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ARTICLE

Biotic Elicitation of Isoflavone Metabolism with Plant Growth Promoting Rhizobacteria in Early Stages of Development in *Glycine max* var. Osumi

Beatriz Ramos-Solano, Elena Algar,* Ana García-Villaraco, Jorge García-Cristóbal, J. Antonio Lucas García, and F. Javier Gutierrez-Mañero

Department of Biology, School of Farmacy, San Pablo CEU University, P.O. Box 67, Boadilla del Monte, 28668 Madrid, Spain

Nine plant growth-promoting rhizobacteria from different backgrounds were assayed on *Glycine max* var. Osumi to evaluate their potential as biotic elicitors to increase isoflavone (IF) levels. Strains were inoculated on 2 day old pregerminated seeds. Six days after inoculation, the seedlings were harvested. Biometric parameters were registered, and IFs were determined. Although only one strain (N21.4) increased total IF contents and only one (M84) caused significant decreases in total IF, five different behaviors were detected when the daidzein and genistein families were analyzed separately. All strains triggered IF metabolism so further studies have to be developed since the different beneficial effects of IF through the diet may be due to the different IF profiles. These are encouraging results from two points of view: (1) N21.4 increases IF in seedlings, and (2) all other beneficial strains trigger IF metabolism differentially; hence, both facts could be used to prepare food supplements or as enriched standardized foods after full development of the biotechnological procedure.

KEYWORDS: Isoflavones; PGPR; biotic elicitation; systemic induction; ISR

INTRODUCTION

Soybean is a grain legume for human and cattle consumption due to its high protein content and to the benefits of isoflavones (IFs) for health (*1*). At present, soybean plays a crucial role in both the field of food and the pharmaceutical industry. In the field of food, benefits are attributed to its high content of functional and nonfunctional components (fiber, complex carbohydrates, vegetable protein, mainly unsaturated fatty acids, vitamins, and minerals). The effects of functional or bioactive components (IF) range from protective properties against the development of many chronic diseases including specific tumors (uterus, breast, and endometrial), cardiovascular disease (atherosclerosis and myocardial infarction), climacteric symptoms (hot flashes, sweats, insomnia, etc.) and postmenopausal osteoporosis (*1*, *2*). IFs have a remarkable therapeutic potential and can be delivered either through the diet (bioactive or nutraceuticals) or as food supplements, and this has opened a new market for industry.

However, because of the inducible nature of secondary metabolism, IF levels change according to environmental conditions (*3*, *4*); therefore, effects on health through the diet are not consistent, since a relation between the dose and the response may not be established. This lack of reproducibility may be overcome by means of elicitation (*5*), that is, triggering plant's metabolism with a molecule, the elicitor, that can be of a different nature (*6*). So far, elicitors have been grouped into two distinct blocks: abiotic factors (light intensity, temperature, and chemicals) (*7*−*10*) and biotic factors (pathogenic bacteria, beneficial bacteria, fungi, and insects) (*4*, *11*, *12*).

Biotic elicitation with plant growth-promoting rhizobacteria (PGPR) is proposed as a useful strategy to improve biomass production and to trigger secondary metabolism at the same time (*13*). Upon recognition of the nonpathogenic biotic agent, a series of metabolic changes are systemically initiated throughout the plant to activate defensive metabolism (*13*, *14*). Using PGPR as elicitors to trigger secondary metabolism has a double advantage: First, in some plant species like soybean, defensive metabolites are bioactive compounds constituting food products with an added value for human health; second, from a physiological point of view, this initial increase in secondary metabolites indicates that the biotic agent would have a *priming* effect on the plant. Primed plants have a specific metabolic state that allows a better performance upon pathogen challenge (*10*, *12*, *15*), although plant growth may be compromised in the first stages (*10*). Although simultaneous induction of growth and accumulation of secondary metabolites are rare in nature, the use of selected PGPR bacteria, some of its cellular components, and biotic factors, in general, to increase levels of some secondary metabolites has been demonstrated in various studies, as in *Digitalis lanata* (*16*), or in soybean for IF (*4*, *17*−*19*), or to enhance defense against pathogens in other plant species such as the model plant *A. thaliana* (*12*, *14*). However, not all efforts

*To whom correspondence should be addressed. Tel: + 34 913 726 411. Fax: +34 91 351 04 96. E-mail: elalgar@ceu.es.

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Table 1. Characteristics of the PGPR Strains Used in This Work: Morphology, Gram Stain, Spore Formation, Origin, Biological Activity, Most Significant Alignment of the 16s RNA Gene Partial Sequence[a]

