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Selection and evaluation of nitrate-tolerant strains of *Rhizobium leguminosarum* biovar *viceae* specific to the lentil

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Abstract Seventeen strains of *Rhizobium leguminosarum* biovar *viceae* specific to the lentil (*Lens culinaris* L.) were screened, using the high-yielding lentil cultivar L 4076, for their tolerance to three levels of NO_3^-: 0, 4, and 8 mM NO_3^-. Preliminary screening of this symbiosis for nodulation and N fixation in the presence of NO_3^- showed significant variations among the strains. The number of nodules decreased and nitrogenase activity was depressed in all strains in the presence of NO_3^-. Strains L-1-87, L-27-89, L-33-89, and L-40-89 tolerated 8 m*M* NO_3^-. Four strains, three tolerant of NO_3^- (L-1-87, L-27-89, and L-33-89), and one sensitive (L-11-89) to NO_3^-, were selected from preliminary screening and used in a pot experiment to assess the symbiosis in the presence of 6 m*M* NO_3^- at three stages of plant growth, viz., 40 days, 60 days, and at the final harvest. In general, the weight of nodules and C_2H_2 reduction activity was significantly higher after 60 days than after 40 days. Inoculation with strain L-1-87 produced the maximum number of nodules, and root and shoot biomass both in the presence and the absence of NO_3^-. Nitrate reductase activity in the tops and nodules was assayed only after 60 days and did not show significant variations among strains and NO_3^- treatments. The grain yields for all strains except L-11-89 were significantly higher in the presence of NO_3^- than in the absence of NO_3^-, indicating that tolerant strains contributed symbiotically fixed N to the plant's N pool, resulting in an additive effect on yield. Inoculation with strain L-1-87 produced the maximum grain yield and this strain appears to have potential use as an inoculant in the presence of high levels of soil N.

Key words Biomass · Grain yield · Lentil · Nitrate reductase · Nitrate tolerance · Nitrogenase · Nodulation · *Rhizobium* · Symbiosis · Total N

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Introduction

The general inhibitory effects of soil N, primarily NO_3^-, on nodulation and N fixation are well known (Gibson and Harper 1985; Nelson 1987). The extent of inhibition varies with the host species or cultivar and the rhizobial strain. Variations in the plant's ability to nodulate and fix N in the presence of NO_3^- have been reported for strains of soybean (Gibson and Harper 1985), alfalfa (Heichel and Vance 1979), and pea (Nelson 1987).

The capacity of the legume-*Rhizobium* symbiosis to maintain optimum levels of N fixation in the presence of high concentrations of externally applied N is particularly important for cropping systems in which legumes are grown mixed or in rotation with crops requiring high levels of N fertilizer. In India, lentils (*Lens culinaris* L) are generally grown alone after rice or millet, but sometimes it is intercropped with barley and/or mustard (Tata and Wadhwani 1992). Thus there is a need to identify *Rhizobium* sp. strains that can nodulate and maintain optimum levels of N fixation in the presence of soil N. The aim of the present investigation was to screen strains of *R. leguminosarum* biovar *viceae* specific for the lentil that were able to nodulate and fix N at high levels of NO_3^-, and to test the performance in terms of the grain yield of selected strains in soils amended with NO_3^--N.

Materials and methods

Bacterial cultures

Seventeen strains of *R. leguminosarum* biovar *viceae* specific to the lentil were obtained from the National Facility for *Rhizobium* spp. germplasm collection, Division of Microbiology, Indian Agricultural Research Institute, New Delhi. These were L-1-87, L-1-89, L-2-89, L-3-89, L-5-89, L-6-89, L-9-89, L-10-89, L-11-89; L-12-89, L-13-89, L-24-89, L-27-89, L-31-89, L-33-89, L-40-89, and L-46-89. The strains were maintained on yeast extract mannitol agar medium.

