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The pleiotropic effects of extract containing rhizobial Nod factors on pea growth and yield

Research Article

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Abstract: Rhizobial lipochitooligosacharides (Nod factors) influence the development of legume roots, including growth stimulation, nodule induction and root hair curling. However, their effect on the green parts of plants is less known, therefore we evaluated seed and foliar application of an extract containing Nod factors on pea growth and yield. Pea plants were examined from emergence to full maturity, including growth dynamics and morphological (nodule number and weight, the quantity and surface area of leaves) or physiological (photosynthesis and transpiration intensity, chlorophyll and nitrogen content) parameters. The foliar application Nod factor extract, or seed dressing followed by foliar application, resulted in the best outcomes. The number and weight of root nodules, the chlorophyll content in leaves, and the intensity of net photosynthesis were all elevated. As a consequence of Nod factor treatment, the dynamics of dry mass accumulation of pea organs were improved and the pod number was increased. A significant increase in pea yield was observed after Nod factor application. Increase of nodule and pod numbers and improved growth of roots appear to be amongst the beneficial effects of Nod factor extract on the activation of secondary plant meristems.

Keywords: *Biological nitrogen fixation • Nod factors • Pea yield • Dynamics of mass accumulation*

1. Introduction

Numerous studies have been conducted in an effort to increase pea yield, mainly focusing on cultivation technology and breeding. Among other strategies, enhancement of symbiotic nitrogen fixation efficiency has been investigated [1,2], an approach that is well-founded both economically and ecologically. Biological nitrogen fixation is of significant importance to various crop and pasture legumes, as this additional nitrogen source often meets more than half of a plant's requirement for nitrogen [3].

As the efficiency of dinitrogen reduction depends on the colonization of plant tissues by rhizobia and on the activity of bacterial nitrogenase, significant resources have been invested in selecting rhizobial strains that are highly effective in symbiotic nitrogen fixation, and in using them as biofertilizers for legume plants. However, any beneficial effect of such practices would be observed only if the introduced strain could compete successfully against autochthonous rhizobia and if it was also well-adapted to a particular climate and soil [4,5]. Fulfilling all these requirements is often problematic, therefore the successful use of rhiozobial inoculants is often limited to legume plants that are introduced into new regions where the presence of compatible rhizobial microsymbionts in the soil is dubious [6].

In soils with numerous rhizobial populations other strategies for enhancing *Rhizobium*-legume symbioses have been investigated, such as the improvement of molecular signaling between plants and bacteria. Some promising results were observed when the inoculant rhizobia were applied together with flavonoid compounds

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[7,8], however, the direct application of rhizobial Nod factors seems to be a more efficient solution.

It is well known that Nod factors, by triggering numerous plant responses (root hair curling, growth of infection threads, induction of nodule primordia), are indispensable for the development of *Rhizobium*-legume symbiosis [9,10]. Recently, these signal molecules were also used for promoting plant growth [11] or enhancement of bacterium-plant symbiosis [12-15]. This enhancement of symbiosis can be obtained by increasing the rhizobial population density in root nodules. Such an increase in microsymbionts in plant tissues may result in increased plant nitrogen supply, even if the individual rhizobial strains possess only average nitrogen fixing ability.

In papers published up to now [11,12,14,15], the effect of Nod factors on plant growth was examined only fragmentarily, at single, arbitrarily-selected growth stages. Here, we present an exhaustive study of the growth and development of pea plants (*Pisum sativum* cv. Muza), where the effects of Nod factor treatment were observed from emergence (BBCH 08) to full ripening of seeds (BBCH 89), and using crude extract containing Nod factors (cNFextract), which can be produced simply and cheaply from rhizobial cultures. Moreover, we evaluate different methods of application of this Nod factor preparation and how it affects the growth, development and yield of pea.

2. Experimental Procedures

2.1 Nod factors production and isolation

Rhizobial Nod factors were isolated from liquid cultures of *Rhizobium leguminosarum* bv. *viciae* GR09 (*Rlv* GR09) strain induced by a plant flavonoid extract [16,17].

In order to obtain flavonoid extract, pea seeds were surface-sterilized by immersion in 0.1% $HgCl_2$ for 3 min, rinsed with sterile distilled water, treated with 70% ethanol for 3 min, and rinsed once again with sterile water. Then, the seeds were shaken in sterile water, in darkness, for 4 days at 28°C. After removal of the sprouted seeds, the supernatant was extracted with ethyl acetate in the ratio 10:1 (v/v). Ethyl acetate was evaporated and the pellet was resolubilized in 95% ethanol and stored at 4°C. The amount of the flavonoids was determined by drying the ethanol extract and weighing the dry mass. The approximate flavonoid concentration in seed exudates was calculated relative to the molecular weight of authentic flavone.

In order to produce rhizobial cNFextract, liquid cultures of *Rlv* GR09 (100 ml aliquots of TY medium in 250 mL flasks) [18] were prepared. After 24 h of growth, logarithmic cultures of *Rlv* GR09 were induced with sterile pea seed flavonoid extract at a final concentration of 10 µmol L^{-1} and incubated at 28°C for 48 h. To isolate Nod factors, one liter of the flavonoid-induced culture was extracted twice with 0.2 volume of *n*-butanol [11]. The organic fraction was separated and dried in a rotary evaporator (Rotavapor–R, Bűchi, Switzerland). The amount of Nod factors was determined by conversion of the amino sugars to methyl glycosides and gas chromatography/mass spectrometry (GC/MS) analysis. To 250 µg sample, 30 µg N-acetylgalactosamine (GalNAc) was added as the internal standard and hydrolyzed in 1 mL of 2 mol L^{-1} trifluoroacetic acid (TFA) at 120°C for 2 h. The TFA was eliminated by washing the samples twice with Millipore water, followed by drying under nitrogen. Free fatty acids were removed from the samples with 0.5 mL 10% (v/v) ether in hexane. This step was repeated three times. Samples were reduced by solid sodium borohydride ($NaBH_4$) at 0°C, in darkness, for 12 h. The excess of $NaBH_4$ was decomposed by few drops of acetic acid, and boric acid was removed by evaporation with 10% acetic acid in methanol. After this, samples were evaporated twice from 500 µL of methanol, using nitrogen stream. Next, the acetylation was performed: 50 µL acetic anhydride and 50 µL pyridine were added to the dry sample and incubated at 85°C for 30 min and then dried under nitrogen. Finally, samples were dissolved in a small volume of chloroform and applied to GC/MS (Hewlett- Packard HP 5890A) equipped with an HP-5MS capillary column coupled to a mass selective detector MSD HP 5971. The temperature program was as follows: initially 150°C for 5 min, then raised to 310°C (5°C/min), and the final temperature 310°C for 10 min. The Nod factor concentration was approximated based on the assumption that a single molecule of a Nod factor contains on average four residues of GlcNAc.