strain	morphology	Gram	spores	source	biological activity	alignment
Aur9 CECT 5399	bacilli	−	−	*Lupinus albus* rhizosphere	• production of auxins (*22*) • ISR in *Arabidopsis thaliana* (*12*), *Solanum lycopersicum* and *Capsicum annuum* (*23*)	*Chryseobacterium balustinum*
Aur6 CECT 5398	bacilli	−	−	*Lupinus albus* rhizosphere	• production of auxins and siderophores (*22*) • ISR in *Solanum lycopersicum* and *Capsicum annuum* (*23*)	*Pseudomonas fluorescens*
BB1 CECT7170	bacilli	+	−	*Pinus pinea* rhizosphere	• production of auxins and siderophores (*24*) • ISR in *Arabidopsis thaliana* (*25*)	*Arthrobacter oxidans*
N5.18	bacilli	−	−	*Nicotiana glauca* rhizosphere	• production of siderophores and chitinases* • ISR in *Arabidopsis thaliana* (*26*)	*Stenotrophomonas maltophilia**
N11.37	bacilli	+	+	*Nicotiana glauca* rhizosphere	• production of siderophores and chitinases* • ISR in *Arabidopsis thaliana* (*26*)	*Bacillus subtilis* HJ19*
N21.4	bacilli	−	−	*Nicotiana glauca* rhizosphere	• production of siderophores and chitinases* • ISR in *Arabidopsis thaliana* (*26*)	*Pseudomonas fluorescens**
N6.8	bacilli	−	−	*Nicotiana glauca* rhizosphere	• production of siderophores and chitinases* • ISR in *Arabidopsis thaliana* (*26*)	*Stenotrophomonas maltophilia* 6B2-1*
M84	bacilli	+	−	*Pinus pinea* mycosphere	• phosphate solubilization (*24*) • ISR in *Arabidopsis thaliana* (*25*)	*Curtobacterium* sp. SG041
L81	bacilli	+	+	*Pinus pinea* rhizosphere	• production of siderophores (*24*) • ISR in *Arabidopsis thaliana* (*25*)	*Bacillus* sp.

[a] ISR, induced systemic resistance. *Submitted.

should address the increase of secondary metabolites for example in soybean. Recent studies have shown that excessive consumption of IF can have adverse health effects (*20*, *21*), which reveals that there is a demand for the food product with a controlled content of IF (*3*), another challenge that can also be achieved by using PGPR.

Therefore, there is a great interest in finding effective biotechnological methods to obtain consistent and reproducible induction of these secondary metabolites in soybean plants with two main purposes: on one hand, to increase IF contents, mainly addressing the preparation of food supplements, and, second, to obtain plants with the normalized IF contents for dietary intake directed to novel food market. On the basis of the foregoing, the objective of this study was to evaluate the ability of nine PGPR from different backgrounds to cause a systemic stimulation of soybean metabolism in early stages of growth, evaluating growth and IF as metabolic markers of the induction.

MATERIALS AND METHODS

Plant Material. *Glycine max* var. Osumi plants belong to the so-called short cycle and were kindly provided by Dra. Rodríguez Navarro at (CIFA) Las Torres-Tomejil, Sevilla.

Bacterial Strains. The bacterial strains used in this study were isolated from natural populations of the rhizosphere or mycosphere of wild plants and have demonstrated their ability to alter the metabolism of the plant or possess some metabolic capabilities that suggest their ability to modify the physiology of plants. In **Table 1**, a brief description of each strain, their origin, and their demonstrated beneficial skills are shown.

Inoculum Preparation. Bacterial strains were maintained at −80 °C in nutrient broth with 20% glycerol. Inoculum was prepared by streaking strains from −80 °C onto plate count agar (PCA) plates, incubating plates at 28 °C for 24 h, and scraping bacterial cells off the plates into sterile 10 mM SO_4Mg buffer. Inoculation was done by soil drench with a bacterial density such to achieve 10^8 ufc/mL substrate.

Experimental Design. The nine bacterial strains were tested in four independent experiments, each with its own control. Strain N21.4 was tested in experiment A. In experiment B, N5.18 and Aur6 were tested. The third experiment (experiment C) was conducted with Aur9, M84, and BB1, and, finally, in experiment D, N6.8, N11.37, and L81 were evaluated.

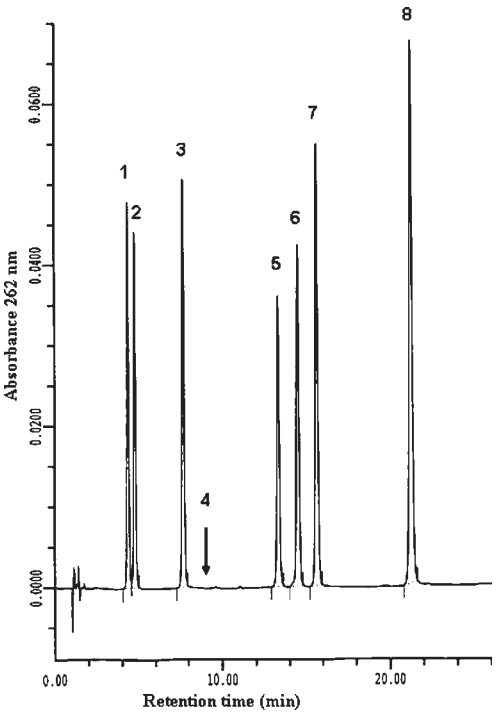

Figure 1. HPLC chromatogram of the IF standards used. Peaks: 1, daidzin; 2, glycitin; 3, genistin; 4, malonyl daidzin (retention time identified by HPLC/MS); 5, malonyl genistin; 6, daidzein; 7, glycitein; and 8, genistein.

