND CHLOROPHYLL CONTENTS

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ABSTRACT

Astragalus nitidiflorus is an endangered legume endemic to the southeast of the Iberian Peninsula. This species develops symbiotic relationships with N-fixing bacteria. However, the problem of isolating its rhizobia has not been solved. Because poor N fixation in plants can be corrected by fertilization, the effect of N-P-K fertilizers on growth, leaf chlorophyll and mineral ions was studied. Plants of *A. nitidiflorus* were grown in 100%-substrate with different N-P-K fertilizer rates (mg l^{-1}): 1-1-8 (S0), 69-29-35 (SL), 144-43-131 (SM) and 245-58-235 (SH). A treatment with substrate plus soil from the natural habitat and no fertilizers (T0) was included. The reference foliar contents of N, P and K were 42.5, 3.5 and 36.5 mg g^{-1}, respectively. Although the species did not form root nodules when grown in substrate, T0 plants produced active nodules that allowed the plants to grow properly without fertilization. In the absence of nodules, both N fertilization (~144 mg l^{-1}) and Fe fertilization (>12 mg l^{-1}) are vital, as is, to a lesser extent, K fertilization (~75 mg l^{-1} K_2O). The S0 and SL reduced leaf chlorophyll, while SM prevented its degradation.

Key words: nutrition, nodulation, native plants, substrate, pot, nursery

INTRODUCTION

The main characteristic of legumes is their ability to change atmospheric N into a form of N that is available for the plant. This process is mediated only by N-fixing rhizobia bacteria, which live in small growths on the roots (nodules). Within these nodules, the bacteria carry out N fixation, and the plant can easily absorb the ammonia that they produce [Castroviejo et al. 1986]. *Astragalus nitidiflorus* is a legume of the Fabaceae family. In the Iberian Peninsula, there are 41 described species of *Astragalus*. Among them, *A. nitidiflorus* Jiménez Mun. et Pau, is a endemism of Murcia (SE Spain) that is listed as "critically endangered" [Martínez-Sánchez et al. 2011, Vicente et al. 2016].

In the case of endangered species, one of the ways in which to avoid their loss is to introduce specimens in the natural habitat, which requires producing high quality plants. So, nursery techniques need to be optimized. Previous experiments have demonstrated the inability of this species to grow properly in substrate when cultivated in pots, while the addition of soil from the habitat to the substrate induced nodule formation in roots and led to good development [Vicente et al. 2016]. However, although the rhizobium of *A. nitidiflorus* has been isolated and identified as *Mesorhizobium* sp., there is currently no protocol for inoculating seeds or seedlings, as can be done in the case of other endangered legumes [Navarro et al.

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2014]. Therefore, the only way to inoculate plants in the nursery is by adding soil from of the habitat to the growing medium, which is an expensive task and alters the natural habitat of the species. One way to solve this problem could be to use fertilizer, although the response of *A. nitidiflorus* to fertilizers is still unknown.

Since the growth of this species depends on the symbiosis of plants with root-nodule bacteria and since the inoculation of such symbiotic bacteria is a limiting factor, the effect of fertilization on growth was investigated. For this, an experiment was carried out to study the effect of increasing N-P-K concentrations provided in the nutrient solution on the aerial growth, and leaf chlorophyll and mineral ion contents of *A. nitidiflorus*.

MATERIALS AND METHODS

Plant material and culture conditions. Seeds of *A. nitidiflorus* were planted in 60 multi-pot forest trays, each pot (240 cm^3 volume) with an inverted pyramid form. The trays were filled with a substrate containing black peat (45%), coconut fibre (45%) and perlite (10%), pH 6.3 and EC of 1.0 dS m^{-1}. The substrate contained 1N-0.68P_2O_5-0.84K_2O-0.37CaO-0.68MgO at a rate of 190 mg l^{-1}. In December 2015, six week-old seedlings were transplanted in PVC pots (2.8 l volume), which were filled with the substrate described above (100%) or with a mixture of substrate and soil from the habitat of *A. nitidiflorus* (15%, volume). The experiment took place in a greenhouse with a semicircular cover located at the Agricultural Experimental Station of the Technical University of Cartagena (Cartagena, Spain). The pots were kept in place in holes in a metal grid made of corrugated bars (8 mm ϕ), 80 cm off the ground. The average air temperature in the greenhouse was 6.8°C (minimum) and 28.8°C (maximum); relative humidity was 36.8% minimum and 83.6% maximum.

