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A Raffin, P Roumet. Shoot-root control of nitrate tolerance of N₂ fixation in spontaneously tolerant soybean lines: reciprocal grafting experiments. *Agronomie*, EDP Sciences, 1994, 14 (7), pp.473-480. <hal-00885653>

HAL Id: hal-00885653

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Submitted on 1 Jan 1994

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Shoot-root control of nitrate tolerance of N₂ fixation in spontaneously tolerant soybean lines: reciprocal grafting experiments

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(Received 10 May 1994; accepted 25 August 1994)

Summary — A characterization of spontaneous nitrate tolerance of nitrogen fixation in soybean was carried out by means of 2 reciprocal grafting experiments, with a top cross and a diallel type design. Shoot-root control of nitrate tolerance was studied in a spontaneous tolerant line (Tielingbaime), a non-tolerant cv (Kingsoy), and 5 progeny lines derived from their cross. Both experiments demonstrated a shoot control for nitrate tolerance of nodulation. Nitrate tolerance was expressed as the maintenance of a higher number and a greater specific mass of nodules in the presence of nitrate. Regulation of nitrogen fixation in the presence of nitrate also seemed to be related to shoot factors. However, diallel analysis of reciprocal grafts indicated that interaction effects with root control could not be excluded for this trait.

nitrogen fixation / nitrate tolerance / soybean / reciprocal grafting / diallel analysis

Résumé — Contrôle par les parties aériennes et racinaires de la tolérance au nitrate de la fixation symbiotique de l'azote chez des lignées de soja : étude top cross et diallèle de greffes réciproques. Une caractérisation de la tolérance au nitrate de la fixation de l'azote chez le soja a été réalisée dans 2 expérimentations de greffes réciproques, avec des dispositifs de types top cross et diallèle. Le contrôle de la tolérance au nitrate par les parties aériennes et racinaires de la plante a été étudié chez une lignée tolérante (Tielingbaime), un cultivar sensible (Kingsoy), et 5 lignées issues de leur croisement. Les 2 expérimentations ont démontré l'existence d'un contrôle par la partie aérienne de la tolérance au nitrate de la nodulation. Le caractère de tolérance permet le maintien de nodosités en plus grand nombre et de masse spécifique supérieure en présence de nitrate. La régulation de la fixation de l'azote en présence de nitrate serait également dépendante de facteurs de la partie aérienne. Cependant, l'analyse diallèle des greffes réciproques indique que, pour ce caractère, des effets d'interaction avec un contrôle racinaire ne sont pas à exclure.

fixation de l'azote / tolérance au nitrate / soja / greffes réciproques / analyse diallèle

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INTRODUCTION

Increasing symbiotic nitrogen fixation potential in legumes could provide possibilities to improve crop yield (Buttery *et al*, 1992). A number of environmental factors reduce the contribution of N₂ fixation to legume nitrogen nutrition. An interesting approach to improving N₂ fixation could exploit genotypic tolerance to limiting factors such as the presence of high amounts of nitrate in the soil.

Inhibition of symbiotic nitrogen fixation by nitrate is common to all legumes with variations between and within species (Hardarson *et al*, 1984; Harper and Gibson, 1984; Jensen, 1987; Park and Buttery, 1989). The physiological mechanisms involved in this inhibition have not been completely elucidated (Vessey and Waterer, 1992; Parsons *et al*, 1993). Since variability for the level of inhibition seems to be more dependent on the plant genotype than on the microsymbiont strain (McNeil, 1982; Gibson and Harper, 1985), sources of tolerance have been searched for among plant hosts. Plant genotypes that can maintain a nitrogen fixing activity in the presence of high amounts of nitrate (tolerant genotypes) could provide a useful model for the investigation of the inhibition mechanisms. Two sources of partially tolerant genotypes were described: lines obtained through induced mutagenesis in soybean (Carroll *et al*, 1985; Gremaud and Harper, 1989), pea (Jacobsen and Feenstra, 1984; Duc and Messenger, 1989), and common bean (Park and Buttery, 1988); and lines issued from spontaneous variability in soybean (Betts and Herridge, 1987; Serraj *et al*, 1992).

