

Modification of symbiotic interaction of pea (*Pisum sativum* L.) and *Rhizobium leguminosarum* by induced mutations

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Summary In pea (*Pisum sativum* L.), mutants could be induced, modified in the symbiotic interaction with *Rhizobium leguminosarum*. Among 250 M₂-families, two nodulation resistant mutants (K₅ and K₉) were obtained. In mutant K₅ the nodulation resistance was monogenic recessive and not *Rhizobium* strain specific. Out of 220 M₂-families one mutant nod₃ was found which could form nodules at high nitrate concentrations (15 mM KNO₃). This mutant nodulated abundantly with several *Rhizobium* strains, both in the absence and presence of nitrate. Probably as the result of a pleiotropic effect, its root morphology was also changed. Among 1800 M₂-families, five nitrate reductase deficient mutants were obtained and one of them (mutant E₁) was used to study the inhibitory effect of nitrate on nodulation and nitrogen fixation.

The results of the present investigation show that pea mutants which are modified in their symbiosis with *Rhizobium leguminosarum*, can readily be obtained. The significance of such mutants for fundamental studies of the legume-*Rhizobium* symbiosis and for applications in plant breeding is discussed.

Introduction

In pea, natural variability has been found for symbiotic performance with *Rhizobium leguminosarum*. It is restricted to a few major genes controlling nodulation^{6,10,15} and N₂-fixation^{10,15}. This paper reports on the induction, selection and characterisation of mutants with a modified symbiotic behaviour. The mutants were obtained either directly by selection for non-nodulation as recently described by Kneen and LaRue¹⁴, and for persistent nodulation in the presence of high amounts of nitrate, or indirectly by selection for nitrate reductase deficiency. The importance of host plant mutants for fundamental research of the legume-*Rhizobium* symbiosis and the potential value of certain mutants for agronomic research is discussed.

Materials and methods

The mutagenic treatment of seeds is a critical step in mutation work. Based on information from literature¹ and on our experiences with *Arabidopsis*³ seeds of cv Rondo (*Pisum sativum* L.; kindly supplied by CeBeCo, The Netherlands) were treated with EMS (ethyl methanesulphonate) as described earlier⁴. Seeds from each M₁-plant were harvested separately to ensure

that mutants which were mutated in the same gene but were found in different M_2 -families originated from different mutation events. The mutation frequency in the M_2 -generation was determined by screening for chlorophyll deficiency. In the mutagenic treatment used in the present study 3–4% of the M_2 -families contained chlorophyll deficient mutants, indicating that sufficient mutations were induced.

The nitrate reductase deficient mutants were found indirectly by selection for chlorate resistance⁴, and the nodulation-tolerant mutant *nod₃* was selected, after inoculation with *Rhizobium*, by its persistent nodulation in the presence of nitrate^{11,13}.

Seeds were germinated in vermiculite, and the plants were grown in aerated solutions containing nitrogen-free standard mineral solution (SMS)⁴ alone or SMS complemented with KNO_3 or KCl. In nodulation experiments, plants were inoculated with *Rhizobium leguminosarum* strain PF₂, TOM or PRE. For selection of non-nodulating host plant mutants, M_2 -plants were inoculated and grown during 3–4 weeks on nitrogen-free SMS in a temperature controlled glasshouse and further characterisation of mutants was performed in a temperature controlled chamber⁵.

The nitrogenase activity of nodules was based on acetylene reduction⁵; nitrate content and nitrate reductase activity in young leaves were determined as described earlier⁴.