2.2 Plant growth conditions – preliminary experiments

Two small-scale plant tests were performed to confirm the biological activity of Nod factor extract and to determine its optimal concentration. Due to limitations concerning plant growth, vetch (*Vicia villosa* cv. Wista) was used as a plant model instead of pea.

In the first experiment, vetch seeds were soaked in serially diluted cNFextract (from 10^{-9} to 10^{-12} mol L^{-1}) or in distilled water (control group) for 1 hour, and then transferred into plastic pots with unsterile soil (500 g of soil and 5 seeds per pot). Pots were watered every two days with tap water and kept in a laboratory at room temperature with a 16h/8h day/night regimen. After 6 weeks of growth, plants were harvested, the fresh mass of shoots were estimated and root nodules were counted. The experiment was carried out twice, with 4 replicates.

In the second experiment, vetch seeds were sown into plastic pots with unsterile soil (500 g of soil and 5 seeds per pot). Ten days after sowing, young vetch plants were sprayed with serially diluted cNFextract (from 10^{-9} to 10^{-12} mol L^{-1}) or with distilled water (control group). Pots were watered every two days with tap water and kept in a laboratory at room temperature with a 16h/8h day/night regimen. After 6 weeks of growth, plants were harvested, fresh mass of shoots were estimated and root nodules were counted. The experiment was carried out twice, with 4 replicates.

2.3 Root hair deformation assay

To confirm the biological activity of Nod factor extract, a root hair deformation test was performed on vetch (*Vicia villosa* cv. Wista). Surface-sterilized seeds of vetch were germinated on Fahraeus agar medium [19] in Petri dishes in darkness at 28°C. After two days of incubation, individual seedlings were transferred to microscope glass slides covered with 1 mL 0.4% Fahraeus medium supplemented with serially diluted cNFextract (from 10^{-9} to 10^{-12} mol L^{-1}). Slides were placed in a moist chamber and incubated at 28°C in darkness. After 24 and 48 h, the growth and deformation of individual root hairs was assessed under a light microscope (Olympus BX41). The morphology of root hairs after Nod factor treatment was compared to the morphology of root hairs curled after the addition of live cells of *Rhizobium leguminosarum* bv. *viciae* GR09 into 0.4% Fahraeus medium instead of cNFextract.

2.4 Plant growth conditions – main experiment

All plant experiments were conducted at Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy. Mitscherlich pots were filled with a mixture of 5 kg of arable soil (loamy sand: pH_{H2O} 7.6, pH_{KCl} 7.4, Mg – 0.22 g / 100 g of soil, P_2O_5 - 56.76 g / 100 g of soil, K_2O – 17.13 g / 100 g of soil, granulometric composition: 2.0-1.0 mm – 2%, 2.0-0.1 mm – 65%, 0.1-0.02 mm – 22%, <0.02 mm – 13%) and 2 kg of sand. The number of autochthonous rhizobia in the soil was estimated by the most probable number method [20] as 0.4 x 10^2 cells g^{-1} of soil. Ten pea seeds (*Pisum sativum* cv. Muza, afila type) were sown into each pot. After the phase of full emergence (i.e. 15 days after sowing), some seedlings were thinned and finally five plants were cultivated in each pot. Thinning of pea seedlings was performed randomly, *i.e.* seedlings in each pots were numbered, and then numbers of seedlings intended for thinning were randomized. After that, the potted plants were cultivated in Heraeus climatic chambers, at (day/night, respectively): 24°C / 14°C, 75% / 60% humidity and 14h / 10h, with PAR illumination of 300 µmol $m^2 \cdot s^{-1}$. The position of pots in the chambers was randomized.

Each experimental group consisted of four pots. The plants from one pot per group were harvested at flowering (BBCH 60), another pot per group – at green pod phase (BBCH 75), and two pots per group – at full maturity (BBCH 89). A randomized method was used for choosing the pots for analyses at BBCH 60 and BBCH 75.

The fertilization used in this experiment (g/pot) was as follows: N - 0.1, P - 1.1, and K - 1.4. Liquid fertilizers were applied during watering once, at pea plant emergence. The plants were watered using demineralized water twice a day (by gravimetric method). The soil humidity was kept at field water capacity: 30% (from the beginning of the experiment to the phase of 3rd-4th leaf), 60% (from the phase of 3rd-4th leaf to green pod phase) and 30% (from green pod phase to full maturity of seeds).

The experiment was repeated three times with the same results, and the mean of these three independent experiments, repeated in the same growth chambers, is presented.

2.5 Experimental factors

The experiment was planned as a complete randomized design (2 x 2 factorial design). The first experimental factor was dressing of seeds by soaking in distilled water or a solution of rhizobial Nod factor extract, whereas the second experimental factor was spraying the plants with distilled water or a solution of cNFextract. Therefore, four plant groups were included in this experiment: 1 – not treated with Nod factors (control), 2 – grown from seeds soaked in solution of cNFextract (SS), 3 – sprayed with solution of cNFextract (SP), 4 - grown from seeds soaked in and sprayed with solution of cNFextract (SSSP).

2.6 Application of Nod factor extract

The concentration of Nod factors in the water solution applied to seeds or sprayed onto the plants (using Mercury Pro+ Super360 technical sprayer – Kwazar Sp. z o.o., Jaktorów, Poland) was 10^{-11} mol L^{-1}. Pre-sowing seed dressing consisted of their soaking in solution of cNFextract or distilled water for 1 hour (100 ml of Nod factor preparation or distilled water was used per 1 kg of seeds). Spraying of plants was performed at the three leaf stage (BBCH 13), using 25 ml of Nod factor preparation or distilled water per pot.

