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Metabolomic Profile and Feeding Preference of Diatraea Saccharalis in Sugarcane Co-inoculated With a Diazotrophic Consortium

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Abstract

Sugarcane is cultivated in over 100 countries and was introduced to Brazil in 1532. It has since become a major crop in the Brazilian economy. However, it is susceptible to pests, particularly *Diatraea saccharalis*. Applying microorganisms, such as plant growth-promoting rhizobacteria (PGPBs), affects plant metabolism and resistance, helping plants cope with pests. These microorganisms promote differential growth and induce resistance, phytohormone production, volatile organic compound production, and nitrogen fixation in plants. *Gluconacetobacter diazotrophicus* and *Azospirillum* sp. are examples of PGPBs that promote plant growth via biological nitrogen fixation, phytohormone production, phosphate and zinc solubilisation, and protection against biotic and abiotic stress. This study aimed to characterise the metabolism of sugarcane co-inoculated with a consortium of two diazotrophic PGPBs, (*G. diazotrophicus* and Azospirillum sp)., and to compare the metabolic profiles of co-inoculated and non-inoculated plants. We used nuclear magnetic resonance to identify soluble metabolites. We evaluated weight gain and D. saccharalis preference using choice and no-choice feeding bioassays, respectively. In the free-choice feeding bioassay, the preference for *D. saccharalis* was higher for the control plants than for the co-inoculated plants. Higher concentrations of soluble metabolites, including carbohydrates, aromatic compounds, amino acids, and organic acids, particularly gallic acid, which are used to combat biotic stress in plants, were detected in co-inoculated plants.

1 INTRODUCTION

Plant growth-promoting bacteria (PGPBs) are microorganisms that enhance plant growth through direct and indirect mechanisms. Direct mechanisms include the production of phytohormones, such as auxins and gibberellins (Deka et al. 2014; Gupta et al. 2022). Indirect mechanisms vital to the plant include organic acid production, which aids in the solubilisation of inorganic phosphate, as seen in phosphate-solubilizing bacteria that produce gluconic, citric, malic and other organic acids that solubilize bound phosphates in the soil (Vyas and Gulati 2009), and siderophores, which compete with pathogenic microorganisms for iron ions (Saraf et al. 2014). Plant defence systems against herbivorous insect attacks are also influenced by plant–PGPB interactions (Brock et al. 2018) through direct and indirect defence strategies (Naeem et al. 2018; Pineda et al. 2012). *Gluconacetobacter diazotropicus* and *Azospirillum* spp. are examples of PGPBs that promote plant growth through biological nitrogen fixation, phytohormone production, phosphate and zinc solubilisation, and protection against biotic and abiotic stress (Devi et al. 2022; Yasuda et al. 2022). *Gluconacetobacter diazotropicus* was initially isolated from the roots and stems of sugarcane varieties cultivated in northeastern states (Pernambuco, PE and Alagoas, AL) and southeastern states (São Paulo, SP and Minas Gerais, MG), Brazil (Cavalcante and Dobereiner 1988) and was later found in other plants. *Gluconacetobacter diazotropicus* is an endophyte that colonises the root tissues and aerial parts of sugarcane (Rodriguez et al. 2019). On the root surface, these bacteria concentrate in regions where lateral roots are joined. Inside the root, they are found in the epidermal cells and apoplast of the sugarcane stem. The bacteria are also present inside the xylem vessels, migrating to the aerial parts via plant transpiration (Rangel de Souza et al. 2016).

Azospirillum spp. colonises several plant species and improve their growth, development, and productivity (Zarea 2017) through the production and metabolism of phytohormones and other plant growth-regulating molecules. They are also facultative endophytes that can colonise leaves and roots to control sugarcane stalks. Inoculation with *Azospirillum* spp enhances water and nutrient absorption by promoting extensive root system development, increasing the area of soil explored by the roots (Curá et al. 2017). They can also trigger resistance to pathogenic fungi by activating the host plant's innate immune system (Fujita et al. 2017), thus protecting the plant from disease (Parmasi et al. 2019).

To our knowledge, there are no reports in which PGPBs (*G*. *diazotropicus* and Azospirillum spp.) have been used to manage *Diatraea saccharalis*. Sugarcane is cultivated in over 100 countries (Vandenberghe et al. 2022) and was introduced in Brazil in 1532. Since then, it has become a crop of great importance to the Brazilian economy, both in exports and domestic consumption. The 2024–2025 Brazilian production of this crop reached 676.95 million tonnes, an increase of 4.4% compared to 2021–2022, cultivated on 8.62 million hectares (CONAB 2022). Sugarcane is exposed to aggressive biological agents, mainly pests, the most significant of which are *D*. *saccharalis* Fabricius (Lepidoptera: Crambidae), *Migdolus fryanus* Westwood (Coleoptera: Cerambycidae) and the giant sugarcane borer *Telchin licus* (Drury) (Lepidoptera: Castniidae) (Dinardo-Miranda et al. 2012, 2013). These pests cause severe economic damage to sugarcane crops (Cursi et al. 2022). In Brazil, annual economic losses caused by sugarcane borer (*Diatraea saccharalis*) infestations are estimated at up to R$ 8 billion (approximately US$ 1.5 billion), directly compromising stalk yield and industrial processing (Cursi et al. 2022). Uncontrolled infestations lead to estimated reductions of up to 12.1% in sugarcane biomass, 3.8% in sugar recovery, and 2.7% in ethanol yield (Dinardo-Miranda and Fracasso 2013; Cursi et al. 2022).

Therefore, it is essential to develop alternative strategies for controlling *D*. *saccharalis*. The application of endophytic microbes is a promising strategy for managing insect pests, which has spurred research into their ecology in agricultural ecosystems. To determine whether PGPBs modulate herbivore behaviour, choice and no-choice feeding tests were conducted. These tests evaluated both the attractiveness and suitability of inoculated plants as hosts for *D*. *saccharalis*, providing an indirect but functional measure of the induction of bacterial-mediated defence mechanisms.

Using nuclear magnetic resonance (NMR), this study aimed to carry out metabolic characterisation of sugarcane plants (variety 'SP 79-1011') co-inoculated a bacterial consortium composed of two diazotrophic PGPBs (*G. diazotrophicus* and *Azospirillum* sp.), previously selected for their nitrogen fixation capacity and to investigate the influence of this co-inoculation on plant defence against attack by *D*. *saccharalis*. To observe the responses of *Diatraea* to sugarcane co-inoculation, feeding experiments with and without a choice and comparisons of the metabolomes between co-inoculated and non-inoculated plants were conducted.

2 MATERIAL AND METHODS

2.1 Plants

Sugarcane plants (cv. SP79-1011) were obtained from the mill industry, Usina Triunfo Agroindustrial Ltd (Boca da Mata, Alagoas State, Brazil), at an altitude of 132 m and geographic coordinates of 90°38’27.6” S and 360°13’12” W. Sugarcane of the SP 79 1011 variety was manually collected following the procedure adopted by the research team. The plants were collected from selected plots, with a uniform stage of development and without visible mechanical or phytosanitary damage. Harvesting was carried out by manually cutting close to the ground using a machete, followed by manual removal of the leaves, keeping only the intact stems. Subsequently, the stems were sectioned into standardized pieces, placed in clean and properly identified containers, and immediately transported to the Laboratory for Research in Chemistry and Natural Resources (LPQRN) for subsequent preparation and analysis steps. The aim was to minimize the interval between harvesting and laboratory processing to preserve the chemical integrity of the plant material. This variety was selected because it had previously been adopted as a model by our group and shown to be harmful to *D. saccharalis* development (Sabino et al., 2019). The stalk of each cane was sectioned into pieces, each with one bud. For NMR metabolomic analysis, leaves were collected from plants at 60–70 days of development.

2.2 Microorganisms

The selected bacterial strains used in this study were *G. diazotrophicus* (BR 11281) and *Azospirillum* sp. (BR 11145). The Culture Collection of CRB-JD supplied them at Embrapa Agrobiology (Seropédica, RJ, Brazil). They were cultured as described below.

2.3 Polymeric inoculant carrier

To prepare the carrier, xanthan gum (5.0 $g \bullet L^{-1}$), a microbial polysaccharide used as a thickener, polyvinylpyrrolidone (PVPP, 5.0 $g \bullet L^{-1}$), a stabilising agent, and trehalose (1.0 $g \bullet L^{-1}$), a cell protectant, were mixed. This carrier mixture was dispersed in 50 mL of distilled water to prepare colloidal liquid suspensions (Scheidt et al. 2019) and subsequently sterilised in an autoclave at 121°C in polyethylene high-density polyethene flasks.

