# Changes in metabolic profiling of sugarcane leaves induced by endophytic diazotrophic bacteria and humic acids

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Plant growth promoting bacteria (PGPB) and humic acids (HA) have been used as biostimulants in field conditions. The complete genomic and proteomic transcription of Herbaspirillum seropedicae and Gluconacetobacter diazotrophicus is available but interpreting and utilizing this information in the field to increase crop performance is challenging. The identification and characterization of metabolites that are induced by genomic changes may be used to improve plant responses to inoculation. The objective of this study was to describe changes in sugarcane metabolic profile that occur when HA and PGPB are used as biostimulants. Inoculum was applied to soil containing 45-day old sugarcane stalks. One week after inoculation, the methanolic extracts from leaves were obtained and analyzed by gas chromatography coupled to time-of-flight mass spectrometry; a total of 1,880 compounds were observed and 280 were identified in all samples. The application of HA significantly decreased the concentration of 15 metabolites, which generally included amino acids. HA increased the levels of 40 compounds, and these included metabolites linked to the stress response (shikimic, caffeic, hydroxycinnamic acids, putrescine, behenic acid, quinoline xylulose, galactose, lactose proline, oxyproline and valeric acid) and cellular growth (adenine and adenosine derivatives, ribose, ribonic acid and citric acid). Similarly, PGPB enhanced the level of metabolites identified in HA-treated soils; e.g. 48 metabolites were elevated and included amino acids, nucleic acids, organic acids, and lipids. Co-inoculation (HA+PGPB) boosted the level of 110 metabolites with respect to non-inoculated controls; these included amino acids, lipids and nitrogenous compounds. Changes in the metabolic profile induced by HA+PGPB influenced both glucose and pentose pathways and resulted in the accumulation of heptuloses and riboses, which are substrates in the nucleoside biosynthesis and shikimic acid pathways. The mevalonate pathway was also activated, thus increasing phytosterol synthesis. The improvement in cellular metabolism observed with PGPB+HA was compatible with high levels of vitamins. Glucuronate and amino sugars were

stimulated in addition to the products and intermediary compounds of tricarboxylic acid metabolism. Lipids and amino acids were the main compounds induced by co-inoculation in addition to antioxidants, stress-related metabolites, and compounds involved in cellular redox. The primary compounds observed in each treatment were identified, and the effect of co-inoculation (HA+PGPB) on metabolite levels was discussed.

1              Title

# **Changes in metabolic profiling of sugarcane leaves induced by endophytic diazotrophic bacteria and humic acids**

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13   **Running head:** Foliar metabolic changes induced by inoculation

14   **Highlights**

15   • Metabolic profile of sugarcane leaves was changed by inoculation

16   • Tricarboxylic acid, shikimic acid and mevalonate pathways were induced

17   • Compounds related to the plant stress response were detected

18   • Humic acids amplified the metabolic response induced by diazotrophic bacteria

19   **Abstract**

20        Plant growth promoting bacteria (PGPB) and humic acids (HA) have been used as

21   biostimulants in field conditions. The complete genomic and proteomic transcription of

22   *Herbaspirillum seropedicae* and *Gluconacetobacter diazotrophicus* is available but interpreting

23   and utilizing this information in the field to increase crop performance is challenging. The

24   identification and characterization of metabolites that are induced by genomic changes may be

25   used to improve plant responses to inoculation. The objective of this study was to describe

26   changes in sugarcane metabolic profile that occur when HA and PGPB are used as biostimulants.

27   Inoculum was applied to soil containing 45-day old sugarcane stalks. One week after inoculation,

28   the methanolic extracts from leaves were obtained and analyzed by gas chromatography coupled

29   to time-of-flight mass spectrometry; a total of 1,880 compounds were observed and 280 were

30   identified in all samples. The application of HA significantly decreased the concentration of 15

31   metabolites, which generally included amino acids. HA increased the levels of 40 compounds,

32   and these included metabolites linked to the stress response (shikimic, caffeic, hydroxycinnamic

33   acids, putrescine, behenic acid, quinoline xylulose, galactose, lactose proline, oxyproline and

34   valeric acid) and cellular growth (adenine and adenosine derivatives, ribose, ribonic acid and

35   citric acid). Similarly, PGPB enhanced the level of metabolites identified in HA-treated soils;

36   e.g. 48 metabolites were elevated and included amino acids, nucleic acids, organic acids, and

37   lipids. Co-inoculation (HA+PGPB) boosted the level of 110 metabolites with respect to non-

38   inoculated controls; these included amino acids, lipids and nitrogenous compounds. Changes in

39   the metabolic profile induced by HA+PGPB influenced both glucose and pentose pathways and

40   resulted in the accumulation of heptuloses and riboses, which are substrates in the nucleoside

41   biosynthesis and shikimic acid pathways. The mevalonate pathway was also activated, thus

42   increasing phytosterol synthesis. The improvement in cellular metabolism observed with

43   PGPB+HA was compatible with high levels of vitamins. Glucuronate and amino sugars were

44   stimulated in addition to the products and intermediary compounds of tricarboxylic acid

45   metabolism. Lipids and amino acids were the main compounds induced by co-inoculation in

46   addition to antioxidants, stress-related metabolites, and compounds involved in cellular redox.

47   The primary compounds observed in each treatment were identified, and the effect of co-

48   inoculation (HA+PGPB) on metabolite levels was discussed.

49    **1. Introduction**

50    Plant inoculation technology is a secure and environmentally-friendly technology that
51    saves approximately 3 billion dollars per year in Brazil by decreasing the application of nitrogen
52    fertilizers in soybean and others leguminous plants (Alves et al., 2003). This technology has
53    considerable potential for non-leguminous plants (Reis et al., 2008). Current scientific efforts are
54    focused on the isolation and selection of plant-beneficial microorganisms and their application in
55    soil-plant systems in controlled conditions (Vassilev et al. 2015).

56    Despite the genomic sequencing efforts of *Herbaspirillum seropedicae* and
57    *Gluconacetobacter diazotrophicus* (Bertalan et al., 2009; Pedrosa et al., 2011), the field
58    responses of sugarcane inoculation with plant growth promoting bacteria (PGPB) are far from
59    those results obtained in experimental conditions (Oliveira et al., 2009; Carvalho et al., 2014;
60    Schultz et al., 2014). Da Silva et al. (2017) applied a suspension containing humic acids (HA)
61    and PGPB on sugarcane foliage and verified a 37% increase in productivity (26 tons ha$^{-1}$) during
62    the first year as compared to the control. The positive growth promotion in sugarcane co-
63    inoculated with HA and *H. seropedicae* persisted for three years (sugarcane plant and two
64    ratoons). Furthermore, an experiment utilizing large pots containing inoculated sugarcane
65    (emulating commercial plantations) showed larger yields than non-inoculated plants for three
66    consecutive years, further proving the benefits of co-inoculating with HA and PGPB.

67    Field efficiency is a key aspect for bioinoculant acceptance and wider agricultural use
68    (Bhattacharyya and Jha, 2012; Owen et al., 2015). Olivares et al. (2017) reviewed the use of HA
69    and PGPB on non-leguminous plants and showed different morphological adaptations and
70    physiological changes induced by co-inoculation. The study of plant metabolite levels has
71    contributed significantly to our understanding of plant physiology, particularly from the
72    viewpoint of small chemical molecules that reflect the end point of biological pathways (Hong et
73    al., 2016). Changes in the metabolic profile of plants treated with inoculants may further
74    decrease the gap between field and laboratory results. Furthermore, the recognition of cell
75    metabolites altered by inoculation is fundamental for the identification of specific molecular
76    targets. Therefore, the aim of this work was to characterize the changes in the metabolic profile
77    of sugarcane co-inoculated with HA and *H. seropedicae* and *G. diazotrophicus*.

78    **2. Materials and methods**

79    Humic acids (HA) and plant growth promoting bacteria (PGPB)

80         Humic substances were extracted and purified as reported elsewhere (Aguiar et al.,
81    2013). Briefly, 10 volumes of 0.5 mol L$^{-1}$ NaOH were mixed with one volume of earthworm
82    compost, under a N$_2$ atmosphere and shaken overnight. After 12 h, the suspension was
83    centrifuged at 5,000 $g$ and acidified to a pH of 1.5 using 6 mol L$^{-1}$ HCl. The HAs were
84    solubilized in 0.5 mol L$^{-1}$ NaOH and precipitated three times. The sample was repeatedly washed
85    with water until a negative test against AgNO$_3$. The HAs were titrated to a pH of 7.0 with 0.1
86    mol L$^{-1}$ KOH then dialyzed against deionized water using a 1 kD cutoff membrane (Thomas
87    Scientific, Swedesboro, NJ) before they were lyophilized.

