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INDUCTION OF ROOT HAIR AND NODULE PRIMORDIA ON SOYBEAN AND VIGNA BY THE NOD SIGNALS LCOS EXTRACTED FROM *Bradyrhizobium japonicum*

BY

Selim, Sh. M.
Unit of Biofertilizers, Microbiology Department, Faculty of Agriculture, Ain – Shams University, P.O. Box 68, Hadayek shoubra 11241, Cairo, Egypt.

ABSTRACT

Root exudates of soybean and vigna seedlings grown for 14 days in water culture with N free nutrient solution were extracted and concentrated. A 25 µl of either soybean or vigna root exudates were added into shaked culture of *Bradyrhizobium japonicum* USDA 110 and the lipo chitin oligosaccharide Nod signal was extracted and purified with High- performance Liquid chromatography. Un–induced *Bradyrhizobium japonicum* or 10 µl of either of the two purified LCOs, obtained from root exudates induced *Bradyrhizobium japonicum* culture, was inoculated into water culture of soybean and vigna seedlings. The purified LCOs produced by induced *B. japonicum* due to treatment with soybean or vigna root exudates were shown to induce a little difference in the number of bands and peaks profiles in thin – layer – chromatography (TLC) and HPLC analyses, respectively. The purified LCOs also induced root hair deformation and nodule primordia on soybean and vigna seedling roots.

Key words: *Bradyrhizobium japonicum*, Soybean, Vigna, Root exudates, Nod signal, Root hair deformation, Nodule primordia .

INTRODUCTION

The symbiotic relationship between legumes and root nodule bacteria i.e. *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Azorhizobium* is exemplified in the formation of nitrogen – fixing root nodules. The development of legume root nodules is largely controlled by reciprocal signal exchange between the macro- and microsymbiont. Legume roots are known to secrete flavonoids, which induce expression of nodulation genes of rhizobia (Schultze *et al.*, 1994; Selim, 2000 and Broughton *et al.*, 2003). Many of these genes are involved in the synthesis and secretion of lipo- chitin- oligosaccharides (LCOs) (Schultze *et al.*, 1994 and Song & Lin, 1999). The LCOs molecules have been characterized from several *Rhizobium* species and described to date as oligomers which are closest of three to five N- acetylglucosamine residues with an amide linked fatty acyl moiety on the non reducing terminal residue (Carlson *et al.*, 1994 and Broughton *et al.*, 2003) . Specific modifications of the LCOs produced by different rhizobia have

been shown in several cases to determine host specificity (Denarie *et al.*, 1994 and Spaink *et al.*, 1994).

Purified LCOs have been shown to induce several responses in plant root which are also developed by rhizobial infection. The responses include root hair deformation, root hair curling, formation of pre-infection threads and nodule primordial (Van Brussel *et al.*, 1992; Stokkermans *et al.*, 1995 and Nasr & Selim, 1997).

The host range of root nodule bacteria can vary from a restricted (*R. leguminosarum* bv. *viceae*, *R. leguminosarum*. bv. *trifolii*, *R. leguminosarum*. bv *phaseoli* and *R. meliloti*) to a broad host range (*Bradyrhizobium japonicum*). Nodulation studies showed that soybean, vigna and peanut plants are nodulated by *Bradyrhizobium japonicum* strains but not by most other *Rhizobium* strains. *Bradyrhizobium japonicum* is also known to produce a large diversity of specific lipo-chitin – oligosaccharides (LCOs) which are necessary for root infection and nodule formation.(Denarie *et al.*, 1994)..

The aims of this study was to study the effect of extracted and purified LSOs obtained from root exudates induced *Bradyrhizobium japonicum* on root hairs responses in soybean and vigna roots.

MATERIAL AND METHODS

Bacterial strain

Bradyrhizobium japonicum USDA 110 was provided from the Unit of Biofertilizers, Fac. Agric., Ain – Shams Univ., Cairo, Egypt to be used in this study.

