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Changes in Phenolic Profile and Total Phenol and Total Flavonoid Contents of *Guadua angustifolia* Kunth Plants under Organic and Conventional Fertilization

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David A. Villamarin-Raad,[#] Hair S. Lozano-Puentes,[#] Luis Carlos Chitiva, Geison M. Costa, Sergio A. Díaz-Gallo, and Lucía A. Díaz-Ariza*

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ABSTRACT: Agronomic management of a crop, including the application of fertilizers and biological inoculants, affects the phenol and flavonoid contents of plants producing these metabolites. *Guadua angustifolia* Kunth, a woody bamboo widely distributed in the Americas, produces several biologically active phenolic compounds. The aim of this study was to evaluate the effect of chemical and organic fertilizers together with the application of biological inoculants on the composition of phenolic compounds in *G. angustifolia* plants at the nursery stage. In 8-month-old plants, differences were observed in plant biomass (20.27 ± 7.68 g) and in the content of total phenols and flavonoids (21.89 ± 9.64 mg gallic acid equivalents/plant and 2.13 ± 0.98 mg quercetin equivalents/plant, respectively) when using the chemical fertilizer diammonium phosphate (DAP). No significant differences were found owing to the effect of the inoculants, although the plants with the application of *Stenotrophomonas* sp. on plants fertilized with DAP presented higher values of the metabolites (24.12 ± 6.72 mg gallic acid equivalents/plant and 2.39 ± 0.77 mg quercetin equivalents/plant). The chromatographic profile of phenolic metabolites is dominated by one glycosylated flavonoid, the concentration of which was favored by the application of the inoculants *Azospirillum brasilense*, *Pseudomonas fluorescens*, and *Stenotrophomonas* sp. In the case study, the combined use of DAP and bacterial inoculants is recommended for the production of *G. angustifolia* plant material with a high content of promising biologically active flavonoids or phenolics.
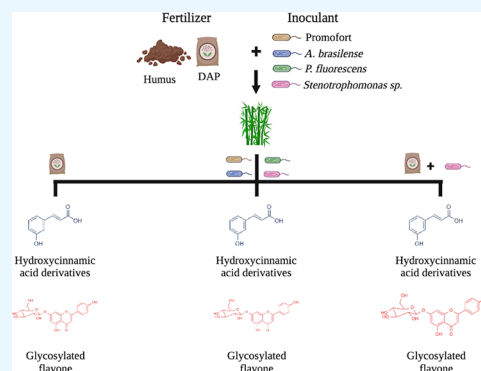
1. INTRODUCTION

Guadua angustifolia is a bamboo species with high economic value for Colombia.[1] The culm has been the main organ of interest for this plant because it can be used for the manufacture of structures, handicrafts, flooring, and woodwork.[2] Likewise, it has been found that byproducts of the culm production process, such as chips and leaves, are used for pulp and biochar production.[3,4] Additionally, its chemical composition and biological activity, associated with secondary metabolites present in the culms and leaves of this plant, have been studied;[5,6] furthermore, phenolic compounds such as caffeoylquinic acid and caffeic acid 3-glucoside and flavonoids such as quercetin 3,7-dirhamnoside, kaempferol-3-*O*-rutinoside, violanthin, vitexin 6″-(3-hydroxy-3-methylglutarate), and kaempferol 7-sophoroside have been reported in these leaves,[7] showing the potential of this plant as a source of secondary metabolites of interest in different industries.

In addition, the use of agronomic management that improves the content of phenolic compounds is a strategy for crop production with greater phytochemical value.[8,9] For this purpose, the use of different fertilizers has been evaluated, such as organic fertilizers[10] and conventional fertilizers,[11,12] which have been effective in increasing the content of phenols and flavonoids in different plant species.[13−15] In addition, inoculants can encourage the formation of phenolic compounds, which are naturally occurring poisons that help protect plants from harmful organisms.[16] Bacteria, such as *Azotobacter* sp., *Azospirillum* sp., *Bacillus* sp., and *Pseudomonas* sp., also increase the content of phenolic compounds. These organisms can act in conjunction with fertilizers to promote higher phenolic and flavonoid contents, as well as higher biomass.[14,17−19]

Table 1. Leaf NPK Content in Fertilized and Inoculated *G. angustifolia* Plants[a]