Results and discussion

Selection of host plant mutants and their use in studies of the symbiosis

1. Nodulation resistant mutants

Poor nodulation has been found in the primitive pea collected from the gene centers¹⁵. This character is *Rhizobium* strain specific¹⁵ and its inheritance is semi-dominant¹⁵, or recessive¹⁰, depending on the *Rhizobium* strain and on culture conditions. Non-nodulating plants were selected from M_2 -material after inoculation with *Rhizobium leguminosarum* strain PF₂. In 250 M_2 -families, two non-nodulating plants were found (K_5 and K_9). Since all M_3 - and M_4 -progenies of both plants were non-nodulating it was concluded that these plants were mutants. Mutant K_5 was investigated in more detail. The F_1 hybrid between mutant K_5 and cv Rondo nodulated as well as the wild type, and in the F_2 -generation a segregation of 3:1 was found (nod^+ : $nod^- = 37:14$, $\chi^2_{1(3:1)} = 0.14$). Therefore, the inheritance of this character is likely to be monogenic and recessive. In contrast with cv Afghanistan¹⁵, mutant K_5 did not produce nodules with *R. leguminosarum* strain TOM. This probably indicates that different genes are involved in mutant K_5 compared to cv Afghanistan. This has to be confirmed in tests of allelism. In the wild type, the root morphology was found to change as a result of inoculation with *Rhizobium* bacteria (Fig. 1). In mutant K_5 , similar macroscopic changes in root morphology were observed after inoculation with *Rhizobium*. This may suggest that root infection had occurred but that the subsequent nodulation process was blocked. Cytological studies on inoculated roots of this mutant showed root hair curling and infection threads, and occasionally nodules were found on lateral roots in a late developmental stage, indicating that the resistance to nodulation is not complete.

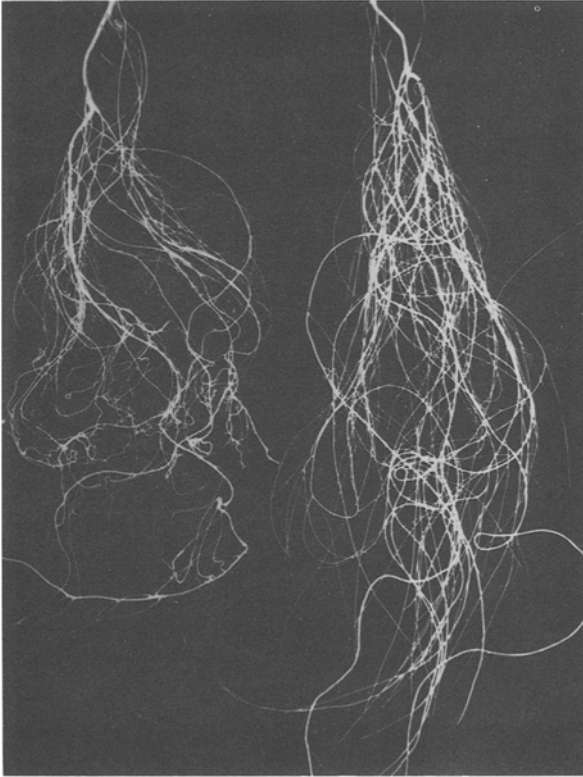


Fig. 1. Root morphology of pea cv Rondo grown on nitrogen-free medium non-inoculated (right), and 14 days after inoculation with *Rhizobium leguminosarum* strain PF₂ (left).

2. Nitrate-reductase (NaR) deficient mutants

Among 1800 M₂-families 5 NaR deficient pea mutants were obtained. Unfortunately, all of them appeared to be non-viable in soil, but in aerated SMS complemented with either NH₄NO₃ or with (NH₄)₂SO₄ plant growth and seed multiplication was possible. The inheritance of NaR deficient mutant E₁ which was studied in more detail, was monogenic and recessive. Biochemical characterisation of E₁ showed inhibited NaR activity (in vitro NaR activity was 5% of that of cv Rondo)⁴ probably caused by disturbance of the cofactor of the NaR enzyme¹² and, as a consequence of this, high nitrate concentrations in the leaves.

The inhibitory effect of nitrate on nodulation and acetylene reduction in legumes is well documented and appears to be plant specific^{7,16}. It is not clear whether nitrate per se, its reduction or both are involved in this inhibition. Therefore, mutant E₁ provides a good tool in a study of this effect.

Table 1. The effect of 15 mM KNO₃ on nodule number and nodule mass in pea cv Rondo and nitrate reductase deficient mutant E₁ compared with nodulation of both genotypes on nitrate-free medium

Genotype	Nitrate-free		Nitrate-containing	
	Nodule number	Nodule mass (mg)	Nodule number	Nodule mass (mg)
cv Rondo	71.3 ± 42.3*	189 ± 54	11.8 ± 1.0	12 ± 7
mutant E ₁	81.5 ± 64.2	175 ± 67	11.9 ± 5.1	12 ± 5