88         Mixed inoculum containing *Gluconacetobacter diazotrophicus* strain PAL5 and
89    *Herbaspirillum seropedicae* strain HRC54 was used in this experiment, both originally isolated
90    from sugarcane in Brazil and characterized as endophytic diazotrophs (Cavalcante and
91    Dobereiner, 1988; Olivares et al., 1996), and being part of the five-species inoculant
92    recommended for sugarcane in Brazil (Schultz et al., 2016). For pre-inoculum preparation, both
93    bacteria species were grown in 5mL liquid DIGYS medium at 30 °C for 36 hours at 140 rpm in
94    rotatory shaker (Baldani et al., 2014). Later, 50 µL of each bacterial species was inoculated in
95    separated in 500 mL erlenmeyer containing freshly liquid DIGYS at the same growing condition
96    for 48 hours. Bacteria biomass produced was centrifuged at 5.000 g for 10 minutes and
97    resuspended in sterilized water, being adjusted for a cell density of 10$^8$ cells. mL$^{-1}$ that
98    correspond to O.D. of 1.0 at 440 ηm. The inoculant was prepared by diluting 100 mL of each
99    bacterial suspension in 800 mL of distilled water (bacteria treatment) or in 800 mL humic acid
100   solution at pH 6.5 (bacteria + humic acid treatment), as well 200 mL of distilled water plus 800
101   mL of humic acid solution (humic acid treatment) and 1000 ml of distilled water (control
102   treatment).

103   Plant assay

104        Before the planting, we perform the heat treatment and cane stalks were immersed water
105   (50º C) by 2 h. The sugarcane cultivar RB 96 7515 was used for pot experiments. We use the
106   superficial layer (0–20 cm) of a typical soil used to sugarcane plantation in Rio de Janeiro state
107   and classified as Inceptisol. The soil chemical properties were analyzed according to Embrapa
108   methods described in Claessen et al., (1997): pH (H$_2$O) = 5.5; Al= 3 mmol$_c$ dm$^{-3}$ (titration

109  against NaOH); Ca= 7 mmol$_c$ dm$^{-3}$ and Mg= 4 mmol$_c$ dm$^{-3}$ (titration against EDTA); P= 4.0 mg

110  dm$^{-3}$ (Mehlich 1 extraction and colorimetric determination); K= 33 mg dm$^{-3}$ (Mehlich 1

111  extraction and flame photometric determination). The randomized complete block design

112  (RCBD) was used as experimental design with four treatments and three replicates. The

113  treatments consisted of a final bacteria suspension at 4 x10$^8$ cell mL$^{-1}$, humic substances at 50

114  mg C L$^{-1}$ or its combination which were applied at the same time directly in the soil at 45 days

115  after germination leaf bud at rate of 400 mL per pot. Control treatment was performed applying

116  water at the same volume. The plants were irrigated to keep soil moisture at field capacity. The

117  leaves were collected after 7 days of treatments application to metabolomic profile analyze.

118  Sugarcane sample processing and metabolite extraction

119      Sugarcane leaves were collected under controlled conditions, ground in the N$_2$

120  homogenizer and stored at -80º C. Metabolites were extracted using 20 mg of fresh weight

121  sample adding 1 mL of pre-chilled extraction solution (80:20 v/v solvent mixture of

122  methanol/water). The samples were vortex for 10s and shake for 6 minutes followed

123  by centrifugation for 10 min at 10,000 $g$. We remove the whole supernatant in two 500 µL

124  portions, saving one as a backup. One portion was dried and submitted to derivatization.

125  GC–TOF mass spectrometry

126      Derivatization was performed as described previously (Fiehn et al., 2008). In summary,

127  2 µL of a C8-C30 FAME mixture was used to convert retention times to retention index (RI).

128  Carbonyl groups were protected by 10 µL of a solution of 20 mg mL$^{-1}$ methoxyamine in pyridine

129  at 30º C for 90 min. Ninety microliters of MSTFA and 1% TMCS was added for

130  trimethylsilylation of acidic protons at 37º C for 30 min. After derivatization 0.5 µL samples

131  were injected in randomized sequence into a Gerstel cold injection system (Gerstel, Muehlheim,

132  Germany) and Agilent 7890A gas chromatograph (Santa Clara, CA) in split less mode. The

133  system was controlled by the Leco ChromaTOF software versus 2.32 (St. Joseph, MI). A 30 m

134  long, 0.25 mm i.d. RTX 5Sil-MS column with 0.25 lm 5% diphenyl/ 95% dimethyl polysiloxane

135  film and additional 10 m integrated guard column was used (Restek, Bellefonte, PA). Injection

136  temperature was 230º C, the interface was set to 280º C. Helium flow was 1 mL min$^{-1}$. After a 5

137  min solvent delay time at 50º C, oven temperature was increased at 20º C min$^{-1}$ to 330º C, 5 min

138   isocratic, cool down to 50º C and additional 5 min delay, afterwards. Liners were exchanged

139   automatically every 10 samples. A Leco Pegasus IV time-of-flight mass spectrometer was

140   operated at a transfer line temperature of 280º C, ion source was adjusted at 250º C and -70 V

141   electron impact ionization. Mass spectra were acquired at mass resolving power R = 600 from

142   m/z 85 to 500 at 17 spectra $s^{-1}$. The results files were exported to a data server with absolute

143   spectra intensities and further processed by a filtering algorithm implemented in the

144   metabolomics Bin-Base database (Fiehn et al., 2005).

145        Quantification was reported as peak height using the unique ion as default, unless a

146   different quantification ion was manually set in the BinBase administration software

147   Bellerophon. Metabolites were unambiguously assigned by the Bin-Base identifier numbers,

148   using retention index and mass spectrum as the two most important identification criteria. All

149   database entries in BinBase were matched against the Fiehn mass spectral library of 1,200

150   authentic metabolite spectra and the NIST05 commercial library (Kind et al., 2009). A

151   quantification report table was produced for all database entries that were positively detected in

152   more than 50% of the samples of a study design class as defined in the SetupX database (Scholz

153   and Fiehn, 2007).

154

155   Multivariate analysis

156        The metabolite data were then normalized based on the cell dry weight in each sample.

157   The resulting data sets, which comprised 12 samples and 285 variables, were imported into The

158   Unscramble software (version 10.1, Camo Co., Norway) for multivariate statistical and analysis

159   (Fiehn et al., 2008; Lee and Fiehn, 2008) and univariate analysis (Anova and Dunnet mean test

160   against the control).

161   **3.  Results**

162        We found 1,885 different compounds in sugarcane leaves extracts and 280 were found in

163   all samples and with accurate identification according similarity to chemical standard of Fiehn

164   library used in this work (Lee and Fiehn, 2008). The identified compounds were mainly of

165   carbohydrates (30%), organic acids (16%), lipids (17%), amino acids (9%), aromatics (11%),

166   others nitrogenous compounds besides amino acids (12%) and other compounds (2%). Identified

167  compounds were sorted according its chemical classification. The values represent percentage

168  difference of the concentration in relation to the control according to the significance analysis of

169  the variance (Table 1-6). In general, humic acid (HA treatment) induced decreased amount of

170  larger number of compounds in respect to control, except for aromatics compounds. For bacteria

171  inoculation (PGPB treatment), remarkable increase of amino acids metabolism and nitrogenous

172  biosynthesis related to control plants were observed. Combined application (HA + PGPB

173  treatment) displayed the highest number of compounds related to the control (Figure 1).

174  Hereafter, we expose the main results according the biochemical category of leaves metabolites.

175  *Carbohydrates (Table 1)*

176      All treatments induce the concentration of these sugars: glucose, glucose-1-P, galactose,

177  levanbiose, lactobionic acid, mannose, hexopyranose, deoxyerythriol and sialicin (amino sugar).

178  In the HA treatments we found nine carbohydrates that have its concentration reduced in respect

179  to control. With the inoculation this number was reduced to six and PGPB+HA to zero. The

180  main compounds decreased by HA were: 1,5 anhydrous gluciol (-80%), leucrose (-66%), two

181  generic disaccharides described by numbers 200391 and 553367 in the Fiehn library (-36 and -

182  52%, respectively), melibiose (-61%) and fucose (-60%). sucrose-6-phosphate (-42%). The sugar

183  decreased by inoculation were 2-ketogulonic acid, the carbohydrate 208845 and the raffinose (-

184  50%). The carbohydrates founded in larger concentration with HA treatment were raffinose and

185  ribose (548%), lactose (+510%), xylulose (+458%), salicin (+211%), among others with less

186  increase. The sugars enhanced by bacteria inoculation were erythrol lactone, gluco heptulose,

187  melibiose, 1-deoxy pentiol and two disaccharides (267647 and 285065). The PGPB+HA enhance

188  the concentration of ribito (+825%), isomaltose (+586%), melezitose (+533%), glucose-6-

189  phosphate (+413%), sophorose (+297%), glycerol-gluco heptose (+258%), enolpyruvate

190  (+246%), tagalose (+220%), hexose amino-2-deoxy (192%), erythrol (+169%), trehalose

191  (+133%), galactose-6-P (+107%) and fucose (+44%). It was notable the increase of melibiose

192  with inoculation enhancing from 943% to 4143% when the inoculation was done in the presence

193  of HA.