treatments	nitrogen content (mg N/plant)	phosphorus content (mg P/plant)	potassium content (mg K/plant)
control−A. brasilense	194.35 ± 146.28	35.65 ± 26.52	242.51 ± 199.95
control−P. fluorescens	210.90 ± 142.07	32.12 ± 19.68	209.02 ± 123.62
control−*Stenotrophomonas* sp.	138.79 ± 84.19	21.60 ± 13.61	141.32 ± 89.06
control−control	136.49 ± 76.61	23.14 ± 12.82	147.05 ± 94.20
control−Promofort	173.32 ± 87.60	27.14 ± 11.70	187.76 ± 85.45
DAP−A. brasilense	334.72 ± 130.64	47.85 ± 23.84	303.56 ± 148.30
DAP−P. fluorescens	338.52 ± 109.25	48.08 ± 16.80	321.99 ± 97.23
DAP−*Stenotrophomonas* sp.	309.64 ± 113.44	48.41 ± 17.58	308.59 ± 114.05
DAP−control	375.51 ± 116.92	52.01 ± 18.15	331.69 ± 117.61
DAP−Promofort	350.88 ± 151.52	49.85 ± 19.82	366.18 ± 153.89
humus−A. brasilense	186.48 ± 127.82	28.51 ± 19.94	201.02 ± 164.16
humus−P. fluorescens	141.13 ± 97.52	27.68 ± 17.66	159.80 ± 99.29
humus−*Stenotrophomonas* sp.	144.52 ± 99.33	23.20 ± 14.21	150.03 ± 99.84
humus−control	154.94 ± 124.52	22.81 ± 16.15	160.65 ± 134.05
humus−Promofort	166.08 ± 66.71	29.41 ± 14.37	174.84 ± 79.72
Fertilizante			
control	169.98 ± 111.13 b	27.84 ± 17.99 b	184.85 ± 127.94 b
DAP	342.67 ± 122.43 a	49.34 ± 18.68 a	325.73 ± 124.55 a
humus	159.27 ± 104.31 b	26.33 ± 16.41 b	169.76 ± 118.18 b
Inoculante			
A. brasilense	233.86 ± 147.84	36.40 ± 24.22	245.46 ± 174.11
Consorcio P. fluorescens	230.18 ± 141.57	35.96 ± 19.75	230.79 ± 125.26
cocultivo *Stenotrophomonas* sp.	193.85 ± 124.72	30.47 ± 19.17	196.26 ± 124.73
control	226.04 ± 153.80	33.06 ± 20.97	216.00 ± 143.15
Promofort	219.11 ± 129.51	34.16 ± 17.77	231.72 ± 133.38
ANOVA (*p* value)			
fertilizer	<0.001	<0.001	<0.001
inoculant	0.468	0.398	0.292
fertilizer × inoculant	0.703	0.700	0.680

[a]Values are mean ± standard deviation (SD) obtained in quadruplicate. DAP, diammonium phosphate. Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

The use of conventional fertilizers, like diammonium phosphate (DAP), improves the nitrogen and phosphorus content in the soil, increasing the chlorophyll content[20] and plant yield.[21,22] Also, organic fertilizers have a beneficial impact on the soil microbiome, since they may prime microbial activities and act as a biologically active agent and/or enhance synergistic interactions within the soil microbiome in enhancing plant biomass.[23] In addition, biological inoculants improve soil fertility and crop production,[24−26] by enhancing beneficial elements like nitrogen, phosphorus, potassium, vitamins, and proteins.[27−30]

Moreover, the combination of conventional and/or organic fertilizers with microbial inoculants has been a strategy in sustainable agriculture to reduce the impact of the excessive application of chemical fertilizers to promote plant growth.[31−34] These types of strategies have not yet been evaluated in *G. angustifolia*, so our hypothesis is that the combination of fertilizers with bacterial inoculants can improve the growth and content of secondary metabolites in *G. angustifolia* leaves in the nursery. The aim of this study was to evaluate the effect of an organic fertilizer, San Rafael humus, and the conventional fertilizer, DAP, together with four bacterial inoculants, Promofort, a coculture of *Stenotrophomonas* sp., a consortium of *Pseudomonas fluorescens*, and *Azospirillum brasilense*, on the composition of phenolic metabolites in *G. angustifolia* plants at the nursery stage.

2. RESULTS

2.1. Plant Dry Weight and Leaf NPK. The average biomass (20.27 ± 7.68 g) of fertilization with DAP increased significantly compared to fertilization with humus (10.50 ± 6.70 g) (*p* value <0.001) and biomass from control plot plants (11.39 ± 7.33 g) (*p* value <0.001). In the case of inoculants, there were no significant differences on biomass, owing to their application (Table S1).

Similarly, it was found that DAP significantly enhanced the leaf NPK content: 342.67 ± 122.43 mg N/plant, 49.34 ± 18.68 mg P/plant, and 325.73 ± 124.55 mg K/plant, with respect to humus and control, obtaining *p* values inferior to 0.001 in every case. The plants fertilized with humus showed contents of 159.27 ± 104.31 mg N/plant, 26.33 ± 16.41 mg P/plant, and 169.76 ± 118.18 mg K/plant. The values obtained for leaf nutrients of control plants were 169.98 ± 111.13 mg N/plant, 27.84 ± 17.99 mg P/plant, and 184.85 ± 127.94 mg K/plant. The nutrient content did not show significant differences by inoculants (Table 1).

2.2. Total Content of Phenolic Compounds and Flavonoids. The application of fertilizers had significant effects on the content of phenols and flavonoids in leaf extracts of *G. angustifolia* (Table S2). There is a higher content of total phenols in plants fertilized with humus (5.99 ± 1.44 mg GAE/g DM) compared to the control (5.33 ± 1.68 mg GAE/g DM) (*p* value = 0.042). The results of DAP (5.42 ± 1.46 mg GAE/g DM) were not significantly different with respect to the humus and the control. The plants that showed the highest values of

phenols corresponded to those of the control inoculated with the *Stenotrophomonas* sp. (5.87 ± 1.47 mg of GAE/g DM). Among the plants fertilized with DAP, those inoculated with *Stenotrophomonas* sp. presented a high phenol content, 5.83 ± 1.20 mg GAE/g DM, followed by those inoculated with *A. brasilense* and *P. fluorescens*, with values very similar to each other, 5.60 ± 1.51 and 5.60 ± 1.67 mg GAE/g DM, respectively. In the case of the plants fertilized with humus, those inoculate with *A. brasilense* showed values of 7.05 ± 1.13 mg GAE/g DM, followed by the ones inoculated with *Stenotrophomonas* sp. with 6.04 ± 1.20 mg GAE/g DM.