Seeds

Seeds of soybean (*Glycine max* L.) and vigna (*Vigna sinensis*) were kindly supplied by the Dept. of Legume Research, ARC, Giza, Egypt

Collection of root exudates

Seeds of soybean and vigna, selected for uniformity in size and viability (92%), were surface –sterilized with ethanol (95%) and Hg Cl_2 (0.1%) for 30 min and then washed several times with sterile distilled water (Gyorgpal *et al.*, 1991). Surface sterilized seeds were germinated on moist sterile filter paper for 3 days at 28°C. Seedlings with root lengths of about 1.5–2.5 cm were transferred into tubes (4× 25 cm) contained sterilized N-free Hoagland nutrient solution (Hoagland and Arnon, 1938) at a rate of 25 ml / tube (Nasr and Selim, 1997). One seedling was placed aseptically in each tube. The tubes were covered with dark paper to protect the root system from light and kept in growth chamber for 14 days. The nutrient solutions of ten soybean or vigna seedlings were collected, filtrated and then mixed with 15 ml of isobutanol and left overnight on a rotary shaker (100 rpm) at 28 ± 2°C. The last step was repeated twice using 15ml of isobutanol for 5 min (Selim, 2000). The alcoholic layer was collected, evaporated and the residue was resuspended in 250 µl of isobutanol.

Extraction of Nod metabolites (LCOs)

Activated *Bradyrhizobium japonicum* USDA 110 was inoculated into B-liquid medium (Spaink *et al.*, 1989), supplemented with 25 µl of root exudates of either soybean or vigna seedling and then shaked at 28°C until an absorbance of 0.6-0.8 was obtained at 620 nm. The culture was shaken overnight together with 40% n-butanol. The butanol layer was collected, dried and the residue was resuspended in 60% acetonitrile in water. The mixture was then purified by HPLC waters (Millipore) 510 conneted with waters 486 tunable a µ Bondapak C-18 column (3.9 × 30° nm), using the following gradient system: 5 min isocratic elution with 20 % acetonitrile, 30 min isocratic elution with 30 % acetonitrile, 30 min isocratic elution with 40 % acetonitrile, 15 m in isocratic elution with 60 % acetonitrile, and 10 min from 60 to 100% acetonitrile. The HPLC elution was performed at flow rate of 0.7 ml / min., and the eluens were monitored at 206 nm. The purified LCOs obtained from induced *Bradyrhizobium japonicum* USDA 110 i.e., treated with the soybean or vigna root exudates were analyzed by High-performance Liquid chromatography (HPLC) according to Hartwing *et al.* (1990). A 15 µl of the same materials were spotted on TLC plastic sheets of Silica Gel 60 with F_{254} (Merk) with a separation solvent consisted of Toluen: diethyl formate: formic acid at a ratio of 5:4:1 TLC plates were exposed to X-ray and developed bands were photographed as shown by (Spaink *et al.*, 1992)

Plants Culture

Developed soybean and vigna seedlings were treated with 10 µl of purified LCOs, obtained from *Bradyrhizobium japonicum* cells grown in the presence of soybean or vigna root exudates or 1 ml of *Bradyrhizobium japonicum* culture. Ten replicates were made for each treatment .After 2–3 days root were stained with methylene blue as described by Truchet *et al.* (1991) and microscopically examined. Changes in root hair morphology were photographed by Canon power Shot A60 digital camera.

RESULTS AND DISCUSSION

Production of LCOS by *B. japonicum*

The production of LCOs was assayed using thin-layer chromatography (TLC) analysis of n-butanol extracts of *Bradyrhizobium japonicum* grown in the presence of soybean or vigna root exudates as a nod D genes inducer.

In the absence of root exudates treatment no nod metabolites was detected (Fig 1 lane A), but and the reverse was true in the presence of soybean or vigna roots exudates (Fig. 1 lanes B & C) where several inducible spots were developed on silica TLC plate, based on their migration behavior. There was a littler difference between the number of spots produced by *B. japonicum* treated with root exudates of soybean and those treated with the root exudates of vigna .