2.4 Microorganisms

2.4.1 Preparation of microorganisms and inoculants

The strains were grown individually in BP medium (Bioprocesses- Embrapa Agrobiologia – Campinas, SP, Brazil) liquid culture medium (5.0 $g \bullet L^{-1}$ glycerine, 5.0 $g \bullet L^{-1}$ yeast extract, 0.5 $g \bullet L^{-1}$ K_2HPO_4, 0.5 $g \bullet L^{-1}$ $MgSO_4$) as described by Scheidt et al. (2019). Cultivation was performed in 1.0-L Erlenmeyer flasks in an orbital shaker (Tecnal Equipamentos Científicos, Piracicaba, SP, Brazil) at 120 rpm and 30°C. When the stationary phase of the growth curve was reached, the turbidity (OD_{600}) of the cell broth was adjusted to 1.5 units with fresh sterile medium, corresponding to a cell count of around 5.0×10^9 $UFC \bullet mL^{-1}$ for both strains. Correlation plots between medium turbidity (OD_{600}) measurements and cell counts ($UFC \bullet mL^{-1}$) were produced for each strain. The two cell broths were mixed, and the carrier (described in Section 2.3) was added in a 1:1 (v:v) ratio. The final cell concentration was approximately 2.0×10^9 $UFC \bullet mL^{-1}$; cell

counts were confirmed using a Petroff–Hausser-type bacteria counting chamber (Hausser Scientific Company, Horsham, PA, USA). Subsequently, this inoculant formulation was transferred to 50-mL polypropylene conical tubes for storage and further use. The transfer was performed in a vertical laminar-flow hood after disinfection with 70% ethanol and ultraviolet light for 50 min.

2.4.2 Inoculation with diazotrophic bacteria

Inoculation was performed according to a protocol adopted by the sugarcane industry. Following this protocol, sugarcane stalks were cut into 5–10 cm lengths with one bud. The stalks were immersed in the inoculum solution in a 2-L beaker. This solution consisted of 1 L of drinking water and 40 mL of inoculum (diazotrophic bacteria). The stalks were incubated in a water bath at 34 ± 1°C for 10 min and then planted. The control followed the same steps as the treatment except for inoculum addition.

2.4.3 Obtaining sugarcane plants

After inoculation, the stalks were propagated via internode cuttings and cultivated in 500-mL plastic pots containing a coconut fibre substrate (Bioplant, Bioplant Misturadora Agricola Ltd., Nova Ponte, MG, Brazil). Sugarcane was grown in a greenhouse at 25 ± 2°C and 70% relative humidity (RH). The plants were watered once or twice daily, as required. The plastic flowerpots had holes to allow excess water to drain. The sugarcane plants inoculated with PGPB were referred to as the inoculated group, and the non-inoculated plants were termed the control group.

2.4.4 *Diatraea saccharalis*

Insects were obtained from the Biological Control Lab of the Sugar Industry, Central Açucareira Santo Antonio (Alagoas, Brazil). Young *D*. *saccharalis* larvae were individually reared in Petri dishes on an artificial diet (Hensley and Hammond, 1968) and maintained at 26 ± 5°C and 70 ± 5% RH, with a 12-/12-h light/dark photophase, until required for the infestation experiments. Third-instar caterpillars were used for the experiments, with and without choice, as they have previously been shown to be the most active in the laboratory. These caterpillars were kept in glass tubes with an artificial diet for up to 3 days before the experiment began. Thereafter, the caterpillars were deprived of food but could access cotton moistened with distilled water. Food deprivation ensured that the caterpillars fed on sugarcane throughout the experiment.

2.5 No-choice feeding experiment

To evaluate the no-choice feeding action, the caterpillar growth rate was measured in a no-choice feeding experiment, as modified from Sadeghi et al. (2007). The experiment was conducted by placing the caterpillar at the base of the plant and allowing it to feed for 5 days. Subsequently, the caterpillars were removed from the stalks using tweezers. The caterpillar growth rate was calculated from their weights before and after introduction to the plants. Additionally, a screen cage was used to maintain the caterpillars at the base of the plant stalk. Each replicate consisted of 10 plants from each treatment, with one caterpillar per plant. Data were analysed using a t-test ($p < 0.05$).

2.6 Choice-feeding experiment

The preference of herbivorous insects was investigated in a glass arena with the option to choose between plants from two treatments (control and inoculated), as described by Peñaflor et al. (2011) with modifications. The glass arena consisted of a cube-shaped structure measuring 40 × 25 × 26 cm (l × w × h). There were two circular openings at the base, arranged 15 cm apart, through which the plants were carefully inserted to prevent them from being damaged. The bottom of the stalk was at the height of the base of the arena. The caterpillars were released in the centre of the arena, one at a time, and their preference for inoculated and non-inoculated plants was evaluated. The observation period for each choice was 30 min. When there was no choice, the caterpillars were removed from the arena, sanitised, and not counted. The experiments were performed under infrared light, with bioassays consisting of 18 replicates (9 plants per treatment). Data were analysed using the Wilcoxon test ($p < 0.05$).

2.7 Nuclear magnetic resonance analysis (1D and 2D homonuclear and heteronuclear)

Non-volatile metabolites were identified by analysing the ¹H NMR (hydrogen NMR) spectra. Metabolites were extracted from plant leaves that were removed using scissors previously sanitised with commercial ethanol (700 g/L, Usina Coruripe, Coruripe AL, Brazil) and immediately frozen in liquid N_2. The leaves were placed in an ultra-freezer at − 80°C and stored until extraction.

Extraction was performed according to the methodology described by H. K. Kim and Verpoorte (2010), adapted for this study. Fresh leaves (50 mg) were lyophilised and transferred to a 2-mL Eppendorf® tube (Eppendorf AG, Hamburg, Germany) with 500 µL of methanol - d4 (Cambridge Isotope Laboratories, Thaxted, UK) and 500 µL of phosphate buffer in deuterated water with a pH of 6.0, containing 0.01% trimethylsilyl propionic acid (TSP) sodium salt (Cambridge Isotope Laboratories, London, UK). The material was then ultrasonicated for 20 min and centrifuged at 5000 rpm (2236 × g) for 15 min. Subsequently, the supernatant was collected and transferred to a 5-mm NMR tube.

The spectra were obtained at 20°C using a Bruker AVANCE spectrometer (Bremen, Germany) operating at 400 MHz. Each ^{1}H-NMR spectrum consisted of 128 pulses (10 min and 26 s) with an acquisition time of 5 s, a pulse width of 90° = 9.75 µs, and a relaxation delay (RD) of 2 s. A water signal suppression technique was used to reduce the signal-to-noise ratio. The J-resolved spectrum was acquired using 32 pulses with 128 increments at 8K data points, with a spectral length of 5.208 kHz and a spin-spin coupling of 60 Hz, and an RD of 1.5 s. The COSY ^{1}H-^{1}H spectrum was obtained with 32 pulses in 512 increments at using 2K data points, with a spectral length of 5.208 kHz in both dimensions and RD = 1.5 s. The HMBC ^{1}H-^{13}C and HSQC ^{1}H-^{13}C spectra were acquired with 64 pulses in 512 increments at 4K data points, with spectral widths of 5.208 kHz for 1H and 22.149 kHz for 13C, with RD = 1.5 s.

The spectra were processed using Bruker's TopSpin® software (version 2.1), with phase correction, baseline correction, and calibration using TSP sodium salt as the internal standard at 0.0 ppm. The optimised ¹H-NMR spectra were reduced (0.3–10.0 ppm), divided into broad regions (bucket size =

0.0004 ppm), normalised, and aligned using MATLAB version 2014a. The δ 4.7–5.0 and δ 3.24–3.33 areas were excluded from the analysis due to residual water and methanol signals.

The metabolites in the plant leaves were identified by comparing chemical shifts and coupling constants with those reported in the literature, Heteronuclear Multiple Bond Correlation (HMBC 1H-13C) and by analysing 2D NMR spectra, such as J-resolved, Correlation Spectroscopy (COSY 1H-1H), Total Correlation Spectroscopy (TOCSY), HMBC, and Heteronuclear Single Quantum Coherence (HSQC ^{1}H-^{13}C) with the chemical standard. Statistical Total Correlation Spectroscopy (STOCSY) and Chenomx software version 8.2 were used to assign metabolites.

2.8. Multivariate statistical analysis

The ^{1}H-NMR spectra were optimised, reduced (δ 0.7–9.5 ppm), divided into wide standard regions (bucket width = 0.0005 ppm), aligned, and normalised by the total area using MATLAB (version 2014A). Additionally, the regions of δ 4.7–5.0 and δ 3.24–3.33 were excluded from the analysis. Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed using SIMCA-P software (version 14.0, Umetrics, Umeå, Sweden) with unit-variance (UV) scaling. A paired *t*-test and boxplots were performed in RStudio version 4.4.1. We performed supervised OPLS-DA analysis to compare control and treated plants and identify discriminant metabolites in response to *D. saccharalis* herbivory on sugarcane. Principal component analysis (PCA) was performed to identify outliers and clustering trends between the control and inoculated plants (Fig. 1).

3 RESULTS

3.1 Metabolic profile of sugarcane plant leaves

NMR is a powerful tool for metabolomic studies and a universal detector for a wide range of molecules, requiring minimal sample preparation. It can also reproduce data even with versatile spectrometers operating at different frequencies and offers a range of 1D, 2D, and pseudo-2D experiments (pulse sequences) for structural elucidation. Another advantage of NMR is the ease of data digitisation for chemometric methods (Leiss et al., 2011). This technique is not sample-specific, as it can be applied to different classes. Using this strategy, we identified a total of 29 metabolites belonging to different chemical classes, including amino acids, organic acids, sugars, phenolic compounds, and alkaloids.

