Identification of Plant Compounds Involved in the Microbe-Plant Communication During the Coinoculation of Soybean with *Bradyrhizobium elkanii* and *Delftia* sp. strain JD2

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Delftia sp. strain JD2 is a betaproteobacterium characterized as a plant growth-promoting bacterium with a ‘helper’ function, enhancing the performance of rhizobial inoculant strains during the coinoculation of alfalfa and clover. In this work we analyzed i) the effect of the coinoculation with *Bradyrhizobium elkanii* and *Delftia* sp. strain JD2 strains on the performance of soybean plants and ii) the production of a few secondary plant metabolites that would explain the positive effect of coinoculation on the growth and development of soybean plants. The results showed a beneficial effect of coinoculation on soybean growth, nodulation rate, and pulse yield, with the concomitant benefit for the agricultural economy. In addition, based on a metabolomics approach, we demonstrated that a different pattern of plant metabolites is being produced at different stages of plant growth. The new information suggests that the coinoculation of soybean changes the primary and secondary metabolism of the plant, including changes in the metabolic status of main and secondary nodules within the plant. The relevance of producing a different pattern of photosynthetic and photoprotective pigments, flavonoids, organic acids, and carbohydrates are discussed. Finally, we propose that JD2 could be used together with bradyrhizobia to manipulate the chemical composition of plant tissues, promoting the nutritional benefits and health of soybean.

Delftia sp. strain JD2 is a Cr(VI)-reducing betaproteobacterium (family Comamonadaceae) that was isolated by our working team many years ago (Morel et al. 2011). JD2 was characterized as a plant growth-promoting bacterium with a ‘helper’ function, enhancing the performance of rhizobial inoculant strains during the coinoculation of alfalfa and clover

(Morel et al. 2011, 2015; Ubalde et al. 2012). This bacterium fixes atmospheric nitrogen when growing in N-free semisolid medium and produces the phytohormone indole-3-acetic acid (IAA) and siderophores (Morel et al. 2011). The analysis of the draft genome from JD2 suggests that IAA is produced by the indole-3-acetamide pathway and that JD2 has the genetic machinery to produce and uptake the siderophore pyoverdine but also has the machinery for the acquisition of Fe-pyochelin and Fe-enterobactin (Morel et al. 2016).

Experiments of coinoculation of alfalfa with *Sinorhizobium meliloti* U143 (an indigenous strain used as inoculant in Uruguay) (Castro-Sowinski et al. 2002) and JD2 showed an increase of shoot and root dry matter (measured as dry weight) up to 13 and 34%, respectively, in greenhouse conditions, compared with single rhizobial inoculation (Morel et al. 2015). The results support the idea that the coinoculation with JD2 improves agronomic expectations compared with single-rhizobial inoculation, as it has also been shown with the coinoculation of leguminous plants with rhizobial and azospirilla strains (Burdman et al. 1997; Hungria et al. 2013, 2015; Itzigsohn et al. 1993).

The positive effect of *Azospirillum* (alphaproteobacterium; family Rhodospirillaceae) in the nodulation and nitrogen fixation by rhizobia strains on several pulse and forage legumes has been extensively reported (increase in shoot length and weight, number of root hairs, root diameter, number of root nodules, and the percentage of infected root hairs), thus resulting in increased legume yields (Morel et al. 2012). This plant growth-promoting effect has been explained by the production of plant hormones by *Azospirillum* spp.; however, the mode of action is currently better explained as an additive and selective effect (Cassán and Díaz-Zorita 2016). Most azospirilla produce auxin, a phytohormone that promotes root development and proliferation, leading to enhanced nutrient uptake and increasing the number of potential niches for beneficial microbial infection (Castillo et al. 2015; Morel and Castro-Sowinski 2013). Even though both *Delftia* and *Azospirillum* spp. strains belong to different classes, they may display similar mechanisms of plant-microbe interaction.

The aims of this work were to analyze the performance of soybean plants subjected to coinoculation with *Bradyrhizobium elkanii* and *Delftia* sp. strain JD2 and to contribute to the understanding of the plant response during various stages of plant growth. We conducted gnotobiotic and greenhouse experiments, showing the beneficial effect of coinoculation in plant

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growth, nodulation rate, and pulse yield; in addition, based on a metabolomics approach, we showed that different patterns of plant metabolites are produced at different stages of plant growth.

RESULTS

Does the coinoculation of soybean with bradyrhizobia and JD2 have an effect on plants growth in gnotobiotic and greenhouse conditions?