Lines obtained through mutagenesis produce a large number of nodules in the presence and absence of nitrate (supernodulating mutants). Reciprocal grafting studies in soybean showed that the lack of nodulation autoregulation in the mutant type involves the shoot of the plant (Delves *et al*, 1986; Cho and Harper, 1991), and especially the leaf (Delves *et al*, 1992). Shoot control was also responsible for supernodulation in pea (Duc and Messenger, 1989) and common bean (Buttery and Park, 1990). A model of nodulation autoregulation was proposed, involving a root-derived signal linked to the process of nodulation, and a response-inhibiting signal from the shoot to the root (Gresshoff *et al*, 1988, 1991). Additionally, nitrate treatment may interact with the latter signal, and enhance the inhibitory response (Day *et al*, 1989). In the mutant type, the shoot-derived autoregulation signal would be either altered or absent, inducing a supernodu-

lant and nitrate-tolerant (for nodulation) phenotype. In terms of nitrogen fixation, supernodulating mutants showed a low specific activity (C₂H₂ reduction / nodule biomass), and this activity was reduced in the presence of nitrate in the same proportion as for the wild type parent (soybean: Hansen *et al*, 1990; Wu and Harper, 1990; pea: Jacobsen and Feenstra, 1984; common bean: Buttery and Park, 1990). This last result was not always confirmed for soybean; a lower inhibition of specific activity was observed for the mutant type (Schuller *et al*, 1988).

Unlike supernodulating mutants, nitrate-tolerant soybean lines issued from germplasm screening showed normal nodulation in the absence of nitrate (Betts and Herridge, 1987; Serraj *et al*, 1992). A partially tolerant line Tielingbaime was described for physiological characteristics (Serraj *et al*, 1992). Spontaneous nitrate tolerance was related to a low inhibition of nodulation and nitrogen fixation in the presence of nitrate, and possibly involved an adaptation of nodule enzymatic activities (low induction of nodule cytosolic nitrate reductase). To further characterize spontaneous nitrate tolerance, our purpose in this study was to determine the effects of shoot and root factors on the expression of this character by means of reciprocal grafting experiments (using 'top cross' and 'diallel' designs) with the tolerant line Tielingbaime, the non-tolerant cv Kingsoy, and their progeny.

MATERIALS AND METHODS

Plant material

Soybean line Tielingbaime (nitrate tolerant, maturity group III), cv Kingsoy (non-tolerant, maturity group II), and 5 F7 lines (tolerant line 21, and intermediate lines 26, 57, 76, 89: maturity group III) derived from Tielingbaime (female) x Kingsoy (male) cross were used.

Two grafting experiments were carried out. A 'top cross' design was used for the first experiment (reciprocal grafts of the 5 progeny lines plus Tielingbaime, with Kingsoy as a common tester), and a 'diallel' design for the second experiment (all possible reciprocal grafts between 4 lines: Kingsoy, Tielingbaime, 21, 57).

Grafting procedure and growth conditions

Plants were grown under greenhouse conditions at the Station de Génétique et d'Amélioration des Plantes,

INRA, Montpellier, France (43°34' N, 3°57' E), from June to October. The temperature was maintained between 28°C (day) and 14°C (night). No artificial light was used. The seeds were germinated in vermiculite. Eight days after sowing, plants were grafted using a V-shaped graft below the cotyledons. Grafts were supported by parafilm covering the graft union, and a plastic sleeve. Immediately after grafting, plants were placed under a misting system functioning 1 min every 10 min for 10 d, to prevent the desiccation of the scion. Plants were then transferred in 4-l pots (1 per pot) containing vermiculite, and inoculated with an aqueous suspension of *Bradyrhizobium japonicum*, commercial strain G49 (originating from SB16, IARI, New Delhi).

Plants were watered 5 times a day with a complete nutrient solution (KH₂PO₄, 0.16 mM; KCl, 2.08 mM; MgSO₄, 1 mM; CaCl₂, 1.46 mM; H₃BO₃, 4 µM; MnSO₄, 6 µM; ZnSO₄, 0.9 µM; CuSO₄, 1 µM; Na₂MoO₄, 0.1 µM; Fe (ferric EDTA) 9 µM). This solution contained either a KNO₃ concentration maintained throughout the experiment (N treatment), or no mineral nitrogen source (ON treatment) except for 2 mM urea for the first 3 weeks, to prevent a lack of nitrogen before the installation of N₂-fixing root nodules. For the N treatment, the concentration in KNO₃ was 4 mM for the top cross experiment, and 2.5 mM for the diallel experiment. For the diallel experiment, K¹⁵NO₃ was also added to the nutrient solution, so that total N available for plants was enriched in ¹⁵N up to 0.1%.

