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REGULAR ARTICLE

Effect of the presence of the plant growth promoting rhizobacterium (PGPR) *Chryseobacterium balustinum* Aur9 and salt stress in the pattern of flavonoids exuded by soybean roots

Marta S. Dardanelli · Hamid Manyani · Sergio González-Barroso · Miguel A. Rodríguez-Carvajal · Antonio M. Gil-Serrano · Maria R. Espuny · Francisco Javier López-Baena · Ramon A. Bellogín · Manuel Megías · Francisco J. Ollero

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Abstract In this work we studied how biotic and abiotic stresses can alter the pattern of flavonoids exuded by Osumi soybean roots. A routine method was developed for the detection and characterization of the flavonoids present in soybean root exudates using HPLC-MS/MS. Then, a systematic screening of the flavonoids exuded under biotic stress, the presence of a plant growth promoting rhizobacterium, and salt stress was carried out. Results obtained indicate that the presence of *Chryseobacterium balustinum* Aur9 or 50 mM NaCl changes qualitatively the pattern of flavonoids exuded when compared to control conditions. Thus, in the presence of *C. balustinum* Aur9, soybean roots did not exude quercetin and naringenin and, under salt stress, flavonoids daidzein and naringenin could not be detected. Soybean root exudates obtained under saline conditions showed a diminished capacity to induce the expression of the *nodA* gene in comparison to the exudates obtained in the absence of salt. Moreover, lipochitooligosaccharides (LCOs) were not detected or weakly detected when *Sinorhizobium fredii* SMH12 was grown in the exudates obtained under salt stress conditions or under salt stress in the presence of *C. balustinum* Au9, respectively.

Keywords Plant groth promoting rhizobacteria (PGPR) · Soybean · Flavonoids · Exudation · Saline stress · Nod gene expression

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M. S. Dardanelli · H. Manyani · M. Megías
Departamento de Microbiología y Parasitología,
Facultad de Farmacia, Universidad de Sevilla,
c/ Profesor García González 2,
41012 Sevilla, Spain

S. González-Barroso · M. A. Rodríguez-Carvajal ·
A. M. Gil-Serrano
Departamento de Química Orgánica, Facultad de Química,
Universidad de Sevilla,
c/ Profesor García González,
41012 Sevilla, Spain

M. R. Espuny · F. J. López-Baena · R. A. Bellogín ·
F. J. Ollero (✉)
Departamento de Microbiología, Facultad de Biología,
Universidad de Sevilla,
Avenida Reina Mercedes 6,
41012 Sevilla, Spain
e-mail: fjom@us.es

M. S. Dardanelli
Departamento de Biología Molecular,
Facultad de Ciencias Exactas, Físico-Químicas y Naturales,
Universidad Nacional de Río Cuarto,
Ruta Nacional N° 36 km 601,
Cp X5804BYA Córdoba, Argentina

Introduction

Rhizobia are soil bacteria that induce the formation of nitrogen-fixing nodules on legumes. Plant roots exude

phenolic compounds, called flavonoids, which bind to the bacterial protein NodD. The complex NodD-flavonoid binds to highly conserved DNA promoter regions (*nod* boxes) and activates the transcription of the nodulation (*nod*) genes. As a consequence, the bacterium synthesizes and secretes a family of molecules, called nodulation factors or lipochitooligosaccharides (LCOs), which trigger a series of biological responses in the root cells that lead to the formation of the nodule.

Flavonoids are the products of the central phenylpropanoid pathway. After their biosynthesis, flavonoids are generally stored in a sugar-conjugated form in plant vacuoles (Aoki et al. 2000). Flavonoids play a key role in nodulation specificity, since a legume will induce the biosynthesis of LCOs in a rhizobial strain only if any of the flavonoids secreted is able to activate, via NodD, the transcription of the *nod* genes.

Analysis of the soybean cv. Amsoy 71 root exudates resulted in the identification of the flavonoids daidzein and coumestrol (D'Arcy-Lameta 1986). Soybean cultivars Peking and McCall root exudates also contain picomolar concentrations of daidzein, genistein, coumestrol, and glycitein (Pueppke et al. 1998). Other authors have isolated, from soybean cv. Maple Arrow root exudates, 4, 2′, 4′-trihydroxychalcone (isoliquiritigenin), which possesses a strong inducing activity of the *nod* genes in *Bradyrhizobium japonicum* (Kape et al. 1992). Root and seed soybean exudates also contain derivatives of genistein, glycitein, and daidzein with glucose, malonyl, and acetyl groups attached (Smit et al. 1992). Inoculation with compatible rhizobia influences plant root exudation. Thus, when Williams soybeans are inoculated with *B. japonicum* USDA110, the root exudates contain higher concentrations of daidzein, genistein, and coumestrol in comparison with non inoculated plants (Cho and Harper 1991).

