Enhanced specialized metabolite, trichome density, and biosynthetic gene expression in *Stevia rebaudiana* (Bertoni) Bertoni plants inoculated with endophytic bacteria *Enterobacter hormaechei*

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Stevia rebaudiana (Bertoni) Bertoni is a plant of economic interest in the food and pharmaceutical industries due its steviol glycosides (SG), which are rich in metabolites that are 300 times sweeter than sucrose. In addition, *S. rebaudiana* plants contain phenolic compounds and flavonoids with antioxidant activity. Endophytic bacteria promote the growth and development and modulate the metabolism of the host plant. However, little is known regarding the role of endophytic bacteria in the growth; synthesis of SG, flavonoids and phenolic compounds; and the relationship between trichome development and specialized metabolites in *S. rebaudiana*, which was the subject of this study. The 12 bacteria tested did not increase the growth of *S. rebaudiana* plants; however, the content of SG increased with inoculation with the bacteria *Enterobacter hormaechei* H2A3 and *E. hormaechei* H5A2. The SG content in leaves paralleled an increase in the density of glandular, short, and large trichome. The image analysis of *S. rebaudiana* leaves showed the presence of SG, phenolic compounds, and flavonoids principally in glandular and short trichomes. The increase in the transcript levels of the *KO*, *KAH*, *UGT74G1*, and *UGT76G1* genes was related to the SG concentration in plants of *S. rebaudiana* inoculated with *E. hormaechei* H2A3 and *E. hormaechei* H5A2. In conclusion, inoculation with the stimulating endophytes *E. hormaechei* H2A3 and *E. hormaechei* H5A2 increased SG synthesis, flavonoid content and flavonoid accumulation in the trichomes of *S. rebaudiana* plants.

1 **Enhanced specialized metabolite, trichome density, and biosynthetic gene**
2 **expression in *Stevia rebaudiana* (Bertoni) Bertoni plants inoculated with**
3 **endophytic bacteria *Enterobacter hormaechei***

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21 **Abstract**

22 *Stevia rebaudiana* (Bertoni) Bertoni is a plant of economic interest in the food and
23 pharmaceutical industries due its steviol glycosides (SG), which are rich in metabolites
24 that are 300 times sweeter than sucrose. In addition, *S. rebaudiana* plants contain
25 phenolic compounds and flavonoids with antioxidant activity. Endophytic bacteria
26 promote the growth and development and modulate the metabolism of the host plant.
27 However, little is known regarding the role of endophytic bacteria in the growth;
28 synthesis of SG, flavonoids and phenolic compounds; and the relationship between
29 trichome development and specialized metabolites in *S. rebaudiana*, which was the
30 subject of this study. The 12 bacteria tested did not increase the growth of *S.
31 rebaudiana* plants; however, the content of SG increased with inoculation with the
32 bacteria *Enterobacter hormaechei* H2A3 and *E. hormaechei* H5A2. The SG content in
33 leaves paralleled an increase in the density of glandular, short, and large trichome. The
34 image analysis of *S. rebaudiana* leaves showed the presence of SG, phenolic
35 compounds, and flavonoids principally in glandular and short trichomes. The increase in
36 the transcript levels of the *KO*, *KAH*, *UGT74G1*, and *UGT76G1* genes was related to
37 the SG concentration in plants of *S. rebaudiana* inoculated with *E. hormaechei* H2A3
38 and *E. hormaechei* H5A2. In conclusion, inoculation with the stimulating endophytes *E.
39 hormaechei* H2A3 and *E. hormaechei* H5A2 increased SG synthesis, flavonoid content
40 and flavonoid accumulation in the trichomes of *S. rebaudiana* plants.
41 **Keywords:** *Stevia rebaudiana*, Endophytic bacteria, Trichomes, Specialized metabolite,
42 Steviol glycosides, Biosynthetic genes.

43
44

45 **Introduction**

46 Plant–microbiome interactions are beneficial because they enhance the acquisition of
47 mineral nutrition and provide protection against abiotic and biotic stresses in plants
48 (Asaf et al., 2017; Wang et al., 2015). The study of these interactions has great potential
49 for application in biotechnology and agriculture because the use of microorganisms
50 improves the plant growth and development of food crops (Lodewyckx et al., 2002;
51 Rosenblueth & Martínez-Romero, 2006).
52 Endophytic bacteria are an important group of microorganisms that are found in
53 different plant tissues, such as in the roots (rhizosphere), leaves (phylloplane), stems
54 (laimosphere and caulosphere), fruits (carposphere), seeds (spermosphere) and flowers
55 (anthosphere) (Brader et al., 2017). In this relationship, plants and endophytic bacteria
56 form a unique interaction with the ability to provide alternative sources of active
57 metabolites such as enzymes, biofunctional chemicals, phytohormones and nutrients
58 and to facilitate the distribution and production of secondary metabolites (Hardoim et al.,
59 2015; Santoyo et al., 2016). The host plant provides a protective environment for the
60 bacteria, in which the microorganism can grow and reproduce, but with no adverse
61 effects that negatively affect plant growth and health (Shahzad et al., 2018). Bacteria
62 also enhance the accumulation of secondary metabolites and modulate the
63 accumulation profile and the expression patterns of several biosynthetic pathways in
64 many plant species (Tiwari et al., 2013, 2010; Yang et al., 2019; Zhou et al., 2016). For
65 example, isolated bacteria from *Lycoris radiata (L'Hér.)* Herb promote Amaryllidaceae
66 alkaloid accumulation in the host plant (Liu et al., 2020), and *Pseudomonas fluorescens*
67 induces sesquiterpenoid accumulation in *Atractylodes macrocephala* Koidz plants
68 (Yang et al., 2019).
69 Trichomes are epidermal structures where various secondary metabolites are
70 synthesized and accumulated and are associated with the chemical defense of the plant
71 (Li et al., 2020; Werker, 2000). Trichomes are classified according to their morphology
72 into glandular and nonglandular groups. In particular, glandular trichomes play an
73 important role in the deposition of many secondary metabolites, such as alkaloids,
74 polyketides, phenylpropanoids, phenolic compounds and terpenoids (Li et al., 2021).
75 *Stevia rebaudiana* (Bertoni) Bertoni is a perennial shrub species of the Asteraceae
76 family and is an economically important crop due its ability to accumulate specialized
77 metabolites called steviol glycosides (SG), including isosteviol, stevioside,
78 rebaudiosides (A, B, C, D, E and F), steviolbioside and dulcoside A, which are used as
79 low-calorie sweeteners (Sarmiento-López et al., 2020; Rajasekaran et al., 2008). The
80 sweet taste of *S. rebaudiana* leaves depends on the contents of stevioside and
81 rebaudioside A, which are approximately 250–300 times as sweet as sucrose (Geuns,
82 2003). Due to the high content of sweet glycosides, *S. rebaudiana* is considered a
83 valuable source of natural sweeteners for the growing food market (Goyal & Goyal,
84 2010). In addition, the leaves of *S. rebaudiana* contain phenolic compounds, which are
85 a family of antioxidant metabolites, including stilbenes, flavonoids and phenolic acids
86 (Lemus-Mondaca et al., 2012).
87 Brandle & Telmer (2007) proposed that SG biosynthesis begins with geranylgeranyl-di-
88 phosphate (GGDP) synthesis through the methyl-erythrol-4-phosphate (MEP) route.
89 GGDP is transformed to kaurene by two cyclization steps carried out by terpene
90 cyclases and later converted to steviol by four additional enzyme actions: (EC 5.5.1.13)
91 copalyl diphosphate synthase (*CDPS*), (EC 4.2.3.19) kaurene synthase (*KS*), (EC