Soybean seeds were sterilized in 70% ethanol by stirring for 1 min, 5% bleach for 6 min, and five washes with distilled water. Then, sterilized seeds

Table 2. Fresh Weight (Shoots, Cotyledons, Roots, and Total) (g) and Shoot Length (cm) of 8 Days Old Seedlings Inoculated with the Nine PGPR and Noninoculated Controls in the Four Experiments[a]

		fresh weight (g)				
		shoot	cotyledons	root	total	shoot length (cm)
experiment A	control	0.317 ± 0.014 a	0.475 ± 0.024 a	0.172 ± 0.018 a	0.949 ± 0.042 a	11.071 ± 0.221 a
	N21.4	0.278 ± 0.010 b	0.443 ± 0.019 a	0.246 ± 0.019 b	0.974 ± 0.040 a	10.393 ± 0.220 b
experiment B	control	0.258 ± 0.006 a	0.478 ± 0.019 a	0.226 ± 0.014 a	0.944 ± 0.029 a	9.531 ± 0.196 a
	Aur6	0.247 ± 0.010 a	0.453 ± 0.017 a	0.260 ± 0.012 a	0.963 ± 0.030 a	9.811 ± 0.272 a
	N5.18	0.236 ± 0.009 a	0.453 ± 0.013 a	0.217 ± 0.013 a	0.907 ± 0.022 a	9.160 ± 0.268 a
experiment C	control	0.254 ± 0.011 a	0.498 ± 0.020 a	0.481 ± 0.019 a	1.241 ± 0.038 a	7.707 ± 0.187 a
	BB1	0.264 ± 0.008 a	0.502 ± 0.023 a	0.437 ± 0.015 a	1.208 ± 0.031 a	7.813 ± 0.181 a
	Aur9	0.265 ± 0.012 a	0.484 ± 0.020 a	0.457 ± 0.017 a	1.210 ± 0.040 a	7.800 ± 0.169 a
	M84	0.240 ± 0.011 a	0.482 ± 0.032 a	0.474 ± 0.023 a	1.221 ± 0.054 a	7.500 ± 0.181 a
experiment D	control	0.283 ± 0.012 a	0.462 ± 0.019 a	0.444 ± 0.017 a	1.186 ± 0.041 a	8.544 ± 0.202 a
	N6.8	0.280 ± 0.011 a	0.472 ± 0.023 a	0.458 ± 0.015 a	1.215 ± 0.040 a	8.359 ± 0.235 a
	N11.37	0.276 ± 0.010 a	0.441 ± 0.018 a	0.489 ± 0.016 a	1.210 ± 0.036 a	8.707 ± 0.220 ab
	L81	0.296 ± 0.010 a	0.475 ± 0.017 a	0.468 ± 0.016 a	1.233 ± 0.035 a	9.286 ± 0.189 b

[a] Data are the means ± SE (n = 21). Different letters indicate significant differences between treatments within each experiment for each of the evaluated parameters according to the LSD test ($p < 0.05$).

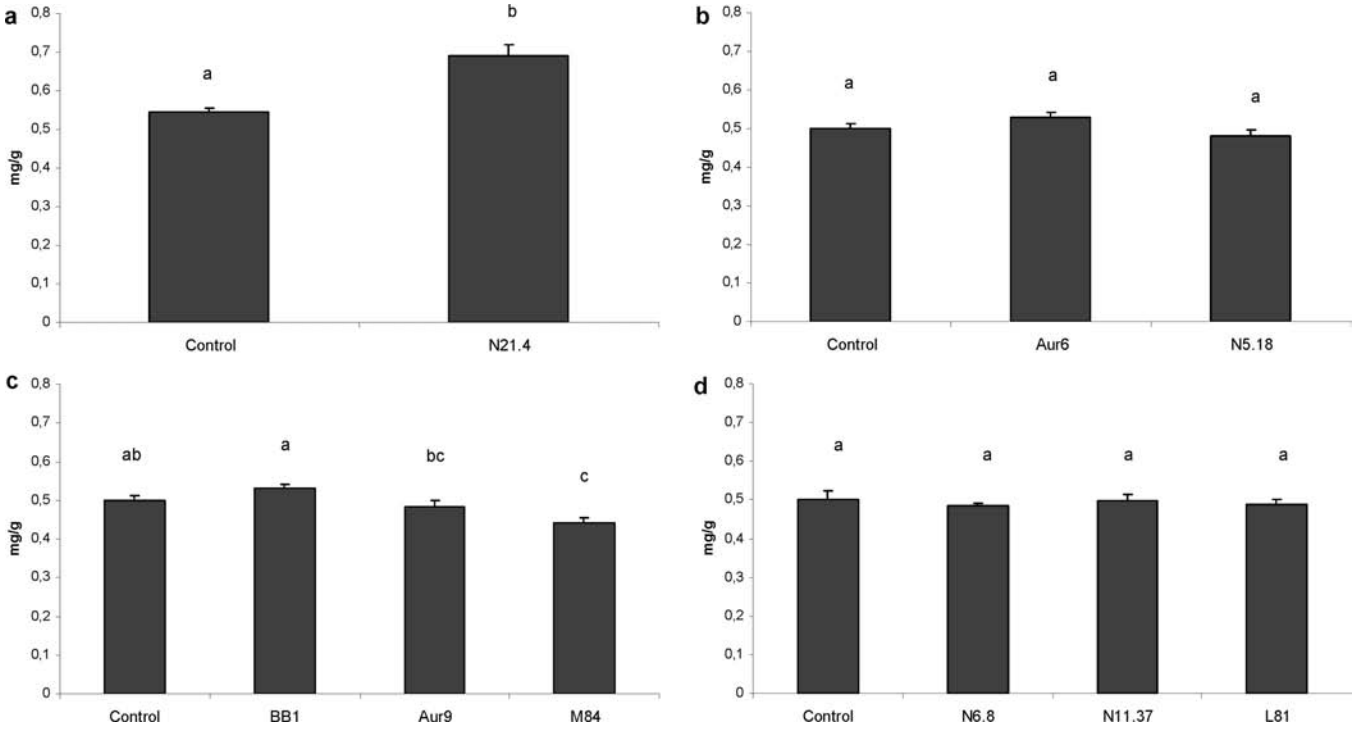

Figure 2. Concentration (mg/g) of IFs in 8 day old soybean seedlings inoculated with the nine PGPR and noninoculated controls in the four different experiments. Data are the means ± SE (n = 3). Different letters indicate significant differences between treatments within each experiment according to LSD test ($p < 0.05$).