Experimental design

The pots were arranged in a randomized complete block design with 5 and 4 replications, for top cross and diallel experiments, respectively. In the top cross experiment only, the experimental design included self-grafts of each line in each block, plus a ON treatment (self-grafts only) arranged in a separate design with 5 replications in total randomization. Graft combinations were referred to as y/z, where y = line used for the shoot, z = line used for the root. Lines Kingsoy and Tielingbaime were referred as K and T, respectively.

Measured parameters

Top cross experiment

Symbiotic nitrogen fixation was estimated at stages R4 and R5 (Fehr and Caviness, 1977) by acetylene reducing activity (ARA), according to an *in situ* method (Balandreau and Dommergues, 1971). Pots were placed individually in a gas-tight bag comprising a septum. Acetylene was added to internal atmosphere up to 10%. After incubation (30 min), 4 samples of 5 ml were taken every 25 min, and ethylene concentration was measured with a Delsi 30 gas chromatograph equipped with a flame ionisation detector (Porapak T column 1.2 m x 3.2 mm; oven temperature 80°C; carrier gas N₂, 30 ml min⁻¹). The ARA was calculated as

the slope of ethylene accumulation, expressed in µmol C₂H₄ evolved per hour per plant. Nitrogen fixation index was computed as follows: index = (ARA (R4) + ARA (R5)). At pod filling (R6), plants were harvested and dried (70°C for 48 h). Shoot, pod, and nodule dry weights were measured.

Diallel experiment

Plants were harvested at maturity (R8), and the seed dry weight was determined. The nodules were senescent at this stage and so nodulation intensity was evaluated by nodule number. Seed N content and ¹⁵N abundance were determined on a 2 mg sample of finely ground seeds for each plant, by mass spectrometry analysis (autoanalyser Roboprep CN, mass spectrometer Tracermass, Europa Scientific). The proportion of seed N derived from atmosphere (%Ndfa) was calculated using the standard isotope dilution equation, where the abundance of the nutrient solution was used as reference for N derived from assimilation.

Statistical analysis

Data were submitted to analysis of variance, after verification of the normality of residuals and the homogeneity of residual variances. Mean separations were accomplished using the Tukey–Kramer test (a pairwise comparisons test based on the studentized range). The Kramer test minimizes the type 1 error and is adapted to unequal cell size (Dagnelie, 1975). To study shoot and root effects on measured characters, a diallel analysis was carried out according to Griffing (1956): a model with constant effects, where reciprocals but not 'parents' (self-grafts) are included. Reciprocal effects, as combining ability effects, were split into general and specific effects (Demarly, 1977). When effects were significant, individual values were computed. For general combining ability and reciprocal effects respectively, individual values were defined as follows: $g_i = (a_i + b_i) / 2$ and $m_i = (a_i - b_i) / 2$, where a_i is the effect of line i as a shoot, and b_i the effect of line i as a root ($\sum g_i = \sum m_i = 0$). The diallel analysis was computed under interactive matrix language (SAS Institute, 1985).

RESULTS

Top Cross Experiment

The inhibition of nodulation and nitrogen fixation measured on self-grafts in the presence of 4 mM NO₃ was very high: the average rates of inhibition reached 80–90% for nodulation measures, and above 95% for nitrogen fixation indices, compared with the ON treatment.

In the absence of nitrate, there were no differences between self-grafts for growth parameters, nodulation and nitrogen fixation, except for relative nodule dry weight (per g of total shoot dry weight). Graft 21/21 was significantly superior to K/K ($p = 0.04$, table I).

In the presence of nitrate, more differences were expressed between self-grafts; total and relative nodule dry weight, and nitrogen fixation index showed significant differences between self-grafts. For all nodulation and fixation parameters, graft K/K showed lowest values and 21/21 highest values.

Shoot and root effects were first analysed in lines 21 (tolerant) and Kingsoy (non-tolerant) separately (table I). For all nodulation parameters, graft combinations with tolerant line 21 as a shoot showed significantly greater values than graft combinations with non-tolerant cv Kingsoy as a shoot, regardless of root stock genotype. For nitrogen fixation index, similar results were observed, but distinction between grafts 21/21 and 21/K, and K/21 and K/K, was less clear. Tolerant line 21 as a shoot also induced a greater shoot dry weight, but no effect on pod dry weight was detected.