The ability of *Delftia* sp. strain JD2 to improve biomass production and nodulation during the inoculation and coinoculation of alfalfa and clover plants was already shown by Morel et al. (2011; 2015) and Ubalde et al. (2012); thus, in this work, we evaluated the effect of the coinoculation of soybean plants with *B. elkanii* strains and JD2 in shoot, root, and pod or seed yields and nodulation rate. Results from gnotobiotic plant experiments showed that, in both inoculation and coinoculation treatments, the first nodule appears 7 days after bacterization, but the nodulation rate was 1.2 and 2.0 nodules per plant per day, respectively, suggesting a positive effect of coinoculation on the bradyrhizobial-plant interaction (Fig. 1). The highest number of nodules per plant was achieved 22 days after bacterization, but coinoculated plants (23 nodules per plant) reached a higher number of nodules per plant, as compared with bradyrhizobial single-inoculated plants (17 nodules per plant). In addition, when plants grown in greenhouse conditions were collected at the V4 growth stage, only coinoculated plants showed nodules, suggesting an early nodulation induced by JD2. These results suggest that coinoculation with JD2 induces early nodulation and increases the number of nodules per plant.

The ability of *Delftia* sp. strain JD2 to improve soybean yield during inoculation and coinoculation experiments was assessed as shown in Table 1. In greenhouse conditions at the V4 stage, coinoculation increased shoot dry matter, plant height, and root dry matter up to 18, 34, and 80%, respectively, over bradyrhizobia-inoculated plants (Fig. 2B). Interestingly, single JD2 inoculation increased root dry matter by 43% compared with a single inoculation with bradyrhizobia, suggesting that JD2 affects soybean root development, at least during the first stages of growth. At R2 stage, results showed an increase of 4 and 14% in shoot dry matter and plant height but a 10% reduction in dry root dry matter, suggesting that the most important effect of coinoculation is detected at the first stages of growth. Reinforcing this result, the data obtained from

experiments in gnotobiotic conditions also support a positive effect of coinoculation on shoot dry matter and plant height (Table 1). In addition, results obtained by micro Kjeldhal experiments and the determination of ureides showed that the total content of total nitrogen and ureides of coinoculated plants were significantly higher than JD2- or bradyrhizobia-inoculated plants (Table 2), suggesting that coinoculation positively affect the acquisition of nitrogen. Nodules collected from R2 plants were cut in half, showing a reddish interior (Fig. 2D). This observation suggests that they are functionally active nodules.

Probably, the most important effect was observed in the harvest index (HI) (Table 1). Compared with the control treatment, results showed an increase of 35, 12, and 64% in HI of plants inoculated with JD2, bradyrhizobia, and coinoculated, respectively, suggesting that the coinoculation has an important beneficial effect on the most productive parameter of soybean, the HI.

Does coinoculation alter the profile of plant-produced metabolites?

We measured some plant performance characteristics, such as chlorophyll and carotenoid content (Table 3; Fig. 2C). A general overview of the table shows that plants from V4 present a much higher content of chlorophylls compared with plants collected at the R2 stage (flowering stage), but the carotenoid content was much higher in plants from the R2 stage. Results also support that coinoculation increased the pigment content of plants (chlorophylls and carotenoids) at the R2 stage, compared with single-inoculated plants. Both the chlorophyll a and chlorophyll b content increased 84 and 66%, respectively, in coinoculated plants compared with bradyrhizobial-inoculated ones (Table 3). The occurrence of a few sets of flavonoids (daidzein, genistein, luteolin, coumestrol), commonly involved in the soybean-bradyrhizobia interaction, was searched on roots and nodules of plants collected at the V4 and R2 stages, grown in greenhouse conditions (Fig. 2C) (the chromatographic profile can be seen in Supplementary Figure S1). These flavonoids were not detected in nodules, and a different pattern of flavonoids (presence/absence) was detected in roots of plants from different treatments.

At the V4 stage, we did not detect the presence of these flavonoids in the root extracts of noninoculated plants (control plants) and bradyrhizobia-inoculated plants showed a minor accumulation of daidzein. However, the accumulation of

Fig. 1. Data averages for nodules in **A**, main roots and **B**, secondary roots. Each point represents the average of all the plants at each date of recording. Upward pointing triangles (▲) represent plants inoculated with bradyrhizobia, downward pointing triangles (▼), coinoculated plants, and black squares (■), uninoculated plants.

daidzein, genistein, luteolin, and coumestrol was observed in the roots of JD2-inoculated and coinoculated plants. The relative integrated area of peaks showed that roots from coinoculated plants have a higher content (tenfold, data not shown) of

flavonoids rather than JD2- or bradyrhizobial-inoculated plants, suggesting that, in response to coinoculation, the soybean plants could modify their metabolism for the production of a higher amount of these flavonoids.