were sown in 50 mL pots filled with sterile vermiculite (40 min at 121 °C). Two days after they were sown, each PGPR was inoculated by soil drench; the control group was mock-inoculated with 10 mL of $MgSO_4$ (10 mM) buffer. Plants were watered upon inoculation and 3 days after with 5 mL of distilled water. They were kept in a growth chamber SANYO (Growth Cabinet MLR-350H) under controlled conditions (16/8 h light/dark, 30/27 °C, 60% relative humidity, and light intensity of 350 μE m^{-2} s^{-1}). Six days after inoculation, plants were harvested, biometric parameters were determined, and the IFs present in the shoots, cotyledons, and roots were analyzed by high-performance liquid chromatography (HPLC).

Biometrical Parameters Measurement. Seedling growth was determined 6 days after inoculation, evaluating the following parameters: shoot length (cm), shoot fresh weight (g), cotyledons fresh weight (g), root fresh weight (g), and total fresh weight (g) (n = 21).

Extraction and Determination of IFs. Plants from each treatment (n = 21) were split in three groups, and each constituted a replicate. Each replicate consisted of the shoots, cotyledons, or roots of seven plants. Replicates were powdered with liquid nitrogen and kept at −80 °C, until extraction (1 g) with 30 mL of 80% methanol under continuous shaking (145 rpm) for 15 h at 40 °C. Then, samples were centrifuged at 4500 rpm for 20 min at 20 °C, supernatants were filtered through a 0.45 μm nylon membrane, and methanolic extracts were injected onto HPLC.

Identification and quantification of IF were performed on a Beckman HPLC provided with a two-pump 125 solvent module and a 168 diode array detector. Chromatographic conditions were as follows: UV detection, 262 nm; C18 Phenomenex Luna column (5 μm, 150 mm × 4.6 mm), kept at 30 °C with a Gecko-2000 30−80 °C thermostat; the mobile phase

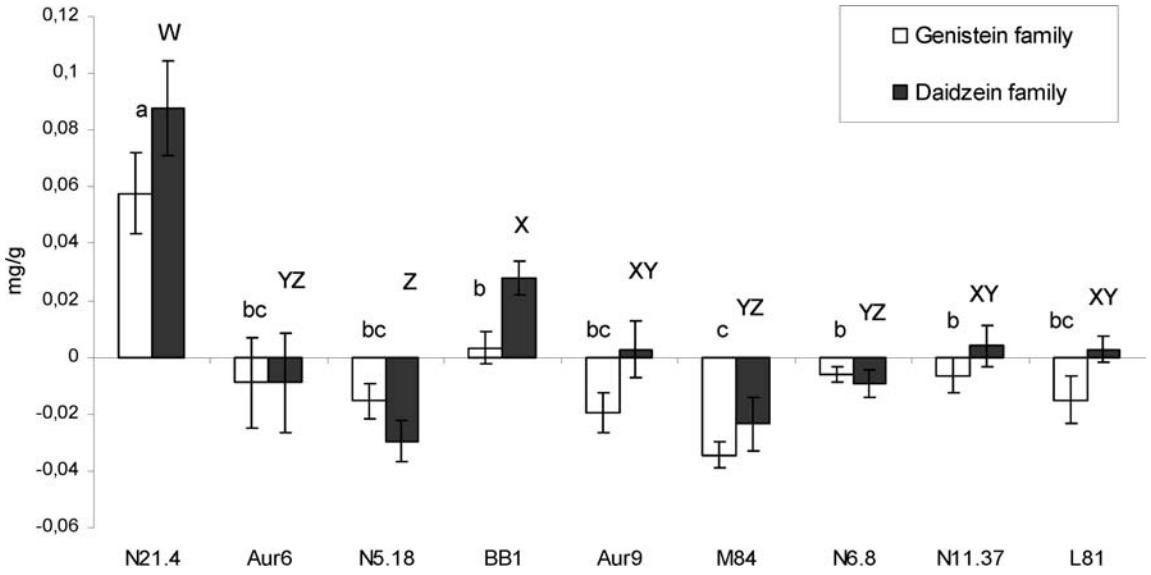

Figure 3. Variation of IFs concentration (mg/g) in 8 day old soybean seedlings inoculated with the nine PGPR with respect to their noninoculated controls. Data are the means ± SE ($n = 3$). Different letters indicate significant differences between treatments on the genistein family (a, b, and c) or the daidzein family (x, y, and z) according to LSD test ($p < 0.05$).