Plant growth

All the strains were tested for nodulation and N fixation at three levels of NO_3^-, 0, 4, and 8 m*M*, in a growth room experiment. Seeds of the lentil cultivar L 4076 were surface-sterilized with 1% $HgCl_2$ and 70% alcohol and washed several times with sterile water. The seeds were sown in small plastic cups filled with 200 g of washed and sterilized river sand. NO_3^- levels were maintained at sowing by externally applying KNO_3. At the beginning of the experiment, each cup contained two plants and approximately 2×10^8 rhizobia. Each treatment was replicated five times. The plants were watered at regular intervals using Jensen's N-free nutrient solution (Jensen 1942). They were maintained in a growth room with a photo period of 12 h, a temperature of 25°C and a light intensity of 12000 lux. The plants were harvested 40 days after emergence. The number of nodules per pot, both pink and white separately, was recorded. Nitrogenase activity in the root nodule system was measured by C_2H_2 reduction assay with gas chromatography. The roots and shoots were oven-dried at 80°C and the dry matter yield was recorded separately.

Pot culture experiment

A pot culture experiment was conducted to study the symbiotic performance of the selected NO_3^--tolerant and NO_3^--sensitive strains of lentil. Seeds of the lentil cultivar L 4076 were sown in earthen pots filled with 10 kg unsterilized soil (sandy loam, pH 7.2, electrical conductivity 0.9 dS m^{-1}, total N 0.95%). The treatments included (1) no NO_3^- amendment and (2) 6 m*M* NO_3^- as KNO_3. Twelve replicates per treatment were maintained. The pots were inoculated with an appropriate *Rhizobium* sp. strain at 2×10^9 cells per pot. Uninoculated controls were invariably maintained. Three replicates of each treatment were harvested 40 and 60 days after sowing for determinations of the C_2H_2 reduction rate, and the nodule, root, and shoot fresh weight. After 60 days of growth, the nitrate reductase activity in the tops (Nicholas et al. 1976) and nodules (Manhart and Wong 1980) was assayed. The NO_2^- content was expressed as nmol NO_2 g^{-1} fresh weight of tops or nodules.

Six replicates were maintained until the final harvest and grain yield data were recorded. The total N content in shoots was determined on a Tecator Kjeltec Autoanalyzer.

Results

The preliminary screening for nodulation and N fixation in the presence of NO_3^- showed significant variations among the 17 strains of lentil-specific *Rhizobium* sp. tested. The number of efficient pink nodules produced was severely depressed for all the strains in the presence of NO_3^-. The proportion of inefficient white nodules increased with the NO_3^- level. Strains that produced more pink nodules up to 8 m*M* NO_3^- were classified as NO_3^--tolerant and included L-1-87, L-27-89, L-33-89, and L-40-89. Strains L-9-89 and L-10-89 were moderately tolerant and produced pink nodules only up to 4 m*M* NO_3^-. The other strains were sensitive and produced either no nodules or only white nodules up to 4 m*M* NO_3^- (Table 1). Data on nitrogenase activity confirmed this categorization and suggested that NO_3^- inhibited components of N fixation. Although strain L-1-87 showed the highest activity in the 8 m*M* NO_3^- treatment, this still represented 71% inhibition relative to the controls (Table 2).

In all NO_3^- treatments, the plant shoot dry weight was significantly greater than in the non-NO_3^- controls (Table 3). However, doubling the NO_3^- level from 4 to 8 m*M* did not increase the shoot dry weight correspondingly. On the contrary, for 10 strains, L-5-89, L-9-89, L-10-89, L-12-89, L-13-89, L-27-89, L-33-89, L-37-89, L-40-89, and L-46-89, the shoot dry weight was lower with 8 m*M* NO_3^- than with 4 m*M* NO_3^->. The root dry weight decreased with increasing concentrations of NO_3^- for all strains.

Table 1 Effect of NO_3^- on the number of nodules induced by strains of *Rhizobium leguminosarum* biovar *viceae* (growth-room experiment) *LSD* least significant difference