92 1.14.14.86) kaurene oxidase (*KO*), and kaurenoic acid hydroxylase (*KAH*) (Kim et al.,
93 1996). Different SG are formed by steviol glycosylation by specific glucosyltransferases;
94 the enzyme (EC 2.4.1.17) *UGT74G1* is involved in the conversion of steviolbioside to
95 stevioside, while the enzyme (EC 2.4.1.17) *UGT76G1* is involved in the conversion of
96 stevioside to rebaudioside A (Shibata et al., 1991 ; Shibata et al., 1995). Some studies
97 have been carried out in *S. rebaudiana* to evaluate the effect of plant growth-promoting
98 rhizobacteria (PGPR) and mycorrhizal fungi on growth, secondary metabolite
99 accumulation, and the expression of biosynthetic genes. Mamta et al. (2010) and
100 Vafadar et al. (2014) reported that inoculation with different PGPR improved plant
101 growth, photosynthetic parameters, and the accumulation of stevioside and
102 rebaudioside A. Likewise, Sarmiento-López et al. (2020) reported that arbuscular
103 mycorrhizal (AM) symbiosis with *Rhizophagus irregularis* improves growth and
104 photosynthetic activity. Additionally, they reported the upregulation of the biosynthetic
105 genes *KO, UGT74G1* and *UGT76G1.* Furthermore, it has been proposed that the
106 synthesis and accumulation of SG take place in trichomes (Bondarev et al., 2010).
107 Recently, Sarmiento-López et al. (2021) reported that AM symbiosis with *R. irregularis*
108 induced a significant increase in the accumulation of phenolic compounds, related to the
109 high number of trichomes, and reported that these metabolites were localized
110 specifically in the secretory cavity of glandular trichomes.
111 Endophytic bacteria are microorganisms that can live inside plant tissues, providing
112 advantages over other rhizospheric microorganisms. However, little is known regarding
113 their role in secondary metabolism, plant growth, and the relationship between trichome
114 development and specialized metabolites (SG and phenolic compounds) in *S.*
115 *rebaudiana*. In this study, we hypothesize that 1) inoculation with endophytic bacteria
116 promotes the accumulation of specialized metabolites and the expression of their
117 biosynthetic genes in *S. rebaudiana* and 2) endophytic bacteria induce the development
118 of trichomes in relation to the accumulation of specialized metabolites. Thus, the
119 objective of this work was to evaluate the accumulation of specialized metabolites and
120 the expression of their biosynthetic genes in *S. rebaudiana* and the development of
121 trichomes in response to inoculation with endophytic bacteria of *S. rebaudiana* plants.
122
123 **Materials and methods**
124 **Growth of *Stevia rebaudiana* plants**
125 *S. rebaudiana* plants were grown under greenhouse conditions at Centro de Desarrollo
126 de Productos Bióticos (CeProBi-IPN) in Morelos, México, according to the methodology
127 described by Sarmiento-López et al. (2021). Briefly, to obtain rooted plants, one apical
128 shoot (three to five cm long) was planted for each 1 dm^3 pot containing a mixture of
129 60:20:20 (w:w:w) sterilized turf, perlite, and vermiculite with an initial pH of 5.6 ± 0.5 and
130 a porosity of 85%. This substrate was sterilized at 121 °C and 15 psi for 2 h. The plants
131 growing in this substrate showed root formation at 15 days.

132 **Endophytic bacterial culture**
133 The endophytic bacteria were isolated from different tissues of *S. rebaudiana* plants:
134 leaf, stem, and roots (Table 1). The tissues were rinsed with sterile water and surface
135 sterilized using 70% ethanol (10 min) and 2% sodium hypochlorite (20 min). The
136 fragments of each tissue were seeded in Petri dishes with LB medium and agar
137 (Sigma–Aldrich, St. Louis, Missouri, USA); the Petri dishes were incubated at 25 ± 1 °C

138 for 24 h. Axenic cultures were obtained and cryopreserved in glycerol at 20% (v/v) at -
139 80 °C. The 16S rDNA sequences of the isolates were compared to the GenBank
140 database using BlastN and a phylogenetic analysis using the MEGA 6 program
141 according to Montes-Salazar et al. (2018). The bacterial inoculum was grown in 250
142 cm^3 flasks with a volume of 100 cm^3 of liquid LB medium and incubated on a rotary
143 shaker (Infors HT, Minitron, Switzerland) at 200 rpm for 48 h at 25 °C.

Inoculation of *S. rebaudiana* with endophytic bacteria

146 Fifteen-day-old *S. rebaudiana* plants with five-cm-long roots and two leaves were used.
147 Plants were disinfected by using 70% ethanol for 1 min, followed by 2% sodium
148 hypochlorite for 1 min, and subsequently rinsed three times with sterile distilled water for
149 2 min. The plants were planted in 1 dm^3 pots containing the same substrate mentioned
150 above. One day after being planted (time 0 from the start of the experiment), the plants
151 were inoculated at the root with 5 cm^3 of culture broth of each of the 12 isolates. The
152 concentration was adjusted to 0.2 OD at 600 nm (approximately 1 x 10^8 cells cm^{-3})
153 (Botta et al., 2013). The plants were grown at 28 °C, with a photoperiod of 16 h light/8 h
154 darkness. Ten plants per treatment were considered, and two independent experiments
155 were carried out. The control was noninoculated plants. All plants were watered every
156 other day with a 50% Steiner solution (Rodríguez-García, 2015). The pots were placed
157 in the nursery in a random arrangement, and no pruning was performed during the
158 evaluation time.

Evaluation of plant growth

161 The *S. rebaudiana* plants inoculated with the endophytic bacteria were collected at 30
162 days postinoculation (dpi). With a Vernier caliper, the plant height was measured from
163 the surface of the substrate to the apex of the plant, and root size was measured from
164 the base of the stem to the root apex. The numbers of leaves and shoots were
165 recorded, and roots were separated and dried in an oven (RiossA E-33, Monterrey,
166 México) at 50 °C. The dry tissue was weighed on an analytical balance, and the dry
167 weight (DW) was recorded. For the biochemical determinations, the collection of leaves
168 of inoculated and noninoculated plants was carried out following the method previously
169 reported by Sarmiento-López et al. (2021).

Determination of steviol glycoside (SG) concentration

172 In the leaves of inoculated and noninoculated plants, the SG concentration was
173 determined according to the methodology reported by Villamarin-Gallegos et al. (2020).
174 Briefly, the leaves were dried in an oven (RiossA E-33) at 65 °C for 48 h. Dry tissue (0.1
175 g) was extracted with 1 cm^3 of methanol (J.T. Backer, Phillipsburg, USA) in 1.5 cm^3
176 microtubes, according to Woelwer-Rieck et al. (2010). The microtubes were stirred for 3
177 min, allowed to stand for 24 h without stirring, and then centrifuged at 1300 x g at 4 °C
178 for 10 min. The supernatant was recovered, placed in fresh microcentrifuge tubes, and
179 stored at 4 °C until analysis by high-performance thin layer chromatography (HPTLC,
180 CAMAG, Muttenz, Switzerland). Stevioside and rebaudioside A concentrations were
181 expressed as mg g DW^{-1}. For each treatment, three plants were evaluated, and two
182 independent experiments were performed.

183

Determination of phenolic compound and flavonoid concentrations

Samples (0.1 g) of dry leaves from plants not inoculated and inoculated with endophytic bacteria were extracted with 1 cm^3 of 75% ethanol and centrifuged at 1300 x g at 4 °C for 10 min. The supernatant was recovered in 1.5 cm^3 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 reaction was performed on a microplate incubated at room temperature in the dark for 2 h. The absorbance was measured at 760 nm on a spectrophotometer (Multiscan Go, Thermo Fisher Scientific, Massachusetts, USA) equipped with SkanIt Software version 1.00.40. Gallic acid (Sigma–Aldrich, St. Louis, Missouri, USA) was used as a standard, and the curve was constructed with serial dilutions (5, 10, 15, 20, 25 µg cm^{-3}) in distilled water. The standard curve had a correlation value R^2 = 0.995. The results were expressed as mg equivalents of gallic acid (GAE) g DW^{-1}.

The flavonoid concentration was determined according to Villamarín-Gallegos et al. (2020) and adapted from Chang et al. (2002). The assay mix was performed on a microplate of 96 wells and incubated at room temperature in the dark for 30 min. Absorbance was monitored at 415 nm on a spectrophotometer (Multiscan Go, Thermo Fisher Scientific) equipped with SkanIt Software version 1.00.40. Serial dilutions (5, 10, 15, 20, 25 µg cm^{-3}) of quercetin (Sigma–Aldrich) in distilled water were used to construct the standard curve; the correlation value of the standard curve was R^2 = 0.995. The results were expressed as mg equivalents of quercetin (QE) g DW^{-1}.

Trichome analysis by environmental scanning electron microscopy (ESEM) and confocal laser scanning microscopy

The trichome density of leaves was analyzed with an environmental scanning electron microscope (Carl Zeiss, EVO LS10, Germany) according to the methodology reported by Sarmiento-López et al. (2021). Fully developed leaves close to the apical meristem from plants that were noninoculated and those inoculated with the endophytic bacteria were collected. A leaf was placed in aluminum stubs with double-sided conductive carbon tape and 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 trichome density in 0.255 cm^2 (trichome leaf area^{-1}) and the type of trichomes (short, large and glandular) were determined by image analysis using ImageJ editing software 2.0 from micrographs obtained by ESEM.