2.7 The dynamics of pea emergence

The number of plants in each pot was counted at 12-hour intervals, since the emergence of the first plant in a pot, and it was conducted as long as new seedlings were not observed. Only normally germinating seeds and fully developed plants were counted. An index of

emergence dynamics (E_d) was calculated from the following formula:

$E_d = N_e/N_s \cdot 100\%$ (%), where:

N_e- number of plants after emergence,

N_s – number of sown seeds

2.8 The dynamics of pea weight increase

Plants were harvested at: flowering (BBCH 60), green pod stage (BBCH 75) and full maturity (BBCH 89). After each harvest, the fresh and dry weights of a particular plant's organs were determined. The roots were weighed after rinsing on thick metal sieves. Nodules were harvested, weighed (for fresh mass) and then air-dried for dry mass determination. The dynamics of weight increase were fixed to the base of relative growth rate (RGR) using Evans (1972) formula:

$RGR = (\ln W_2 - \ln W_1)(T_2 - T_1)^{-1}$ (g g^{-1} day^{-1}), where:

W_1- dry weight of plants at the beginning of the measurement period,

W_2- dry weight of plants at the end of the measurement period,

T_1–the beginning of the measurement period,

T_2- the end of the measurement period

2.9 Measurement of leaf area

Measurement of leaf area was performed at three phenological steps: flowering (BBCH 60), green pod (BBCH 75) and full maturity (BBCH 89). Leaves were plucked and put on a special foil and then the measurement was done using Leaf Area Scanner AM 300 (ADC BioScientific Ltd., UK).

2.10 Measurement of SPAD index

SPAD, which is also called "leaf greenness index", was measured three times: at flowering (BBCH 60), green pod (BBCH 75) and full maturity (BBCH 89), using a Minolta chlorophyll meter SPAD-502. For each examined plant SPAD index was scored for the first, second and third fully-developed leaf counting from the top of a plant. Each SPAD index presented here is a mean of 30 independent measurements.

2.11 Measurement of gas exchange parameters

Measurement of gas exchange parameters - net photosynthesis intensity (Pn), transpiration intensity (E), stomatal conductance (Gs) and water-use efficiency coefficient (WUE) - were performed at flowering stage using a CIRAS-2 device (Portable Photosynthesis System). Radiation intensity used in these measurements was 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and CO_2 value amounted 380 ppm. Water use efficiency coefficient was calculated from a formula:

WUE = Pn/E (µmol CO_2 $mmol^{-1}$ H_2O)

Measurements were performed on the first fully-developed leaf counted from the top of a plant. Results which are presented are mean of 3 independent measurements.

2.12 Seed yield and yield structure

Seeds were collected at green pod (BBCH 75) and full maturity (BBCH 89) stages and weighed. The number of pods per plant, the number of seeds per pod and the number of seeds per plant were also estimated. The weight of one thousand seeds was determined as an indicator of seed size and degree of fullness. The seed yield obtained at full maturity was recalculated to 14% moisture.

2.13 Determination of nitrogen content of vegetative organs and seeds of pea

Nitrogen concentration was determined using flow spectrophotometer method (PN-92/R-04014), at Laboratory of Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy, Poland. Nitrogen uptake with yield (U_Y) was counted from a formula:

$U_Y = C_N \times Y/100$ ($g \cdot pot^{-1}$), where:

C_N – concentration of nitrogen at yield (%),

Y – yield ($g \cdot pot^{-1}$)

2.14 Other observations and measurements

During the entire vegetation period, detailed observations of growth and development of plants and their infestation by diseases and pests were carried out. Measurements of plant height were performed at flowering (BBCH 60), green pod (BBCH 75) and full maturity (BBCH 89) stages.

2.15 Statistical analyses

Statistical calculations relating to analysis of variance and regression were performed with Statgraphics ver. 5.1 program. Differences between means were compared using Tukey`s test at significance level P=0.05.

3. Results

3.1 The characteristics of crude Nod factor extract

Before the principal experiment testing the effect of Nod factor extract on pea growth, development and yield, some preliminary experiments were performed to confirm the presence of rhizobial Nod factors in the cNFextract as well as to determine the appropriate concentration of this extract in further experiments.

GC/MS analysis (Figure 1a-c) revealed the presence of glucosamine as the main sugar component in the extract, as well as numerous fatty acids (*i.e.* $C_{16:0}$, $C_{18:0}$,

