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Effect of simulated acid rain on nodulation and nitrogen metabolism in *Vigna radiata* cultivars

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Abstract

Nodulation was inhibited in plants of green gram (*Vigna radiata*, cvs. ADT-1 and CO-5) exposed to different levels of simulated acid rain using a mixture of H_2SO_4, HNO_3 and HCl (6:3:1) of pH 2.5, 4.0 and 5.5 in comparison with control (pH 7.0). Protein content of leaves increased in cv. CO-5 but decreased in cv. ADT-1 whereas the nitrate content of leaves increased in cv. ADT-1 but lowered in cv. CO-5. Nitrate reductase activity was increased in the nodular roots of cv. ADT-1 but was decreased in leaves. In cv. CO-5 it was increased in leaves but was insignificantly reduced in the nodules at pH 2.5. The nodule nitrogenase activity increased at pH 4.0 and 2.5 in cv. ADT-1.

Additional key words: acid mixture, green gram, nitrate reductase, nitrogenase, proteins.

Introduction

Studies on the impact of acid rain on crop plants, mostly using sulphuric acid mists, have mainly concentrated on growth and yield. A few authors have reported the effect of simulated acid rain (SAR) on nitrogen metabolism, analysing the process of nodulation (Oden 1968, Gates and Muller 1979, McLaughlin and Shriner 1980, Shriner and Johnston 1981, Norby *et al.* 1986) and nitrate reductase activity (Muthuchelian *et al.* 1993, 1995).

Hence, experiments employing an acid mixture of sulphuric, nitric and hydrochloric acids were designed under the prevailing tropical conditions *1*) to assess the effect of SAR on nodulation after *Rhizobium* inoculation into the soil, *2*) to

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analyse the distribution of nitrogenous compounds, *3*) to follow the activities of two key enzymes, nitrate reductase in leaves and roots, and nitrogenase in nodules, and *4*) to assess the cultivar differences in responses, if any.

Materials and methods

Plants: Two green gram [*Vigna radiata* (L.) Wilczek] cultivars ADT-1 and CO-5 were raised in earthenware pots in greenhouse conditions [day/night temperature 36 ± 2/18 ± 2 °C; relative humidity 60 ± 5 %; photoperiod 12 - 14 h; maximum irradiance (PAR) 400 μmol(photon) m^{-2} s^{-1}]. Ten-day-old seedlings in each pot were inoculated with 200 mg of the commercial preparation of *Rhizobium* (cowpea strain) inoculum suspended in 1 cm^3 of water and poured on the surface of the soil as suggested by Shriner and Johnston (1981).

Treatment: Deionized double distilled water was adjusted to different pH levels (5.5, 4.0 and 2.5) using a diluted mixture of H_2SO_4, HNO_3 and HCl in the molar ratios of 6:3:1. Since the mean rain pH is around 7.0 in the tropics, glass distilled water, adjusted to pH 7.0 with 0.1 M NaOH served as control.

Starting from 15 d after sowing (DAS) plants were sprayed daily for 10 min for 10 d with acid mixture using a rain-generating device (Kohno and Kobayashi 1989) at an effective flow rate of 7.8 mm h^{-1}. The rain drop size ranged from 0.35 to 1.35 mm^3. Showers were applied to the top of the plant from a height of 1.2 m and no efforts were made to prevent the run off to soil.

Assessments were made at two stages, 5 and 15 d after beginning the treatment (DAT); stage 1 plants had received five showers while stage 2 corresponded to 5 d after termination of the treatment. Plants were carefully uprooted and the number of nodules were counted at 30, 40 and 50 DAS corresponding to 15, 25 and 35 DAT, respectively. Soluble proteins were estimated using Folin phenol reagent method (Lowry *et al.* 1951). Nitrate content was determined using naphthylamine salt-mixture (Woolley *et al.* 1960).

Nitrate reductase activity (NRA): *In vivo* NRA was assayed by the method of Jaworski (1971) with suitable modifications (Muthuchelian *et al.* 1993). Leaf bits/nodules (100 mg f.m.) were washed and placed in vials containing 5 cm^3 of incubation medium prepared by mixing 0.1 M KNO_3 (1 cm^3), 0.1 M phosphate buffer of pH 7.5 (3.75 cm^3), 0.1 % of Triton X-100 (0.01 cm^3) and 1 % propanol (0.25 cm^3) and incubated in dark for 1 h at room temperature (28 ± 2 °C) with occasional shakings. Aliquots (0.2 cm^3) from the incubated mixture were analysed for nitrite using 3 % sulphanilamide in 3 M HCl and 1 cm^3 of 0.02 % n-(1-naphthyl ethylene-diamine dihydrochloride). After 15 min of incubation in darkness, the absorbance was read at 540 nm using a spectrophotometer (*Shimadzu*, Kiyoto, Japan).