Many studies have shown that the presence of microbes or microbial components, like cell wall components, can elicit changes in the expression of enzymes involved in the biosynthesis of flavonoids. In some cases, an enhanced expression of plant biosynthetic genes has been correlated with qualitative and quantitative changes in root exudation. Phytopathogens and associative and non-symbiotic plant growth promoting rhizobacteria (PGPR) also elicit changes in the pattern of flavonoids exuded (for a review see Shaw et al. 2006). In other cases, flavonoids may represent a carbon source for rhizosphere microorganisms that posses the appropriate catabolic enzymes. Several studies have demonstrated an aerobic flavonoid biodegradation for a number of bacterial species, including rhizobia (Rao and Cooper 1994, 1995).

Salinity induces osmotic and ionic stresses in plants, and plants respond inducing a defense response. Legumes show a low salt tolerance that limits their capacity to grow in many saline soils (Zahran 1999). Moreover, salt stress affects the symbiotic process established between rhizobia and legumes (Tejera et al. 2005). Several authors have demonstrated that salt stress reduces the nodulation capacity and nitrogen fixation by a reduction in the number of nodules (Tu 1981; Bekki et al. 1987), reduction of the leghemoglobin levels (Delgado et al. 1994) or reduction of the nitrogenase activity (Tejera et al. 2004).

Chryseobacterium balustinum Aur9 (hereafter Aur9) is a PGP bacterium that promotes germination and increases root surface, total nitrogen content, and biological nitrogen fixation in *Lupinus albus* seedlings (Gutiérrez-Mañero et al. 2003). Previous works have demonstrated that inoculation of Osumi soybean plants with Aur9 increases plant nitrogen concentration, nitrogen absorption, and aerial growth (Lucas-García et al. 2004). Co-inoculation of Aur9 and the *Sinorhizobium fredii* strains HH103 and SMH12 led to a significant increase in the number of nodules and root growth of Osumi soybean plants under saline or non-saline conditions (Estévez et al. 2009). *S. fredii* SMH12 (hereafter SMH12) is a rhizobial strain that produces seed yields that are not different to those produced by *B. japonicum* USDA110, a highly effective soybean inoculant (Rodríguez-Navarro et al. 2002). Moreover, Aur9 can trigger an induced systemic response in Williams soybean (Gutiérrez-Mañero, personal communication) and protect *Arabidopsis* (Ramos-Solano et al. 2007) and soybean plants against salt stress (Gutiérrez-Mañero, personal communication).

The aim of this work was to determine whether soybean plants under biotic or abiotic stresses modify their pattern of exuded flavonoids. Thus, we studied whether the presence of Aur9 influences the exudation of flavonoids by soybean roots. We have observed qualitative changes in the flavonoids secreted by soybean in the presence of this PGPR strain. This finding could be interesting for the formulation of

mixed inoculants PGPR-rhizobia. We have also studied whether salt stress (50 mM NaCl) affects the pattern of flavonoids exuded by Osumi soybean in the presence or absence of Aur9. This is the first time that a systematic screening of flavonoids exuded by soybean plants has been carried out in the presence of a PGPR bacterium and under salt stress. Moreover, the capacity of the soybean exudates to induce *nod* gene expression and LCOs production in the soybean symbiont SMH12 has been studied.

Materials and methods

Bacterial strains and media

Sinorhizobium fredii SMH12 (Rodríguez-Navarro et al. 1996) was grown at 28ºC in B^- medium (Spaink et al. 1992). *Chryseobacterium balustinum* Aur9 (Gutiérrez-Mañero et al. 2003) was grown at 28ºC in B^- medium with glucose as carbon and energy source instead of mannitol, and supplemented with hydrolyzed casaminoacids (1 gl^{-1}).