Table 1. Effects of different treatments on yield components of soybean^a

		Treatment			
Stage	Plant characteristics	Control	Inoculation with JD2	Inoculation with bradyrhizobia	Coinoculation
Greenhouse conditions					
V4 stage	SDM (mg per plant)	6,750 ± 91 a	6,900 ± 107 a	6,566 ± 130 a	7,733 ± 183 a
	PH (cm)	33.5 ± 4.7 ab	30.3 ± 3.6 bc	26.4 ± 3.1 c	35.3 ± 3.2 a
	RDM (mg per plant)	195.7 ± 36.9 ab	206.0 ± 40.4 ab	143.3 ± 44.6 b	256.6 ± 38.8 a
R2 stage	SDM (mg per plant)	4,294.0 ± 0.9 b	4,586.0 ± 0.5 b	5,772.0 ± 1.0 ab	5,997.0 ± 0.7 a
	PH (cm)	38.61 ± 10.90 b	39.84 ± 9.75 ab	44.32 ± 7.18 ab	50.57 ± 8.42 a
	RDM (mg per plant)	1,764 ± 662 a	2,002 ± 453 a	1,917 ± 618 a	1,714 ± 841 a
R8 stage	Harvest index	0.042 ± 0.002 c	0.057 ± 0.003 b	0.047 ± 0.002 c	0.069 ± 0.003 a
Gnotobiotic conditions					
V4 stage	SDM (mg per plant)	204.2 ± 36.7 b	183.4 ± 31.4 b	144.2 ± 50.7 b	231.5 ± 40.3 a
	PH (cm)	38.6 ± 10.9 b	39.8 ± 9.7 ab	44.3 ± 7.2 ab	50.5 ± 8.4 a

^a Experiments were performed in greenhouse (harvesting times: V4, R2, and R8) and gnotobiotic conditions. Means followed by different letters are statistically different ($P \leq 0.05$). SDM = shoot dry matter; RDM = root dry matter; and PH = plant height.

Fig. 2. Gnotobiotic and greenhouse experiments. **A**, The gnotobiotic system. **B**, Plants collected at the V4 stage (from greenhouse experiments: 1 is a bradyrhizobia-inoculated plant and 2 is a coinoculated plant). **C**, Bradyrhizobia-inoculated (left) and coinoculated (right) plants, in 5-kg pots, at the R2 stage. **D**, Reddish interior from nodules from the main root of plants grown in greenhouse conditions and collected at the R2 stage (the right side shows a zoom of the nodules).

The biochemical analysis of roots collected at the R2 stage showed a different composition of flavonoids compared with roots from plants collected at V4 stage. At the R2 stage, daidzein, genistein, and coumestrol were detected in noninoculated control plants but at low levels; no flavonoids were detected in JD2-inoculated plants, suggesting that they would have already been degraded or excreted by the plant; an increased level of daidzein was detected in bradyrhizobia-inoculated plants, accompanied by the detection of low amounts of genistein, luteolin, and coumestrol; and in roots from the coinoculated plants, daidzein, genistein, luteolin, and coumestrol were detected but at lower levels than those detected in plants collected during stage V4 (when nodulation was already established in plants from greenhouse experiments). The relative integrated area of peaks showed a similar abundance of flavonoids in bradyrhizobia-inoculated and

coinoculated plants (daidzein and coumestrol as the major flavonoids, two- and tenfold higher than genistein and luteolin, respectively).

Results of analysis by gas chromatography coupled to mass spectrometry (GC-MS) are shown in Tables 4, 5, and 6. Many organic acids (including fatty acids and amino acids) and carbohydrates were detected in all treatments. A general overview of results suggests that roots from coinoculated plants contain a few compounds that were not found on control or single-inoculated plants (bradyrhizobia or JD2-inoculated plants). In other words, a small set of compounds were only found in coinoculated plants and could contribute to the understanding of how coinoculation may contribute to a better plant performance. At growth stage V4, the fatty acids caproic and pentadecanoic, the amino acids glutamine and aspartate, and the sugars fructose and galactose were detected only in coinoculated root plants; meanwhile, oleic, linolenic, arachidonic, and monopalmitin acids, hydroxybutyric acid, glycerol, proline, and glutamine were found only in roots collected at growth stage R2.