194  *Amino acids (Table 2)*

195     Twenty-six amino acids were identified, and the HA reduce the concentration of two

196     including asparagine (-85%) and glutamine (-72%) and enhance significantly lysine (+306%), 5-

197     aminovaleric acid (+211%), isoleucine (+45%), proline (+278%) and oxoproline (+26%). The

198     PGPB and PGPB+HA enhanced the amino acid metabolism increasing mostly including aspartic

199     acid (from 122 to 629%), asparagine (177 to 937%), glutamic acid (254%), glutamine (1038% to

200     595%), homoserine (216 to 640%), isoleucine (88 to 135%), N-methyl alanine (100%), ornithine

201     (247% to 425%), hydroxyproline (94 to 369%), proline (1031 to 1155%), oxoproline (192 to

202     320%), citrulline (480 and 446%), lysine (429% to 677%) and methionine (208 to 264%).

203     *Organic acids (table 3)*

204     All treatments induced the concentration of following acids: citric, alpha ketoglutaric,

205     maleic, ribonic, oxalic, 2-deoxytetronic, lactic and idonic acid. The HA reduce the concentration

206     of four organic acids (pyruvic acid -61%; itaconic -50%, mucic acid -47% and succinic acid, -

207     23%) and enhance the concentration of saccharic acid and isocitric lactone; PGPB decrease the

208     concentration of only succinic acid (-27%) and no organic acids significantly decreased in

209     respect to control in HA+PGPB treatment. The inoculation enhanced the acids 2-

210     hydroxyglutaric, aminomalonic, gluconic acid lactone, isothreonic, itaconic, mannonic, mucic

211     and pantothenic. The acids pyruvic (29%), fumaric acid (126%), 2-oxogluconic acid (730%),

212     hexuronic acid (305%), ascorbic acid (368%), 3-hydroxypropionic acid (87%), 2-hydroxyadipic

213     acid (292%) were found in higher concentration in respect to control plants in the PGPB+HA

214     treatment.

215     *Nitrogenous compounds (Table 4)*

216     The nucleosides were the most nitrogenous compounds other than amino acids produce in

217     large amount. Adenine was induced by all treatments and as also others nitrogenous compounds

218     like ethanolamine, putrescine and N-acetyl-D-hexosamine. The HA treatments reduce the

219     concentration of thymide (-56%) while enhance 4-hydroxyquinoline-2-carboxylic acid (+650%),

220     1,3-diaminopropane (+131%), 2-deoxyadenosine (+736%). The PGPB enhance 2-

221     deoxyguanosine (+1179%), adenine (+3817%), guanine (+777%) and cytosin (368%),

222     butirolactan (+800 %), isonicotic acid (+260 %), thymidine (+125 %), thymine (216%) and

223    uracil (+213 %), while PGPB+HA induce the carnitine (+384%), maleimide (+527%), orotic

224    acid (+214%), spermidine (+749%), and 5'-deoxy-5'-methylthioadenosine (+157%).

225    *Lipids (Table 5)*

226         Four lipids were inhibited by HA in respect to control including the fatty acids palmitic,

227    2-monoolein and 6-hydroxycaproate and the plant sterol phytol while monoolein was inhibit by

228    PGPB+HA. The 2-monoolein, lauric, methlylhexadecanoic and perlagonic acids and β-

229    sistosterol were found in larger concentration in all treatments. The 6-hydroxycaproate dimer,

230    dihydrosphingosine, 2-monoolein and palmitic acid were enhanced by inoculation while

231    monoolein, behenic acid, eicosanoic and capric acid and cholesterol by HA. The following lipids

232    were found in larger concentration only in PGPB+HA treatment: 2-monopalmiitn,1-

233    monopalmiteolin, cerotic acid, linoleic mand linolenic acid, myristic acid, palmitoleic acid,

234    pimelic acid, and the plant sterols phytol and the terpene squalene.

235    *Aromatic compounds (Table 6)*

236         Two aromatics compounds decrease its concentration (pipecoluc acid and coniferin) due

237    HA treatment while PGPB+HA reduce the level of 4-hydroxycinnamic acid. The concentration

238    of shikimic acid, caffeic acid, 3,4-hydroxybenzoic acid and 2,3-dihydroxybenzoic acid were

239    enhanced by all treatments. The inoculation induces the following compounds: chlorogenic,

240    pipecolic and phtahlic acid while HA enhance the concentration of arbutin and 4-

241    hydroxycinnamic acid. The PGPB+HA enhance the concentration of parabanic acid, 4-

242    hydroxybenzoate, 4-hydroxybenzaldehyde and coniferin.

243    *Multivariate analysis*

244         The Figure 2 shown the linear combination of original variables. The two first principal

245    components captured 95% of total variance. As the principal components are orthogonal, it was

246    possible to observe the relationship among the treatments and variables by the scores and loading

247    graphics (Figure 2). The first principal component captured 73% of total variance and separated

248    PGPB+HA from other treatments while PC2 captured 22% of total variance and grouped HA and

249    PGPB+HA treatments. The main compounds responsible for segregate PGPB+HA from others

250   in PC1 were those from lipids metabolism while the changes on shikimic pathway put together

251   HA and HA+PGPB treatments in PC2 axis.

252   **4. Discussion**

253        The CG-MS TOF is one of most powerful tools for metabolomics approach and allows

254   the compounds identification about five-fold than sugarcane metabolomics previously reported

255   by Glassop et al., (2007). The effect of treatment on sugarcane leaf metabolites concentration

256   will be discussed considering the main biochemical class of compounds and then an integrate

257   view is presented (Figure 3).

258        The glucose and glucose-1-P concentrations increase in response of inoculation in

259   comparison with controls plant. Glucose-1-P is a key intermediate in several major carbon

260   anabolic fluxes, such as sucrose, starch and cellulose biosynthesis while Glucose-6-P was found

261   in higher concentration only in PGPB+HA showing effect on oxidative pentose phosphate cycle.

262   Therefore, we observed enhance of erythritol in PGBP+HA treatment. Erythritol is an important

263   nutrient for $N_2$-fixing plant endosymbionts and larger concentration of fructose-1-P is compatible

264   with the erythritol biosynthesis (Barbier et al., 2014). Lactobionic acid, a disaccharide formed

265   from gluconic acid and galactose was induced by inoculation and may be formed also by

266   oxidation of lactose. Galactose is a member of the raffinose family of oligosaccharides and is the

267   major carbohydrate translocated in many plants. High free concentration of galactose is toxic in

268   leaves reducing cell wall loosening (Cheung and Cleland, 1991). The inhibition of β-

269   galactosidases release galactose from cell wall polysaccharides and could occurs as growth

270   response induced by auxins (Thorpe et al., 1999). It is well documented that HA and PGPB have

271   auxin-like activity. It was also found high level of lactose and lactobionic acid and this former

272   compound were associate to the metabolic signature related to high plant growth rate in

273   *Arabidopsis* by Meyer et al., (2007).

274        The effect of inoculation on plant carbohydrates was accentuated. In Gram negative

275   bacteria, the inner core oligosaccharide typically contains residues of heptose (Kosma, 2009).

276   The role of oligoliposaccharides in bacteria-host interaction is well known and described

277   (Nwodo et al., 2012) and we found a huge increase of glycerol gluco heptose in plants treated

278   with PGPB+HA. Other characteristic from the intensification of carbohydrate metabolism by

279    inoculation was the increase on aditols concentration in respect to control-plants (e.g., glucicol,

280    ribitol, desoxypentiol, erythritol, Table 1). The alditols are products of photosynthesis and are

281    more reduced their corresponding sugars revealing metabolic boost induced by inoculation.