The effect of the inoculation of plants with endophytic bacteria on specific metabolite accumulation was visualized with a confocal laser scanning microscope (Carl Zeiss, model LSM 800, Germany) according to the methodology reported by Sarmiento-López et al. (2021). The maximum fluorescence of secondary metabolites (SG, 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

230 The transcript accumulation levels of the genes for kaurene oxidase (*KO*), kaurene
231 hydroxylase (*KAH*) and (UDP)-glycosyltransferases (*UGT74G1* and *UGT76G1*) were
232 evaluated in leaves of noninoculated plants (control) and leaves of plants inoculated
233 with the selected endophytic bacteria *E. hormaechei* H2A3, *E. hormaechei* H5A2, and
234 *E. xiangfangensis* R7A2. Expression of each gene was normalized against the
235 expression levels of the housekeeping gene *GAPDH*. Frozen leaf samples (0.5 g DW)
236 were ground to a fine powder with liquid nitrogen. Total RNA was obtained using TRIzol
237 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. First-strand
238 cDNA synthesis was performed as previously reported by Sarmiento-López et al.
239 (2020).
240 The primers corresponding to the *KO* gene were SrKOF 5´-
241 TCTTCACAGTCTCGGTGGTG-3´ and SrKOR 5´-GGTGGTGTCGGTTTATCCTG-3´;
242 the primers corresponding to the *KAH* gene were SrKAHF 5´-
243 CCTATAGAGAGGCCCTTGTGG-3´ and SrKAHR 5´-TAGCCTCGTCCCTTTGTGTC-3´;
244 the primers corresponding to the glycosyltransferase *UGT74G1* gene were
245 SrUGT74G1F 5´-GGTAGCCTGGTGAAACATGG-3´ and SrUGT74G1R
246 5´CTGGGAGCTTTCCCTCTTCT – 3´; and the primers corresponding to the
247 glycosyltransferase *UGT76G1* gene were SrUGT76G1F 5´-
248 GACGCGAACTGGAACTGTTG-3´ and SrUGT76G1R 5´-
249 AGCCGTCGGAGGTTAAGACT – 3´. qRT–PCR was performed using SYBR Green
250 (QIAGEN, California, USA) and quantified on a Rotor-Gene Q (QIAGEN, California,
251 USA) real-time PCR thermal cycler. qRT–PCR was programmed for 35 cycles, with
252 denaturing at 95 °C for 15 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s.
253 Three biological replicates with three technical replicates per treatment were evaluated.
254 Primer specificity was verified by regular PCR and melting curve analysis. The primers
255 for the *S. rebaudiana* glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene
256 SrGAPDHF 5´-TCAGGGTGGTGCCAAGAAGG-3´ and SrGAPDHR 5´-
257 TTACCTTGGCAAGGGGAGCA- 3´ were used as internal controls for normalization,
258 and the quantitative results were evaluated by the $2^{-\Delta CT}$ method described by Livak &
259 Schmittgen (2001). Three plants per treatment were evaluated.
260
261 **Statistical analysis**
262 Raw data from each analysis were used to obtain the central tendency measures
263 (means and standard deviations). Data on each parameter for the noninoculated and
264 inoculated plants were analyzed by using one-way analysis of variance (ANOVA), and
265 significant differences were analyzed using Tukey's test, with a *P* value < 0.05. All the
266 data were checked for normality using Shapiro–Wilk's test before statistical analysis. All
267 statistical analyses were performed using the statistical software Minitab® for Windows,
268 version 19.1 (United States, LLC), and figures were made using GraphPad Prism for
269 Windows, version 6.0 (GraphPad Corp, San Diego, CA, USA).
270
271 **Results**
272 **Effect of endophytic bacterial inoculation on *S. rebaudiana* growth, steviol**
273 **glycosides (SG), phenolic compounds, and flavonoid accumulation in the leaves**
274 The inoculation with endophytic bacteria did not promote the growth of *S. rebaudiana*
275 plants, since plant and root length, number leaves and root dry weight were not different

276 from those of noninoculated plants (Table 2). However, the results of the effect of
277 inoculation with endophytic bacteria on the accumulation of specialized metabolites,
278 showed that there are stimulating endophytes (*Enterobacter hormaechei* H2A3, *E.*
279 *hormaechei* H5A2) and nonstimulating endophytes. For the stimulating endophytes, it
280 was observed that in plants inoculated with *Enterobacter hormaechei* H2A3, there was
281 a significant increase in the concentrations of total SG (Fig. 1A), rebaudioside A (Fig.
282 1B), and stevioside (Fig. 1C), with values 2.2, 2.2 and 2.1- fold greater, respectively,
283 than those in noninoculated plants. The same trend was found with the inoculation with
284 *E. hormaechei* H5A2, where the concentrations of total SG, rebaudioside A, and
285 stevioside increased significantly by 1.5, 1.5, and 1.4-fold in comparison with those in
286 the noninoculated plants. Inoculation with *E. bacterium* H7A1 did not significantly
287 increase the concentration of specialized metabolites in relation to noninoculated plants,
288 but the concentration of metabolites was similar to that found in plants inoculated with
289 *E. hormaechei* H5A2. Plants inoculated with other bacteria (nonstimulating endophytes)
290 did not present significant changes in the concentration of specialized metabolites (Fig.
291 1).
292 In *S. rebaudiana* plants inoculated with *E. hormaechei* H5A2, there was a significant
293 increase of 1.4-fold in the flavonoid concentration in comparison to that in noninoculated
294 plants (Fig. 2A), while the concentration of phenolic compounds was similar to that in
295 noninoculated plants (Fig. 2B). Inoculation with other bacteria did not increase the
296 concentration of flavonoids and phenolic compounds, while inoculation with *E.*
297 *xianfangensis* R7A2 significantly decreased the concentration of phenolic compounds.
298 Based on the screening results with endophytic bacteria, the selected bacteria used to
299 continue this work were *E. hormaechei* H2A3 *and E. hormaechei* H5A2. Additionally, *E.*
300 *xianfangensis* R7A2 was used as an additional treatment because it did not show
301 induction of specialized metabolites or growth promotion. These bacteria were used to
302 analyze the effect on trichome density in leaves as well as the expression of genes of
303 the SG biosynthesis pathway.
304
305
306 **Trichome density in *S. rebaudiana* leaves by scanning electron and confocal**
307 **microscopy**
308 Photomicrographs (SEM) of the leaves from noninoculated plants and plants inoculated
309 with the selected bacteria showed three types of trichomes: glandular, large, and short
310 (Fig. 3, see labels G, L and S). In plants inoculated with *E. hormaechei* H2A3, the
311 photomicrographs showed the presence of a greater number of trichomes that were
312 short, large and glandular in comparison with those in noninoculated plants (Fig. 3B,
313 see labels S, L and G), while in leaves of plants inoculated with *E. hormaechei* H5A2
314 and *E. xiangfangensis* R7A2, the photomicrographs did not show a visual effect on the
315 trichome number in relation to that in noninoculated plants (Fig. 3C-D).
316 In the leaves of noninoculated and inoculated plants, short trichomes were the most
317 abundant (2000 to 6000 trichomes/cm^2), followed by glandular (1000 to 3000
318 trichomes/cm^2) and large trichomes (200 to 800 trichomes/cm^2) (Fig. 4). Trichome
319 density showed that *E. hormaechei* H2A3 induced a significant increase in glandular,
320 large, and short trichomes of 1.7, 4.3, and 1.5-fold those in noninoculated plants,
321 respectively (Fig. 4A-C). However, *E. hormaechei* H5A2 did not induce any effect on

322 trichome density (Fig. 4A-C) and *E. xiangfangensis* R7A2 had significantly lower
323 number of short and did not induce effect on glandular and large trichomes compared to
324 control (Fig. 4A-C).
325 The location of SG, phenolic compounds, and flavonoids in the trichomes of *S.*
326 *rebaudiana* leaves by autofluorescence using confocal microscopy is shown in Fig. 5. In
327 the red channel, the autofluorescence of chlorophylls is shown in epidermal and
328 mesophyll leaf cells (Figs. 5 A-D), while in the blue channel, the autofluorescence of
329 SG, phenolic compounds, and flavonoids is shown in the trichomes (Fig. 5 E-H).
330 Inoculation with *E. hormaechei* H2A3 and *E. hormaechei* H5A2 increased the intensity
331 of the autofluorescence signal in the blue channel, particularly in glandular and short
332 trichomes (Figs. 5 F and G, see labels G and S), while in the noninoculated plants (Fig.
333 5E) and those inoculated with *E. xiangfangensis* R7A2 (Fig. 5H, see labels L, G, and S),
334 the autofluorescence signal was lower.
335 Notably, strong blue fluorescence was exhibited in the secretory cavity of the glandular
336 trichomes and on the short trichomes of inoculated plants with *E. hormaechei* H2A3 and
337 *E. hormaechei* H5A2 (Fig. 5N and O, see labels S and G) in comparison to the
338 noninoculated plants (Fig. 5M) and those inoculated with *E. xiangfangensis* R7A2 (Fig.
339 5P).
340
341 **Effect of endophytic bacterial inoculation on differential SG biosynthetic gene**
342 **expression in *S. rebaudiana* plants**
343 The results of the differential expression of SG biosynthesis genes in *S. rebaudiana*
344 plants inoculated with endophytic bacteria are presented in Fig. 6. The transcription
345 level of the *KO* gene increased significantly with *E. hormaechei* H5A2 bacteria (21.3-
346 fold) and *E. xiangfangensis* R7A (42.3-fold) compared to that inoculated with *E.*
347 *hormaechei* H2A3 and that in noninoculated plants (Fig. 6A). The *KAH* transcript level
348 increased significantly by 52.3-fold with *E. hormaechei* H5A2 (Fig. 6B). The transcript
349 levels of the *UGT74G1* gene were significantly increased with inoculation with *E.*
350 *hormaechei* H2A3 (11.3-fold) and *E. hormaechei* H5A2 (17.2-fold), while *E.*
351 *xiangfangensis* R7A increased (6.0-fold) but was not significant with respect to that in
352 noninoculated plants (Figs. 6C). Finally, the transcript levels of the *UGT76G1* gene
353 were significantly increased by 3.2-fold with the addition of *E. hormaechei* H2A3 (Figs.
354 6D).
355
356 **Discussion**
357 Interactions between plant and endophytic microorganisms have been proposed as a
358 strategy to improve plant growth and stimulate secondary metabolism (Afzal et al.,
359 2019; Hardoim et al., 2015; Hardoim et al., 2008). However, in this work, reinoculation
360 with endophytic bacteria isolated from different tissues of *S. rebaudiana* did not
361 significantly promote plant growth. These results suggest that growth promotion is not
362 associated with endophytic bacterial reinoculation and that the bacteria did not
363 negatively affect plant growth. It is possible that the plant could divide the nutrients for
364 primary metabolism or provide the nutrients required for bacterial growth. This behavior
365 has also been observed in different plant–microorganism interactions, such as *Ocimum*
366 *basilicum* L. inoculated with *Glomus mosseae* (Copetta et al.,2006) and *Ocimum*
367 *gratissimum* L. inoculated with *Glomus intrarradices* (Hazzoumi et al., 2017).

