Mineral nitrogen effect on nodulation and nitrogen fixation of the stem-nodulating legume *Aeschynomene afraspera*

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Summary – Zusammenfassung

The effect of increasing amounts of nitrogen (nitrate or urea) on the nitrogen fixing capacity (acetylene reduction assay = ARA), growth (fresh and dry weight) and the number of stem- and root-nodules of the tropical legume *Aeschynomene afraspera* was studied in hydroponic cultures (in growth cabinet) as well as in pot experiments (field conditions). The experiments were carried out at Dakar in the rainy season of 1985. Plants were grown in the presence of 6 nitrate concentrations (0,3,6,9,12 and 15 mM N/l) in hydroponic solution and with 4 urea concentrations (0,50,100 and 200 kg N/ha) in pots. In both types of experiments, root nodulation and ARA were strongly inhibited by increasing amounts of mineral nitrogen. Stem nodulation and potential nitrogen fixation of stems, however, remained unaffected. Lower amounts of mineral nitrogen even enhanced growth as well as nitrogen fixation. The possible future of this remarkable plant as green manure or fodder in low input countries of the tropics is discussed.

Einfluß von mineralischem Stickstoff auf die Knöllchenbildung und Stickstoffbindung der stengelknöllchenbildenden Leguminose *Aeschynomene afraspera*

Aeschynomene afraspera ist eine rasch wachsende, weitverbreitete Leguminose Senegals und verfügt über Wurzel- und Stengelknöllchen zur symbiotischen Stickstoffbindung (mit *Rhizobium* sp.). In den vorliegenden Untersuchungen wurde der Einfluß steigender Mengen an Stickstoff (Harnstoff oder Nitrat) auf die Pflanzenentwicklung (Frisch- oder Trockengewicht), die Anzahl Knöllchen (an Wurzeln und Stengeln) sowie die potentielle Fähigkeit zur Stickstoffbindung (Acetylen-Reduktions-Test = ARA) von *A. afraspera* in hydroponischen Nährlösungen sowie in Gefäßversuchen untersucht. Die Versuche fanden in der Regenzeit (April bis Juli 1985) auf dem Versuchsgelände des Instituts in Dakar statt. Die Pflanzen wurden aus sterilisiertem Samen gezogen und in hydroponischer Lösung mit steigenden Nitratgaben (0,3,6,9,12 und 15 mM Nitrat-N/l) sowie mit steigenden Mengen an Harnstoff (0,50,100 und 200 kg N/ha) in einem Gefäßversuch (mit einem sandigen Lehm) unter Freilandbedingungen untersucht. In beiden Versuchsserien wurde die Wurzelknöllchenbildung und die ARA signifikant durch steigende Mengen an Stickstoff vermindert. Die Stengelknöllchen und ihre potentielle Fähigkeit zur

Introduction

Optimal growth and yield of cultivated plants cannot be obtained by symbiotically fixed nitrogen only. In crop production, plant growth is highly affected by the amount of nitrogen supplied by the soil. For energetic reasons legumes prefer soil derived rather than biologically fixed nitrogen (*Houwaard,* 1979). The application of mineral nitrogen, however, is known to reduce both nodulation and the rate of nitrogen fixation by legumes. The degree of inhibition varies with the form of the N-compound, plant species, cultivar, strain of *Rhizobium,* growth season, light intensity, temperature and other nutritional and soil conditions (*Eaglesham* et al., 1983). The mechanisms of nitrogen interaction are not completely understood, and need not occur in all legumes. Thus, potential nitrogen fixation (ARA) by the stemnodulating legume *Sesbania rostrata* remained unaffected by mineral nitrogen (*Dreyfus* and *Dommergues,* 1980). Even 17 mM N/l did not affect the formation of stem nodules of *Aeschynomene scabra (Eaglesham* and *Szallay,* 1983). On *Aeschynomene* plants stem nodules were first reported in 1982 by *Hagerup (Dreyfus* et al., 1984). Several other reports have been published since then (*Alazard,* 1985). These stem nodules are usually formed by specific rhizobia (*Rhizobium* spp.) under waterlogged conditions (*Eaglesham* and *Szallay,* 1983). Due to the presence of chloroplasts in the cortical cells of stem nodules, these structures differ both in shape and colour from those of the roots of the same plant. *Alazard* (1985) described this phenomenon of stem nodulation with *A. afraspera*, a fast growing annual tropical legume widespread in southern Senegal. Usually, the stem nodules of *A. afraspera* are 2–3 mm high, hemispheric protuberances with a diameter of 3–8 mm and contain the red pigment of leghemoglobin. The objective of the paper presented was to determine whether mineral nitrogen may affect nitrogen fixation with *A. afraspera* and what levels of N should be applied for optimal N_2-fixation. In addition to the effect of increasing concentrations of $Ca(NO_3)_2$ on nodulation, growth and nitrogen fixation by hydroponically grown plants, pot experiments were carried out to study the influence of urea on growth and nitrogen accumulation with *A. afraspera.*

