5-\(n\)-alkylresorcinol-based metabolic response of rice to the interaction with \textit{Burkholderia glumae}: a chemical characterization of the temporal and spatial variations depending on environmental conditions

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ABSTRACT

\textit{Burkholderia glumae} is a relevant worldwide pathogen of rice. \textit{B. glumae} unfavorably affects the throughput of rice crops provoking seedling death and blight panicles. Different intrinsic and extrinsic factors influence the interaction, the host physiological response and its metabolic performance, e.g. biosynthesis of protecting metabolites such as 5-\(n\)-alkylresorcinols (AR). However, the metabolic responses of rice interacting with \textit{B. glumae} and involving different factors are still unraveled. Thus, AR variations in two Colombian rice genotypes, grown in two climatically different locations and inoculated independently with two \textit{B. glumae} strains were evaluated through total AR content and LC-MS-based characterization. AR accumulated in rice plants at different levels depending on genotypes, plant parts and phenological stages. Rice genotypes were affected by pathogen inoculation, promoting AR variations. Such pathogen-mediated influence was also affected by the tested factors. This AR-based rice response against \textit{B. glumae} constitutes a contribution to understand better this biotic interaction.

1. Introduction

Rice (\textit{Oryza sativa} L.) is currently one of the most important staple food crops around the world due to its enormous nutritional value, representing globally 20\% of the human diet (FAO 2004; Gnanamanickam 2009). Rice crop has increased over the years, reaching 508.7 million tons in 2020, which was 1.5\% greater than 2019 (FAO 2020). Such a rise is a consequence of the expansion of rice crops in Asia, the rice harvest in the United States and the improvement of crops in Latin America (FAO 2020). Colombia is the third-largest rice-producing country in Latin America with around 2.9 million tons in 2020 according to national data (FEDEARROZ 2021). However, production is constantly limited by phytopathogens (e.g. viruses, bacteria, and fungi), which cause diseases that decrease rice yield and/or even lead to plant death.

\textit{Burkholderia glumae} is a relevant pathogen in rice plants because it causes several diseases in different stages of the life cycle of plants. Some growth stages of rice plants such as seedling, floral buds, and flowering are more susceptible to infection. \textit{B. glumae} causes Bacterial Seedling Rot (BSR) in seedling stages and Bacterial Panicle Blight (BPB), also named Bacterial Grain Rot (BGR), in reproductive stages (Cottyn et al. 1996). BPB-derived symptoms comprise linear lesion in-leaf sheath and brown spots on panicles, altering even the proper filling of grains and, during extreme cases, provoking blight (Tsushima 2011; Li et al. 2016). Accordingly, the yield reduction of BPB-affected rice crops can reach variable values that depend on genotype susceptibility and severe conditions of climate (Echeverri et al. 2019; Shew et al. 2019; Echeverri-Rico et al. 2021). BPB is one of the most widespread rice diseases in the world, especially in tropical and subtropical countries. \textit{B. glumae} has been detected in Central and Andean regions in Latin America, including Colombia (Beltran-Molina et al. 2013; Zhou-qi et al. 2016; Echeverri-Rico et al. 2021).

The primary inoculum of \textit{B. glumae} is transmitted through the seed where it can multiply and boost the host colonization (Ham et al. 2011). The bacteria spreading to infect the rice plant is considered as polycyclic dissemination, which comprises two phases of infection. First, the primary inoculum of bacteria multiplies exponentially through the plant, mainly colonizing the spikelets. Second, the bacterial population spreads horizontally on crops (Tsushima 2011). \textit{B. glumae} uses several virulence factors that promote and support the infection process such as chemicals (e.g. toxollavin), flagella-dependent motility, and extracellular polymeric substances (EPS) to cause disease (Sato et al. 1989; Devescovi et al. 2007; Ham et al. 2011; Karki et al. 2012). Thus, the bacteria may break down the plant defenses and initiate the successful invasion within the plant tissue.

Despite there are no fully effective control methods against BPB, some strategies to prevent this disease have been considered, including the use of pathogen-free seeds, chemical and biological control (e.g. oxolinic acid and bacteria strains, respectively), cultural practices, and plant breeding towards resistant cultivars (Ham et al. 2011; Pérez
et al. 2013; Zhou-qi et al. 2016). These strategies seek to reduce the initial inoculum in the field to limit the multiplication and dissemination of the bacterial population. However, to obtain and propose highly effective control methods, it is essential to know in depth the interaction between rice- \textit{B. glumae}, which is still unclear. Initially, the characterization of the diverse array of mechanisms used by the plant against bacteria is then required. In this sense, the tolerance or resistance capacity of rice plants to overcome the attack of the pathogen including environmental factors should be disclosed (Zipfél and Oldroyd 2017).

The biosynthesis of metabolites is one of the mechanisms that plants employ to defend themselves against the attack by pathogens. Thus, the ability to biosynthesize chemical agents with antimicrobial activity (Taiz and Zeiger 2010), involving their over-accumulation, is closely related to the plant response to the presence of a particular pathogen (Gomez-Casati et al. 2013). 5-n-alkylresorcinols (AR) are phenolic lipids highly occurring in Poaceae plants, including rice, whose production/accumulation is associated with defense against phytopathogens (Miché et al. 2003). They have demonstrated potential antimicrobial activity on commercially-important fungi such as \textit{E. coli} (Zarnowskí et al. 1999; Miché et al. 2003; Marentes-Culma et al. 2019). To study such a biochemical response related to AR production/accumulation in \textit{B. glumae}-inoculated rice plants at different growth stages, emerging holistic strategies are very useful for this purpose such as metabolomics-based approaches (Castro-Moretti et al. 2020; Vo et al. 2021).