368   The stimulating endophytes *E. hormaechei* H2A3 and *E. hormaechei* H5A2 increased
369   the concentrations of stevioside and rebaudioside A in the leaves of *S. rebaudiana*. *E.*
370   *hormaechei* H5A2 increased the concentration of flavonoids, which indicates that these
371   bacteria play an important role in the biosynthesis of the specialized metabolites, SG
372   and flavonoids in *S. rebaudiana.* Similarly, other studies have shown that bacteria may
373   have a differential effect on the biosynthesis of secondary metabolites in crops such as
374   *Oryza sativa* L. (Andreozzi et al., 2019; Balachandar et al., 2006), *Beta vulgaris* L. (Shi
375   et al., 2010), *Artemisia annua* L. (Li et al., 2012; Tripathi et al., 2020), *Catharanthus*
376   *roseus* (Nirmal) (Tiwari et al., 2013), *Salvia miltiorrhiza* Bunge (Yan et al., 2014),
377   *Fragaria ananassa* (Duch) Macarena (Guerrero-Molina et al., 2014), *Glycine max* (L.)
378   Merr (Asaf et al., 2017), *Glycyrrhiza uralensis* F (Li et al., 2018), *L. radiata* (Liu et al.,
379   2020), and *Camellia oleifera* Abel (Xu et al., 2020). The effect of inoculation with
380   bacteria and fungi on the growth and synthesis of metabolites in *S. rebaudiana* plants
381   has been reported. Vafadar et al. (2014) reported that bacteria isolated from the
382   rhizosphere (*Bacillus polymixa*, *Pseudomonas putida* and *Azotobacter chroococcum)*
383   inoculated in *S. rebaudiana* plants significantly increased root and shoot biomass and
384   the concentrations of stevioside, chlorophyll, and macronutrients (nitrogen, phosphorus
385   and potassium) in plants. Kilam et al. (2015) reported that the bacterium *A.*
386   *chroococcum* improved the growth, antioxidant activity and SG content of *S. rebaudiana*
387   plantlets grown in vitro. Several fungi, including *G. intraradices, Piriformospora indica,*
388   *Rhizoglomus irregulare,* and *Rizophagus intraradices,* have been reported as other
389   inoculant microorganisms of *S. rebaudiana,* and several results have demonstrated that
390   these fungi can enhance plant growth and stevioside accumulation (Kilam et al., 2015;
391   Mandal et al., 2013a; Mandal et al., 2015a Vafadar et al., 2014; Sarmiento-Lopez et al.,
392   2020; Tavarini et al., 2018). A synergistic relationship between bacteria and fungi has
393   been proposed to improve the plant growth of *S. rebaudiana* and the accumulation of
394   SG (Kilam et al., 2015; Vafadar et al., 2014). Nowogórska & Patykowski's (2015)
395   findings support the idea that sequential inoculation with bacteria, fungi, or a
396   combination of both does not always yield synergistic effects. However, this issue
397   should be investigated in the future. To our knowledge, this is the first report of
398   inoculation of *S. rebaudiana* with endophytic bacteria from the *Enterobacter* genus as a
399   strategy to improve the biosynthesis of their specialized metabolites. The results of our
400   study indicate that the synthesis of specialized metabolites is achieved with the
401   inoculation of endophytic bacteria without fungal coinoculation.
402   Trichomes are plant structures that accumulate secondary metabolites, and their
403   presence in plant leaves is associated with defense mechanisms of the plant against
404   pathogens, insects, and adverse environmental conditions (Champagne & Boutry, 2016;
405   Tian et al., 2017; Werker, 2000). The trichomes observed in the leaves of *S. rebaudiana*
406   were short, large, and glandular. This trichome morphology was typical of those
407   previously reported (Bondarev et al., 2003; Bondarev et al., 2010; Cornara et al., 2001;
408   Monteiro et al., 2001). Our results showed that inoculation with endophytic bacteria
409   caused a significant increase in trichome density in *S. rebaudiana* leaves. This
410   anatomical response in the increased trichome density has been observed in other
411   plants, such as *A. annua* inoculated with *R. intraradices* (Mandal et al., 2015) and *A.*
412   *annua* inoculated with *Glomus macrocarpum* and *Glomus fasciculatum* (Kapoor et al.,
413   2007). Inoculation with the endophyte *E. hormaechei* H2A3 generated a higher density

414   of trichomes in *S. rebaudiana* leaves as well as a higher concentration of SG and
415   flavonoids in comparison to the control. However, the results of a Pearson analysis
416   between the concentration of specialized metabolites and trichome density did not show
417   a correlation between the variables ($R^2 < 0.53$). These results are in contrast with
418   Bondarev et al. (2010); the authors suggest a positive relationship between the number
419   of glandular trichomes and the accumulation of SG; however, they did not present a
420   quantitative analysis of the correlation between SG accumulation and trichome density.
421   In other plants that accumulate secondary metabolites in trichomes, a relationship
422   between the number of trichomes in the leaves and the accumulation of secondary
423   metabolites induced by inoculation with different fungi was reported. Kapoor et al.
424   (2007) and Mandal et al. (2015b) described that the inoculation of beneficial fungi
425   (*Glomus macrocarpum*, *Glomus fasciculatum* and *Rhizophagus intraradices*) in *A.*
426   *annua* plants enhanced the accumulation of artemisinin in trichomes and reinforced the
427   idea that beneficial interactions, including endophytic bacteria, induce several
428   biochemical and physiological responses for the benefit of crops.
429   The use of confocal microscopy tools used in this work allowed the localization of the
430   specialized metabolites in the trichomes of *S. rebaudiana* leaves by detecting their
431   autofluorescence (Agati et al., 2002; Talamond et al., 2015; Vidot et al., 2019). In this
432   work, autofluorescence in the blue spectrum, which is indicative of the accumulation of
433   these metabolites, was found in trichomes of inoculated *S. rebaudiana* by *E.*
434   *hormaechei* H2A3 and *E. hormaechei* H5A2. In plants, the accumulation of different
435   specialized metabolites has been observed in trichomes (Agati et al., 2002; Conéjéro et
436   al., 2014; Hutzler et al., 1998; Talamond et al., 2015). Recently, Sarmiento-López et al.
437   (2021) reported that *S. rebaudiana* plants colonized with arbuscular mycorrhiza fungi *R.*
438   *irregularis* showed fluorescence in the trichomes and that this was related to the
439   increase in phenolic compounds and flavonoid accumulation. Taken together, these
440   results suggest that a similar mechanism for metabolite induction and accumulation
441   occurs in both endophytic bacterial and fungal interactions with plants. It is well known
442   that specialized metabolites such as terpenes and phenolic compounds have an
443   important function in the priming response by activating systemic resistance, enabling
444   plants to respond more effectively to attacks from pathogens and herbivores
445   (Cervantes-Gámez et al., 2016; Pozo & Azcón-Aguilar, 2007; Santos et al., 2017). In
446   this work, we observed a significant increase in trichome development, as well as in the
447   accumulation of SG and phenolic compounds, which can be related to the induction of
448   systemic resistance by the inoculation of endophytic bacteria in *S. rebaudiana* plants in
449   a manner similar to that observed in other plant species under different plant–
450   microorganism interactions (Kapoor et al.,2017; Mandal et al.,2015). However, in *S.*
451   *rebaudiana,* further experimental studies are needed to prove this hypothesis.
452   Kaurene oxidase and kaurenoic acid hydroxylase are important enzymes in SG
453   biosynthesis and represent the principal branch point in the catabolism of the central
454   backbone (steviol) of SG. In fact, steviol is glycosylated by the conjugation of glucose by
455   UDP-glycosyltransferases (*UGTs*), where *UGT74G1* is responsible for synthesizing
456   stevioside, while *UGT76G1* is required to produce rebaudioside A (Brandle & Telmer,
457   2007). Our results of gene expression analysis of the SG biosynthesis pathway in *S.*
458   *rebaudiana* leaves showed that the *KO* gene was upregulated with *E. hormaechei* H5A2
459   and *E. xiangfangensis* R7A2; the *KAH* gene was upregulated with *E. hormaechei* H5A2.

460 Likewise, the *UGT74G1* gene was upregulated with the inoculation of *E. hormachei*
461 H2A3 and *E. hormachi H2A3* and *E. hormaechei* H5A2, which was consistent with the
462 high stevioside concentration, whereas the *UGT76G1* gene was upregulated with *E.*
463 *hormachei* H2A3 inoculation, which may be directly related to the rebaudioside A
464 concentration determined in *S. rebaudiana* leaves. Inoculation with *E. hormachei* H5A2
465 also stimulated rebaudioside A accumulation, but it was not reflected in the expression
466 of genes involved in their metabolite synthesis. Although the transcript levels in plants
467 inoculated with *E. hormachei* H5A2 were low, it is possible that the enzymatic activity of
468 (UDP)-glycosyltransferases synthetized by the *UGT76G1* gene could be similar to that
469 in plants inoculated with *E. hormachei* H2A3. However, further complementary studies
470 of enzymatic activity are necessary to confirm this hypothesis.
471 Previously, other rhizospheric microorganisms inoculated in *S. rebaudiana* plants
472 showed improved SG accumulation, and the effect was associated with the high
473 expression of their biosynthesis genes, *KO, KS, KHA, UGT74G1* and *UGT76G1* (Kilam
474 et al., 2015; Mandal et al., 2013; Tavarini et al., 2018; Vafadar et al., 2014). In other
475 plants, inoculation with endophytic bacteria also increased the content of secondary
476 metabolites and the expression of genes in their biosynthetic pathway. For example,
477 *Pseudonocardia* species induce the production of artemisinin in *A. annua* (Li et al.,
478 2012), and *Acinetobacter* sp. induces abscisic acid (ABA) and salicylic acid (SA)
479 production in *Atractylodes lancea* (Thunb.) DC. (AL) (Wang et al., 2014). The findings of
480 the present work show that the use of the endophytic bacteria *E. hormachei* H2A3 and
481 *E. hormachei* H5A2 can be considered a biotechnological strategy to increase the
482 concentration of specialized metabolites in *S. rebaudiana.*
483
484

Conclusions

486 Endophytic bacteria inoculated in *S. rebaudiana* plants did not promote plant growth,
487 but the stimulating endophytes *E. hormaechei* H2A3 and *E. hormaechei* H5A2
488 increased the SG content and stimulated the density of trichomes in the leaves as well
489 as the accumulation of specialized metabolites in trichomes. The increase in the
490 transcript levels of the *KO, KAH, UGT74G1*, and *UGT76G1* genes was correlated with
491 SG concentration by inoculation with *E. hormaechei* H2A3 and *E. hormaechei* H5A2.
492 These results suggest the potential use of *E. hormaechei* H2A3 and *E. hormaechei*
493 H5A2 to increase the content of SG and flavonoids in *S. rebaudiana* plants.
494

Author contributions

496 Dumas G. Oviedo-Pereira conceived and designed the experiments, performed the
497 experiments, prepared figures and/or tables, authored or reviewed drafts of the paper,
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499 Melina López-Meyer analyzed the data, prepared figures and/or tables, authored or
500 reviewed drafts of the paper, and approved the final draft.
501 Silvia Evangelista-Lozano performed the experiments and prepared the figures and/or
502 tables.
503 Gabriela Sepúlveda-Jiménez authored or reviewed drafts of the paper and approved the
504 final draft.

505 Luis G. Sarmiento-López conceived and designed the experiments and performed the
506 experiments.
507 Mario Rodríguez-Monroy conceived and designed the experiments, analyzed the data,
508 reviewed and edited drafts of the paper, and approved the final draft.
509
510 **Declarations**
511 **Conflict of interest:** The authors declare that they have no conflicts of interest.
512

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799

Figure 1

Content of total steviol glycosides (A), stevioside (B) and rebaudioside A (C) in *Stevia rebaudiana* leaves of noninoculated (control) and inoculated plants with endophytic bacteria.

Bars represent the mean ± standard deviation of three individual plants collected to 30 days postinoculation. Different letters indicate a significant difference according to Tukey's test (P value of less than 0.05).

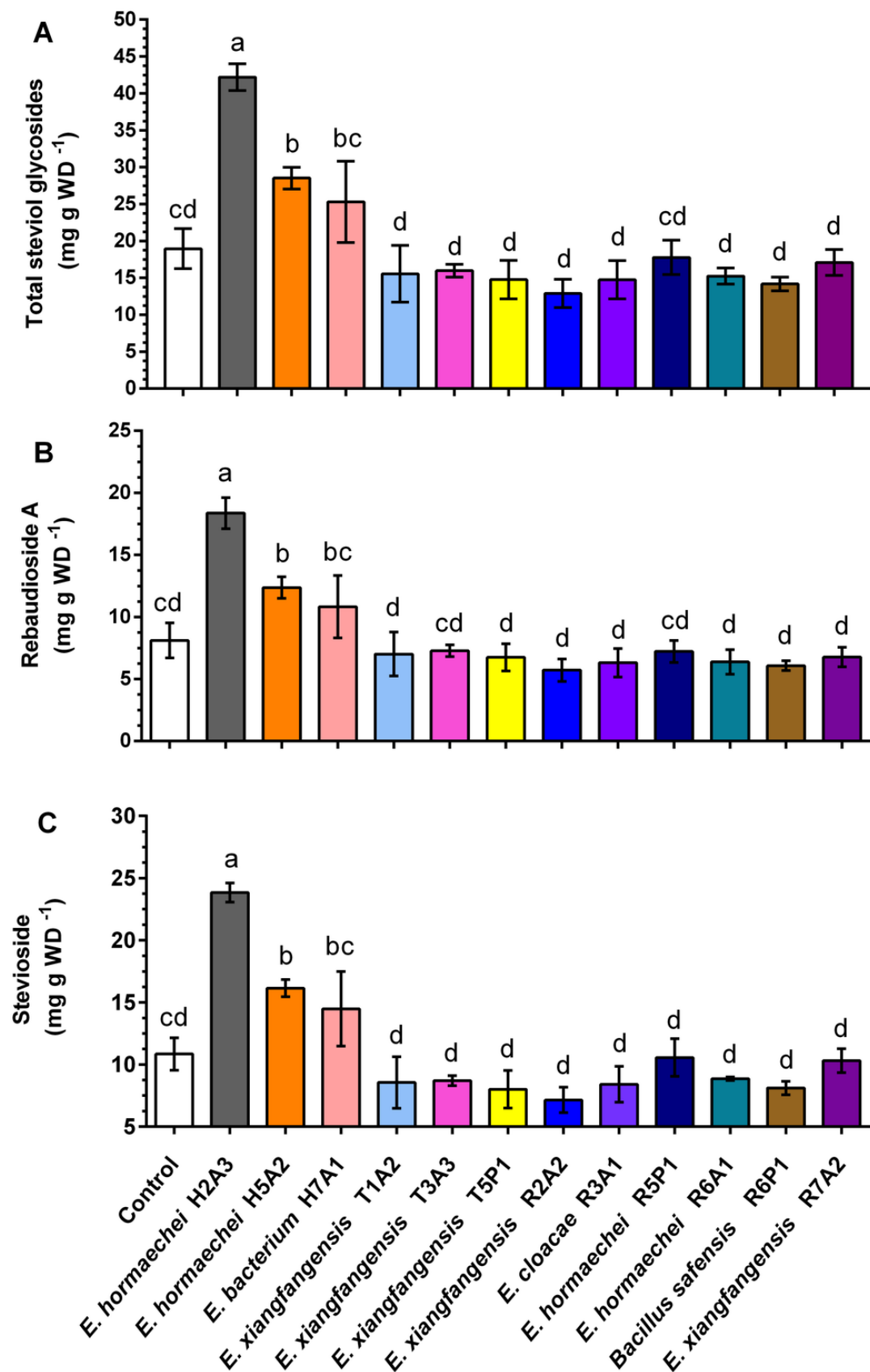



Figure 2

Content of flavonoids (A) and phenolic compounds (B) in *Stevia rebaudiana* plants inoculated with endophytic bacteria.

Bars represent the mean ± standard deviation of three individual plants collected to 30 days postinoculation. Different letters indicate a significant difference according to Tukey's test (*P* value of less than 0.05).

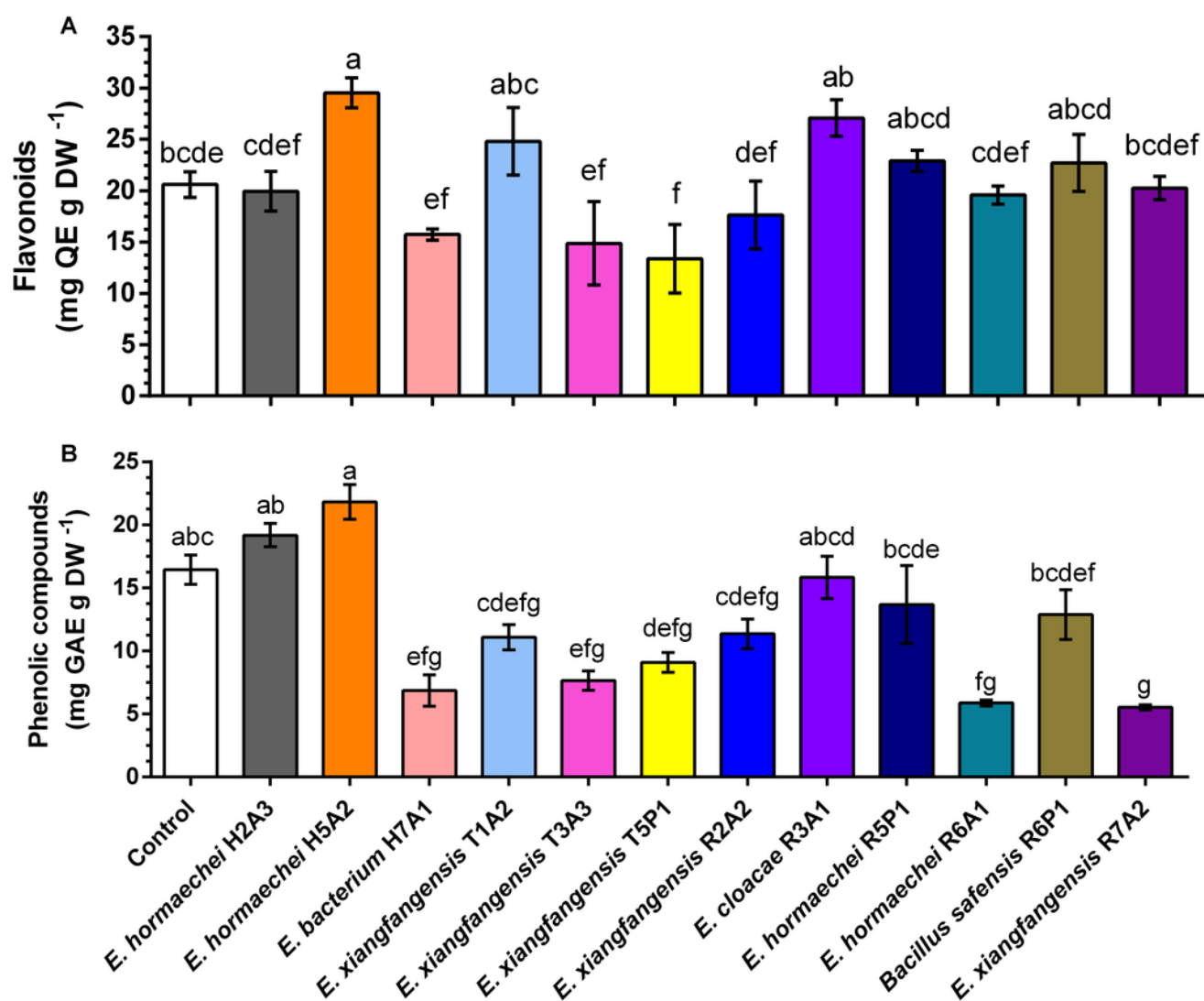



Figure 3

Photomicrographs obtained with environmental scanning electron microscopy of trichomes in *Stevia rebaudiana* leaves.

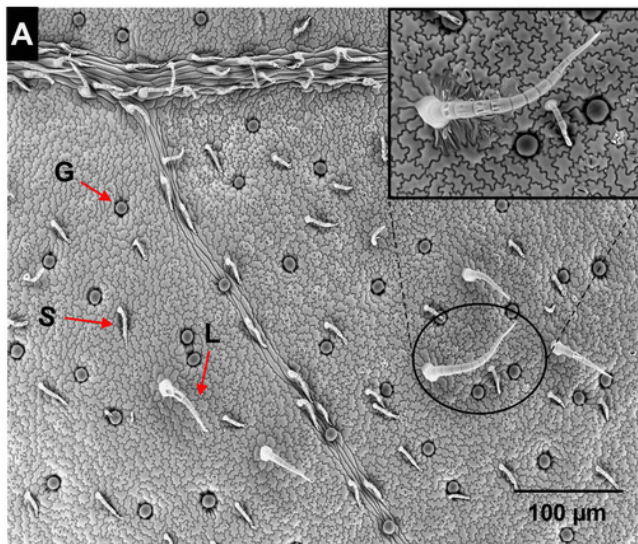
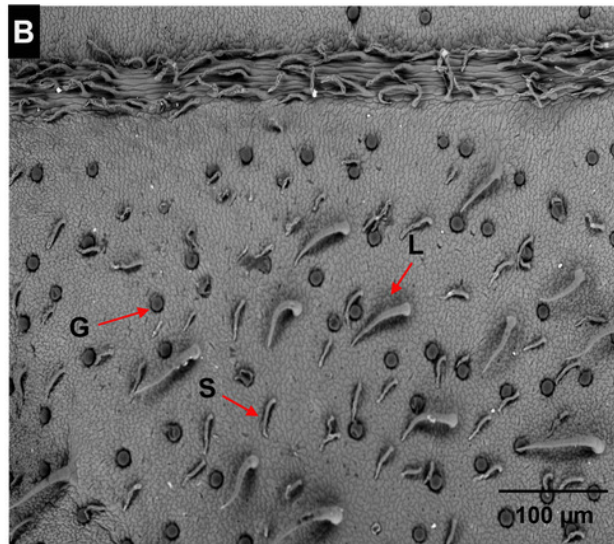
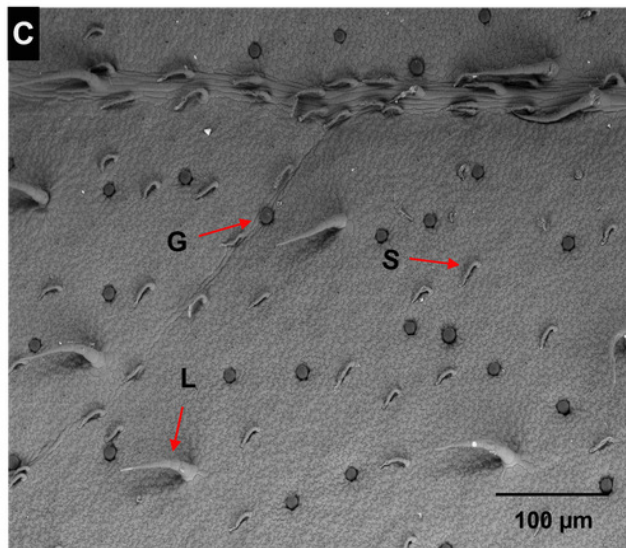
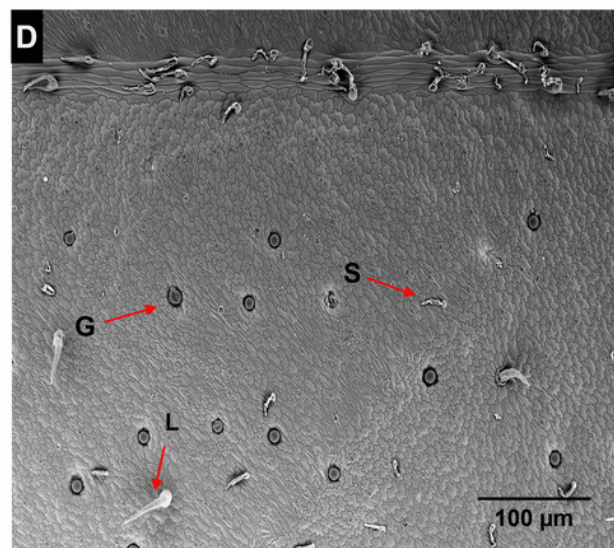
Trichomes in leaves of (A) noninoculated and inoculated plants with (B) *E. hormaechei* H2A3, (C) *E. hormaechei H5A2* and (D) *E. xiangfangensis* R7A2. 4x magnified Panel A corresponds to the image of trichome morphology of the uninoculated leaves. In all panels, the letter "S" is used to indicate short trichomes, "L" to indicate large trichomes and "G" to indicate glandular trichomes.

Control

***E. hormachei* H2A3**

***E. hormachei* H5A2**

***E. xiangfangensis* R7A2**

Figure 4

Effect of endophytic inoculation on the trichome density of *Stevia rebaudiana* leaves. (A) Glandular, (B) large, and (C) short trichome density in leaves.

Bars represent the mean ± standard deviation of four individual plants collected 30 days postinoculation. The different letters indicate a significant difference according to Tukey's test (P value of less than 0.05).

off
off

A

B

C

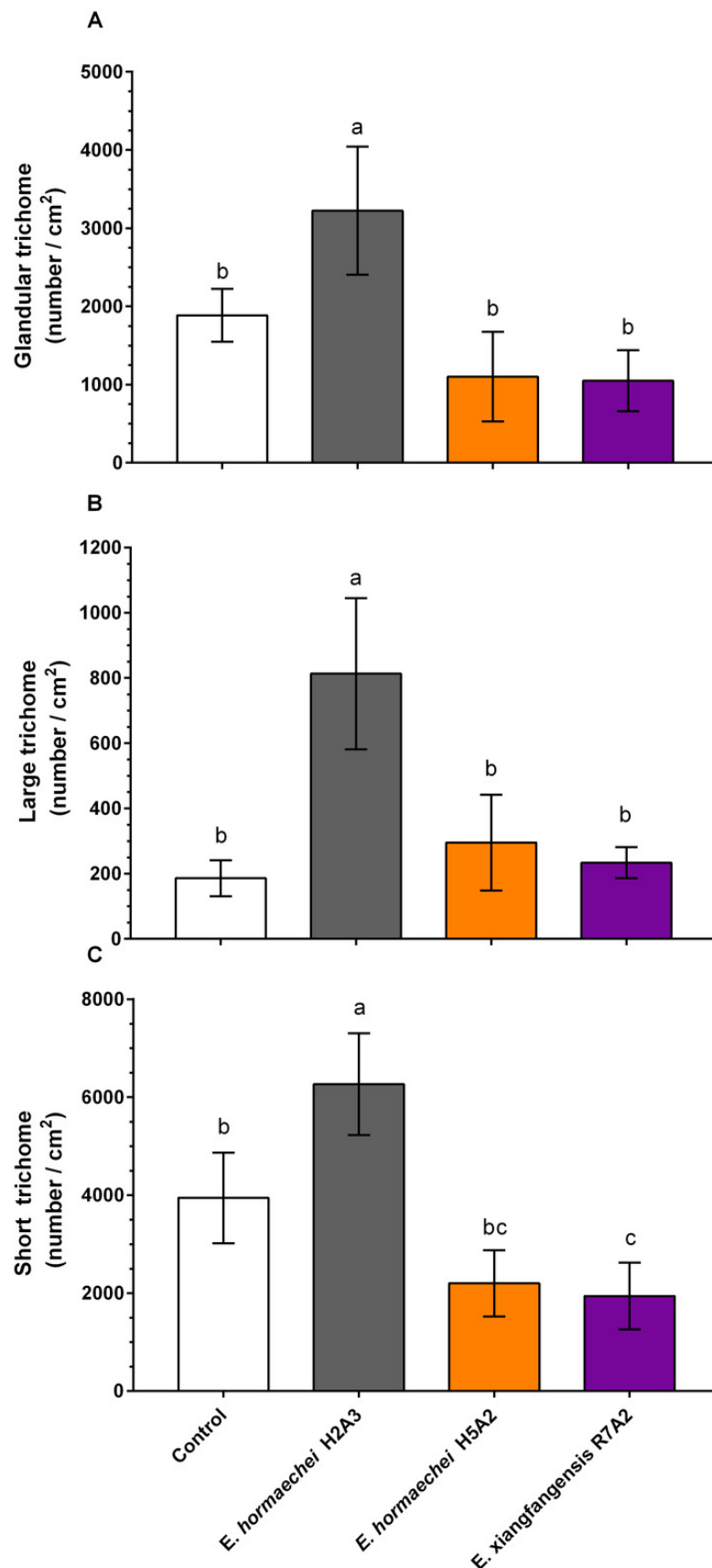



Figure 5

Localization of the specialized metabolites steviol glycosides, phenolic compounds, and flavonoids in *Stevia rebaudiana* trichomes.

Chlorophyll autofluorescence (red channel) of (A) noninoculated and inoculated plants with (B) *E. hormaechei* H2A3, (C) *E. hormaechei* H5A2), and (D) *E. xiangfangensis* R7A2. Specialized metabolite autofluorescence (blue channel) of (E) noninoculated and inoculated plants with (F) *E. hormaechei* H2A3, (G) *E. hormaechei* H5A2, and (H) *E. xiangfangensis* R7A2. Colocalization of chlorophyll and specialized metabolite autofluorescence in (I) noninoculated and inoculated plants with (J) *E. hormaechei* H2A3, (K) *E. hormaechei* H5A2, and (L) *E. xiangfangensis* R7A2. Panels M-P correspond to a magnification of trichomes in noninoculated and inoculated plants with *E. hormaechei* H2A3, *E. hormaechei* H5A2, and *E. xiangfangensis* R7A2, respectively. The chlorophyll and specialized metabolite fluorescence emission spectra were obtained at 630–685 nm (red channel) and 435–485 nm (blue channel), respectively. In all panels, the letter "S" is used to indicate the shot trichomes, "L" to indicate the large trichomes and "G" to indicate the glandular trichomes.

Red channel

A

B

C

D

Blue channel

E

F

G

H

Mergue

I

J

K

L

Mergue

M

N

O

P

Control

E. hormaechei H2A3

E. hormaechei H5A2

E. xiangfangensis R7A2

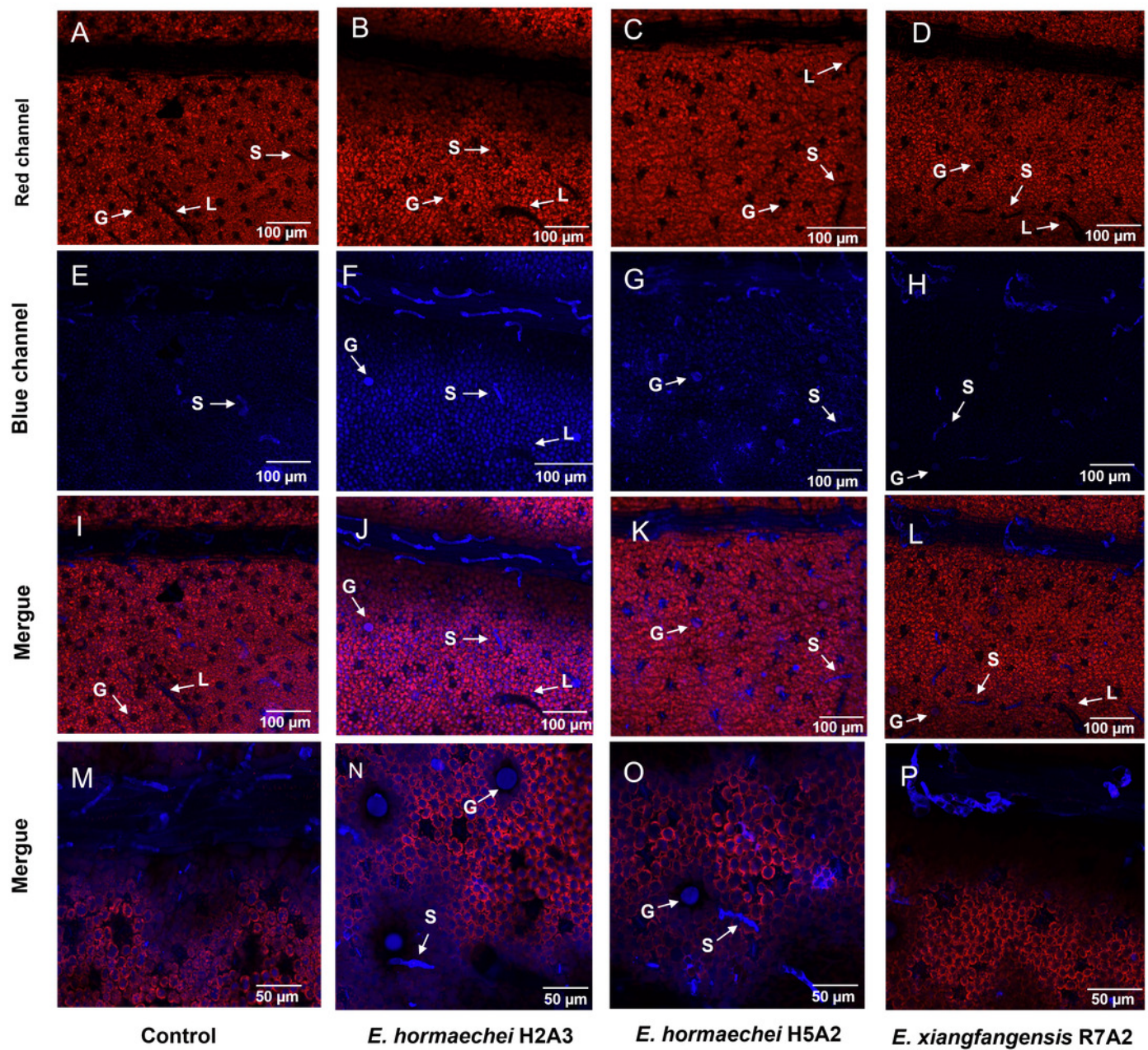
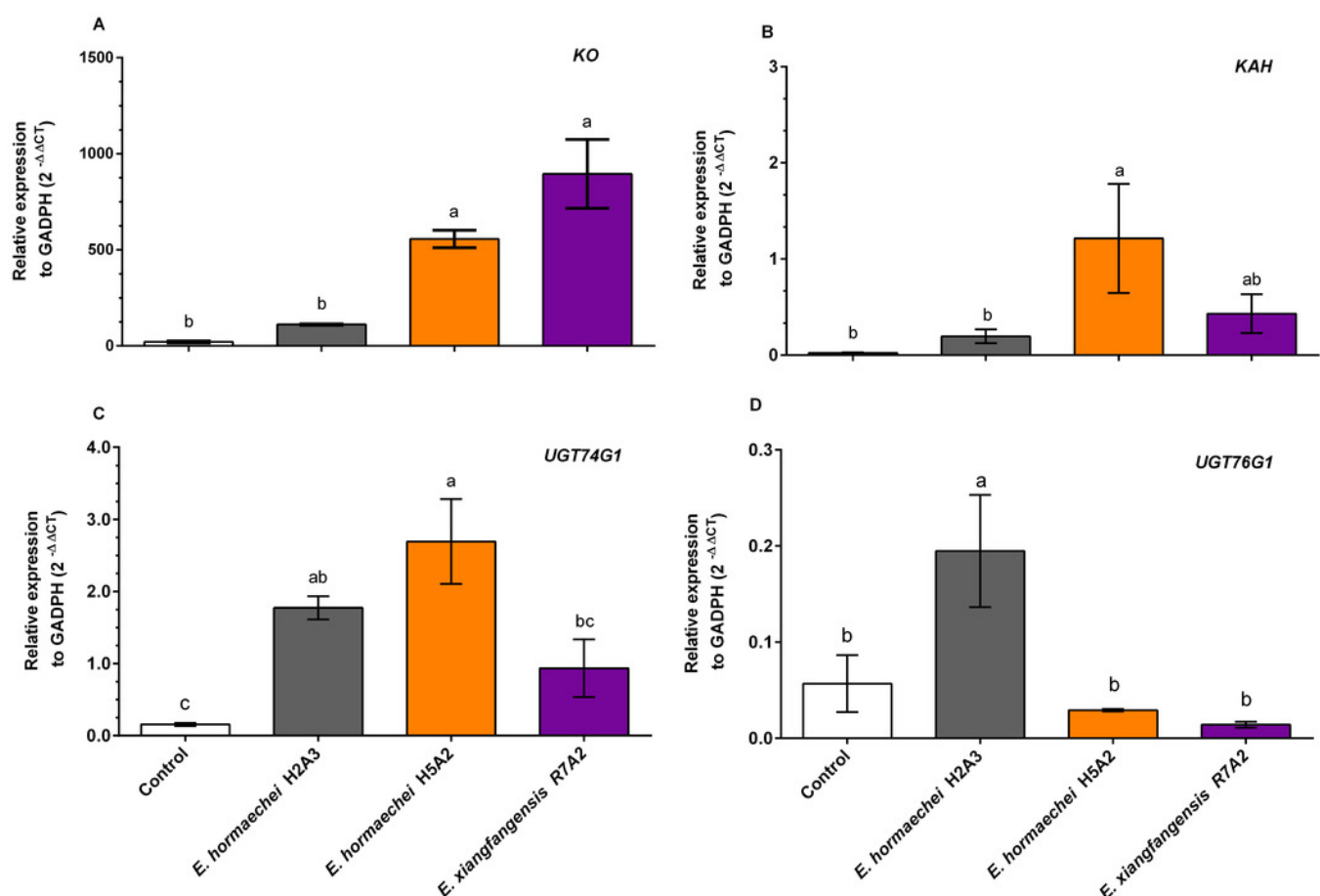



Figure 6

Relative expression analysis of key genes in the steviol glycoside biosynthesis pathway of *Stevia rebaudiana* leaves noninoculated and inoculated with endophytic bacteria. (A) *KO*, (B) *KAH*, (C) *UGT74G1*, (D) *UGT76G1*.

The relative expression of *GAPDH* was calculated. The $2^{-\Delta CT}$ method was used. Bars represent the mean ± standard deviation of three biological and three technical replicates collected 30 days postinoculation. Different letters indicate significant differences according to Tukey's test (*P* value of less than 0.05).

Identification of endophytic bacteria isolated from *Stevia rebaudiana* plants.

Tissue	Isolate	Most similar strain NCBI database	Access code	Identity
Leaf	H2A3	*Enterobacter hormaechei strain* C15	CP042488.1	99.7%
Leaf	H5A2	*Enterobacter hormaechei strain* ER48	MT124573.1	*100%*
Leaf	H7A1	*Enterobacter bacterium strain E*Mt 5	EU863187.1	*90%*
Stem	T1A2	*Enterobacter xiangfangensis strain* KV7	MH200641.1	*99.7%*
Stem	T3A3	*Enterobacter xiangfangensis strain* SitB416	KY880912.1	*99.8%*
Stem	T5P1	*Enterobacter xiangfangensis strain* KV7	MH200641.1	*99.7%*
Root	R2A2	*Enterobacter xiangfangensis strain* KV7	MH200641.1	*99.7%*
Root	R3A1	*Enterobacter cloacae strain* MB0-11	MH041191.1	*100%*
Root	R5P1	*Enterobacter hormaechei strain* EGYMCRVIM	CP052870.1	*99.3%*
Root	R6A1	*Enterobacter hormaechei strain* C45	CP04255.1	*99.7%*
Root	R6P1	*Bacillus safensis strain* F14	MH065717.1	*100%*
Root	R7A2	*Enterobacter xiangfangensis strain* KV7	MH200641.1	*99.7%*

Growth parameters (length, number of leaves, leaf dry weight, root length and root dry weight) of *Stevia rebaudiana* plants inoculated with endophytic bacteria.

Data represent the mean ± standard deviation of ten individual plants harvested to 30 days postinoculation. The same letter indicates no statistically significant differences between growth parameters (*P* value of less than 0.05).

Treatment	Plant length (cm)	Number of leaves	Leaves dry weight	Root length (cm)	Root dry weight
Control	23.6 ± 2.5 a	28.5 ± 3.3 a	0.117 ± 0.03 a	19.1 ± 2.2 a	0.128 ± 0.05 a
E. hormaechei H2A3	19.7 ± 1.3 a	27.2 ± 2.5 a	0.082 ± 0.02 a	18.9 ± 2.0 a	0.116 ± 0.04 a
E. hormaechei H5A2	23.4 ± 1.5 a	29.9 ± 3.7 a	0.088 ± 0.01 a	20.8 ± 2.4 a	0.146 ± 0.04 a
E. bacterium H7A1	25.2 ± 7.3 a	29.9 ± 3.7 a	0.105 ± 0.05 a	21.6 ± 1.2 a	0.129 ± 0.07 a
E. xiangfangensis T1A2	21.9 ± 2.6 a	28.6 ± 2.9 a	0.103 ± 0.05 a	23.9 ± 5.7 a	0.107 ± 0.04 a
E. xiangfangensis T3A3	24.9 ± 3.1 a	30.2 ± 3.5 a	0.095 ± 0.02 a	21.2 ± 2.9 a	0.107 ± 0.05 a
E. xiangfangensis T5P1	25.8 ± 3.1 a	31.0 ± 2.0 a	0.108 ± 0.04 a	21.1 ± 1.7 a	0.125 ± 0.04 a
E. xiangfangensis R2A2	20.7 ± 2.1 a	28.2 ± 1.9 a	0.087 ± 0.03 a	22.2 ± 4.7 a	0.092 ± 0.03 a
E. cloacae R3A1	23.6 ± 1.7 a	29.4 ± 0.7 a	0.116 ± 0.03 a	21.2 ± 2.7 a	0.114 ± 0.04 a
E. hormaechei R5P1	24.5 ± 2.6 a	28.6 ± 2.8 a	0.099 ± 0.03 a	23.0 ± 3.4 a	0.104 ± 0.03 a
E. hormaechei R6A1	23.3 ± 3.7 a	26.6 ± 5.0 a	0.093 ± 0.01 a	26.0 ± 6.3 a	0.101 ± 0.02 a
Bacillus safensis R6P1	22.3 ± 1.3 a	26.7 ± 1.8 a	0.121 ± 0.02 a	20.0 ± 2.3 a	0.115 ± 0.02 a
E. xiangfangensis R7A2	23.3 ± 3.3 a	29.7 ± 2.5 a	0.087 ± 0.02 a	21.6 ± 2.3 a	0.102 ± 0.03 a