3.2 Multivariate analysis of sugarcane plants from the control and inoculated groups

Eighteen ^{1}H NMR spectra from control and inoculated samples were aligned and scaled using the UV scaling method prior to multivariate analysis. Principal component analysis (PCA) was initially performed to explore intrinsic data patterns, with the first two principal components (PC1 and PC2) explaining 42.5% of the total variance. Subsequently, orthogonal partial least squares discriminant

analysis (OPLS-DA) was applied to maximize group discrimination. The OPLS-DA score plot (Fig. 1) revealed a clear separation between control and inoculated samples

The supervised statistical method OPLS-DA was used to identify discriminatory metabolites. Method validation was performed using cross-validation and analysis of variance ($p < 0.05$), whereas discriminatory metabolites were selected using Pearson's correlation coefficient ($r > | 0.576 |$). Pearson's correlation measures the shared variance between two variables, where a one-unit increase or decrease in variable X has the same impact on Y (Figueiredo Filho and Silva Júnior 2009). Table 1 presents the metabolites that discriminate between control and inoculated plants, as identified using the S-line (Fig. 2) generated in SIMCA-P (OPLS-DA loads). Peaks with a high correlation are highlighted in red. In contrast, low-correlation peaks are represented in blue.

The alanine, gallic acid (GA), and sucrose concentrations increased, whereas the (E)-aconitic acid, malic acid, and proline contents decreased in the inoculated plants.

The signals in the centre of the figure are in a sugar-dominant region. The large number of these signals and high intensities make it challenging to identify metabolites.

Table 1

Sugarcane metabolites associated with inoculated plants compared to control plants.

m, multiplet; t, triplet; d, doublet; s, singlet

The levels of discriminating metabolites in bacteria-induced sugarcane are shown in boxplots in Fig. 3, which were constructed from the integrated areas of 1H-NMR chemical shifts obtained from MATLAB-processed data. A t-test was used to verify the significance of the selected discriminatory metabolites in the OPLS-DA results. The boxplots corroborated the OPLS-DA results, showing elevated alanine, GA, and sucrose contents and reduced (E)-aconitic acid, malic acid, and proline contents in the inoculated plants.

The boxplots show the relative concentrations of the discriminating metabolites (on the y-axis) for the control and inoculated plants. Median-normalised metabolite concentrations were log-transformed to ensure a Gaussian distribution and scaled using the Pareto scale before statistical analysis, including PCA, which was performed using SIMCA software.

3.3 Metabolic pathway enrichment analysis

a)

The discriminant metabolites identified between control and PGPB-inoculated plants were subjected to enrichment analysis in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Metabolomic Pathway Relationships Database (RaMP-DB) to identify the metabolic pathways most affected by inoculation. KEGG analysis revealed that the metabolites were primarily enriched in the citric acid cycle (TCA cycle) and pyruvate metabolism, indicating changes in central energy metabolism associated with

PGPB-induced metabolic modulation (Fig. 4a, b). In the RaMP-DB database, 25 pathways were enriched, 3 of which were statistically significant ($p < 1e-5$): urea cycle and associated pathways; proton-coupled transport of neutral amino acids; and transport of cations, inorganic anions, and amino acids (Fig. 4c, d). These pathways reflect potential changes in the dynamics of nitrogen metabolism and cellular transport of organic compounds in response to inoculation

Figure 4 Differential enrichment pathways of soluble metabolites. a, b KEGG enriched pathways for differential metabolites in two pathways: citric acid cycle (TCA cycle) and pyruvate metabolism, c, d. RaMP-DB enrichment analysis revealed that the 6 differentially expressed compounds were significantly enriched in 25 pathways, predominantly those involved in the urea cycle and associated pathways, proton-coupled transport of neutral amino acids, and transport of cations, inorganic anions, and amino acids.

3.4 Experiment with and without choice feeding.

Feeding experiments with and without choice

A no-choice feeding experiment was conducted to evaluate weight gain in *D. saccharalis*. In this experiment, insects were offered single food source per arena (either a control cane or a co-inoculated cane). The results of the no-choice feeding, comparing *D. saccharalis* fed on plants from the co-inoculated with those fed on control plants, showed no significant differences in insect weight gain (Wilcoxon test, df = 1, p = 0.20042). The analysis showed that the average weight of the caterpillars fed on the control plants was 0.3176 ± 0.007727 mg, whereas that of the caterpillars fed on co-inoculated plants was 0.3235 ± 0.002959 mg (Fig. 5a).

A choice experiment was conducted to assess the preference of *D. saccharalis* for the control and co-inoculated canes. In the choice experiment, in which *D. saccharalis* could choose between control and co-inoculated plants, we found that these insects preferred the control plants (Wilcoxon test, df = 1, p = 0.03260). The mean number of choices of *D. saccharalis* for the control plants was 0.77778 ± 0.146986 (SD), whereas that for the inoculated plants was 0.33333 ± 0.16667 (SD) (Fig. 5b).

4 DISCUSSION

4.1 Metabolomic analysis of sugarcane plants from the control and inoculated groups

Metabolomic analysis by ^{1}H-NMR of leaf tissue from sugarcane plants co-inoculated with a diazotrophic bacterial consortium (*G. diazotrophicus* and *Azospirillum* sp.) and from non-inoculated plants proved to be a method capable of detecting metabolites differentially expressed in sugarcane (Sabino et al., 2019). As a result, multivariate analysis of NMR data discriminated between plants subjected to different treatments. The sucrose content dominated the NMR spectra profiles; however, the identification of discriminatory secondary metabolites was possible. As a result, the OPLS-DA model based on NMR data

discriminated inoculated sugarcane plants from those not inoculated due to the variability in sucrose, alanine, and GA present in higher concentrations and aconitic acid, malic acid, and proline in lower concentrations in the co-inoculated plants.

The proline concentrations decreased in the leaves of plants co-inoculated with PGPBs with PGPBs. Proline accumulates in many plant species under a wide range of stress conditions, playing essential roles in mitigating these environmental challenges. It contributes to osmotic adjustment and participates in cellular signalling pathways that help plants cope with adverse conditions, including pathogen attack (Szabados and Savouré 2010; Z. Wang et al. 2020). In sugarcane plants, for example, proline accumulation occurs under water stress, especially when inoculated with rhizobacteria (Rampazzo et al. 2018).

Proline can be catabolised in plants and converted to α-ketoglutarate, a key intermediate in the Citric acid cycle TCA, directly integrating it into mitochondrial energy metabolism. This conversion occurs in two steps: the oxidation of proline to pyrroline-5-carboxylate (P5C) by proline dehydrogenase (ProDH), followed by the conversion of P5C to glutamate by P5C dehydrogenase (P5CDH). Glutamate is deaminated to α-ketoglutarate, integrating proline directly into mitochondrial energy metabolism (Miller et al. 2009; Schertl et al. 2014).

In *Arabidopsis thaliana*, a mitochondrial complex involving ProDH1, P5CDH, and ornithine δ-aminotransferase (OAT) has been demonstrated, channelling P5C and facilitating its efficient conversion into glutamate, which prevents its toxicity and contributes electrons to the respiratory chain for redox balance (Zheng et al., 2024). In rice (*Oryza sativa*), P5CDH also exhibits significant activity, with high specificity for NAD^+ as a reducing cofactor, reinforcing its role in mitochondrial energy production (Forlani et al. 2015). By generating glutamate and subsequently α-ketoglutarate, proline oxidation contributes to the supply of Krebs cycle intermediates, connecting nitrogen metabolism to carbon metabolism and ATP generation, providing energy for the maintenance of photosynthesis (Ludwig 2016) and precursors for chlorophyll and protein synthesis (Hosseinifard et al. 2022). PGPBs can utilise amino acids, including proline, as a carbon and nitrogen source, or reduce the levels of these metabolites under conditions of high energy demand (Cipriano et al. 2021; Curá et al. 2017; Gontia-Mishra and Sharma 2012; Nautiyal et al. 2010; Naveen and Balachandar 2019; Pedrosa et al. 2011). In the present study, proline levels were reduced in sugarcane co-inoculated with plant growth-promoting bacteria (PGPB). Tejera et al. (2006) and Weston et al. (2012) observed changes in the free amino acid profile in sugarcane and *Arabidopsis thaliana* after inoculation with endophytic bacteria, with a tendency to reduce leaf amino acid content.