In the case of flavonoid from the extract (mg of QE/g of DM), no significant differences were observed between fertilizers or between inoculants (Table S2). The plants from the control plot but with *Stenotrophomonas* sp. had 0.63 ± 0.22 mg QE/g DM, followed by plants inoculated with Promofort (0.62 ± 0.24 mg QE/g DM), plants fertilized with DAP and *A. brasilense* (0.62 ± 0.25 mg QE/g DM), and those fertilized with DAP and *Stenotrophomonas* sp. (0.59 ± 0.18 mg QE/g DM). Finally, the flavonoid content in plants fertilized with humus and *A. brasilense* was 0.60 ± 0.15 mg QE/g DM and that in plants with humus and no inoculant application was 0.57 ± 0.21 mg QE/g DM.

In the case of phenolic compound content per plant, total phenols were significantly increased in DAP (21.89 ± 9.64 mg GAE/plant) compared to the control (13.68 ± 7.66 mg GAE/plant, p value <0.001) and humus (12.32 ± 7.55 mg GAE/plant, p value <0.001), which showed no differences between them (Table 2). In the DAP plot with *Stenotrophomonas* sp., the content was 24.12 ± 6.72 mg of GAE/plant while the content in the control plot with the bacteria was 16.03 ± 8.57 mg GAE/plant. Finally, the content of phenolic compounds for the humus plot with *A. brasilense* was 15.25 ± 10.36 mg GAE/plant.

Regarding total flavonoids per plant, the trend was similar to that from total phenols per plant, since DAP had significantly higher flavonoid content (2.13 ± 0.98 mg QE/plant), followed by the control and humus with 1.50 ± 0.95 and 1.22 ± 0.81 mg QE/plant (p value <0.001, in both cases). *Stenotrophomonas* sp. favored the content of flavonoids in the plots where DAP was applied and in those from the control, with values of 2.39 ± 0.77 and 1.94 ± 1.20 mg QE/plant, respectively, while in the case of humus, it was its application without any inoculant where there was a higher content (1.43 ± 0.85 mg QE/plant).

2.3. Chromatographic Analysis.
The qualitative profile of the phenolic compounds determined by UPLC-PDA showed no differences between the samples, where all of them presented the same chromatographic fingerprint (Figure 1). The standards used do not show the same retention time (Figures S1 and S2). There were differences in the intensity of the peaks, indicating changes in the concentrations of some metabolites due to the effects of the treatments. Three predominant peaks were observed in all chromatograms: peak 1, with a retention time at minute 3.90, peak 2, with a retention time at minute 6.25, and peak 3, with a retention time at minute 7.25 (Figure 1A). UV absorption spectra and MS data of the three main metabolites in the chromatogram of *G. angustifolia* leaves enabled the identification of their metabolite class. Peak 1 (Figure 1B and Figure S3) corresponded to a hydroxycinnamic acid derivative, while peaks 2 and 3 (Figure 1 and Figure S4 and Figure 1 and Figure S5, respectively) were glycosylated flavonoids.

Table 2. Total Phenol and Flavonoid Content per Plant in Fertilized and Inoculated *G. angustifolia* Plants[a]

treatments	total phenols (mg GAE/plant)	total flavonoids (mg QE/plant)
control–A. brasilense	13.77 ± 7.91	1.34 ± 0.71
control–P. fluorescens	11.56 ± 6.43	1.35 ± 1.16
control–*Stenotrophomonas* sp.	16.03 ± 8.57	1.94 ± 1.20
control–control	13.85 ± 7.11	1.33 ± 0.73
control–Promofort	13.12 ± 8.94	1.52 ± 0.89
DAP–A. brasilense	21.13 ± 10.12	1.97 ± 0.93
DAP–P. fluorescens	22.97 ± 10.70	2.22 ± 1.11
DAP–*Stenotrophomonas* sp.	24.12 ± 6.72	2.39 ± 0.77
DAP–control	21.61 ± 8.29	2.09 ± 0.85
DAP–Promofort	19.79 ± 12.64	1.97 ± 1.27
humus–A. brasilense	15.25 ± 10.36	1.37 ± 0.92
humus–P. fluorescens	8.89 ± 4.62	0.93 ± 0.60
humus–*Stenotrophomonas* sp.	12.37 ± 3.63	1.39 ± 0.94
humus–control	12.95 ± 6.31	1.43 ± 0.85
humus–Promofort	12.12 ± 9.87	1.00 ± 0.67
Fertilizer		
control	13.68 ± 7.66 b	1.50 ± 0.95 b
DAP	21.89 ± 9.64 a	2.13 ± 0.98 a
humus	12.32 ± 7.55 b	1.22 ± 0.81 b
Inoculant		
A. brasilense	16.72 ± 9.80	1.55 ± 0.88
coculture *Stenotrophomonas* sp.	17.70 ± 8.17	1.91 ± 1.04
control	16.14 ± 8.10	1.62 ± 0.86
Promofort	15.12 ± 10.95	1.50 ± 1.03
consortium *P. fluorescens*	14.05 ± 9.46	1.46 ± 1.09
ANOVA (p value)		
fertilizer	<0.001	<0.001
inoculant	0.501	0.265
fertilizer × inoculant	0.767	0.864

[a]Values are mean ± standard deviation (SD) obtained in triplicate. DAP, diammonium phosphate. Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

A preliminary analysis of the MS/MS spectra revealed logical losses for peaks 2 and 3. We identified the deprotonated compounds a $[M-H]^-$ at m/z 471 and 571, as shown in Table 3.

