Development of the nodulated soybean plant after flooding of the root system with different sources of nitrogen

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Flooding leads to hypoxia, a stress to which symbiotic N_2 fixation is especially sensitive. The response of fully nodulated soybean plants to a 21-day period of flooding was studied by measurements of growth parameters and xylem transport of organic nitrogenous components to the shoot, in the presence and absence of NO_3^- and NH_4^+ in the medium. Flooding was found to seriously impair N_2 fixation, irrespective of the N source, as indicated by strongly reduced xylem ureide levels. In the absence of a source of N, growth was strongly reduced during flooding while accumulation of N in the shoot was virtually abolished. Flooding in the presence of 5 mM NO_3^- or NH_4^+ led to the accumulation of total N in the shoot but only NO_3^- promoted increases in total dry matter, plant height and leaf area above that found in the absence of N. The accumulation of N, however, was lower than that of the non-flooded control for both NO_3^- and NH_4^+. The increases in total dry matter, plant height and leaf area with NO_3^- was as high as those of the non-flooded control. These data clearly show the beneficial effects of NO_3^- during a prolonged period of flooding of the nodulated root system of soybean.
Key words: *Glycine max*, anaerobiosis, oxygen deficiency.

Desenvolvimento da planta de soja nodulada após o alagamento do sistema radicular com diferentes fontes de nitrogênio: O alagamento leva à hipoxia, um estresse ao qual a fixação simbiótica de N_2 é muito sensível. Estudou-se a resposta da planta de soja nodulada ao alagamento do sistema radicular por 21 dias, na presença e na ausência de NO_3^- e NH_4^+ no meio. Avaliaram-se parâmetros de crescimento da planta e componentes nitrogenados orgânicos transportados à parte aérea através do xilema. A inundação diminuiu drasticamente a fixação de N_2, independentemente da sua fonte, como indicaram os níveis de ureídeos na seiva do xilema. Na ausência de fonte de N, o desenvolvimento foi fortemente reduzido durante o alagamento e seu acúmulo na parte aérea foi pequeno. O alagamento na presença de 5 mM de NO_3^- ou NH_4^+ proporcionou acumulação de N total na parte aérea, mas somente o NO_3^- promoveu aumento na matéria seca total, estatura e área foliar das plantas acima do tratamento inundado sem N. Entretanto, seu acúmulo na parte aérea dos tratamentos inundados com NO_3^- e NH_4^+ foi menor que a testemunha não inundada. O incremento na matéria seca total, estatura e área foliar das plantas do tratamento com NO_3^- foi similar ao da testemunha não inundada. Os dados demonstram claramente o efeito benéfico do NO_3^- durante um período prolongado de alagamento do sistema radicular nodulado da soja.
Palavras-chave: *Glycine max*, anaerobiose, deficiência de oxigênio.

INTRODUCTION

In many soils under aerobic conditions, NO_3^- and NH_4^+ are the predominant sources of N available to the plants. However, the concentrations of NH_4^+ in aerobic soils are usually very low and often 10-1000 times less than those of NO_3^- (Marschner, 1995). Nevertheless, the relative rate of uptake of each form may have no bearing on the amount available (Wirén et al., 2000), since many species preferentially absorb NH_4^+ (Gojon et al., 1986; Gazzarrini et al., 1998) probably due to its assimilation requiring less energy than NO_3^- (Wirén et al., 2000). The uptake of NH_4^+ by roots can involve passive mechanisms or diffusion through channels when external concentrations are high, or can involve active mechanisms, through transport systems, at low external concentrations. The uptake of NO_3^- on the other hand only occurs by active mechanisms (Williams and Miller, 2001). Usually, growth is optimal when plants are supplied with both NH_4^+ and NO_3^- (Bloom et al., 1993). However, few species present satisfactory development when NH_4^+ is the sole or predominant source of N and many show symptoms of toxicity under moderate or high levels of NH_4^+ (Marschner, 1995; Britto et al., 2001). Many hypotheses have been proposed to explain the toxic effects of NH_4^+ in plants, but none are considered satisfactory (Britto et al., 2001).