In this context, LC-MS-based analysis makes possible to identify and quantify a specific metabolite group, even its metabolic network, which could be consistent with the attack of pathogens (Gomez-Casati et al. 2013). Consequently, as part of our research on rice- \textit{B. glumae} interaction, the variations of AR levels of two rice genotypes are disclosed for the first time using LC-MS-based characterization under different environment and climate characteristics. The research was carried out in two FEDEARROZ experimental centers that have suitable rice-growing conditions but have different environment and climate characteristics. The first location was the Centro Experimental La Victoria-CELV (Montería, Colombia, 8.75° N, 75.9° W). According to the Köppen-Geiger climate classification system, this location is classified as Tropical (A) and Savanna (w), i.e. Aw, 20 m.a.s.l., mean annual temperature = 28.4 ± 5.1°C, mean relative humidity = 74 ± 7%, mean annual precipitation = 1225 mm, ultraviolet (UV) index = 6 ± 1. The second experimental location was the Centro Experimental Las Lagunas-CELL (Saldaña, Colombia, 3.93° N, 75.0° W) and classified as Tropical (A) as well as Monsoon (m), i.e. Am, 303 m.a.s.l., mean annual temperature = 27.2 ± 7.9°C, mean relative humidity = 54 ± 15%, mean annual precipitation = 1541 mm, UV index = 6 ± 1. The sowing date in each location was chosen to synchronize the flowering stage with the climate conditions that favored BPB development (i.e. from June to October in Montería and from August to December in Saldaña). The experiment was carried out in pots located inside a frame structure (2.8 × 20 × 16 m, h × l × w) covered only with a transparent plastic roof (polyethylene, 4 mm thickness) with high light transmission (>85%). The pots were filled with soil and sand (ratio 50:50). Plants were irrigated with previously filtered water using a microbiological pore filter (0.22 μm) to avoid microbial contamination.

### 2. Experimental locations and growth conditions of rice plants

The research was carried out in two FEDEARROZ experimental centers that have suitable rice-growing conditions but have different environment and climate characteristics. The first location was the Centro Experimental La Victoria-CELV (Montería, Colombia, 8.75° N, 75.9° W). According to the Köppen-Geiger climate classification system, this location is classified as Tropical (A) and Savanna (w), i.e. Aw, 20 m.a.s.l., mean annual temperature = 28.4 ± 5.1°C, mean relative humidity = 74 ± 7%, mean annual precipitation = 1225 mm, ultraviolet (UV) index = 6 ± 1. The second experimental location was the Centro Experimental Las Lagunas-CELL (Saldaña, Colombia, 3.93° N, 75.0° W) and classified as Tropical (A) as well as Monsoon (m), i.e. Am, 303 m.a.s.l., mean annual temperature = 27.2 ± 7.9°C, mean relative humidity = 54 ± 15%, mean annual precipitation = 1541 mm, UV index = 6 ± 1. The sowing date in each location was chosen to synchronize the flowering stage with the climate conditions that favored BPB development (i.e. from June to October in Montería and from August to December in Saldaña). The experiment was carried out in pots located inside a frame structure (2.8 × 20 × 16 m, h × l × w) covered only with a transparent plastic roof (polyethylene, 4 mm thickness) with high light transmission (>85%). The pots were filled with soil and sand (ratio 50:50). Plants were irrigated with previously filtered water using a microbiological pore filter (0.22 μm) to avoid microbial contamination.

### 2.2. Inoculation of rice genotypes with \textit{B. glumae}

\textit{B. glumae} strains (43A and 33C) were independently grown in a Luria–Bertani (LB) broth for 18 h at 30°C, using constant shaking at 150 rpm (OD$_{600}$>0.7). Considering $10^5$ CFU/mL as the initial unit of absorbance, the bacterial dispersion of every strain was then adjusted to a final concentration of $10^7$ CFU/mL. Subsequently, pathogen-free seeds from both genotypes (P3085 and FL68) were inoculated with the pathogen in a ratio of 15 seeds/25 mL (1 h), while the treatment control seeds were soaked in sterile water (1 h).

### 2.4. Experimental design and sample collection

The experimental design comprised six treatments such as two non-inoculated treatments (T1) used as independent controls for pathogen-free seeds and four \textit{B. glumae}-inoculated treatments (T2 for 33C and T3 for 43A), two of them for both test rice genotypes (i.e. P3085 and FL68). The treatments were placed using a randomized complete block design, with different randomizations for each treatment.
design. Hence, a total of 720 plants were employed to collect triplicates of plants from T1-T3 treatments and both rice genotypes along five phenological stages. A summary of the experimental conditions of the present study is outlined in Scheme 1, including the workflow for the extraction and analysis of AR.

Complete rice plants were randomly collected in triplicate from each of the following five growth stages: 1. tillering; 2. panicle initiation; 3. heading; 4. flowering; and 5. milk stage (Duquette and Kimball 2020). Collected plants were quenched by freezing by placing the plant materials into a container with liquid nitrogen (−196°C) until their further processing in the laboratory. Six or more plants were collected (particularly stages 1–3) to comply with the required biomass for 5-n-alkylresorcinols (AR) detection. Each plant was separated into different existing plant parts considering their development stage: leaves and roots were obtained in all of the stages (1–5); sheaths were obtained from stages 2 to 5; stems and panicles were obtained from stages 3 to 5. All of these plant materials were finally crushed using liquid-nitrogen until obtaining a fine powder. These samples were lyophilized and then stored at −20°C until metabolite extraction.

2.5. Metabolite extraction

The dried and ground plant material (1 g) of each collected sample was soaked using acetonitrile (10 mL) through three 15-min cycles of ultrasound-assisted extraction. Subsequently, the solvent was removed in a vacuum concentrator at room temperature and the resulting dry residues were recovered to afford the AR-containing raw extracts. They were then stored at −20°C until chemical analyses to measure the AR total content and record the AR profiles.

2.5.1. Total 5-n-alkylresorcinols (AR) content measurement

Total AR content was measured by the Fast-Blue RR (FBRR®) microcolorimetric method (Sampietro, Jimenez et al. 2013). Briefly, the entire pool of resulting AR-containing raw extracts was separately reconstituted using methanol to obtain the respective extract solutions (5 mg/mL). The extract solutions were added to a 96-well microplate (30 µL each well) and mixed with 1% K2CO3 (20 µL), and 0.1 mg/mL FBRR® (180 µL). The resulting mixture was incubated in darkness for 20 min. Once the incubation time was reached, the absorbance at 480 nm was measured using a Varioskan™ Lux multimode microplate reader VLB000D0 (Thermo Fisher Scientific, Waltham, Ma, USA). These measurements were performed by triplicate. Absorbance data were interpolated in an Olivetol-based standard curve. Thus, total AR content was measured as mg AR equivalent Olivetol per g dried material (mg OE/g DM).