Furthermore, for peak 2, two highly intense fragments were observed at m/z 453 $[M-H-18]^-$ and m/z 349 $[M-H-122]^-$, and for peak 3, at m/z 439 $[M-H-132]^-$ and m/z 298 $[M-H-173]^-$, possibly the loss of water and sugar moieties. Statistical analysis showed no significant differences in peak intensities (Table S3). The intensities of the phenolic acid peaks in DAP plots inoculated with *Stenotrophomonas* sp. and *P. fluorescens* were 0.036 ± 0.01 and 0.036 ± 0.02 AU, respectively. Peak 2 and peak 3 intensities increased significantly in the control plot but inoculated with Promofort (0.048 ± 0.01 AU) and the control plot but inoculated with *A. brasilense* (0.103 ± 0.05 AU), respectively (Table S3).

Glycosilylated flavonoid (peak 3) was the most abundant in all treatments (Table 4). Plants fertilized with humus and control plants, when inoculated with *A. brasiliense*, *P. fluorescens*, and *Stenotrophomonas* sp., showed a higher abundance of the metabolite compared to those inoculated with Promofort and without inoculant.
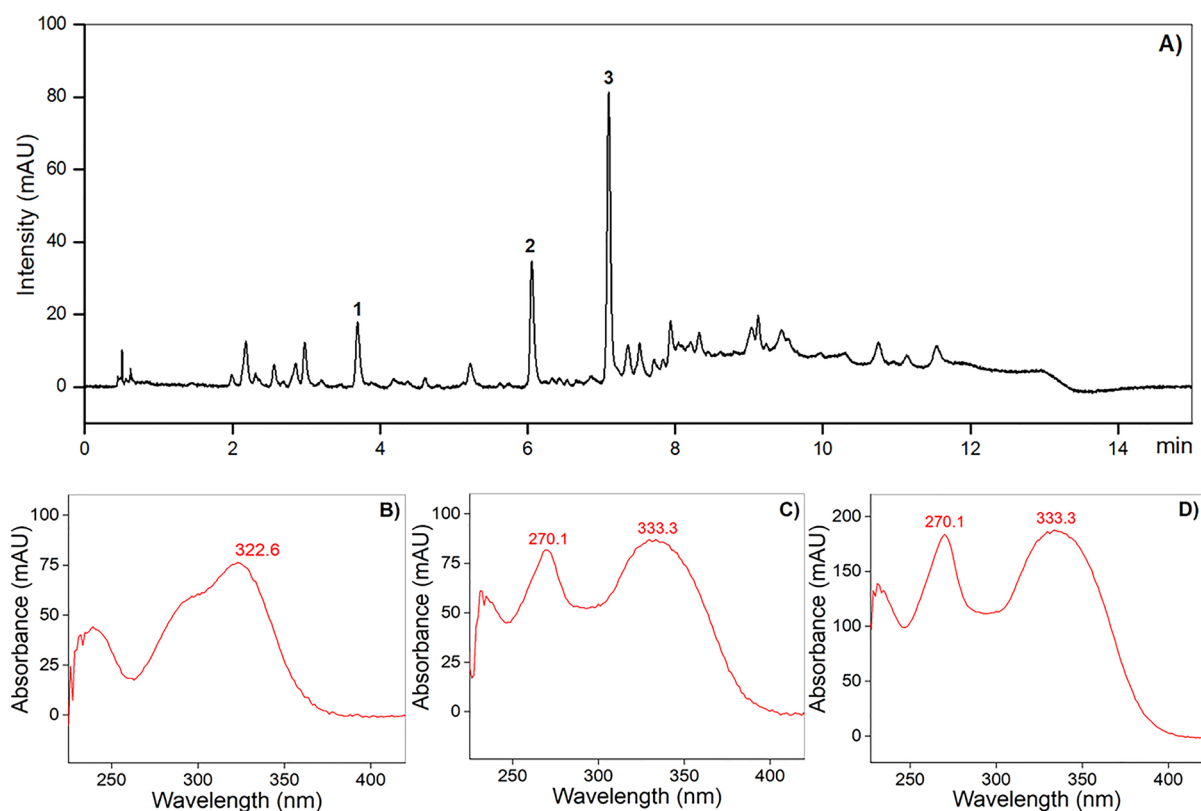
Figure 1. Fingerprint chromatogram of *G. angustifolia* leaf extract (region of interest 3.4−7.2 min). The graph corresponds to plot control without inoculant. (A) The peaks labeled 1, 2, and 3 correspond to the predominant phenolic compounds in the extract. UV absorption spectrum of peak 1 (B), peak 2 (C), and peak 3 (D).

Table 3. Compounds Tentatively Identified in *G. angustifolia* Leaves by UPLC-PDA and LC-QToF-MS

peak number	RT (min)	class	compound	UV (nm)	molecular formula	experimental Mass	error (ppm)[a]
1	3.88	phenolic acid	hydroxycinnamic acid derivatives	322.6		337.0882	5
2	6.10	flavonoid	glycosylated flavone	270.1, 333.3		451.0502	2
3	7.03	flavonoid	glycosylated flavone	270.1, 333.3		575.1365	7

[a]Maximum mass error of 10 ppm. RT, retention time.