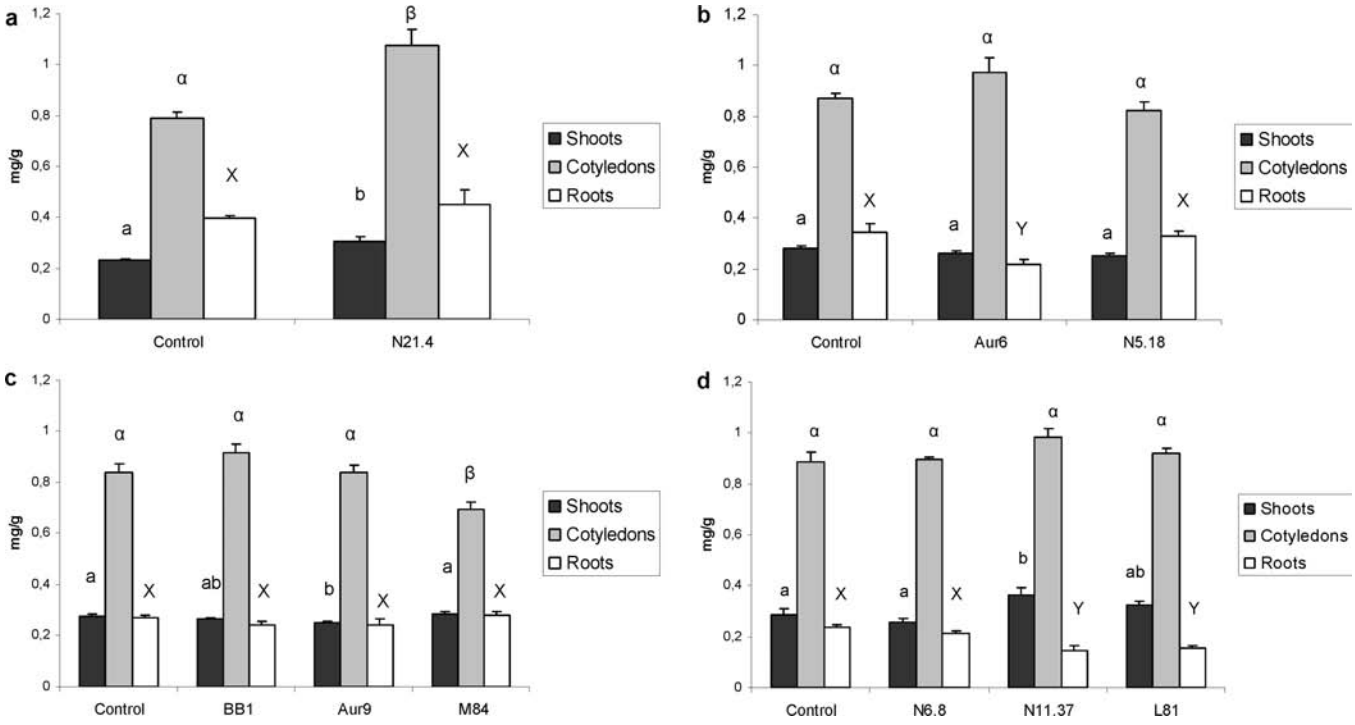

Figure 4. Concentration (mg/g) of IF in shoots, cotyledons, and roots of 8 day old soybean seedlings inoculated with the nine PGPR in the four experiments. Data are the means ± SE ($n = 3$). Different letters indicate the existence of significant differences according to LSD test ($p < 0.05$) between the different treatments on shoots (a, b, and c), cotyledons (α, β, and γ), and roots (x, y, and z).

was solvent A = water + 0.1% acetic acid and solvent B = acetonitrile + 0.1% acetic acid, with the following gradient: 15−45% B in 40 min and then increased to 100% B in 1 min and remaining at this composition for 9 min, after which it decreased to the initial conditions (15% B) in 1 min and was kept constant for 9 min to restore initial conditions. The flow was set at 1.5 mL/min, and the injection volume was 10 μL. IF quantification was done by interpolating relative area counts into indirect calibration curves for each, done with a commercial standard. The indirect calibration curves were constructed with the commercial IF (LC Laboratories): daidzein, daidzin, genistein, genistin, and malonyl genistin (**Figure 1**). The calibration curve of malonyl daidzin was the same as daidzin. Because the malonyl group does not contain an ultraviolet chromophore, it was hypothesized that the absorption properties of the β-glucoside structures at 262 nm should not be modified by a malonyl conjugation and that their response factor only depended on their molecular weight (*3*).

Statistical Analyses. To evaluate bacterial effects on growth, and IF content, one-way analysis of variance was performed. When differences were significant, the least significant differences (LSD) posthoc test was also performed (*27*) with the software Statgraphics plus 5.1, for Windows.

RESULTS AND DISCUSSION

Although elicitation of IF metabolism in soybean has been approached in different ways, this is the first time that free-living