Treatments. The following treatments were studied: substrate + soil and no fertilizers (T0, soil control), substrate and no fertilizers (S0, substrate control), substrate and low fertilizer rate (FR) (0.43 dS m^{-1}) (SL), substrate and medium FR (0.81 dS m^{-1}) (SM), substrate and high FR (1.23 dS m^{-1}) (SH). Table 1 shows the nutrient concentrations and ratios in the applied nutrient solutions, which were made up using local irrigation water. The nutrient solutions were made by adding ammonium nitrate, potassium nitrate, and phosphoric acid to the irrigation water. Nitric acid was also added to neutralize the alkalinity, resulting in an endpoint solution of pH 6.1. A fertilizer (35 mg l^{-1}), which contained microelements, and an iron EDTA chelate (9 mg l^{-1}) were added. Nutrient supplementation began on 28 December 2015 and ended on 31 May 2016.

Irrigation management. The plants were irrigated using a pump connected to five 250 l tanks containing the different nutrient solutions. This pump was controlled by solenoid valves opened and closed by a Hunter XC irrigation programmer (Hunter Industries, CA, USA). Each pot had two emitters (1.2 l h^{-1}) connected to two spaghetti tubes. All treatments were irrigated in the same day. The total volume of water applied during the experimental period was the same in all treatments (26.12 ±0.9 litres per pot).

Table 1. Nutrient concentration, nutrient ratio and electrical conductivity (EC) in the nutrient solutions (pH 6.1 ±0.2) and irrigation water

Treatments	Concentration (mg l^{-1})			EC (dS m^{-1})	
	N	P_2O_5	K_2O	Fertilizer	Solution
Substrate + soil and no fertilizers (T0)	1	1	8	0	0.94
Substrate and no fertilizers (S0)	1	1	8	0	0.94
Substrate and low fertilizer rate (SL)	69	29	35	0.43	1.37
Substrate and medium fertilizer rate (SM)	144	43	131	0.81	1.75
Substrate and high fertilizer rate (SH)	245	58	235	1.23	2.17

Plant growth, development, and SPAD values. At the end of the experiment (the last week of May 2016), the dry weight (DW) of the aerial part, the number of shoots per plant, the number of leaves per plant, the number of leaflets per leaf and the number of fruits per plant were determined in six plants per treatment. In order to check the presence of nodules in the roots, four photographs were taken of the roots (the four sides of the root ball) with a digital camera. SPAD values were measured using a SPAD-502 chlorophyll meter (Konica Minolta Sensing Inc., Osaka, Japan), with the light transmitted through the leaf at 650 nm and at 940 nm. All leaves of the main shoot in each plant were measured. In each leaf, the SPAD value in the fifth leaf pair from the bottom was measured. The value of foliar SPAD for each treatment was the average of the values obtained on all leaves of one shoot. The gradient of greenness in leaves along the mean shoot was studied by measuring the SPAD values according to the position of the leaf on the shoot.

Determination of nutrients in leaves. Chlorotic and green leaves from six plants growing in their natural habitat were randomly collected. At the end of our experiment, leaves from the central part of the main stem were collected from six plants grown in each treatment. Four samples of leaves per treatment were selected at random to analyse the mineral ion concentrations. Dried plant tissue samples were ground and 0.2 g of each tissue was added to 50 ml of distilled water. Each solution was mixed for 30 min by shaking on a magnetic stirrer at 117 rpm and 27°C then filtered and passed through a 0.45 μm nylon membrane. Ten ml of each filtered solution were analysed in a Metrohm 850 Ion chromatography system equipped with a conductometric detector and an autosampler (Metrohm 815 Robotic USB Sample Processor XL), which also filtered the samples in-line through a 0.20 μm pore ϕ cellulose acetate membrane filter. Anion separation was carried out using a Metrosep A Supp 5–50 column (Metrohm AG) with carbonate-bicarbonate eluent (3.2 mM Na_2CO_3 1.0 mM $NaHCO_3$) at a flow rate of 0.7 ml min^{-1}. Cations were separated on a Metrosep C3-100 column (Metrohm AG) with 3.5 mM nitric acid as eluent at a flow rate of 1.0 ml min^{-1}.

Statistical analysis and experimental design. The experiment was arranged in a randomised complete block design on crop benches with three blocks of seven plants per treatment. The data collected were subjected to analysis of variance (one-way ANOVA). When the ANOVA indicated significant effects, means were separated by the least significant difference (LSD) test ($P < 0.05$). The regression analysis was made using Sigma-Plot 10.0 (Systat Software Inc., CA, USA).