The presence of low molecular-weight compounds in nodules (main and secondary ones) collected at R2 stage is shown in Table 5. Interestingly, the composition of metabolites in main and secondary nodules from the same treatment showed a different pattern of compounds, no matter what treatment we consider, bradyrhizobia-inoculated or coinoculated plants. In addition, we detected a differential pattern of compounds between nodules from different treatments. However, a few compounds, such

Table 2. Total nitrogen and ureides content of plants collected at R2 stage (greenhouse experiments)^a

Treatment	Total nitrogen (mg/g of SDM)	Ureides (mg/g of SDM)
Control	7.938 ± 1.203 c	0.190 ± 0.157 b
Inoculation with JD2	12.721 ± 0.722 bc	0.301 ± 0.122 b
Inoculation with bradyrhizobia	13.904 ± 1.379 b	0.359 ± 0.139 b
Coinoculation	20.632 ± 4.021 a	0.942 ± 0.011 a

^a Means followed by different letters are statistically different ($P \leq 0.10$). SDM = shoot dry matter.

Table 3. Chlorophylls and carotenoid content in soybean plants, at V4 or R2 stage, grown in greenhouse conditions^a

Treatment	Total chlorophyll	Chlorophyll a	Chlorophyll b	b/a ratio	Carotenoids
V4 stage					
Control	5.267 a	2.009 a	3.258 b	1.622 b	0.999 b
Inoculation with JD2	5.712 a	1.893 a	3.819 b	2.018 a	1.056 b
Inoculation with bradyrhizobia	6.483 a	2.334 a	4.149 a	1.778 b	1.240 a
Coinoculation	4.956 b	1.869 a	3.087 b	1.651 b	0.991 b
R2 stage					
Control	0.409 b	0.305 c	0.104 c	0.341 b	14.30 b
Inoculation with JD2	0.477 b	0.336 c	0.141 b	0.419 a	16.30 b
Inoculation with bradyrhizobia	0.556 b	0.418 b	0.138 b	0.330 b	20.60 a
Coinoculation	1.418 a	1.189 a	0.229 a	0.193 c	30.50 a

^a Values are expressed in milligrams of pigment per gram of fresh leaf. Different letters indicate significant differences between treatments ($P \leq 0.05$).

Table 4. Chemical composition of roots collected at V4 growth stage during greenhouse experiments^a

Chemical family	All treatments ^a	Inoculation with bradyrhizobia	Inoculation with JD2	Coinoculation
Fatty acids	Palmitic acid	...	...	Caproic acid
	Valeric acid	...	...	Pentadecanoic acid
	Stearic acid	...	...	...
Carboxylic acid and derivatives	Succinic acid	...	Butyric acid	...
	Malic acid	...	Malonic acid	Malonic acid
	Propanoic acid	...	Gluconic acid	Gluconic acid
	Threonine acid	...	...	...
Amides, amines, amino acids, and other N-compounds	Butanamine	Proline	Proline	Glutamine
	Alanine	Urea	Urea	Aspartic acid
	Valine	...	Tyrosine	...
	Serine	...	...	...
	Threonine	...	...	...
Carbohydrates	Arabinose	Xylose	Ribose	Ribose
	myo-Inositol	...	...	Fructose
	Mannose	...	...	Galactose
	Glucose	...	...	...
	Xylitol	...	...	...
	Sucrose	...	...	...
Phytosterols	...	...	Stigmasterol	...
	...	...	Sitosterol	...

^a Compounds detected in all treatments, including nonbacterized controls. Compounds exclusively found in a specific treatment are indicated in the corresponding column. Experiments were done at least three times, showing similar trends, but results shown are from one of three independent experiments.

as hydroxybutyric and malic acids were found in both main and secondary nodules in all tested conditions.

DISCUSSION

The use of plant growth-promoting microorganisms in agricultural systems improves the growth and development of plants, including the yield of many commercial crops and seed stocks. Among others, there are many reports on the beneficial effect of the combined use (coinoculation) of bacteria of the genus *Azospirillum* and rhizobia on a variety of plants, due to their ability to promote the growth of plants through one or both the production of phytohormones (IAA and cytokinins) and the ability to fix atmospheric nitrogen (Morel et al. 2012). The coinoculation may also change the composition of root-exuded molecules such as flavonoids, as described by Morel et al. (2015) and Volpin et al. (1996).

In this work, we worked under the hypothesis that proposes that the coinoculation of soybean seeds with *Delftia* sp. strain JD2 and *B. elkanii* strains improves the nodulation and productivity of plants compared with single inoculation and that the production of some secondary metabolites would explain the positive effect of coinoculation on the growth and development of soybean plants. As a first step, we faced bacterial antagonist tests. The results showed that they (JD2, U1301, and U130) are not antagonist to each other (data not shown).

Experiments performed in gnotobiotic and greenhouse conditions support that the coinoculation of soybean with bradyrhizobia and *Delftia* sp. strain JD2 induce early nodulation, a higher number of nodules per plant, and improved soybean yield, including the accumulation of total nitrogen, ureides, and the HI. A study done by the United States Department of Agriculture Economic Research Service in 2011 showed a large increase in grain yield of 23.4 kg per hectare per year between

Table 5. Chemical composition of roots collected at R2 growth stage during greenhouse experiments^a

Chemical family	All treatments	Inoculation with bradyrhizobia	Inoculation with JD2	Coinoculation
Fatty acids	Stearic acid	...	Linoleic acid	Oleic acid
	Palmitic acid	...	...	Linolenic acid
	...	...	...	Arachidonic acid
	...	...	...	Monopalmitin
Carboxylic acid and derivatives	Propanoic acid	Benzoic acid	Butanoic acid	Benzoic acid
	Malic acid	...	Gluconic acid	Gluconic acid
	...	...	Glucuronic acid	Hydroxybutyric
	...	...	Malonic acid	...
	...	...	Maleic acid	...
	...	...	Lactic acid	...
	...	...	Cinnamic acid	...
Amides, amines, amino acids and others N-compounds	Proline	Alanine	...	Alanine
	Serine	Glycine	...	Glycine
	Aspartic acid	Valine	...	Glutamine
	Lysine	...	...	Proline
	Butanamine	Urea	...	Urea
Carbohydrates	Ribose	...	Glycerol	Dulcitol
	Inositol	...	Maltose	Glycerol
	Galactose	...	Mannose	...
	Glucose	...	Xylose	...
	Arabinose	...	Xylitol	Xylitol
	Fructose	...	Melibiose	...
Phytosterols	Stigmasterol	Campesterol	...	Campesterol
	Sitosterol	...	...	...

^a Compounds listed in the All-treatments column were detected in all treatments, including nonbacterized controls. Compounds exclusively found in a specific treatment are listed in the corresponding column. Experiments were done at least three times, showing similar trends, but results are from one of three independent experiments.

Table 6. Chemical composition of nodules collected at R2 growth stage, during greenhouse experiments^a

Chemical family	Nodules on the main root		Nodules on the secondary roots	
	Inoculation with bradyrhizobia	Coinoculation	Inoculation with bradyrhizobia	Coinoculation
Fatty acids	Tetracosenoic acid	...	Propanodioic acid	Palmitic acid
	...	...	...	Propanoic acid
Carboxylic acid and derivatives	Acetic acid	Malonic acid	Palmitic acid	Keto d-gluconic acid
	Keto-d-gluconic acid	...	...	Glucuronic acid
	Hexanoic acid	...	...	...
	Pentonic acid	...	...	...
	Altronic acid	...	...	...
	Ribonic acid	...	...	...
Amides, amines, amino acids and other N-compounds	...	Glutamine	Urea	Proline
	...	...	...	...
Carbohydrates	Galactose	Melibiose	Melibiose	Galactofuranose
	...	Mannose	Ribose	Turanose
	...	...	Lactose	Glucose
	...	...	...	Maltose

^a Compounds exclusively found in a specific treatment are listed in the corresponding column. Experiments were done at least three times, showing similar trends, but results are from one of three independent experiments.

1924 and 2011 in the United States but this high yield would not meet the demands predicted by 2050 (Ray et al. 2013). Thus, the data obtained in greenhouse experiments show that coinoculation with JD2 increases soybean grain yield, overcoming agronomic expectations, and probably increasing agricultural commodities, helping buyers and sellers to trade in commodity and, ultimately, benefiting the farmers.