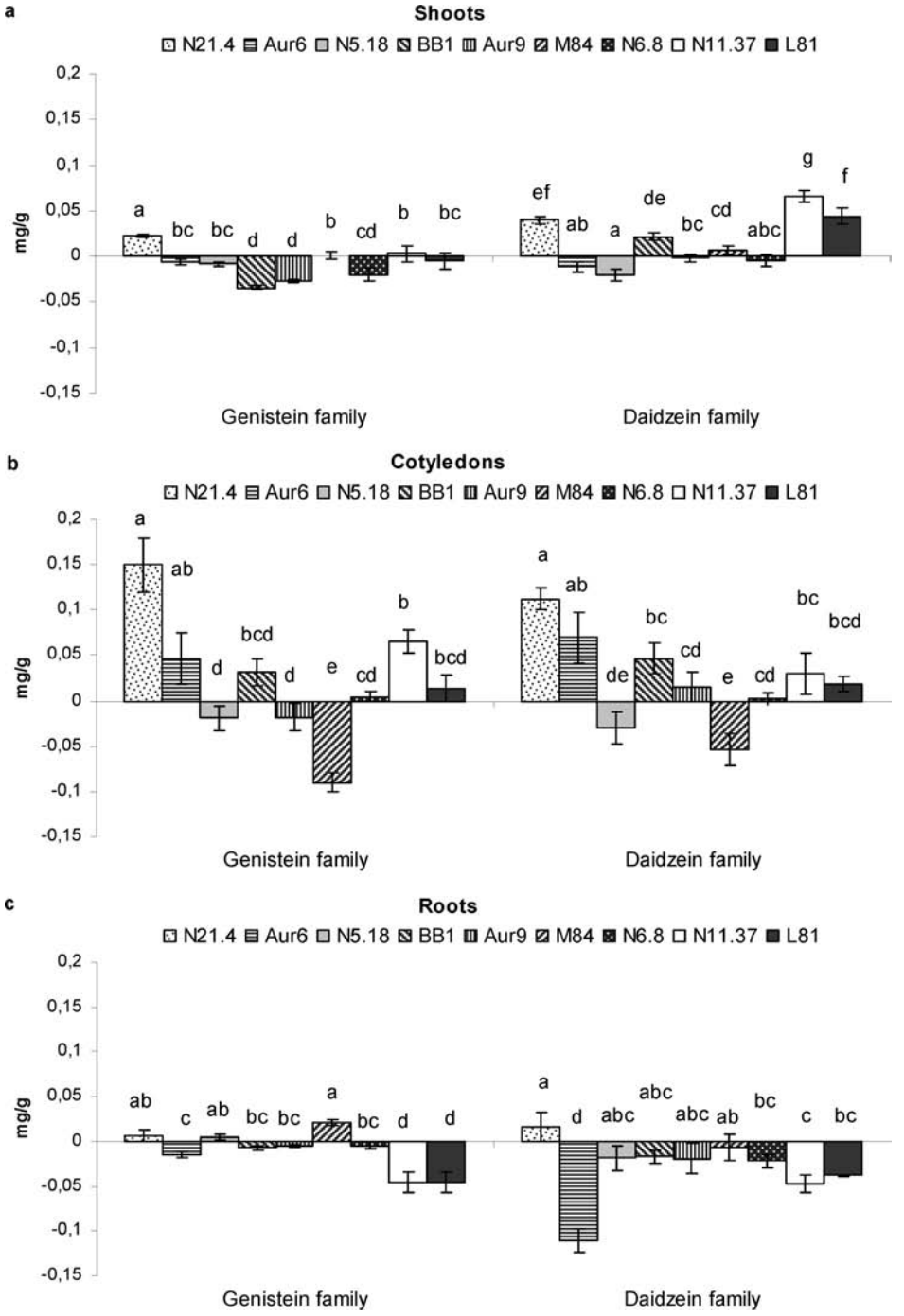

Figure 5. Variation of IF of the genistein and daidzein family concentration (mg/g) on shoots (**a**), cotyledons (**b**), and roots (**c**) of 8 day old soybean seedlings inoculated with the nine PGPR with respect to their noninoculated controls. Data are the means ± SE ($n = 3$). Different letters indicate significant differences according to the LSD test ($p < 0.05$) between treatments in each IF family.

nonpathogenic beneficial bacteria have been used for this purpose in soybean. The effects of the nine bacterial strains on soybean seedling growth appear in **Table 2**. The total fresh weight averaged 1.1 g, and this parameter was not affected by any strain in this short experiment, in which growth depended mostly on cotyledons, which accounted for almost 60% of total weight (0.5 g), while shoots and roots accounted for the remaining 40% (0.2 g of shoots and 0.3 g of roots). Only N21.4 significantly decreased shoot weight. The shoot length ranged from 7 to 11 cm in the four experiments; despite the difference, this parameter was consistent within each experiment. Only two strains significantly affected shoot length: N21.4 caused a decrease, while L81 increased it. Despite the apparent lack of effect on growth in this experiment, different results may appear on longer experiments in which PGPR may exhibit other mechanisms involved in nutrient improvement.

It has been shown that upon certain stimuli, plants detour energetic resources to secondary defensive metabolism, compromising growth, but in turn, these plants will perform better upon stress challenge (*10*, *15*). Therefore, on the basis of this effect and considering the role of IF in plant defense (*17*), an increase in IF could be expected on N21.4-treated plants since it was the only treatment that significantly decreased growth parameters.

Hence, IFs were analyzed with a double aim: as secondary metabolites involved in plant's defense (*17*) and because of their relevance to human health (*1*). The experimental design was set up

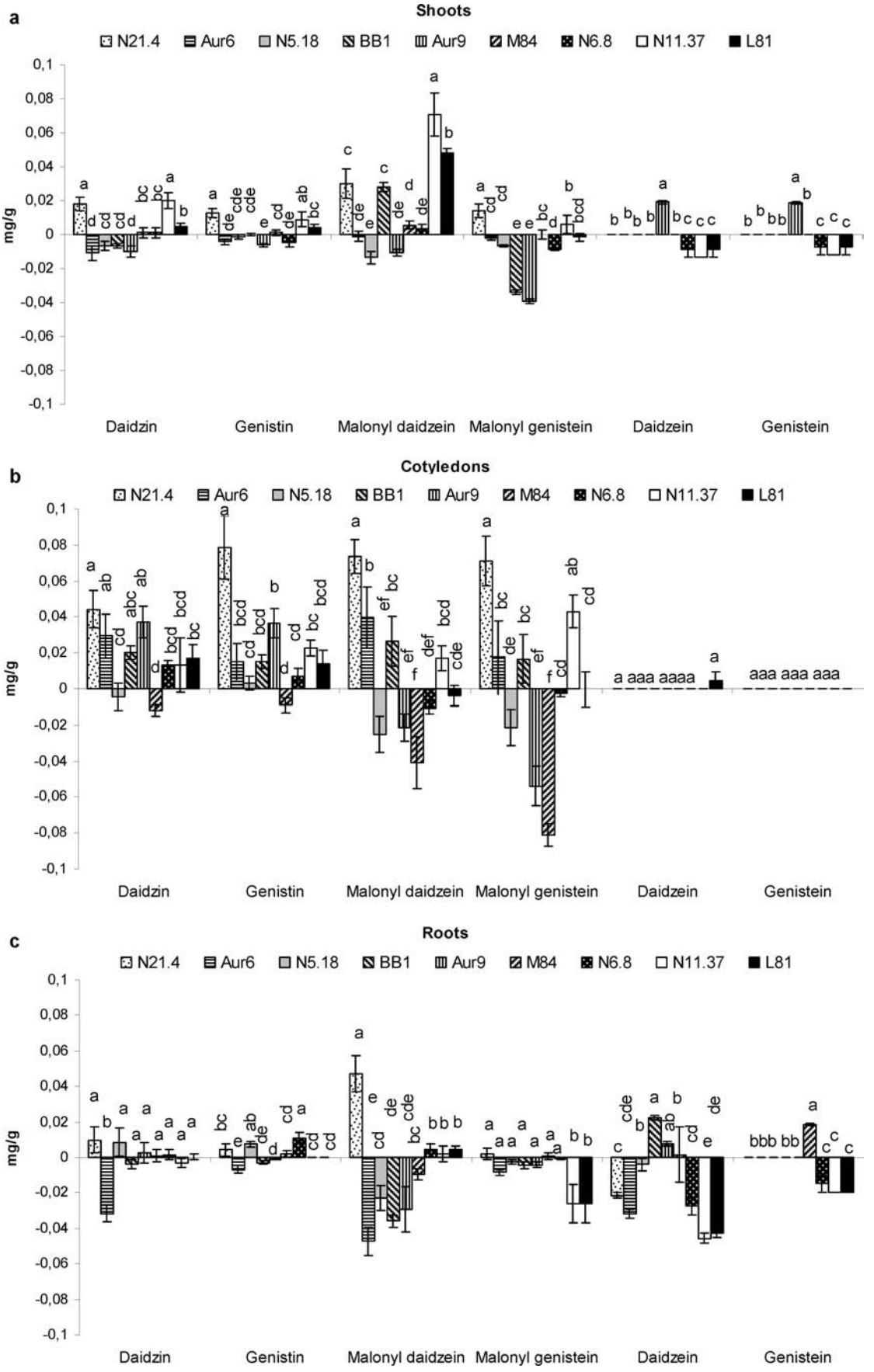

Figure 6. Variation of the three chemical species of daidzein and genistein (daidzin, malonyl daidzin, daidzein, genistin, malonyl genistin, and genistein) concentration (mg/g) on shoots (**a**), cotyledons (**b**), and roots (**c**) of 8 day old soybean seedlings inoculated with the nine PGPR with respect to their noninoculated controls. Data are the means ± SE ($n = 3$). Different letters indicate significant differences according to the LSD test ($p < 0.05$).

to detect (1) if any of the strains would increase total IF content, (2) if the daidzein and genistein families would be affected to the same extent, and (3) in what part of the plant these changes would occur and which chemical forms would be affected to a greater extent, since the overall IF profile and content may affect health differentially (*4*, *28*−*30*). The IF content was determined in each of the three parts defined (cotyledons, shoots free of cotyledons, and roots). Then, results were combined, and data are presented to answer several of the questions set in the working hypothesis.

Figure 2 shows the total IF content per plant. The total IF content on 8 day old soybean seedlings used in these experiments is around 0.5 mg per g of plant, as seen in the four panels in **Figure 2** in the noninoculated controls. It should be pointed out that only two strains of the nine tested were able to significantly affect the total content on IF: a positive effect caused by strain N21.4 and a decrease caused by M84, despite the decrease in growth detected for both strains, which was only significant under N21.4 (**Table 2**). There are many factors that are able to increase IF in early stages of development including wounding, pathogens, or elicitors (*17*, *31*−*34*), which is consistent with the increase detected on N21.4-treated plants. However, the decrease on IF caused by M84 has not been reported before. It has been shown in different plant tissues that IFs are rapidly hydrolyzed to aglycons upon fungal pathogen challenges; therefore, the decrease on IF following inoculation with M84 could be attributed to the rapid transformation of the aglycons on other phytoalexins such as glyceollins (*33*). This hypothesis suggests that M84 behaves as an avirulent pathogen since it triggers this transformation more actively than all other strains and opposite to N21.4, which seems to trigger IF biosynthesis instead of transformation on other defensive compounds.