282    Other intense change on carbohydrate profile includes the production of osmoregulators

283    compounds and sugars linked to cell wall rigidity. Melibiose, galactose, salicin and trehalose

284    were previously described that their concentration enhances under abiotic or biotic stress (Peña

285    et al., 2004). The larger concentration of mannose is compatible with high levels of galactose

286    (Herold and Lewis, 1977). All treatments showed larger levels of salicin or 2-(hydroxymethyl)

287    phenyl-$O$-β-D-glucopyranoside, one phenolic glycoside precursor of salicylic acid that have

288    important roles in the ecological survival of plants against biotic and abiotic stress (Mahdi,

289    2014). Finally, it was founded higher levels of hexosamines a precursor of the synthesis of

290    several lipids. It is not surprising, therefore, to find changes on lipids profile induced by

291    inoculation. In addition, as nitrogen metabolism is closely linked to carbohydrates synthesis, the

292    amino acid profile was dramatically changed by the treatments compared with control. The

293    general effect of HA was the decrease in the concentration of most of amino acids in respect to

294    control (Figure 1). On the other hand, the cell concentration of acid amino acids was increased

295    by PGPB including glutamine, asparagine and aspartic acid, the most abundant amino acids in

296    leaves. The accurate determination of amino acids concentrations in the metabolome studies is a

297    tricky question due massive number of compounds and some chemical transformation that may

298    occur during the derivatization process. For instance, glutamine and glutamate can be partially

299    converted to pyroglutamate during derivatization procedures as demonstrated by Kanani and

300    Klapa (2007) and Purwaha et al., (2014). However, we use in this work the data correction for

301    such biases changes developed by platform of primary metabolism identification from Genome

302    Center (UC-Davis, USA) which considers these possible changes (Niehaus et al., 2017).

303        Interestingly, an increase in alpha ketoglutarate derived from the citric acid cycle was

304    noticed in all bacteria treatments that provides carbon skeleton for ammonium assimilation and

305    biosynthesis of amino acids through the GS/GOGAT nitrogen assimilation pathway (EC 6.3.1.2

306    and EC 1.4.1.14, respectively). GS catalyzes the ATP-dependent assimilation of $NH_4^+$ into

307    glutamine, using glutamate as a substrate, and it functions in a cycle with GOGAT

308    (glutamine-2-oxoglutarate aminotransferase), which catalyzes the reductive transfer of the amide

309    group from glutamine to α-ketoglutarate (2-oxoglutarate), forming two molecules of glutamate

310    (Richards et al., 2010). Modulation of GS activity in sugarcane leaves have been demonstrated

311    by inoculation with diazotrophic bacteria and explain partially nitrogen fixation abilities

312    associated with sugarcane genotypes (de Matos Nogueira et al., 2005). In addition, changes in

313    amino acid pools were previous demonstrated on sugarcane plants inoculated with diazotrophic

314    bacteria that modulates nitrogen fixation and ammonium release by the bacteria (Loiret et al.,

315    2009).

316        The proline and oxoproline concentration was larger in inoculate treatments and the role

317    of these amino acids in cell osmoregulation is well documented. The biotic stress induced by

318    inoculation can promote a general plant response that includes increase of 5-hydroxy norvaline

319    production and citrulline (Yokota et al., 2002). A general view, plant inoculation sparks amino

320    acids production especially that related to urea cycle (ornithine cycle). The 5-aminovaleric acid,

321    isoleucine and proline were induced by HA. In plants, stress initiates a signal-transduction

322    pathway, in which increased cytosolic $Ca^{2+}$ activates $Ca^{2+}$/calmodulin-dependent glutamate

323    decarboxylase activity and GABA synthesis. Elevated $H^+$ and substrate levels can also stimulate

324    glutamate decarboxylase activity. GABA accumulation probably is mediated primarily by

325    glutamate decarboxylase (Shelp et al., 1999). Ramos et al., (2015) describe a mechanism

326    involved $Ca^{2+}$ pulse uptake by plants stimulated by HA and describe a dynamic interaction

327    between $Ca^{2+}$/$H^+$ efflux-influx as well as the induction on CDPK (calcium dependent

328    phosphokinase) activity and $Ca^{2+}$ channels. Furthermore, sugarcane transcriptomic study showed

329    that protein kinases are main genetic response against drought and $N_2$-fixing inoculation (Rocha

330    et al., 2007). It is possible conclude based in metabolic profile analysis that the treatments

331    improve the mechanism of adaptation based on ornithine cycle.

332        The inoculation enhances the synthesis of acidic amino acids and consequently it was

333    possible to observe a significant enhance on nucleoside concentration (Table 2 and 4). The

334    interdependence of threonine, methionine and isoleucine metabolism in plants is well described

335    Joshi et al., (2010). Isoleucine is synthesized from threonine and methionine, which are derived

336    from aspartate via enzymes located in the plastids and homoserine kinase (EC 2.7.1.39), which

337    catalyzes the formation of O-phosphohomoserine from homoserine, leads to the formation of

338    either threonine or methionine (Hildebrandt et al., 2015). PGPB and PGPB+HA enhance both

339    the homoserine and isoleucine concentration.

340    The basic components of the citric acid cycle have their concentration enhanced by the
341    treatments including oxalic, citric and α-ketoglutaric acid while succinic and fumaric acids
342    decreased in respect to control (Table 3). High amount of α-keto glutaric acid results in higher
343    concentration of glutaric acid and the glutamate/glutamine synthesis. This is coherent with
344    increase of ornithine, citrulline and proline and oxoproline production in plants inoculated.
345    Another important evidence is the higher concentration of polyamines a direct product from
346    ornithine transformation. Several intermediary products of TCA pathways were accumulated in
347    higher concentration including dihydroxymalonic acid, isocitric lactone, 2-oxogluconic acid,
348    succinate semialdehyde. Ascorbic acid was found in high concentration. Ascorbate is a major
349    metabolite in plants. It is an antioxidant and, in association with other components of the
350    antioxidant system, protects plants against oxidative damage resulting from aerobic metabolism,
351    photosynthesis and a range of pollutants (Colville and Smirnoff, 2008). This increase is
352    compatible with enhance of glucoronate metabolism observed (Table 3) and galactonic,
353    gluconic, gluconic acid lactone, glutaric and glycolic acid concentration since a biosynthetic
354    pathway of ascorbic acid via GDP-mannose, GDP-L-galactose, L-galactose, and L-galactono-
355    1,4-lactone has been proposed (Smirnoff, 1996). Pantothenic acid also was induced by
356    inoculation and is a water-soluble vitamin (vitamin B5) essential for the synthesis of CoA and
357    ACP, cofactor in energy yielding reactions including carbohydrate metabolism and fatty acid
358    synthesis (Coxon et al., 2005).

359    Nucleoside and nucleoside derivatives (cytosine, guanine, adenine and thymine)
360    participate in the bioenergetics process (ATP) and macromolecules synthesis including
361    polysaccharides, phospholipids and glycolipids. The nucleosides synthesis was stimulated by
362    treatments (Table 4). The growth cell acceleration must be preceded by carbohydrates
363    metabolism and by the units necessary to build cell nuclei. The enhance of inosine concentration
364    was observed in HA treatment. This compound is linked to cell growth activation. Furthermore,
365    it was previously observed enhance of root growth of different plants with exogenous use of
366    inosine (Tokuhisa et al., 2010). The HA like cytokinin activity was previously described (Mora
367    et al., 2012) and the precursors of cytokinin was increased by HA treatment (3-hidroxyimethyl
368    glutaric acid, oxoadenosine and inosine). Ethanolamine is a serine derivative (Rontein et al.,

369    2001) and substrate to choline synthesis and phosphatydiletanolamine and phosphadidilcoline the
370    main lipids in plant cell membranes.

371          The lipids metabolism was strongly affected by PGPB and responsible for the main
372    differentiation among the other treatments according PCA analysis (Figure 2, Table 5). The
373    cytosine and 2-deoxyguanosine were huge increased with PGPB inoculation. The butyrolactam
374    is synthesized from GABA cyclization by GABA-cysteine hydrogenase. This pathway may have
375    been active by PGPB due the presence of high concentration of oxoproline. This amino acid is
376    synthesized using glutamine-cysteine by cyclization catalyzed by (glutamiltransferase, EC
377    2.3.2.4) producing cysteine and oxoproline. This pathway was previously described in rice as
378    response to *Xanthomonas* infection (Sana et al., 2010). In the treatment with PGPB+HA one
379    imide (maleimide) was found in high concentration and it is a well-known cytotoxic compound.
380    The main effect of maleimide is block the vesicular transport mainly galactose transport
381    (Uemura et al., 2004). Spermidine and putrescine were found in high concentration with PGPB
382    and PGPB+HA and are involved in cell growth inducing root growth and normally detected in
383    high concentration in active tissue under stress (Takahashi and Kakehi, 2009). Previous report
384    compares the action of HA with polyamines (Young and Chen, 1997; Mora et al., 2012). Larger
385    concentration of isonicotinic acid was found in PGPB+HA treatment as well as the orotic acid a
386    precursor for the synthesis of water soluble B13 vitamin. The vitamins have important role in
387    cell redox status and are enzymatic cofactor of metabolic reaction (Asensi-Fabado and Munné-
388    Bosch, 2010).