RESULTS

Aerial growth and development. Increasing the fertilizer rate (FR) increased the aerial part DW of *A. nitidiflorus*, by 26% (SL) and around 50% (SM and SH) compared with the S0 treatment (tab. 2). However, the T0 treatment produced the highest aerial DW (68.22 g per plant). Similarly, the number of shoots per plant responded positively to the FR (tab. 2). The SH treatment produced plants with three times more stems compared with those grown under S0. The T0 had the same effect as SH. Both fertilization (SL, SM, and SH) and the presence of soil in the substrate (T0) significantly increased the number of leaves per plant compared with S0. The SL treatment almost doubled the number of leaves, while SM and SH increased it by 2.3 times. T0 produced 2.7 times more leaves than S0. All treatments increased the number of leaflets per leaf in a similar way (tab. 2). The S0 treatment did not produce fruits, while T0 presented the highest number of the same (32 per plant). The three fertilizer rates used increased the number of fruits per plant in a similar proportion compared with S0, producing around 14 fruits (tab. 2).

Root nodules. Nodules were seen to be present on the roots of the plants grown under T0, while no nodules were observed with 100% substrate. The area of the nodules was 1.5% of the total lateral area of the root ball.

Foliar SPAD. Increasing the FR significantly increased the SPAD value in leaves (tab. 2). The lowest FR (SL) increased foliar SPAD by 39% compared with S0, while higher rates raised the percentage by around 51. The T0 treatment had a similar effect to SM and SH. A linear decrease in SPAD in leaves along the shoots was determined in all treatments, falling from a value of around 75 at the apex to nearly zero at the base (fig. 1).

Table 2. Effects of fertilizer rate on growth, development and foliar SPAD

Parameters	Treatments				
	S0	SL	SM	SH	T0
Aerial part DW (g)	32.62a	42.31b	58.01c	60.02c	68.22d
Number of shoots	6.83a	10.21b	13.50c	16.66d	17.71d
Number of leaves	55.13a	90.71b	124.11c	130.77c	146.08d
Nº of leaflets per leaf	21.64a	24.56b	25.31b	25.82b	26.03b
Number of fruits	0.16a	15.06b	13.74b	14.66b	32.19c
Foliar SPAD	33.35a	54.51b	68.78c	69.11c	67.97c

Different letters in the same row indicate statistically significant differences between means at $P < 0.05$ according to LSD test. Explanation of abbreviations as in Table 1

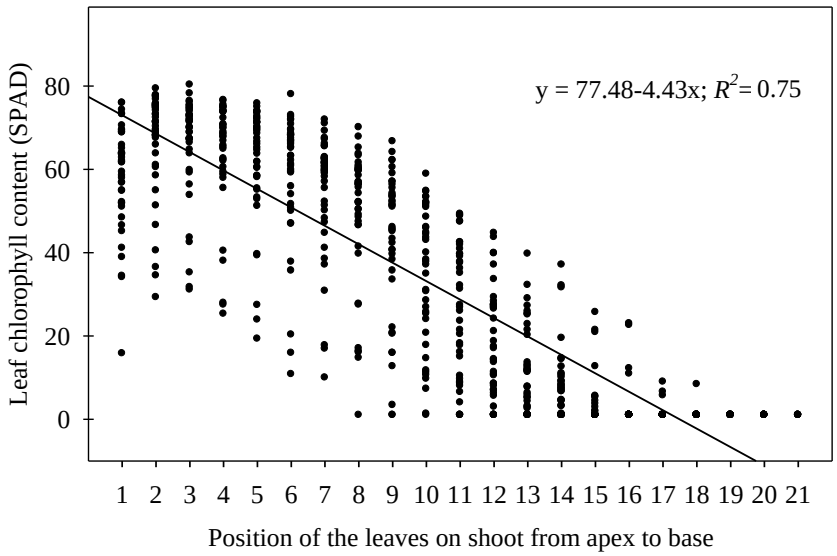

Fig. 1. Linear relationship ($P < 0.001$) between foliar SPAD values and the position of the leaves on the shoot. All treatments are included

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Reference concentrations of nutrients in leaves. The concentration of N in green leaves of plants grown in their natural habitat was 42.5 mg g^{-1}, while the chlorotic leaves of the same plants had approximately half this value (fig. 2). P concentration was 3.5 mg g^{-1} in green leaves, and 2 mg g^{-1} in chlorotic leaves. The K in green leaves was 36.5 mg g^{-1}, which is 40% higher than that recorded in chlorotic leaves. The contents of Ca, Mg and Na were similar in green and chlorotic leaves. The Fe in chlorotic leaves was