In corn (*Zea mays* L.) plants subjected to chromium or salinity stress, inoculation with PGPBs reduced the proline level by up to 30%, suggesting that these microorganisms mitigate stress (Islam et al. 2016; Rojas-Tapias et al. 2012; Zafar-ul-Hye et al. 2021). In non-inoculated corn plants under drought stress, proline accumulation was high, as expected under adverse conditions; however, plants inoculated with *Azospirillum* sp. and *Herbaspirillum* sp. showed significantly lower proline levels, even under drought

stress (Curá et al. 2017). In sugarcane, Cipriano et al. (2021) reported that bacterial inoculation reduced typical stress compounds, such as proline, putrescine, and aspartate, but increased others, such as glycine and alanine. Based on the results of these studies, it can be speculated that, in the context of the present investigation, the observed reduction in proline may be the result of the plant's reduced need for protection against biotic stress, the active consumption of this amino acid by PGPBs, which can use proline as a carbon and nitrogen source (Christgen and Becker 2019; Liu et al. 2017), or the oxidation of proline to glutamate and citric-acid-cycle intermediates in the plant (Szabados and Savouré 2010; Verslues and Sharma 2010). These findings reinforce the hypothesis that, in the current investigation, PGPBs cause co-inoculated plants to experience lower stress levels compared to non-inoculated plants, reprogramming plant metabolism by directing compounds, such as proline, to other metabolic pathways, activating anabolic processes that are essential for growth and ATP production, and enhancing the photosynthetic process, resulting in amino acid biosynthesis and carbohydrate accumulation. Collectively, this suggests that the observed reduction in proline levels in our co-inoculated sugarcane plants is likely a multifaceted response, driven both by plant stress mitigation and the direct metabolic activity of PGPBs.

Malic acid and aconitic acid concentrations also decreased in the co-inoculated plants. Malic acid (2-hydroxybutanedioic acid) is a four-carbon dicarboxylic acid that plays a key role in plant functions, including maintaining the cytoplasmic pH balance, stress responses (Finkemeier and Sweetlove 2009; Ye et al. 2017), and stomatal closure (Sasaki et al. 2022). Malic acid is also involved in the respiration of rhizobia, which are nitrogen-fixing bacteria that form nodules in legumes (Booth et al. 2021). This organic acid is common in root exudates and plays a fundamental role in communication between PGPBs and plant roots (Rekha et al. 2018). It contributes to the solubilisation of inorganic phosphate (Vyas and Gulati 2009), improving fruit quality and nutrient uptake in grape (Si et al. 2022). Malic acid functions as a chemical signal that attracts beneficial soil bacteria, facilitating the establishment of symbiotic interactions that promote plant growth (Rudrappa et al. 2008; Zhuang et al. 2024). This signal plays a key role in attracting and initiating PGPR colonisation of plant roots (Yan et al. 2017). Furthermore, malic acid contributes to cellular homeostasis and is associated with increased plant resistance to environmental stress and promotes young tissue development (Ghazijahani et al. 2018; Sun et al. 2019).

Previous studies have reported increased production of this metabolite in sugarcane inoculated with *Bacillus subtilis* B9 (Di et al. 2023) and in *Combretum lanceolatum* plants associated with endophytic fungi (Lacerda et al. 2021). Malic acid can be metabolised by beneficial microorganisms (Ikeda et al., 2013; Jha et al., 2012; Jones et al., 2009) or converted into pyruvate, a precursor of alanine, especially during photosynthetic metabolism in C4 plants (Heldt and Piechulla 2011). Low malic acid concentrations in plants co-inoculated with PGPBs may reflect their intense consumption during the symbiotic process, as many PGPBs, such *as Bacillus subtilis* (Xie et al. 2015), utilise malate as a carbon source and a substrate for growth and colonisation of plant tissues (Rekha et al., 2018).

Aconitic acid (trans-aconitic acid, TAA) is one of the most prevalent organic acids in Poaceae plants, such as sugarcane, sorghum (Bruni and Klasson 2022), and corn (Eprintsev et al. 2015). It is an intermediate in the TCA cycle, originating from the isomerisation of citrate by the action of the enzyme aconitase, which converts citrate to cis-aconitate (Z-isomer), which is subsequently transformed into trans-aconitate (Igamberdiev and Eprintsev 2016). It can be found in exudates from root inoculated with PGPBs, acting primarily to improve nutrient uptake (Ain et al. 2024), plant growth, and the chemoattraction of *Rhizobium leguminosarum* bv. *viciae* 3841 and *Azospirillum brasilense* (Nisha et al. 2024). The application of mycorrhizal fungi in conjunction with PGPBs increased the organic acid concentration in the rhizosphere soil around corn plant roots, with significant increases in malic acid and aconitic acid in all treatments, directly improving nutrient uptake (Ain et al. 2024).TAA is known to accumulate in plants, such as corn (Igamberdiev and Eprintsev 2016); however, its accumulation impaired critical physiological processes in *Glycine max*, causing damage to the root system and photosynthetic apparatus, thereby significantly reducing plant growth (Bortolo et al. 2018).

PGPBs use TAA as a carbon source, and this assimilation constitutes a widespread bacterial mechanism for adapting to different environmental conditions. This compound, which is naturally present in various ecosystems, serves as an alternative carbon source that can be metabolised by some bacterial species (Zheng et al., 2024). *Bacillus velezensis* FZB42, a vital PGPB, possesses a TAA assimilation system. The ability to assimilate TAA confers a survival and growth advantage to bacteria in environments where TAA is present, such as soil. This suggests that using TAA as a carbon source may be an essential strategy for the adaptation and competitiveness of PGPBs in the plant environment (Zheng et al., 2024).

Based on these studies, the reduction in malic acid and aconitic acid levels observed in sugarcane plants co-inoculated with *G. diazotrophicus* and *Azospirillum sp.* indicates that these bacteria significantly interfere with primary metabolism, even in the absence of overt stress. This suggests a priming process, in which the plant is metabolically prepared to respond more quickly and efficiently to future biotic or abiotic stressors. In this scenario, co-inoculation promotes early metabolic reprogramming, redirecting TCA cycle intermediates to metabolic pathways involved in the biosynthesis of defence and signalling compounds. This behaviour is consistent with studies on wheat inoculated with PGPBs, which have also reported a progressive decrease in malate and aconitate levels in primed plants compared to controls (Mashabela et al. 2023). These results suggest that beneficial bacteria modulate biological nitrogen fixation and act as biostimulants, triggering a metabolic redirection that prepares the plant to respond more efficiently to future environmental challenges. The decrease in the levels of these organic acids may reflect their consumption in the biosynthesis of aromatic amino acids or phenolic compounds, as part of the induced systemic resistance mechanism, or as a carbon source for PGPBs. Thus, co-inoculation with *G. diazotrophicus* and *Azospirillum sp.* promotes plant growth and activates metabolic responses associated with defence, even in the absence of pathogens, reinforcing their potential as promising tools for more sustainable agriculture and reduced dependence on chemical inputs.

Sucrose is more than just a product of photosynthetic metabolism; it links carbon assimilation to plants' adaptive responses to the environment. The increased sucrose concentration in leaves may indicate an

overall improvement in photosynthesis (De Souza et al. 2008; Kumar et al. 2017) especially when associated with beneficial interactions with microorganisms. This improvement is indicative of metabolic health and represents a crucial energy base for activating defence mechanisms. There is a close interdependence between photosynthesis and resistance processes to biotic and abiotic stressors (Göhre 2015; Trotta et al. 2014; Wielkopolan et al. 2022) since the energy generated by carbon assimilation is necessary to sustain physiological, structural, and biochemical responses to herbivores and pathogens. Attacks by insects and phytopathogenic microorganisms can significantly reduce the plant's photosynthetic rate, compromising photoassimilate accumulation and growth maintenance (Kerchev et al. 2012). In this context, plants associated with PGPBs, such as those used in this study, may have an adaptive advantage against biotic stress, since the presence of these microorganisms is often associated with increased photosynthesis and, consequently, the sucrose concentration. This sugar is an energy reserve and plays a signalling role in various metabolic processes and in activating pathways associated with plant defence. Therefore, sucrose actively participates in modulating gene expression and directing metabolic flow through secondary pathways, including those involved in the synthesis of phenolic compounds, such as flavonoids and phenylpropanoids (Bolouri-Moghaddam et al. 2010).

Previous studies offer experimental support for this hypothesis. In strawberry, for example, sucrose treatment activated the pentose phosphate and shikimate pathways, which are both precursors of the phenylpropanoid pathway that culminate in the production of flavonoids with antioxidant and defensive functions (Li Dong et al. 2019). The shikimate pathway depends on precursors from carbohydrate metabolism, such as erythrose-4-phosphate and phosphoenolpyruvate, which are more readily available at high sucrose concentrations (Lyu et al. 2022; Wei et al. 2024). In *Arabidopsis thaliana*, sucrose has also been shown to activate the jasmonic acid signalling pathway, promoting phenol accumulation by inducing genes associated with secondary metabolism (Morkunas et al. 2011). These data suggest that rather than being merely a consequence of enhanced photosynthetic activity, increased sucrose may be a central trigger in the metabolic reconfiguration that underpins plant tolerance.

In sugarcane, this same pattern appears to be repeated. Plants colonised by *G. diazotrophicus* showed a positive correlation between the sucrose concentration and the activation of genes in the shikimate pathway, particularly phenylalanine ammonia-lyase (PAL), a key enzyme in the conversion of phenylalanine into phenolic acids, which are precursors of flavonoids (Nogueira et al., 2001). These compounds play essential roles in protection against herbivory, tissue healing, and regulation of oxidative stress, reinforcing the role of sucrose as a pivot between primary and secondary metabolism. In the present study, the higher sucrose accumulation in co-inoculated plants compared to the control may be associated with the coordinated induction of defence and signalling pathways, mediated by both energy-dependent pathways and hormonal networks.