Preparation of soybean root exudates

Glycine max (L.) Merrill cv. Osumi seeds were surface sterilized for 30 s in ethanol followed by gentle shaking for 6 min in a solution of 6% sodium hypochlorite. Sterilized seeds were soaked at least six times in sterile demineralized water. Then, seeds were placed in Petri dishes containing TY medium solidified with 0.8% agar. Twenty five pre-germinated seeds were individually transferred to a stainless-steel lattice placed in a glass cylinder containing 130 ml of a modified nitrogen-free Rigaud-Puppo solution 0.25X ($CaCl_2 \cdot 2H_2O$ 25 mg l^{-1}, $MgSO_4 \cdot 7H_2O$ 50 mg l^{-1}, KH_2PO_4 50 mg l^{-1}, $NaHPO_4 \cdot 2H_2O$ 18.75 mg l^{-1}, Gibson solution 1 ml l^{-1}, sequestrene 2.5 mg l^{-1}, pH 6.8). Seedling roots grew through the holes of the lattice into the nutritive solution. This hydroponics system was placed into a sterilized 5-litre container and incubated for 7 days in a climate-controlled growth chamber (25°C, 70% relative humidity, 16 h daylight). The exudates were centrifuged at 10000 rpm for 20 min (5ºC), checked for sterility, and finally filtered using a 0.25 µm cellulose nitrate filter. The hydroponics systems were inoculated with *C. balustinum* Aur9 at about 10^5 cells ml^{-1} when required. The different exudates were also obtained in the presence of the nitrogen-free Rigaud-Puppo solution supplemented with 50 mM NaCl. Four different soybean root exudates were then obtained: soybean root exudates uninoculated, soybean root exudates inoculated with *C. balustinum* Aur9, soybean root exudates uninoculated but in the presence of 50 mM NaCl, and soybean root exudates inoculated with Aur9 and in the presence of 50 mM NaCl.

Determination of the β-galactosidase activity

SMH12 harboring plasmid pMP240, that contains the *nodA* promoter of *R. leguminosarum* fussed to the *lacZ* gene (de Maagd et al. 1988), was used to investigate the capacity of the different soybean root exudates and single flavonoids to induce the expression of nodulation genes. Flavonoids were dissolved in ethanol and used at 1 µg ml^{-1}, which gave final concentrations between 3.0 µM (quercetin) and 6.2 µM (umbelliferone). β-galactosidase assays were carried out as previously described (López-Baena et al. 2008). For the experiments with exudates, 900 µl of the different soybean root exudates and 100 µl of the rhizobial suspension were used. The strains were induced for a maximum period of 72 h at 28ºC with shaking (150 rpm), and bacterial growth was monitored by measuring turbidity at 660 nm. β-galactosidase assays were carried out when cultures reached an $OD_{660\ nm}$ of about 0.3–0.4. Each experiment was repeated at least three times with three replicates each time to determinate the reproducibility of the results.

Effect of the soybean root exudates on some surface characteristics of *S. fredii* SMH12

To study changes in the surface characteristics of the rhizobial colonies, SMH12 was grown in solid soybean root exudates (solidified with 2% agar) and mixed with B^- medium in a 1:1 proportion. The same medium supplemented with either Congo red (25 µg m^{-1}) or calcofluor (200 µg ml^{-1}. Fluorescent Brightener 28, Sigma) was also used. After incubation at 28ºC for 3 to 5 days, the smooth/rough aspect of the colonies, the capacity to accumulate Congo red, and the brightness under UV illumination when calcofluor was present in the medium, were observed. As controls, B^- medium and B^- mixed with the nutritive plant solution of Rigaud-Puppo in a 1:1 proportion were used.

Reverse phase thin layer chromatography (RP-TLC) analysis of lipochitooligosaccharides (LCOs)

For every experiment, SMH12 was grown in 900 μl of soybean root exudates and 100 μl of the rhizobial suspension (O.D.$_{600\,nm}$=1.0) or in mineral B$^-$ medium as control with or without the inducer flavonoid genistein (3.7 μM) and in the presence of *N*-acetyl [^{14}C] glucosamine (7.4×10^3 Bq μl^{-1}, specific activity 2×10^3 Mbq mmol^{-1}, Amersham). Each experiment was repeated at least three times with three replicates each time, to determinate the reproducibility of the results. The ^{14}C-labeled LCOs were analyzed by reversed-phase thin-layer chromatography (RP-TLC), as previously described (Crespo-Rivas et al. 2007).

Analysis of lipopolysaccharides

To determine the lipopolysaccharide (LPS) profiles, bacterial cultures were grown on solid TY medium. Bacterial cells were washed in 0.9% NaCl and pelleted by centrifugation. Treatments applied to the bacterial pellets, electrophoresis of crude bacteria extracts, and the silver-staining procedures were carried out as previously described (Buendía-Clavería et al. 2003).

Flavonoids sample preparation and analysis

One sixth of the lyophilized solid soybean exudates, from five independent hydroponics soybean cultures (300 ml), was resuspended in 50 ml of water and passed through a Resprep C18 solid phase extraction cartridge (Restek corp., Bellefonte, USA). Flavonoids were released with 5 ml elution of 50%, 80%, and 100% methanol. These fractions were combined and freeze-dried. For HPLC analysis, samples were resuspended in 1 ml 50% methanol and 100 μl aliquots were injected in a HPLC-ESI system (Ríos et al. 2005). Sixteen standard flavonoids (supplied by Fluka or Sigma-Aldrich) were used: flavanones as naringenin and hesperitin; flavonols as kaempferol, fisetin, morin, and quercetin; isoflavones as genistein and daidzein; flavones as 7, 4′-dihydroxyflavone, apigenin, chrysin, and luteolin; chalcones as isoliquiritigenin; coumaronochromones as lisetin; coumestans as coumestrol, and coumarins as umbelliferone. The flavonoid-glycosides genistin, daidzin and naringin were also used.

