EFFECT OF FERULIC ACID ON CHEMOTAXIS AND NODULATION OF *Bradyrhizobium japonicum*

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ABSTRACT. The effect of three concentrations of ferulic acid on chemotaxis and nodulation of *B. japonicum* ICA 8001 was studied in this work. The effect of this hydroxycinnamic acid obtained from vainillin was also evaluated and its induction capacity on nodulation genes by Nod factor synthesis detection. A positive but not strong chemotactic activity was obtained and only at 10 mM when it was put in the culture medium it showed a positive influence on nodulation. The ferulic acid from vainillin increased all nodulation parameters. The *nod* induction activity of this acid was evident by the production of four different lipochitinoligosaccharide structures.

Key words: *Bradyrhizobium japonicum*, *Glycine max*, ferulic acid, chemotaxis, nodulation

RESUMEN. En este trabajo se estudió el efecto de tres concentraciones de ácido ferúlico sobre la quimiotaxis y la nodulación de *B. japonicum* ICA 8001. También se evaluó el efecto de este ácido hidroxicinámico obtenido a partir de vainillina, así como su capacidad de inducción sobre los genes de nodulación mediante la detección de factores Nod sintetizados. Se obtuvo una actividad quimiotáctica positiva pero no fuerte y solamente 10 mM como componente del medio de cultivo mostró una influencia positiva sobre la nodulación. El ácido ferúlico sintetizado a partir de vainillina incrementó todos los parámetros de la nodulación. La actividad *nod* inductora de este ácido se evidenció con la producción de cuatro estructuras de lipoquitinoligosacáridos.

Palabras clave: *Bradyrhizobium japonicum*, *Glycine max*, ácido ferúlico, quimiotaxis, nodulación

INTRODUCTION

Hydroxycinnamic acids are universally present in higher plants and they have an important function as intermediates in the isoflavonoid biosynthesis. Ferulic acid (4-hydroxy-3-methoxy-cinnamic acid) has been found in hydrolyzed soybean root extracts (1). Therefore, it is interesting to investigate its role in the interaction with *Bradyrhizobium*.

Chemotaxis is one of the first phenomena to be taken into account in plant-microorganism interaction, since it is involved in the spreading of bacteria through soil, infection localization and the competition between different microorganisms.

Inoculation of legumes under field conditions with rhizobia does not always result in the desired yield increase and it is frequently related with the no occupancy of nodules by the inoculum, which must compete with the indigenous population (2). Several factors attempt to a successful inoculation (3). In a homogeneous environment (no gradients), cells swim in a three-dimensional random walk consisting of alternating periods of smooth swimming and regular tumbles. However, the presence of a chemotactic gradient leads to excitation and changes the swimming behavior of the cell. Increasing concentrations of an attractant or decreasing concentrations of a repellent lead to less frequent tumbling of the cells which continue to swim in the favorable direction. Increasing repellent or decreasing attractant concentrations, on the other hand, will increase the tumbling frequency and the cells will change their swimming direction (4).

Chemotaxis is controlled by a complex signal transduction pathway (5) and numerous citoplasmatic proteins are involved (6).

Several works about chemotaxis have been carried out with *B. japonicum* (7, 8) and all of them make evident that *B. japonicum* responds chemotactically to gradients of certain chemicals. In some cases, a strong correlation was observed between the *nod* gene-inducing and the chemotaxis-eliciting activities of the phenolic compounds, but in other cases this correlation was less pronounced or even totally absent, and host phenolics served only as attractants or inducers. Eventhough, an effect on rhizobial selection by chemotaxis toward these compounds was suggested.

A strong chemotactic effect of ferulic acid on *B. japonicum* 110 spc4 at 10 mM was reported (7) and a *nod* gene induction as strongly as coumestrol at 1 mM.

In this paper the effect of different concentrations of ferulic acid on the chemotactic behavior and nodulation capacity of *Bradyrhizobium japonicum* ICA 8001 was studied. The effect of ferulic acid obtained from vainillin was also studied and its *nod* induction capacity.

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MATERIALS AND METHODS

Strain and growing conditions. *Bradyrhizobium japonicum* ICA 8001 strain was used and cultivated in YEM medium at 28ºC (9).