A particularly relevant aspect is the role of sucrose in modulating the production of galic acid (GA), a phenolic compound with broad antioxidant and antimicrobial activity. The relationship between sucrose and GA is multifaceted and strongly depends on the tissue type, light availability, and sugar

concentration. In pomegranate callus cultures grown in the dark, high sucrose concentrations induced GA, ellagic acid, and punicalagin accumulation. Shikimate levels decreased, suggesting metabolic competition between the pathways that produce anthocyanins (shikimate-dependent) and hydrolysable tannins (GA derivatives) (Habashi et al. 2019). These data suggest that sucrose functions as a regulator of precursor allocation in the shikimate pathway, favouring the accumulation of GA derivatives under certain conditions. Similar results have been observed in other species. In potato, sucrose treatment resulted in a significant reduction in the content of chlorogenic acid (CGA), a derivative of caffeic acid. Gene expression analysis showed that this metabolic alteration may be associated with the induction of caffeoyl shikimate esterase (CSE) and the repression of hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT), redirecting shikimate flux (Yang et al. 2025). In *Melissa officinalis*, a sucrose concentration of 300 mM led to trans-caffeic acid accumulation, indicating that high sucrose levels may intensify the production of specific phenols of the phenylpropanoid pathway (Kim et al. 2011). These findings support that sucrose modulates the shikimate pathway's metabolic fate, directly influencing the phenolic profile of plants.

In the present study, the increased GA concentration observed in plants co-inoculated with diazotrophic bacteria may reflect sucrose-mediated metabolic reconfiguration. Considering the examples above, sucrose accumulation associated with bacterial colonisation may facilitate the channelling of shikimate precursors for the synthesis of GA and its derivatives, with a possible impact on biotic stress tolerance. By integrating physiological, metabolic, and transcriptomic evidence from the literature with experimental results, sucrose is proposed to play a central role in mediating plant–microorganism interactions, influencing both energy production and the profile of defence compounds.

In C4 plants, malate plays a crucial role in photosynthesis, serving as a CO_2 transporter between mesophyll and bundle sheath (BSH) cells. Initial CO_2 fixation by phosphoenolpyruvate carboxylase (PEP) generates oxaloacetate, which is converted to malate for transport to BSH, where malate decarboxylation releases CO_2 for the Calvin cycle and generates pyruvate, a precursor for alanine synthesis via alanine aminotransferase (ALT). This process integrates carbon and nitrogen metabolism, with alanine being synthesised from pyruvate and amino nitrogen (Heldt & Piechulla, 2011) microorganisms (Heldt and Piechulla 2011). The higher alanine concentration observed in inoculated plants suggests that microbial demand for malate alters plant metabolic flux. Rhizobacteria (Ikeda et al. 2013) and endophytic fungi (Zhang et al. 2017) remove malate, increasing PEP carboxylase activity and pyruvate production in plants, which is converted to alanine. Microorganisms, such as *Pseudomonas* and *Fusarium*, utilise malate exuded by roots, converting it to pyruvate via malate dehydrogenase, which can stimulate plants to enhance CO_2 fixation and increase carbon flux in the C4 cycle (Jha et al. 2012). Alanine synthesis is favoured by the greater availability of pyruvate and the additional supply of nitrogen in the form of ammonia (NH_3) released by microorganisms and incorporated into alanine via transamination. (Zhang et al. 2017) confirmed that endophytic fungi alter the amino acid profiles of plants, particularly alanine. In corn inoculated with *Azospirillum*, a 20–30% increase in the leaf alanine concentration was observed, associated with increased PEP carboxylase and ALT activity. Furthermore, alanine may play an important role in plant defence, participating in the biosynthesis of volatiles, such as

aldehydes, which act against herbivorous insects (Dudareva et al. 2013; Shi et al. 2019), and aliphatic glucosinolates, which are also important in defence against herbivores (Chhajed et al. 2020). The higher alanine concentration in pathogen-tolerant citrus varieties suggests its role in disease resistance (Killiny and Hijaz 2016). Similarly, (Cipriano et al. 2021) found that inoculation with PGPBs increased alanine and glycine contents and reduced proline and aspartate contents, suggesting a reduction in osmotic stress. These results indicate that bacterial co-inoculation favoured a more balanced physiological state in sugarcane plants, with a higher alanine concentration, possibly due to metabolic modulation by rhizosphere microorganisms. This increase can be explained by increased PEP carboxylase activity and pyruvate production, a direct precursor of alanine.

The increase in sucrose levels observed in plants co-inoculated with diazotrophic bacteria may be directly related to the induction of secondary metabolic pathways, such as the shikimate and phenylpropanoid pathways, culminating in the production of defence compounds, such as GA. This relationship has been described in different plant species. The shikimate pathway utilises intermediates derived from carbohydrate metabolism, such as erythrose-4-phosphate and phosphoenolpyruvate (Wei et al. 2024), which explains why an increased sucrose content favours this pathway (Lyu et al. 2022). Activation of the jasmonic acid pathway by sucrose has also been described in *Arabidopsis*, increasing phenol production via phenylpropanoids (Morkunas et al. 2011). In sugarcane, this mechanism explains the induction of genes related to the shikimate pathway, such as PAL, in plants colonised by *G. diazotrophicus* (Nogueira et al., 2001). These findings suggest that sucrose accumulation is a primary metabolic response and a signal that induces the biosynthesis of defence compounds. This connection is vital in the case of GA, a phenolic compound derived from benzoic acid whose biosynthesis occurs from 3-dehydroshikimic acid, an intermediate in the shikimate pathway. Previous studies have shown that GA plays a central role in protecting against abiotic stressors, such as salinity, heavy metals, cold, excess micronutrients, and radiation, acting in the elimination of reactive oxygen species and the modulation of osmotic and ionic homeostasis (Rahman et al. 2022; Saidi et al. 2021). In addition to its role in abiotic stress, GA and its derivatives exert antimicrobial and insecticidal functions, participating in defence against pathogens, such as *Alternaria solani* (El-Nagar et al. 2020), nematodes (Seo et al. 2013), and pests, such as *Ectropis obliqua*, *Spodoptera litura*, and *Aphis craccivora* (Ling et al. 2022; Punia et al. 2021; War et al. 2012; Zhang et al. 2022). For example, in mango fruit, GA conferred constitutive resistance to *Bactrocera dorsalis* infestation (Shivashankar and Sumathi 2022). These protective effects are often mediated by the conversion of GA to hydrolysable tannins (gallotannins), which reinforce the plants' physicochemical barriers against microorganisms and insects. Transcriptomic studies indicate that sugarcane colonisation by diazotrophic bacteria is associated with the induction of Expressed Sequence Tags (ESTs) encoding enzymes, such as chalcone synthase (initialising flavonoid biosynthesis) and the three initial enzymes of the phenylpropanoid pathway (PAL, C4H, and 4CL), supporting the hypothesis that these pathways are activated by sucrose and plant-microorganism signalling (Nogueira et al., 2001). Data from pomegranate callus cultures grown in the dark under high sucrose concentrations showed that GA, ellagic acid, and punicalagins accumulated significantly, whereas shikimate levels decreased (Habashi et al. 2019). This suggests a possible redistribution of the

shikimate pathway's metabolic flux toward the production of hydrolysable tannins (from GA) at the expense of anthocyanin production (which also requires shikimate), especially under certain physiological and environmental conditions. In potato, sucrose application reduced the content of CGA, another phenolic metabolite, possibly by inducing CSE expression and suppressing HQT (Yang et al. 2025). Thus, the high sucrose concentration in inoculated plants may act as a regulator of secondary metabolism. This could direct the flow of the shikimate pathway toward the synthesis of compounds, such as GA, which actively participate in plant adaptation to stress and defence against pathogens and herbivorous insects.

4.2 Experiments with and without choice

Enrichment analysis of the metabolite set revealed significant involvement of several metabolic pathways in the NMR-spectroscopy-identified discriminating compounds. The pathways enriched in the KEGG database included starch and sucrose metabolism, the citric acid (TCA) cycle, selenium compound metabolism, and pyruvate metabolism.

Starch and Sucrose Metabolism

The starch and sucrose metabolism pathway was the most significantly enriched, reflecting the central role of sucrose as a metabolic marker in the co-inoculated plants. The higher concentration of this sugar in co-inoculated plants indicates changes in energy flow and metabolic signalling in response to bacterial consortium co-inoculation, with implications for growth (Miret et al. 2024), energy metabolism, and photosynthesis (Wang & Wu, 2023). These observations corroborate previous studies linking sucrose to stress responses and plant–microorganism interactions.