In waterlogged soils there is an accumulation of NH_4^+ due to the absence of O_2 which interrupts nitrification. NO_3^- is the first compound reduced in the soil that is utilized by anaerobic microorganisms under such conditions (Marschner, 1995). However, the lack of O_2 in waterlogged soils imposes a serious stress on the root system of the plant, resulting in reduced rates of respiration (Drew, 1997). In the case of soybean, for example, the hypoxic conditions found in flood water not only inhibit nitrogen fixation but also the uptake of N and other minerals, leading to reduced growth of roots and nodulation (Sallam and Scott, 1987). Consequently, the transport of N and minerals to the shoot may be inadequate, resulting in yellowing of leaves and a reduction in leaf number. Furthermore, reduced rates of leaf photosynthesis occur, attributed in part to diminished stomatal conductance (Oosterhuis et al., 1990). Reduced growth rates and a lower grain yield have been reported for soybean and attributed to a decline in rates of net photosynthesis and leaf expansion (Linkemer et al., 1998).

Many terrestrial plants undergo morphological adaptations under flooding, with the formation of adventitious roots and aerenchyma (Perata and Alpi, 1993). Such adaptations facilitate the diffusion of oxygen to the flooded root system and are important for plant survival under these conditions. In soybean, adventitious roots and aerenchyma appear within a few days of flooding (Bacanamwo and Purcell, 1999a) and are associated with flooding tolerance. Nitrogen fixation is especially sensitive to flooding of the root system, leading to declines in the accumulation of both total plant N and biomass, but principally the former (Bacanamwo and Purcell, 1999b). The tolerance of plants to flooding is known to be increased by the presence of nitrate (Malavolta, 1954; Trought and Drew, 1981; Priol and Guyot, 1985), although the mechanism is still highly speculative (Sousa and Sodek, 2002). In soybean, the presence of nitrate during flooding of non-nodulated soybean grown on nitrate was also shown to be advantageous in terms of growth (Bacanamwo and Purcell, 1999b), which raises the question whether nitrate and possibly other sources of inorganic N might be beneficial during a period of flooding for nodulated plants.

The objective of this study was to compare the development of the nodulated soybean plant during a period of prolonged flooding (21 days) in the presence of inorganic sources of N (NO_3^-, NH_4^+ and NH_4NO_3).

MATERIAL AND METHODS

Plant material: Soybean plants (*Glycine max*), cv. FT-Abyara, inoculated on sowing with *Bradyrhizobium elkanii,* strain SEMIA 5019, were grown in a greenhouse under natural light (summer) and temperature conditions (mean max-min temperatures were 38°C-19°C). Plants were grown in pots of 2 L, two plants per pot, with vermiculite as substrate. N-free nutrient solution (Hoagland and Arnon, 1950) was supplied at the rate of 200 mL twice a week up to the V6-7 growth stage (plants at the vegetative stage with 5 or 6 trifoliolate leaves, according to Costa and Marchezan, 1982). At this point the pots were placed inside 3L non-perforated pots and flooded with N-free nutrient solution at 1/3 strength. At the same time the treatments with different sources of N were applied, by addition of N in the form of NO_3^-, NH_4^+ and NH_4NO_3 to a final concentration of 5 mM (equivalent to the N content of Hoagland and Arnon´s complete solution at 1/3 strength). The source of NO_3^- was KNO_3 and NH_4^+ was $(NH_4)_2$ SO_4. N-free controls were also set up.

The water level of the nutrient solution was maintained at 2-3 cm above the surface of the vermiculite by the daily addition of the solution, representing a total volume of 1,8 L per flooded pot. The plants were kept in these conditions for 21 days and material harvested for analysis on days 0, 5, 14 and 21.