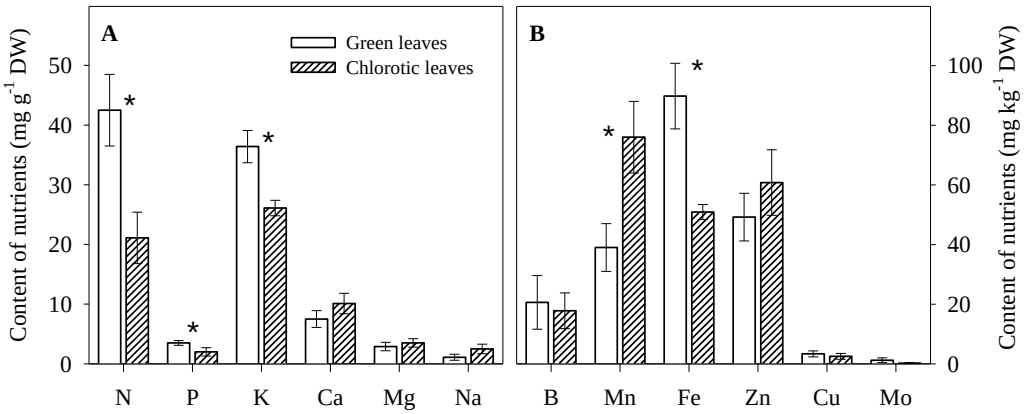

Fig. 2. Concentration of nutrients in green and chlorotic leaves of *Astragalus nitidiflorus* plants grown in their natural habitat. Macronutrients (A) and micronutrients (B). For each nutrient, the presence of an asterisk indicates significant differences between green and chlorotic leaves according to LSD test ($P < 0.05$). The vertical bars indicate ± the standard error of the means

Table 3. Effects of fertilizer rate on concentration of nutrients in leaves

Ions	Treatments				
	S0	SL	SM	SH	T0
			mg g^{-1} DW		
N	18.22 a	22.77 a	36.32 b	41.4 c	35.13 b
P	3.30 b	3.53 c	4.40 d	5.30 d	2.44 a
K	28.27 a	31.35 a	39.13 b	37.02 b	29.78 a
			mg kg^{-1} DW		
B	48.13 b	57.93 c	47.47 b	42.83 a	44.33 ab
Fe	59.89 a	73.12 b	76.69 b	67.76 ab	95.11 c
Mn	20.56 a	22.82 ab	29.41 2b	59.49 2d	38.72 c

Explanation of abbreviations as in Tables 1 and 2

almost half that measured in green leaves (98.8 mg kg^{-1}). By contrast, the Mn of chlorotic leaves was double that of the green leaves. The contents of B, Zn, Cu and Mo were statistically similar in green and chlorotic leaves.

Foliar concentrations of mineral nutrient in treatments. Leaf N in the S0 treatment was 18.22 mg g^{-1}, while both the SM and SH increased it by 2 and 2.3 times, respectively (tab. 3). The presence of soil (T0) led to a foliar N concentration of 35.13 mg g^{-1}, which was similar to that of the SM treatment and lower than that obtained with SH. The SM and SH treatments increased leaf P concentration by 25% and 38% compared to S0 (3.30 mg g^{-1}), respectively, whereas the SL had no effect. The T0 treatment led to 2.44 mg g^{-1}, which was the lowest P content in leaves. The lowest concentrations of leaf K were observed in S0 and SL (around 30 mg g^{-1}), which is not very different from the T0 value. The application of SM maximized the accumulation of K in leaves (39.13 mg g^{-1}). No treatment significantly affected the contents of Ca, Mg and Na in leaves (data not shown). The SL treatment increased foliar B by 17% compared with S0, while a reduction of 11% was observed with the SH. The T0, S0 and SM treatments produced similar foliar B (tab. 3). The leaf

Fe clearly increased with the presence of soil; hence, T0 treatment produced 62% more leaf Fe than S0 and 42% more than SH. Fertilizing with SL or SM treatment increased the leaf Fe compared with S0, while SH led to a non-statistically significant increase. T0 treatment doubled the Mn leaf, while SH tripled the same compared with S0. The SL treatment produced the same content of leaf Mn as S0, whereas SM increased it by 28%. Neither the FR nor the presence of soil significantly affected the concentrations of Zn, Cu and Mo in the leaves of *A. nitidiflorus* (data not shown).

