Enhanced secondary metabolite, trichome density and biosynthetic gene expression in *Stevia rebaudiana* Bertoni plants inoculated with endophytic bacteria

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Abstract

Stevia rebaudiana is considered a plant of economic interest in the food and pharmaceutical industries due to its steviol glycosides (SG). SG are compounds that are 300 times sweeter than sucrose; and produce phenolic compounds and flavonoids with antioxidant activity. Endophytic bacteria associate with plants through a mutualistic interaction, which plays an important role in the growth and development of the host plant. The objective of this study was to investigate the effect of culturable endophytic bacteria of *S. rebaudiana* on plant growth, trichome density in leaves, and secondary metabolite accumulation, and the expression of genes associated with SG biosynthesis. The 12 bacteria tested herein showed no effect on plant growth; however, secondary metabolites were increased with the inoculation of *Enterobacter hormaechei* H2A3 and *Enterobacter hormaechei* H5A2. This accumulation of secondary metabolites in leaves paralleled an increase in the glandular, short and long trichome density. Additionally, image analysis in *S. rebaudiana* leaves showed strong autofluorescence (blue channel, 440 nm), mainly in glandular and short trichomes, indicating the presence of SG, phenolic compounds, and flavonoids. Finally, *E. hormaechei* H2A3 and *E. hormaechei* H5A2 bacteria treatment resulted in the upregulation of *KO, KHA, UGT74G1*, and *UGT76G1* genes according to the increase in GSs accumulation. These results represent a finding for the use of endophytic bacteria to favor the increase in the SG content in *S. rebaudiana*.

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Introduction

Bacteria-plants associations are beneficial because they enhance the acquisition of mineral nutrition, and provide protection against abiotic and biotic stresses (Asaf et al., 2017; Wang et al., 2015). Furthermore, it is well known that interactions can induce plant growth and development in many agroeconomic crops (Lodewyckx et al., 2002; Rosenblueth & Martínez-Romero, 2006).

Endophytic bacteria are an important group of microorganisms that live within plant tissue and can enhance several biochemical and physiological processes, such as metabolite biosynthesis and accumulation, as well as growth-promoting activity in plants. Previous studies have shown that these bacteria enhance the accumulation of secondary metabolites, and modulate the profile accumulation and the expression patterns of several biosynthesis pathways in many plant species (Tiwari et al., 2013, 2010; Yang et al., 2019; Zhou et al., 2016). For example, isolated bacteria from *Lycoris radiata* promote Amaryllidaceae alkaloid accumulation in the host plant (Liu et al., 2020), and *Pseudomonas fluorescens* induces sesquiterpenoid accumulation in *Atractylodes macrocephala* Koidz plants (Yang et al., 2019).

Stevia rebaudiana Bertoni is a perennial shrub species of the Asteraceae family and is an economically important crop due to its ability to accumulate low-calorie sweeteners called steviol glycosides (SG), including isosteviol, stevioside, rebaudiosides (A, B, C, D, E and F), steviolbioside and dulcoside A (Sarmiento-López et al., 2020; Rajasekaran et al., 2008). The sweet taste of Stevia leaves depends on the contents of stevioside and rebaudioside A, which is approximately 250–300 times as sweet as sucrose (Geuns, 2003). Due to the high content of sweet glycosides, Stevia is considered a significant source of natural sweeteners for the growing food market (Goyal & Goyal, 2010). On the other hand, the leaves of *S. rebaudiana* also contain other phytochemical constituents, such as phenolic compounds, which are a family of antioxidant metabolites, including stilbenes, flavonoids and phenolic acids (Lemus-Mondaca et al., 2012).

Brandle and Telmer (2007) proposed that SG biosynthesis begins with geranylgeranyl-di-phosphate (*GGDP*) synthesis through the methyl-erythrol-4-phosphate (MEP) route. *GGDP* is transformed to kaurene by two cyclization steps carried out by terpene cyclases and then later converted to steviol by four additional enzyme actions: copalyl diphosphate synthase (*CDPS*), kaurene synthase (*KS*), kaurene oxidase (*KO*), and kaurenoic acid hydroxylase (*KAH*) (Kim et al., 1996). Different SG are formed by steviol glycosylation by specific glucosyltransferases, the enzyme *UGT74G1* is involved in the conversion of steviolbioside to stevioside, while the enzyme *UGT76G1* is involved in the conversion of stevioside to rebaudioside A (Shibata et al., 1991 ; Shibata et al., 1995).