Besides the four treatments with flooding (presence of NO_3^-, NH_4^+ and NH_4NO_3 together with the N-free control), non-flooded controls were also set up, feeding 200 mL per pot of N-free solution 3 times a week. Each pot received, therefore, a total of 600 mL of normal strength solution added per week, equivalent to the 1.8 L of 1/3 strength solution used in the flooded treatments. Water was supplied as necessary.

Physical and biochemical measurements: Plant height was measured from the base of the stem and leaf area using a LI-COR model LI-3100 area meter. Dry mass was obtained after the plant material dried to constant weight in a forced-draught oven at 60°C. The temperature of 60°C was used to avoid losses by volatilization of N.

Oxygen concentration in the nutrient solution was measured with a portable O_2 densitometer (JENWAY, model 9071).

Xylem bleeding sap was collected as described by McClure and Israel (1979). Total free amino acids in the sap was determined from the total amino acids recovered after separation and analysis by HPLC, as derivatives of OPA, according to Jarret et al. (1986). Uriedes were determined according to Vogels and Van Der Drift (1970).

Total N of the shoot was determined by the Kjeldahl method according to Tedesco et al. (1995). NO_3^- was determined by the method of Cataldo et al. (1975) and NH_4^+ according to Mitchell (1972), as modified by Felker (1977).

Experimental design and statistical analysis: Treatments were arranged in a completely randomized design with three replicates. Each replicate was composed of the pooled material from the two plants from a single pot. Data were subjected to an analysis of variance and when F was significant, the means compared by Duncan´s multiple range test at 5 % probability.

RESULTS

The concentration of O_2 in the nutrient solution used to flood the pots declined from approximately 6.7 mg O_2.L^{-1} on flooding to 0.7 mg O_2.L^{-1} after 1 day, reaching levels as low as 0.4 mg O_2.L^{-1} over the 21 day period of flooding (data not shown). This indicates that hypoxic conditions were present throughout the experiment.

Plant development was evaluated through measurements of plant height, dry mass and N accumulation during the 21-day experimental period. All plants showed an increase in height over the experimental period (table 1). Plant height of flooded plants with NO_3^- present did not differ from the non-flooded controls at days 5, 14 or 21, while for all other treatments height was significantly lower. The lowest plant height (at day-21) was recorded when NH_4^+ was present. Although this was not statistically different from the flooded N-free control at day-21, it was significantly lower than this control at days 5 and 14, suggesting that NH_4^+ exerted a stronger effect during the earlier phase of flooding. Even when NH_4^+ was together with NO_3^- (in the form of NH_4NO_3) plant height was lower compared to plants flooded with NO_3^-.

The data for leaf area (table 1) revealed a similar response to the treatments seen with plant height. The presence of NO_3^- during flooding again led to a value similar to that found for the non-flooded controls at days 5, 14 and 21, where the highest values were found. Flooding in the presence of NH_4^+ or without N resulted in the lowest values, while again NH_4NO_3 produced an intermediate value between the treatments NO_3^- and NH_4^+ alone at day-21.

Total plant dry mass (table 2) showed no significant difference between any treatment at day-5 but by day-14 the treatment with NH_4^+ present during flooding had a lower dry mass than both the non-flooded controls and the treatment with NO_3^-. By day-21 it was lower than all other treatments except for the N-free flooded control. In fact, the NH_4^+ treatment accompanied the N-free flooded control quite closely throughout the experiment. From the data for root and shoot dry mass (table 2) it is clear that both NH_4^+ and N-free control treatments produced their effects first on

Table 1. Height and leaf area of nodulated soybean plants with root systems flooded at the V6-7 growth stage[a] for 5, 14 and 21 days with nutrient solution free of N (N-free) or with NO_3^-, NH_4^+ and NH_4NO_3, and in non-flooded plants (NF) cultivated with the N-free nutrient solution