389          The enhance of a range of cytosolic metabolites including glucose 6-P, malate
390    derivatives, phosphoenolpyruvate and pyruvate support high rates of fatty acid synthesis by
391    inoculated plants (Pleite et al., 2005). The long chain fatty acids as docosanoic acid (behenic
392    acid), 2-monolein and hexadecanol were found in high concentration in HA treated plants. These
393    long chain lipids are involved in membrane structure and dynamics regulating cell size but also
394    the division and differentiation process (Zheng et al., 2005). Sphingolipids were found in larger
395    concentration in HA and HA+PGPB plants. Elevated levels of complex sphingolipids were
396    associated with cell apoptosis, terminal differentiation, or cell cycle arrest (Du Granrut and
397    Cacas, 2016). In another way the decrease on sphingolipids levels can lead to cell
398    proliferation increase (Bach and Faure, 2010).

399    Sterols are synthesized by the mevalonate pathway and was possible to observe enhance of
400    sterols concentration induced by treatments in respect to control. β-sitosterol is the main plant
401    sterols involved into cellulose elongation chain and its increase were observed in HA and
402    HA+PGPB treatments. Tocopherol is a precursor of main lipids soluble vitamin (vitamin E) and
403    its concentration is regulated by biotic and abiotic stress (Munne-Bosch, 2005). The cholesterol
404    concentration is often low in cell plants but the GC-MS TOF was sensible to detect significant
405    variation in its concentration according the treatments. Phytol was abundant in HA+PGPB
406    treatment and is the main precursor to brassinosteroids synthesis. Other lipid in abundance in
407    HA+PGPB was the terpene squalene ($C_{30}H_{50}$) precursor from major plant sterols (Amarowicz,
408    2009). The azelaic acid is a dicarboxylic acid with nine carbons and in the plant synthesis the
409    even-numbered chains are privileged due acetyl-coA mechanism reactions. This fatty acid was
410    present only in PGPB and HA+PGPB treatments. Previously it was found enhance in azelaic
411    acid concentration in Arabidopsis infected by *Pseudomonas syringe* starting the plant defense
412    against pathogen by activation of salicylic acid pathway (Jung et al., 2009).

413    In plants, the biosynthesis of isoprene units and common precursors for isoprenoid
414    biosynthesis involves two distinct pathways. According (Hemmerlin et al., 2006), the
415    mevalonate (MVA) is utilized for the biosynthesis of non-plastidial isoprenoids (phytosterols,
416    prenylated proteins, sesquiterpenoids), whereas plastidial isoprenoids (carotenoids,
417    plastoquinone, diterpenes, monoterpenes, etc.) are synthesized via the alternative 2C-methyl-D-
418    erythritol 4-phosphate (MEP) pathway. This pathway uses 1-Deoxy-D-xylulose 5-phosphate
419    synthase (DXS) and thiamine to produce isoprenoids. We identify a significant enhance in the
420    xylulose (+1263%) as well in the thymine (+4970%) concentration in a clear indicative of
421    isoprenoids pathways stimulation by PGPB. Isoprenoids, represent the chemically and
422    functionally most diversified class compounds including electron carriers (quinones), membrane
423    constituents (sterols), vitamins (A, D, E and K), plant hormones (side chain of cytokinin, abscisic
424    acid, gibberellins and brassinosteroids), and photosynthetic pigments (chlorophyll, phytol and
425    carotenoids) (Hemmerlin et al., 2006). In fact, we observe an increase of *p*-hydroquinone, several
426    sterols (β-sitosterol) phytol, tocopherol, stigmasterol, cholesterol) cytosine, inosine and squalene
427    in sugarcane treated with PGPB+HA. Furthermore, the accumulation of methylerytrol not
428    discharge the enhance of isoprenoids products using a non-mevalonate pathway (MEP/DOXP)

429  that use 2-methyl-D-erythritol 4-phosphate (MEP) and deoxy-xylulose 5 phosphate (DOXP)

430  rote.

431     The shikimic pathway was also staggeringly changed by inoculation. Phenylpropanoids

432  are a class of phenylalanine derivatives with a basic C6-C3 (phenyl-propane) skeleton (Iriti and

433  Faoro, 2009). In turn, the essential amino acid phenylalanine arises from the shikimate pathway,

434  as well as the other aromatic amino acids tyrosine and tryptophan. Precursors of this pathway are

435  phosphoenolpyruvate, from glycolysis, and erythrose 4-phosphate from pentose phosphate (Tzin

436  and Galili, 2010). Both compounds were its concentration enhanced by the treatments.

437  Enolpiruvate enhance 22%, 85% and 246% with HA, PGPB and PGPB+HA treatment,

438  respectively in respect to control while several derivatives of erytrose were found (erythritol,

439  erythronic acid lactone, erythrose) in increased amount in inoculated plants. Than we expected

440  an influence on phenylpropanoids compounds and it was confirmed by influence on multivariate

441  analysis (PCA2, Figure 3). The first step to shikimic branch pathway is the accumulation of

442  aromatic amino acids. It was also observed an increase of phenylalanine concentration and

443  consequently the production of hydroxyl cinnamic derivatives. The main aromatic compounds

444  induced by PGPB are derivatives of phenyl propanoids units as ferulic acids. The enhance of

445  concentration on aromatics compound is often associated to improve redox status and

446  antioxidative cell protection. Phenylalanine (tyrosine) ammonia-lyase (PAL/TAL; EC 4.3.1.5)

447  catalyzes the first committed step in the biosynthesis of phenolics by converting phenylalanine to

448  trans-cinnamic acid and tyrosine to p-coumaric acid. Phenylalanine usually is the preferred

449  substrate, but the monocot enzyme can use both phenylalanine and tyrosine. (Schiavon et al.,

450  2010) observed enhance of PAL/TAL expression in maize treated with HA and therein the

451  concentration of total phenolic compounds. Here we provide an extensive list of aromatic

452  compounds induce by HA. However, as the enhanced promoted in aromatic amino acids

453  phenylalanine and tyrosine by PGPB and PGPB+HA was larger is expected increase on aromatic

454  moieties. Actually, a number of hydroxybenzoic acid were found including a direct derivative of

455  salicylic acids like gentisic or quinic acids (caffeic acids) both involved in plant disease

456  resistance. The improvement on the aromatic compounds synthesis induced by endophytic

457  bacteria inoculation was previously related in sugarcane (França et al., 2001).

458  **Conclusion**

459    Here we describe the changes in the metabolite profile in sugarcane inoculated with
460    PGPB and HA. The glycolysis pathway was activated with high production of glycolysis and
461    derivatives providing substrate to pentose pathway and tricarboxylic acid cycle. The erithroses
462    and heptuloses provide substrate do xylose and ribose synthesis and the nucleotides and acid
463    shikimic acids was improved as well as the mevalonate pathway with enhance of phytosterols.
464    The sugar aminoacids were found in larger concentration as well as sphingolipids. Tricarboxylic
465    acid cycle improvement also provides sources for lipids and amino acid metabolism the main
466    routes changed by inoculation. The cell growth acceleration was compatible with high
467    nucleosides synthesis as well as water and lipid soluble vitamins. In general, a several
468    compounds that as an anti-oxidative agent and osmoprotectors were produced in inoculated
469    plants showing a rapid metabolic response to infection. Particularly, the treatment with HA
470    decrease the number of compounds with high cellular levels. This is compatible with previous
471    proteome (Carletti et al., 2008) and transcriptome (Trevisan et al., 2011) studies that reported
472    inhibition of differential expression for both protein and genes in response of HA. The HA
473    increase the metabolic response of inoculation with PGPB. The levels of glucuronic acids and
474    threonic acids increased when plant was inoculated by PGPB+HA. Finally, analytical results
475    obtained using GC-TOF MS clearly demonstrated the enhance of TCA activity showing
476    accumulation of α-Ketoglutaric acid a central metabolic substrate to glutamine and glutamic acid
477    synthesis precursor of amino acids ornithine, citrulline, proline, oxoproline, hydroxynorvaline
478    and the polyamines (spermidine and putrescine). The level of fatty acids as strongly modified by
479    PGPB+HA.

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722  **Figure 1** – Schematic representation of the effect of the treatments on the levels of amino acids

723  in respect to control. Rectangles containing aminoacids pools that derivates from pathways

724  conversions of 3-phosphoglycerate (PGA), P-enolpyruvate, pyruvate., oxaloacetate and α-

725  ketoglutarate. Aminoacids with significant higher values related to control had shown color

726  circles. The color circles represent the treatments (HA: black; PGPB: blue, HA+PGPB: red) and

727  the magnitude of influence of each treatment in respect to control is represented by the circle

728  size.