2.5.2. Liquid chromatography-mass spectrometry (LC-MS) analysis

AR profiles were recorded by liquid chromatography coupled to mass spectrometry (LC-MS)-based analysis. Hence, AR-containing raw extract solutions (5 mg/mL) were filtered through a PTFE membrane (0.22 µm). Those solutions were analyzed by an ultra-high-performance liquid chromatography (UPLC) system (Shimadzu Prominance UFLC®) coupled to a diode array detector (DAD), and also by an LCMS2020 mass spectrometer equipped with quadrupole analyzer and electrospray ionization (ESI) (Shimadzu, Columbia, MD, USA). Each extract solution (30 µL) was then injected into a reverse-phase C18 Kinetex® column (150 × 4.6 mm and 2.6 µg particle size, Phenomex Inc.), using a flow of 0.6 mL/min. The binary mix used as mobile phase comprised MeOH:H2O (8:2) (phase A) and MeOH:IPA (7:3) (phase B). A 37-min gradient method was used as follows: 0–2 min 0% B, 27–32 min 100% B, and 34–37 0% B. ESI was simultaneously operated in positive (1.7 kV) and negative (1.9 kV) ion modes within the range of 150–2000 m/z. Other parameters for mass spectrometry (MS) were solvation-line temperature at 185°C, heat block at 400°C, and nitrogen flow both as nebulized gas at 1.3 L/min and as dry gas at 8 L/min.

2.6. Data analysis

Total AR content was expressed as a mean ± standard deviation (SD). A Shapiro–Wilks test was employed to examine the data normality. Once the normal distribution was verified, an analysis of variance (ANOVA) and a subsequent post-hoc Tukey test were implemented using the R command package in R Study Desktop version 1.3.1093 at \( p < 0.05 \) level of significance.

On the other hand, the obtained LC-MS dataset was initially filtered by the UV spectra according to the AR maximum absorption (i.e. 275 nm) (Knödler et al. 2008). The filtered AR-related features (i.e. variables) were employed to retrieve the feature intensities per test plant sample (i.e. observations) from the LC-MS data in order to create an...
AR-based feature intensity table (FIT). The FIT dataset was then pre-processed, through normalization by sum and autoscaling, to achieve the relative intensities. The resulting preprocessed AR-based FIT data were analyzed using the web-based platform Metaboanalyst 4.0 (Chong et al. 2019) to explore and scrutinize the AR variations through univariate/multivariate statistics using the webtool’s default parameters. Therefore, a distribution visualization (heatmap) and a random forest test were performed using the statistical analysis module. ANOVA Simultaneous Component Analysis (ASCA) and Multivariate Empirical Bayes Analysis of Variance (MEBA) were performed to evidence the variation from experimental factors such as treatments (i.e. B. glumae-rice interactions) and phenological stages (i.e. time-variable) using the time series tool. The ASCA was graphically presented in two levels: model and submodel plots showed both the time vs. treatments (from now on treatments) and the treatments vs. time (from now on time) interactions. Model plots present the overview of the associated patterns to the evaluated factors and the submodel plots represent the individual contribution of each sample. Additionally, a Multivariate Empirical Bayes Analysis of Variance (MEBA) was performed to plot each AR compound and rank them based on Hotelling’s T2 value. These plots were built for each rice genotype and plant parts along component 1, whose scores were found to be >90% of variation explained (VE).

3. Results

Based on previous field experiments to classify different rice genotypes (n = 550) according to the exposure of such plants against B. glumae, two rice genotypes with contrasting disease response were selected for the present study (i.e. P3085 and FL68). P3085 was established as the moderately tolerant genotype and FL68 was the susceptible genotype (Pérez and Higuera 2013). Additionally, the test bacterial strains (33C and 43A) were also chosen regarding previous in vitro assessments of virulence factors (data not shown). Thus, 33C was classified as a moderate-virulence strain in the onion test whereas 43A was the severe-virulence strain. As above-mentioned, two independent experiments were conducted in two Colombian locations (i.e. Monteria and Saldaña towns) to recognize the influence of climate conditions over AR response in rice genotypes. These locations were selected since they are placed in suitable Colombian areas for rice cultivation, having different environmental conditions (i.e. Aw vs Am, respectively, see Material and Methods). In summary, a total of 720 plant samples were collected comprising triplicates of two controls, namely T1 (i.e. pathogen-free plants of two rice genotypes) and four treatments (i.e. 2 x 2 B. glumae strain-rice genotype pairs, namely T2 and T3 for each genotype) along five phenological stages and different plant parts (see Scheme 1). Hence, total AR contents and LC-MS-based characterization was performed to determine the AR response against B. glumae.

3.1. Total 5-n-alkylescorcinols (AR) content in non-inoculated and B. glumae-inoculated rice genotypes

The influence of B. glumae on the total AR content of rice plants was independently analyzed in different growth stages and plant parts. ARs were detected in all test samples, except root samples at the first stage (tillering) in both Monteria and Saldaña locations (Table 1), indicating AR are constitutive metabolites in rice plants. The resulting total AR content was statistically analyzed using a post-hoc Tukey multiple-comparison test to define trends among total AR content on the test variables. Consequently, samples of each treatment (separated in each column in Table 1) were compared to find differences in the total AR content between plant parts and phenological stages. Overall, some leaf samples of both genotypes and both locations exhibited a total AR content over 3.00 µg olivetol equivalents/g DE, whereas the total AR content of most panicle samples of Monteria was found to be over 1.00 µg Olivetol equivalent/g DE. In contrast, the total AR content of roots and stem samples resulted in the lowest values. On the other hand, samples of each phenological stage and plant parts (separated in each row in Table 1) were also compared to find differences in the total AR content between rice genotypes and locations. In general, the inoculated treatments (T2 and T3) exhibited higher total AR content than their respective control (i.e. non-inoculated plants, T1) in all plant parts. This behavior was specifically observed in samples inoculated with B. glumae 43A in both rice genotypes and both locations. However, samples from Saldaña exhibited higher values than those from Monteria in both rice genotypes, mainly when inoculated with FL68 (comparing T3 in Table 1 in both locations).