Citric Acid Cycle and Pyruvate Metabolism

The enrichment of the citric acid (TCA) cycle and pyruvate metabolism indicates necessary alterations in central energy metabolism. In control plants, the higher concentrations of malic acid and aconitic acid—intermediates of the TCA cycle—suggest robust basal metabolic activity. This pattern is consistent with a physiological state of vegetative growth without stress, in which energy is primarily directed to mitochondrial respiration for ATP production, as described by (Nunes-Nesi et al. 2013; Zheng et al. 2025). In contrast, the significant reduction in these metabolites in co-inoculated plants suggests carbon flux redistribution. This may be a response to metabolic signalling induced by PGPBs, which divert central precursors, such as pyruvate, malic acid, and aconitic acid, to the biosynthesis of aromatic amino acids and phenolic compounds associated with plant defence and adaptation (Jakubowska et al. 2025; Kloepper et al. 2004). Similar results were reported by Mashabela et al., (2023), who found that inoculating wheat plants with PGPBs and infecting them with *Puccinia striiformis* decreased TCA cycle intermediates and increased secondary metabolites of the shikimic acid pathway, such as phenylalanine and tryptophan. This reinforces the role of bacterial co-inoculation in modulating plant metabolic architecture, redirecting carbon to pathways associated with resistance and physiological performance.

Alanine, Aspartate, and Glutamate Metabolism

The alanine, aspartate, and glutamate metabolism pathways were also enriched primarily due to the presence of alanine, which was found in higher concentrations in the co-inoculated plants. This modulation in amino acid metabolism may be a response to biotic stress or an induced metabolic adaptation, as alanine is a direct precursor of pyruvate and is involved in defence and signalling (Heinemann and Hildebrandt 2021; Hildebrandt et al. 2015; Xu and Fu 2022).

4.3 Feeding experiments with and without choice

In feeding experiments conducted with and without choice, we sought to investigate how the metabolic changes induced a diazotrophic bacterial consortium in sugarcane plants affect the feeding preferences and performance of specialist herbivore *D. saccharalis*. The no-choice experiment showed that the average weight of caterpillars fed on co-inoculated plants did not differ significantly from those fed on control plants, suggesting that co-inoculation with PGPBs did not affect the nutritional quality of plant tissue for insect development. Similar results were reported by Crawford et al., (2010), who observed that feeding *Spodoptera frugiperda* with inoculated *Lolium arundinaceum* did not alter caterpillar weight. (Clement et al. 2011) found no impact on the weight of *Oulema melanopus* fed on endophyte-inoculated plants. However, studies with other microorganisms have shown that PGPBs can control herbivorous insects. For example, Naeem et al. (2018) observed a significant reduction in aphid populations on wheat treated with PGPBs. (Pineda et al. 2013) showed positive effects on the performance of the generalist aphid *Myzus persicae* but not on the specialist *Brevicoryne brassicae* (Gadhave and Gange 2016).

In the choice experiment, *D. saccharalis* caterpillars preferred control plants over inoculated ones. Similarly, Li et al., (2015) observed a preference for non-inoculated Hosta plants by *S. frugiperda*. Crawford et al., (2010) reported that aphids more frequently chose non-inoculated grasses under field and laboratory conditions, and (Clement et al. 2011) found an average preference for non-inoculated plants in the herbivores they studied. These findings suggest that bacterial co-inoculation may alter plant chemical cues, such as the accumulation of defensive secondary metabolites and potentially the emission of volatile organic compounds (VOCs), thereby influencing herbivore host choice. It should be noted that VOCs were not directly measured in the present study, and their role in the observed repellence or non-preference remains a hypothesis based on existing literature. Future studies specifically characterizing the VOC profile of co-inoculated sugarcane plants via gas chromatography are required to validate this mechanism..

This discrepancy between feeding performance and preference highlights the complexity of plant–insect–microorganism interactions. Although the nutritional quality of co-inoculated plants remains suitable for larval development, the chemical changes induced by bacterial co-inoculation appear to act as indirect defences, modulating herbivore behaviour. This response is particularly relevant for specialist herbivores, such as *D. saccharalis*, whose host selection reflects fine-tuned adaptations to altered chemical signals. Therefore, our results contribute to understanding how co-inoculation with beneficial microorganisms supports plant growth and nutrition and while influencing ecological interactions

meaningful to integrated biological pest control, highlighting the multifunctional role of PGPBs in sustainable agriculture.

Embora essas descobertas forneçam valiosos *insights* mecanísticos sobre a defesa vegetal mediada por microrganismos, certas limitações experimentais devem ser consideradas. A alta concentração natural de carboidratos inerente ao tecido foliar da cana-de-açúcar gera sobreposição espectral no perfil de RMN de ^{1}H, o que pode mascarar metabólitos secundários de baixa abundância — limitação aqui mitigada por meio de experimentos de RMN 2D. Além disso, este estudo foi conduzido sob condições controladas de laboratório e casa de vegetação utilizando uma única cultivar comercial e um bioensaio alimentar de 5 dias. Embora esse delineamento controlado tenha sido essencial para isolar as respostas metabólicas e garantir a reprodutibilidade biológica, futuros ensaios de campo com múltiplos genótipos acompanhando todo o ciclo da cultura são necessários para validar a persistência e a viabilidade agronômica desse consórcio diazotrófico em programas de manejo integrado de pragas.

4.4 Final considerations

We demonstrated that NMR metabolomics efficiently detects metabolic variations from interactions between sugarcane anda diazotrophic bacterial consortium. These rhizobacteria alter plant primary and secondary metabolism, inducing the synthesis of sucrose, alanine, and GA, which are metabolites that improve photosynthetic and energetic processes and support plant defence against biotic stress. Considering our findings, we suggest that co-inoculating sugarcane plants alters the plant metabolome and provides better defence against *D*. *saccharalis*, as such plants were less preferred by *D*. *saccharalis* in choice feeding experiments. These plants also produced higher concentrations of defence-related compounds, such as GA. Our results contribute to the understanding of endophyte–plant interactions and demonstrate the viability of G. diazotrophicus and Azospirillum spp. as co-inoculant consortium to improve sugarcane productivity and defence.

5. CONCLUSION

The treatment of sugarcane with a diazotrophic bacterial consortium (*G. diazotrophicus* and *Azospirillum* sp.) caused significant changes in the behaviour of *D*. *saccharalis* and the plant metabolome, as shown by the choice-feeding experiment. Primary and secondary metabolism underwent modifications, with 29 metabolites identified across plants subjected to both treatments. Six metabolites were identified as discriminators between the two treatments, with three showing higher concentrations in the control plants and three in the co-inoculated plants. The presence of these metabolites was likely linked to photosynthetic processes and plant defences. These results are consistent with previous studies showing that changes in plant metabolic profiles and their effects on growth, development, and defence processes occur following co-inoculation with diazotrophic microorganisms.

Declarations

Author Contribution

D.J.A.O. A.E.G.S.; and H.F.G. conceptualization, T.F.L.R.; F.S.L,S.;and K.A.L.R.J. investigation, A.R.S.; C.I.M.A.F. and L.H.B.S. data curation and methodology, J.G.C.; D.J.A.O.; H.F.G. writing—original draft preparation, D.J.A.O. and A.E.G.S. writing—review and editing, A.E.G.S.; and H.F.G. supervision and funding acquisition.

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Table 1

Table 1 is available in the Supplementary Files section.