Preparation of different concentrations of ferulic acid. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (Riedel-de-Haen) was dissolved in buffer HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethane sulfonic acid) (Merck) to 1 mM, 10 mM and 100 mM. pH was adjusted to 7 with NaOH . They were sterilized by filtration through 0.2 μm filters.

Ferulic acid obtained by Perkin condensation from vainillin (10) was used at 10 mM in another experiment.

Chemotaxis assay. The chemotaxis assay was done with ferulic acid reagent (11), with the following modification: cells harvested in log phase were diluted in buffer HEPES to 2.5 x 10^6 CFU (Colony Forming Units). mL^{-1} to make the counting in the capillaries easier. The percentage of attracted cells was estimated with respect to the total cells in the assay.

In vitro nodulation assay. Two experiments were carried out with ferulic acid reagent. In one of them different concentrations of ferulic acid were included as a component of the culture medium where bacteria grew up. In the other one, ferulic acid was applied to the seed at the same moment that bacteria were inoculated. YEM medium without ferulic acid was used as control. The bioassay conditions were similar to those used by other authors (8).

Results were statistically analyzed by a Randomized Complete Design using Duncan's Multiple Range Test to discriminate differences between media.

A third experiment was carried out with ferulic acid synthesized from vainillin and the number of nodules per plant; its fresh weight and the acetylene reduction activity were evaluated.

In vivo nodulation assay. To compare the effect of different concentrations of ferulic acid reagent on nodulation in soil conditions, an experiment was carried out in pots, containing 1 kg of Red Ferralitic Compact and Saturated soil. The culture medium used was the one reported (8), where ferulic acid was added. Results were compared with the same medium without ferulic acid.

Determination of Nod factor profile induced by ferulic acid. Ferulic acid 10 mM was used as inducer. Nodulation factors were radioactively labelled and they were isolated by following a slightly modified protocol (12). 100 μL from *Bradyrhizobium* cultures, growing for two nights, were inoculated in 900 μL of each fresh culture medium and the concentration was adjusted to $5x10^8$ CFU per medium milliliter. They were pre-incubated at 30°C with agitation, during one hour. Each sample was supplemented with genistein 10 μM as inducer and incubated during two hours at the same temperature and agitation. After induction, the isotopic label was carried out adding 125 μL of ^{14}C [2-^{14}C] acetic acid as sodium salt, and 20 μL of [S]-sulfate to medium A only. Cells were labelled for 36 h. Nodulation factors were isolated 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_{245}, Merck). H_2O/acetonitrile (1:1, vol/vol) was used as the mobile phase. The radioactivity was visualized by autoradiography using Hyperfilm- β max (Amershan Life Sciences) after four days of exposure.

RESULTS AND DISCUSSION

The effect of different ferulic acid concentrations on the chemotaxis of *B. japonicum* is shown in Table I.

Table I. Chemotaxis of *B. japonicum* ICA 8001 to different ferulic acid concentrations

Ferulic acid concentration	Chemotactic response	% of attracted cells
1 mM	1.41 ab	6.32
10 mM	1.85 a	8.32
100 mM	1.30 b	5.84
Es x*	0.06	

*p<0.05

All ferulic acid concentrations used were chemoattractants for *B. japonicum* ICA 8001. Similar results were found (7) but comparing the values we also observed a positive chemotaxis but not so strong like the previous authors did. Meanwhile, higher than 5.8 % total cells were attracted by the assayed concentrations.

10 mM showed the highest attraction but not statistically different from 1 mM with 8.3 and 6.3 % of attracted cells, respectively. 100 mM attracted 5.84 % from the total cells, with a lowest percentage.

Like in this case, not always the increment in the concentrations of an attractant lead to a higher migration. When ferulic acid concentration increased from 1 to 10 mM, the number of attracted cells increased too. However, when the concentration increased to 100 mM, capillary migration diminished.

The chemotactic effect of hydroxycinnamic acids could be probably related with their function as nutrient for *Bradyrhizobium*.

In spite of the chemoattractant effect obtained by the three concentrations, only an important effect of ferulic acid 10 mM was found on the nodulation when it was employed as culture medium component. The three concentrations applied to the seed at the inoculation moment and 1 and 100 mM used in the culture medium were not significatively different with YEM medium (Table II).