* N = 9

2.1. *Nodulation and N₂-fixation of NaR deficient mutant E₁*. In the absence of nitrate, nodulation of mutant E₁ was similar to that of the parent cultivar Rondo (Table 1). In the presence of 15 mM KNO₃ nodule number and nodule mass were reduced significantly in both genotypes showing that despite differences in nitrate reductase activity between both genotypes the nitrate content in the plant and/or in the medium may affect nodulation adversely. A nitrate uptake deficient mutant could give conclusive information. The observations on mutant E₁ confirm the results obtained in physiological studies with *Lens esculentum*¹⁸ and *Glycine max*⁹. Nitrate is known to decrease N₂-fixation of actively fixing root nodules. Two days after addition of nitrate to plants, which had been nodulated on N-free medium, the N₂-fixation of the wild type was strongly inhibited (47%), whereas this effect was weak and not significant (19%) in mutant E₁. Obviously, nitrate per se did not inhibit N₂-fixation but rather its reduction or its reduced products caused such an inhibition. This means that N₂-fixation of a nodulated NaR deficient mutant is nitrate-tolerant.

3. A nitrate-tolerant nodulation mutant

The presence of nitrate in the soil can prevent nodulation. Host plant genes which facilitate nodulation in the presence of nitrate have not been described in the literature.

Among 220 M₂-families a mutant has been selected which nodulates in the presence of nitrate and this was found to inherit in a monogenic and recessive fashion^{13,11}. The gene was tentatively designated nod₃².

3.1. *Nodulation and N₂-fixation of mutant nod₃*. Mutant nod₃ has two exceptional characters: (1) nodulation on N-free SMS is increased compared to the wild type and (2) nodulation is abundant despite the presence of a high concentration of nitrate (15 mM KNO₃)

Table 2. Nodulation of mutant *nod₃* and pea cv Rondo, inoculated with *Rhizobium leguminosarum* strain PF₂, PRE or TOM on nitrate-free and nitrate containing medium (15 mM KNO₃)

Strain	Total nodule number			
	cv Rondo		Mutant <i>nod₃</i>	
	- NO ₃ ⁻	+ NO ₃ ⁻	- NO ₃ ⁻	+ NO ₃ ⁻
PF ₂	75*	11	> 300	185
PRE	51	8	95	138
TOM	99	28	> 300	> 300

* n = 5

(Table 2). Therefore, mutant *nod₃* nodulates vigorously when cultured on nitrate-free as well as on nitrate containing media. Originally, this mutant was selected after inoculation with *Rhizobium leguminosarum* strain PF₂¹³. Table 2 shows that the mutant character is expressed with at least two other strains. The nodulation performance of *nod₃* and cv Rondo on nitrate-free and nitrate containing medium is illustrated in Fig. 2A + B, whereas Fig. 3 shows that mutant *nod₃* still nodulates vigorously in heavily manured soil. Cv Rondo did not produce any nodules under that growth condition.

Acetylene reduction activity of nodulated plants grown on SMS was more than 3 times higher in *nod₃* than in cv Rondo, whereas the specific activity (per g of fresh nodule weight) was somewhat lower. After nodulation of cv Rondo on a nitrate containing medium the acetylene reduction activity was inhibited more than 90%, whereas that of *nod₃* was less inhibited¹³. In both genotypes the specific nodule activity was equally decreased (50%) by nitrate. Therefore, the very low acetylene reduction activity of cv Rondo, after growth and nodulation on nitrate containing medium, was mainly due to the decrease of nodule mass.

3.2. The root system of mutant *nod₃*. As illustrated in Fig. 2A + B and Fig. 3, the root size of highly nodulated mutant *nod₃* was different from that of cv. Rondo. The root system of non-inoculated plants, cultured in nitrate-free medium is shown in Fig. 4A. Fig. 4B shows the primary lateral roots of both genotypes in detail, demonstrating that also in the nodule-free root systems of *nod₃* the branching intensity is higher. A difference in tap root length found between nodulated root systems of *nod₃* and cv Rondo (*nod₃*: 27 cm, cv Rondo: 43 cm) was again observed on the root systems of both genotypes when without nodules (*nod₃*: 36 cm, cv Rondo 46 cm). This means that in mutant *nod₃* not only the nodulation ability has been changed but also the root morphology, even under nodule-free conditions, and that both properties could be pleiotropic. This assumption is



Fig. 2A. Nodulation of pea cv Rondo (*left*) and mutant nod_3 (*right*) after growth on nitrate-free medium and inoculation with *Rhizobium leguminosarum* strain PRE.

supported by the segregations observed in more than hundred F_2 plants, after crossing this mutant with the wild type. A larger number of F_2 plants has to be screened for both characters before the alternative hypothesis of two linked mutated genes can be excluded.