	Height (cm)				Leaf area ($cm^2.plant^{-1}$)			
	0 days	5 days	14 days	21days	0 days	5 days	14 days	21days
NF	52.0	62.0 ab[b]	77.3 a	96.6 a	900	1334 a	2199 a	3220 a
N-free	–	65.0 a	77.0 a	81.0 c	–	1132 b	1642 bc	1581 c
NO_3^-	–	63.0 a	77.3 a	92.6 a	–	1307 a	2012 ab	3001 a
NH_4^+	–	55.3 c	66.6 b	77.3 c	–	1219 ab	1458 c	1689 c
NH_4NO_3	–	57.0 bc	68.6 b	87.0 b	–	1198 b	1640 bc	2504 b

[a] Plants at the vegetative stage with 5 or 6 trifoliolate leaves. [b] Means followed by different letters within columns are significantly different by Duncan´s multiple range test (P ≤ 0.05).

the roots (at day-14) and only later on the shoots (at day-21). The beneficial effect of NO_3^- during flooding is clearly shown by the similar dry mass increases to the non-flooded control. In contrast to the data for height and leaf area (table 1), NH_4^+ when together with NO_3^- did not diminish the beneficial effect of NO_3^- in terms of dry mass (table 2).

An analysis of the xylem bleeding sap revealed substantial increases in the levels of total amino acids (table 3) in plants flooded with NH_4^+ at days 5, 14 and 21. NO_3^- also led to an increase relative to the non-flooded controls but nevertheless this was some 8 to 9-fold lower than that seen for NH_4^+ at days 14 and 21. The NH_4NO_3 treatment had an intermediary effect. The N-free treatment presented the lowest levels but these were not significantly different from the non-flooded controls. Xylem bleeding sap ureide levels (table 3) were reduced by all flooding treatments to very low values soon after flooding and these were maintained until the end of the experiment.

Total N in the shoot (table 4) for all flooding treatments was lower than in the non-flooded controls at day-14 and in some cases at day-5. By day-14 all sources of inorganic N present during flooding led to total shoot N accumulation that was superior to the N-free control but inferior to the non-flooded control. By day-21 those treatments containing NO_3^- (NH_4NO_3 or NO_3^- alone) contained higher levels of N than the NH_4^+ treatment, but, nevertheless, lower than the non-flooded controls. It is noteworthy that there was no significant difference between any of the flooded treatments with added N in terms of %N. The N-free flooded treatment however resulted in essentially no input of N throughout the experiment with a large decline in %N due to dry mass accumulation up to day-14 (see table 2).

Other measurements taken were pH and inorganic N content of the nutrient solutions after flooding (data not shown). Changes in pH during the flooding treatments would not appear to be a determining factor for any of the

Table 2. Dry mass of the shoot, root and total plant of soybean plants with root systems flooded at the V6-7 stage[a] for 5, 14 and 21 days with nutrient solution without N (N-free) or with NO_3^-, NH_4^+ and NH_4NO_3, and in non-flooded plants (NF)

	Shoot dry mass (g.planta^{-1})				Root dry mass (g.planta^{-1})[c]				Total dry mass (g.planta^{-1})			
	0 days	5 days	14 days	21 days	0 days	5 days	14 days	21days	0 days	5 days	14 days	21 days
NF	3,2	5.1 b[b]	10.9 a	14.9 a	0,7	1.0 a	2.8 a	3.4 a	3,9	6.1 a	13.7 a	18.3 a
N-free	_	5.3 ab	10.1 a	10.4 b	_	0.8 a	1.7 b	1.3 b	_	6.1 a	11.8 ab	11.7 b
NO_3^-	_	6.1 a	11.4 a	15.4 a	_	0.9 a	2.6 a	4.2 a	_	7.0 a	14.0 a	19.6 a
NH_4^+	_	5.6 ab	9.4 a	9.8 b	_	0.9 a	1.2 b	1.7 b	_	6.4 a	10.6 b	11.5 b
NH_4NO_3	_	5.3 ab	9.9 a	13.6 a	_	0.9 a	1.6 b	4.6 a	_	6.2 a	11.5 ab	18.2 a

