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Study of the Inducer Effect of Molasses and Soybean Cake on Synthesis and Excretion of Nodulation Factors in Different Strains of *Bradyrhizobium japonicum*

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ABSTRACT. It is known that the synthesis of nodulation factors by the bacteria within the genera *Rhizobium* is induced by different compounds, mainly of flavonoid nature exudated by the legume plants. The capacity of different compounds to act as inducers of *nod* genes on three *Bradyrhizobium japonicum* strains was studied in this paper and this effect was compared using two concentrations of the inducer. Induced Nod factors were observed among the strains exposed to the inducers. The profiles and amount of induced Nod factors depend on the type and concentration of the inducer, and the strains.
Key words: Nodulation factors, *Bradyrhizobium japonicum*, nodulation factors, inducer.

RESUMEN. Se conoce que la síntesis de los factores de nodulación por bacterias del género *Rhizobium* se induce por diferentes compuestos de naturaleza flavonoide que son exudados por la planta leguminosa. En este trabajo se estudia la capacidad de diferentes compuestos de actuar como inductores de los genes de nodulación en tres cepas de *Bradyrhizobium japonicum* y se compara el efecto cuando se emplean dos concentraciones del inductor. Se presentan los perfiles de factores Nod sintetizados por las diferentes cepas ante cada inductor. El número de estructuras diferentes sintetizadas y su concentración dependen del tipo de inductor, de su concentración y de la cepa empleada.
Palabras claves: Factores de nodulación, *Bradyrhizobium japonicum*, inductor.

INTRODUCTION

The symbiosis established among *Rhizobium* and legume plants requires a complex interchange of molecular signals between both symbionts. In this way compounds like flavonoids produced by the plants induce the synthesis and excretion of lipochitinoligosaccharides or Nod factors as signals that activate the nodule organogenesis on roots of legume plants.

All the Nod Factors are known as substituted chitin oligomers, usually consisted of four to five *N*-acetylglucosamine (GlcNAc) residues, mono-*N*-acylated at both the reducing and non-reducing terminal GlcNAc residues. Each rhizobial species produces a variety of Nod factors with specific substitutions.[2]

The chemical specificity of Nod signal action has been extensively studied.[3] Recently, Stacey et. al. shown the need of a mixture of these Nod signals to elicit the nodule formation.[15]

The nature of the compounds, which induce the activation of nod genes in *Rhizobium*, depends on the specie itself. It is stated that the most potent inducers for *nod* genes of *Bradyrhizobium japonicum* are the genistein, daidzein and isoliquiritigenin.[14]

The presence of a specific inducer at right concentration could induce the *nod* gene transcription and therefore increase nodulation, facilitate N_2 fixation and increase crop yields.[4]

It has been shown that some cultivars seem to supply limited quantities of flavonoids,[7] so the inclusion of these inducers on the culture medium could be an important alternative in the production of high quality biofertilizers.

Zhang and Smith found in 1995 that preincubation of *B. japonicum* with genistein increased the amount of nodules and the nitrogen fixation level. Concentration of the isoflavonoid is increased by the early development of the root and decreases after the fixation process has begun.[6]

Previous results using soybean cake and molasses as medium ingredients for *B. japonicum* ICA 8001,[12] show a clear induction on *in vitro* soybean nodulation. In this work, the effect of these compounds on Nod signals production in different *B. japonicum* strains was studied.

MATERIALS AND METHODS

Strains. *B. japonicum* strains used in this work were: ICA 8001 (from the Institute of Animal Science, Cuba), USDA 136 (USDA, Belstville, MD) and SEMIA 5019 (Brazil). All strains were grown on a yeast extract mannitol broth (YEM) according to Vincent, 1970 at a temperature of 28 °C and in orbital shaking conditions.

Induction of *nod* genes. Three compounds including genistein (Sigma), molasses (byproduct from Sugar Mill) and defatted soybean cake (Oil enterprise) were used as inducers.

Two concentrations of the inducers were used to compare the Nod profiles production.