HPLC-MS/MS

Chromatographic separation was performed using a Pelkin Elmer Series 200 HPLC system (Waltham, USA) coupled to a 2000 QTRAP hybrid triple-quadrupole-linear trap mass spectrometer (Applied Biosystem, Foster City, USA) equipped with an electrospray ion source.

HPLC analyses were performed on a 250×2.1 mm Tracer Spherisorb ODS2 C18 reversed-phase column with a particle size of 5 μm (Teknokroma, Spain). The flow rate was 0.3 ml min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) 50:50 (v/v) acetonitrile:methanol. Both components contained 0.1% formic acid (v/v). The elution profile was: isocratic for 5 min with 5% B, linear for 15 min up to 55% B, linear for 25 min up to 100% B, and isocratic for 5 min (100% B).

Mass spectrometric detection was performed in the negative ion mode after electrospray ionization. For HPLC-MS analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, ionspray voltage −4500 V, source temperature 300ºC, source gas 20 psi, declustering potential −70 V, and entrance potential −10 V. Collision-induced dissociation (CID) was performed with the following parameters: collision cell exit potential −15 V, and collision energy −35 V.

Results

Characterization of the soybean root exudates in the presence or absence of the PGPR Aur9 and/or salt stress

Soybean root exudates were obtained using a hydroponics system as described in the Materials and Methods section. The initial and final pH values of the different root exudates were determined and, in the case of the exudates obtained in the presence of Aur9, also the initial and final number of bacteria (Table 1). Thus, soybean root exudates acidified the nutritive solution in the presence or absence of Aur9 (Table 1). In contrast, when soybean plants were exposed to salt stress, the exudates did not modify the initial pH. Finally, Aur9 was able to grow in the root exudates two orders of magnitude with or without salt stress (Table 1).

Table 1 Final pH values and growth of *Chryseobacterium balustinum* Aur9 in the different soybean root exudates

Condition	Cells ml^{-1} at time 0 day	Cells ml^{-1} at time 7 days	Initial pH	Final pH
Soybean root exudates	–	–	6.8	5.3
Soybean root exudates + Aur9	1×10^5	1.7×10^7	6.8	5.8
Soybean root exudates + NaCl (50 mM)	–	–	6.8	6.7
Soybean root exudates + Aur9 + NaCl (50 mM)	6.2×10^5	4.2×10^7	6.8	6.5

Soybean plants showed a normal root development after 7 days of cultivation in the hydroponics system. However, when plants were exposed to 50 mM NaCl, root development was severely affected and roots were much shorter than those cultivated without salt stress (data not shown).

Effect of the different soybean root exudates in the morphological and physiological characteristics of *S. fredii* SMH12

In order to study the effects of the different exudates on SMH12, a strain that induces the formation of nitrogen-fixing nodules on soybean, various studies were conducted to analyze their influence in its surface characteristics. However, SMH12 could not grow properly when inoculated in the different liquid soybean root exudates. Therefore, to analyze the influence of the different exudates in the bacterial surface, they were mixed with B$^-$ medium in a 1:1 proportion and solidified with agar to inoculate the bacteria in Petri dishes. No differences were observed in the surface of SMH12 colonies when inoculated in the soybean root exudates obtained in the presence of Aur9 or salt stress and supplemented with Congo red or calcofluor in comparison to colonies grown in the non inoculated exudates (data not shown). SMH12 colonies grown in the different exudates were mucoid, absorbed Congo red and were not fluorescent in the presence of calcofluor. In addition, no differences were observed between the LPS profiles of SMH12 grown in the different soybean root exudates and in B$^-$ medium (data not shown).

Capacity of the soybean root exudates to induce the expression of nodulation (*nod*) genes

To elucidate whether the different soybean root exudates were able to induce the expression of the *nodA* gene harbored in plasmid pMP240, which carries a *nodA*::*lacZ* fusion, β-galactosidase activity assays using strain SMH12 (pMP240) were carried out. As shown in Fig. 1 (lanes 1, 4, and 6), soybean root exudates obtained in the presence or absence of Aur9 induced 3.5-fold the expression of *nodA* when compared to the values obtained under control conditions (B$^-$ medium).

