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Nod factors improve the nitrogen content and rhizobial diversity of faba bean and alter soil dehydrogenase, protease, and acid phosphomonoesterase activities**

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A b s t r a c t. Nod factors produced by rhizobia are one of the most important signals involved in symbiotic associations involving legumes. A field trial was performed to assess the symbiotic activity, rhizosphere biological parameters, and plant biomass of faba bean (*Vicia faba* L.) treated with Nod factors. The soil was a Haplic Luvisol derived from loess. The faba bean seeds (cv. Granit) were soaked with an Nod factors solution (260 nM) or water (control) and sown. At the flowering stage, the genetic diversity of rhizobia (based on PCR-RFLP profiles and the sequencing of the 16-23S rDNA and *nodD* gene), nitrogenase activity (acetylene reduction assay), and nodule biomass were evaluated. Nitrogen yield and plant biomass were determined at the flowering and maturity stages. Rhizosphere soil was examined during plant growth in relation to the activities of dehydrogenase, protease, urease, and acid phosphomonoesterase. The results indicated that the application of the Nod factors improved nitrogenase activity (by 74-80%, depending on the parameter analysed) and increased the genetic diversity of rhizobia inhabiting root nodules, plant nitrogen content (by 16.8%, at maturity), and seed protein yield (by 14.6%). The rhizobial population became more heterogeneous under the influence of the Nod factors than it was for the control (12 and 7 specific genotypes, respectively). At the flowering stage, Nod factors enhanced dehydrogenase, protease, and acid phosphomonoesterase activities by 46, 36 and 9%, respectively. The results revealed the positive effect of Nod factors at reducing water deficiency effects during a growing season with a short-term rainfall deficit.

K e y w o r d s: legume, rhizobial diversity, root nodules, biological nitrogen fixation, soil enzymes

INTRODUCTION

Faba bean is grown worldwide in cropping systems as a grain and green-manure legume and is a protein source in food and feed. Among the legumes cultivated in the EU, faba bean occupies the third position with respect to crop production, while it lies in second place regarding the amount of N fixed. Faba bean exhibited the most significant N balance (N fixed – N offtake) (19.8 kg of N per tonne of grain production) among grain legumes cultivated in the EU (Baddeley *et al.,* 2013). The integration of faba bean into cropping systems has revealed many potential advantages in terms of increasing yield and quality, reducing diseases and pests, and influencing soil microbial activity and functions (Köpke and Nemecek, 2010; Li *et al.,* 2013; Wahbi *et al.,* 2016). It was shown that the introduction of faba bean into crop rotation increases microbial populations (bacteria and fungi) and enzyme activities (dehydrogenase, phosphatase, arylsulfatase, β-glucosidase and arylamidase), and also increases the rate of carbon mineralization, as well as modifying microbial populations and the catabolic capability of soil microorganisms (Aschi *et al.,* 2017; Lupwayi and Kennedy, 2007). Moreover, the use of faba bean in crop rotation positively affected mycorrhizal colonization, which improves plant nutrient and water uptake, and enhances drought tolerance (Wahbi *et al.,* 2016). The beneficial effects of legume influence on soil microbial activity could be connected with

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the biochemical composition and quantity of crop residues, including the higher amounts of organic carbon and nitrogen content incorporated into the soil through legume residues, as compared with crop rotation without legumes.

Apart from nitrogen from the soil, legumes may also utilize nitrogen from the atmosphere in the process of symbiotic nitrogen fixation, which is carried out by *Rhizobium* (Anglade *et al.,* 2015). Legume-rhizobium symbiosis is a major contributor to the global nitrogen cycle and has great economic and ecological importance. In this interaction, in response to host plant root exudates containing flavonoids, nodulation genes are activated in rhizobia for the synthesis and secretion of lipochitooligosaccharides (LCO), named Nod factors (NF) (Janczarek *et al.,* 2015). NF were found to induce infection and nodule organogenesis processes (D'Haeze and Holsters, 2002; Kelly *et al.,* 2018).

The genetic diversity of rhizobial populations may be very large, even in individual plants which could be infected by numerous strains differing at a genetic as well as on a phenotypic level (Wielbo *et al.,* 2010), also there are some suggestions that such diversity has a beneficial effect on plant growth (Wielbo *et al.,* 2011).