On the other hand, the total AR content of non-inoculated plants (control treatment, T1) was also compared according to rice phenotypes, location, plant parts, and phenological stage (Figure S1). In this way, the total AR content and its dynamics in all samples, organized according to their phenological stages, were firstly compared between rice genotypes but subdivided into locations. Generally, there was no observed difference between both rice genotypes in Monteria, although sheaths and panicles evidenced small variations and some of them (e.g. stages 2 and 4 for sheaths in T1 and T3, and all stages for panicles in T1-T3) exhibited statistically significant differences (p < 0.05). In contrast, some leaf and root samples of FL68 in Saldaña (e.g. stages 1–5 for leaves and stages 2 and 4 for roots) had a significant increase (p < 0.05) in total AR content. For instance, the sample of the first stage (tillering) from Saldaña showed a 7-fold increase in comparison to the same sample from Monteria. In this regard, although there was an enhancement of total AR content for other samples, such variations were not so drastic. Additionally, the total AR content and its dynamics in all samples, organized according to phenological stages, were then compared between locations but separated by rice genotypes. In general, the samples did not have significant differences for P3085 between locations, except for some samples in leaves and panicles. Nevertheless, some FL68 samples from Saldaña showed a higher total AR content than those of Monteria, specifically, the leaf samples at the first and second stages, and the root samples at the second stage.

The observed pattern of total AR content according to phenological stage represents an estimate of the AR-based behavior related to the ontogenic course of rice plants. Therefore, the production/accumulation of ARs was differentially observed in each plant part and phenological stage. Overall, the total AR content in non-inoculated samples tended to decrease as the phenological stages in plant parts progressed; with the exception of panicles, whose AR content
Table 1. Total S-n-alkylresorcinols content for each processed sample of non-inoculated and B. glumae-inoculated rice plants.

| Plant parts | SPS * | P3085 | FL68 | P3085 | FL68 |
|-------------|-------|-------|------|-------|------|
|             | T1 b  | T2 b  | T3 b | T1 b  | T2 b  | T3 b |
| Leaves      |       |       |      |       |       |      |
| 1           | 3.17 ± 0.2 A | 4.51 ± 0.54 A | 8.33 ± 0.03 A f | 1.76 ± 0.03 C g | 7.65 ± 0.38 A f | 20.52 ± 3.13 A d |
| 2           | 3.24 ± 0.32 A | 5.01 ± 0.31 A | 4.38 ± 0.2 E g | 3.49 ± 0.47 B g | 4.49 ± 0.36 B g | 2.49 ± 0.28 E g |
| 3           | 0.49 ± 0.11 G f | 1.77 ± 0.13 C e | 0.4 ± 0.18 f | 3.31 ± 0.27 B e | 2.17 ± 0.53 C e | 1.87 ± 1.4 E e |
| 4           | 1.55 ± 0.26 C g | 2.3 ± 0.44 C g | 1.67 ± 0.42 H g | 5.63 ± 0.75 A f | 5.49 ± 0.91 B f | 4.82 ± 0.76 B C f |
| 5           | 3.44 ± 0.24 E a | 3.4 ± 0.19 B e | 5.23 ± 0.11 D d | 4.67 ± 0.26 A d | 4.46 ± 0.51 B d | 6.33 ± 0.9 B b |
| Roots       |       |       |      |       |       |      |
| 1           | 0 ± 0 J a | 0.5 ± 0 F a | 0 ± 0 K a | 0 ± 0 H a | 0 ± 0 G a | 0 ± 0 A a |
| 2           | 0.15 ± 0.14 GH c | 0.13 ± 0.11 F c | 0.09 ± 0.03 K c | 0.24 ± 0.06 EF c | 0.64 ± 0.78 E b | 0.28 ± 0.11 G c |
| 3           | 0.44 ± 0.11 G a | 0.22 ± 0.02 G a | 0.57 ± 0.13 D a | 0.6 ± 0.65 E a | 0.15 ± 0.08 G a | 0.54 ± 0.05 A e |
| 4           | 0.28 ± 0.1 G cd | 0.31 ± 0.04 F cd | 0.58 ± 0.15 J c | 0.34 ± 0.29 DE a | 0.41 ± 0.04 E c | 0.28 ± 0.03 G cd |
| 5           | 0.22 ± 0.04 GH a | 0.31 ± 0.1 F a | 0.49 ± 0.29 A a | 0.35 ± 0.38 DE a | 0.2 ± 0.07 EF a | 0.27 ± 0.04 G a |
| Sheaths     |       |       |      |       |       |      |
| 1           | 1.28 ± 0.05 CD f | 1.11 ± 0.09 D f | 4.05 ± 0.24 E c | 0.61 ± 0.18 GE e | 1.52 ± 0.14 D f | 3.15 ± 0.3 D d |
| 2           | 0.36 ± 0.07 B c | 0.03 ± 0.03 H c | 0.58 ± 0.14 J b | 0.6 ± 0.13 DE b | 0.69 ± 0.24 E b | 0.76 ± 0.16 F b |
| 3           | 0.72 ± 0.1 F d | 0.58 ± 0.13 D e | 3.11 ± 0.4 F a | 0.45 ± 0.06 DE e | 0.6 ± 0.48 E d | 0.67 ± 0.08 F d |
| 4           | 0.27 ± 0.03 GH ab | 0.26 ± 0.05 F ab | 0.43 ± 0.02 K a | 0.39 ± 0.02 EF a | 0.33 ± 0.09 G a | 0.33 ± 0.08 G a |
| 5           | 0.32 ± 0.01 G D | 1.04 ± 0.06 D C | 2.28 ± 0.13 G a | 0.41 ± 0.12 D e | 0.7 ± 0.37 E c | 0.85 ± 0.22 F c |
| Stems       |       |       |      |       |       |      |
| 1           | 0.82 ± 0.19 EF bc | 0.63 ± 0.15 DE bc | 0.93 ± 0.25 B b | 0.29 ± 0.03 EF a | 0.22 ± 0.01 EF a | 0.3 ± 0.01 G cd |
| 2           | 0.13 ± 0.02 H i | 0.31 ± 0.08 F h | 0.34 ± 0.04 K CD | 0.14 ± 0.02 EF g | 0.12 ± 0.04 EF f | 0.02 ± 0.07 CD f |
| 3           | 1.93 ± 0.07 B c | 2.04 ± 0.04 C c | 5.65 ± 0.12 A c | 0.94 ± 0.26 D f | 2.45 ± 0.69 C f | 4.34 ± 0.51 BC c |
| Panicles    |       |       |      |       |       |      |
| 1           | 0.10 ± 0.17 D E | 5.18 ± 0.17 A B | 7.21 ± 0.06 B a | 0.61 ± 0.24 D f | 1.1 ± 0.06 E e | 2.52 ± 0.37 E c |
| 2           | 0.22 ± 0.02 GH b | 0.8 ± 0.33 D D | 7.07 ± 0.66 B a | 0.06 ± 0.04 EF g | 0.0 ± 0.01 G b | 0.12 ± 0.01 G F |
| 3           |       |       |      |       |       |      |