Table 4. Relative Abundance of the Predominant Phenolic Compounds in the Plant Extract of *G. angustifolia*[a]

treatments	phenolic acid (peak 1)	flavonoid (peak 2)	flavonoid (peak 3)
control−A. brasilense	17.54%	22.22%	60.23%
control−P. fluorescens	14.50%	26.72%	58.78%
control−Stenotrophomonas sp.	20.83%	22.22%	56.94%
control−control	17.90%	26.54%	55.56%
control−Promofort	18.23%	26.52%	55.25%
DAP−A. brasilense	24.19%	28.23%	47.58%
DAP−P. fluorescens	24.00%	24.67%	51.33%
DAP−Stenotrophomonas sp.	22.93%	24.84%	52.23%
DAP−control	25.17%	23.08%	51.75%
DAP−Promofort	17.99%	28.78%	53.24%
humus−A. brasilense	15.53%	27.33%	57.14%
humus−P. fluorescens	16.67%	25.64%	57.69%
humus−Stenotrophomonas sp.	19.86%	24.66%	55.48%
humus−control	20.47%	24.56%	54.97%
humus−Promofort	23.18%	23.84%	52.98%

[a]DAP, diammonium phosphate.

3. DISCUSSION

Guadua angustifolia plants showed higher dry biomass and leaf NPK content when they were fertilized with DAP. Similarly, Díaz-Ariza and Sandoval[35] determined that the application of DAP favored the content of leaf nitrogen, as well as the content of chlorophyll in leaves, compared to plants in which vermicompost was applied. This entails that DAP favored the uptake of NPK and the biomass of the *G. angustifolia* plants, as reported for other species of bamboo like *Phyllostachys pubescens*, in which there was an increase in aboveground biomass due to the application of these nutrients.[36]

The application of fertilizers resulted in significant differences in the total contents of phenolic compounds and flavonoids in the leaves of *G. angustifolia*. A higher content of total phenols was in the plants that were fertilized with humus. This is consistent with the results of Zahid et al.,[37] who observed that the application of organic fertilizers on *Fragaria × ananassa* Duch increased the content of total phenols, total flavonoids, and anthocyanins in the fruits, compared to strawberries fertilized with urea and strawberries without any fertilizer. Similarly, the application of vermicompost increased the content of total phenols and flavonoids in leaves of

Withania somnifera (L.) Dunal by 40%.[38] The application of organic fertilizers on *Rubus idaeus* contained significantly more phenolic acid and flavonoids, including myrycetin, quercetin, luteolin, and quercetin-3-*O*-rutinoside.[39] The use of organic fertilizers that stimulate the compounds was linked to the shikimic pathway by increasing phenylalanine ammonia-lyase enzyme activity.[40]

However, when the content of total phenols and flavonoids per plant was analyzed, plants fertilized with DAP showed a higher content. This can be attributed mainly to the increase in available nitrogen. About nitrogen fertilization, the results of several research studies showed an influence of nitrogen fertilization on the content of phenolic compounds in plants. However, the findings are inconclusive.[41] For some species, the application of fertilizers reduced the content of phenolic compounds, mainly attributed to the availability of nutrients to increase growth (increase in biomass), decreasing the concentration of nonstructural carbohydrates and consequently lowering the content of carbon-based secondary metabolites, e.g., phenylpropanoids.[42] In contrast, in *Passiflora alata* , greater nitrogen availability due to the application of the fertilizer increased seedling height, number of leaves and phenol, and flavonoid content.[43] Furthermore, conventional fertilization with nitrogen improved the content of phenolic compounds in *Helianthus tuberosus* L.[41] The influence of nitrogen on the content of phenolic compounds, since genes are responsible for the synthesis of flavonoids such as quercetin, kaempferol, myricetin, and their glycosylated derivatives, increased their activity compared to unfertilized seedlings.[44] Thus, our results suggest that fertilizer application improves the flavonoid production in *G. angustifolia*.

Plants inoculated with *Azospirillum brasilense*, *Pseudomonas fluorescens*, and *Stenotrophomonas* sp. presented higher values in the total content of phenolic compounds and flavonoids. Changes in the content of phenolic compounds following the application of inoculants have been reported previously. *Stenotrophomonas* sp. improves the content of phenols, flavonoids, and carotenoids and antioxidant activity in plants of economic and medicinal importance such as *Arachis hypogea*[45] and *Carthamus tinctorius* L.[46] *A. brasilense* increases the content of phenols and flavonoids in tomato plants under salinity stress conditions, as well as in plants that were not exposed to salinity stress.[47] In contrast, on plants of *Coriandrum sativum* L. inoculated with *Bacillus halotolerans*, the content of cinnamic acid derivatives decreased[48] and the inoculation on *Cichorium endivia* L. showed significantly lower levels of cichoric and caffeoylquinic acids.[49] Changes in phenolic compound levels in plants may be due to specific inoculant−plant interactions, such *Rhizobium*−rice, *Azospirillum*−maize, and *Azospirillum*−*Pseudomonas*−*Glomus*−maize.[50] For instance, *Azospirillum* sp. induced the accumulation of glycosylated flavones in *Oryza sativa*.[51] Similarly, inoculation with *A. brasilense*, *P. fluorescens*, and *Stenotrophomonas* sp. improved the accumulation of glycosylated flavones in *G. angustifolia*.

