

## Biological activity of Nod factors\*

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**Chemically, the Nod factors (NFs) are lipochitooligosaccharides, produced mainly by bacteria of the *Rhizobium* genus. They are the main signaling molecules involved in the initiation of symbiosis between rhizobia and legume plants. Nod factors affect plant tissues at very low concentrations, even as low as 10–12 mol/L. They induce root hair deformation, cortical cell division, and root nodules' formation in the host plant. At the molecular level, the cytoskeleton is reorganized and expression of genes encoding proteins called nodulins is induced in response to Nod factors in the cell. Action of Nod factors is highly specific because it depends on the structure of a particular Nod factor involved, as well as the plant receptor reacting with it.**

**Key words:** Rhizobium, Nod factor, nod genes, symbiosis

**Received:** 26 April, 2020; **revised:** 29 June, 2020; **accepted:** 29 June, 2020; **available on-line:** 22 October, 2020

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\*Presented at the XLVII Winter School of the Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University "Molecules, Pathways, and Games", February 8–12, 2020, Zakopane, Poland.

**Acknowledgements of Financial Support:** The authors (D.K., K.S., M.K. and A.S.-B.) acknowledge financial support of the National Centre for Research and Development within the Lider VIII programme LIDER/11/0070/L-8/16/NCBR/2017. A.S.-B. would like to acknowledge Foundation for Polish Science within the Reintegration grant (POIR.04.04.00-00-4398/17-00). J.M. acknowledges Ministry of Science and Higher Education in Poland within statutory activity of the Medical University of Lublin (DS47/2020).

**Abbreviations:** ATP, adenosine triphosphate; *hsn nod* genes, host-specific nod genes; IT, infection thread; Lj, *Lotus japonicus*; LRR, leucine rich repeats; LysM, lysin motif; Mt, *Medicago truncatula*; NFs, Nod factors; Ps, *Pisum sativum*; pSym, symbiotic plasmid; RNA, ribonucleic acid; TAR2, tryptophan aminotransferase-related

### INTRODUCTION

The symbiosis between *Rhizobium* and legume plants is established under conditions of limited nitrogen availability in the soil, in a form available for plants. Atmospheric nitrogen fixation by rhizobia occurs only in highly specialized plant organs, the so-called root nodules. Selection of both symbiosis partners is specific, which is possible due to mutual exchange and recognition of molecular signals released into the rhizosphere by potential symbiosis partners: the plant and the bacterium. For example, *Rhizobium leguminosarum* *bv.* *trifolii* can establish symbiosis only with clover (*Trifolium* spp.) (Perret *et al.*, 2000). Exceptionally, the strain *Sinorhizobium* sp. NGR234 is capable of initiating symbiosis with about 112 different types of legumes (Pueppke & Broughton, 1999; Black *et al.*, 2012). Initiation of symbiosis is a mul-

tistage process. Plants secrete specific flavonoid compounds into the soil which act on rhizobia on the basis of positive chemotaxis (Caetano-Anolles *et al.*, 1988; Dharmatilake & Bauer, 1992). Flavonoids attract bacteria to the roots and cause expression of their specific genes, called nodulation genes, that are responsible for synthesis of the Nod factors. Biological activity of the Nod factors boils down to initiation of cell division in the roots of the host plant and formation of an infection thread through which rhizobial cells penetrate into the forming root nodules. Generally, bacterial symbionts provide the plant with nitrogen compounds and the plant provides bacteria with carbon sources.

### SYNTHESIS AND STRUCTURE OF THE NOD FACTORS

The specificity of choosing a plant partner in symbiosis depends on structure of the Nod factors produced by a given specie, and even on the *Rhizobium* strain. Rhizobial *nod* genes are organized into operons which are most often located within the symbiotic plasmid (pSym), or occasionally on the bacterial chromosome forming the so-called symbiotic islands (Sullivan *et al.*, 2002; Turlough, 2002; Nandasena *et al.*, 2007), e.g. in *Mesorhizobium lotii* (Kaneko *et al.*, 2000; Ramsay *et al.*, 2009) or *Bradyrhizobium japonicum* (Kaneko *et al.*, 2002). Specific plant flavonoids act as positive inducers of the *nod* genes' transcription. After binding of the rhizobial NodD protein to a specific flavonoid, there is a change in the spatial structure of the *nod* gene promoter, attachment of RNA polymerase and initiation of the *nod* genes' transcription (Chen *et al.*, 2005).