729  **Figure 2** – PCA scores for CG-MS data indicating good separation of treatments in different

730  groups according to inoculation. The loadings and their coefficient weight that indicates the

731  position of the variables along the PCs and consequently their importance for that PC are showed

732  in supplementary file.

733  **Figure 3** - Representation of the most important metabolites identified on main sugarcane

734  biochemical pathways. The color squares represent the treatments (HA: black; PGPB: blue,

735  HA+PGPB: red) and the increase of concentration level (% in respect to control) was ranking

736  according to the color-coded scale bar pointed that range from 100 to 500 x times concentration

737  related to control plants.

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Carbohydrates identified on sugarcane methanolic leaves extracts by GC-MS TOF

The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

**Table 1.** Carbohydrates identified on sugarcane methanolic leaves extracts by GC-MS TOF. The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

| metabolite | HA | PGPB | PGPB+HA |
|---|---|---|---|
| 1,5-anhydroglucitol | -80** | -13* | 42** |
| 199177 carbohydrate | 58 ns | 110 ns | 187 ns |
| 1-deoxyerythritol | 104** | 1664** | 1231** |
| 1-desoxypentitol | -28 ns | 320** | 897** |
| 1-kestose | 79 ns | 25 ns | 74 ns |
| 200391 disaccharide | -36** | 8 ns | 81** |
| 200509 carbohydrate | 92** | -4 ns | 54** |
| 200658 carbohydrate | -19 ns | 28 ns | 346** |
| 201952 carbohydrate | 46 ns | 24 ns | 271** |
| 202573 carbohydrate | 18 ns | -1 ns | 871** |
| 202832 disaccharide | -13 ns | 21 ns | 49** |
| 208658 carbohydrate | 19 ns | 175 ns | 779 ns |
| 208845 carbohydrate | -19** | -27** | -48** |
| 214402 carbohydrate | -11 ns | 202 ns | 120 ns |
| 225906 disaccharide | 33 ns | 16 ns | 83 ns |
| 231180 carbohydrate | 61 ns | 29 ns | 240* |
| 231210 disaccharide | 64 ns | 79 ns | 182 ns |
| 233289 carbohydrate | -19 ns | 188 ns | 911** |
| 238267 trisaccharide | 14 ns | -5 ns | -44 ns |
| 267647 disaccharide | -8 ns | 671** | 2169** |
| 285065 disaccharide | -25 ns | 74** | 59** |
| 288331 trisaccharide | 55 ns | 44 ns | 100 ns |
| 288365 trisaccharide | 12 ns | 81 ns | 168 ns |
| 2-deoxyribose | 39 ns | 35 ns | 112 ns |
| 2-ketoglucose dimethylacetal | -11* | 59** | -4 ns |
| 2-ketogulonic acid | -32 ns | -84* | -78* |
| 304945 carbohydrate | 58** | 66** | 15 ns |
| 328803 carbohydrate | 14 ns | 15 ns | 79** |
| 400671 carbohydrate | 71** | -2 ns | 50 |
| 424905 carbohydrate | -46 ns | -69* | 179** |
| 506414 carbohydrate | 49** | 184 ns | 323** |
| 553367 disaccharide | -52** | -37** | 43** |
| arabitol | -22 ns | 65 ns | 147 ns |
| beta-glycerolphosphate | 17 ns | 40 ns | 146 ns |
| beta-hexopyranose 1,6-anhydro | 122 ns | 90 ns | 157* |
| **Table 1** (continuation) | | | |

| | | | |
|---|---|---|---|
| digitoxose | -25 ns | -47 ns | -35 ns |
| enolpyruvate | 22 ns | 85 ns | 246* |
| erythritol | -2 ns | 132 ns | 169* |
| erythronic acid lactone | -18 ns | 154** | 50** |
| erythrose | -30 ns | 35 ns | 65 ns |
| fructose 1 phosphate | 101 ns | 257 ns | 738** |
| fructose-6-phosphate | -17 ns | 36 ns | 75 ns |
| fucose | -51** | 0 ns | 44** |
| galactose | 160** | 975** | 1876** |
| galactose-6-phosphate 1 | -5 ns | 27 ns | 107** |
| glucoheptose | -7 ns | -5 ns | 107 ns |
| glucoheptulose | 7 ns | 152* | 263** |
| glucose | 85** | 173** | 299** |
| glucose-1-phosphate | 38* | 57** | 116** |
| glucose-6-phosphate 2 | -21 ns | 129 ns | 413* |
| glycero-guloheptose | -13 ns | -36 ns | 7891 ns |
| hexose amino-2-deoxy | -16 ns | 83 ns | 192** |
| inulotriose | 120 ns | -6 ns | 36 ns |
| isomaltose | -77 ns | 95 ns | 586** |
| lactobionic acid | 110** | 152** | 247** |
| lactose | 510** | 168 ns | 359** |
| leucrose | -66** | -30 ns | -56** |
| levanbiose | 107** | 90** | 116** |
| levoglucosan | -22 ns | 8 ns | 7 ns |
| maltose | 132* | -16 ns | 12 ns |
| maltotriose | 9 ns | 20 ns | 76 ns |
| mannose | 148** | 22** | 429** |
| melezitose | 188 ns | 134 ns | 422 ns |
| melibiose | -62** | 943** | 4143** |
| methylhexose | 45** | 2 ns | 252** |
| N-acetyl-D-hexosamine | 43** | 37** | 73** |
| pentitol | 29 ns | 49 ns | 80 ns |
| propane-1,3-diol | -8 ns | 2 ns | 15 ns |
| raffinose | 162** | -50** | 16 ns |
| ribitol | 96 ns | 47 ns | 825** |
| ribonic acid gamma-lactone | -6 ns | -14 ns | 92 ns |
| ribose | 548** | 71 ns | 385** |
| salicin | 211* | 336** | 1782** |
| **Table 1** (continuation) | | | |
| sialicin | 179 ns | -18 ns | 98 ns |
| sophorose | 49 ns | 127 ns | 297** |

| | | | |
|---|---|---|---|
| sucrose-6-phosphate | -42 ns | 79 ns | 185 ns |
| tagatose | -24 ns | 22 ns | 175 ns |
| threitol | -15 ns | 111 ns | 418 ns |
| trehalose | -33 ns | -17 ns | 133** |
| furanose | 54 ns | 21 ns | 164 ns |
| xylitol | 91** | 155 ** | 146** |
| xylonolactone | 41 ns | 32 ns | 71 ns |
| xylulose | 458** | 228 ns | 1264** |

ns: no significant; * $p < 0.05$; ** $p < 0.01$

Amino acids identified on sugarcane methanolic leaves extracts by GC-MS TOF

The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

**Table 2.** Amino acids identified on sugarcane methanolic leaves extracts by GC-MS TOF. The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

| metabolite | HA | PGPB | PGPB+HA |
|---|---|---|---|
| 3-aminoisobutyric acid | 12 ns | 1 ns | -20 ns |
| 4-hydroxyproline | 1 ns | 94** | 369** |
| 5-aminovaleric acid | 212** | 24 ns | 64 ns |
| 5-hydroxynorvaline | -26 ns | 154** | 52 ns |
| asparagine | -85** | 177 ns | 937 ns |
| aspartic acid | 16 ns | 122** | 629** |
| β-alanine | 15 ns | -21 ns | 0 ns |
| citrulline | -18 ns | 359* | 276 ns |
| cyano-L-alanine | -3 ns | 155 ns | 186 ns |
| glutamic acid | 14 ns | 254** | 250** |
| glutamine | -72 ns | 1038** | 595** |
| glycine | -14** | 0 ns | 107** |
| homoserine | -38 ns | 216* | 640** |
| isoleucine | 45** | 88** | 135** |
| lysine | 202 ns | 220 ns | 830 ns |
| methionine | -42 ns | 161 ns | 199 ns |
| N-acetylornithine | 17 ns | 95 ns | 237 ns |
| N-hexanoylglycine | 184 ns | 404 ns | 646 ns |
| N-methylalanine | -40 ns | 101** | 99** |
| O-acetylserine | 16 ns | 10 ns | 94 ns |
| oxoproline | 26** | 192** | 320** |
| phenylalanine | 126 ns | 1004** | 819** |
| proline | 278** | 1031** | 1155** |
| serine | -3 ns | 253** | 81** |
| threonine | 34 ns | 414** | 101** |
| thymine | -15 ns | 216** | 367** |

ns: no significant; * $p <0.05$; ** $p <0.01$

Organic acids identified on sugarcane methanolic leaves extracts by GC-MS TOF.