The nodulation factors were radioactively labeled and they were isolated by following a slightly modified protocol of Laeremans *et. al*., 1998.[10] 100 µL from *Bradyrhizobium* cultures, growth for two nights, were inoculated in 900 µL of YEM medium containing the inducer and the concentration was adjusted to $5x10^8$ CFU per milliliter. They were pre-incubated at 30 °C with agitation for 1 h. Each sample was supplemented with genistein 10 µM as inducer and incubated for 2 h at the same temperature with agitation. After the induction the isotopic label was carried out by adding 125 µl of [$2-^{14}C$] sodium acetate. The cultures were labeled for 36 h. The nodulation factors were extracted twice with 500 µl n-butanol and washed with ethyl acetate. The solution was vacuum-dried and samples were applied on reverse-phase TLC plates (RP-18 F_{254}, Merck). H_2O/acetonitrile (1:1, vol./vol.) was used as the mobile phase. The induced Nod factor patterns were visualized by autoradiography using Hyperfilm-βmax (Amershan Life Sciences) after 4 days of exposure.

RESULTS AND DISCUSSION

Study of the inducer effect of the different compounds. The qualitative analysis of the synthesized Nod factors shows a very different performance for each strain against the different inducers (Fig. 1).

ICA 8001 outstands in the induction with molasses and soybean cake with a production of at least three well defined bands structures and in lipooligosaccharide concentrations. In the induction with genistein the highest production was observed in SEMIA 5019 strain.

In spite of the high inducing capability of the genistein on the *B. japonicum* species, the concentrations here studied resulted to be less powerful than the other studied compounds. Therefore we think that in the studied compounds can act more than one inducer, maybe another flavonoids or maybe another inducer's family, not exactly flavonoids.

SEMIA 5019 strain produced two bands against to genistein, three to molasses and four to soybean cake. In all cases the two bands were induced by genistein, and new ones appear, but the main spot which is repeated in all profiles is mainly observed in genistein.

Table 1. Chemical composition of soybean cake and molasses (%).

Soybean cake. Isolation of water unextractable solids[6]		Molasses[1]	
Proteins	57.3	Water	15.9
Starch	1.0	Sucrose	35.5
Non-starch polysaccharide	15.4	Glucose	9.9
Acetic acid groups	1.1	Fructose	10.04
Methanol groups	0.3	Inferment-able reducers	4.01
Fructose	0.6	Ashes	9.5
Sucrose	5.4	Gums and colloids	8.06
Raffinose	0.8	Waxes	0.1-1
Stachyose	4.9	Proteins	0.5-4.5
		Aminoacids	0.3-0.5
		Vitamins and other organic acids	Variable

The strain USDA 136 poorly shows induction with genistein, two bands appear with molasses and the same two with soybean cake. The performance of this strain was very similar in these two latter inducers either qualitatively or quantitatively, just slightly superior in soybean cake.

The strain ICA 8001 showed to be the most strongly induced strain although it did not show a clear induction to genistein. Molasses inducted at least three well-defined structures and in a higher concentration and soybean cake inducted the synthesis of at least four spots, also in a higher concentration. This suggests a relation host-strain genome on specificity of *nod* gene inducing.[9]

In all cases it can be seen the presence of a main spot that aims to be the most important of the Nod factors synthesized by this species.

These results also explain previously founded results using these compounds as culture medium components for *B. japonicum* ICA 8001, where a higher amount of nodules were found in soybean cake and molasses what at that time was associated to an inducer *nod* effect.[12] But, who is the