Nitrogenase activity: Nodular nitrogenase activity was determined by the acetylene reduction technique (Stewart *et al.* 1967). Enzyme assay was carried out with 500 mg

root sample placed in 7 cm^3 bottles fitted with rubber serum stoppers. The assay was initiated by injecting 0.6 cm^3 (10 %) acetylene using a disposable hypodermic syringe. The bottles were incubated for 30 min and the reaction terminated by injecting 0.2 cm^3 of 10 % TCA. One cm^3 of gas phase was withdrawn and the ethylene formed was measured in a gas chromatograph (*Aimil Nucon 5700* with *FID* using *Porapak T* column model). Nitrogen was used as a carrier gas and the flow rate was adjusted to 40 cm^3 min^{-1}. Ethylene standard was used as reference.

Statistical analysis: The values were analysed by Tukey's multiple range test (TMRT) at 5 % level of significance (Zar 1984).

Results and discussion

Both the green gram cultivars had fewer nodules due to SAR treatment and the degree of inhibition increased with the acidity levels (Fig. 1). Shriner and Johnston (1981) found a suppression of nodulation in *Phaseolus vulgaris* and *Pisum sativum* exposed to acid rain of pH 3.2. Employing an acid mixture similar to our study (H_2SO_4:HNO_3:HCl), Kohno and Kobayashi (1989) confirmed these trends in *Pisum sativum* at pH 3.0 and below.

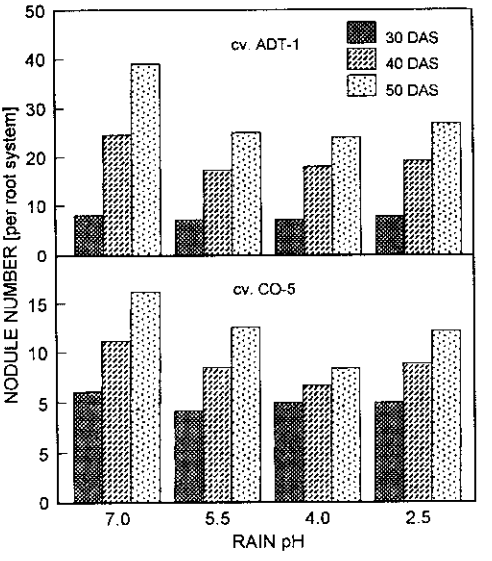

Fig. 1. Effect of simulated acid rain on the number of nodules in roots of *Vigna radiata* cvs. ADT-1 and CO-5.

In cv. ADT-1, nitrate was present in larger amounts at pH 4.0 and 2.5 at both stages but not in cv. CO-5 where the rain of pH 2.5 reduced the nitrate content at both stages (Table 1). But comparatively meagre amounts of nitrite were found in both cultivars at all stages (data not shown). Since leaves are the major sites of nitrate

assimilation in plants including legumes where nitrate is first reduced to nitrite and then immediately to ammonia, free nitrite is rarely found (Beevers and Hageman 1969).

Table 1. Nitrate, protein content and NR activity in the first trifoliate leaves of green gram cultivars (*Vigna radiata* cvs. ADT-1 and CO-5) exposed to simulated acid rain. Values in a column followed by different letters significantly differ according to Tukey's HSD multiple range test at 5 % level of significance (n = 10). DAS = days after sowing.