The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

**Table 3.** Organic acids identified on sugarcane methanolic leaves extracts by GC-MS TOF. The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

| metabolite | HA | PGPB | PGPB+HA |
|---|---|---|---|
| 2,3-dihydroxybutanoic acid | 30 ns | 31 ns | 66 ns |
| 2,5-furandicarboxylic acid | 8 ns | 19 ns | 85 ns |
| 2-deoxyribonic acid | -11 ns | -11 ns | 28 ns |
| 2-deoxytetronic acid | 95 ns | 95 ns | 100 ns |
| 2-hydroxyadipic acid | 68 ns | 7 ns | 185 ns |
| 2-hydroxyglutaric acid | 262 ns | 77 ns | 57 ns |
| 2-ketoadipic acid | -37 ns | 8 ns | -36 ns |
| 2-oxogluconic acid | 65 ns | 264 ns | 730** |
| 3-hydroxy-3-methylglutaric acid | -23 ns | -4 ns | 5 ns |
| 3-hydroxypropionic acid | 27 ns | 31 ns | 87** |
| 4-hydroxybutyric acid | 27 ns | 25 ns | 50 ns |
| 5-hydroxymethyl-2-furoic acid | -12 ns | -3 ns | 33 ns |
| adipic acid | 8 ns | 8 ns | 26 ns |
| alpha ketoglutaric acid | 7* | 22* | 83** |
| aminomalonic acid | 36 ns | 116 ns | 83 ns |
| ascorbic acid | 114 ns | 155 ns | 368** |
| citramalic acid | -8 ns | -27 ns | -49 ns |
| citric acid | 98** | 416** | 748** |
| dihydroxymalonic acid | -72 ns | 0 ns | 298** |
| fumaric acid | 2 ns | -47 ns | 126** |
| galactonic acid | -39 ns | 34 ns | 37 ns |
| gluconic acid | 4 ns | 34 ns | 77 ns |
| gluconic acid lactone | 105 ns | 330** | 412** |
| glutaric acid | 12 ns | 179 ns | 184 ns |
| glycolic acid | -16 ns | 1 ns | 19 ns |
| hexuronic acid | -9 ns | 85 ns | 305* |
| idonic acid | 150* | 150* | 268** |
| isocitric lactone | 145** | 10 ns | 140** |
| isothreonic acid | 30 ns | 103** | 211** |
| itaconic acid | -50** | 31** | 28** |
| lactic acid | 76** | 22** | 43** |
| maleic acid | 305** | 263** | 122** |
| mannonic acid | 27 ns | 160 ns | 173 ns |
| methylmaleic acid | -11 ns | 44 ns | 60 ns |
| mucic acid | -47** | 127** | 408** |
| **Table 3 -** continuation | | | |
| **metabolite** | **HA** | **PGPB** | **PGPB+HA** |

| | | | |
|---|---|---|---|
| oxalic acid | 745** | 18** | 259** |
| pantothenic acid | 11 ns | 81** | 307** |
| propane-1,2,3-tricarboxylate /carballylic acid | 80 ns | 55 ns | 174 ns |
| pyruvic acid | -61** | 0 ns | 29** |
| ribonic acid | 33* | 46** | 155** |
| saccharic acid | 133** | -13 ns | 235** |
| salicylic acid | -26 ns | 44 ns | 72* |
| succinate semialdehyde/4-oxobutanoic acid | -4 ns | 31 ns | 48 ns |
| succinic acid | -23* | -27** | -15 ns |

ns: no significant; * $p < 0.05$; ** $p < 0.01$

Nitrogenous (other than amino acids) identified on sugarcane methanolic leaves extracts by GC-MS TOF.

The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

**Table 4.** Nitrogenous (other than amino acids) identified on sugarcane methanolic leaves extracts by GC-MS TOF. The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

| Nitrogenous (other than amino acids) | HA | PGPB | PGPB+HA |
|---|---|---|---|
| 1,3-diaminopropane | 131* | 47 ns | 196** |
| 2-deoxyadenosine | 736* | -14 ns | 65** |
| 2'-deoxyguanosine | 108 ns | 1179** | 997** |
| 3,6-dihydro-3,6-dimethyl-2,5-bishydroxypyrazine | 4 ns | -13 ns | -1 ns |
| 3-hydroxypyridine | 20 ns | 175 ns | 356** |
| 4-hydroxyquinoline-2-carboxylic acid | 650** | -40 ns | 255** |
| 5'-deoxy-5'-methylthioadenosine | 4 ns | 56 ns | 157** |
| 5-methylcytosine | 31 ns | 349 ns | 503* |
| 6-hydroxynicotinic acid | 35 ns | 26 ns | 21 ns |
| adenine | 377** | 3817** | 4971** |
| adenosine | -77** | 226** | 116** |
| biuret | -4 ns | 72 ns | 94 ns |
| butyrolactam | -4 ns | 800** | 708** |
| carnitine | 10 ns | -10 ns | 384** |
| cytidine | 15 ns | 19 ns | 151 ns |
| cytosin | 5 ns | 368* | 517** |
| ethanolamine | 192** | 86** | 94** |
| guanine | -50 ns | 777 ns | 2119* |
| guanosine | 14 ns | 1 ns | 19 ns |
| inosine | 52 ns | -19 ns | 12 ns |
| isonicotinic acid | 34 ns | 260** | 544** |
| maleimide | -16 ns | 60 ns | 527** |
| nicotinamide | 34 ns | 17 ns | 57 ns |
| orotic acid | 19 ns | 47 ns | 214** |
| putrescine | 75** | 123** | 405** |
| pyrrole-2-carboxylic acid | 25 ns | 30 ns | 108 ns |
| spermidine 2 | 4 ns | 106 ns | 749* |
| taurine | 16 | -3 | 21 |
| thymidine | -56** | 125** | 247** |
| uracil | -30 ns | 213** | 81** |
| xanthine | -9 ns | -25 ns | 114 ns |
| xanthosine | 11 ns | -21 ns | -5 ns |
| xanthurenic acid | 7 ns | 6 ns | 63 ns |

ns: no significant; * $p < 0.05$; ** $p < 0.01$

Lipids identified on sugarcane methanolic leaves extracts by GC-MS TOF.

The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

**Table 5.** Lipids identified on sugarcane methanolic leaves extracts by GC-MS TOF. The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

| metabolite | HA | PGPB | PGPB+HA |
|---|---|---|---|
| 1-hexadecanol | 52 ns | 17 ns | 136 ns |
| 1-monopalmiteolin | 9 ns | -5 ns | 123 ns |
| 1-monopalmitin | 2 ns | 30 ns | 120 ns |
| 223494 fatty acid methyl ester | 21 ns | -16 ns | 54 ns |
| 2-hydroxyvaleric acid | -28 ns | 7 ns | 10 ns |
| 2-monoolein | -59** | 25* | 43** |
| 2-monopalmitin | 113 ns | -35 ns | 357** |
| 2-monostearin | -4 ns | 3 ns | 168* |
| 2-palmitoleic acid | -22 ns | -20 ns | 133 ns |
| 6-hydroxycaproate dimer | -82** | 103** | 134** |
| 6-hydroxycaproic acid | 49 ns | 70 ns | 99 ns |
| arachidic acid | 9 ns | -28 ns | 4 ns |
| azelaic acid | -36 ns | 14 ns | 105 ns |
| behenic acid | 193** | -36 ns | 439** |
| beta-sitosterol | 460** | 191** | 765** |
| caffeic acid | 28 ns | 37 ns | 65 ns |
| capric acid | 60** | 12 ns | 47* |
| cerotic acid | 8 ns | 46 ns | 464** |
| cholesterol | 222* | -7 ns | 342** |
| cis-4-decene-1,10-dioic acid | 142* | 106 ns | 429** |
| dihydrosphingosine | 47 ns | 210* | 692** |
| dodecane | 54 ns | 12 ns | 11 ns |
| dodecanol | 8 ns | 22 ns | 7 ns |
| eicosenoic acid | 167** | -37 ns | 417** |
| gamma-tocopherol | 14 ns | -1 ns | 123* |
| isopalmitic acid | 18 ns | 43 ns | 55 ns |
| lauric acid | 179** | 165** | 202** |
| lignoceric acid | -13 ns | -61** | 61** |
| linoleic acid | 2 ns | 76** | 286** |
| linoleic acid methyl ester | 8 ns | 42 ns | 99** |
| linolenic acid | -6 ns | 51 ns | 597** |
| methylhexadecanoic acid | 34* | 364** | 579** |
| monoolein | 43** | -7* | -41** |
| montanic acid | -3 ns | -19 ns | 15 ns |
| myristic acid | 100 ns | 20 ns | 175* |
| octadecanol | -11 ns | 0 ns | -8 ns |
| **Table 5** (continuation) | | | |