A common feature of all lipochitooligosaccharides is a backbone made of three to five N-acetylglucosamine residues, which are N-acylated at the non-reducing end (Fig. 1) (Long, 1996; Perret *et al.*, 2000; Spaink, 2000; D'Haeze & Holsters, 2002). In bacteria, the common *nodABC* genes are responsible for synthesis of the lipochitooligosaccharide core. The *nodC* gene encodes chitolipooligosaccharide synthase, called N-acetyl-D-glucosamine transferase, which combines N-acetylglucosamine monomers into a single molecule using  $\beta$ -1,4 glycosidic bonds (Spaink, 2000; D'Haeze & Holsters, 2002). In turn, product of the *nodB* gene is a deacetylase which removes acetyl residues at the non-reducing end of the Nod factor. N-acyltransferase, encoded by the *nodA* gene, attaches a fatty acid at the non-reducing end. Common genes are necessary for the symbiosis process because their mutations result in the Nod<sup>-</sup> phenotype, i.e. the inability of bacteria to produce Nod factors and to establish symbiosis (Jacobs *et al.*, 1985; Debelle *et al.*, 1986). These common genes are

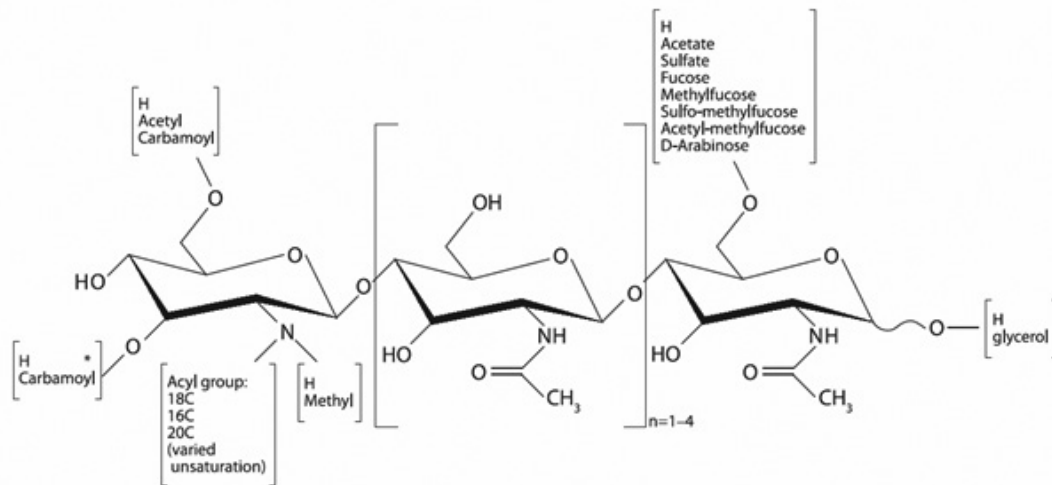


Figure 1. Chemical structure of Nod factors (Long, 1996; Perret *et al.*, 2000; Spaink, 2000; D'Haese *et al.*, 2002).

extremely conserved and found in all rhizobia, except for photosynthetic *Bradyrhizobium* (Giraud *et al.*, 2007). However, the individual Nod factors differ by the presence of various additional chemical groups, for example fucose, sulfate, acetate, etc., for which the host-specific *nod* genes (*hsn nod* genes) are responsible (Mergaert *et al.*, 1997). The *hsn nod* genes also correspond to fatty acid modifications, which, depending on the rhizobium strain, may differ in chain length and saturation level. Examples of enzymes encoded by the *hsn nod* genes are: beta-ketoacyl synthase (*nodE*), O-acetyltransferase (*nodL*), glucosamine synthase (*nodM*), ATP-sulfurylase (*nodP*), sulfotransferase (*nodH*), O-acetyltransferase (*nodX*) (Heidstra & Bisseling, 1996; Downie, 1998). The specific substituents on the chitin skeleton of Nod factors are a kind of key that allows formation of the root nodules and their infection by a proper *Rhizobium* strain (Yates *et al.*, 2011).

Mutations in host specificity genes do not exclude the synthesis of Nod factors, but rather change their structure. This may result in a change in the specificity of the mutated strain relative to the plant host (Lerouge *et al.*, 1990; Schultze *et al.*, 1994; Dénarié *et al.*, 1996).