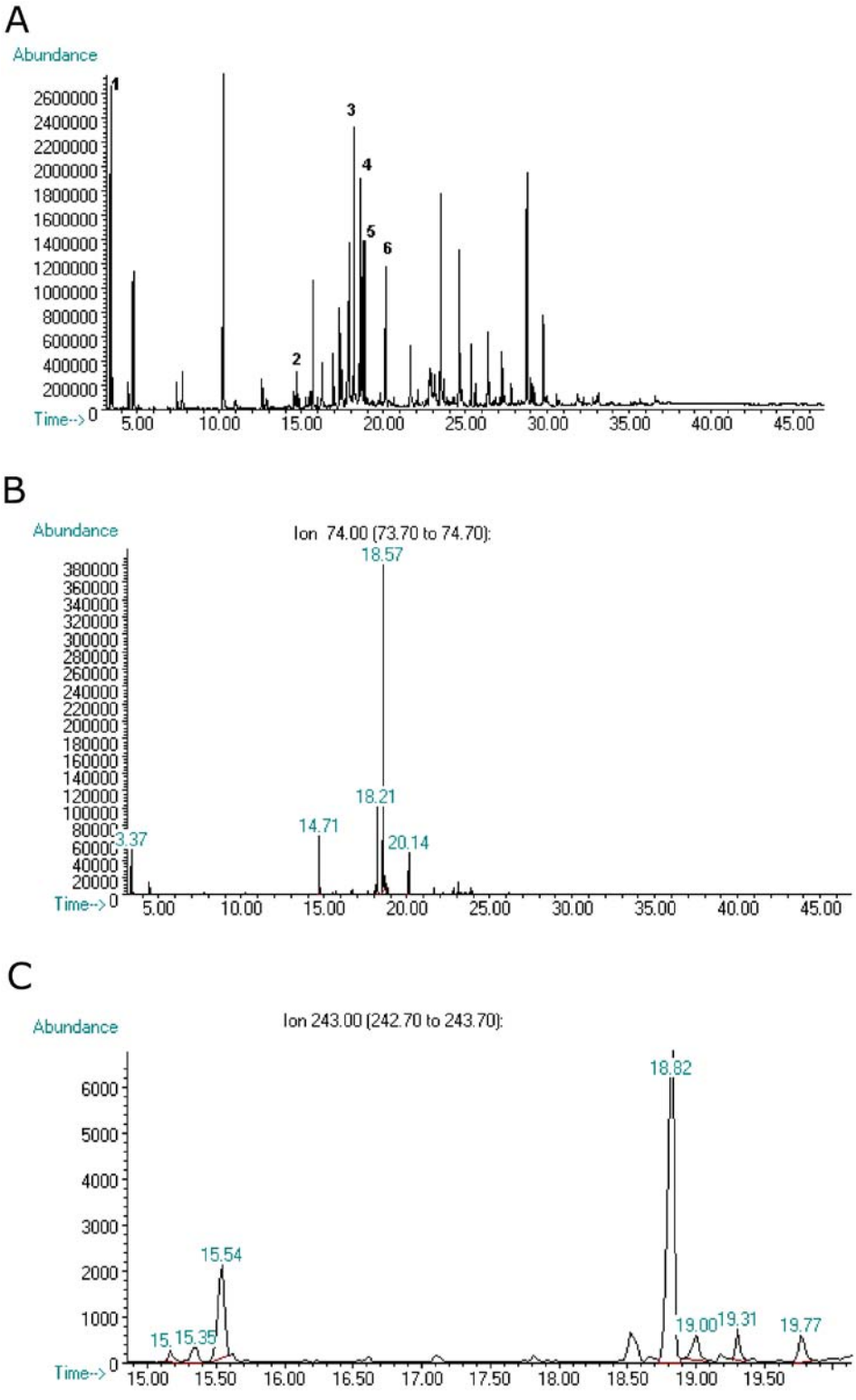

Figure 1. Chemical composition of cNFextract. A: chromatogram (sample after methanolysis: 4 mol L^{-1} HCl/100°C/4h, N-acetylation, redH, peracetylation); peaks: 1- glycerol; 2, 3, 4, 6 – fatty acids: $C_{16:0}$, $C_{18:1}$, $C_{18:0}$, $C_{19:1}$, respectively; 5 – glucosamine. B: ionogram for m/z 74 typical for methyl esters of fatty acids; peaks t_R = 14.71 min ($C_{16:0}$), t_R = 18.21 min ($C_{18:1}$), t_R = 18.57 min ($C_{18:0}$), t_R = 20.14 ($C_{19:1}$). C: ionogram for m/z 243 typical for methyl glycosides (derivatives of sugars) – fragment of analysis, the extension in range 14.00 – 22.00 min; peaks: t_R = 15.17, 15.35, 15.54 min - hexoses, $t_{R\ =}$ 18.82, 19.00, 19.31 and 19.77 min – glucosamine.

$C_{18:1}$, $C_{19:1}$), which are also considered to be components of rhizobial Nod factors [21]. The approximate estimation of Nod factor concentration in the cNFextract based on the results of GC/MS analysis was about 250 nmol L^{-1}.

Moreover, the biological activity of cNFextract was studied in root hair curling test (Figure 2). Except for the lowest dilution of cNFextract (~10^{-9} mol L^{-1} Nod factors and 1% butanol), where the growth of root hairs was probably impaired by butanol, for all studied dilutions (~10^{-10} mol L^{-1}, ~10^{-10} mol $L^{-1}$and ~10^{-10} mol L^{-1} of Nod factors) more (Figure 2b, 2c) or less intense (Figure 2d) curling of root hairs was observed. Similar root hair curling was obtained when live cells of compatible microsymbiont were present in the vicinity of the roots (Figure 2a).

Finally, the optimal concentration of cNFextract was determined in two small-scale laboratory plant tests (Table 1). Irrespective of the method of cNFextract application, the highest nodule numbers were scored when cNFextract solutions containing about 10^{-10} mol L^{-1} and 10^{-11} mol L^{-1} of Nod factors were applied, therefore the dilution containing about 10^{-11} mol L^{-1} was applied in subsequent experiments.

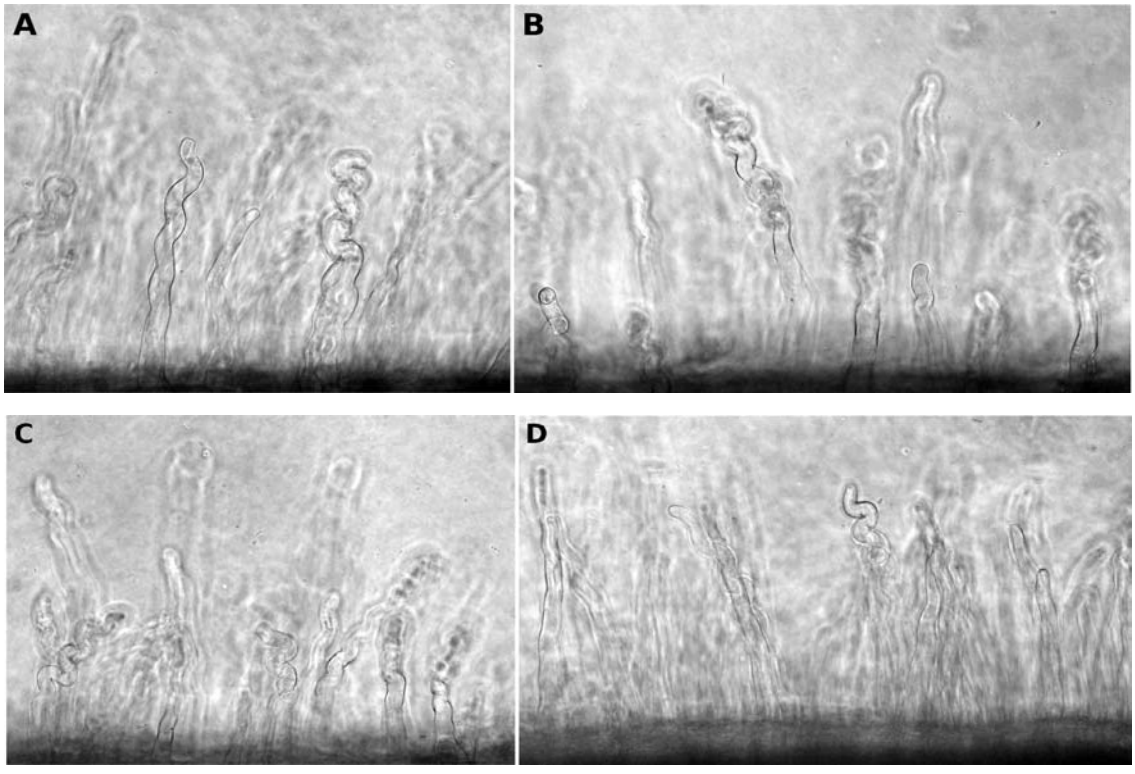

Figure 2. Curling of vetch root hairs resulting from (A) contact with rhizobia, or application of cNFextract containing: (B) ~10^{-10} mol L^{-1} of Nod factors, (C) ~10^{-11} mol L^{-1} of Nod factors, (D) ~10^{-12} mol L^{-1} of Nod factors.