However, when the root exudate was obtained in the presence of 50 mM NaCl (with or without Aur9) the expression of the *nodA* gene increased only 1.5-fold with respect to the control conditions (Fig. 1, lanes 2, 5, and 7). Therefore, the presence of salt in the nutritive plant solution diminished the capacity of the soybean root exudates to induce the expression of *nodA*. Moreover, when the root exudate obtained in the presence of Aur9 was supplemented with genistein (3.7 μM), the expression of *nodA* was lower than that obtained in B$^-$ medium and in soybean root exudates without Aur9, both also supplemented with genistein (Fig. 1, lanes 3, 6, and 9).

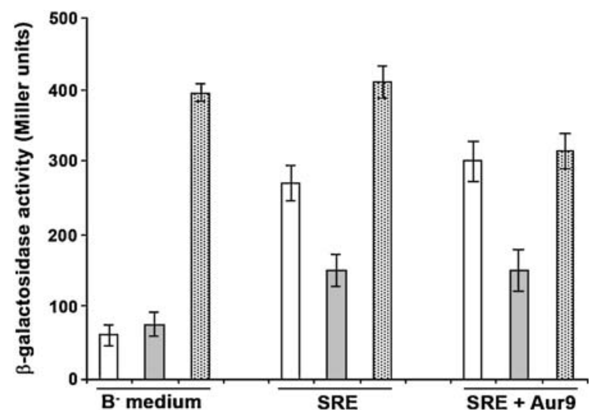

Fig. 1 β-galactosidase activity of the strain *S. fredii* SMH12 harbouring plasmid pMP240. Lanes 2, 5, and 8, cultures grown in the presence of 50 mM NaCl. Lanes 3, 6, and 9, cultures induced with 3.7 μM genistein

LCO profiles of *S. fredii* SMH12 grown in the different soybean root exudates

Reverse-phase thin layer chromatography (RP-TLC) was used to analyze the LCOs obtained from cultures of SMH12 grown in the different soybean root exudates or induced with genistein. At least three spots were resolved from the extracts of SMH12 grown in B^- medium supplemented with genistein (Fig. 2, lane 1). The same spots were detected when the LCOs were produced by SMH12 grown in soybean root exudates and soybean root exudates + Aur9 (Fig. 2a, lanes 4 and 6). However, in the latter case very weak spots were detected. No spots were observed when SMH12 was grown in soybean root exudates + 50 mM NaCl (Fig. 2b, lane 4), and very weak spots were detected in soybean root exudates + 50 mM NaCl + Aur9 (Fig. 2b, lane 6). However, the exudates obtained in the presence of NaCl were able to induce weakly the expression of the *nodA* gene (Fig. 1). These results are in agreement with the minor capacity of activation of *nod* gene expression detected in the soybean root exudates obtained in the presence of 50 mM NaCl (Fig. 1, panels A and B). In general, inoculation of the soybean root exudates with Aur9 decreased the intensity of the spots that correspond to the LCOs produced by SMH12 in comparison to the profiles obtained when grown in non inoculated soybean exudates.

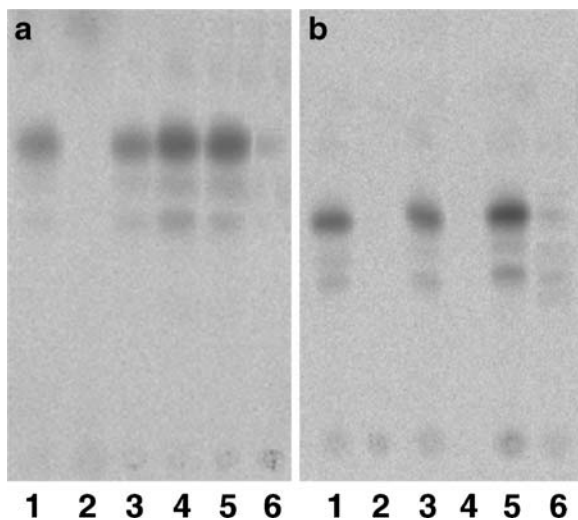

Fig. 2 LCO profiles of *S. fredii* SMH12 **a** without salt stress, and **b** with NaCl 50 mM. *S. fredii* SMH12 was grown in B^- medium, lanes 1 and 2; soybean root exudates, lanes 3 and 4; and soybean root exudates + Aur9, lanes 5 and 6. Lanes 1, 3 and 5: LCOs obtained in the presence of 3.7 μM genistein

Identification of flavonoids secreted by soybean roots in the presence and absence of *C. balustinum* Aur9 and 50 mM NaCl

The number of known flavonoids is extremely large: more than 4000 (Strack and Wray 1994), even almost 6500 (Rauha et al. 2001). Thus, their chemical identification is difficult to determine. Besides this, the amounts of each analyte and its response to the detection method can be very different, which leads to additional problems in dynamic range. Moreover, the method must identify them in very complex mixtures, where other secondary metabolites such as organic acids, amino acids, sugars, etc, can be found (Bais et al. 2006). In these conditions, the application of tandem LC-MS-MS enables specific compounds to be detected in complex mixtures due to their specific and characteristic fragmentation patterns. This technique has been recently applied in our research group for the identification of flavonoids in bean exudates (Dardanelli et al. 2008). The compound to identify must be known and previously well-characterized before this type of experiment is undertaken. The MS/MS spectra obtained allowed us to identify each flavonoid, whereas its retention time indicates whether it is glycosilated or not (Dardanelli et al. 2008). An example of the analysis of soybean exudates for detection of flavonoids is shown in Fig. 3.