Strain	Nodule type	NO_3^- (m*M*)		
		0	4	8
L-1-87	White	5	2	6
	Pink	32	12	4
L-1-89	White	3	1	0
	Pink	4	0	0
L-2-89	White	1	1	0
	Pink	9	1	0
L-3-89	White	2	4	8
	Pink	25	2	0
L-5-89	White	2	7	1
	Pink	24	1	0
L-9-89	White	1	4	5
	Pink	20	6	1
L-10-89	White	6	6	5
	Pink	25	2	1
L-11-89	White	3	0	0
	Pink	8	0	0
L-12-89	White	4	4	1
	Pink	43	1	0
L-13-89	White	3	9	10
	Pink	20	0	1
L-24-89	White	3	4	1
	Pink	19	1	0
L-27-89	White	3	4	1
	Pink	5	1	1
L-31-89	White	5	5	2
	Pink	25	3	0
L-33-89	White	3	4	4
	Pink	22	12	6
L-37-89	White	6	3	1
	Pink	19	0	1
L-40-89	White	4	2	2
	Pink	4	2	0
L-46-89	White	2	1	0
	Pink	6	0	0
LSD (P=0.05)		White	Pink	
Strain (S)		2.07	3.34	
NO_3^- (N)		0.87	1.40	
S×N		3.60	1.33	

Pot culture experiment

Four strains, tolerant (L-1-87, L-27-89, L-33-89) and sensitive (L-11-89) to NO_3^-, were selected and used in a pot experiment to assess their symbiosis in the presence of 6 m*M* NO_3^-. Nodulation was adversely affected by the presence of NO_3^- (Table 4). The native rhizobial population showed 63 and 56% inhibition of nodule formation with 6 m*M* NO_3^- after 40 and 60 days of growth. However, in the presence of L-1-87, the tolerant strain, the inhibition

Table 2 C_2H_2 reduction (nmol C_2H_4 h^{-1} pot $^{-1}$) by a lentil cultivar as influenced by NO_3^- and *Rhizobium* sp. strains (growth-room experiment). *LSD* least significant difference

Strain	NO_3^- (m*M*)		
	0	4	8
L-1-87	51.24	20.64	12.21
L-1-89	9.19	3.68	6.76
L-2-89	10.82	4.74	5.15
L-3-89	6.98	3.78	4.58
L-5-89	6.51	2.18	1.85
L-9-89	10.32	7.30	6.92
L-10-89	25.48	5.11	9.02
L-11-89	25.60	6.07	4.77
L-12-89	95.77	14.03	10.19
L-13-89	11.25	0.00	0.00
L-24-89	21.37	0.00	0.00
L-27-89	15.65	11.68	9.05
L-31-89	94.98	5.90	14.96
L-33-89	24.06	12.76	7.04
L-37-89	52.23	12.24	9.08
L-40-89	10.12	0.00	0.00
L-46-89	11.70	4.98	3.02
LSD (P=0.05)			
Strain (S)	16.60		
NO_3^- (N)	6.98		
S×N	28.76		

was only 50 and 44% after 40 and 60 days, respectively. The other tolerant strains, L-27-89 and L-33-89, showed better nodulation (approximately 50% of control) only after 60 days. The sensitive strain, L-11-89, showed even greater inhibition of nodulation than the native rhizobial population (uninoculated control). Similar trends were observed in total nitrogenase activity after 40 and 60 days (Table 4). Strain L-1-87 was the most tolerant and efficient, maintaining 64% activity of the control in the presence of NO_3^-.

In this study we found no significant differences in nitrate reductase activity in the tops and nodules of the different strains at any of the three levels of nitrate tested.

The NO_3^- treatments produced a greater shoot biomass in all strains except L-11-89. Again, L-1-87 produced a higher biomass both in the presence and absence of NO_3^- (Table 5). The grain yields from all strains except L-11-89 were higher with than without NO_3^- at any level (Table 6). The grain yield for treatments inoculated with L-27-89 was lower than the control, indicating the poor efficiency of this strain.

Discussion

The process of symbiotic N fixation is adversely affected by combined N, especially NO_3^-. In order to select legume-*Rhizobium* combinations that can fix N and give optimal yields under these conditions, it is necessary to carry out preliminary screening of *Rhizobium* sp. strains in the presence of the host and of NO_3^-. The strains used in the present study showed significant variations with regard to nodulation and N fixation in the presence of NO_3^-. Increasing the concentration of NO_3^- in the rooting medium restricted the ability of all strains to induce effective nodules on the roots and to fix N. The inhibitory effects of NO_3^- on infection, nodule development, and N fixation are well known (Gibson and Jordan 1983; Nelson 1983, 1987). However, the exact mechanism of inhibition is not very clear. The theories proposed include a diminished supply of photosynthate available to nodules following its use for the assimilation of NO_3^- in the shoots and roots (Gibson and Harper 1985); inhibition of root-hair curling due to catalytic destruction of indole acetic acid (Thornton 1936; Tanner and Anderson 1964); inhibition of a MoFe protein of nitrogenase due to binding of the NO_2^- produced from NO_3^- (Trinchant and Rigaud 1980); decreased production of lectin and the formation of attachment sites for these lectins on the root hairs (Dazzo et al. 1981).