Figures

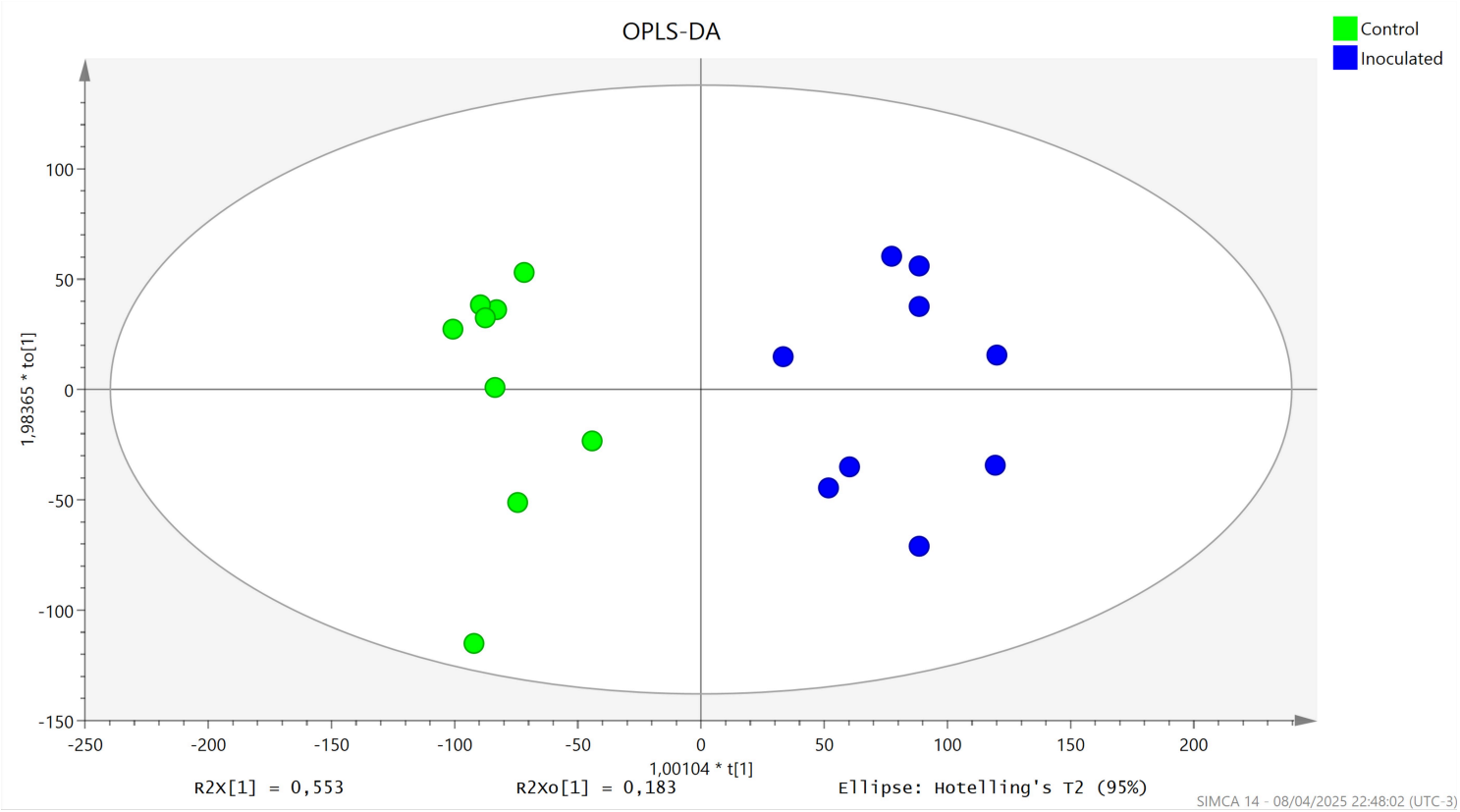


Figure 1

OPLS-DA model with two components (one predictive and one orthogonal component), $R^2 = 0.93$, $Q^2 = 0.82$.

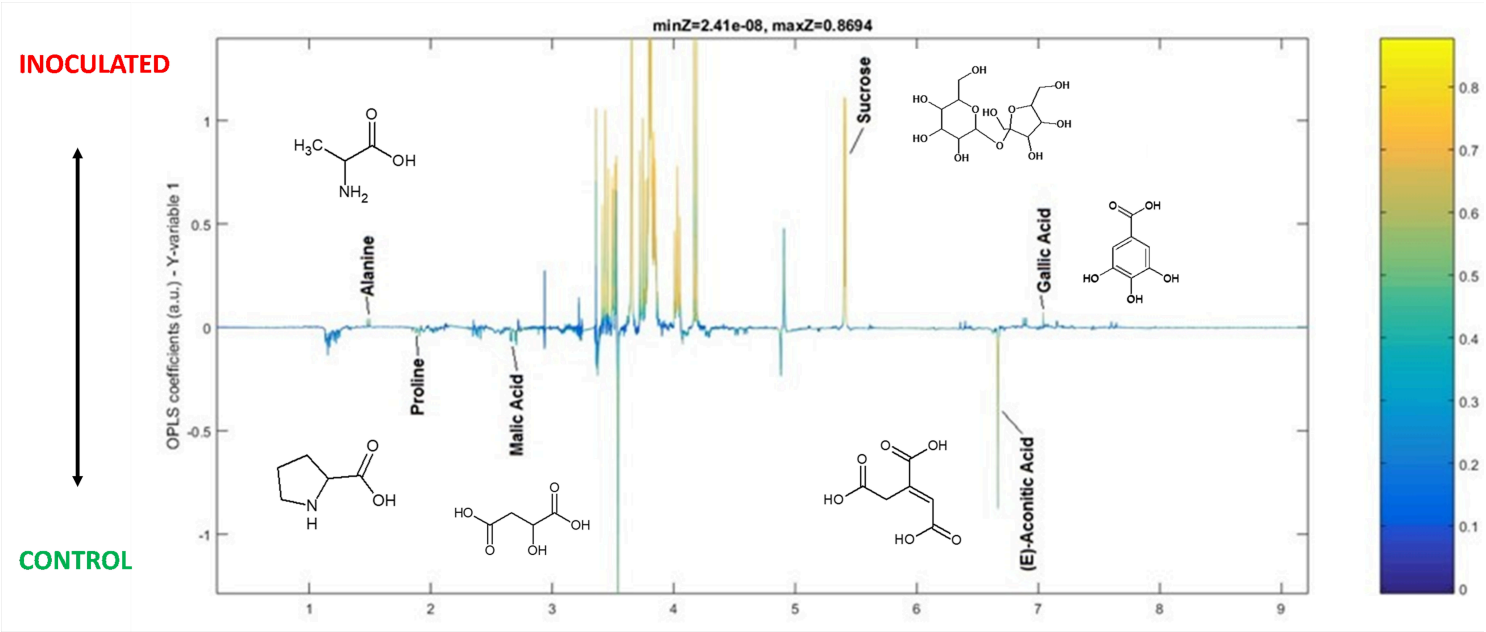


Figure 2

Orthogonal projections to latent structures-discriminant analysis (O-PLS-DA) loading S-line plot. Sugarcane leaf metabolites with higher abundance in plants inoculated with *Gluconacetobacter diazotrophicus* and Azospirillum sp (up) compared to control plants (down). The colour bar indicates the Pearson correlation coefficient (r) between the NMR spectral variables and the Y vector encoding class. OPLS-DA model with two components (one predictive and one orthogonal component), $R^2 = 0.93$, $Q^2 = 0.82$.

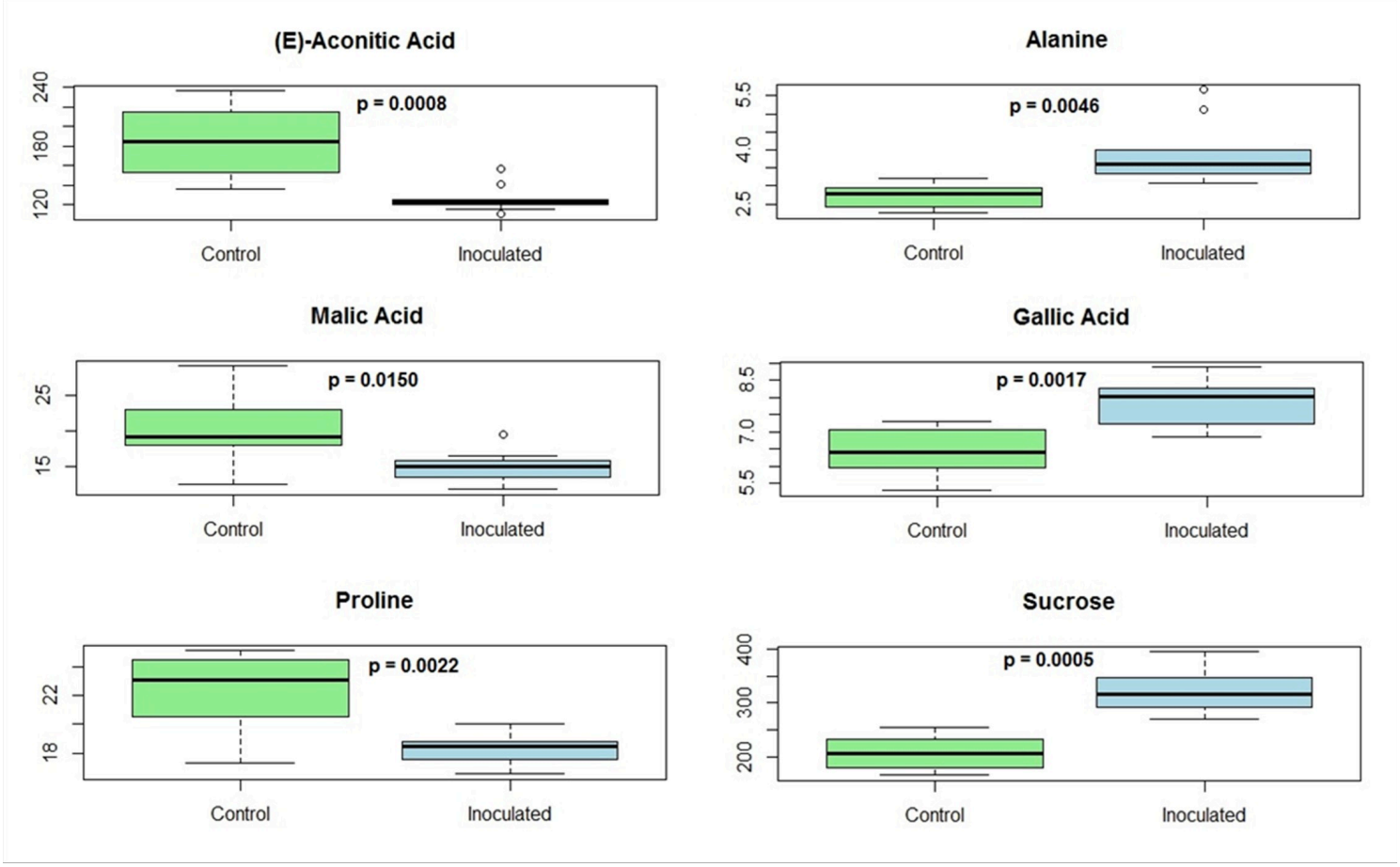


Figure 3

Boxplot of the relative concentrations of discriminating metabolites in response to sugarcane inoculation with PGPBs; the statistical significance was determined by a paired *t*-test, $p < 0.05$; empty circles represent extreme values