3.3 Nitrate reduction and nitrate content in young leaves of mutant nod_3 . The persistent nodulation of mutant nod_3 in the presence of high amounts of nitrate may be due to nitrate uptake deficiency.

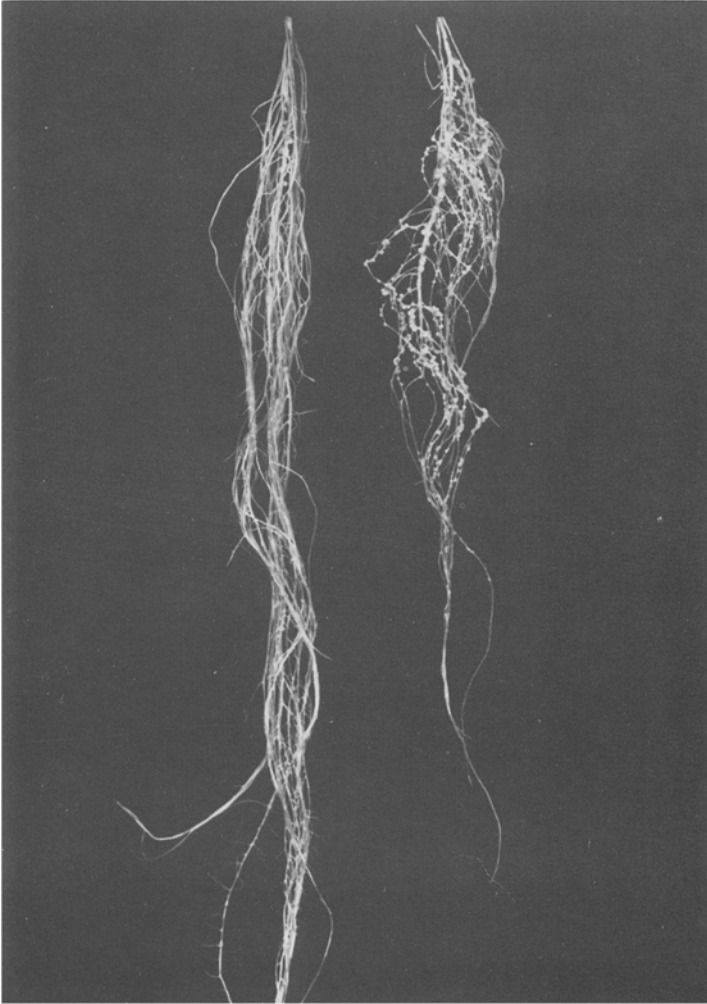


Fig. 2B. Nodulation of pea cv Rondo (*left*) and mutant nod_3 (*right*) after growth on nitrate containing medium and inoculation with *Rhizobium leguminosarum* strain PRE.

of the plant. Therefore, the induction of NaR enzyme and the increase of nitrate content in young leaves of plants, pregrown in N-free medium, were determined 1 and 2 days after transfer of these plants to a nitrate-containing medium (Table 3). The results show that both *in vitro* NaR activity and nitrate content in leaves are increased in nod_3 plants when grown on nitrate containing medium in the same way as in cv Rondo. Therefore, it is unlikely that mutant nod_3 is deficient in nitrate uptake.

Table 3. Nitrate reductase (NaR) activity and nitrate content in young leaves of mutant *nod*₃ and *cv* Rondo 1 resp 2 days after transfer of plants from nitrate-free to nitrate containing medium (15 mM KNO₃)

Genotype	Days on nitrate containing medium	<i>in vitro</i> * NaR activity	Nitrate content**
<i>cv</i> Rondo***	0	0.2	nd
<i>nod</i> ₃ ***	0	0.3	nd
<i>cv</i> Rondo	1	1.8	5.6
<i>nod</i> ₃	1	1.4	4.4
<i>cv</i> Rondo	2	5.2	12.8
<i>nod</i> ₃	2	4.2	7.1