The external application of NF had an impact on plant development and growth. The beneficial effect of NF was observed with regards to legume germination, the photosynthesis rate, nodulation, and plant growth parameters (Khan *et al.,* 2008; Kidaj *et al.,* 2012; Podleśny *et al.,* 2014). The effects of NF were noted in relation to non-legume plants such as barley, rice, cotton, corn, canola and *Arabidopsis thaliana* (Prithiviraj *et al.,* 2003; Miransari and Smith, 2009; Marks *et al.,* 2015; Schwinghamer *et al.,* 2016). The improvement in germination and the early growth of seedlings (plant biomass, leaf area, root length) may be attributable to the stimulation of cell cycle genes and the morphogenetic capacity of NF.

The beneficial effects of NF application on roots, plant physiology, growth, and yields were observed under some stressful conditions such as low pH and temperature as well as water and salt stress (Duzan *et al.,* 2004; Prudent *et al.,* 2016; Nandhini *et al.,* 2017). The positive effects of NF on pea growth and symbiotic activity were noted in compacted soils (Siczek *et al.,* 2013). In field conditions, NF generally improved nitrogenase activity and pea yield in relatively dry but not during wet growing seasons (Siczek *et al.,* 2014).

The literature review showed that studies concerning the effects of Nod factors were restricted to some legume species such as soybean, red clover, pea, and vetch, whereas faba bean has not been investigated as yet. Therefore, we hypothesized that the application of NF on faba bean seeds would improve the symbiotic activity, the genetic diversity of rhizobia inside nodules, plant growth, and soil enzyme activity. The aim of this study was to evaluate the effects of Nod factors on nitrogenase activity, nitrogen yield, the genetic diversity of *Rhizobium*, plant biomass and the rhizosphere enzyme activity of field-grown faba bean.

MATERIALS AND METHODS

The study was conducted in Lublin, Poland (51°15′N, 22°35′E). The soil was classified as a Haplic Luvisol (FAO, 1998) derived from loess with clay, silt, and sand content in the 0-20 cm soil layer of 70 g kg^{-1}, 290 g kg^{-1}, and 640 g kg^{-1}, respectively. The experiment was set up in 2015 in a field that was mouldboard ploughed to a 20-cm depth before plot establishment. The soil pH was 6.1 (H_2O) and the organic carbon content was 8.97 g kg^{-1}. The total N content was 0.75 g kg^{-1} (Kjeldahl method (1883)) and the available K, P, Mg contents were 153, 114, and 39 mg kg^{-1}, respectively in the 0-20 cm soil layer. Faba bean (*Vicia faba* L.) (cv. Granit) seeds were soaked for 30 min with a 260 nM solution of the Nod factors (NF, Nod factors) or water (C, control) (500 ml of NF solution or distilled water was used per 1 kg of seeds) and planted thereafter. Before sowing, the faba bean seeds were inoculated with an inoculum of *R. leguminosarum* bv. *viciae* (Nitragina) produced by the Institute of Soil Science and Plant Cultivation (IUNG, Puławy, Poland). The plots (2 m × 3 m) were randomly organized into three replicates.

The Nod factors were isolated from *R. leguminosarum* bv. *viciae* strain GR09. Flavonoids from faba bean seed exudates were added to induce the synthesis of the Nod factors. The surface-sterilized seeds of faba bean were germinated in water for 4 days and then the supernatant was extracted with ethyl acetate. The ethyl acetate was evaporated, and the resulting pellet was resolubilized in 95% ethanol. In order to determine the flavonoids content, the ethanol extracts were dried and weighed. The approximate flavonoid concentration in seed exudate was calculated relative to the molecular weight of authentic flavone.

Logarithmic cultures of *R. leguminosarum* bv. *viciae* strain GR09 were induced with sterile faba bean seed exudate at a final concentration of 10 μM. After 48 h of incubation, the culture was extracted with 0.2 volume of n-butanol (Prithiviraj *et al.,* 2003). The organic fraction was separated and dried under vacuum. The amount of Nod factors was determined by the conversion of the amino sugars to methyl glycosides and gas chromatography/mass spectrometry (GC/MS) analysis. The concentration of the Nod factors was estimated based on the assumption that a single molecule of Nod factor contains on average four residues of N-acetylglucosamine (GlcNAc). The content of GlcNAc in the Nod factor preparation was 260 nM. The procedure for NF isolation was the same as that described in Siczek *et al.* (2013).