It has been shown that some legumes and non-legumes can also establish symbiosis with fungi, and this relationship is initiated by fungal lipochitoooligosaccharides called the Myc factors (Maillet *et al.*, 2011). Myc factors have an almost identical structure to the rhizobial Nod factors and differ only in additional substituents. Mycorrhiza is already about 400 million years old, and the symbiosis of legume plants with *Rhizobium* appeared less than 60 million years ago, hence the supposition that the rhizobial *nod* genes originated from the fungal *myc* genes (Sprent & James, 2007; Maillet *et al.*, 2011).

## PERCEPTION OF THE NOD FACTORS

The Nod factors not only act on legumes, but studies on receptors for lipochitoooligosaccharides are conducted mainly on this group of plants. Such plants as *Lotus japonicus* (Lj) and *Medicago truncatula* (Mt) are the best known in this respect (Oldroyd & Downie, 2004). Membrane receptors that bind the Nod factors contain the LysM domain, e.g. LjNFR1 and LjNFR5 (*Lo-*

*tus japonicus*), PsSYM10/PsSYM2A (*Pisum sativum*), and MtNFP/MtLYS3/4 (*Medicago truncatula*) (Limpens *et al.*, 2003; Madsen *et al.*, 2003; Radutoiu *et al.*, 2003; Arrighi *et al.*, 2006; Indrasumuran, 2007; Indrasumuran *et al.*, 2009). The LysM domain is characteristic for chitin binding proteins, and chitin is also the core of the Nod factor structure (Steen *et al.*, 2003). The intracellular receptor domain is a kinase, which in the subsequent phosphorylation cycle transmits the signal initiated by the Nod factor to other proteins, also of the kinase nature. Next, transmembrane proteins (MtDMI2, LjSYM19 or PsSYM19) containing a leucine rich region (LRR – leucine rich repeats) are activated (Endre *et al.*, 2002; Stracke *et al.*, 2002; Mitra *et al.*, 2004; Capoen *et al.*, 2005; Limpens *et al.*, 2005; Indrasumaran, 2007). As a result, calcium channels are opened in the cell membrane. The influx of  $Ca^{2+}$  ions leads to local depolarization of the root hair cell membrane (Felle *et al.*, 1999).  $Ca^{2+}$  ions interact with cytoplasmic proteins (MtDMI3, PsSYM9) (Lévy *et al.*, 2004; Mitra *et al.*, 2004), which are probably transported to the cell nucleus, where they activate the NSP1 and NSP2 transcription factors in *Medicago truncatula* (Kaló *et al.*, 2005; Smit *et al.*, 2005; Hirsh *et al.*, 2009), LjNIN in *Lotus japonicus* (Schauser *et al.*, 1999; Borisov *et al.*, 2003), and PsSYM35 in *Pisum sativum* (Lee & Hirsh, 2006; Ferguson *et al.*, 2010). In this way, transcription of genes encoding the plant early nodulin proteins (ENODs) is initiated.

## THE NOD FACTORS ACTIVATE SOME PLANT GENES

Plant genes induced by the Nod factors are involved in the early stages of symbiosis and the root nodule formation. These genes encode proteins from the nodulin group (Sköt, 2003). The so-called early nodulins include, among others, ENOD12 and ENOD5 proteins, which are present in root hair cells and in the infection zone of the forming nodules (Lee & Hirsh, 2006). In turn, genes called late nodulins are activated after completion of the nodule morphogenesis and are involved in binding of the atmospheric nitrogen by bacteroids. An example of late nodulin is oxygen-binding leghemoglobin, which prevents the  $N_2$  reduction process from stopping. In addition, glutamine synthetase uses  $NH_4^+$ , the  $N_2$  reduction product, for glutamine synthesis. Oth-

er nodulins, such as the sucrose synthase, export photosynthesis products to bacteroids (Sköt, 2003).

### NOD FACTORS INDUCE MITOTIC DIVISIONS IN PLANT TISSUES

Already 15 minutes after perception of the Nod factors, the cell membrane is depolarized and  $\text{Ca}^{2+}$  ions oscillate in epidermal cells (Erhardt *et al.*, 1996; Sieberer *et al.*, 2009; Murray, 2011).