Table 3 Effect of NO_3^- on root and shoot biomass (mg pot^{-1}) of the lentil cultivar L 4076 inoculated with strains of *Rhizobium leguminosarum* biovar *viceae* (growth-room experiment). *LSD* least significant difference

Strain	Biomass	NO_3^- (m*M*)		
		0	4	8
L-1-87	Root	67	65	44
	Shoot	61	65	72
L-1-89	Root	69	59	30
	Shoot	53	64	66
L-2-89	Root	84	57	30
	Shoot	39	68	71
L-3-89	Root	55	48	41
	Shoot	55	69	70
L-5-89	Root	54	46	40
	Shoot	56	75	66
L-9-89	Root	76	78	64
	Shoot	56	69	55
L-10-89	Root	55	65	64
	Shoot	42	68	62
L-11-89	Root	76	81	53
	Shoot	44	69	71
L-12-89	Root	72	82	49
	Shoot	60	80	78
L-13-89	Root	68	62	51
	Shoot	58	74	62
L-24-89	Root	85	66	52
	Shoot	48	67	71
L-27-89	Root	76	56	51
	Shoot	43	82	77
L-31-89	Root	72	56	51
	Shoot	51	74	78
L-33-89	Root	64	68	35
	Shoot	80	60	50
L-37-89	Root	50	75	34
	Shoot	50	79	67
L-40-89	Root	77	57	37
	Shoot	48	89	84
L-46-89	Root	93	82	47
	Shoot	47	65	63
LSD (P=0.05)		Root	Shoot	
Strain (S)		9.9	14.7	
Nitrate (N)		4.1	6.2	
S×N		17.6	NS	

Table 4 Effect of NO_3^- on nodulation and N_2 fixation by the lentil cultivar L 4076 inoculated with each of four strains of *Rhizobium leguminosarum* biovar *viceae* (pot experiment). Nodule weight is given as fresh weight (mg pot^{-1}). *ARA* C_2H_2 reduction activity (nmol C_2H_4 h^{-1} pot^{-1}), *LSD* least significant difference

Strain	NO_3^- (m*M*)	After 40 days		After 60 days	
		Nodule weight	ARA	Nodule weight	ARA
L-1-87	0	60	37.61	85	375.5
	6	30	24.19	48	106.1
L-11-87	0	61	35.12	92	259.1
	6	21	9.52	21	19.7
L-27-89	0	50	16.70	70	119.5
	6	12	11.83	33	43.1
L-33-89	0	72	36.09	85	236.4
	6	16	16.77	41	46.8
Control	0	31	19.35	81	167.7
	6	11	9.88	33	69.5
		Nodule weight		ARA	
LSD (P=0.05)		40 days	60 days	40 days	60 days
Strains (S)		7	12	9.57	68.9
NO_3^- (N)		4	8	6.05	43.6
S×N		10	17	13.54	97.4

Table 5 Effect of NO_3^- on root and shoot fresh weight of a lentil cultivar inoculated with each of four strains of *Rhizobium leguminosarum* biovar *viceae* (pot experiment). *LSD* least significant difference