Approximate concentration of Nod factors	Seeds soaked in cNFextract solution		Plants sprayed with cNFextract solution	
	Fresh mass of shoots (g / pot)	Number of nodules (per pot)	Fresh mass of shoots (g / pot)	Number of nodules (per pot)
10^{-9} mol L^{-1}	5.57 ± 0.56^{ab}	41.3 ± 7.3^{ab}	5.80 ± 0.27^{a}	46.8 ± 6.0^{ab}
10^{-10} mol L^{-1}	6.37 ± 1.15^{a}	51.5 ± 8.2^{a}	6.28 ± 0.78^{a}	49.8 ± 6.2^{ab}
10^{-11} mol L^{-1}	6.13 ± 0.44^{a}	52.8 ± 17.9^{a}	6.08 ± 0.51^{a}	53.3 ± 6.0^{a}
10^{-12} mol L^{-1}	4.87 ± 1.29^{b}	31.0 ± 10.4^{b}	5.84 ± 0.86^{a}	42.8 ± 9.2^{ab}
Control (no cNFextract)	5.00 ± 1.02^{b}	33.8 ± 4.6^{b}	5.00 ± 1.02^{a}	33.8 ± 4.6^{b}

Table 1. Fresh mass of shoots and nodule number of vetch treated with different amounts of Nod factors contained in cNFextract

^{a,b} in colums, values followed with the same superscript letter are not significantly different at P=0.05

3.2 The emergence and growth of plants

The emergence of pea plants which grew from non-dressed seeds (control) and from seeds dressed with solution containing Nod factors (SS) occurred after 6 and 4 days after sowing, respectively. Quicker plant emergence was observed for seeds treated with cNFextract, as compared to the control seeds (Figure 3). Considerable differences of emergence dynamics associated with Nod factors use were found between 8th and 11th day after sowing, *i.e.* at a stage of full pea emergence. Moreover, cNFextract application increased the uniformity of pea plant emergence.

The application of cNF extract significantly affected plant height in the period between flowering and full maturity (Table 2). Spraying of plants plus dressing of seeds with solution containing Nod factors (SSSP) had greater effect than seed soaking (SS) alone. The increase in pea plant height resulting from Nod factor treatment was greater at flowering and green pod than at full maturity, and amounted to 13.5%, 14.2% and 9.4%, respectively.

3.3 Morphological changes

During the studied period of plant growth and development, considerable changes in leaf surface area were observed (Table 2). Generally, from flowering to green pod phase, total leaf area increased by 16.7%, while from green pod to maturity the total leaf area decreased by 58.6%, respectively. This decrease was caused by the loss of dry leaves. The use of cNFextract resulted in larger leaf area, and during flowering, green pod and maturity stages this increase was 14.5%, 12.1% and 7.9%, respectively, compared to control plants. Spraying of plants with solution of cNFextract (SP), or seed dressing together with plant spraying (SSSP), resulted in a significantly greater increase in leaf area than soaking of seeds alone (SS) (Table 2).

3.4 The dynamics of dry mass accumulation

The application of cNFextract affected the accumulation of mass in pea vegetative and generative organs (Figure 4), and the most significant effect was observed at green pod phase (BBCH 75), *i.e.* during the rapid growth of plants.

The total yield of stems and leaves in response to seed dressing, plant spraying and seed dressing together with plant spraying (SSSP) with Nod factor extract was higher by 14.2%, 25.0% and 28.2%,

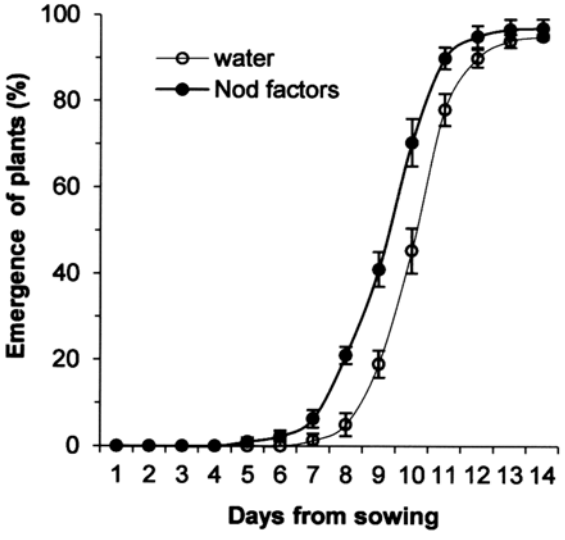

Figure 3. The dynamics of pea seed germination after soaking in water or cNFextract.

Developmental phases of plants	Soaking	Spraying: height of plants (cm)			Spraying: leaf area ($cm^{-2} \cdot plant^{-1}$)			Spraying: SPAD		
		control	SP	mean	control	SP	mean	control	SP	mean
Flowering (BBCH 60)	control	44.7^{aA}	50.0^{aB}	47.3^{a}	560^{aA}	660^{aB}	610^{a}	446^{aA}	525^{aB}	485^{a}
	SS	49.4^{bA}	54.9^{bB}	52.1^{b}	623^{bA}	683^{aB}	653^{b}	517^{bA}	534^{aA}	525^{b}
	mean	47.0^{A}	52.4^{B}		592^{A}	671^{B}		481^{A}	529^{B}	
Green pod (BBCH 75)	control	48.5^{aA}	56.8^{aB}	52.6^{a}	683^{aA}	797^{aB}	740^{a}	642^{aA}	697^{aB}	669^{a}
	SS	55.5^{bA}	57.3^{bB}	56.4^{b}	754^{bA}	780^{aA}	767^{b}	686^{bA}	707^{aA}	696^{b}
	mean	52.0^{A}	57.0^{B}		719^{A}	788^{B}		664^{A}	702^{B}	
Full maturity (BBCH 89)	control	53.2^{aA}	60.2^{bB}	56.7^{a}	441^{aA}	482^{aB}	462^{a}	498^{aA}	552^{aB}	525^{a}
	SS	56.4^{bA}	59.4^{aA}	57.9^{a}	457^{aA}	499^{aB}	478^{a}	540^{bA}	560^{aB}	550^{b}
	mean	54.8^{A}	59.8^{B}		449^{A}	490^{B}		519^{A}	556^{B}	

Table 2. Height of plants (cm), leaf area and SPAD values at period from flowering to harvest of pea plants.

Results of LSD range test are shown. Values followed by similar superscript letters are not significantly different at the 5% probability level. Capitals concern data placed in lines, and small letters concern data placed in columns.