[a] Plants at the vegetative stage with 5 or 6 trifoliolate leaves. [b] Means followed by different letters within columns are significantly different by Duncan´s multiple range test ($P \leq 0.05$). [c] Nodules not included in root mass

Table 3. Total free amino acids and ureides in the xylem bleeding sap of soybean plants with root systems flooded at the V6-7 stage[a] for 5, 14 and 21 dias with nutrient solution free of N (N-free) and with NO_3^-, NH_4^+ and NH_4NO_3, and for non-flooded plants growing on the N-free nutrient solution (NF)

	Amino acids (μmol.mL^{-1})				Ureídes (μmol.mL^{-1})			
	0 days	5 days	14 days	21days	0 days	5 days	14 days	21days
NF	1.4	1.2 c[b]	1.3 c	0.7 b	2.8	2.3 a	2.7 a	2.5 a
N-free	–	0.6 c	1.1 c	0.9 b	–	0.6 b	1.0 c	0.2 d
NO_3^-	–	3.8 b	1.4 c	2.2 b	–	0.1 c	0.8 b	0.8 b
NH_4^+	–	6.1 a	11.5 a	21.9 a	–	0.1 c	0.5 bc	0.7 b
NH_4NO_3	–	4.7 b	6.3 b	1.9 b	–	0.1 c	0.2 c	0.4 c

[a] Plants at the vegetative stage with 5 or 6 trifoliolate leaves.
[b] Means followed by different letters within columns are significantly different by Duncan´s multiple range test ($P \leq 0.05$).

observed differences between treatments, since nutrient solution pH was approximately 6.8 for the N-free solution, 6.8 for NH_4NO_3, 7.2 for NO_3^- and 6.4 for NH_4^+, irrespective of the period of flooding. No accumulation of NO_3^- or NH_4^+ occurred in the nutrient solutions despite the daily replenishment of the solutions. After 21 days of flooding 1.5 to 2.0 mM of NO_3^- remained in the treatment with NO_3^-, 1.5 mM of NH_4^+ remained in the treatment with NH_4^+, and about 1 mM of NH_4^+ and 1.5 mM of NO_3^- remained in the treatment with NH_4NO_3. Considering a starting concentration of 5 mM N, evidently a substantial proportion of the N in the medium was taken up by the plant.

DISCUSSION

The beneficial effects of NO_3^- during flooding are clear from the data presented here. As the period of flooding prolonged to 14 and 21 days, plants flooded in the presence of NO_3^- grew as well as the non-flooded controls as measured by stem height, leaf area (table 1) and dry mass accumulation of the shoot and root (table 2). These data are consistent with other studies that show increased tolerance of plants to flooding in the presence of nitrate (Malavolta, 1954; Trought and Drew, 1981; Prioul and Guyot, 1985, Bacanamwo and Purcell 1999a,b). However, the accumulation of N in the shoot (table 4) of plants flooded with NO_3^- did not accompany that of the non-flooded controls. Nevertheless, it is evident that a substantial amount of N did accumulate in the shoot throughout the 21-day period of flooding in the presence of NO_3^-. It is not known how nitrate increases flooding tolerance of plants, although several hypotheses have been proposed (Sousa and Sodek, 2002). In the case of soybean, Bacanamwo and Purcell (1999a) found that NO_3^- inhibits the formation of aerenchyma, suggesting that its beneficial effect was not related to increased availability of oxygen to the submerged roots through this morphological response to root hypoxia.

Plants flooded in the absence of N practically stopped growing after 14 days of flooding. These data are consistent with the very low levels of xylem sap ureides in flooded plants throughout the experiment (table 3). Since xylem ureides are known to correlate strongly with N_2 fixation in soybean (McClure et al., 1980; Herridge and Peoples, 1990), low ureide levels indicate that N_2 fixation was seriously impaired under flooding and would explain the almost total absence of N accumulation in the shoot when no other source of N was available (table 4). Bacanamwo and Purcell (1999b) also found that flooding strongly reduced N accumulation in nodulated soybean plants flooded for 21 days without addition of N.