Strain	NO_3^- (m*M*)	After 40 days		After 60 days	
		Root (g pot^{-1})	Shoot (g pot^{-1})	Root (g pot^{-1})	Shoot (g pot^{-1})
L-1-87	0	2.18	1.06	2.92	5.30
	6	1.91	1.24	2.94	5.57
L-11-89	0	1.31	1.30	2.66	6.65
	6	1.86	0.76	2.10	4.21
L-27-89	0	1.29	0.81	2.48	4.01
	6	1.38	0.82	2.99	5.23
L-33-89	0	1.31	0.97	1.94	4.39
	6	1.75	1.33	1.92	4.39
Control	0	1.31	0.94	1.91	3.66
	6	1.47	1.09	2.18	4.61
		Root		Shoot	
LSD (P=0.05)		40 days	60 days	40 days	60 days
Strain (S)		0.18	0.26	0.25	1.08
NO_3^- (N)		NS	0.16	0.16	0.70
S×N		0.26	0.36	0.35	1.53

Table 6 Effect of NO_3^- on N content and grain yield (g pot^{-1}) of a lentil cultivar inoculated with each of four strains of *Rhizobium leguminosarm* biovar *viceae* (pot experiment). *LSD* least significant difference

Strain		NO_3^- (m*M*)	
		0	6
L-1-87	N (%)	2.08	2.21
	Yield	10.03	11.18
L-11-89	N (%)	1.76	3.32
	Yield	9.38	9.40
L-27-89	N (%)	2.37	2.78
	Yield	7.03	7.87
L-33-89	N (%)	2.19	2.37
	Yield	7.61	8.74
Control	N (%)	2.11	2.31
	Yield	7.65	8.27
LSD (P=0.05)		N	Yield
Strain (S)		NS	0.39
NO_3^- (N)		NS	0.25
S×N		NS	0.56

The ability of four strains to tolerate 8 m*M* NO_3^- in association with a common host cultivar suggests that there may be some potential for combining the improved lentil cultivar L 4076 with NO_3^--tolerant rhizobial isolates that are more efficient. The results of the present investigation showed that of the tolerant strains L-1-87 was the most efficient where as L-27-89 was a comparatively poor N fixer. Inoculation of a lentil crop with strain L-1-87 in the presence of NO_3^- should improve the symbiosis in terms of growth and yield.

The main objective of any screening study is to test the selected strains under natural soil conditions for biomass and yield increases. Thus three NO_3^--tolerant strains differing in effectiveness (L-1-87 efficient; L-33-89 moderate; L-27-89 poor) and one sensitive but efficient strain (L-11-89) was used in the pot culture experiment. The weight of nodules and C_2H_2 reduction activity was significantly higher after 60 days compared to 40 days of plant growth. One reason for this result may be that the experiments were of the 'rundown' type, that is, the NO_3^- was applied once, at the beginning of the experiment. Thus there was a gradual depletion of NO_3^--N from the soil as the plant grew, thus diminishing the NO_3^- stress.

Strain L-1-87 performed well in unsterilized soil amended with 6 m*M* NO_3^- and produced the highest nodule, root, and shoot biomass. These results were in accord with the growth-room screening experiment. N responses measured by the weight of seedlings in the greenhouse do not reliably indicate N responses in seed yield (Hinson 1975). The grain yield from all the four strains in the presence and absence of NO_3^- showed that inoculation with L-1-87 in the absence of NO_3^- increased the seed yield by 8% over an uninoculated NO_3^--amended control. This shows that inoculation with an efficient strain provides sufficient N through fixation to obtain maximum growth. The grain yields from all the strains except L-11-89 and the uninoculated control were significantly higher with than without NO_3^-. The grain yield with L-1-87 as the inoculant in treatments with NO_3^- was 11% higher than without NO_3^-. It appears that the N fixed symbiotically by the NO_3^--tolerant strains also augmented the N pool of the plant, resulting in an additive effect on the yield of the plant. This suggestion is supported by the grain yields obtained with and without NO_3^- in pots inoculated with L-11-

89, a sensitive strain. No variation in the grain yield was observed in either the presence or the absence of NO_3^-, indicating that no efficient nodules were induced in the presence of NO_3^- to contribute symbiotically fixed N. The data on C_2H_2 reduction activity and the shoot N content confirm the inefficiency of this strain in the presence of NO_3^-.

The present results suggest that screening of strains against NO_3^- stress in association with the host is worthwhile. Strains to be used in the presence of combined N should be efficient, tolerant of NO_3^-, and competitive against the native rhizobial population. Strain L-1-87 appears to have potential for use as an inoculant in the presence of soil N.

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