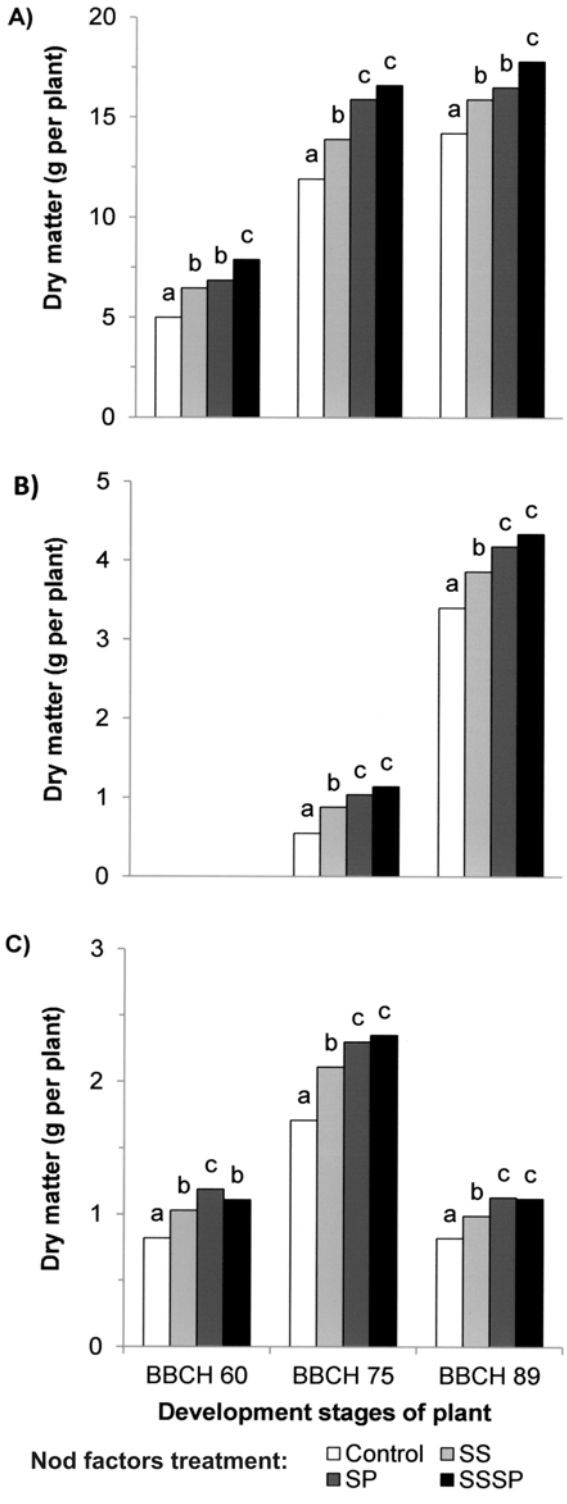

Figure 4. Dry matter of pea organs after cNFextract use. A: stems+leaves+siliques, B: seeds, C: roots. Control – seeds and plants not treated with cNFextract, SS- seeds soaked in the cNFextract, SP- plants sprayed with the cNFextract, SSSP – seeds soaked in and plants sprayed with the cNFextract. Values followed by the same letters are not significantly different at the 5% probability level.

respectively, compared to control plants. Moreover, the increase in seed mass at full maturity reached 11.9%, 18.7% and 19.1%, respectively.

The relative growth rate (RGR) reached the highest values at flowering and pod setting, and then decreased with plant maturity to the previous level (Table 3). RGR values increased after application of cNFextract, especially at the period of intensive weight gain, *i.e.* at flowering and pod setting.

3.5 Number and weight of root nodules

The use of cNFextract significantly affected the number and weight of pea root nodules (Table 4). The dressing

RGR ($g \cdot g^{-1} \cdot day^{-1}$)	Soaking	Spraying		
		control	SP	mean
BBCH (0–60)	control	0.087^{aA}	0.096^{aA}	0.091^{a}
	SS	0.093^{aA}	0.103^{aB}	0.098^{a}
	mean	0.090^{A}	0.099^{B}	
BBCH (60–75)	control	0.155^{aA}	0.185^{aB}	0.170^{a}
	SS	0.170^{bA}	0.187^{aB}	0.178^{b}
	mean	0.162^{A}	0.186^{B}	
BBCH (75–89)	control	0.017^{aA}	0.027^{aB}	0.022^{a}
	SS	0.028^{bA}	0.029^{aA}	0.028^{b}
	mean	0.023^{A}	0.028^{B}	

Table 3. Relative Growth Rate (RGR) of pea plants.

Results of LSD range test are shown. Values followed by similar superscript letters are not significantly different at the 5% probability level. Capitals concern data placed in lines, and small letters concern data placed in columns.

Description	Soaking	Spraying		
		control	SP	mean
Number of root nodules per plant	control	9.8^{aA}	13.5^{aB}	11.6^{a}
	SS	11.4^{bA}	13.1^{aB}	12.2^{b}
	mean	10.6^{A}	13.3^{B}	
Fresh weight of nodules (g per plant)	control	1.04^{aA}	1.46^{aB}	1.25^{a}
	SS	1.12^{bA}	1.40^{aB}	1.26^{b}
	mean	1.08^{A}	1.43^{B}	
Dry weight of nodules (g per plant)	control	0.21^{aA}	0.35^{aB}	0.28^{a}
	SS	0.24^{bA}	0.36^{aB}	0.30^{a}
	mean	0.22^{A}	0.35^{B}	

Table 4. Number and weight of pea nodules at green pod phase (BBCH 75).

Results of LSD range test are shown. Values followed by similar superscript letters are not significantly different at the 5% probability level. Capitals concern data placed in lines, and small letters concern data placed in columns.

of seeds, spraying of plants or both treatments together resulted in an increase in nodule number, which, compared to the control group, was higher by 17.2%, 27.1% and 28.0%, respectively. As a consequence, nodule fresh weight increased by 21.6%, 29.2% and 25.8%, and nodule dry weight by 9.2%, 12.4% and 15.5%, respectively.

3.6 SPAD index and gas exchange parameters

The leaf greenness index (SPAD), which corresponds to the chlorophyll content in leaves, was highest at the green pod stage (BBCH 75) (Table 2). The use of cNFextract significantly elevated SPAD index by 6.5%, 7.9% and 9.2%, for SS, SP and SSSP plants, respectively.

The application of Nod factor extract also affected the gas exchange parameters of the pea leaves (Table 5). A significant increase in photosynthesis intensity was observed, which may result in improved productivity of plants, and the most beneficial effect was observed in the SSSP group. Other parameters of gas exchange, *i.e.* transpiration intensity, stomatal conductance and water use efficiency (WUE) coefficient, were also improved by Nod factor treatment (Table 5).