Material and Methods

Plants and inoculum

The seeds used were selected from wild *A. afraspera* found in the Siné-Saloum region of west Senegal, surface sterilized with concentrated H_2SO_4 for 30 min and rinsed abundantly with sterile water. This treatment stimulates germination. The seeds were germinated for 48 h at 30° on water agar in darkness and transfered at a root length of 15 to 20 mm to test tubes or experimental pots. A well growing *Rhizobium* sp. strain (ORS 322) isolated by *Alazard* (1985) from a stem nodule was used in the studies. Bacteria were grown in darkness in 5 ml tubes containing yeast-mannitol-broth (YMB; *Vincent,* 1970). The tubes were continuously shaken (30°C) to give a suspension of approximately 10^8 cells/ml after 3 days. Plants were grown hydroponically (growth cabinet) and inoculated by adding one drop of the broth culture to the nutrient solution (root inoculation) or by applying the bacterial suspension to the stems (stem inoculation) with a sterile brush at an age of 30 days. To inoculate field grown plants, the bacterial culture was diluted (50 %) in sterile water and

sprayed with a commercially available vaporizer on the soil surface one day before transplanting (root inoculation) or on the stems of 30 day-old plants (stem inoculation).

Acetylen reduction activity (ARA)

At an age of 50 days, plants were taken from the test tubes or from the pots and weights of fresh- and dry matter (after 2 days at 60°C) determined. The N-concentration was analyzed by micro-Kjeldahl method (after oxidation with H_2O_2). Potential nitrogen fixation was estimated by the acetylene reduction assay (*Hardy* et al., 1968). Stems (chopped into 10 cm segments) and roots were incubated separately in the dark (30° C) in 145 ml (hydroponically grown plants) or in 575 ml (field grown plants) gas-proof serum bottles with 10 vol % acetylene in the atmosphere. After 30 and 60 min, 2 samples (0.5 ml) were taken with a 1 ml polypropylene syringe and the ethylene produced measured gaschromatographically (Varian Aerograph 1400, flame-ionization-detector, aluminia column, column-temperature: 60° C, carrier gas: nitrogen).

Growth cabinet experiments

The growth cabinet (constant temperature of 28° C) was illuminated continuously by fluorescent tubes (Mazda-Fluor, 38 W) giving a light-intensity of approximately 26000 Lux in a distance of 20 cm. The nutrient solution used was obtained from *Hewitt* (1966) and contained per l:108.8 mg KH_2PO_4, 117.8 mg $CaCl_2x2H_20$, 77.2 mg $MgSO_4x2H_2O$, 0.450 mg $MnSO_4x4H_2O$, 0.050 mg $CuSO_4x5H_2O$, 0.058 mg $ZnSO_4x7H_2O$, 0.620 mg H_3BO_3, 1.2 mg NaCl, 0.025 mg $Na_2MoO_4x2H_2O$ and 10 mg NaFe-EDTA. The pH was adjusted to 6.5 with 0.1 N NaOH and the solution sterilized for 20 min at 120° C. All plants received a hydroponical solution with 0.5 mM N during the first 3 weeks in order to stimulate development. After 23 days of growth, increasing amounts of nitrogen (sterile $Ca(NO_3)_2$-solution) were added to give 0,3,6,9,12 and 15 mM N, respectively. The nutrient solution was changed once a week until inoculation and then every two days. As culture containers 125 ml glass tubes were used, equipped with an opening at the bottom for solution discharge and another in the top of the tube (sealed with aluminium foil) for refilling. The openings were closed by plastic plugs. The tubes were sterilized for 20 min at 120° C and then filled with sterile nutrient solution. The middle of the aluminium foil was punched with a needle and the germination root introduced. Homogeneous plant material was grown with the shoots above the aluminium foil cap. The experiments were carried out in 6 treatments (0,3,6,9,12 and 15 mM N) and 8 repetitions each (48 test-plants).