At the flowering stage (BBCH 65), nitrogenase activity was assessed based on an acetylene reduction assay (C_2H_2). Ten plants, which were treated as replicates, were taken randomly from both the control and Nod factors treatments. Cleaned roots with attached nodules were placed in bottles (one plant per bottle) and 10% (v/v) of the gas from the bottles was replaced with acetylene. After 30 min incubation,

the gas samples from the bottles were analysed for ethylene concentration by gas chromatography using a Shimadzu GC14-B with FID (Flame Ionization Detector). Detailed information is provided in Siczek *et al.* (2013). The roots with nodules were collected from the 0-15 cm soil layer. The amount of ethylene (μmol h^{-1}) was converted to the dry weight of one nodule (specific nitrogenase activity) and individual nodule. The number and dry mass (65°C, 24 h) of nodules were determined for each plant separately.

R. leguminosarum strains were isolated at the end of the flowering stage (BBCH 69). Root nodules were surface-sterilized, crushed and plated on TY medium (5 g Bacto trypton, 3 g yeast extract, and 1.3 g $CaCl_2$ per litre). Isolates were purified by the successive re-streaking of single colonies. The level of genetic diversity was determined based on the number of different PCR-RFLP profiles. Total DNA was isolated from the rhizobial cells, and the 16-23S rDNA fragment (primers FGPS1490 5'-TGCGGCTGGATCACCTCCTT-3' and FGPL132 5'-CC GGGTTTCCCCATTCGG-3') as well as the *nodD* gene (primers: NBA12 5'GGATSGCAATCATCTAYRGMRTGG-3' and NBF12 5'-GGATCRAAAGCATCCRCASTATGG-3') (Laguerre *et al.,* 1996) were amplified. The PCR reaction was performed in the following conditions: initial denaturation at 95°C for 3 min, denaturation at 94°C for 1 min, 35 cycles: primer attachment at 55°C for 1 min, elongation at 72°C for 2 min, and final elongation at 72°C for 5 min. The PCR products obtained were digested with enzymes *Bsu*RI, *Msp*I, *Bsp*131I (for one hour at 37°C), and *Taq*I (for one hour at 65°C). The restriction profiles were analysed after the electrophoretic separation of amplicons in a 3% agarose gel (100 V, 2.5 h).

For 32 selected strains representing different PCR-RFLP profiles, the sequencing of the 16-23S rDNA and *nodD* amplicons was carried out. In the RFLP analysis, all restriction fragments >100 bp were compared, named, and attributed to the analysed strains; this was followed by agglomerative clustering performed using Statistica software (StatSoft Inc., Tulsa, OK, USA, 1997). The sequence analysis of the 16-23S rDNA and *nodD* amplicons as well as phylogenetic tree construction was carried out with DNASTAR Lasergene software (DNASTAR, Inc., Madison, Wisconsin, USA) using a Clustal W algorithm.

Plant biomass was determined in three replicates at the flowering (BBCH 65) and maturity (BBCH 99) stages. The harvested area was 0.5 m^2 and the plant material collected at the flowering stage was dried at 65°C for 24 h. The nitrogen content was determined separately for the leaves, stems, flowers, roots, and nodules (at flowering) and in the stems, pods, roots, and seeds (at maturity) with the Kjeldahl method (1883) in three replications and afterwards the values were summarized separately for both stages. At both stages, faba bean roots were collected from the 0-15 cm soil layer. The seed protein yield was calculated based on the seed yield and the nitrogen content of the seeds.

Faba bean rhizosphere soil samples (the soil adhering tightly to the roots was collected by first shaking off the loosely adhering soil) from the 0-15 cm layer were taken three times during the vegetative period, at T1 corresponding to the 5-6 leaf stage (BBCH 15-16), T2 – flowering (BBCH 65), and T3 – development of the fruit (BBCH 79) of the faba bean. The number of days from sowing to harvest was 46, 61 and 102 for T1, T2 and T3, respectively. The soil used for enzymatic analyses was sieved through a 0.2 cm mesh.

Dehydrogenase activity was measured using the Thalmann method (1968) modified by Alef (1995), urease activity was assessed with the method developed by Zantua and Bremner (1977), and protease activity was determined with the method proposed by Ladd and Butler (1972) and modified by Alef and Nannipieri (1995). The Tabatabai and Bremner (1969) method was used for the determination of acid phosphomonoesterase activity. Each analysis was performed in four replicates. The results were converted into the dry (105°C) weight of the soil.