Pea plants grown from cNFextract-treated seeds or plants sprayed with it contained more water in their main organs than non-treated plants (Table 6), and they reached maturity after 72 days (compared to 68 days for control plants) from emergence.

3.7 Pea yield and yield structure

Increases in pea seed yield (Figure 4) as well as improvement in plants' structural features (Table 7) were observed as a result of changes in the growth and development of pea after the application of cNFextract.

Organ of plant	Soaking	Spraying		
		control	SP	mean
Leaves	control	57.4^{bB}	41.2^{aA}	49.3^{b}
	SS	48.9^{aA}	45.6^{aA}	47.2^{a}
	mean	53.1^{B}	43.4^{A}	
Stems	control	46.9^{bB}	42.7^{aA}	44.8^{b}
	SS	40.5^{aA}	43.3^{aB}	41.9^{a}
	mean	43.7^{A}	43.0^{A}	
Roots	control	78.4^{bB}	64.7^{aA}	69.7^{a}
	SS	65.2^{aA}	65.1^{aA}	61.1^{a}
	mean	70.0^{B}	64.9^{A}	
Hulls	control	87.3^{bB}	77.1^{aA}	82.2^{b}
	SS	76.4^{aA}	76.3^{aA}	76.3^{a}
	mean	81.8^{B}	76.7^{A}	
Seeds	control	90.9^{bB}	84.3^{aA}	87.6^{b}
	SS	82.8^{aA}	84.0^{aA}	83.4^{a}
	mean	86.8^{A}	84.1^{A}	

Table 6. Dry matter content (%) at pea plants during full maturity (BBCH 89).

Results of LSD range test are shown. Values followed by similar superscript letters are not significantly different at the 5% probability level. Capitals concern data placed in lines, and small letters concern data placed in columns.

Gas exchange parameters	Soaking	Spraying		
		control	SP	mean
Transpiration intensity (T) (mmol $H_2O \cdot m^{-2} \cdot s^{-1}$)	control	2.6^{aA}	3.8^{aB}	3.2^{a}
	SS	3.2^{bA}	3.4^{aA}	3.3^{a}
	mean	2.9^{A}	3.6^{B}	
Stomatal conductance (Gi) (mmol $H_2O \cdot m^{-2} \cdot s^{-1}$)	control	584^{aA}	920^{aB}	752^{a}
	SS	854^{bA}	1115^{bB}	985^{b}
	mean	719^{A}	1018^{B}	
Intensity of net photosynthesis (Pn) (μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$)	control	15.5^{aA}	17.7^{aB}	16.6^{a}
	SS	17.2^{bA}	19.1^{aB}	18.1^{b}
	mean	16.3^{A}	18.4^{B}	
Water-use efficiency (WUE) (μmol $CO_2 \cdot$ mmol^{-1} H_2O)	control	5.96^{aA}	4.66^{aB}	5.31^{a}
	SS	5.37^{bA}	5.62^{bA}	5.49^{a}
	mean	5.66^{A}	5.14^{B}	

Table 5. Values of gas exchange parameters at flowering (BBCH 75).

Results of LSD range test are shown. Values followed by similar superscript letters are not significantly different at the 5% probability level. Capitals concern data placed in lines, and small letters concern data placed in columns.

The increase in seed yield was a consequence of the increase in pod number per plant, seed number per plant and weight of 1000 seeds. On the other hand, the number of seeds per pod, which is admittedly a low-variance feature, did not change significantly after Nod factor treatment.

3.8 Nitrogen uptake and content

Significant differences in nitrogen concentration during flowering were found between control and SP plants as well as SS plants, and the use of cNFextract increased N content both in plants and in seeds. Moreover, significant difference in N uptake between control and experimental groups was found for SP plants at flowering and for SS plants at maturity (Table 8).

4. Discussion

Studies on the beneficial potential of the stimulation of signaling in the *Rhizobium*-legume symbioses have been conducted for years. The early concepts involved the use of flavonoids as stimulators for Nod factor synthesis by rhizobia, which in turn should lead to better nodulation of the plant host [2,7,8,22,23]. However, the rapid loss of flavonoids in the rhizosphere [2], often before activation of the rhizobial *nod* genes, necessitated the search for another solution. Therefore, the direct use of Nod factors seemed a more efficient method than induction of bacteria by flavonoids. In experiments focused on defined stages of plant growth, it has been demonstrated that exogenous Nod factors can promote germination and early growth of leguminous and non-leguminous plants [4,11], increase nodulation and the growth of roots [12,14,15,24], or temporarily improve photosynthetic intensity [25].

In our experiments, using a crude extract containing Nod factors obtained from rhizobial culture, we observed a pleiotropic effect of Nod factor treatment on the growth

Organ of plant	Soaking	Spraying		
		control	SP	mean
Number of pods per plant	control	6.0^{aA}	7.0^{aB}	6.5^{a}
	SS	$6.9b^{A}$	7.5^{aA}	7.2^{a}
	mean	6.9^{A}	7.2^{A}	
Number of seeds per pod	control	3.5^{aA}	3.5^{aA}	3.5^{a}
	SS	3.6^{aA}	3.5^{aA}	3.5^{a}
	mean	3.5^{A}	3.5^{A}	
Number of seeds per plant	control	21.0^{aA}	24.5^{aB}	22.7^{a}
	SS	24.8^{bA}	26.2^{aA}	25.5^{b}
	mean	22.9^{A}	25.3^{B}	
Weight of 1000 seeds (g)	control	210^{aA}	221^{aB}	215^{a}
	SS	225^{bA}	232^{aB}	223^{a}
	mean	217^{A}	226^{B}	

Table 7. Features of pea yield structure at full maturity (BBCH 89).

Results of LSD range test are shown. Values followed by similar superscript letters are not significantly different at the 5% probability level. Capitals concern data placed in lines, and small letters concern data placed in columns.

Description		Soaking	Spraying		
			control	SP	mean
Plants (BBCH 60)	Content (C_N)	control	2.66^{bA}	3.06^{aB}	2.86^{a}
		SS	3.01^{bA}	3.04^{aA}	3.02^{b}
		mean	2.83^{A}	3.05^{A}	
	Uptake (U_y)	control	171.5^{aA}	209.9^{aB}	190.7^{a}
		SS	194.3^{aA}	240.0^{bB}	217.1^{b}
		mean	182.9^{A}	224.9^{B}	
Seeds (BBCH 89)	Content (C_N)	control	3.78^{aA}	3.85^{aA}	3.81^{a}
		SS	3.89^{bA}	3.91^{aA}	3.90^{a}
		mean	3.83^{A}	3.88^{A}	
	Uptake (U_y)	control	129.0^{aA}	160.0^{aB}	144.5^{a}
		SS	152.0^{bA}	164.4^{aA}	158.2^{a}
		mean	140.5^{A}	162.2^{B}	

Table 8. Content (%) and uptake (mg•plant^{-1}) of nitrogen in pea plants (BBCH 60) and in seeds at full maturity (BBCH 89).