Table II. Effect of different ferulic acid concentrations on *in vitro* nodulation

Treatment	Number of nodules.plant^{-1}	Fresh weight of nodules.plant^{-1}	Dry weight of nodules. plant^{-1}
Control	2.9 b	0.0070 b	0.0011 b
1 mM - seed	1.7 b	0.004 b	0.00032 b
10 mM - seed	2.1 b	0.002 b	0.0003 b
100 mM - seed	2.0 b	0.0037 b	0.00066 b
1 mM - culture medium	3.2 b	0.005 b	0.0012 b
10 mM - culture medium	8.0 a	0.03 a	0.006 a
100 mM - culture medium	1.7 b	0.008 b	0.0004 b
ES x	0.31	0.00	0.00
p<	0.05	0.001	0.001

10 mM in the culture medium (treatment VI) caused a higher number of nodules, a higher fresh weight and a higher dry weight of nodules per plant. But the highest effect was on the number of nodules per plant. In all treatments the size of nodules was small; that is why the values of weight were so low. All nodules were effective.

We hope that placing ferulic acid on the seed, the positive chemotaxis turned out in a positive effect on nodulation, but it did not happen for any case. Then, putting it in the culture medium at 10 mM caused a good result in nodulation and the other determinations related. These results make us think that ferulic acid functioned more as a nutrient or as a *nod* inducer than as a chemoattractant. It is also possible that the controlled conditions of nodulation bioassay, where no other microorganisms are present and the fact of putting the product localized on the seed, do not permit to express the chemotactic activity. In this sense, it could be interesting to reproduce this experiment under field conditions, where not only our inoculum is present.

Table III. Effect of ferulic acid synthetized from vainillin on nodulation and nitrogen fixation

Culture medium	Number of nodules.plant^{-1}	Fresh weight of nodules.plant^{-1}	Acetylene reduction activity (µmol.plant^{-1})
Control	0 c	0 c	0 c
YEM	26.8 b	0.20 b	3.32 b
YEM + ferulic acid	32.2 a	0.27 a	6.10 a

The three parameters evaluated were higher when ferulic acid was used but mainly the nitrogen fixation activity.

The effect of this ferulic acid was more pronounced than the reagent and the result of ARA made evident that it not only increases the number and nodule weight but has a positive contribution on nitrogen fixation.

Table IV. Effect of different ferulic acid concentrations on *in vivo* nodulation

Treatment	Number of nodules.plant^{-1}	Fresh weight of nodules.plant^{-1}	Dry weight of nodules.plant^{-1}
Medium without acid	28.4 b	0.29 b	0.23
Ferulic acid 1 mM	29.1 b	0.31 ab	0.17
Ferulic acid 10 mM	35.2 a	0.49 ab	0.24
Ferulic acid 100 mM	40.3 a	0.66 a	0.25
	P<0.001	P<0.05	Ns

In spite of the fact that nodule mass dry results were not significantly different, the results of *in vivo* nodulation show a positive effect on the fresh mass and number of nodules per plant when ferulic acid was applied. 100 and 10 mM show the highest values in all determinations. This result could explain the *in vitro* behaviour obtained. The presence of another microorganism living in the surrounding area could allow the total expression of the chemotaxis phenomena.

Because of the presence of ferulic acid in the culture medium, we are not sure if the response of nodulation is due to a *nod* inducer activity or its role as nutrient.

Taking into account the chemotaxis and the results of nodulation, 10 mM seems to be the best concentration of ferulic acid to be used.

The profile of Nod factors produced by *B. japonicum* induced with ferulic acid is shown in Figure 1.

Figure 1. Nod factors produced by *B. japonicum* using ferulic acid 10 mM as inducer

There is a clear evidence that this compound induces *nod* gene activation in this strain. At least four different structures were produced and one of them at a high concentration.

This result explains what was previously obtained in nodulation response and if it is possible for this acid to act as nutrient too; it is sure that it is an inducer for this strain.

Present results permit us to include ferulic acid as a component of biofertilizers for soybean, mainly if we think that it can be obtained from some raw materials in our country.

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