* SPS (selected phenological stages): 1. Tillering; 2. Panicle initiation; 3. Heading; 4. Flowering; and 5. Milk stage. + Total AR content is given as Orotol equivalent/100 dry extract (DE), for those samples obtained from non-inoculated (T1) and inoculated (T2 and T3) plants. T1=control; T2=Plants inoculated with 33C8 glumae strain; T3=Plant inoculated with 43A8 glumae strain. Different **uppercase bold letters** next to the Total AR content and treatment columns evidence statistically significant differences according to Tukey’s test between samples (throughout phenological stages and plant parts) for each treatment (T1-T3) in both locations (Montería and Saldaña). Different *lowercase italic letters* next to the Total AR content but along table rows evidence statistically significant differences according to Tukey’s test between samples throughout treatments (T1-T3) and plant parts (T1=T2) in both locations (Montería and Saldaña) for each phenological stage (1-5) per plant part.
did not decrease but was similar in the third and the fifth stages (Figure S1). Nevertheless, leaf samples of FL68 from both locations had a different trend: the total AR content in Monteria was the lowest in the third stage, whereas the total AR content in Saldaña was lower in the first stage than the middle stages (stages 2–4).

Finally, the samples of B. glumae inoculated treatments (T2 and T3) were compared with their respective control (T1), separating the dataset into plant parts, locations, and rice phenotypes (Figure S2). Overall, the total AR content increased in leaf samples of the FL68 genotype in comparison to those of P3085 in both Monteria and Saldaña locations. On the contrary, panicle-derived AR content of FL68 was found to be lower regarding P3085 cultivated in Monteria, but not in Saldaña. Roots, sheaths, and stems kept similarly their total AR content regardless of the location. In addition, the AR content was higher in most of the inoculated treatments; therefore, a higher AR content than their respective controls was evidenced, especially in plants inoculated with B. glumae 43A. This trend is more evident in those samples from Saldaña (mostly in leaves and panicles) considering the P3085 genotype. Sheaths and stems also exhibited significant differences between inoculated treatments and their respective controls, but such differences were not as marked as in leaves and panicles. The root samples did not show significant differences between inoculated and controls, with the exception of the samples in the second and fourth phenological stages of FL68 from Saldaña.

3.2. LC-MS-based analysis of constitutive AR of both rice genotypes

The control samples (T1 treatments) were analyzed by HPLC-DAD-ESI-MS using a chromatographic method previously developed to increase the selectivity of AR (Marentes-Culma et al. 2019) with some modifications. Thus, three AR-type compounds were detected and annotated, whose structural identities were deduced from MS data (Ross et al. 2004). Such compounds were related to a homologous series of C19, C23, and C25 alkyl side chains. Their distribution and variations among test factors were analyzed based on their observed relative intensities obtained from spectral data after creating the feature intensity table (FIT). In this sense, the global distribution of AR relative intensities was visualized as a heatmap after feature autoscaling (Figure 1(a)). Such comparative levels revealed that 5-n-tricosylresorcinol (AR-C23) was usually the most abundant in several samples, with the exception of leaves and sheaths from Monteria and panicles from Saldaña. In contrast, 5-n-nonadecanylicosyrnesorcinol (AR-C19) had the lowest abundance in most samples, whereas 5-n-pentacosylresorcinol (AR-C25) accumulated the most in particular samples such as sheaths from Monteria and panicles from Saldaña.

This AR global distribution was also classified through a random forest algorithm. Hence, the top-fifteen representative experimental conditions (i.e. variables) were ranked by the relative intensity of each AR-compound (i.e. features) according to the mean decrease in classification accuracy (i.e. variable importance) when variables were permuted. Therefore, each top-ranked sample was related to each AR-compound from the highest to the lowest influence in the classification test (Figure 1(b)). In other words, this random forest-based classification is an indication of those conditions that favored higher production/accumulation of AR in rice. In general, the AR compound suffering the most influence by top-ranked experimental conditions was 5-n-tricosylresorcinol (AR-C23), whereas 5-n-pentacosylresorcinol (AR-C25) had an intermediate impact and 5-n-nonadecanylicosyrnesorcinol (AR-C19) showed the lowest influence. According to the top-ranking, the two samples of the FL68 genotype in the fourth and fifth phenological stages from Saldaña had the highest representativeness (mean decrease accuracy > 0.013). However, leaves of P3085 at the fourth phenological stage from Monteria showed the highest influence regarding the accumulation of 5-n-tricosylresorcinol (AR-C23). A similar impact was observed for the next five samples in the top-ranking. Such a trend is different in two samples of FL68 from Saldaña (S_F_4_P and S_F_5_P) and one from Monteria (M_F_5_S), whose accumulation is related to 5-n-pentacosylresorcinol (AR-C25).