In a second analysis, the 4 intermediate lines were tested as a group with Kingsoy as a non-tolerant tester in the presence of nitrate (table II). The intermediate group as a shoot induced a greater total and relative nodule dry weight compared with graft combinations with Kingsoy as a

shoot. Analysis of variance on fixation index indicated a significant interaction between graft combination and block effects: mean comparisons were not computed. For shoot and pod dry weights, no specific effect of shoot or root was found.

Both analyses with tolerant line 21 and the intermediate group, indicated that shoot genotype is determinant for nitrate tolerance of nodulation. Nitrate tolerance allowed for the maintenance of a greater nodule dry weight per g of shoot biomass compared with non-tolerant plants, partly due to the development of nodules of a greater size. However, no significant effect of shoot or root on nitrate tolerance of nitrogen fixation was found.

Diallel experiment

The nitrate concentration of the nutrient solution in this second experiment was decreased to 2.5 mM, to ensure a higher level of nitrogen fixation. The proportion of seed N derived from atmosphere (%*Ndfa*) ranged from 51 to 62% (average 58%).

Each graft combination was considered as a cross between 2 lines in a diallel design. A first model of analysis of variance was used to test for combination and block effects (table III). The combination effect was significant for nodulation and fixation parameters, thus allowing for the

Table I. Shoot and root effects on plant biomass, nodulation and nitrogen fixation in the presence (N treatment) and absence (ON treatment) of 4 mM KNO₃, in shoot/root grafts between nitrate-sensitive cv Kingsoy (K) and nitrate-tolerant line 21 (top cross design).

Treatment and graft combination	Plant biomass		Nodulation			N ₂ fixation index (Σ ARA)
	Shoot DW (g)	Pod DW (g)	Total nodule DW (mg)	Specific nodule DW (mg)	Relative nodule DW (per g of total shoot DW) (mg/g)	
<i>N treatment</i>						
21/21	6.2 ^a	7.3 ^a	93 ^a	1.8 ^a	6.9 ^a	4.0 ^a
21/K	5.3 ^a	6.2 ^a	93 ^a	1.5 ^a	8.1 ^a	3.7 ^{ab}
K/21	3.0 ^b	6.0 ^a	25 ^b	0.8 ^b	2.8 ^b	0.7 ^b
K/K	2.8 ^b	5.1 ^a	22 ^b	0.9 ^b	2.7 ^b	1.0 ^{ab}
<i>ON treatment</i>						
21/21	6.4 ^a	11.6 ^a	771 ^a	9.0 ^a	41.7 ^a	81 ^a
K/K	5.6 ^a	14.7 ^a	521 ^a	5.8 ^a	25.6 ^b	65 ^a

Means followed by the same letter are not significantly different based on a Tukey–Kramer test at $p = 0.05$.

Table II. Shoot and root effects on plant biomass, nodulation and nitrogen fixation in the presence of 4 mM KNO₃, in shoot/root grafts between nitrate sensitive cv Kingsoy (K: common tester) and intermediate lines 26, 57, 76 and 89 (x) – (top cross design).

Graft combination	Plant biomass			Nodulation		N ₂ fixation index (Σ ARA)
	Shoot DW (g)	Pod DW (g)	Total nodule DW (mg)	Specific nodule DW (mg)	Relative nodule DW (per g of total shoot DW) (mg/g)	
x/x	5.6 ^a	6.5 ^a	62 ^a	1.5 ^a	5.2 ^a	2.0
x/K	4.6 ^b	6.0 ^a	64 ^a	1.2 ^{ab}	6.1 ^a	2.1
K/x	4.0 ^b	5.5 ^a	37 ^b	1.0 ^b	3.9 ^b	1.0

Means followed by the same letter are not significantly different based on a Tukey–Kramer test at $p = 0.05$ (mean comparisons were not computed when block x graft combination interaction was significant).

Table III. Diallel analysis of shoot/root effects on nodulation and nitrogen fixation in the presence of 2.5 mM KNO₃: analysis of variance for graft combination and block effects; and analysis of variance for the decomposition of combination effect in general and specific combining ability (GCA, SCA) and general and specific reciprocal effects (GRE, SRE).