Soybean root exudates obtained in the different experimental conditions were analyzed for their flavonoids content (Table 2). Two flavones (7, 4′-dihydroxyflavone and apigenin), one flavonol (quercetin), a flavanone (naringenin), two isoflavones (daidzein and genistein), a chalcone (isoliquiritigenin) and a coumarin (umbelliferone) were found in the Osumi soybean root exudates. In the other exudation conditions tested, some qualitative differences were observed. For instance, quercetin and naringenin were not detected in the exudates obtained in the presence of Aur9, and daidzein and genistein were not detected in the exudates obtained in the presence of salt stress (Table 2). These results indicate that the presence of 50 mM NaCl reduces dramatically the exudation of isoflavonoids by soybean roots.

The analytical technique allowed the detection of other common soybean flavonoids such as daidzein,

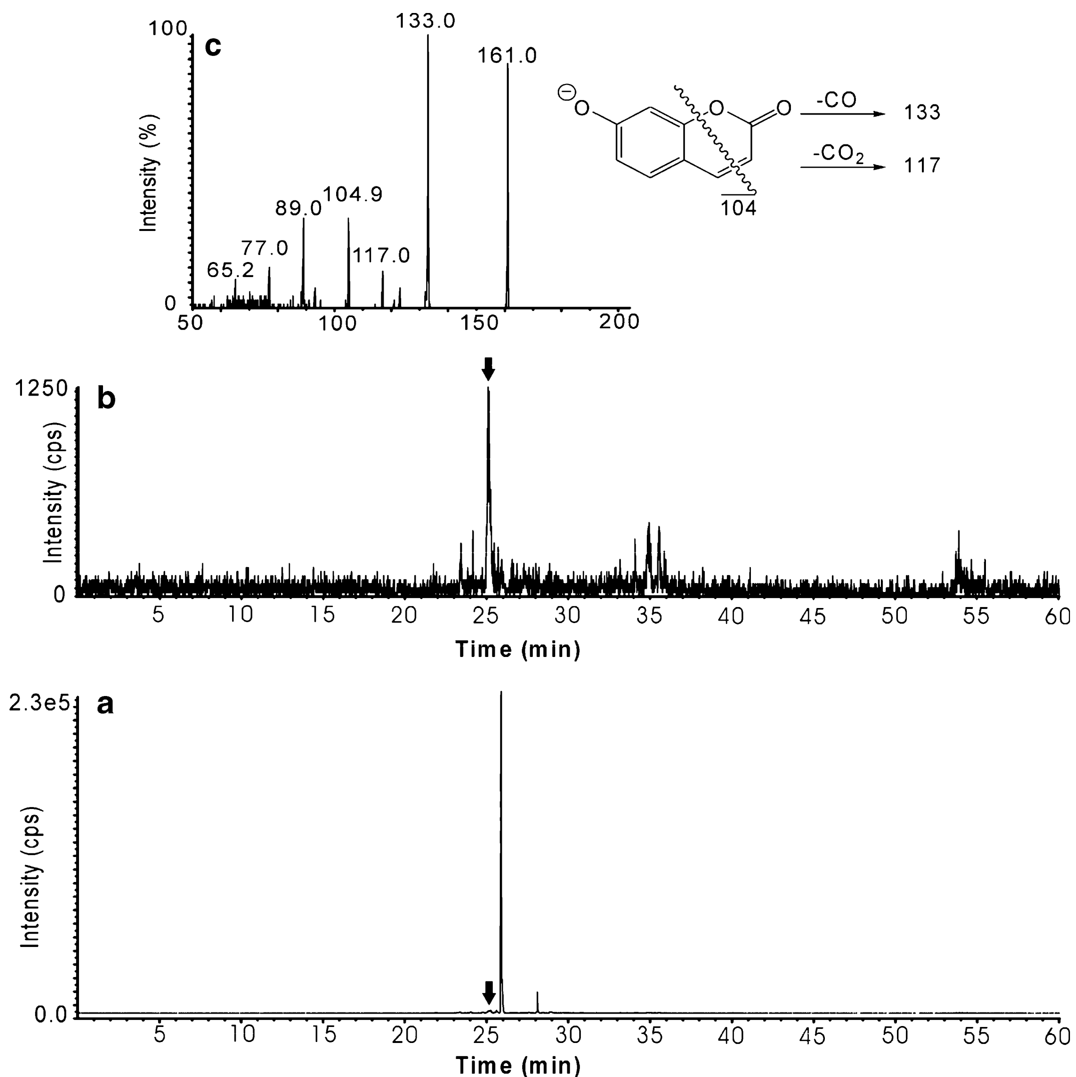

Fig. 3 Analysis of flavonoids present in soybean root exudates. **a** HPLC chromatogram obtained when registering in Total Ion Monitoring mode. The arrow indicates the peak corresponding to umbelliferone. **b** HPLC chromatogram obtained when Multiple Reaction Monitoring is applied (searching for umbelliferone). **c** Collision Induced Decomposition mass spectrum obtained from umbelliferone peak. Product ions are assigned and indicated in the structure

genistein, or apigenin, and even others more structurally complicated as umbelliferone. Apparently, in the conditions of the experiments, Osumi soybean roots do not exudate, or they do at very low concentrations, the flavonoids coumestrol, chrysin, fisetin, hesperitin, kaempferol, lisetin, luteonin, and morin.

The different flavonoids exuded by Osumi soybean roots in the different culture conditions were assayed individually using SMH12 (pMP240) as a reporter strain. All flavonoids analyzed, with the exception of quercetin, induced the expression of the *nodA* gene harbored in plasmid pMP240 between 4.68 and 7.23-fold in comparison to non induced cultures (Table 3). Quercetin only induced the expression of *nodA* about 1.64-fold with respect to the control without flavonoids.

Discussion

Plant flavonoids, particularly isoflavonoids, are key components of the defense response in legumes against pathogens by probably inhibiting microbial growth. In addition, they are essential compounds in