Parameter	Rain pH	ADT-1		CO-5	
		20 DAS	30 DAS	20 DAS	30 DAS
Nitrate	7.0	4.01 (a)	2.29 (a)	7.61 (a)	1.10 (a)
[mg g^{-1}(d.m.)]	5.5	5.33 (b)	2.03 (a)	7.28 (a)	1.23 (a)
	4.0	7.25 (c)	3.91 (b)	8.30 (b)	0.69 (b)
	2.5	7.73 (c)	3.40 (b)	6.77 (c)	0.36 (c)
Protein	7.0	2.77 (a)	7.84 (a)	3.55 (a)	8.76 (a)
[mg g^{-1}(f.m.)]	5.5	2.16 (a)	7.65 (a)	5.58 (b)	10.70 (b)
	4.0	1.44 (b)	5.89 (b)	5.13 (b)	10.20 (b)
	2.5	1.52 (b)	5.61 (b)	5.60 (b)	6.73 (c)
NR activity	7.0	3030 (a)	1680 (a)	380 (a)	502 (a)
[nmol(NO_2^-) kg^{-1}(f.m.) s^{-1}]	5.5	2820 (a)	1524 (ab)	800 (b)	520 (a)
	4.0	2800 (a)	1380 (b)	824 (b)	580 (b)
	2.5	2356 (b)	628 (c)	860 (b)	647 (b)

Table 2. Influence of simulated acid rain on nitrate levels and activities of nitrate reductase (NRA) and nitrogenase in roots of *Vigna radiata* cvs. ADT-1 and CO-5. Values within a column followed by different letters significantly differ according to Tukey's HSD multiple range test at 5 % level of significance (n = 10).

Rain pH	Nitrate [mg g^{-1}(d.m.)]	NR activity [nmol (NO_2^-) kg^{-1}(f.m.) s^{-1}]	Nitrogenase activity [µmol(ethylene reduced) g^{-1}(f.m.) s^{-1}]
cv. ADT-1			
7.0	0.56 (a)	1988 (a)	0.66 (a)
5.5	0.22 (b)	2485 (b)	0.61 (a)
4.0	0.27 (b)	2328 (b)	0.98 (b)
2.5	0.23 (b)	2663 (b)	1.06 (b)
cv. CO-5			
7.0	0.76 (a)	688 (a)	1.31 (a)
5.5	0.54 (b)	873 (b)	1.30 (a)
4.0	0.57 (b)	801 (b)	1.26 (a)
2.5	0.58 (b)	654 (a)	1.23 (a)

Whereas the cv. ADT-1 had 25 and 27 % lower protein content at pH 4.0 and 2.5, respectively, at 30 DAS compared to controls, significant increases were found at pH 5.5 and 4.0 in cv. CO-5 at 30 DAS. It was reduced only at pH 2.5 in the post-treatment assessment (Table 1). Changes in protein content were matched by

corresponding changes in nitrates. A similar pattern was observed in *Erythrina* and *Hardwickia* exposed to sulphuric acid mist (Muthuchelian *et al.* 1995).

The activity of foliar nitrate reductase was suppressed at pH 4.0 and 2.5 in cv. ADT-1 (Table 1). On the other hand, at pH 2.5 cv. CO-5 showed enhanced activity of 126 % during the treatment (20 DAS) and 29 % in the post-treatment period (30 DAS). The nodular NRA was stimulated by the acid rain in cv. ADT-1 but was found varying in cv. CO-5. Nodular nitrate contents of SAR-treated plants were lower than that of the controls (Table 2). While contrasting responses were evident in the two cultivars of the present study, seedlings of two other legumes, *Phaseolus mungo* and *Vigna sinensis* and two tree species, *Erythrina* and *Hardwickia* responded to sulphuric acid rain of pH 2.0 by inhibitions of NRA (Muthuchelian *et al.* 1993, 1995). Several reasons like increased membrane permeability or different buffering capacities of the cultivars could be adduced for the differential responses of the cultivars (Shriner and Johnston 1981).

Nitrogenase activity of the root system was higher at pH 4.0 and 2.5 in cv. ADT-1, and no inhibition was evident anywhere (Table 2). As only a small proportion of the SAR contained-nutrients lodged on the foliage find their way into the leaf cells (Evans *et al.* 1981), the unabsorbed chemicals drip along the surface and reach the soil as "through fall". In addition, materials are also leached out of the acid-rain affected cells and reach the rhizosphere, building up a fertilized atmosphere in the soil (Park 1987). Consequently, faced with foliar as well as soil nitrate feeding, the response of the cultivar, by way enhanced or decreased NRA and nitrogenase activity would be determined by its nutrient status. Hence, the two cultivars responded differently.

The data presented here seem to validate the view of Shriner and Johnston (1981) that acid rain events at the canopy level could not only influence the leaves but also the root system.

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