This work showed that the combined application of fertilizers and inoculants presented changes in the total phenol and flavonoid content. Increased nitrogen uptake of leaves due to the dual application of both fertilizers and inoculants can induce increased biomass, which can stimulate the plant secondary metabolism and, consequently, increase phenolic compound content. Likewise, as reported, the application of organic fertilizers and microbial inoculants in *Zea mays* L. has

improved the polyphenolic contents in seeds.[52] Similarly, use of biofertilizers along with organic manures found enhancements in total phenolics of *Fragaria ananassa* Duch. cv Chandler.[53]

To identify the phenolic compounds, the UV absorption spectra and MS spectra obtained were considered. The absorption spectrum of peak 1 agrees with that reported by some authors,[54,55] who reported that phenolic acids, such as caffeic acid, coumaric acid, and ferulic acid, showed a maximum absorption spectrum at 310−325 nm, with a shoulder-shaped absorption pattern. In our case, peak 1 showed characteristic shoulder-shaped absorption spectra, with a maximum absorption at 322 nm. Associated with MS data, we associated this peak with a hydroxycinnamic acid derivative. Regarding the other two peaks, flavonoids have two absorption bands: the A ring, which absorbs at 250−290 nm generically among flavonoids, and the B ring, which absorbs in the range of 310−350 nm when flavones are involved.[56,57] As in the case of peaks 2 and 3 of the chromatogram, both presented two bands with maximum absorption at 270 and 333 nm, which correspond to a flavone-type scaffolds. Once the scaffolds was established, the mass spectrum of the compounds indicated the presence of sugar moieties in each of them, with peak 2 being a monoglycosylflavone and peak 3 a diglycosylflavone. Isolation and purification of these major compounds are necessary for the complete identification of these constituents.

Finally, it was found that inoculation with *A. brasilense*, *P. fluorescens*, and *Stenotrophomonas* sp. favored the relative abundance of peak 3, compared to Promofort. This may suggest a microorganism−plant−flavonoid specificity, since these three strains were isolated from *Cynodon dactylon* and *G. angustifolia* belonging to the Poaceae family, different from the Promofort strains, isolated from *Tectona grandis* belonging to the Lamiaceae family. In *Zea mays*, interaction with the rhizosphere microbiota stimulates the flavone synthase gene, *FNSI2*, inducing flavone production and subsequently influencing the assembly of the rhizosphere microbiota.[58] Additionally, peak 3 was tentatively identified as a diglycosylated flavone. Compounds of this class, such as maisyn, have been reported for the Poacea family in *Guadua* spp.[7] and *Z. mays*.[59] This shows the wide potential for the use of fertilization strategies to produce biologically active compounds in *G. angustifolia*, possibly maisyn, which is known to have high neuroprotective activity.[60−62]

4. CONCLUSIONS

This study shows that the use of fertilizers in combination with bacterial inoculants increases the content of phenolic compounds in the leaves of *G. angustifolia*. The results obtained suggest that *G. angustifolia* leaf extracts could be an important source of high biological value metabolites.

5. MATERIALS AND METHODS

5.1. Location of the Experiment and Weather Conditions. The experiment was conducted in an experimental area located in the municipality of Pacho, Cundinamarca, Colombia (5°10′51.4″ N; 74°11′44.0″ W), with an altitude of 1330 m, an average annual temperature of 18 °C, and an average humidity of 86%.

5.2. Biological Material and Sowing Substrate. *G. angustifolia* plants were propagated from the lateral branches of natural stands in Cundinamarca, Colombia.

The bacterial inoculants used for plant inoculation included strains deposited in the Collection of Microorganisms of the Pontificia Universidad Javeriana (RNC 148, WFCC 857). Four inoculants were produced: *Azospirillum brasilense*, formulated with the strain ATCC 29710 originally isolated from *Cynodon dactylon* (USA); *Pseudomonas fluorescens* corresponds to a consortium of four strains isolated from *G. angustifolia* (Colombia); *Stenotrophomonas* sp. corresponds to a coculture of two isolates obtained from isolates of *G. angustifolia* (Colombia); and Promofort is a consortium of four strains isolated from *Tectona grandis* (Colombia).

The sowing substrate has the following physical and chemical attributes: pH 6.68, electric conductivity 0.99 dS/m, oxidizable organic carbon 7.49%, organic matter 12.9%, total nitrogen 0.624%, available phosphorus 12.2 mg/kg, iron 2.55 mg/kg, copper 0.19 mg/kg, zinc 5.18 mg/kg, boron 0.612 mg/kg, sulfur 37.6 mg/kg, effective cation exchange capacity 26.3 cmol$^+$/kg, exchangeable potassium 0.35 cmol$^+$/kg, exchangeable calcium 19.33 cmol$^+$/kg, exchangeable magnesium 6.60 cmol$^+$/kg, exchangeable sodium 0.24 cmol$^+$/kg, and apparent density 0.789 g/cm^3.

5.3. Experimental Design and Treatments. The experimental design used was a randomized block design with subdivided plots with four blocks. The blocks consisted of 240 plants. Each block was divided into three plots, where the fertilizers were applied. Each plot was divided into five subplots in which inoculants were applied (Table 5).

Table 5. Combination of Treatments Used in the Main Plots and Subplots

plot (fertilizer)	subplot (inoculant)
humus San Rafael (0.5% 100 mL)	A. brasilense (5% 100 mL)
humus San Rafael (0.5% 100 mL)	coculture Stenotrophomonas sp. (5% 100 mL)
humus San Rafael (0.5% 100 mL)	control (100 mL water)
humus San Rafael (0.5% 100 mL)	Promofort (5% 100 mL)
humus San Rafael (0.5% 100 mL)	consortium P. fluorescens (5% 100 mL)
DAP (0.7 g + 0.4 g [15 days] + 0.4 g [30 days]) and 100 mL water	A. brasilense (5% 100 mL)
DAP (0.7 g + 0.4 g [15 days] + 0.4 g [30 days]) and 100 mL water	coculture Stenotrophomonas sp. (5% 100 mL)
DAP (0.7 g + 0.4 g [15 days] + 0.4 g [30 days]) and 100 mL water	control (100 mL water)
DAP (0.7 g + 0.4 g [15 days] + 0.4 g [30 days]) and 100 mL water	Promofort (5% 100 mL)
DAP (0.7 g + 0.4 g [15 days] + 0.4 g [30 days]) and 100 mL water	consortium P. fluorescens (5% 100 mL)
control (100 mL water)	A. brasilense (5% 100 mL)
control (100 mL water)	coculture Stenotrophomonas sp. (5% 100 mL)
control (100 mL water)	control (100 mL water)
control (100 mL water)	Promofort (5% 100 mL)
control (100 mL water)	Consortium P. fluorescens (5% 100 mL)