Table 2 Flavonoids detected in soybean root exudates in the presence or absence of *C. balustinum* Aur9 and salt stress

	Control	50 mM NaCl	Control + Aur9	50 mM NaCl + Aur9
Flavones				
7, 4′-dihydroxyflavone	+[a]	+[a,b]	+[a]	+[a,b]
Apigenin	+[a,b]	+[a]	+[a]	+[a]
Flavonols				
Quercetin	+	+	–	–
Flavanones				
Naringenin	+	+	–	–
Isoflavones				
Daidzein	+[a]	–	+[a]	–
Genistein	+[a]	–	+[a]	–
Chalcone				
Isoliquiritigenin (4, 2′, 4′-trihydroxychalcone)	+[a]	+[a]	+[a]	+[a]
Coumarin				
Umbelliferone (7-hydroxy-2H-1-benzopyran-2-one)	+	+	+	+

The presence (+) or absence (–) of a flavonoid is indicated

[a] Glycosidated flavonoid

[b] Several peaks detected, possibly from different glycosides

the establishment of an effective symbiosis with rhizobia. Plant root exudates consist of a complex mixture of organic acid anions, phytosiderophores, sugars, vitamins, amino acids, purines, nucleosides, inorganic ions, gaseous molecules, enzymes and root border cells which have major direct or indirect effects on the acquisition of mineral nutrients required for plant growth. Phenolic and aldonic acids exuded directly by roots of nitrogen-fixing legumes serve as major signals to rhizobia which form root nodules where N_2 is reduced to ammonia (Dakora and Phillips 2002).

Several authors have shown that different soybean cultivars, namely Amsoy, Marple Arrow, Peking, and McCall, exudate the flavonoids coumestrol, daidzein, genistein, and isoliquiritigenin (D'Arcy-Lameta 1986; Kape et al. 1992; Pueppke et al. 1998). It has been proposed that the presence of microorganisms influences the quality and quantity of flavonoids exuded by a particular legume (Shaw et al. 2006). In this work we have analyzed the presence of sixteen flavonoids in the exudates of soybean cv. Osumi. Eight of these flavonoids were exuded by soybean plants under control conditions (Table 2). Interestingly, the coumestan coumestrol was not detected, although it is commonly identified in soybean root exudates (D'Arcy-Lameta 1986; Cho and Harper 1991; Pueppke et al. 1998). Four of these flavonoids (7, 4′-dihydroxyflavone, apigenin, isoliquiritigenin, and umbelliferone) were exuded in all the conditions assayed. In the exudates obtained in the presence of salt and in those inoculated with Aur9, isoflavonoids daidzein and

Table 3 Expression of the *nodA* gene harbored in strain SMH12 (pMP240) induced with different flavonoids