Pot experiments under field conditions

The containers used were 30 l PVC pots with a diameter of 30 cm and a surface of 700 cm^2. Each pot was filled with material of a loamy sand (pH (N KCl) = 7.0; 0.4 % C; 0.026 % N). P, K and Mg-fertilization was the same in all the pots: 1.36 g KCl (100 kg K/ha), 2.64 g $Ca(H_2PO_4)_2$ (100 kg P/ha) and 1.74 g $MgSO_4$ (50 kg Mg/ha). Three two-day-old seedlings were planted per pot and kept waterlogged after the first week. 4 N-treatments with 5 repetitions each were compared: 0.00, 0.76, 1.53 and 3.06 g urea/pot (0,50, 100 and 200 kg N/ha). Nitrogen fertilization was splitted, 50 % being given before planting and 50 % at stem inoculation. All experiments were performed at ORSTOM Bel-Air experimental station in Dakar/Senegal during the rainy season of 1985.

Statistics

Statistical calculations were done with the SPSS-X ONEWAY program. Significances of results were determined by the multiple range test of Duncan at a risk of erroneous rejection of 5 %.

Results

Influence of nitrate on nodulation and nitrogen fixation (ARA) in growth cabinet

The effect of increasing amounts of nitrate on the performances of *A. afraspera* is shown in Table 1. With increasing doses of N there was a nearly linear decrease in the

Table 2: Influence of increasing amounts of urea on nitrogen fixation and performace of pot grown *A. afraspera* at field conditions
Tabelle 2: Einfluß steigender Harnstoffgaben auf die potentielle Stickstoffbindung und Leistung von *A. afraspera* im Gefäßversuch unter Feldbedingungen

kg N/ha (urea)		0	50	100	200
Freshweight (FW)		86.2 a	102.0b	158.1 d	122.8 c
Dryweight (DW)		32.2 a	37.9 b	56.4 d	43.4 c
% N in dry matter		3.2 a	3.4 b	3.6 b	3.9 c
ARA/h (C_2H_4/h)	Stem	84.9 a	98.0 b	130.5 d	117.3 c
Number of nodules	Stem	170.0 a	199.0 b	259.0 c	212.0 b
Weight of nodules	Stem	910.0 a	1 040.0 a	1 520.0 b	1 470.0 b
ARA/g nodule FW	Stem	89.5 b	87.2 b	86.0 b	68.7 a
ARA/h (C_2H_4/h)	Root	16.4 c	14.8 c	9.7 b	5.6 a
Number of nodules	Root	50.0 b	50.0 b	43.0 a	30.0 a
Weight of nodules	Root	548.0 c	534.0 c	500.0 b	380.0 a
ARA/g nodule FW	Root	28.6 c	29.5 c	20.4 b	9.8 a

Data followed by the same letter are not significantly different (Duncan-test, p = 0.05)

Effects of urea on the performances of A. afraspera in pot experiments

The growth response of *A. afraspera* to increasing amounts of urea under field conditions is presented in Table 2. In general, the reactions of N_2-fixation on mineral nitrogen is nearly the same as in hydroponically grown plants (Table 1). 50 kg N/ha had no negative effect upon the root nodules. Nitrogen fertilization stimulated plant growth, the weight of stem nodules as well as ARA (µM C_2H_4/h). A fertilization of 200 kg N/ha surpassed the amount of N optimal for plant development. This can be understood easily when taking into account that *A. afraspera* is an unselected wild plant being unadopted to high nitrogen concentrations in the soil. Generally there is a close relation

stem nodules. this remarkable phenomenon was already reported with the stem nodulating legume *Sesbania rostrata (Dreyfus* and *Dommergues,* 1980). Even high N-concentrations (15 mM N) did not affect the stem nodule number of *A. afraspera.* The same observations were made for *A. scabra (Eaglesham* and *Szallay,* 1983). A high supply of urea to field grown *A. afraspera* inhibited neither specific ARA nor nodule development. The opposite is even true. 100 kg N/ha increased the ARA per plant distinctly by enhancing plant growth *and* the number of stem nodules. This insensitivity of the nitrogenase complex in the stem nodules towards mineral nitrogen may be explained by the higher illumination and consequently higher carbohydrate supply under field conditions. Further, the exceptional position and the morphology of these structures may account for the results obtained. In fact, these nodules are not directly exposed to soil nitrogen (transported by xylem).

Further, the photosynthetic activity in the cortical tissue of the stem nodules may function as a relatively autonomic source of energy. In general the supply of reduced carbon compounds (photosynthetic products) may be regarded as a limiting factor in biological nitrogen fixation (*Mahon,* 1983). An intensive photosynthetic activity in the cortical tissue of stem nodules could change the C/N-ratio in favour of energetically rich carbon compounds and thus favour N_2-fixation by *Rhizobium* sp., particulary at improved growth. The photosynthetic activity of the cortical tissue may explain also the

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