	Test of combination effect		Decomposition of combination effect			
	Combination	Block	GCA % F	SCA % F	GRE % F	SRE % F
%Ndfa	**	NS	28*	6 NS	45**	21 NS
Total nodule number	**	NS	50**	0 NS	48**	2 NS
Relative nodule number (per g total shoot DW)	*	NS	79**	0 NS	21 NS	0 NS

Significance for the Fisher test (F) is indicated (NS: non-significant, * $p = 0.05$; ** $p = 0.01$). Values shown are percent of total variation explained by each effect.

decomposition of this effect in general and specific combining ability (GCA, SCA), and general and specific reciprocal effects (GRE, SRE), using a second model (table III). As block effect was not significant, this second analysis was carried out on means for each combination.

Significant GRE and GCA effects for %Ndfa and for total nodule number indicated that one part of the plant was preponderant over the other in the control of these characters, and that the 4 tested lines were variable for this expression. GCA effects explained 79% of total variance for relative nodule number, indicating a global effect of both shoot and root on the expression of this character in the presence of nitrate, and the existence of a variability for this effect between the tested lines. Non-significant SCA and SRE effects showed the absence of significant specific interactions between the shoots and roots of the

tested lines for each parameter. However these specific effects were not negligible in %Ndfa and accounted for 27% of total variance.

When effects were significant, individual values were computed for each line: g_i for GCA, and m_j for GRE (table IV). A positive estimation for g_i indicates a GCA of line i as a shoot or a root, which is higher than the mean of the tested lines. A positive estimation for m_j indicates a positive effect of the shoot or a negative effect of the root for line i , compared with the mean of the tested lines. In this analysis for nodulation and fixation parameters, tolerant line 21 showed no GCA of shoots or roots (g_i not different from 0), but high and significant reciprocal effects (positive m_j), whereas opposite results were observed for Tielingbaime (high and significant GCA: positive g_i , and lower reciprocal effects, not different from 0 or positive m_j). For all parameters, these 2 lines had greater g_j and m_j

Table IV. Individual values of general combining (GCA) ability and reciprocal effects (GRE) in the diallel analysis of shoot/root effects on nodulation and nitrogen fixation in the presence of 2.5 mM KNO₃.

Genotype	<i>g_i</i> , individual values of GCA			<i>m_i</i> , individual values of GRE		
	%Ndfa	Total nodule number	Relative nodule number (per g total shoot DW)	%Ndfa	Total nodule number	Relative nodule number (per g total shoot DW)
21	0.07 ^b	0.35 ^b	-0.04 ^b	1.97 ^{**a}	6.56 ^{**a}	0.46
Tielingbaime	1.95 ^{**a}	12.17 ^{**a}	1.65 ^{**a}	-0.41 ^b	4.10 ^a	0.26
Kingsoy	-1.67 ^{*c}	-5.72 ^{*c}	-0.95 ^{*c}	-0.57 ^b	-4.94 ^{**b}	-0.34
57	-0.35 ^b	-6.80 ^{*c}	-0.66 ^{bc}	-0.99 ^b	-5.72 ^{**b}	-0.38

Values were tested for significance (different from 0 at * $p = 0.05$, ** $p = 0.01$). Values followed by the same letter were not significantly different based on

^a Student *t*-test at $p = 0.05$.

values than cv Kingsoy and line 57, for which GCA and reciprocal effects were always negative or not different from 0. Differences in combining ability and reciprocal effects between tolerant (21, Tielingbaime, positive effects) and non-tolerant lines (57, Kingsoy, negative effects) were significant for total nodule number.

For comparison of means, graft combinations were then divided in 4 groups according to tolerance (noted '+') or non-tolerance ('-') of lines used as shoot and root (table V). Combinations with a tolerant shoot developed a significantly greater total nodule number in the presence of nitrate, compared with combinations with a non-tolerant shoot. This effect was less pronounced for relative nodule number, since the graft combinations +/- and -/+ were not significantly different. This was related to a lower accumulation of shoot and seed dry weight for combinations with a tolerant root compared with combinations with a

non-tolerant root, independently of shoot genotype. However differences in shoot and seed dry weight were inconsistent with top cross results, and were not significant. For %Ndfa, the graft combination effect was significant ($p = 0.02$) in the analysis of variance. However, the Tukey–Kramer test did not show significant differences between means at the 0.05 level, indicating a heterogeneity of within-class variances. However, the same classification between combinations was observed for %Ndfa as for nodulation parameters.