In contrast to NO_3^-, NH_4^+ was totally ineffective during flooding with regard to dry matter accumulation and other growth parameters studied here. The plant growth values were no different from those of plants flooded in the absence of N. On the other hand, a substantial amount of N accumulated in plants flooded in the presence of NH_4^+, such that, together with restricted dry mass accumulation, high values of N concentration were found. Indeed, the tremendous increase in the xylem bleeding sap amino acid concentration observed for the NH_4^+-flooded plants is indicative of a large influx and assimilation of NH_4^+ during flooding. Vanlerberghe and Turpin (1990) showed that plant cells could assimilate large amounts of NH_4^+ into amino acids under anaerobic conditions. It would appear that C assimilation did not accompany this large influx of N, in

Table 4. Total and % N in the shoot of soybean plants with root systems flooded at the V6-7 stage[a] for 5, 14 and 21 days with nutrient solution without N (N-free) or with NO_3^-, NH_4^+ and NH_4NO_3, and for non-flooded plants with the N-free solution (NF)

	Shoot N (mg.plant^{-1})				%N in the shoot			
	0 days	5 days	14 days	21days	0 days	5 days	14 days	21days
NF	113	149 a[b]	296 a	453 a	2.9	2.9 a	2.7 a	3.0 a
N-free	_	88 c	124 c	122 d	–	1.6 d	1.2 c	1.2 c
NO_3^-	_	132 ab	225 b	356 b	–	2.2 bc	2.0 b	2.3 b
NH_4^+	_	135 ab	200 b	277 c	–	2.4 b	2.1 b	2.8 ab
NH_4NO_3	_	124 b	204 b	351 b	–	2.3 bc	2.1 b	2.6 ab

[a] Plants at the vegetative stage with 5 or 6 trifoliolate leaves. [b] Means followed by different letters within columns are significantly different by Duncan´s multiple range test ($P \leq 0.05$).

view of the reduced dry matter accumulation. Apparently, NH_4^+, in contrast to NO_3^-, leads to impaired C assimilation. NH_4^+ is known to have a toxic effect on plants but, although several hypotheses have been proposed, none are considered satisfactory (Britto et al., 2001). Britto et al. (2001) have shown that for species susceptible to NH_4^+, high external concentrations of NH_4^+ trigger a costly energy-consuming efflux system resulting in a futile cycling of NH_4^+ in and out of the root cells and propose that this mechanism precedes any toxicity-associated events such as cation displacement or carbohydrate depletion. Nevertheless, our data suggest that any negative effect of NH_4^+ on C assimilation would appear to be neutralized when NO_3^- is present together with NH_4^+, in view of the superior performance of plants flooded with NH_4NO_3 compared with NH_4^+ alone. Indeed, concentrations of amino acids found in the xylem sap of plants flooded for 21 days with NH_4NO_3 were similar to the values found in all treatments where NH_4^+ was absent, contrasting the extremely high concentrations that characterized the NH_4^+ treatment. Overall, the data obtained with NH_4^+ indicate that the beneficial effect of NO_3^- is not simply as a source of N for growth, since NH_4^+ also led to N accumulation in the plant without promoting growth.

Despite the evident beneficial effects of NO_3^- during flooding shown here, under non-flooding conditions NO_3^- is known to strongly inhibit N_2 fixation of nodules (McClure and Israel, 1979; Vessey et al., 1988). This would not be a problem during flooding, since N_2 fixation is in any case impaired by such conditions. Nor should it present a problem when the flooding condition terminates. Theoretically, much of the NO_3^- should drain with the water, thereby allowing N_2 fixation to recover. Nevertheless, this remains to be shown in practice.

In conclusion, our data clearly show the beneficial effect of nitrate during a prolonged period of flooding of the root system of soybean. NH_4^+ on the other hand, despite leading to marked increases in the N content of the flooded plants, was totally ineffective in maintaining plant growth during flooding.

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