Understanding the role that the molecules have during the plant-microbe interaction might help to propose new strategies to increase agronomical practices. Thus, we searched for a set of compounds commonly found in leaves (photosynthetic and photoprotective pigments), roots and nodules (flavonoids, organic acids, sugars). We sought to answer the following question: Does coinoculation change the profile of plant metabolites? If so, may these compounds explain the beneficial effect of coinoculation on soybean?

Chlorophyll a is the main pigment involved in photoelectron transport, the process that provides the energy for photosynthesis (Ripullone et al. 2003). In addition to chlorophyll a, the antenna complex contains other pigments (chlorophyll b, carotenoids, and xanthophylls) that absorb light from different wavelengths, which can also be used for photosynthesis. Chlorophyll b is found in the external antennas of photosystems (Busheva et al. 1991), and the ratio chlorophyll b/a thus allows the estimation of the relative amount of external antennas per reaction center. Carotenoids are involved in the assembling of photosystems and act as light-harvesting antenna complexes for photosynthesis and photoprotection but also have a function in the regulation of plant growth and development (Nisar et al. 2015). The information gathered during this work (higher number of external antennas for each reaction center and increased content of carotenoids in coinoculated plants compared with bradyrhizobial-inoculated ones) suggests that coinoculation may help to increase the nutrient status of plants and to cope with the damaging effects of light. The increase in the content of photosynthetic and photoprotective pigments by the inoculation with *Azospirillum brasilense* of wheat seedlings (Bashan et al. 2006) and *Arabidopsis thaliana* (Cohen et al. 2015) was already reported, reinforcing the hypothesis that some bacteria may influence the content of pigments produced by plants.

Flavonoids are plant-specific compounds that activate the genes involved in the production of the nodulation factor by the rhizobia. These chemical factors trigger the formation of nodules in the roots of the leguminous plants, and daidzein (the most abundant flavonoid in soybean), genistein, and coumestrol are key signal compounds in the establishment of the symbiosis between bradyrhizobia and soybean (Antunes et al. 2006; Juge et al. 2012). We showed that roots from coinoculated plants have tenfold higher content of flavonoids compared with JD2- or bradyrhizobia-inoculated plants (at the V4 growth stage), suggesting that, in response to coinoculation, the soybean plants could modify their metabolism for the production of a higher amount of these flavonoids. The results suggest that coinoculated plants would show a better performance for the development of nodules, mainly at the V4 plant-growth stage. Likely, as shown above, once the nodule is established (R2 growth stage), coinoculated plants slow the production of flavonoid (or raise their degradation) and change their metabolism to produce other molecules with much relevance in the anabolic metabolism. Probably, when nodules and the nitrogen-fixation process are already established the amount of flavonoids within the root is kept at a steady state and they no longer influence the symbiotic processes.

Among the compounds found by GC-MS, the amino acid glutamine is clearly involved in nitrogen fixation. The fixed nitrogen is transformed to ammonium into the bacteroid, then

assimilated into glutamine (and aspartate) and, finally, through the purine pathway, and a series of oxidation and enzymatic steps is converted into ureides. The ureides are transported, via the xylem, to the rest of the plant. It has been suggested that the amount of glutamine (and aspartate) must be balanced in the nodule and this balancing is essential for improving nodule function and nitrogen assimilation (Carter and Tegeder 2016). In addition, the amino acid proline, found in roots from R2 plants, may act as an osmolyte, but proline is also known for stabilizing subcellular structures such cell membranes, scavenging free radicals, and buffering the redox potential of cells (Hayat et al. 2012). Thus, this result could mean that nodules from coinoculated plants are better prepared for nitrogen fixation than are nodules from bradyrhizobia-inoculated plants.

Among the different treatments, we did not find differences in the pattern of fatty acids produced, although the presence of the short-chain fatty acids caproic acid (C6:0) on roots from coinoculated plants must be highlighted. This fatty acid has been linked to membrane fluidity and may indicate that the plant cell membrane is now more fluid and prone to bacterial colonization, as previously suggested by Brechenmacher et al. (2010). The same result was obtained in previous studies performed on alfalfa root exudates, i.e., the presence of caproic acid in 15-day exudates of plants coinoculated with JD2 and *Sinorhizobium meliloti* U143 (Morel et al. 2015).

The different pattern of metabolites found in main and secondary nodules from bradyrhizobia-inoculated and coinoculated plants suggests that both kinds of nodules have either a different function, react differently, or both to the coinoculation. Once again, glutamine shows up, now as a compound only found in the main nodules of coinoculated plants, suggesting that the metabolism of amino acids is still very active in nodules from coinoculated plants at R2.