100 mL of 0.5% San Rafael humus was applied to the organic fertilizer plot. To the conventional fertilizer plot, 1.5 g of fractionated DAP was applied: 0.75 g at sowing and 0.4 g 15 and 30 days after sowing, together with 100 mL of tap water. On the third plot, 100 mL of tap water was applied. This resulted in three fertilizer levels (organic, conventional, and control). Patent no. US11001536 B2 was used to prepare the bacterial inoculants. 100 mL of each inoculant at 5% was applied at sowing. The same volume was reinoculated 15 and

30 days after sowing. In the fifth subplot, 100 mL of tap water was applied. This fertilization was repeated twice, every 3 months and 14 days, and every 1 month and 17 days.

5.4. Plant Dry Weight and Leaf NPK Analysis. Guadua plants were harvested 8 months after sowing. Plant dry matter yield was calculated after the samples of four plants per block were dried in an oven at 80 °C until they reached a constant weight. Subsequently, oven-dried leaves were taken for NPK analysis. N was extracted by Kjeldahl digestion and P and K by acid digestion, and the content was estimated as the methods described.[63]

5.5. Metabolite Extraction and Sample Preparation. The foliage leaves of three plants per block were dried in an oven at 37 °C for 72 h. To extract the plant material, 300 mg of dried and ground samples was mixed with 10 mL of a solvent containing chloroform, methanol, and water (in a ratio of 5:2.5:2.5 *v/v/v*). The resulting mixture was then vortexed for 1 min, sonicated for 20 min at 50 °C, and centrifuged at 5000 rpm and 20 °C for 10 min. The liquid supernatant was filtered using PTFE syringe filters with a pore size of 0.22 μm (Thermo Scientific, Rockwood, Tennessee) and stored at −80 °C until used for analysis (Lozano et al., 2023 unpublished data).

5.6. Determination of Total Phenol Content. Total phenol content was determined by the Folin−Ciocalteu assay method.[64] 0.2 mL of the extract was mixed with 0.6 mL of distilled water. Subsequently, 0.2 mL of Folin−Ciocalteu reagent, 1 mL of sodium carbonate, and 1 mL of distilled water were added. This mixture was stirred and allowed to react for 30 min at room temperature in the dark. The absorbance was measured at 765 nm on a HACH DR6000 UV−vis spectrophotometer and compared with a gallic acid calibration curve (0−100 mg/L) r^2 = 0.9872. The result was expressed as milligrams of gallic acid equivalent per gram of dry matter (mg equiv GAE/g DM), following eq 1:

$$\frac{mg\ GAE}{g\ DM} = \frac{C \times V}{g\ DM}$$

where C = concentration obtained in mg/L; V = volume of extraction (0.005 L); g DM = amount of leaf dry plant material (0.3 g).

The data obtained were then correlated with the total leaf weight to determine the milligram equivalents of gallic acid per plant (mg GAE/plant), following eq 2:

$$\frac{mg\ GAE}{plant} = \frac{mg\ GAE}{gDM} \times \frac{TLW}{plant}$$

where TLW = total leaf weight in gram.

5.7. Determination of Total Flavonoid Content. Total flavonoid content was determined by the colorimetric method with AlCl$_3$.[64] One milliliter of the extract was mixed with 500 μL of 1 M potassium acetate and 1 mL of 2% AlCl$_3$. This mixture was stirred and allowed to react for 60 min at room temperature in the dark. The absorbance was measured at 420 nm in a HACH DR6000 UV−vis spectrophotometer and compared with a quercetin calibration curve (0−30 mg/L) r^2 = 0.9962. The result was expressed as milligrams of quercetin equivalent per gram of dry matter (mg QE/g DM), following eq 3:

$$\frac{mg\ QE}{g\ DM} = \frac{C \times V}{g\ DM}$$

where C = concentration obtained in mg/L; V = volume of extraction (0.005 L); g DM = amount of leaf dry plant material (0.3 g).

The data obtained were then correlated with the total leaf weight to determine the milligram equivalents of quercetin per plant (mg QE/plant), following eq 4:

$$\frac{\text{mg QE}}{\text{plant}} = \frac{\text{mg QE}}{\text{g DM}} \times \frac{\text{TLW}}{\text{plant}}$$

where TLW = total leaf weight in gram.

5.8. Chromatographic Analysis by UPLC-PDA and LC-QToF-MS. The ACQUITY H Class UPLC Waters with an ACQUITY photodiode array detector (PDA), quaternary pump, degasser, and auto sampler was used. The data obtained were processed by using the Empower 3 software. For the analysis of the extract and standard's (gallic acid and rutine 1 mg mL^{-1} Sigma-Aldrich), a Phenomenex Kinetex EVO C18 column (100 mm × 2.1 mm i.d.; 2.6 μm) was used at 30 °C with a run gradient of formic acid at 0.1% in water (solvent A) and acetonitrile (solvent B) as follows: 5% B for 0 to 8 min, 5 to 20% B for 8 to 12 min, 20% B for 12 to 13 min, 20 to 5% B for 13 to 14 min, and 5% B for 14 to 15 min with a run flow rate of 0.5 mL min^{-1} and an injection volume of 3 μL.