Trichomes are epidermal structures where various secondary metabolites are synthesized and accumulated and are associated with the chemical defense of the plant (Werker, 2000; Li et al., 2020). Trichomes are classified according to their morphology into glandular and non-glandular groups. In *S. rebaudiana,* the presence of short and long glandular trichomes has been reported by Sarmiento-López et al. (2021), they increase in leaves by arbuscular mycorrhizal colonization, as well as the accumulation of phenolic compounds. Additionally, it has been

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proposed that SG synthesis and accumulation take place in trichomes (Bondarev et al., 2010), but it has not been determined whether the presence of beneficial endophytic bacteria or fungi could stimulate the development of these structures.

Additionally, some studies have been carried out in *S. rebaudiana* to evaluate the effect of plant growth promoting rhizobacteria (PGPR) and mycorrhiza fungi on growth, secondary metabolite production, and the analysis of biosynthetic gene expression. Mamta et al. (2010) and Vafadar, Amooaghaie & Otroshy (2014), reported that inoculation with different PGPR improved plant growth, photosynthetic parameters, and the accumulation of stevioside and rebaudioside A. Sarmiento-López et al. (2020), reported that AM symbiosis by *Rhizophagus irregularis* improves the growth and photosynthetic activity of plants, as well as the expression of *KO, UGT74G1* and *UGT76G1* biosynthetic genes.

Endophytic bacteria are microorganisms that have evolved and can live inside plant tissues, providing advantages over other rhizospheric microorganisms. Thus, the main objective of this work was to determine whether if endophytic bacteria isolated from *S. rebaudiana* can stimulate plant growth, the expression of SG biosynthesis genes (*KO, KAH, UGT74G1* and *UGT76G1*), the accumulation of SG, and phenolic compounds, and trichome density.

Materials & Methods

S. rebaudiana plant material

Plants of *S. rebaudiana* were cultivated and maintained in Centro de Desarrollo de Productos Bióticos (CeProBi-IPN), Morelos, México, according to the methodology described by Sarmiento-López et al. (2021). Apical shoots were used for rooting and transferred to pots containing a mixture of 60:20:20 sterilized turf, perlite, and vermiculite with an initial pH of 5.6 ± 0.5 and porosity of 85%. This substrate was sterilized at 121 °C and 15 psi for 2 h. The explants were kept in a greenhouse for 15 days.

Endophytic bacteria culture

The endophytic bacteria were isolated from different tissues of *S. rebaudiana* (Table 1) and stored in glycerol at -20 °C. The bacterial inoculum was grown in 250 mL flasks with a volume of 100 mL of liquid LB medium, and incubated on a rotary shaker (Infors HT, Minitron, Switzerland) at 200 rpm for 48 h at 25 °C.

Inoculation of *S. rebaudiana* with endophytic bacteria

S. rebaudiana plants (15 days old) with five cm-long roots, and two leaves were used. For this assay, pots (1 L) were placed in plastic trays with the same substrate as mentioned before. The inoculation was carried out at the beginning of the experiment with a bacterial concentration of 1 x 10^{8} cells mL^{-1} (Botta et al., 2013). Ten plants were sown per treatment and two independent

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experiments were performed. Non-inoculated plants were considered as control, and inoculated and non-inoculated plants were watered every other day with a 50% Steiner solution (Rodriguez-García, 2015). The pots were placed in the nursery in a random arrangement, and no pruning was performed during the evaluation time.

Plant growth promotion parameters

The *S. rebaudiana* plants treated with the endophytic bacteria were collected at 30 days post-inoculation (dpi). Plant height was measured with a Vernier caliper from the surface of the substrate to the apex of the plant (cm), and root size (cm) was measured from the base of the stem to the root apex. The number of leaves was registered, and the aerial part and the root were separated and placed in an oven (RiossA E-33, México) at 50 °C to measure the dry weight (DW). Each part of the plant was weighed on an analytical balance.