Other compounds, such as hydroxybutyric and malic acids were found in main and secondary nodules in all tested conditions. The presence of hydroxybutyrate in soybean nodules is a widely reported feature in bradyrhizobia. The β -hydroxybutyric acid is commonly present in roots and nodules of soybean and is accumulated as polyhydroxybutyrate in the bacteroids and used as carbon source when the supply of carbohydrates from the plant is limited (Wong and Evans 1971). Some authors propose that the degradation of this compound in nodules maintains the oxygen demand of the bacteroids during darkness, preserving the activity of the nodule and the biological nitrogen fixation until the stage of grain development (Bergersen et al. 1991).

Malic acid is the main carbon source transported to the bacteroids (Udvardi and Poole 2013). It is possible that the malic acid present in nodules and roots of the inoculated and coinoculated soybean comes from the Krebs cycle. The Krebs cycle, as a central metabolic pathway in rhizobia, provides reduction equivalents (NADH and FADH₂) for the production of energy and metabolic intermediates for the production of amino acids and other biosynthetic pathways. In summary, the detection of intermediary metabolites of the Krebs cycle in roots of coinoculated plants as well as of metabolites related to the assimilation of nitrogen suggests that these plants are actively producing reducing equivalents and energy, which could be used for biomass production, including grains.

In summary, the new information suggests that coinoculation with *B. elkanii* and *Delftia* sp. strain JD2 strains induces a higher yield and harvest rate of soybean compared with the bradyrhizobia single inoculation, with a concomitant benefit for the agricultural economy. The results suggest that the coinoculation of soybean changes the primary and secondary metabolism of the plant. Consequently, JD2 could be used together with bradyrhizobia to manipulate the chemical

composition of plant tissues, promoting the nutritional benefits and health of soybean. Interestingly, an overview of results from GC-MS carried out on nodules showed that main and secondary nodules have a different metabolic status within the plant and that the coinoculation may change the flux (or rate) of metabolic pathways, anabolic or catabolic ones, yielding different chemical products with different benefits. Based on these inferences, future work will focus on the study of the differential expression of proteins (by shotgun of proteins) and genes (by RNA-seq) among the main and secondary nodules from bradyrhizobia-inoculated and coinoculated plants.

MATERIALS AND METHODS

Bacterial strains.

The bacterial strains used in the present study were *Delftia* sp. strain JD2 (Morel et al. 2011) and the microsymbionts of soybean (*Glycine max*) *Bradyrhizobium elkanii* U1301 and U1302 from the commercial formulation LIKUIQ produced by Lage & Cia S.A. (initial microbial concentration of 5×10^9 CFU mL⁻¹). JD2 cells were grown in tryptone-yeast medium (5 g of tryptone, 3 g of yeast extract, and 0.09 g of anhydrous CaCl₂ per liter) at 30°C and 200 rpm. Growth was monitored by measuring optical density at 620 nm. The antagonism was determined by the disk agar diffusion test, by means of the dual confrontation between JD2 and one bradyrhizobium at a time, as described by Hewitt and Vincent (1989).

Gnotobiotic plant experiments.

Surface-sterilized soybean seeds (GE 590ci) (15 min in 20% NaClO, rinsed with and soaked in sterilized water) were pre-germinated in soft agar at 30°C in dark conditions. Then, pre-germinated seeds were sown in 500-ml test tubes (one seed per tube [Fig. 2A]) containing 450 ml of sterile nitrogen-free mineral solution (Somasegaran and Hoben 1985) and 0.8% agar (Sigma; low nitrogen-content agar) and were inoculated with either 100 µl of bacterial suspension of JD2 (10^7 CFU/ml), 100 µl of LIKUIQ bacterial suspension (10^8 CFU mL⁻¹), or both, per seed. All plants were incubated for 32 days at 26°C, under a photoperiod of 16 h of light and 8 h of dark. The light was supplied through Gro-lux tubes (Sylvania). Nodules were counted daily and the main and secondary root nodules were recorded. After 32 days of growth, shoot and root dry matter (SDM and RDM, respectively) were measured after drying at 60°C until constant weight.

Greenhouse experiments.