Statistica 10.0 software (StatSoft Inc., Tulsa, OK, USA, 2011) was used to obtain the statistical analyses. A comparison of means was performed on the basis of analysis of variance (ANOVA) and post-hoc Tukey's HSD test at a $p < 0.05$ significance level.

RESULTS

The nodule number and dry weight did not change significantly ($p < 0.05$) after the application of the Nod factors (Table 1). However, the activity of the nodules increased greatly in NF compared to the control. Nitrogenase activity per one plant, per nodule dry weight, and per one nodule significantly exceeded the values observed in the control (by 80, 77, and 74%, respectively).

Table 1. Parameters of nodules and nitrogenase activity as influenced by Nod factors application with standard deviations (n = 10). Different letters in rows indicate significant differences ($p < 0.05$)

Parameters	Control	Nod factors
Nodules		
Number (plant^{-1})	86.8 (37.0) a	103.0 (23.2) a
Dry weight (mg plant^{-1})	0.085 (0.026) a	0.101 (0.025) a
Nitrogenase activity		
C_2H_4 μmol h^{-1}	4.58 (4.28) b	22.37 (7.56) a
C_2H_4 μmol h^{-1} g nodule^{-1}	51.7 (44.2) b	220.3 (48.7) a
C_2H_4 nmol h^{-1} nodule^{-1}	56.1 (50.9) b	221.1 (74.8) a

At the end of the flowering stage, the root nodules were collected, 42 strains were isolated and 12 genotypes were identified from the control, also 45 strains and 17 genotypes were identified from NF (Fig. 1). In total, 24 different genotypes were identified. In the control and NF, 5 common genotypes were present, however, two of them occurred more frequently than the specific genotypes (19-38%). The NF population was more heterogeneous than the control (12 and 7 specific genotypes, respectively). A greater variation in the

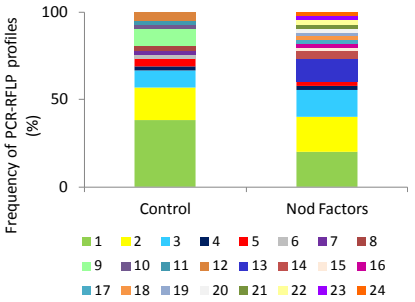

Fig. 1. The effect of Nod Factors on the frequency of different genotypes (1-24) based on PCR-RFLP of 16-23S rDNA and *nodD* gene profiles obtained in the faba bean nodules rhizobial isolates.

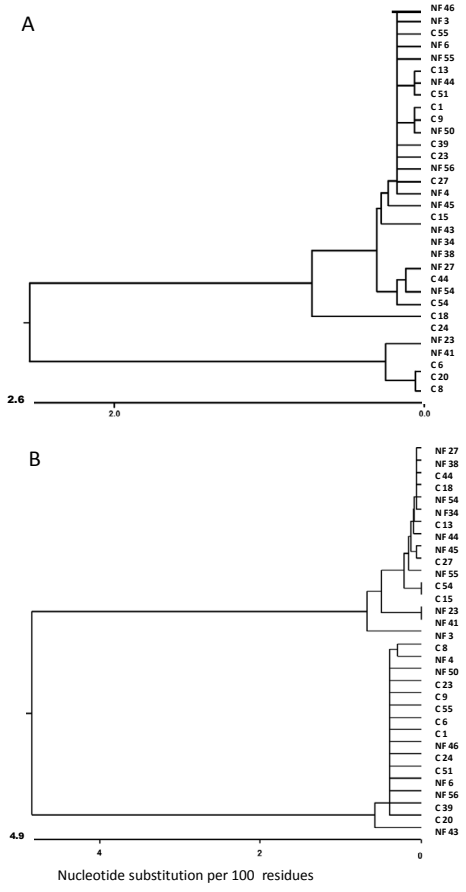

Fig. 2. Phylogenetic trees for 32 selected rhizobial strains from Control (C) and Nod Factors (NF) treatments for 16-23S rDNA (A) and *nodD* amplicons (B). *R. leguminosarum* strains were isolated at the end of the flowering stage from faba bean nodules. The sequence analysis of the 16-23S rDNA and *nodD* amplicons as well as the phylogenetic tree construction was carried out with DNASTAR Lasergene software using a Clustal W algorithm.