DISCUSSION

This is the first attempt to study the influence of the fertilization rate of N, P and K on the growth of *A. nitidiflorus* in the nursery. The research was carried out using a commercial substrate, while the soil control (T0) used soil from the species' natural habitat. The results confirm that the incorporation of 15% in volume of this soil to the growth medium promoted the formation of environmental N-fixing nodules, which did not occur when only commercial substrate was used [Vicente et al. 2016]. The observed effect would be because nodule-forming bacteria are part of the microbial community of the soil, and the physicochemical features of the growing medium are determinants to propitiate their development or not [Bazin et al. 1990]. Here, the fertilization of non-inoculated plants (grown in substrate) was effective for increasing aerial growth. Adding fertilizers of 0.81 dS m^{-1} maximized the aerial DW, which was due to an increase in the number of stems and leaves since the number of leaflets per leaf was hardly affected. Despite not being fertilized, the T0 plants produced the highest aerial growth, suggesting that *A. nitidiflorus* has an efficient physiological mechanism to nourish itself and grow favourably when they develops nodules on their roots. The absence of fertilization (S0) inhibited the formation of fruits, while fertilization slightly favoured fruiting. The presence of soil in the growing medium favoured fructification, probably because the plants experienced earlier leaf development than those of other treatments, giving these plants an advantage in terms of growth and development compared with the other.

The content of nutrients in leaf is often used for the diagnosis of the nutritional status of plants. This content in green leaves of *A. nitidiflorus* that grew in their habitat were considered the reference values for determining a suitable level of nutrition in the plant. The reference value for N was 42 mg g^{-1}, which is slightly higher than that suggested by Sonneveld and Voogt [2009] for several ornamental plants. Perhaps this reference value for N could be lower, because the plants that grew most were those of T0 (35 mg g^{-1} of leaf N). However, 42 mg g^{-1} of leaf N is within the range of reference values recommended for cucumbers and sweet pepper [De Kreij et al. 1992]. In this experiment, the addition of 144 mg l^{-1} and 245 mg l^{-1} of N (tab. 1) led to a leaf N content close to the reference value, as obtained, too, in T0 (tab. 3). By contrast, when they were not fertilized (S0) or had a low level of N (69 mg l^{-1} of N, SL), the N contents were around 20 mg g^{-1}, well below the reference value and reflecting the value found in chlorotic leaves of natural plantations (fig. 2A). The adequate leaf N in T0 plants suggests that the nodules of *A. nitidiflorus* were good fixers of atmospheric N. The ability of legumes to establish a symbiotic relationship with soil bacteria is well known, taking N from the air and fixing it in the form of amino acids, while the plant gives carbohydrates to the bacteria [Castroviejo et al. 1986]. Unfortunately, the absence of a rhizobia inoculation protocol for *A. nitidiflorus* means that soil had to be added to the growth medium to be inoculated, which makes the management of nursery production rather cumbersome besides producing an alteration in the natural ecosystem of the plant.

The SPAD value is a good indicator of the greenness of leaves and leaf chlorosis [Valdés et al. 2015], which can be related to N because this is a key component of chlorophyll. Low levels of SPAD in S0 point to the loss of chlorophyll, which was effectively prevented with fertilization. The addition of 144 mg l^{-1} of N (SM) was sufficient to maximize the leaf chlorophyll. A gradient of leaf chlorosis was observed along the stem of *A. nitidiflorus* in all treatments, even in plants grown in their natural habitat, which shows that this is natural behaviour in this plant. Along the stem, the more apical leaves had a higher chlorophyll, the level was lower the closer

the leaf was to the base of the stem (older leaves). This degradation of chloroplasts is an important process during leaf senescence, which allows the accumulated N in the chloroplasts to be mobilized to other, developing organs [Killingbeck 2004]. So, the senescence trend of basal leaves of the stems may be considered as a nutritional strategy of *A. nitidiflorus* to reduce their need for N.