Steviol glycoside (SG) content in leaves of *S. rebaudiana*

SG concentrations were determined in the leaves of inoculated and non-inoculated plants according to the methodology reported by our group (Sarmiento-López et al., 2020). Briefly, leaves from each plant inoculated and non-inoculated with the endophytic bacteria were dried in an oven (RiossA E-33, México) at 65 °C for 48 h. Leaf tissue (0.1 g DW) was extracted with 1 mL of methanol (J.T. Backer, USA) in microtubes, according to Woelwer-Rieck et al. 2010. The microtubes were stirred for 3 min, allowed to stand for 24 h without stirring, and then centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was recovered, placed in fresh microcentrifuge tubes, and stored at -4 °C until analysis by High-performance Thin Layer Chromatography (HPTLC) (CAMAG, Muttenz, Switzerland). The SG quantification was based on the methodology reported by Villamarín-Gallegos et al. (2020). Stevioside and rebaudioside A concentrations were expressed as mg gDW^{-1}. Six plants per treatment were evaluated, and two independent experiments were performed.

Determination of phenolic compound and flavonoid contents in leaves of *S. rebaudiana*

For phenolic compound determinations, leaves from non-inoculated and inoculated with plants were used. Then, 0.1 g DW of leaves was extracted with 75% ethanol at 0.1% (w/v) and centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was recovered in 1.5 mL microcentrifuge tubes and kept at -4 °C until processing.

The phenolic compounds were determined using the Folin-Ciocalteu reagent as described by Bobo-García et al. (2014). The assay was performed on a microplate and incubated at room temperature in the dark for 2 h. Absorbance was measured at 760 nm on a spectrophotometer (Multiscan Go) equipped with SkanIt Software version 1.00.40 (Thermo Fisher Scientific, Massachusetts, USA). The results were expressed as mg equivalents of gallic acid (GAE) g^{-1} DW.

The flavonoid concentration was determined according to Villamarín-Gallegos et al. (2020) and adapted from Chang et al. (2002). The assay was performed on a microplate of 96 wells and

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incubated at room temperature in the dark for 30 min. Absorbance was monitored at 415 nm on a spectrophotometer (Multiscan Go) equipped with SkanIt Software version 1.00.40. The results were expressed as mg equivalents of quercetin (EQ) g^{-1} DW.

Trichome analysis in *S. rebaudiana* plant leaves by environmental scanning electron microscopy (ESEM) and confocal laser scanning microscopy

To analyze the trichomes from *S. rebaudiana* leaves, samples were assessed via ESEM (Carl Zeiss, EVO LS10, Germany) according to the methodology reported by Sarmiento-López et al.(2021). Leaves close to the apical meristem from non-inoculated and inoculated plants with the endophytic bacteria were used. The leaf was placed in aluminum stubs with double-sided conductive carbon tape, and was observed under ESEM using a voltage of 15 kV. The gas pressure in the ESEM chamber was maintained at 20 Pa by introducing water vapor, and a secondary electron detector was utilized to obtain micrographs. The density of trichomes (trichome leaf $area^{-1}$), by type (short, large and glandular), was determined by image analysis using ImageJ editing software 2.0 from micrographs obtained by ESEM.

To determine the effect of endophytic bacterial inoculation on specific metabolite accumulation on *S. rebaudiana*, observation of the leaves by confocal laser scanning microscopy (Carl Zeiss, model LSM 800, Germany) was analyzed according to the methodology reported by Sarmiento-López et al. (2021). The maximum fluorescence of secondary metabolites (steviol glucosides, phenolic compounds, and flavonoids) was observed in the blue spectrum (435-485 nm) and chlorophylls in the red spectrum (630-685 nm) were detected according to the methodology of Talamond et al. (2015). Micrographs were obtained using Zeiss Efficient Navigation (ZEN) 2.6 Blue edition.

Expression analysis by qRT–PCR

The transcript accumulation levels of the genes for kaurene oxidase (*KO*), kaurene hydroxylase (*KAH*) and (UDP)- glycosyltransferases (*UGT74G1* and *UGT76G1*) were evaluated in plant leaves inoculated with the selected endophytic bacteria. Frozen leaves (0.5 g DW) were ground to a fine powder in liquid nitrogen. Total RNA was isolated from leaves using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. First-strand cDNA synthesis was performed as previously reported by Sarmiento-López et al. (2020).