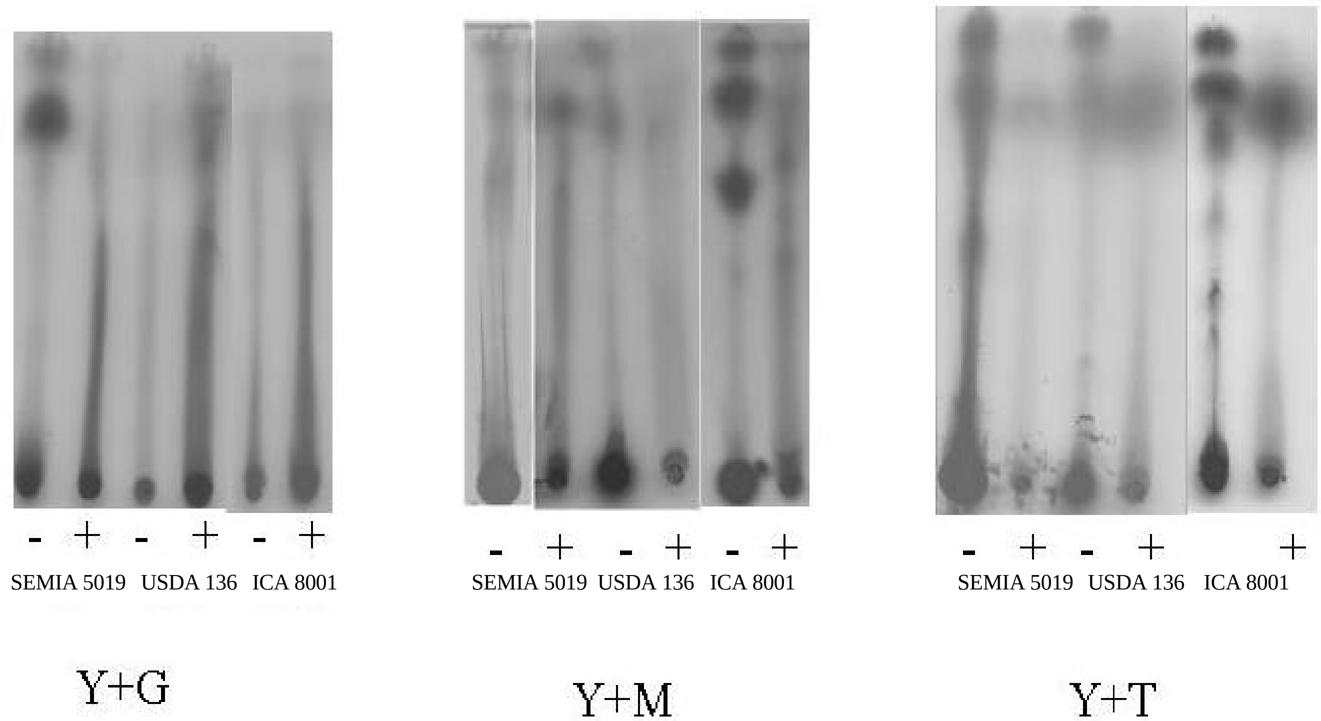

Fig. 1. Induced Nod factor profiles synthesized by different *B. japonicum* strains responding to different compounds. -, non-induced extracts; +, genistein induced extracts; G, genistein; M, molasses; T, soybean cake.

responsible of the inducer effect in these compounds? It is not clear yet. We can think in flavonoids considering that daidzein and genistein are in outstanding biologically concentrations in soybean's seeds and roots.[9] The soybean aqueous extract contains several compounds, some of them could reveal like an inducer. Molasses contain monomeric phenols,[11] which constitute weak *nod* inducers in *B. japonicum*.[8] It contains besides another free acids, then, it is not wrong to think in the presence of aldonic acids, which have recently discovered like a new family of inducer.[5] Therefore, could be possible to think about one or several components as the responsible of such induction activity. It is necessary to carry out a deepest study to determine exactly what elements in soybean cake and molasses are the inducers.

Regularly, the highest amount of spots appeared in the non-induced extracts, what also agrees with that reported by María C. Nápoles *et. al.*, 1999 according to a possible effect of repression due to superinduction.

Comparative study of two concentration of each compound on their capability as inducer. The performance of the studied strains on two concentrations of genistein and soybean cake is shown in Fig. 2. The profiles induced by molasses are not shown, although they are discussed.

The strain SEMIA 5019 shows a similar performance on both genistein concentrations with the same two synthesized structures and in a very similar concentration. It also produced four structures on the two concentration of soybean cake and the performance on molasses (non-showed results) was maintained with the production of the same three structures found for the 10 g/l concentration.

In the case of the USDA 136 strain in which an induction with genistein 10 µM did not appeared it is shown the synthesis of at least two well defined structures when the 5 µM concentration is used. Perhaps for this strain 10 µM constitute an excessive higher concentration, so there is the need to study the effect of lower concentrations. Zhang and Smith found that the addition of 5 µM of genistein was enough to increase the amount of nodules and nitrogen fixation in *B. japonicum* USDA 110.[19] The performance of this strain was similar on the two concentrations of soybean cake, with the clear synthesis of two structures and the slight presence of a third one but the spots had less intensity when the cake lower concentration was used.

A very different response was found when the strain was grown with the different concentrations of molasses. Only two bands were found at the concentration of 10 µM while three Nod factors in outstanding levels and with a very different position to the former structures found.

Strain ICA 8001 produced three light structures when 5µM of genistein was used those which did not appear on the 10 µM concentration.

The performance was similar on soybean cake for both concentrations, but the four spots presents in 10 µM appear in 5 µM in less concentration. Similar situation was

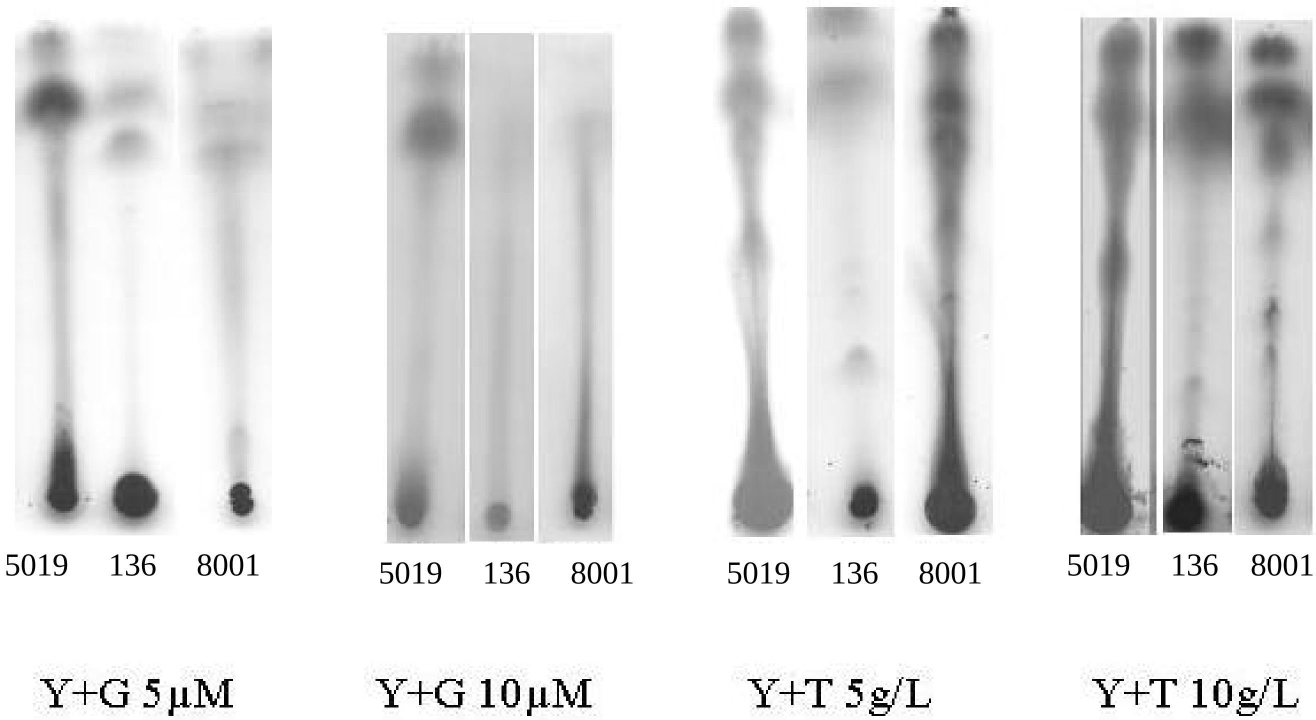

Fig. 2. Nod factor profiles of *Bradyrhizobium* strains induced by genistein and soybean cake at two concentrations. All of them correspond to non-induced extracts. G, genistein; T, soybean cake.

founded when this strain was grown at both concentrations of molasses.

The synthesis of three well-defined bands are maintained but favored by the highest concentration.

This study shows that the Nod factors profile produced and excreted by *Bradyrhizobium* depends on the type of inducer, its concentration and the strain itself. The inducer capacity of molasses and soybean cake is remarked for all studied strains. According to the inducer concentration it is recommended to take into account the strain to be induced.

This study support the use of molasses and soybean cake as *Bradyrhizobium* culture medium ingredients due to its induction capacity on *nod* genes and maybe it constitute the beginning in finding new inducers to *Bradyrhizobium*.

REFERENCES

1. Biart, J. R., P. Serrano and J. Conde. 1982. Study of sugar cane final molasses. Editorial Científico-Técnica. C. Habana. Cuba.
2. Carlson, R. W., N. P. J. Price, and G. Stacey. 1994. The biosynthesis of Rhizobial lipo-oligosaccharide nodulation signal molecules. MPMI 17:684-695.
3. Cohn, J. R., B. Day and G. Stacey. 1997. Legume nodule organogenesis. Trends Plant Sci. 3:105-110.
4. Dakora, F. D. 1995. Plant flavonoids: Biological molecules for useful exploitation. Australian J. Plant Physiol. 22:87-99.
5. Gagnon, H., and R. K. Ibrahim. Aldonic Acids: A Novel Family of *nod* Gene Inducers of *Mesorhizobium loti, Rhizobium lupini,* and *Sinorhizobium meliloti*. 1998. Mol. Plant Microbe Interaction 11:988-998.
6. Huisman, M. M. H., H. A. Schols, and A. G. J. Voragen. 1998. Cell wall polysaccharides from soybean (Glycine max.) meal. Isolation and characterization. Carbohydr Polymers 37:87-95.
7. Hungria, M. and D. A. Phillips. 1993. Effects of a seed color mutation on rhizobial nod-gene inducing flavonoids and nodulation in common bean. Mol. Plant Microbe Interaction 6:418-422.
8. Kape, R., M. Parniske, and D. Werner. 1991. Chemotaxis and *nod* gene activity of *B. japonicum* response to hidroxicynnamic acids and isoflavonoids. Appl. Environ. Microbiol. 57:316-319.
9. Kosslak, R. M., R. Bookland, J. Barkei, H. Paaren, and E. R. Appelbaum. 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from Glycine max. Proc. Natl. Acad. Sci. USA 84:7428-7432.
10. Laeremans, T., N. Coolsaet, C. Verreth, C. Snoeck, N. Hellings, J. Vanderleyden, and E. Martínez Romero. 1998. Functional redundancy of genes for sulphate activation enzymes in *Rhizobium* sp. BR 816. Microbiology 143:3933-3942.
11. Manual of Sugar Cane by-products. 1998. ICIDCA-

GEPLACEA-PNUD. La Habana. Cuba.
12. Nápoles, M. C., A. Gutiérrez, and M. Varela. 1998. Behaviour of a *Bradyrhizobium japonicum* strain in new culture media which contain inducers of the nodulation factors synthesis. Cultivos Tropicales 19: 25-27.
13. Nápoles, M. C., A. Gutiérrez, T. Laeremans and J. Vanderleyden. 1999. The analysis of nodulation factors as a tool in the design of new culture media for *Bradyrhizobium japonicum*. Cultivos Tropicales 20: 79-81.
14. Rao, J. R., and J. E. Cooper. 1995. Degradation and modification of nod gene-inducing flavonoids by rhizobia. *In* Nitrogen Fixation: Fundamentals and Applications. P. 325. Tikhonovich, I., Provorov, N. A., Romanov, V. and Newton, W. E., Eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
15. Stacey, G. *et. al*. 1999. Biology of Plant-Microbe Interactions. Vol. 2. Proceedings of the 9th International Congress on Molecular Plant-Microbe Interactions. Amsterdam, The Netherlands, July 25-30.
16. Vincent, J. M. 1970. A Manual for the practical study of root-nodule bacteria. *In* International Biological Programme Handbook. No. 15. Blackweel Scientific publications, Oxford, England.
17. Zhang, F. and D. L. Smith. 1995. Preincubation of *Bradyrhizobium japonicum* with genistein accelerates nodule development of soybean at suboptimal root zone temperatures. Plant Physiol. 108:961-968.
18. Zhang, F., and D. L. Smith. 1996. Genistein accumulation in soybean [Glycine max (L.) Merr.] root systems under suboptimal root zone temperature. J. Exp. Bot. 47:785-792.
19. Zhang, F., and D. L. Smith. 1998. Genistein addition to the rooting Medium of Soybean at the Onset of Nitrogen Fixation Increases Nodulation. J. Plant Nutrition 21:1631-1639.