Flavonoids	μM	Hydroxylation pattern	Average (Miller units)	n
None			424±46	1.00
7, 4′-dihydroxyflavone	3.9	7, 4′	2263±124	5.34
Apigenin	3.7	5, 7, 4′	3067±135	7.23
Quercetin	3.0	3, 5, 7, 3′, 4′	697±17	1.64
Naringenin	3.7	5, 7, 4′	2394±24	5.65
Daidzein	3.9	7, 4′	2636±118	6.22
Genistein	3.7	5, 7, 4′	2786±115	6.57
Isoliquiritigenin	3.7	4, 2′, 4′	2327±45	5.49
Umbelliferone	6.2	7	1983±55	4.68

Numbers are the mean of at least two independent experiments performed in duplicate

n=fold induction with respect to control without flavonoid

genistein, and quercetin and naringenin, respectively, could not be detected. Under salt stress conditions, the flavonoid biosynthesis pathway could be altered or flavonoids daidzein and genistein could not be stable or secreted in a very low concentration. In the case of soybean plants inoculated with Aur9 the absence of some flavonoids in the soybean root exudates could be due to: i) Aur9 could block the biosynthesis of flavonoids or ii) flavonoids exuded could be used as a carbon and energy source. In fact, some soybean-nodulating rhizobia degrade flavonoids daidzein and genistein via a C-ring fission mechanism (Rao and Cooper 1995).

Genes involved in the phenylpropanoid pathway and in defense are highly regulated during the infection process (Lohar et al. 2006). Thus, chickpea roots inoculated with different *Pseudomonas* spp. PGPR show a marked increase in flavonoids concentration in comparison with roots of untreated seeds (Parmar and Dadarwal 1999). Moreover, previous studies have shown that co-inoculation of *Phaseolus vulgaris* plants with the PGPR *Azospirillum brasilense* Cd and *Rhizobium tropici* CIAT899 increased the number of flavonoids exuded by bean roots 14 days after inoculation, but the number of flavonoids exuded 7 days after inoculation was similar to those secreted by non infected plants (Dardanelli et al. 2008). In contrast, results shown in this work indicate that inoculation of soybean with the PGPR Aur9 reduced the number of flavonoids secreted by plant roots (Table 1). Despite the reduction in the flavonoids exuded, they were able to induce the expression of the *nodA* gene in a level similar to that shown by non inoculated soybean root exudates (Fig. 1). However, these exudates induced very weakly the biosynthesis of LCOs in SMH12 in comparison to those produced when induced by non inoculated soybean root exudates (Fig. 2a, lanes 3 and 6). Thus, there was no correlation between the capacity of the soybean root exudates and those obtained in the presence of Aur9 to induce the expression of *nodA* and the slight biosynthesis of LCOs detected by RP-TLC. Aur9 could somehow modulate plant metabolic pathways that culminate in the synthesis of chitinases or other enzymes that could degrade the LCOs synthesized by rhizobia. The presence of salt (50 mM) in the nutritive nitrogen-free plant solution repressed drastically the capacity of the exudates to induce the expression of *nod* genes (Fig. 1), indicating that salt stress could have a negative influence on the exudation of flavonoids by soybean. In fact, these exudates were not able to induce the biosynthesis of LCOs in SMH12 (Fig. 2b, lane 4). This could be due to the absence in these exudates of the isoflavonoids daidzein or genistein, described as two strong inducers of the *nod* genes in *S. fredii* (Vinardell et al. 2004). However, these isoflavonoids were also absent in the exudates obtained in the presence of salt and Aur9. As previously mentioned, this soybean root exudate could only induce weakly the biosynthesis of LCOs in SMH12 that was not detected in soybean root exudates + NaCl uninoculated (Fig. 2b, lanes 4 and 6). Recent reports have shown that co-inoculation of soybean plants with *S. fredii* SMH12 and Aur9 significantly improved root and shoot growth and increased nodule number and mass in both moderate saline (25 mM NaCl) and non-saline conditions (Estévez et al. 2009). Moreover, under salt stress conditions SMH12 induced a significantly lower number of nodules than those induced in control conditions. These results are in agreement with the results shown in this paper, in which it has been demonstrated that under saline conditions soybean roots did not exude genistein and daidzein. However, soybean plants co-inoculated with Aur9 showed a better symbiotic performance than single inoculations despite the fact that in the presence of this bacterium soybean roots exuded less flavonoids than in control conditions. Thus, the presence of the PGPR Aur9 could trigger in soybean plants a response that could enhance the capacity of SMH12 to nodulate soybean. In fact, some PGPR are able to promote root hair development and hence possible sites for rhizobial entry (Lucas-García et al. 2004; Spaepen et al. 2007). All these results would indicate that there could be other factors involved in the symbiosis between soybean and SMH12, apart from flavonoids and Nod factors, which are also important for nodulation efficiency.

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