About 1–3 hours later, root hair deformation and curling occurs – a necessary stage when rhizobia enter the root system (Heidstra *et al.*, 1994; Sieberer *et al.*, 2009; Murray, 2011). Nod factors induce the development of an infection thread (IT) in the cortical cells of the root; It grows towards the root bark and rhizobia can infect the nodules through it. Regular oscillations of intracellular  $\text{Ca}^{2+}$  concentrations, streaming of the cytoplasm and changes in the cytoskeleton structure cause formation of the infection thread. Rhizobia are enclosed in a space bound by the wall of deformed root hair. The cell wall surrounding bacteria is partially degraded and the cell membrane is concave; around it, a new cell wall of plant origin is produced and finally the tubular structure of the infection thread is formed (Brewin, 2004). Nod factors cause differentiation of the root cortex cells, which leads to resumption of mitotic divisions. In this way, a nodule primordium is created. Rhizobial cells penetrate plant cells by endocytosis. There, they are surrounded by a peribacteroid membrane forming symbiosomes, inside which bacteria undergo physiological and morphological transformation into a bacteroid form (Brewin, 2004).

In addition, it has been observed in laboratory experiments that purified rhizobial Nod factors, thanks to their mitogenic properties, accelerate seed germination and stimulate growth of the root and shoot biomass of legumes (e.g. soybean) and non-legumes (e.g. corn, cotton) (Souleimanov *et al.*, 2002; Prithiviraj *et al.*, 2003). Rhizobial Nod factors have been also proven to be morphogens in *in vitro* plant tissue experiments. These studies were conducted with such plants as *Daucus carota* (de Jong *et al.*, 1993), *Nicotiana tabacum* (Schmidt *et al.*, 1993), and *Picea abies* (Dyachok *et al.*, 2000). Nod factors had demonstrated biological activity at very low concentrations ( $10^{-9}$ – $10^{-12}$  M).

### NOD FACTORS PLAY A ROLE IN THE ROOT NODULE ORGANOGENESIS

Development of a root nodule requires mitotic activation of the cortical root cells, which leads to the formation of a nodule primordium. We distinguish 2 types of nodules in legume plants – determinate (limited) and indeterminate (unlimited) (Popp & Ott, 2011), which depend on proliferative properties of the plant host cells (Terpolilli *et al.*, 2012; Kondorosi *et al.*, 2013). Determinate nodules develop on the roots of *Phaseolus vulgaris* and *Lotus japonicus*, they do not have active meristem and do not show steady growth. In turn, in the apical region of the indeterminate nodules, there is an active meristem, and therefore the nodules continue to grow and lengthen. Indeterminate nodules occur in temperate zone plants, e.g. *Medicago sativa*, *M. truncatula*, *Vicia sativa* and *Pisum sativum*.

This meristem undergoes mitotic divisions throughout the period of the nodule's functioning, thanks to which newly emerging cells can be constantly infected by rhizobia, and the zones are visible in the cross-section of the wart (Vasse *et al.*, 1990; Timmers *et al.*, 2000, Diouf *et*

*al.*, 2003; Kereszt *et al.*, 2011; Popp & Ott, 2011). These zones differ in the degree of advancement of the symbiosis process and the diversity of bacteroids.

### NOD FACTORS ARE THE MANIPULATORS OF PHYTOHORMONES' METABOLISM

Phytohormones are the main regulators of plant growth. According to many studies, hormone biosynthesis is regulated by the Nod factors (Buhian & Bensmihen, 2018). It is known that several auxin signalling genes are activated in plants after a 24 h Nod factor treatment (Breakspear *et al.*, 2014). In *M. truncatula* genome, many auxin-regulated genes are stimulated or antagonized by Nod factors (Herrbach *et al.*, 2017). Generally, Nod factors can manipulate plant hormone levels. There are many synergistic effects of Nod factors and auxins on the transcription process of numerous hormone biosynthesis genes, for example an auxin biosynthesis tryptophan aminotransferase-related (TAR2) homolog (Herrbach *et al.*, 2017).

Nod factor signalling also influences auxin transport. Application of specific Nod factors can modify auxin gradients, which was measured by the GH3:GUS reporter gene in white clover (Mathesius *et al.*, 1998). In the case of *M. truncatula* plants, Nod factor application or infection by *S. meliloti* cells inhibited auxin transport from root to shoot in the first 24 h and this observation coincided with the very earliest cortical cell divisions (Ng *et al.*, 2015). It was suggested that the Nod factors caused inhibition of acropetal auxin transport and probably this is unique to the process of indeterminate nodule organogenesis (Ng & Mathesius, 2018).

Gibberellins were also found to play a role in controlling the early steps of symbiosis. It was shown that after 24 h Nod factor treatment of *M. truncatula* root hairs, both metabolic and biosynthetic genes of gibberellins were induced (Breakspear *et al.*, 2014; Jardinaud *et al.*, 2016). In addition, Nod factors caused activation of gibberellin biosynthesis in soybean roots (Hayashi *et al.*, 2012).

It has been repeatedly demonstrated that cytokinins play a role during the process of nodule organogenesis and infection thread formation (Jardinaud *et al.*, 2016). Accumulation of bioactive cytokinins was also shown in the root of *M. truncatula* after 3 h Nod factor treatment, and this was dependent on the Nod factor signalling gene MtDMI3 (van Zeijl *et al.*, 2015). Several genes from the trans-zeatin metabolic network were modulated in root hairs after a 24 h Nod factor treatment, but bioactive cytokinins' level has not occurred in root hairs after Nod factor application (Jardinaud *et al.*, 2016).

Scientists highlight the regulatory role of ethylene in the early symbiotic steps (Oldroyd *et al.*, 2001; Penmetsa *et al.*, 2008). Nod factors induced transcription of several ethylene biosynthetic genes, for example MtACS1 and MtACS2 in *M. truncatula* (van Zeijl *et al.*, 2015). Also, MtACS3 was synergistically regulated by a 10 h treatment with a combination of Nod factors and auxins (Herrbach *et al.*, 2017). In contrast, NF-dependent negative regulation of ethylene biosynthesis was observed in soybean roots (Hayashi *et al.*, 2012). All of these data show that Nod factors induce synthesis of ethylene which is a negative regulator of rhizobial infection.

### NOD FACTORS IN AGRICULTURAL PRACTICE

Inoculation of legume seeds using rhizobia is one of the oldest agrobiotechnological methods (Lindström *et*

al., 2010). Inoculant bacteria are usually selected from the soil population of a given agricultural region, which is due to the specificity of the symbiosis between rhizobia and legumes.

Traditional rhizobial vaccines containing live rhizobial cells have a beneficial agrotechnical effect only when local rhizobial populations are few or nonexistent due to the lack of a compatible plant host and the vaccine strain does not face strong competition (Streeter, 1994; Skorupska *et al.*, 2010). Legume plants have been cultivated for a long time, and their wild relatives also grow outside agricultural areas, which results in a high number of *Rhizobium* strains in soils. In this case, it is preferable to use isolated, purified Nod factors in the crop. Applying Nod factors directly to seeds increases the number of root nodules, which are an additional habitat for indigenous rhizobia, and accelerates germination and plant growth (Prithiviraj *et al.*, 2003; Macchiavelli & Belles-Marino, 2004; Kidaj *et al.*, 2011; Podlešny *et al.*, 2014; Siczek *et al.*, 2014). This leads to an increase in the amount of bound nitrogen even at low symbiotic yields of individual strains in the soil population.

There is a clear need to select potential vaccine strains characterized by both, high atmospheric nitrogen binding activity and competitiveness against indigenous populations (Brockwell *et al.*, 1995), but also high efficiency in production of the Nod factors (Skorupska *et al.*, 2010).

## CONCLUSIONS

Many years of research into initiation of the Rhizobium – legume plants symbiosis have shown that the symbiosis performance can be stimulated and improved by manipulation of bacterial (Nod factor) and plant (flavonoids) molecular signals. Application of the isolated Nod factors on seeds helps to bypass the problem of competition between strains used in microbial vaccines and indigenous strains. The phenomenon of competition observed inside the soil population of different *Rhizobium* strains is very complex and not all factors affecting it have been already explained. Therefore, it seems reasonable to search for and select *Rhizobium* strains not only highly effective in the process of nitrogen binding, but also in production of the Nod factors. It should be remembered that Nod factors are strong stimulators of mitotic division, also acting in a universal manner on meristems of non-legume plants. Acceleration of seed germination, seedling growth and expansion of the root system due to application of the isolated Nod factors is a rescue approach for agriculture in the face of drought periods that recur every year. Therefore there is a need to test preparations containing Nod factors on numerous non-legume crops.

## Conflict of interest statement

The authors declare no conflict of interest.

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