The primers corresponding to the kaurene oxidase gene were SrKOF 5′-TCTTCACAGTCTCGGTGGTG-3′, and SrKOR 5′-GGTGGTGTCGGTTTATCCTG-3′; the primers corresponding to the kaurenoic acid hydroxylase gene were SrKAHF 5′-CCTATAGAGAGGCCCTTGTGG-3′, and SrKHAR 5′-TAGCCTCGTCCCTTTGTGTC-3′; the primers corresponding to the glycosyltransferase UGT74G1 gene were SrUGT74G1F 5′-GGTAGCCTGGTGAAACATGG-3′, and SrUGT74G1R 5′CTGGGAGCTTTCCCTCTTCT – 3′; and the primers corresponding to the glycosyltransferase UGT76G1 gene were SrUGT76G1F 5′- GACGCGAACTGGAACTGTTG-3′, and SrUGT76G1R 5′-AGCCGTCGGAGGTTAAGACT – 3′. qRT–PCR was performed using SYBR Green

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(QIAGEN, USA) and quantified on a Rotor-Gene Q (QIAGEN, USA) real-time PCR thermal cycler. qRT–PCR was programmed for 35 cycles, with denaturing at 95 °C for 15 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. Three biological replicates with three technical replicates per treatment were evaluated. Primer specificity was verified by regular PCR and melting curve analysis. The primers for the *S. rebaudiana* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene SrGAPDHF 5′-TCAGGGTGGTGCCAAGAAGG-3′; and SrGAPDHR 5′- TTACCTTGGCAAGGGGAGCA- 3′, were used as internal controls for normalization, and the quantitative results were evaluated by the $2^{-\Delta\Delta CT}$ method described by Livak & Schmittgen (2001). Three plants per condition were evaluated.

Statistical analysis

The results were analyzed in the statistical package GraphPad Prism version 6.0 to obtain the mean and standard deviation. To find differences between the treatments, the data were analyzed using one-way analysis of variance (ANOVA). Subsequently, a multiple comparison analysis was performed using Dunnett's test considering as a *p*-value of less than 0.05 ($p < 0.05$).

Results

Effect of endophytic bacterial inoculation on *S. rebaudiana* growth, steviol glycosides (SG), phenolic compounds, and flavonoid accumulation in the leaves

S. rebaudiana plants inoculated with the endophytic bacteria did not have growth-promoting activity, since plant length, root length, number leaves and root dry weight were not different from those of the non-inoculated plants (Table 2).

In the plants inoculated with *Enterobacter hormaechei* H2A3, *E. hormaechei* H5A2 and *E. bacterium* H7A1, the contents of stevioside, rebaudioside A and total SG increased significantly, while in the plants inoculated with other bacteria, the content of SG did not change (Fig. 1). The plants inoculated with *E. hormaechei* H2A3, *E. hormaechei* H5A2, and *E. bacterium* H7A1 show a ratio in the content of rebaudioside A to stevioside that remains near to 0.7, which is similar to that observed in the non-inoculated plants.

Fig. 2 shows that there was an increase of 1.3 times in the phenolic compound accumulation and 1.4 times in the flavonoid content in *S. rebaudiana* plants inoculated with *E. hormaechei* H5A2 in comparison to non-inoculated plants. While *E. cloacae* R3A1 only showed an increase in the flavonoid content of 1.3 times more than the non-inoculated plants. On the other hand, *E. bacterium* H7A1, *E. xianfangensis* T1A2, *E. xianfangensis* T3A3, *E. xianfangensis* R2A2, *E. hormaechei* R6A1 and *E. xianfangensis* R7A2 induced a decrease in phenolic compound content in the plants of *S. rebaudiana* in comparison to non-inoculated plants, but they did not affect flavonoid accumulation. Finally, *E. hormaechei* H2A3, *E. xianfangensis* T5P1, *E. hormaechei* R5P1, and *Bacilus safensis* R6P1 did not affect the accumulation of these metabolites.

Based on the screening results obtained with endophytic bacteria to stimulate the accumulation of SG, phenolic compounds, and flavonoids, the selected bacterial strains to continue this work were *E. hormaechei* H2A3, *E. hormaechei* H5A2, and *E. xianfangensis* R7A2, this bacterium

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was used as control. These strains were used to analyze the effect on trichome density in leaves, as well as the expression of genes of the SG biosynthesis pathway.