3.3. AR-based response of rice genotypes against B. glumae depending on environment

We found contrasting patterns in the model plots between the samples from Monteria (Aw-type) and Saldaña (Am-type) in both rice genotypes (Figure 2, column a, c). The samples of sheaths, stems, and panicles of both-genotypes from Saldaña evidenced differences between treatment 43A and control, while the samples of sheaths and panicles from Monteria kept a few similarities. In addition, roots and leaves of B. glumae-inoculated P3085 from Monteria samples behave differentially to those of the respective control (Figure 2, column a), whereas inoculated FL68 plants exhibited differences only in stems (Figure 2, column c). In P3085 treatments, the inoculated plants showed contrasting behavior related to their respective control in Monteria and Saldaña (Figure 2, column a).

Specifically sheaths, stems, and panicles of the P3085 genotype from Monteria did not evidence clear ASCA-derived scoring differences among treatments (Figure 2, column a). In contrast, leaves and roots from inoculated plants showed particularly scoring variations regarding the corresponding control. Such changes were found to be related to the second to fourth phenological stages (according to the submodel plots) (Figure 2, column b). In the case of leaves, roots, and panicles of the P3085 genotype from Saldaña, a similar scoring performance between plants inoculated with B. glumae-33C and control was noticed; the plants inoculated with B. glumae-43A varied considerably from that of control. Such differences were observed in the respective submodel plots revealing that the fifth stage caused this AR-derived variational trend. Additionally, sheath and stems were found to be substantially different among the three treatments (Figure 2, column b).

On the other hand, roots, sheaths, and panicles of the FL68 genotype from Monteria did not show evident scoring differences between treatments T1, T2, and T3 (Figure 2, column c). However, leaves of plants inoculated with B. glumae-33C had a distinctive trend (observed for all stages according to submodel plots) with that of stems, since their AR-based variation provided distinct scores to those of control (Figure 2, column d). In addition, the submodel plots displayed similar patterns in phenological stages of roots, sheaths, stems, and panicles of those plants from Monteria. However, in such plant parts, the
fourth and fifth stages revealed significant scoring patterns in the samples from Saldaña. In the case of leaves, the observed scores were found to be opposite among locations, since an identical trend resulted between T1, T2, and T3 in samples from Saldaña; meanwhile, a sudden variation was evident in samples from Monteria, specifically for the plants inoculated with B. glumae-33C, whose score evolved in the opposite direction (Figure 2, column d).

The model ASCA plots associated with the phenological stage (time) indicated that the scoring patterns between P3085 and FL68 are similar from the third to the fifth stage of leaves and roots, and the fourth and fifth stages of sheaths (Figure 3, columns a, c). Regarding the P3085 genotype, the scoring trends from the second to the fifth stages were found to be the same, showing that the variation at the fifth stage is more pronounced. However, the stems and panicles behaved differently along phenological stages, since the scores of panicles had a downward slope whereas the scores of stems descended from the third to the fourth stage but increased toward the fifth stage (Figure 3, column a). Submodel plots evidenced that samples from Saldaña influenced such patterns the most. Nonetheless, leaves and roots were influenced by the treatment inoculated with B. glumae-43A; panicles and sheaths were influenced by control samples from Saldaña; and stems were influenced by the treatments inoculated with B. glumae-33C (Figure 3, column b). Unlike P3085, the lowest point in FL68 was different in all plant parts (Figure 3, column c). Hence, the submodel plots showed that samples from Monteria contributed specifically to the AR-derived variational scoring trends, excepting panicles (Figure 3, column d).

In order to analyze the contribution of each AR compound in the rice response against B. glumae, a Multivariate Empirical Bayes Analysis of Variance (MEBA) was then implemented to the entire chemical dataset. This analysis allowed us to graphically compare the changes of normalized intensity-related patterns between inoculated treatments (T2-T3) and the control (T1), as well as to find out the changes through a time scale related to the phenological stage-mediated progress. The MEBA derived profiles were then built independently by plant parts subdividing them into rice genotype and location. Such profiles exhibited different trends, but the most representative variations are presented in Figure 4. In the case of leaves, there was a larger difference in the samples between Saldaña and Monteria (Figure S3). Thus, samples from Saldaña showed a rise in AR abundance for the treatment inoculated with B. glumae-43A at the second and fourth stages (Figure 4(a)). Contrarily, roots did not exhibit clear patterns among test factors, involving a similar behavior between treatments T1, T2, and T3 (Figure S4). Particularly, the intensity of AR-C_{25} for those samples from Saldaña increased in all phenological stages, implying a marked influence regarding the accumulation of this compound by the presence of the bacterial strains (Figure 4(b)). In addition, AR levels of sheaths increased in the first, fourth, and fifth stages in both locations and both inoculated treatments (Figure S5). For instance, the treatment inoculated with B. glumae-33C showed a differential rise at the first, fourth, and five stages, depending on the AR compound (Figure 4(c)).

Finally, the variation of total AR content in stems of the FL68 rice genotype was higher in Monteria than in Saldaña. In contrast, the variation of total AR content of the P3085 rice genotype did not show changes in plants from Monteria, whereas the variation of the samples from Saldaña had a slight increase in the inoculated treatments with B. glumae-33C (Figure S6). However, the total AR content variation was more evident in those samples from Saldaña, specifically regarding AR-C_{25} of FL68 and AR-C_{25} of P3085 (Figure 4(d)). Lastly, the variation of total AR content in samples did not differ between treatments and control, but the P3085 genotype plants grown in Monteria showed a slight increase in the fifth stage (Figure 4(e) and S7).