Results of LSD range test are shown. Values followed by similar superscript letters are not significantly different at the 5% probability level. Capitals concern data placed in lines, and small letters concern data placed in columns.

of pea plants, with numerous pea traits improved after such treatment. This effect lasted from emergence (BBCH 08) until full ripening of seeds (BBCH 89), and resulted in changes in the shoots (increased plant height, leaf surface area or dry mass and pod number) as well as roots (increased dry mass and nodule number). Interestingly, the method of cNFextract application did not affect the incidence of these effects – both seed and foliar application resulted in promotion of shoot and root growth. The precise nature of this phenomenon was not studied, however two different mechanisms could be envisaged. Transport of the Nod factors from the site of their application to the site of the observed effect *via* plant vascular tissues could be responsible for their delivery to Nod factor-susceptible groups of plant cells. Conversely, the transmission of a plant-derived molecular signal in this process cannot be excluded, considering the involvement of similar MADS-box-type transcriptional factors in numerous plant developmental processes, *i.e.* in Nod-induced nodule primordia development [26-28], the development of arbuscular mycorrhiza [29], or in the transition from shoot meristem to floral meristem [30,31].

Irrespective of the method of cNFextract application, a significant increase in the majority of studied plant parameters was observed after Nod factor treatment. Moreover, foliar application of rhizobial cNFextract (SP) gave better results than seed dressing (SS), and almost the same as foliar and seed application together (SSSP). It is plausible that seed application is less efficient due to the diffusion of Nod factors in the soil solution or their degradation by soil microorganisms or plant-derived hydrolases [32,33].

The double application of cNFextract (SSSP) gave better results than the single foliar treatment (SP) in the case of dry mass of shoots and seed mass. On the other hand, there was no difference in numbers of root nodules. In the future, in a situation when the shoot tissues are less susceptible and a greater dose of Nod factors derived from multiple treatments would result in a higher number of pods or an increase in shoot mass, a repeated foliar dosage (more than one dose) of Nod factors-containing solution for young plants should be considered.

One of the most evident outcomes of cNFextract application was the acceleration of plant growth and development. The time of emergence was shortened, and the relative growth rate was increased after Nod factor treatment, especially up to development of seeds (BBCH 75). This resulted in an increase in mass of shoots and roots of up to 20%, and in the mass of seeds up to 60%, respectively, compared to the mass of control plants. During the next stage (full maturity of seeds) these differences were not as pronounced, which may be explained by an increased demand for nutrients by the growing seeds as well as by the excessive mutual shading of leaves [34]. However, the differences between the untreated and cNFextract -treated plants remained significant, and this observation may be useful in the context of plant fresh mass production.

Other effects of the Nod factor treatment of pea plants concern plant meristems. With respect to the role of Nod factors in nodule primordia development, the significant increase in root nodule number after the treatment was similar as earlier reported [12,14,15]. Apart from that, the cNFextract application resulted in the increase in the dry mass of roots. It was demonstrated that rhizobial Nod factors and fungal lipooligosaccharides (similar to bacterial Nod factors) can both stimulate lateral root formation in *Melilotus alba* and *Medicago truncatula* [35-37], or root length and root surface area in *Arabidopsis thaliana* [24]. Thus, it is plausible that in our experiments the increase in root growth as well resulted from the intense induction of lateral root meristems and branching of lateral roots. Both of the mentioned types of meristems (nodule meristems and lateral roots meristems) are secondary meristems, suggesting a specific susceptibility of these types of plant meristems to rhizobial Nod factors.

After cNF extract application the number of pods increased, which may result from a more frequent transformation of the auxiliary bud into floral meristem (also secondary meristem). On the other hand, the number of leaves was unaffected by cNF extract application. Considering that leaf primordia are formed directly from apical meristem (primary meristem), this may also support the hypothesis of the specific activation of secondary meristems by Nod factors. Finally, we found that Nod factor application also affected stomatal conductance at the green pod stage by 46-97%, and these results are supported by data obtained by Almaraz *et al.* [38] in experiments with soybean. If an increase in stomatal conductance results from increased stomata number, this would agree with the hypothesis of stimulation of secondary meristem activity by the rhizobial Nod factors, due to stomata emergence from meristemoids, also classified as secondary meristems.

All of the above changes in plant morphology caused by application of cNF extract may result in a better water supply for the plants (due to better development of the roots), which is very important for obtaining high pea yield [39], and better nitrogen supply (due to the increase in nodule number). It was shown that the net primary production of plants is dependent on the leaf area index, which, in turn, is conditional upon nitrogen supply [40]. In our experiments simultaneous increase

in nodule number, leaf area and SPAD readings was observed, so taking into consideration the relationship between nitrogen supply and plant productivity [40] it can be assumed that the larger area available for photosynthesis stems from higher nitrogen supply from the nodules. The water use efficiency (WUE) values after Nod factor treatment were worse than in the control group, however, suggesting that changes in the water balance were not detrimental to the plants. The percentage of water in the leaves of the control plants was 42.6%, compared to 51.1-58.8% in cNF extract-treated plants. Moreover, despite poorer WUE values, the plants treated with cNF extract had a 20-30% higher yield at full maturity stage (BBCH 89). Therefore it may be supposed that in Nod-treated plants, the water balance is less economical and more water is used for yield production, however, this does not seem to affect these plants in a negative manner, probably due to a better development of the roots [24], which can thus obtain more water from the soil.

The most important change in the yield structure was a significant increase in pod number per plant treated with cNFextract. This resulted in an increase in the number of seeds per plant and in the total yield at green pod stage, and at maturity. Moreover, the mass of 1000 seeds significantly increased, but only in SP and SSSP plants. On the other hand, seed number per pod did not change regardless of the use of Nod factor solution.

Taken together, the application of rhizobial Nod factor extract to pea seeds or pea plants entailed numerous beneficial effects. It accelerated and increased the uniformity of pea plant emergence, resulted in better growth and accumulation of the biomass of vegetative and generative organs of pea, as well as improved pea yield. However, considering some differences in pea responses to the different methods of cNF extract application, this matter requires further investigation.

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