Fig (2) shows the elution profile of purified nod-metabolites (LCOs) obtained from *B. japonicum* untreated or treated with root exudates obtained from soybean and vigna. There were considerable differences in the profiles of LCOs peaks obtained from untreated *B. japonicum* culture (Fig. 2C) and that treated

with root exudates of the two tested hosts (Fig 2 A&B). On the other hand, a little difference in peak numbers and profiles of HLPC analysis of LCOs obtained from *B. japonicum* induced by soybean root exudates compared with that induced by vigna root exudates. The above – mentioned results of TLC and HPLC analyses conclude that *Bradyrhizobium japonicum* can produce a diverse of lipo-chitin oligosaccharides (LCOs) when induced by root exudates of specific hosts (Spaink *et al.*, 1992, Lopez-Lara, *et al.*, 1995; Luis *et al.*, 1998 and Frederic *et al.*, 1999).

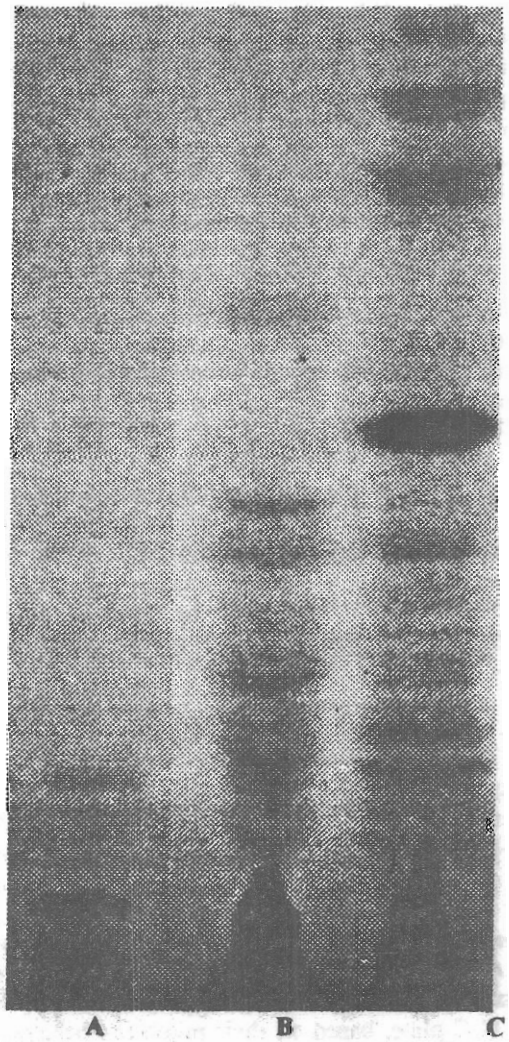

Fig. (1): Thin – layer chromatography (TLC) analysis of nod signal produced by *B. japonicum*, (A) n-butanol extracts of cells grown without induction (control), (B) n-butanol extracts of cells grown in the presence of soybean root exudates and (C) n-butanol extracts of cells grown in the presence of vigna root exudates.

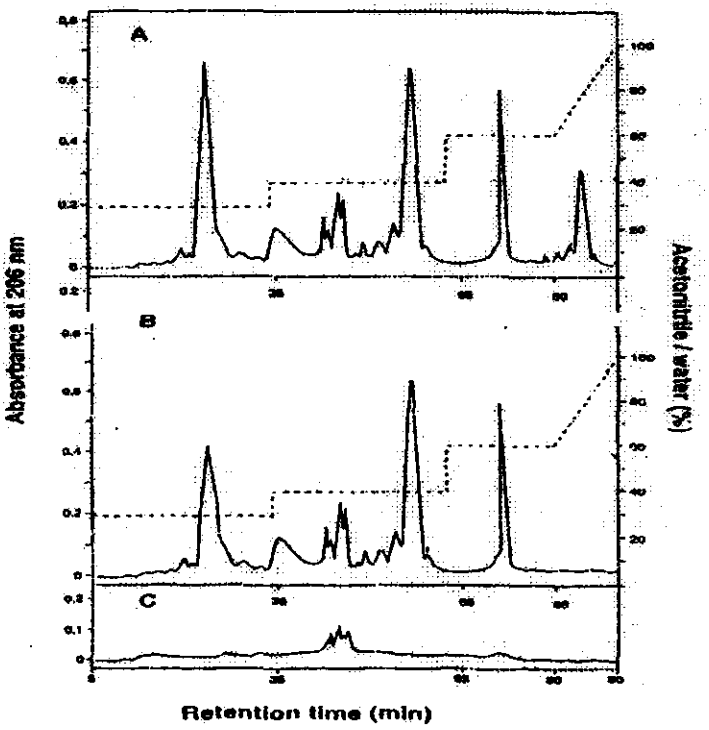

Fig. (2): High – performance liquid chromatography (HPLC) profiles of n-butanol (LCO_s) extracts obtained from *Bradyrhizobium japonicum* grown in
A. The presence soybean root exudates .
B. The presence Vigna root exudates.
C. Uninduced culture

Biological activity of the LCOs

The biological activity of purified LCOs was tested by the response of root hair morphology. Microscopic examination the root systems of soybean and vigna seedlings grown for 2-3 days in the presence of *B. japonicum* (as a positive control), n- butanol (as a negative control) or purified LCOs (obtained from active culture of *Bradyrhizobium japonicum* treated with the root exudates of soybean or vigna seedling). The results showed that *B. japanicum* and LCOs elicit abundant root hair deformation in the 2 tested plants (Fig 3 G, H) which is one of the earliest responses of legumes plant to compatible rhizobial infection, and for their ability to induce the symbiosis (Stokkermans *et al.*, 1995).

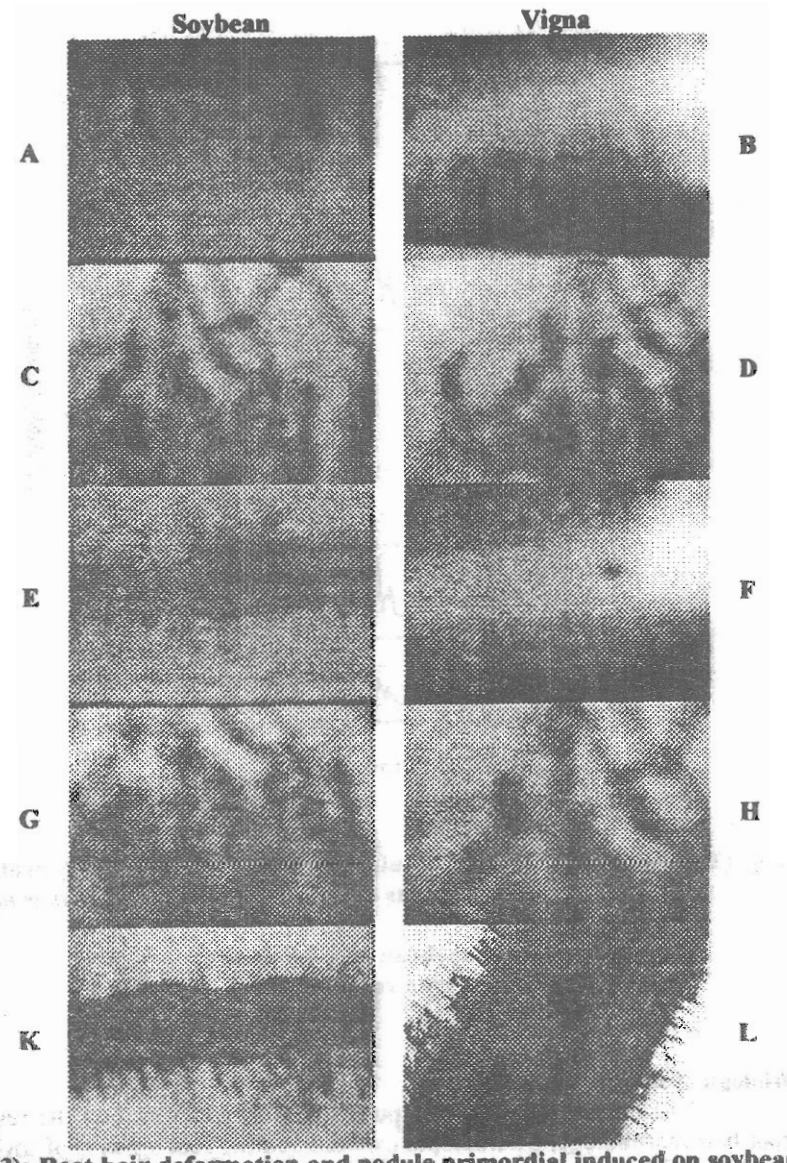

Fig (3): Root hair deformation and nodule primordial induced on soybean or vigna plants: Panels: A, B, untreated plants (control). Panels: C, D, E, F: treated with *Bradyrhizobium japonicum*. Panels: G, K; Treated with purified LCOs extracted from *B. japonicum* grown with soybean root exudates. H,L: treated with purified LCOs extracted from *B. japonicum* grown with vigna root exudates. Panels: C, D, G, H: Root hair deformation. Panels: E,F,K,L: nodule primordial

The purified LCOs was sufficient to induce not only root hair deformation but also nodule primordia Fig. (3 K, L). Thus it seems that the signal molecule induced cell division by changing the phytohormone balance in the root (Franssen *et al.*, 1992). Other studies in the same sequence, also showed that, the zeatin gene is involved in cytokinin synthesis (Long and cooper, 1988), and compounds which are supposed to block the polar transport of auxin, namely naphtylphthalamic acid (NPA) and Triiodobenzoic acid (TIBA) could induce the formation of nodule-like structures on legume root (Franssen *et al.*, 1992).

Conclusively, the LCOs produced from *B. japonicum* treated with the root exudates of soybean or vigna appeared to be able to induce root hair deformation and nodule primordia on a specific host. The interaction of Nod D with flavonoids differs not only between species or strains but also within a single strain among individual Nod D alleles. Two nod D copies were found in *B. japonicum* (Appelbaum *et al.*, 1988), Perhaps, the soybean root exudates activate the first nod D and vinga root exudates activate the second nod D copy.

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تحفيز إنحناء الشعيرات الجذرية وتكوين بيريموردیا العقده فی نباتی فول الصويا واللوبيا بواسطة إشارات الـ Nod ليبوشيتين أوليجو سكريدات (LCOs) المستخلصة من البرادی ريزوبيام جابونكم.

شوقي محمود أحمد المرسي سليم

وحدة التسميد الحيوي – قسم الميكروبيولوجيا –كلية الزراعة – جامعة عين شمس
ص.ب. ٦٨ حدائق شبرا رقم بريدي ١١٢٤١ القاهرة

تم فی هذا البحث استخلاص وتركيز إفرازات جذور بادرات نباتی فول الصويا واللوبيا النامية فی محلول مغذی خالي من النيتروجين لمدة ١٤ يوما ثم أضيفت إفرازات الجذور الخاصة بكل عائل على حده بمعدل ٢٥ ميكروليتر للمزرعة المهتزة للسلالة USDA 110 من البرادی ريزوبيام جابونكم وتم استخلص منها الليبوشيتين أو ليجو سكريدات (LCOs) حيث تم تنقيته بواسطة جهاز High- Performance Liquid chromatography (HPLC) و لوحظ ان LCOs المنقى المتحصل عليه من البرادی ريزوبيام جابونكم المعاملة بافرازات جذور فول الصويا واللوبيا اختلافا بسيطا فی عدد الحزم والمنحنيات المتحصل عليها عند التحليل بواسطة TLC, HPLC على التوالی وعند معاملة بادرات فول الصويا واللوبيا بواسطة خلايا البرادی ريزوبيام جابونكم الغير ملقحة أو (LCOs) المنقاه و المتحصل عليها من السلالة المذكورة المستنبته سواء بافرزات جذور فول الصويا اواللوبيا لوحظ أنها قادرة على تحفيز انحناء الشعيرات الجذرية وتكوين بيريموردیا العقده الجذرية فی بادرات النباتين تحت الدراسة.