DISCUSSION AND CONCLUSION

Both experiments (top cross and diallel design) demonstrated a significant effect of shoots in the control of nitrate tolerance of nodulation, shoots of tolerant lines induced a greater number and

Table V. Shoot and root effects on plant biomass, nodulation and nitrogen fixation in the presence of 2.5 mM KNO₃, in shoot/root grafts (diallel design) between sensitive cv Kingsoy, intermediate line 57 (mean noted -) and tolerant lines Tielingbaime and 21 (mean noted +).

Graft combination	Plant biomass		Total nodule number	Nodulation Relative nodule number (per g total shoot DW)	Nitrogen fixation % Ndfa (%)
	Shoot DW (g)	Seed DW (g)			
+/+	2.3 ^{bc}	6.7 ^b	59 ^a	6.4 ^a	60 ^a
+/-	3.0 ^a	8.1 ^{ab}	56 ^a	5.4 ^{ab}	59 ^a
-/+	2.1 ^c	7.3 ^{ab}	37 ^b	4.0 ^{bc}	56 ^a
-/-	2.8 ^{ab}	8.7 ^a	34 ^b	3.2 ^c	56 ^a

Values followed by the same letter were not significantly different based on a Tukey–Kramer test at $p = 0.05$.

mass of nodules per plant than graft combinations with a non-tolerant line as a shoot. This result is consistent with observations on supernodulating mutants (Delves *et al*, 1986; Cho and Harper, 1991) for which nitrate tolerance of nodulation is also shoot controlled. For these mutants, the lack or the alteration of an autoregulating shoot-derived signal would account for the greater number of nodules compared with the wild type parent, in the absence or presence of nitrate. However, contrary to supernodulating mutants, the nodulation phenotype of a spontaneous tolerant line was distinct from non-tolerant lines in the presence of nitrate only (Serraj *et al*, 1992). In the present study (top cross design), differences in all nodulation parameters between a tolerant and a non-tolerant line were amplified in the presence of nitrate. Nitrate tolerance of nodulation resulted in the maintenance of a greater number of infected sites in the presence of nitrate, but also in the development of nodules of greater specific dry weight, whereas supernodulating mutants develop small-sized nodules compared with their wild type parent (Day *et al*, 1987; Gremaud and Harper, 1989). According to the model of nodulation autoregulation proposed by Gresshoff *et al* (1988, 1991), the tolerant lines observed in the present study display a normal autoregulatory signal, but interaction between this signal and the presence of nitrate (Day *et al*, 1989) may be modified, inducing a lower inhibition of nodulation in the presence of nitrate compared with non-tolerant cv Kingsoy.

For nitrogen fixation in the presence of nitrate (ARA or ¹⁵N dilution), both experiments suggested a shoot control for nodulation parameters, but differences were not significant between grafts. A possible explanation for this result is the presence of an interaction between shoot and root effects; diallel analysis indicated that specific effects were not negligible for %Ndfa. Thus, while nodule mass is determined by shoot factors in the presence of nitrate, the efficiency of nitrogen fixation may depend on shoot and/or root control. Physiological study of the tolerant line Tielingbaime (Serraj *et al*, 1992) suggested that nitrate tolerance was possibly related to the nodule activity (low induction of nodule cytosolic nitrate reductase and availability in energetic supply to support nitrogenase activity), thus involving a root control of this character.

Tolerant lines Tielingbaime and 21 (progeny line) were further characterized in the diallel analysis. These 2 lines displayed a differential response in individual values of GCA. Values were non-different from 0 in line 21 but strictly

positive for Tielingbaime, suggesting the presence of different factors determining nitrate tolerance in Tielingbaime, originating either from the shoot or the root (polygenic character). None of these factors may be transmitted or expressed in the progeny line 21. Whether such factors involve the elicitation or the alteration of a function has yet to be determined. The genotypes observed in the present study should provide interesting experimental material for such investigations.

ACKNOWLEDGMENT

The authors wish to thank the CETIOM (Centre d'Etude Technique Interprofessionnel des Oléagineux Métropolitains) for the grant that supported this work.

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