The reference value for P (3.5 mg g^{-1}) was reached in all the fertirrigated plants, even in the absence of fertilization. One explanation for this could be that the plant absorbs the P existing in the substrate, because the P in the irrigation water was negligible. However, T0 plants had the lowest leaf P levels, perhaps because they grew more and the P had to be distributed through more tissue, and because the soil contained less P than the substrate. However, the leaf P of T0 (2.4 mg g^{-1}) was above the value found in chlorotic leaves from natural plantations (2.0 mg g^{-1}), and was sufficient to maximize growth and fruiting. Consequently, a low rate of phosphate fertilization should be applied in the production of *A. nitidiflorus* in substrate. In fact, the contribution of 29 mg l^{-1} of P_2O_5 (12 mg l^{-1} of P) (SL) is in line with the indications of Van der Boon [1981], authors who recommended 10 mg l^{-1} of P to optimize the growth in ornamental shrubs. However, Kim and Li [2016] suggested the application of 20 mg l^{-1} of P to maintain the optimum growth of *Lantana camara*.

The leaf K increased with 131 mg l^{-1} of K_2O (61 mg l^{-1} of K, SM), reaching a similar value to the reference value (36.5 mg g^{-1}). A high K content in tissues is desirable because its relationship with an improvement in plant quality is well established [Lester et al. 2010] and with the increase of resistance to diseases and abiotic [Yermiyahu et al. 2015]. The SL produced a leaf K content below the reference value, although no sign of K deficiency was observed. Sonneveld and Voogt [2009] indicated that the optimum leaf concentration of K in ornamental crops varies greatly with the species, and suggested an optimal of 8 to 20 mg g^{-1} in azalea, and between 27 and 80 mg g^{-1} in hydrangea. The absorption of K by the plant tends to affect the absorption of cations. For example, Barickman et al. [2016] observed a reduction in the leaf content of B, Mg, S, Ca, and Fe with an increase in P fertilization in lettuce. The leaf contents of Ca, Mg and Na in *A. nitidiflorus* was not affected by the treatments, which showed values within the ranges suggested as suitable for numerous greenhouse crops [De Kreij et al. 1992], probably due to the high content of the three nutrients in the irrigation water.

The chlorotic leaves of natural populations of *A. nitidiflorus* contained much lower Fe than the green leaves, and showed typical symptoms of iron deficiency (interveinal chlorosis). These symptoms have been related with a reduction in chlorophyll, since Fe is essential for the biosynthesis of chlorophyll [El-Jaoual and Cox 1998]. Radhamani et al. [2016] demonstrated that SPAD values were suitable for ascertaining which varieties of sugar cane were deficient in Fe. Here, the application of 12 mg l^{-1} of Fe led to leaf Fe values of around 75 mg kg^{-1}, which is below the reference value (98.8 mg kg^{-1}). However, the Fe of T0 plants was close to the reference value, which can be explained by the contribution of Fe from the soil of the habitat, which was rich in available Fe, and because nodulation activated a plant mechanism that favoured the root absorption of Fe [Terry et al. 1991].

Chlorotic leaves from natural populations of *A. nitidiflorus* accumulated high levels of Mn (76 mg kg^{-1}), which could be close to the toxic level for the plant because some chlorotic leaflets showed reddish-brown spots. Moreover, the initial symptoms of Mn toxicity are related to those of Fe deficiency, because of the antagonism between Mn and Fe [Fageria 1988]. This excess of Mn can be explained by the high Mn content of the soils of the habitat, while the degradation of chlorophyll in the chlorotic leaves releases Mn [Hauck et al. 2003]. Fertilizer treatments included the same amount of Mn, but the leaf Mn was differed, suggesting an interaction of Mn with other nutrients that affected its release [Fageria 2001]. But in no case did this interaction lead to an leaf Mn similar to that found in chlorotic leaves.

CONCLUSION

A growing medium with 15% of soil from the natural habitat of *A. nitidiflorus* encouraged the presence of root nodules, which did not occur when sub-

strate alone was used, regardless of the fertilization rate. The presence of nodules increased the N and Fe contents of the plants grown with soil, which grew well without fertigation. The chlorotic leaves of plants grown in their natural habitat had lower N and Fe than green leaves, and to a lesser extent of K. By contrast, the Mn content of chlorotic leaves was much higher than that of green leaves. In all treatments, a gradient of leaf chlorophyll degradation was observed, suggesting a nutritional mechanism of *A. nitidiflorus* to supply N by itself. Growing in substrate, a concentration of N of around 144 mg l^{-1} in the nutrient solution prevented leaf chlorophyll degradation and maximized aerial growth. Contents higher than 75 mg l^{-1} of K_2O are not necessary because they do not increase the foliar K. Contents lower than 29 mg l^{-1} of P_2O_5 and higher than 12 mg l^{-1} of Fe are deemed appropriate to produce plants in the nursery.

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