4. Discussion

The present study describes constitutive AR and the performance of AR in rice plants induced by two B. glumae strains and two different climate conditions (i.e. Aw vs Am). In this sense, we focused on factors that could impact their activation and production; those factors are ontogeny (Moore et al. 2014), genotype (Bellato et al. 2013), and environmentally-different location (Landberg et al. 2014). Plants respond to these factors by triggering adaptive mechanisms,
such as AR which are secondary metabolites (currently called specialized) that are involved in the communication and interaction between the plant and its environment. According to our results, AR were detected in the non-inoculated plants showing that these metabolites (C19, C23, and C25) can be considered constitutive compounds as they were present at the basal metabolism of rice. Consequently, we can infer that AR are involved in relevant physiological processes of rice plants and likely acting as phytoanticipins (Baerson et al. 2010). Likewise, the independent trend of AR content in each of the two rice genotypes evidenced that the machinery for AR biosynthesis and/or accumulation is present in the five plant parts evaluated. In consequence, a tissue distinction can be prompted as described in rye, where each plant part has a characteristic AR composition that self-regulates the AR biosynthesis and/or accumulation in response to neighboring pressure (Sun et al. 2020). Also, AR can be produced, accumulated, and transported through the plant from AR-producing plant parts to sinks, causing a tissue-specific AR-distribution (Fang et al. 2012).

### 4.1. AR-based response of rice plants induced by B. glumae

The increased total AR contents and the higher AR-homologue relative intensities measured in inoculated treatments showed that such an overproduction might be related to the presence of B. glumae. AR have been documented as antimicrobial compounds, specifically in Poaceae plants as rice. For instance, AR isolated from rice plants have exhibited germination inhibition against aggressive spores of Pyricularia oryzae (Suzuki et al. 1996). When the plants are exposed to pathogenic organisms, they generally produce a chemical response; this response includes specific secondary metabolites as part of the plant

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**Figure 2.** ASCA models associated with treatments shown independently for P3085 (column a shows the model plots and column b shows the submodel plots), and FL68 (column c shows the model plots and column d shows the submodel plots) genotypes along plant parts. Letters on the X-axis of each ASCA plot represent the strain-genotype interaction: \( C\): Control-Monteria (T1); \( 33C\): 33C B. glumae-inoculated from Monteria (T2); \( 43A\): 43A B. glumae-inoculated from Monteria (T3); \( S\): Control-Saldaña (T1); \( 33C\): 33C B. glumae-inoculated from Saldaña (T2); and \( 43A\): 43A B. glumae-inoculated from Saldaña (T3). Phenological stages are represented as time course lines as follows: 1. Tillering; 2. Panicle initiation; 3. Heading; 4. Flowering; and 5. Milk stage. The score in the Y-axis caption is expressed as the percentage of variation explained (VE).
defense mechanisms (Pott et al. 2019). Such production is mediated by detection and recognition of pathogens by plants (Zaynab et al. 2018). The AR up-regulation under the inoculation of *B. glumae* strains (Figure S2) is a plausible indication of a relationship with a defensive response in these rice genotypes against microbial presence (Ji and Jetter 2008; Marentes-Culma et al. 2019). In fact, some previous studies have suggested that AR-homologues in exudates of rice roots are involved in the regulation of the microbial population of the rhizosphere; besides, they have shown close participation in the host–pathogen relationship increasing their levels and production (Miché et al. 2003; Sampietro, Belizán et al. 2013). Therefore, the rice genotypes maintaining or up-regulating higher AR levels can be advantageously better adapted for their antimicrobial response under biotic pressure. Actually, such an AR-related trait can be exploited for genetic improvement to produce resistant genotypes against BPB in Colombian Caribbean region, since other studies have previously suggested that improving plant resistance through breeding programs is the most relevant option for the control of this bacterial disease (Pérez and Saavedra 2011; Wamishe et al. 2014).

### 4.2. AR variations regarding ontogeny

Tillering in leaves and the flowering and milky in panicles were those phenological stages with the highest AR contents. This fact suggests that the physiological and ecological interactions between rice plants and *B. glumae* cause a differentiated AR-based response in the parts of the rice plant where the bacteria causes disease (Tsushima 1996; Moore 2003).
et al. 2014; Li et al. 2016; Pedraza et al. 2018). Consequently, AR biosynthesis in seedlings focuses on the coleoptiles of rice plants, indicating that AR-based response might be involved in some vital processes such as shoot protection (Deszcz and Kozubek 2000). Considering the fact that seeds were the inoculated organs, this finding is very significant since rice plants are severely affected in the seedling stage by B. glumae causing bacterial seedling rot (Uematsu et al. 1976; Maeda et al. 2004; Mizobuchi et al. 2013). However, if root growth increases within the soil while the AR concentration also rises, an ecological role in the root system might be rationalized (Miché et al. 2003).

It was also observed that the fourth and the fifth phenological stages (i.e. flowering and milk stages) from inoculated treatments are important related to AR dynamics (Figure 4). These stages are fundamentals for the life cycle of rice since the panicle is exposed toward the outer of sheaths during the fourth stage and the rice grain is evidenced that C23 had a close relation with Monteria and C25 with Saldaña (Figure 1). In addition, the abundance of C23 can be related to the P3085 genotype. These deeds should be expanded to include other genotypes with contrasting AR composition from B. glumae-inoculated rice plants comprised the same three AR homologues previously described in non-inoculated plants (i.e. C19, C23, and C25), but the AR-homologues ratio changed regarding virulence of B. glumae strains showing B. glumae 43A strain triggered the highest AR levels in both rice genotypes. This fact can be rationalized as the virulence features from each strain (virulence factors) are particular and hence trigger stronger or softer plant responses against pathogens. Such response levels are differentially presented according to the specific traits of the rice varieties as well as the pathogen characteristics that affect the plant (Nandakumar et al. 2007; Karki et al. 2012; Beltran-Molina et al. 2013; Mizobuchi et al. 2013; Lee et al. 2016; Mizobuchi et al. 2016).

4.3. AR changes related to rice genotype

The genotype is a factor that has an influence on the capacity to produce/accumulate AR due to the genetic background that is closely related to the heritability to the next generation. In addition, this feature might be modified according to the genetic selection and/or changes to afford new genotypes with high yield and great agronomical traits (Ziegler et al. 2015). In this sense, P3085 and FL68 genotypes share some agronomic features to raise rice production, but they have a contrasting response against B. glumae (Pérez and Higuera 2013). However, both rice genotypes exhibited AR occurrence in non-inoculated samples, but the AR levels raised in B. glumae-inoculated treatments at distinct ratios.