The LC-QToF-MS was conducted using a Shimadzu Nexera X2 LCMS Q-TOF 9030 instrument with an electrospray interface (ESI). The same chromatographic conditions previously described were employed. Full-scan MS1 and MS/MS data were acquired. Data mass spectra were acquired in negative ionization mode (ESI−), in a mass range of m/z 80−1700 Da in data-dependent acquisition (DDA) mode.

5.9. Mass Spectrometry Data Processing. MZmine 2.53 software (http://MZmine.github.io/) was used to perform the detection of the characteristics of the samples with the MS data (full scan). Data processing was carried out by considering the following criteria: mass detection; retention time: 0−15 min, mass detector: centroid, noise level: 1.0E3, chromatogram builder; minimum time span: 0.01 min, minimum height: 3.0E3, m/z tolerance: 0.0 m/z or 20.0 ppm, chromatogram deconvolution: baseline cutoff: minimum peak height: 2.0E3, peak duration: 0.01−3.0, baseline level: 1.0E3, isotopic peaks grouper: m/z tolerance: 0.0 m/z or 20.0 ppm, retention time tolerance: 0.1 min maximum charge: 3, representative isotope: most intense, alignment with algorithm Join aligner, 0.0 m/z or 20.0 ppm, weight for m/z: 75, retention time tolerance: 0.1 min weight for RT: 25, gap filling, normalization by peak area. For the annotation of the characteristics found, mass accuracy (maximum mass error 10 ppm), isotopic pattern distribution, adduct formation, and elution order of the compounds based on chromatographic conditions were considered and different online public databases such as METLIN (http://METLIN.scripps.edu), KEGG (http://genome.jp/kegg), HMDB (https://hmdb.ca/), PubChem (https://PubChem.ncbi.nlm.nih.gov/), and ChEBI (https://www.ebi.ac.uk/ChEBI/). Compounds from primary metabolism were discarded in this process.

5.10. Statistical Analysis. Initially, diagnostics for variance analysis were performed, testing the normality and homoscedasticity using SPSS software version 28.0. The results were subjected to the F test of analysis of variance (ANOVA) for the studied factors and their interactions. When ANOVA indicated significance for the results ($p < 0.05$), Tukey tests were performed for plant dry weight, foliar NPK content, total phenols content, total flavonoid content, and intensity of the peaks in the chromatograms obtained by UPLC.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c04579.

> Mass spectra of tentatively identified compounds, chromatograms of standards, plant dry weight, content of total phenols and flavonoids per gram of dry leaf material, and intensity (AU) of phenolic acid (peak 1) and flavonoids (peaks 2 and 3) obtained in the chromatogram of fertilized and inoculated *G. angustifolia* (PDF)

■ AUTHOR INFORMATION

Corresponding Author

> **Lucía A. Díaz-Ariza** − *Laboratorio Asociaciones Suelo Planta Microorganismo (LAMIC), Pontificia Universidad Javeriana Sede, Bogotá 110231, Colombia;* ⓞ orcid.org/0000-0001-5736-6335; Email: luciaana@javeriana.edu.co

Authors

> **David A. Villamarin-Raad** − *Laboratorio Asociaciones Suelo Planta Microorganismo (LAMIC), Pontificia Universidad Javeriana Sede, Bogotá 110231, Colombia;* ⓞ orcid.org/0000-0002-1576-4922
>
> **Hair S. Lozano-Puentes** − *Laboratorio Asociaciones Suelo Planta Microorganismo (LAMIC), Pontificia Universidad Javeriana Sede, Bogotá 110231, Colombia;* ⓞ orcid.org/0000-0002-0639-4958
>
> **Luis Carlos Chitiva** − *Grupo de Investigación Fitoquímica Universidad Javeriana (GIFUJ), Pontificia Universidad Javeriana Sede, Bogotá 110221, Colombia;* ⓞ orcid.org/0000-0003-1422-5545
>
> **Geison M. Costa** − *Grupo de Investigación Fitoquímica Universidad Javeriana (GIFUJ), Pontificia Universidad Javeriana Sede, Bogotá 110221, Colombia;* ⓞ orcid.org/0000-0003-2449-1986
>
> **Sergio A. Díaz-Gallo** − *Laboratorio Asociaciones Suelo Planta Microorganismo (LAMIC), Pontificia Universidad Javeriana Sede, Bogotá 110231, Colombia;* ⓞ orcid.org/0009-0000-4274-2983

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.3c04579

Author Contributions

[#]D.A.V.-R. and H.S.L.-P. have contributed equally to this work and share the first authorship.

Notes

The authors declare no competing financial interest.

2018), Convocatoria del Fondo de Ciencia, Tecnología e Innovación del Sistema General de Regalías, en el marco del Programa de Becas de Excelencia Doctoral del Bicentenario, definido en el artículo 45 de la ley 1942 de 2018, Bogotá, Colombia,Ministerio de Ambiente y Desarrollo Sostenible for granting permission to use genetic resources and derived products (Contract number 281/2019; Resolution 2167/2019).Biorender that was used for the elaboration of the graphical abstract, and Geoambiente S.A.S and Juan José Sanchéz Matiz for support in the fieldwork.

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