Trichome density in *S. rebaudiana* leaves by screening electron and confocal microscopy

Photomicrographs (SEM) of the *S. rebaudiana* plant leaves inoculated with the selected bacteria, showed three types of trichomes: glandular (G), large (L), and short (S) (Fig. 3). Inoculated plants with *E. hormaechei* H2A3 showed a significant increase in S, L and G trichomes in comparison with non-inoculated plants (Fig. 3b), while inoculated plants with *E. hormaechei* H5A2 and *E. xianfangensis* R7A2 did not have an effect on the trichomes in relation to non-inoculated plants (Figs. 3 c-d). The number of short, glandular, and large trichomes showed densities of 2000-6000, 1000-3000, and 200-800 trichomes cm^{-2}, respectively (Fig. 4). The trichome density showed that *E. hormaechei* H2A3 produced an increase in glandular, large and short trichomes, of 1.7, 4.3, and 1.5 times in comparison to non-inoculated plants respectively (Figs. 4a-c). However, *E. hormaechei* H5A2 and *E. xianfangensis* R7A2 bacteria, did not have any effect on the trichome density (Figs. 4 a-c).

Fig. 5 shows the location of SG, phenolic compounds, and flavonoids in the trichomes of *S. rebaudiana* leaves by autofluorescence using confocal microscopy. In the red channel, the autofluorescence of chlorophylls is shown (Figs. 5 a-d), while in the blue channel, the autofluorescence of SG, phenolic compounds, and flavonoids accumulates in trichomes (Fig. 5 e-h). Inoculation with *E. hormaechei* H2A3 and *E. hormaechei* H5A2 generated a greater intensity in the autofluorescence signal in the blue channel, particularly in glandular and short trichomes (Figs. 5 f, g), while, in the non-inoculated plants and those inoculated with *E. xianfangensis* R7A2 (Fig. 5e), the autofluorescence signal was lower.

Figs. 5 m-p correspond to the magnification of the glandular (G) and short (S) trichomes. Trichomes have been reported as epidermal structures where different secondary metabolites are synthesized and accumulate in plants. In this work, *E. hormaechei* H2A3 and *E. hormaechei* H5A2 were selected because both induce higher accumulation of SG in leaves; additionally, *E. hormaechei* H5A2 promotes the accumulation of phenolic compounds and flavonoids. Interestingly, trichome density was higher in the plants inoculated with *E. hormaechei* H2A3 but not with *E. hormaechei* H5A2, whereas localization analysis of these secondary metabolites by autofluorescence indicated that such compounds were located in glandular and short trichomes in plants inoculated with both bacteria.

Effect of endophytic bacterial inoculation on differential SG biosynthetic gene expression in *S. rebaudiana* plants

In the *S. rebaudiana* plants inoculated with *E. hormaechei* H2A3, *E. hormaechei* H5A2, and *E. xianfangensis* R7A, the transcript levels of the *KO* gene increased 5.2, 21.3 and 42.3 times, respectively, compared to non-inoculated plants (Fig. 6a). The relative expression of *KAH* increased 52.3 times with the inoculation of *E. hormaechei* H5A2, and 18.6 and 8.4 times with *E. xianfangensis* R7A2 and *E. hormaechei* H2A3, respectively (Fig. 6b).

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The *UGT74G1* and *UGT76G1* genes involved in stevioside and rebaudioside A biosynthesis were upregulated by inoculation with *E. hormaechei* H2A3 (11.3 and 3.2 times, respectively) in comparison with the non-inoculated plants (Figs 6. c and d). *E. hormaechei* H5A2 and *E. xianfangensis* R7A2 induced the upregulated gene expression of *UGT74G1* by 17.2 and 6.0 fold, respectively (Fig. 6c). In contrast, inoculation with *E. hormaechei* H5A2 and *E. xianfangensis* R7A2 did not have any effect on *UGT76G1* gene expression in comparison with the non-inoculated plants (Fig. 6d).

According to the previous results, the application of *E. hormaechei* H2A3 and *E. hormaechei* H5A2 in *S. rebaudiana* suggests that SG biosynthetic genes can be positively regulated in response to endophytic bacterial inoculation. In addition, the accumulation of stevioside and rebaudioside A is consistent with the increase in the relative expression of *UGT74G1* and *UGT76G1,* which are involved in glycosylating the central backbone of SG to convert stevioside into rebaudioside A.

Discussion

Plant - endophytic microorganism interactions have been proposed as a strategy to improve plant growth and stimulate secondary metabolism (Afzal et al., 2019; Hardoim et al., 2015; Hardoim et al., 2008). In this work, the inoculation of endophytic bacterial isolates from *S. rebaudiana* and its reinoculation were proven. Bacterial inoculation in *S. rebaudiana* plants did not induce significant plant growth (Table 2). Interestingly, *E. hormaechei* H2A3, *E. hormaechei* H5A2 and *E. bacterium* H7A1 increased the contents of stevioside and rebaudioside A in the leaves of *S. rebaudiana*. While *E. hormaechei* H5A2 produced an increase in the accumulation of phenolic compounds and flavonoids*, E. cloacae* R3A1 produced an increase in the accumulation of flavonoids. These results suggest that growth promotion is not associated with endophytic bacterial reinoculation and indicate for the first time that this bacterium plays an important role in the biosynthesis of secondary metabolites (steviol glycosides and phenolic compounds) in *S. rebaudiana.* Previous studies in other species such as: *Oryza sativa* (Andreozzi et al., 2019; Balachandar et al., 2006), *Beta vulgaris* L (Shi et al., 2010), *Artemisia annua* (Li et al., 2012; Tripathi et al., 2020), *Catharanthus roseus* (Tiwari et al., 2013), *Salvia miltiorrhiza* (Yan et al., 2014), *Fragaria ananassia* (Guerrero-Molina et al., 2014), *Glycine max* (Asaf et al., 2017), *Glycyrrhiza uralensis* F (Li et al., 2018), *Lycoris radiata* (Liu et al., 2020), and *Camellia oleifera* (Xu et al., 2020), indicate that bacteria may have a differential effect in promoting plant growth or in the biosynthesis of secondary metabolites, as observed in our study. To our knowledge, this is the first report where the effect of endophytic bacteria of *S. rebaudiana* is reported as a strategy to improve their growth or accumulation of their secondary metabolites. Previously, Vafadar, Amooaghaie & Otroshy (2014), reported that bacteria isolated from the rhizosphere (*Bacillus polymixa*, *Pseudomonas putida* and *Azotobacter chroococcum*) and inoculated in *S. rebaudiana* plants significantly increased root and shoot biomass as well as stevioside, chlorophyll, and NPK content in plants. Kilam et al. (2015), reported that *A. chroococcum* improves the growth, antioxidant activity and steviol glycoside content of *in vitro S. rebaudiana*

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plantlets. Several fungi, including *Glomus intrarradises, Piriformospora indica, Rhizoglomus irregulare,* and *Rizophagus intraradices* have been reported as other inoculant microorganisms of *S. rebaudiana,* and the results demonstrated that they have the ability to enhance plant growth and stevioside accumulation (Vafadar, Amooaghaie, & Otroshy, 2014; Kilam et al., 2015; Tavarini et al., 2018; Sarmiento-Lopez et al., 2020; Mandal et al., 2013a; Mandal et al., 2015a). A synergistic relationship between bacteria and fungi has been proposed to improve the plant growth of *S. rebaudiana* and the accumulation of SG (Kilam et al., 2015; Vafadar, Amooaghaie & Otroshy, 2014). However, little is known about the use of endophytic bacteria to enhance secondary metabolism in *S. rebaudiana*. In this work, it was found that bacteria can colonize the plant tissue and live within the plant.

Trichomes are plant structures where the accumulation of secondary metabolites has been reported, and their presence in plant leaves is associated with defense mechanisms of the plant against pathogens, insects and adverse environmental conditions (Champagne & Boutry, 2016; Tian et al., 2017; Werker, 2000). The *S. rebaudiana* trichomes observed in the leaves in the present study were short, large and glandular. This trichome morphology was consistent with those previously reported by *S. rebaudiana* (Bondarev et al., 2003; Bondarev et al., 2010; Cornara et al., 2001; Monteiro et al., 2001). In the present work, inoculation with the endophyte *E. hormaechei* H5A2 generated a higher density of trichomes in *S. rebaudiana* leaves, which paralleled a higher content of SG, phenolic compounds and flavonoids. These results are in accordance with Bondarev et al. (2010). These authors proposed a positive correlation between the number of trichomes and the contents of SG. Similarly, in the working group, Sarmiento-López et al. (2021) reported that the inoculation of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* on *S. rebaudiana* plants induces the formation of trichomes causing an increase in phenolic compound and flavonoid contents in leaves. In other plants that accumulate secondary metabolites in trichomes, a relationship between the number of trichomes in the leaves and the accumulation of secondary metabolites produced by inoculation with different fungi has also been observed. Kapoor et al. (2007) and Mandal et al. (2015b), described that the inoculation of beneficial fungi (*Glomus macrocarpum*, *Glomus fasciculatum* and *Rhizophagus intraradices*) in *A. annua* plants enhanced the accumulation of artemisinin in trichomes.

The use of confocal microscopy tools used in this work, allowed the localization of the compounds of interest in the trichomes. This is a novel tool used in other studies for secondary metabolite localization in plants (Agati et al., 2002; Talamond et al., 2015; Vidot et al., 2019). This is based on the autofluorescence of those metabolites when excited by a beam of light, and this fluorescence is characterized by emission bands in the blue (440 nm), green (520 nm) and red (690 nm) spectra (Buschmann et al., 2001; García-Plazaola et al., 2015). In plant leaves, secondary metabolites such as alkaloids, terpenes, phenolic compounds and flavonoids, are located by their autofluorescence emission in epidermal tissues and vascular bundles (Agati et al., 2002; Conéjéro et al., 2014; Hutzler et al., 1998; Talamond et al., 2015). In this work, autofluorescence in the blue spectrum was found in inoculated *S. rebaudiana* leaf trichomes by endophytic bacteria. These results agree with the increase in trichome density, SG content,

phenolic compound content and flavonoid content observed in the leaves of *S. rebaudiana*. Recently, Sarmiento-López et al. (2021), reported the use of these techniques to show that *S. rebaudiana* leaves inoculated with arbuscular mycorrhiza fungi *Rhizophagus irregularis* show fluorescence in the trichomes and that this is related to the increase in phenolic compounds and flavonoid accumulation. This suggests that a similar mechanism for metabolite induction and accumulation occurs in both endophytic bacterial and fungal interactions with plants.

The results of gene expression analysis of SG biosynthesis pathway in *S. rebaudiana* leaves, showed that *KO* and *KAH* genes were upregulated when the plants were inoculated with *E. hormaechei* H2A3, *E.hormaechei* H5A2 and *E. xianfangensis* R7A2. Likewise, the *UGT74G1* gene was upregulated with the inoculation of these bacterial strains, which was consistent with the high stevioside accumulation, whereas the *UGT76G1* gene was upregulated with the *E. hormachei* H2A3 inoculation, which may be directly related to the rebaudioside A content determined in *S. rebaudiana* leaves. On the other hand, *E. hormachei* H5A2 inoculation also stimulated rebaudioside A accumulation, but it was not reflected in the gene expression involved in their metabolite synthesis. Previously, other microorganisms inoculated in *S. rebaudiana* plants showed improved in the SG accumulation, and the effect was associated with the high expression of their biosynthesis genes. Several authors have reported the use of different rhizosphere microorganisms. Kilam et al. (2015), Mandal et al. (2013), Tavarini et al. (2018), and Vafadar, Amooaghaie & Otroshy (2014), used *Rhizophagus fasciculatus*, *Bacillus polymixa*, *Pseudomonas putida*, *Azotobacter chroococcum*, *Glomus intraradices*, *Piriformospora indica*, and *Rhizoglomus irregulare,* which generated an increase in SG and were associated with *KO, KS, KHA, UGT74G1* and *UGT76G1* expression. Endophytic bacteria have also reported to increase their secondary metabolite content and the expression of genes in their biosynthetic pathway. For example, *Pseudonocardia* species induce the production of artemisinin in *Artemisia annua* (Li et al., 2012), and *Acinetobacter* sp. induces the abscisic acid (ABA) and salicylic acid (SA) production in *Atractylodes lancea* (Wang et al., 2014).

The findings of this work show that the use of the endophytic bacteria *E. hormachei* H2A3 and *E. hormachei* H5A2 can be considered as a biotechnological strategy to increase the content of secondary metabolites in *S. rebaudiana*.

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Conclusions

Endophytic bacteria inoculated in *S. rebaudiana* plants did not promote plant growth, but the bacterial strains *E. hormaechei* H2A3 and *E. hormaechei* H5A2 increased the SG content and stimulated the density of trichomes in the leaves, as well as the accumulation of secondary metabolites in trichomes. SG accumulation by inoculation with endophytic bacteria was related to the upregulated of the *KO, KHA, UGT74G1* and *UGT76G1* genes. These results represent a finding for the use of endophytic bacteria to favor the increase in the SG content in *S. rebaudiana*.

Acknowledgements

We owe special thanks to Dr. Luis Cardenas Torres from Instituto de Biotecnología (Universidad Nacional Autonoma de México) for access to laboratory to perform molecular biology tests of *S. rebaudiana* tissue. The authors are grateful to Msc. Daniel Tapía Maruri for providing technical assistance in producing the environmental scanning electron microscope and confocal laser scanning microscope images.

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