In this sense, such distinct ratios are related by the presence of specific molecular machinery in rice plants because they will be activated to overcome biotic or abiotic stress, which may influence the ratio of AR homologues to be biosynthesized (Sun et al. 2020). We found out that there was no clear relationship for the AR homologues ratio, although panicle samples evidenced that C23 had a close relation with Monteria and C25 with Saldaña (Figure 1). In addition, the abundance of C23 can be related to the P3085 genotype. These deeds should be expanded to include other genotypes with contrasting response against B. glumae, centralizing the efforts on the fourth and the fifth phenological stages and leaves and panicles.

Additionally, the AR composition from B. glumae-inoculated rice plants comprised the same three AR homologues previously described in non-inoculated plants (i.e. C19, C23, and C25), but the AR-homologues ratio changed regarding virulence of B. glumae strains showing B. glumae 43A strain triggered the highest AR levels in both rice genotypes. This fact can be rationalized as the virulence features from each strain (virulence factors) are particular and hence trigger stronger or softer plant responses against pathogens. Such response levels are differentially presented according to the specific traits of the rice varieties as well as the pathogen characteristics that affect the plant (Nandakumar et al. 2007; Karki et al. 2012; Beltran-Molina et al. 2013; Mizobuchi et al. 2013; Lee et al. 2016; Mizobuchi et al. 2016).

4.4. AR changes regarding environmentally-different location

As mentioned above, variations of AR levels and AR homologue ratios were generally more evident in the sample set
depending on test location (Table 1 and Figure 1). Some abiotic factors, i.e. environmental conditions, seasonality, and climate, impact the production of phytochemicals (Andersson et al. 2010) and these factors are associated with each location where plants are sowed, shifting the performance over ecological interaction (Landberg et al. 2014; Moore et al. 2014). Under this context, the locations in our study, i.e. Monteria and Saldaña, were selected since they are suitable for rice growing but have particular differences in certain environmental conditions, such as the climate type (i.e. Aw vs. Am), temperature (28.4 ± 5.1°C vs. 27.2 ± 7.9 °C), relative humidity (74 ± 7% vs. 54 ± 15%), and precipitation (1225 mm vs 1541 mm), respectively. Thus, we observed that rice plants from Saldaña exhibited a higher AR amount than plants from Monteria, especially in some plant parts such as leaves. According to the Köppen-Geiger climate classification system, Monteria has an isothermal climate whereas the temperature in Saldaña is more variable, but Monteria has a wetter environment and lower precipitation than Saldaña. Such a behavior had been documented in rye and wheat, whose differences in AR levels and homologues change according to the climate conditions of each location (Ross et al. 2003; Landberg et al. 2014). This climate variations appears to have a significant impact on the production of AR increasing their levels especially in leaves. In addition, the accumulation of C23 was more closely related to Saldaña conditions in both rice genotypes, whereas FL68 is more related to C25 in Monteria, which expose a particular influence on the regulation of specialized type III polyketide synthases (namely alkylresorcinol synthases) in rice by temperature and relative humidity increase to favor the extension the alkyl unit (Baerson et al. 2010).

Our experiment was set up during a favorable annual period in both locations that favors the colonization and propagation of B. glumae in rice plants. Hence, the increase of total AR levels (especially in rice plants inoculated with B. glumae 43A strain) were considerably evident in samples from the Am-type climate (i.e. Saldaña) (Figure S2), even the most representative MEBA plots show that those samples containing higher levels of AR homologues came from Saldaña (Figure 4), due to the higher precipitation and lower relative humidity impacting the metabolic-based plant response (Landberg et al. 2014). However, the relative AR homolog composition was interestingly similar in both locations (excepting FL68 leaves from Monteria and panicles of both genotypes from Saldaña), being C23 the most occurred in rice samples (excepting sheaths from Monteria and panicles from Saldaña) (Figure 1). Homologue C19 was the lowest abundant AR in all test rice-derived samples under study, although this homologue C19 had been previously found as the most frequent AR in wheat plants (Andersson et al. 2010).

5. Conclusions

In this study, we performed a chemical exploration to examine the AR-mediated response of plant parts from two rice genotypes to the B. glumae presence depending on climate conditions. Our findings indicated that the test rice genotypes (P3085 and FL68) produce AR in all plant parts (leaves, roots, sheaths, stems, and panicles) during all evaluated physiological stages (i.e. tillering, panicle initiation, heading, flowering, and milk stage) as constitutive metabolites possibly having physiological and/or ecological roles. Additionally, the presence of B. glumae influences the total AR content and AR homologue-based composition in the two rice genotypes evaluated (P3085 and FL68), but such an influence depended on the plant part related to ontogeny, B. glumae strain, and location, indicating a temporarily, spatially and environmentally-dependent AR-based metabolic regulation to face B. glumae. The Am-type climate influenced the AR accumulation (particularly the homologue C23), favored by temperature, lower relative humidity, and higher precipitation in roots, sheaths and panicles. In this regard, AR are an alternative to study the expression of metabolite-mediated responses in rice plants against this economically important pathogen in rice cultivation. These findings would provide a reference information for growth regularity, disease monitoring, metabolic traits, and the further quality evaluation of rice genotypes. In addition, these results provide an initial background to understand the interaction between host-pathogens, which may support new approaches to develop strategies of integrated bacterial disease management in rice crops as well as genetic improvement to produce resistant genotypes against BPB.

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Availability of data and material

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Author contributions

E. Coy-Barrera and C. Cuellar-Cuestas designed the experiments and supervised the project. R. Marentes-Culma performed the experiments and analyses. R. Marentes-Culma and E. Coy-Barrera integrated the data and wrote the draft. R. Marentes-Culma, C. Cuellar-Cuestas, H.A. Ardila and E. Coy-Barrera finalized the data interpretation and the manuscript. All authors reviewed and approved the manuscript.

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