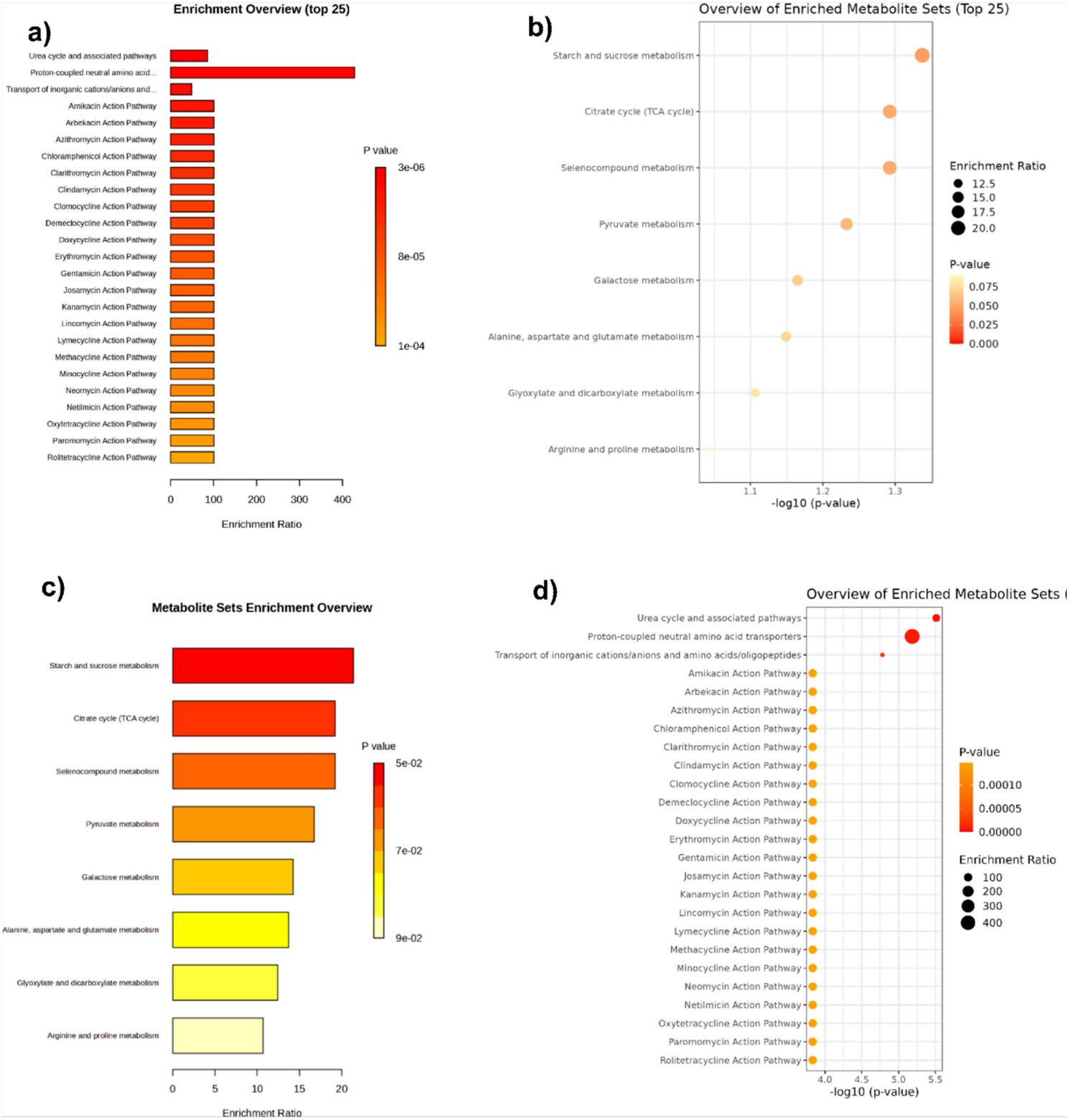


Figure 4

Differential enrichment pathways of soluble metabolites. a, b KEGG enriched pathways for differential metabolites in two pathways: citric acid cycle (TCA cycle) and pyruvate metabolism, c, d. RaMP-DB enrichment analysis revealed that the 6 differentially expressed compounds were significantly enriched in 25 pathways, predominantly those involved in the urea cycle and associated pathways, proton-coupled transport of neutral amino acids, and transport of cations, inorganic anions, and amino acids.

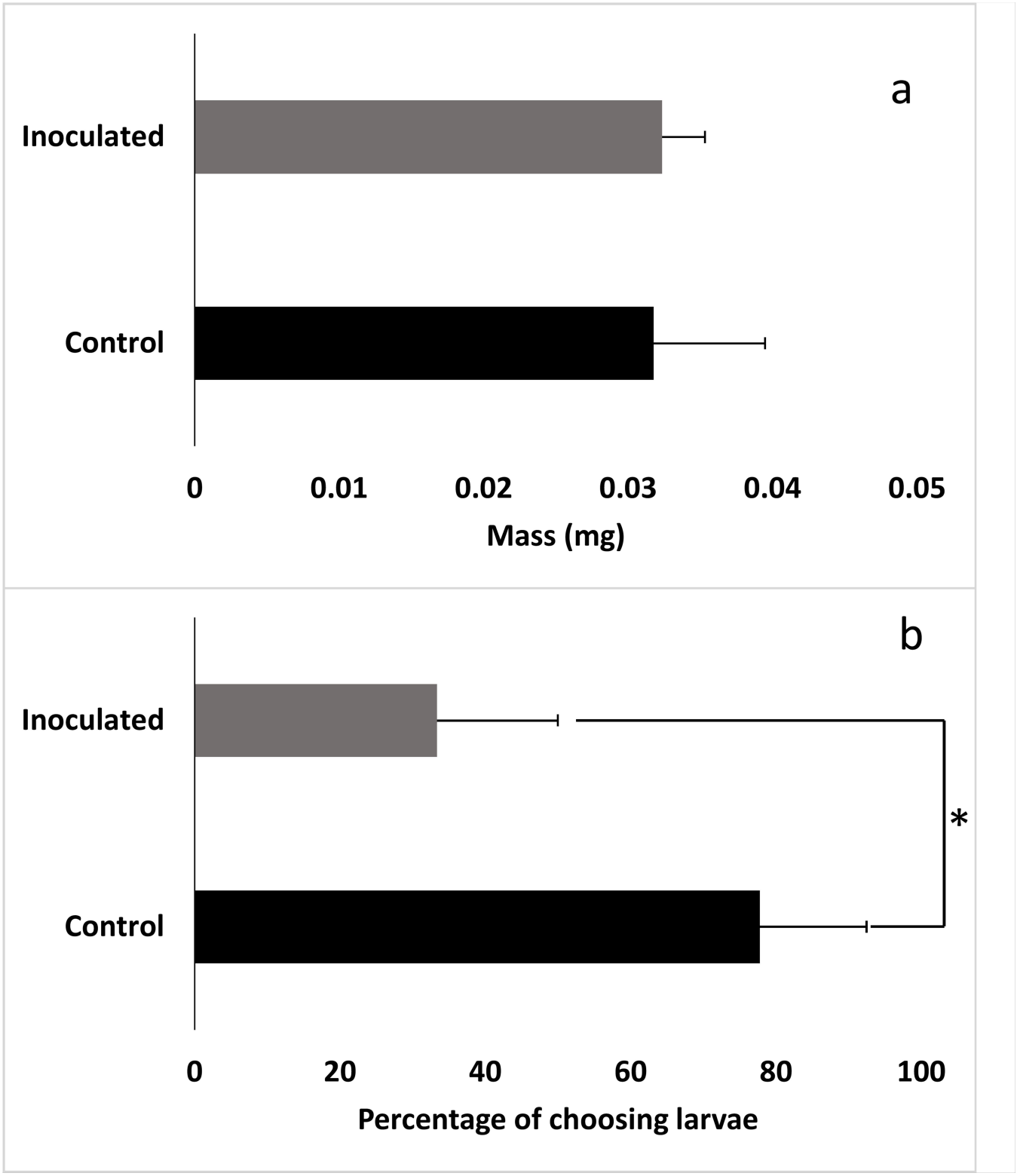


Figure 5

Behavioral and feeding responses of *D. saccharalis* larvae. (a) Weight gain of *D. saccharalis* caterpillars fed sugarcane co-inoculated with a diazotrophic bacterial consortium and control plants (Wilcoxon test, p= 0.20042). (b) Preference of *D. saccharalis* caterpillars between co-inoculated and control sugarcane plants in a dual-choice assay (Wilcoxon test, p = 0.03260). The numbers on the x-axis represent the mean choice percentage.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx