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Growth Media Modulating the Symbiotic Efficiency of *Bradyrhizobium elkanii*

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Abstract

The biosynthesis of Nod factors is specific to rhizobial species and is induced by appropriate signals, mostly flavonoids, excreted by the host plant. Nod factors from numerous different strains have been characterized and the molecular and biochemical mechanisms of Nod factor production have been elucidated in detail. Here, we report the influence of different culture media on Nod factor biosynthesis and symbiotic efficiency of two strains of *Bradyrhizobium elkanii*. The different growth media were validated by using them to grow the inoculants for soybean on two different field sites in Habana Province, Cuba. The effect on nodulation parameters and soybean yield was evaluated. Depending on the culture medium used to grow the inoculant, the number of nodules obtained per plant and the corresponding nitrogen fixation activity differed. Consequently, the results under field conditions showed different soybean yield, depending on the media used. In addition, thin-layer chromatography analysis revealed that different mixtures of Nod factors were produced when different media were used.

Keywords: Molasse, soybean cake, genistein, inoculant

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1. Introduction

Soil bacteria, belonging to the family of Rhizobiaceae, have the unique ability to induce nitrogen-fixing nodules on the roots or stems of leguminous plants. The process of nodule formation involves an intensive exchange of signal molecules between microbial and plant partner. Flavonoid signal molecules produced by the plant are recognized by the rhizobia, a process which is mediated through the regulatory NodD protein. In the presence of the correct flavonoids, NodD activates the transcription of the *nod*, *nol* and *noe* genes. The corresponding gene products generate specific signal molecules (Nod factors - NF), which are specifically recognized by the host plant (Perret et al., 2000). NFs are lipo-chitooligosaccharide molecules acting as morphogens, initiating the nodulation program of the host plant (Schultze and Kondorosi, 1998; D'Haeze and Holsters, 2002). Rhizobia produce NFs that are different in their fine structure and the corresponding host plants respond only to NFs with a defined structural composition. Therefore, NFs are major determinants of host specificity.

NFs from several rhizobial species have been characterized and their structures have been determined. For some species, it is also known which flavonoids are the best inducers of nod gene expression (Vlassak and Vanderleyden, 1997). For instance, the isoflavones daidzein and genistein, the main components present in soybean root extracts, are responsible for inducing the *nod* genes of *Bradyrhizobium japonicum* (Kosslak et al., 1987). However, it is quite obvious that NF biosynthesis by rhizobia in the soil is dependent on (unknown) other factors as well. Therefore, the composition of the medium in which the rhizobia are grown is likely to affect NF production in a qualitative and quantitative manner. A positive effect of genistein addition to *B. japonicum* inoculants on soybean grain and protein yield (Zhang and Smith, 1996), nodulation efficiency (Pan et al., 1998), N_2 fixation and total N yield at low root zone temperatures (Zhang and Smith, 1997) has been reported.

In this study, the influence of the culture medium on rhizobial NF production and excretion and rhizobial symbiotic efficiency was evaluated. The bacterial strain tested, *Bradyrhizobium elkanii* ICA 8001, is commonly used in Cuba for soybean inoculation. As a reference, *B. elkanii* strain LMG 6134 was used in parallel to compare the effect on NF production.

2. Materials and Methods

Bacterial strains and growth conditions

Bradyrhizobium elkanii ICA 8001 (Nápoles, 2003), a strain isolated from

Cuban soil, was grown at 30°C in three different media. The media differed basically in carbon and nitrogen source. As a reference (Medium A), yeast extract mannitol broth (YEM, Vincent, 1970) was utilized. Medium B (López, 1990) contains molasses 10 $g.l^{-1}$ (by-product from sugar-mill) and yeast extract 5 $g.l^{-1}$ as C and N source, and Medium C (Nápoles and Gutiérrez, 1998) contains defatted soybean cake 10 $g.l^{-1}$ (Oil enterprise) and molasses 5 $g.l^{-1}$ as C and N source. Both media B and C contain the same mineral composition: 0.5 $g.l^{-1}$ $(NH_4)_2SO_4$, 0.5 $g.l^{-1}$ K_2HPO_4, 0.2 $g.l^{-1}$ $MgSO_4$, 0.1 $g.l^{-1}$ NaCl and 1 $g.l^{-1}$ $CaCO_3$. A reference strain, *B. elkanii* LMG 6134, was used as a standard for NF analysis and was grown at the same temperature.

Experimental set-up for in vitro evaluation of nodulation

Two assays were carried out, in which strains were grown in different media A, B and C, and adjusted to the same cell density by counting in a Neubauer Chamber and confirmed by colony counting on plate (5×10^8 Colony Forming $Unit.ml^{-1}$). Soybean seeds (*Glycine max* variety William'82, Vincent, 1970), were surface sterilized by immersion in 70% ethanol during 30 seconds and $HgCl_2$ 0.2% (v/v) for 90 seconds. Subsequently, they were washed several times with sterile water.

Assay 1: After germination, the soybean seedlings were planted in tubes containing semisolid Norris and Date medium (Norris and Date, 1976). For each treatment, 5×10^8 $CFU.ml^{-1}$ was inoculated on the seedling. In parallel, control seedlings were inoculated with 1 ml of filter-sterilized inoculant supernatant (0.22 µm-pore size). These were used as a control together with non-inoculated seedlings. Five replicates were made per treatment. Plants were grown in a plant-growth chamber at 28°C, 9400 lux and 42% of relative humidity for 30 days. At harvest, the nodule number was determined. A complete randomized block design with factorial decomposition together with Duncan's Multiple Range Test was used to discriminate differences between treatments.

Assay 2: *Glycine max* cv. "William 82" seedlings were planted in 250 ml flasks with 150 ml semisolid Norris and Date medium (Norris and Date, 1976). Seedlings were inoculated with 200 µl of inoculant (1.5×10^8 $CFU.ml^{-1}$). As a control, non-inoculated treatments were used. Ten replicates were made per treatment. Plants were grown in the plant-growth chamber with a 12-h photoperiod (day/night temperature 26°C/22°C, relative humidity 70%) and placed in a complete randomized block design. Four weeks after inoculation, nitrogen fixation activity of the inoculated plant was determined by means of the acetylene reduction assay (ARA) using a gas-chromatograph (5890 A; Hewlett-Packard, equipped with a "PLOT fused silica" column) as described by Michiels et al. (1998). Other parameters such as the number of nodules and

fresh and dry weights of nodules per plant were determined. Data were analysed as described by Snoeck et al. (2003).

Experimental set-up of field trials

ICA 8001 was grown in Medium B or C (until density equivalent of 10^8 CFU.ml^{-1}) and was used as inoculant on the seeds prior to sowing in three different field experiments. Soybean seeds var. Tapachula were used in summer season. The experiments were conducted following a randomized block design, on two different experimental areas belonging to the National Institute of Agricultural Sciences, La Habana, Cuba.

For field experiments 1 and 2, each treatment was replicated six times in parcels of 11 m^2. At harvest (90 days after sowing), plant height, number of pods, overall yield and weight of 100 grains were determined. In addition, for experiment 1, ten plants per parcel were used to determine the nodulation parameters (number, fresh weight and dry weight of nodules per plant).

Field experiment 1: The trial was carried out on the central agricultural area "Las Papas" in San Jose de Las Lajas, La Habana (23°N and 82°12'W, 138 m above sea level, annual average precipitation of 101.85 mm and annual average temperature of 23.38°C) on 0.5 ha of soil with medium to high fertility (red, ferralitic compact and saturated soil type with 3.2% organic matter, pH 7.2 with high level of P - 417 ppm).

Field experiment 2: The trial was carried out on the experimental farm of sustainable agriculture "Bainoa", in Jaruco, La Habana (23°00'33"N and 81°55'22"W, 100 m above sea level, annual average precipitation of 117.23 mm and annual average temperature of 22.94°C) on 0.5 ha of soil with medium to low fertility (similar soil type as that used in experiment 1 but unsaturated, with lower pH 6.6 and lower levels of P - 29.2 ppm).

Field experiment 3: The trial was carried out on the same area as experiment 2, but on a larger scale, being 2 ha. In this experiment, four replicates per treatment in parcels of 14 m^2, were used to evaluate the effect of the different media on culture yield.

In all field experiments, the seeds were inoculated following the methodology proposed by Gómez et al. (1996), using a dosage of 500 g.ha^{-1} solid bioprepared. Here, 250 ml of inoculant was mixed with 500 g of peat as carrier. The culture irrigation was carried out according to the Technical Instruction (Technical Manual, 1998).

Radioactive labelling and detection of Nod metabolites by Reversed Phase Thin Layer Chromatography (TLC)

The NFs were radioactively labelled *in vivo* using the isotope [^{14}C]-acetate.

Subsequently, they were isolated using a slightly modified protocol of Laeremans et al. (1998). *B. elkanii* (ICA 8001 and LMG 6134) cultures were grown for 48 hrs, brought to the same OD (600 nm) and diluted ten times in fresh culture medium to a final concentration of approximately $5x10^8$ CFU.ml^{-1}. These cultures were pre-incubated at 30°C with agitation, during 1 h. When appropriate, samples were supplemented with genistein, 10 µM, as inducer and incubated during 2 hours under the same conditions. Subsequently the isotopic label [^{14}C]-acetate was added to all samples and cultures were grown for 36 hours. The NFs were extracted twice with 500 µl n-butanol and washed with ethyl acetate. The solution was vacuum-dried and samples were dissolved in 50% methanol and subsequently applied on reverse phase TLC plates (RP-18 F_{254}, Merck) with H_2O/acetonitrile (1:1, vol./vol.) as the mobile phase. Radioactivity was visualized by autoradiography using Hyperfilm-β max (Amershan Life Sciences) after 4 days of exposure.

3. Results and Discussion

The effect of inoculants on in vitro nodulation

To test the effect of the different bacterial culture media on nodulation efficiency, *Glycine max* seedlings were inoculated with *B. elkanii* ICA 8001 grown in different growth media (Medium A, B or C). As a control, seedlings were inoculated with the filtered supernatant of each inoculant. The number of nodules formed on five independent replicates of each treatment was evaluated. Significant differences between the treatments were observed. Inoculants obtained in medium C induced on average 18 nodules per soybean plant. This was significantly higher than the number of nodules formed by the inoculant prepared in either medium A or B. Nevertheless, medium B was superior to medium A with respect to number of nodules (12 versus 10), possibly due to the molasses present in medium B. Molasses contain monomeric phenols (Manual of Sugar Cane By-Products, 1988), which constitute weak *nod* gene inducers for *B. japonicum* (Kape et al., 1991). Remarkably, the number of nodules induced by non-filtered and filtered inoculants were similar. Inoculation with plain medium did not induce nodules, so the observed nodules are induced by the NFs present in the filtrate. As previously reported, NFs by themselves can induce the formation of nodule-like structures on legume roots (see review Geurts and Bisseling, 2002). Interestingly, the number of nodules formed by the filtered inoculants grown in medium C were significantly higher than the number formed by the filtered inoculants grown in medium A or B (20 vs. 5 and 8).

In a second experiment, the nodulation efficiency was also analysed (Table 1). In addition, the nodule number, the nodule dry weight and the nitrogen fixation activity (ARA) of the plants were also determined. The obtained results favor inoculants grown in medium C since not only more nodules were formed, but also a higher ARA was measured.

Table 1. Effect of different media on nodulation and nitrogen fixation.

Culture medium	Number of nodules per plant	Fresh weight of nodules per plant (g)	Dry weight of nodules per plant (g)	ARA (μmolethylene/ plant/h)
Medium A	13.3 c	0.16 c	0.02 b	1.9 c
Medium B	26.3 b	0.21 b	0.05 b	4.1 b
Medium C	55.0 a	0.37 a	0.09 a	7.8 a
Control	0 d	0 d	0 c	0 d

Values followed by the same letter are not significantly different ($P \leq 0.05$).

Table 2. Field experiment 1: Effect of different culture media on soybean yield, analysed in a red ferralitic, compact and saturated soil.

Treatments	Height (cm)	Pod number per plant	100 grains weight (g)	Yield ($t.ha^{-1}$)
Medium B	70.67 a	30.40 a	12.0 b	1.26 b
Medium C	76.47 a	40.87 a	16.0 a	1.46 a

Values followed by the same letter are not significantly different ($P \leq 0.05$).

Table 3. Field experiment 2: Effect of different culture media on soybean yield analysed in a red ferralitic, compact and unsaturated soil.

Treatments	Height (cm)	Pod number per plant	100 grains weight (g)	Yield ($t.ha^{-1}$)
Medium B	109.92 b	53.40 b	11.75 b	2.08 b
Medium C	114.12 a	66.82 a	15.5 a	2.38 a

Values followed by the same letter are not significantly different ($P \leq 0.05$).

Field trials

For the field trials, three independent experiments on two field sites were conducted. The fields had a history of sugar cane cultivation for many years and the last crop grown prior to the experiment was maize. As a result, no natural nodulation appeared on the control plots of all three field experiments. However, the different field trials (with different soil types) showed different soybean yields. Yield was lower in San Jose de Las Lajas (experiment 1) compared to Jaruco (experiments 2–3). This difference possibly results from the higher soil fertility in San Jose de Las Lajas, where nitrogen may inhibit the nitrogenase activity, resulting in lower nitrogen fixation and consequently lower yields (Streeter, 1988).

In experiment 1, ICA 8001 grown in medium C induced a higher nodule number than inoculants grown in medium B (37 on average vs. 24, respectively). Furthermore, differences were observed for fresh (48 vs. 30 mg respectively) and dry weight (0.39 vs. 0.27 mg respectively) of the nodules. These results confirm the data obtained with the *in vitro* nodulation experiment.

When evaluating yield, significant differences were found between the treatments for each experiment. The superior effectiveness of medium C was corroborated in the first two experiments. Table 2 shows a 15.8% yield increase when medium C was applied in the saturated soil type. In the case of unsaturated soil, a 14.4% yield increase was observed (Table 3). When an extended area was evaluated, plots inoculated with medium C grown cultures gave a yield increase of 34% compared to plots with medium B grown cultures (2.8 $t.ha^{-1}$ vs. 2.08 $t.ha^{-1}$, respectively). The yield increased with 34%, obtaining 2.80 $t.ha^{-1}$ of culture seeds (in comparison with 2.08 $t.ha^{-1}$ when inoculated with inoculants grown in medium B). Clearly, the positive effect of the culture medium on nodulation resulting in higher N_2-fixation per plant, is reflected by a higher yield in field trials.

Effect of different culture media on the synthesis of NFs

The NFs produced in the different growth media were evaluated by thin layer chromatography. Both *B. elkanii* strains produce NFs of which the profile on TLC differs depending on the culture media used.

Strain ICA 8001 (Fig. 1-I) produces three different Nod compounds in medium B (lane 1). When adding genistein, the concentrations of these compounds increased (lane 2) and a new spot appeared. In medium C (lane 3), the strain excreted additionally a minimum of four different spots. Remarkably, addition of genistein appeared to inhibit the formation of most of these additional spots (lane 4).

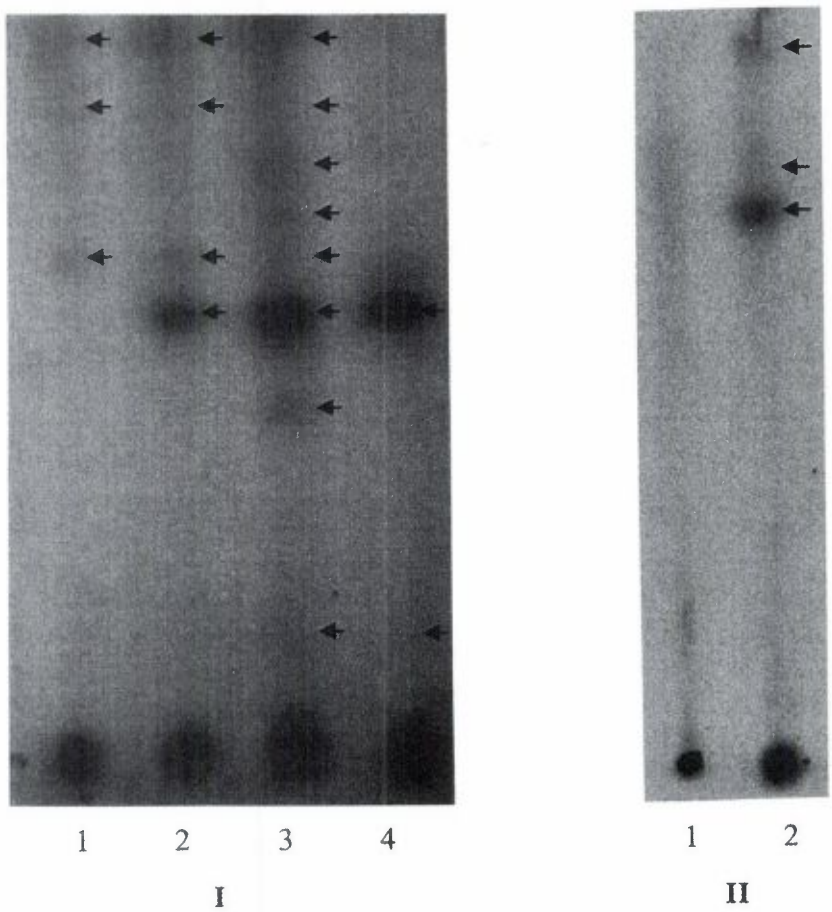

Figure 1. Autoradiogram of ethylacetate extracts of culture supernatant of *Bradyrhizobium elkanii* ICA 8001 (I) and LMG 6134 (II) grown in medium B or medium C. Possible NFs are indicated with black arrows. I: Lanes with odd numbers are samples without extra addition of genistein, lanes with even numbers are samples with extra addition of genistein. Lanes 1 and 2 represent samples taken from medium B, lanes 3 and 4 represent samples taken from medium C. II: Lane 1 represents a sample taken from cells grown in medium B, lane 2 represents a sample taken from cells grown in medium C. No genistein was added.

B. elkanii LMG 6134 (Fig. 1-II) produces less compounds than the ICA 8001 strain. LMG 6134 grown in Medium B (lane 1) barely showed production of Nod compounds, while growth in Medium C (lane 2) induced one clear intense and two weak compounds. Apparently for both strains, the growth medium induces similar effects on NF production.

While medium B consists basically of molasses, yeast extract and some salts, medium C in addition contains soybean cake. Supplementing the culture medium with soybean cake not only adds extra carbon and nitrogen sources but most likely also adds inducers for NF biosynthesis. The isoflavonoids daidzein and genistein and their glycosylated derivatives are present in high concentration in the soybean seeds (Porter et al., 1985). They are the major compounds of soybean root extracts and are responsible for induction of the nod gene(s) of *B. japonicum* (Kosslak et al., 1987; Kape et al., 1991).

In both media (B and C), the NF secreted in the highest amount is the one that migrates the slowest on the TLC plate. This spot could represent a pentasaccharide, according to Sanjuan et al. (1992) and Carlson et al. (1993). Work carried out by Staehelin et al. (1994) showed that in the case of *Sinorhizobium meliloti*, NFs with the highest polymerization degree have the strongest activity for triggering nodule formation.

Remarkably when genistein was added to medium C, the synthesis of NFs was repressed (lane 4, Fig. 1-I). Possibly, the excess of inducers inhibits NF synthesis. Similar observations were reported by Kape et al. (1991) where the activation of *nod* genes increased with addition of genistein up to 10 μM, while the addition of 100 μM genistein showed a significant reduction in inducing activity. In our study, the induction of nod genes by compounds present in the soybean cake (lane 3) was superior to the genistein effect (lane 2). Similar results were reported by Sanjuan et al. (1994), who detected higher NF production upon inducing a wild strain of *B. japonicum* with soybean seed extract as compared with genistein.

The presence of newly formed compounds in the extracts of cultures grown in medium C suggests that soybean cake contains inducer metabolites other than genistein, which could act synergistically in the synthesis of NFs. We reported previously on the effect of the active compounds in Medium C on the NF production of different strains of *Bradyrhizobium* (Nápoles et al., 2001). Different NF profiles were obtained on TLC when different compounds were used as inducer in the culture medium. In addition, the hypothesis on the influence of culture medium composition on NF production was confirmed in another experiment with ICA 8001 (results not shown). We compared the effects of nitrogen and carbon concentrations of media B and C on NF biosynthesis. In all cases, the main NF produced was the proposed pentasaccharide, together with seven other molecules that appeared or disappeared depending on the carbon and nitrogen source/concentration used. Therefore it seems that, besides the earlier reported influence of aeration and growth temperature on host specific NF modifications (Mergaert et al., 1997; Olsthoorn et al., 2000), carbon and nitrogen source may also influence NF synthesis.

The presented data suggest that the medium induced change in NF profiles could be correlated with the differences in nodulation and consequently with yield. The increase of effective NFs can also be concluded indirectly from the higher number of uninfected nodules when inoculating soybean seedlings with the supernatant filtrate of inoculant grown in medium C versus medium B (see above). However, it cannot be excluded that the growth medium also affects other properties of the bacterial cells that might be relevant to nodulation and nitrogen fixation.

As an overall conclusion, we consider that it is important to keep in mind the effects reported in this study, when formulating *Bradyrhizobium elkanii* inoculants. The higher soybean yields obtained with ICA 8001 inoculants grown in medium C, urged our Institute to use medium C for the preparation of all soybean inoculants in the region La Habana, Cuba.

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