Because it has been shown that effects on health may change depending on the relative amounts of IF (*4*), our next hypothesis was if any of the tested strains would differentially affect the two IF families: daidzein or genistein. For this purpose, data from the three daidzein species (daidzein, daidzin, and malonyl daidzin) and from the three genistein species (genistein, genistin, and malonyl genistin) were combined, and to evaluate if there were differences among bacterial strains, increases or decreases on IF content between treated plants and their own control were calculated (**Figure 3**). Five different effects were detected as follows: (1) N21.4 increased both families, with the increase in daidzein family being more marked; (2) BB1 increased only the daidzein family; (3) M84 decreased both, although it decreased the genistein family to a greater extent, which rules out transformation to glyceollins that derive from daidzein; (4) N5.18, with the opposite effect, decreased both, although it was more marked on the daidzein family; and (5) there was a slight decrease on the genistein family and a slight increase on the daidzein family involving Aur9, N11.37, and L81. From **Figure 2**, only two different behaviors were detected. However, a deeper insight has shown five groups. These five groups suggest that these bacteria trigger soybean metabolism differentially, so elicitors and induced pathways should be studied on one hand, and second, each could be used to produce soybean with different IF profiles addressing different human targets, to be determined in further studies.

The next question to answer was in which of the three parts analyzed where the observed changes in IF take place. For this purpose, data were presented in the four different experiments as distribution of IF in the three parts (**Figure 4**).

IF in shoots and roots averaged 0.3 mg/g each, while cotyledons accounted for 0.9 mg/g. Increases found for N21.4 occurred in shoots and cotyledons, while decreases in M84 took place in cotyledons. These results are consistent with data from total IF (**Figure 2**) that showed significant increases in N21.4-treated plants and significant decreases in M84-treated plants. These changes point out that in the first 8 days of development, N21.4 is stimulating synthesis of IF de novo and translocation to shoots, while M84 is blocking IF synthesis de novo, since the decrease of IF in cotyledons is not coupled to increases in shoots or roots (**Figure 4**). Interestingly, N11.37 and L81 do not affect de novo synthesis but enhance translocation of IF to shoots based on the decrease detected in roots, which is not detected under BB1. These data suggest the existence of different mechanisms underlying the asymmetric distribution induced by each of the strains that do not affect de novo biosynthesis, as in N21.4. The increase on IF in shoots suggests that N11.37 and L81 cause IF mobilization toward the upper part of the plant, where they may play a role in defense, as suggested for these compounds (*19*, *33*, *35*−*37*). Although Aur6 was not outstanding on total IF content, it is worth mentioning that the decrease detected on roots could be correlated with the role of IF on nodulation, since for soybean to nodulate with their rhizobia, IFs need to be released to soil (*38*). If this was the case, Aur6 could be considered as a nodulation-helper bacteria as shown before by Lucas Garcia et al. (*39*) on coinoculation studies. Also, there was a significant decrease on IF contents on shoots of Aur9-treated plants, although it was not coupled to other changes. Although it has been said that IFs are defensive metabolites, it has also been shown that they represent a form of accumulation that will be immediately transformed in the real active defensive compound the pterocarpans glyceollins (*4*). Hence, it could be hypothesized that the decrease in IF caused by Aur9, a strain with a good background on activation of defensive metabolism (*12*, *22*, *23*), could be coupled to enhancement of other defensive compounds that have not been analyzed and could have effects on human health (*4*, *29*, *30*).

Once changes in IF were identified within the plant; the next question was if they could be attributed to daidzein or genistein families. IF increases detected in shoots of L81-, N11.37-, and N21.4-inoculated seedlings (**Figure 4a**,**d**) were due to increases in the daizein family except for N21.4 that also increased genistein family (**Figure 5a**); increases were due mostly to glucosides and malonylglucosides (**Figure 6a**), speaking of transport forms (*33*, *37*). Significant changes in cotyledons caused by N21.4 and M84 (**Figure 4a**,**b**) were due to both families (**Figure 5b**), and changes were detected in the glucosides and malonylglucosides, suggesting transport or accumulation forms, while the putative active forms in defense, the aglycons, showed nonsignificant changes at all (**Figure 6b**). This situation is consistent with information in the literature that reports that raw soybeans contain predominately the glucoside forms of the IF and a low percentage of the aglycone forms (*40*). Interestingly, decreases caused by M84 in cotyledons were due to decreases in malonylglucosides (**Figure 6b**). With regard to significant decreases detected in roots (**Figure 4b**,**d**), there were different behaviors between the strains. While Aur6 decreased mainly the daidzein family (**Figure 5c**) in the three chemical forms (**Figure 6c**), N11.37 and L81 decreased both families to the same extent (**Figure 5c**), affecting only the aglycons and the malonyl genistin (**Figure 6c**).

In summary, among the nine strains tested, five different behaviors were detected, one increased total IF content, and the other four triggered IF metabolism differentially, causing an asymmetric distribution throughout the plant. The nonpathogenic bacteria used here as biotic elicitors appear as good candidates to trigger soybean defensive responses and with a good potential application in sustainable field production due to the involvement of IF in defense and symbiosis establishment; furthermore, the differential ability to trigger IF metabolism and

distribution may have a dramatic impact on agriculture and health, since beneficial bacteria can be used as biocontrol agents or biofortificants, different from other biotic elicitors with a fungal nature. This data should be further studied to develop two products: first, soybean as a functional food with standarized IF contents, and, second, high-quality food supplements more profitable for industry since the same seedlings would have higher and diverse IF contents.

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