Soybean seeds (GE 590ci) were surface-sterilized and were transferred to 5-kg pots (for harvesting of plants at V4 or R2 growth stages) or 8-kg pots (for harvesting of plants at physiological maturity, growth stage R8) (10 pots per treatment; three seeds per pot) filled with nonsterile soil, sand, and vermiculite (2:2:1). A completely randomized block experiment was designed. Seeds were inoculated with one or both *B. elkanii* and JD2 (10^7 and 10^6 CFU per seed, respectively). Ten days later, two plants per pot were thinned out, and the plants that showed uniformity of growth appearance were used for further experiments. Plants were grown at 30°C (16 h light and 8 h dark), using Gro-lux lamps (Sylvania), and were irrigated every 3 days (to soil water capacity) with water, 10-fold diluted Jensen medium (Vincent 1970), or calcium hyperphosphate solution (1 g per liter), alternately. As control treatment uninoculated, nitrogen-free plants were used. Soybean plants were cut 1 cm above the pot soil at V4 and R2 growth stages (30 and 80 days after inoculation, approximately). SDM and RDM were measured after drying at 60°C until constant weight. When plants reached the R8 stage

(approximately 180 days after sowing), grain yield was determined. The number, weight, and length of pods and the number and weight of grains were determined. The HI was also determined as the ratio between the grain yield and the total aerial biomass of the crop (total aerial part including grains), according to Koester et al. (2014). The determination of total nitrogen in plants was done by micro Kjeldhal, using the service provided by Ecotech. The content of ureides in soybean plants was analyzed as described by Vogels and Van der Drift (1970).

Chlorophyll and carotenoid content.

Fresh leaves (250 mg) of plants grown under greenhouse conditions were collected up to stages V4 and R2, were macerated in 12.5 ml of 80% acetone, on an ice bed (low temperature) and in dark conditions, and were centrifuged at $10,400 \times g$ for 3 min at 4°C. The content of chlorophyll a and b and total carotenoids were determined at 663, 647, and 470 nm, respectively (Pompelli et al. 2013). The contents of pigments were determined using the Arnon equations (Arnon 1949), but based on Pompelli et al. (2013) modifications.

Preparation of samples for chromatographic experiments.

Leaves, roots, and nodules from plants collected in stages V4 and R2 were lyophilized and, then, ground with liquid nitrogen. The extraction of methanol (flavonoids), methanol/chloroform/water (12:5:3) (amino acids and carbohydrates) and acetone/ethanol (1:1) soluble compounds was performed as described by Sérandour et al. (2008) and Juge et al. (2012). Compounds were solubilized in 50 µl of acetonitrile and derivatized by silylation, using 50 µl of N-methyl-N-(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich), as described by Sérandour et al. (2008) and Morel et al. (2015).

Identification of low molecular weight metabolites.

Low molecular-weight metabolites, including flavonoids, amino acids, and carbohydrates were identified by GC-MS using GCMS-QP 2010 Ultra equipment (Shimadzu) with an auto-injector (AOC-20i; Shimadzu). For analyses, a deactivated guard column (5 m length $\times$ 0.25 mm ID; Restek) and an analytical capillary column HP-5MS (30 m length $\times$ 0.25 mm ID $\times$ 0.25 µm film thickness; Agilent, J & W Columns) were employed. The carrier gas was helium at a flow rate of 1.0 ml per minute. The oven temperature program was as follows: kept to 80°C for 2 min, then increased to 290°C (8°C min⁻¹), maintained at 290°C for 3 min, then increased to 310°C (20°C min⁻¹), and finally, maintained at 310°C for 10 min. The temperature of the injector and detector was 300°C during all analyses. Sample aliquots of 1 µl (prepared as described above) were injected in splitless mode and were analyzed (total ion current) using the GC-MS Solutions software from Shimadzu, ionization energy, 70 eV and mass scan range, 50 to 850 *m/z*.

The identification of the metabolites was performed by comparison (at least 95% match) with standard mass spectra stored in commercially available libraries (NIST and Wiley). To confirm the chemical identity of the compounds, linear retention indices (LRIs) were calculated by injection of a solution of n-alkanes (C7 to C36; Supelco). In all cases, differences between theoretical and experimental calculated LRI values were smaller than 10 units, confirming the MS identification.

Statistics.

All experiments were performed at least three times and the data were subjected to one-way analysis of variance (ANOVA), using PAST software version 1.56, with post hoc pair-wise comparison based on Tukey's honestly significant difference test when the ANOVA showed significant inequality of means.

Statistical significance was determined at $P < 0.05$. Homogeneous groups were designated by superscript letters.

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