* Assayed *in vitro* expressed as $\mu\text{mol NO}_2^-$ per g of fresh weight per hour

** Expressed as nmol NO_3^- per g of fresh weight; nd: not determined.

*** Plants were non-nodulated.

General discussion and concluding remarks

Symbiotic behaviour of the host plant can be modified by means of induced mutations. It is possible to select for altered symbiotic performance by searching for poor or non-nodulation, high nodulation, or for nodulation ability in the presence of nitrate. The present results on mutant *E*₁ show that the inhibiting effect of nitrate on acetylene reduction of actively fixing root nodules can be decreased by nitrate reductase deficiency. It is noteworthy that the frequency of mutants with a modified symbiosis is high (see section 1; 2 and 3). The same has been observed during the selection of non-nodulating pea mutants by Kneen and LaRue¹⁴, indicating that several different genes are involved in the symbiotic process directly or indirectly. Crosses have been made in order to combine the mutant character of *nod*₃ with that of NaR deficient mutant *E*₁ in one genotype. It is expected that the recombinant, with two mutated genes, persistently nodulates in a nitrate containing medium and that also the inhibitory effect of nitrate on acetylene reduction is decreased. NaR deficient mutant *E*₁, nodulation resistant mutants as *K*₅ and *K*₉, and nodulation-tolerant mutant *nod*₃ are of importance for fundamental research on host plant controlled factors which are directly or indirectly involved in the symbiotic interaction with *Rhizobium*. The mutants with persistent nodulation and/or with persistent acetylene reduction in the presence of nitrate could additionally be of interest in agronomic research and in plant breeding.

In further investigations on *nod*₃, questions have to be answered about the effect of other forms of combined nitrogen on nodulation,

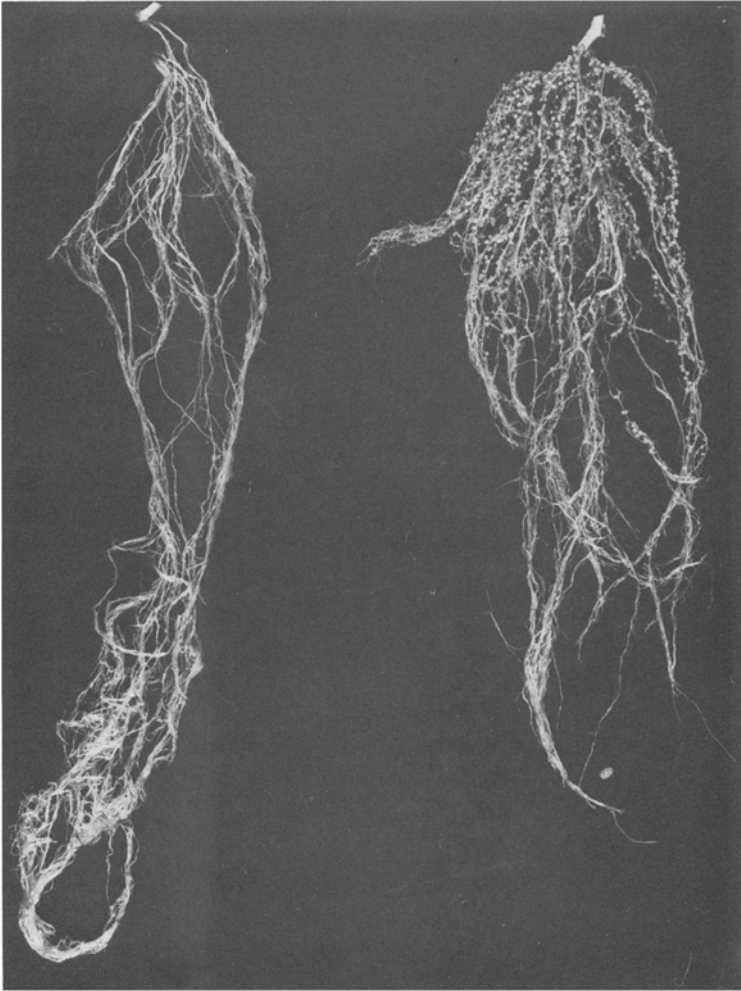


Fig. 3. An abundantly nodulated root system of mutant nod_3 (right) in contrast with that of pea cv Rondo (left), after growth of both genotypes in heavily manured soil and inoculation with *Rhizobium leguminosarum* strain PF₂.

and about the physiological and morphological basis of its modified nodulation ability. Mutant nod_3 might be used as a tool in applied agronomic research, and it is potentially of interest for plant breeding under 2 different environmental conditions: (1) high nodulation in soils with low levels of nitrate, a situation which is mainly found in soils with a low input of N-containing fertilizers and (2) persistent high nodulation in soils with relatively high levels of nitrate, as a result of using high amounts of N-containing fertilizers.