| metabolite | HA | PGPB | PGPB+HA |
|---|---|---|---|
| oleic acid | -8 ns | 22 ns | 92 ns |
| palmitic acid | -11** | 9** | 32** |
| palmitoleic acid | 41 ns | 71 ns | 297** |
| pelargonic acid | 38** | 117** | 69** |
| pentadecanoic acid monoacylglycerol ester | -5 ns | -2 ns | 94 ns |
| phytol | -84** | 17 ns | 454** |
| pimelic acid | -30 ns | -37 ns | 104** |
| squalene | -43 ns | 5 ns | 124** |
| stigmasterol | -29 ns | -2 ns | 76 ns |
| suberyl glycine | -20 ns | -19 ns | 39 ns |
| α-tocopherol | 8 ns | 2 ns | 49* |

ns: no significant; * $p < 0.05$; ** $p < 0.01$

Aromatics compounds identified on sugarcane methanolic leaves extracts

The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

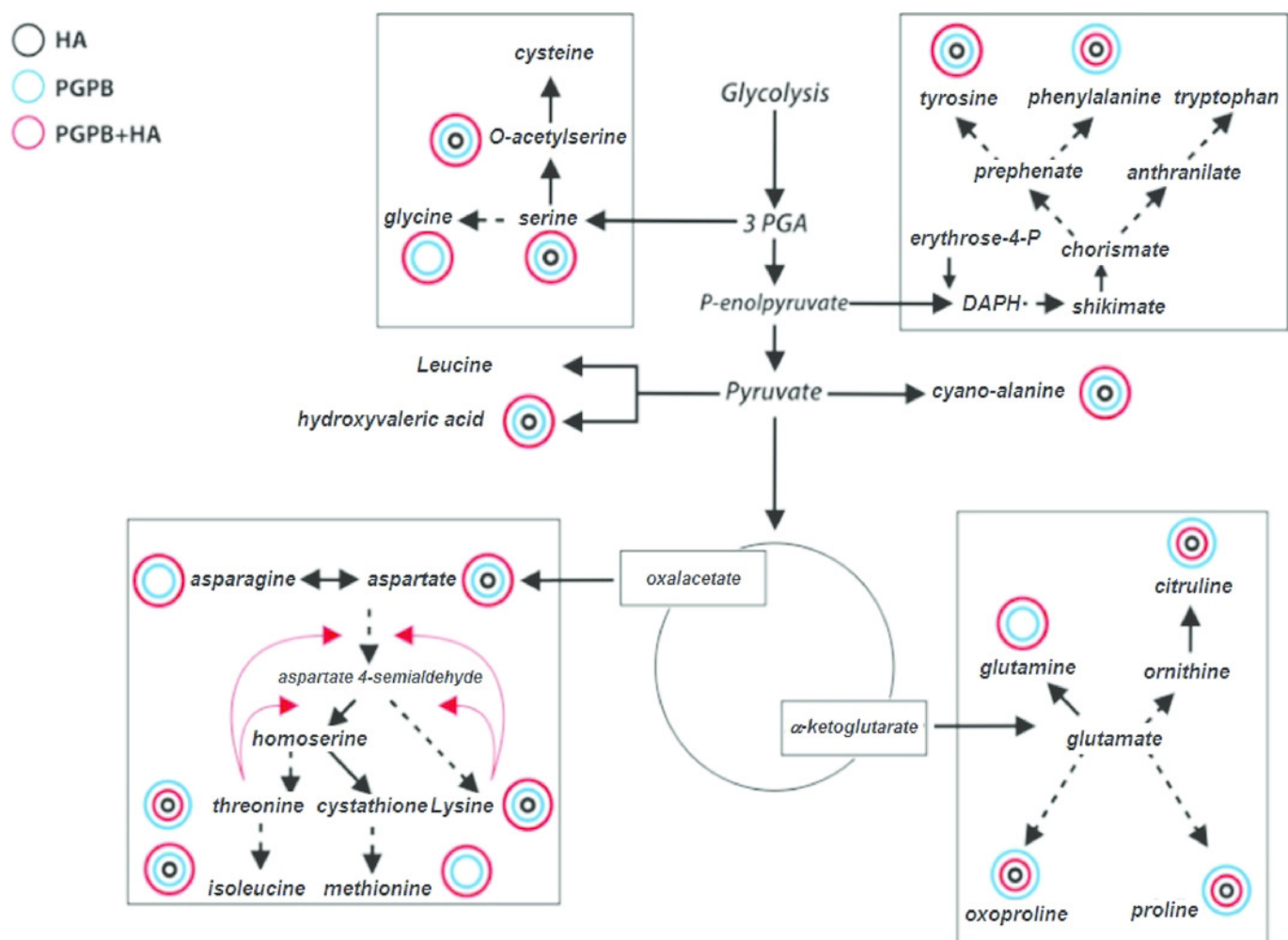
**Table 6.** Aromatics compounds identified on sugarcane methanolic leaves extracts. The treatments were inoculation with *G. diazotrophicus* and *H. seropedicae* (PGPB), bacteria and humic acid (PGPB+HA) or only humic acid (HA) isolated from vermicompost. The data were expressed in respect to control treatment (%).

| metabolite | HA | PGPB | PGPB+HA |
|---|---|---|---|
| vanillic acid | 18 ns | 24 ns | 60* |
| syringic acid | 46 ns | -11 ns | 83* |
| shikimic acid | 311** | 22** | 385** |
| pyrogallol | -9 ns | 39 ns | 64 ns |
| pyrazine 2,5-dihydroxy | 60 ns | 85 ns | 189 ns |
| pipecolic acid | -77** | 197** | 450** |
| p-hydroquinone | 29 ns | 77 ns | 122 ns |
| phthalic acid | -29* | 72** | 117** |
| phenylethylamine | -63 ns | -13 ns | 4 ns |
| phenylacetic acid | 2 ns | -9 ns | 29 ns |
| parabanic acid | 13 ns | 62 ns | 137** |
| oxamic acid | 69 ns | 50 ns | 92 ns |
| gentisic acid | 45 ns | -1 ns | 35 ns |
| ferulic acid | -28 ns | 59 ns | 175 ns |
| dihydroabietic acid | -29 ns | 17 ns | 78 ns |
| coniferin | -77* | -33 ns | 201** |
| cis-caffeic acid | 294** | 138* | 494** |
| chlorogenic acid | -20 ns | 133* | -30 ns |
| arbutin | 204** | 21 ns | 127** |
| 4-hydroxyphenylacetic acid | 6 ns | 12 ns | 12 ns |
| 4-hydroxymandelic acid | -45 ns | 17 ns | -24 ns |
| 4-hydroxycinnamic acid | 100** | -6 ns | -19** |
| 4-hydroxybenzoate | 19 ns | 1 ns | 98** |
| 4-hydroxybenzaldehyde | 63 ns | -48 ns | 87** |
| 3-hydroxybenzoic acid | -5 ns | 27 ns | -20 ns |
| 3,4-dihydroxybenzoic acid | 46** | 58** | 29** |
| 2-phenylpropanol | 47 ns | 10 ns | 15 ns |
| 2-(4-hydroxyphenyl)ethanol | -11 ns | -12 ns | 108** |
| 2,3-dihydroxypyridine | 1 ns | -9 ns | -3 ns |
| 2,3-dihydroxybenzoic acid | 12** | 52** | 80** |
| 1,2,4-benzenetriol | 14 ns | -9 ns | 39 ns |

ns: no significant; * $p < 0.05$; ** $p < 0.01$

# Figure 1

Schematic representation of the effect of the treatments on the levels of amino acids in respect to control

Schematic representation of the effect of the treatments on the levels of amino acids in respect to control. Rectangles containing aminoacids pools that derivates from pathways conversions of 3-phosphoglycerate (PGA), P-enolpyruvate, pyruvate., oxaloacetate and α-ketoglutarate. Aminoacids with significant higher values related to control had shown color circles. The color circles represent the treatments (HA: black; PGPB: blue, HA+PGPB: red) and the magnitude of influence of each treatment in respect to control is represented by the circle size.

# Figure 2

PCA scores for CG-MS metabolic profile data

PCA scores for CG-MS data indicating good separation of treatments in different groups according to bacteria inoculation (PGPB) , humic acid (HA) application or its combined use (HA + PGPB) for metabolomic profiles, as well good agreement among biological replicates. The loadings and their coefficient weight that indicates the position of the variables along the PCs and consequently their importance for that PC are showed in supplementary file.

# Figure 3

General view of the most important metabolites identified on main sugarcane biochemical pathways.

Representation of the most important metabolites identified on main sugarcane biochemical pathways. The color squares represent the treatments (HA: black; PGPB: blue, HA+PGPB: red) and the increase of concentration level (% in respect to control) was ranking according to the color-coded scale bar that range from 100 to 500 x times concentration related to control plants .