Table 2. Plant growth parameters of faba bean as influenced by Nod factors application with standard deviations (n = 3). Different letters in rows indicate significant differences ($p < 0.05$)

Plant parameters	Control	Nod Factors
	Flowering	
Plant biomass (g m^{-2})	617 (40.4) a	699 (32.6) a
Total plant N (g m^{-2})	14.5 (1.25) a	16.3 (0.93) a
	Maturity	
Plant biomass (g m^{-2})	1057 (53.2) a	1126 (56.4) a
Total plant N (g m^{-2})	24.1 (0.70) b	28.2 (2.05) a
Seed protein yield (g m^{-2})	120 (6.3) b	137 (5.9) a

nodD gene sequence than that of the 16-23S rDNA sequence (maximum > 4 and > 2 substitutions/100 bp, respectively) was noted. Most of the 16-23S rDNA region sequences were grouped within a cluster with an internal difference of less than 1 substitution/100 bp (Fig. 2A). The sequences of the *nodD* gene were grouped into two clusters (Fig. 2B) characterized by a slight internal variation; the sequences from the NF group strains were predominant within one cluster and those from group C dominated within the other.

Although higher in the NF than in the control, plant biomass did not differ significantly between the treatments at the flowering and maturity stages (Table 2). The N content in faba bean biomass at maturity and seed protein yield increased ($p > 0.05$) by 16.8% and 14.6%, respectively, after the seeds were treated with the Nod factors.

The effect of NF on soil enzymatic activity was beneficial ($p < 0.05$) at the flowering stage with respect to dehydrogenase, protease and acid phosphomonoesterase (NF increased their activity by 46, 36 and 9%, respectively) (Table 3). Urease activity did not differ between treatments for all the growth stages.

DISCUSSION

In this study, the Nod factors improved soil biological parameters by increasing the activities of the enzymes involved in the N and P cycle (protease and acid phosphomonoesterase) and total activity (dehydrogenase). This enhancement of protease and acid phosphomonoesterase activities indicate respectively a high release of protein N (Paul and Clark, 1996; Schimel and Bennett, 2004) as well as inorganic phosphorus from organic phosphomonoesters (Alef *et al.,* 1995). In turn, dehydrogenase plays a pivotal role in the oxidation of soil organic matter by hydrogen transfer from the organic substrate to inorganic acceptors (Zhang

Table 3. Soil enzyme activity as influenced by Nod factors application with standard deviations (n = 4). Different letters in columns indicate significant differences ($p < 0.05$)

Growth stage	Treatment	Dehydrogenase (cm^3 H_2 kg^{-1} d^{-1})	Protease (mg tyrosine kg^{-1} h^{-1})	Urease (mg kg^{-1} h^{-1})	Acid phosphomonoesterase (mg PNP kg^{-1} h^{-1})
5-6 leaves	Control	18.3 (1.07) e	3.1 (0.37) b	1.1 (0.06) b	38.9 (1.21) ab
	Nod Factors	17.1 (0.82) e	3.8 (1.00) b	1.4 (0.04) ab	41.1 (2.28) a
Flowering	Control	37.7 (1.29) d	3.8 (0.61) b	1.1 (0.08) b	36.6 (1.09) b
	Nod Factors	70.5 (2.15) a	5.9 (0.55) a	1.8 (0.73) ab	40.3 (1.71) a
Development of fruit	Control	53.1 (5.38) b	2.9 (0.11) b	2.1 (0.53) a	40.3 (1.69) a
	Nod Factors	45.5 (3.97) c	2.7 (0.31) b	1.6 (0.30) ab	40.4 (0.98) a

et al., 2010), thus the enhanced activity of this enzyme by NF may be connected with the higher rate of organic matter mineralization. The enhanced activities of the enzymes suggest an increase in nutrient availability to plants and microbes induced by the application of NF and thus may provide a favourable environment for faba bean growth. This beneficial effect was mainly observed at the flowering stage, which is one of the most critical stages for plant yield potential. Enhanced enzyme activity may be connected with the beneficial influence of NF on plant root growth (Kidaj *et al.,* 2012; Podleśny *et al.,* 2014; Marks *et al.,* 2015).

The beneficial effect of NF on nodule activity was connected with the increased genetic diversity of the rhizobial strains inside the nodules of the NF-treated plants. This suggests that NF reduce the competitive pressure between the rhizobial strains and this increases the number of nodules which can be colonized by rhizobia. The competitiveness of *R. leguminosarum* strains is under the influence of different bacterial traits (Wielbo *et al.,* 2007; Maj *et al.,* 2010; Wielbo *et al.,* 2012) and is not associated with symbiotic effectiveness. Nevertheless, it may be speculated that the increase in nodule number observed after Nod factors treatment allow for the infection of plants by strains with high symbiotic effectiveness and low competitiveness, and this is the reason for higher nitrogenase activity. The influence of NF on the genetic diversity of rhizobia colonizing pea and vetch nodules was previously observed by Kidaj *et al.* (2012) in a laboratory experiment. However, this beneficial effect on genetic diversity was observed to be greater in our study under field conditions.

The N content in plant biomass did not differ significantly between treatments at the flowering stage, however, at the maturity stage, it was significantly ($p < 0.05$) improved by NF. This implies the long-term effect of NF. These results are supported by data obtained in a pot experiment by Podleśny *et al.* (2014), who noted the improved N content in pea plants after NF application. The beneficial effect of NF on plant growth could be partly due to its influence on the rhizospheric microbial community. As indicated by Siczek *et al.* (2015) NF significantly increased the *Bacillus* and *Pseudomonas* population. It has been shown that some species of these bacteria positively affected legume growth, yield and symbiotic activity (Zahir *et al.,* 2011).

In a field experiment conducted with pea plants under the same climatic conditions (Siczek *et al.,* 2014), the application of NF increased nitrogenase activity ($p < 0.05$) and plant growth in a relatively dry season, but this effect was not observed during the wet growing season. A similar conclusion may be drawn from the present study. With reference to the long-term average (572 mm) (Siczek *et al.,* 2014), the yearly precipitation of 2015 was similar (531 mm) as measured by the meteorological station close to the experimental field. However, the amount of rainfall during the faba bean growing season (April-August) was considerably lower in 2015 than the long-term average (by 39%). It was demonstrated previously, in a laboratory study by Prudent *et al.* (2016) that NF application under water stress conditions increased the amount of N fixed and nodule parameters over the control. This NF effect is connected with an increase in the content of cytokinins (Prudent *et al.,* 2016), which are known to promote nodule organogenesis (Gamas *et al.,* 2017) as well as lateral root formation (Kiba *et al.,* 2011). Improved root growth due to NF, as indicated in earlier studies (Siczek *et al.,* 2013; Podleśny *et al.,* 2014) may result in improved water and nutrient absorption and thus the enhancement of nodule activity and N yield.

In the case of Nod factors there are several possible effects: the "primary effect" is an increase in the number of nodules due to the induction of more meristems. As Nod factors are signalling molecules, a change in their concentration in the environment probably interferes with the process of signal exchange between the rhizobia and plants, and therefore various "secondary effects" like the change in the composition of populations of rhizobia colonizing nodules are observed.

Similar results concerning the application of Nod factors for the improvement of plant productivity have been obtained for other legumes, such as soybean and pea (Smith *et al.,* 2015). However, to date, there is no research data available in relation to the faba bean. The results suggest that the application of Nod factors may have a beneficial effect on faba bean symbiotic activity and productivity for the years with lower precipitation during the growing season. Such a response may also have a positive effect on future faba bean productivity in light of the progressive decrease in precipitation during the growing season in Poland (Denisow and Malinowski, 2016). Further studies are intended to assess these effects over the course of a long-term field experiment.

CONCLUDING REMARKS

This study has shown that the treatment of faba bean seeds with Nod factors (NF) influenced both symbiotic nitrogen fixation and the rhizobia population in nodules, soil microbial activity, and faba bean yields.

1. The application of Nod factors before sowing improved the nitrogenase activity and changed the genetic diversity of rhizobia inhabiting root nodules.

2. At the flowering stage, Nod factors increased dehydrogenase activity as well as the protease and phosphomonoesterase activity connected with the N and P cycles.

2. Nitrogen and seed protein yields were improved by NF in comparison with the control.

3. The results of this study demonstrate that the addition of Nod factors may exert a positive influence on faba bean growth during seasons with a short-term rainfall deficit by increasing the symbiotic nitrogen fixation efficiency and therefore, the nitrogen yield.

Conflict of interest: The authors declare that they have no conflict of interest.

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