As shown previously, the character of vigorous nodulation of nod_3



Fig. 4A. Non-nodulated root systems of pea cv Rondo (*left*) and mutant nod_3 (*right*) 3 weeks after growth on nitrogen-free medium.

is controlled by a single recessive gene. Therefore, after crossing of nod_3 with other agronomically important breeding lines, 25 percent of the F_2 progeny plants remained highly nodulating. This means that mutations which positively influence nodulation ability, such as found in mutant nod_3 , offer perspectives in breeding for plants fixing N_2 effectively. In literature, two genes of pea (nod_1 and nod_2^2), have been described causing "high nodulation". In agronomic studies with the combined "highly nodulating" line $nod_1 nod_2$, it has been shown that nodule mass and seed yield are positively correlated⁶. Van Mil¹⁷ found that during plant growth nodule mass increases

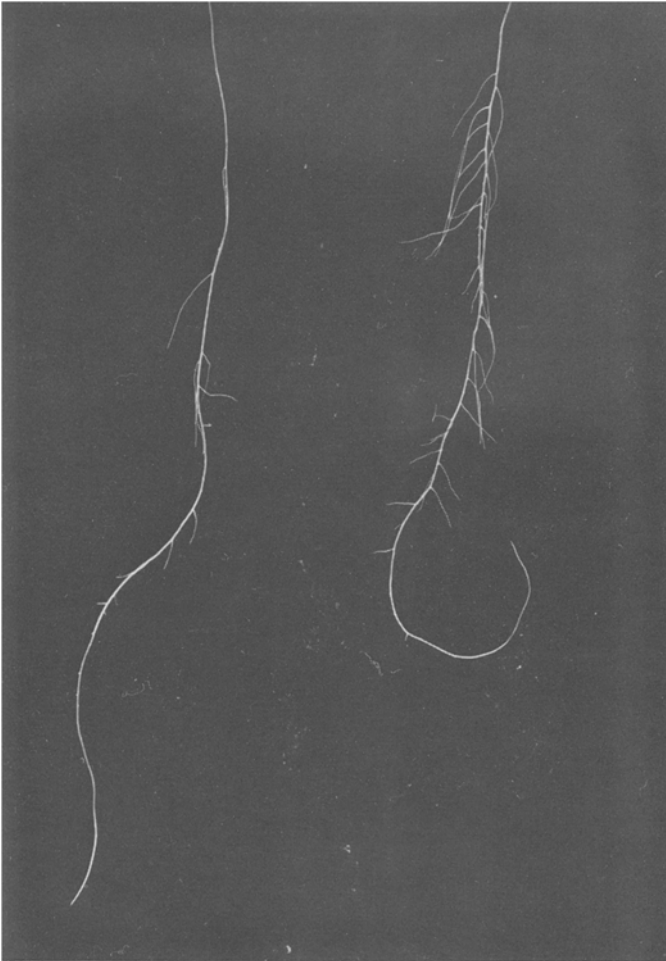


Fig. 4B. Primary lateral roots of non-nodulated root systems of pea cv Rondo (*left*) and mutant nod_3 (*right*) 3 weeks after growth on nitrogen-free medium.

despite of a suboptimal nitrogenase activity. He suggested that the amount of N_2 -fixation per plant is ultimately determined by nodule mass more than by specific nodule activity. A high nodule mass has been found in mutant nod_3 (Table 2;¹³), even in the presence of nitrate. Therefore, the combined effect of nitrate and N_2 -fixation in the plant on the yield can be investigated simultaneously by using mutant nod_3 . After inoculation of mutant nod_3 and cv Rondo with *Rhizobium leguminosarum*, application of ^{15}N labelled fertilizers could show the proportion of the bound nitrogen originating from N_2 -fixation and that originating from nitrate fertilisation in the harvested plant or seeds⁸.

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