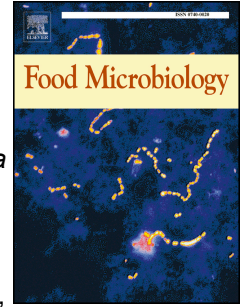# Journal Pre-proof

Mycofumigation of postharvest blueberries with volatile compounds from *Trichoderma atroviride* IC-11 is a promising tool to control rots caused by *Botrytis cinerea*

Fernando Bello, Ivana Dalila Montironi, María Belén Medina, Martín Sebastián Munitz, Flavia Vanina Ferreira, Celia Williman, Daniel Vázquez, Laura Noelia Cariddi, Matías Alejandro Musumeci

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1 **Mycofumigation of postharvest blueberries with volatile compounds from *Trichoderma***

2 ***atroviride* IC-11 is a promising tool to control rots caused by *Botrytis cinerea***

3

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Abstract

Botrytis cinerea, the causal agent of the gray mold, is a filamentous fungus that infects blueberries and can cause important production losses in postharvest storage. Considering that the use of synthetic fungicides is not allowed on blueberries in postharvest conditions, alternative and natural strategies are needed to control gray mold. The objective of this work was to evaluate the capability of volatile organic compounds (VOCs) produced by *Trichoderma atroviride* IC-11 to control *B. cinerea* growth in blueberries after harvest.

These VOCs inhibited almost completely *B. cinerea* growth *in vitro*. The most abundant volatile compound was 6-pentyl-α-pyrone (6PP). *In vitro* assays with pure 6PP confirmed its antifungal activity. The incidence of gray mold was evaluated in blueberries inoculated with *B. cinerea* and exposed to volatiles of *T. atroviride* IC-11. Gray mold incidence among those stored in air at 20 ℃ for 14 days was 100%, while the incidence among the volatile-treated fruit was 17%. Gray mold incidence among those stored in air at 4 ℃ for 31 days was 82%, while the incidence among the volatile-treated fruit was 11%. *T. atroviride* IC-11 VOCs inhibited mycelial growth and conidia germination of *B. cinerea*. The binding of VOCs to the surface of hyphae caused their vacuolation and deterioration. Selective cytotoxicity of 6PP on *B. cinerea* was observed but not on human intestinal cells at specific concentrations that controlled gray mold.

The postharvest mycofumigation of blueberries with *T. atroviride* IC-11 VOCs is a promising approach to protect these fruits from gray mold.

48 **1.   Introduction**

49   Blueberry (*Vaccinium* spp.) is a perennial shrub that produces fruits rich in anthocyanins

50 and other phytochemicals, which have anti-inflammatory and antioxidant activities that provide

51 different benefits for the human health such as prevention of DNA oxidation, anti-tumoral

52 activity, modulation of immune system, reduction in body weight and fat accumulation (anti-

53 obesity) and prebiotic potential (Silva et al., 2020). Besides, the regular consumption of

54 blueberries was associated with reduced risk of cardiovascular disease and type 2 diabetes,

55 neuroprotection and prevention of urinary tract disease (Kalt et al., 2020). Considering these

56 benefits, the demand for blueberries has increased progressively during the last twenty years and

57 consequently boosted their production (Ștefănescu et al., 2020).

58   Different fungal pathogens such as *Colletotrichum acutatum*, *Botrytis cinerea* and

59 *Alternaria* spp. infect blueberry in the field and prevail during postharvest storage, where they

60 spread and develop decays, causing important production losses (Rivera et al., 2013; Wharton et

61 al., 2008; Zhu et al., 2015). *B. cinerea*, the causal agent of the gray mold disease, is considered

62 the primary postharvest pathogen that infects blueberry fruits during storage and transport at low

63 temperatures (Rodriguez et al., 2016). This pathogen is mainly managed by the application of

64 chemical substances and synthetic fungicides, which present different limitations. Some

65 chemicals such as sulfur dioxide and methyl bromide can harm fruit quality (Bell et al., 2021;

66 Thang et al., 2016). In addition, intensive applications of sulfur dioxide could contribute to

67 environmental pollution (Craig, 2019). On the other hand, excessive use of chlorine dioxide

68 produces chlorates with prejudicial effects for the human health (Xu et al., 2016). Considering

69 that the application of chemical fungicides on blueberries is not allowed after harvest, these

70 compounds are only applied in the field. This practice decreases the population of fungal crop

71 symbionts and contaminates soil and water (Lloyd et al., 2021). Furthermore, the intensive usage

72 of fungicides has favored the proliferation of *B. cinerea* strains with resistance to one or more

73 fungicides (Rupp et al., 2017; Saito et al., 2016). The resistance of *B. cinerea* to fungicides is

74 highly persistent even after interrupting application for long time (Amiri et al., 2018). The

75 emergence of *B. cinerea* strains with resistance to multiple fungicides is a major concern that

76 affects not only to blueberries but also other crops such as raspberries, strawberries, grapes, stone

77 fruits and ornamental flowers (Rupp et al., 2017; Weber, 2011). The rise of multi-resistant *B.*

78 *cinerea* populations has reduced considerably the efficacy of fungicides to control the gray mold;

79 therefore alternative strategies are imperative to face this challenge. These strategies must ensure

80 the absence of harmful substances on blueberries to care the health of consumers and meet the

81 requirements of profitable markets.

82 Currently, there are no commercially available postharvest fungicides or biofungicides for

83 the control of postharvest diseases on blueberries (Wang et al., 2021). Strategies based on the

84 control of physical variables such as temperature, humidity, modified atmospheres and UV

85 radiation have been applied (Bell et al., 2021; Hu et al., 2021). These methods usually require the

86 complementation with other approaches to increase the antifungal effect (Bell et al., 2021).

87 Natural approaches involving the application of generally recognized as safe (GRAS) substances

88 such as ethanol vapor, chitosan and essential oils; and the use of microbial antagonists (biological

89 control) have also been applied to control *B. cinerea* after harvest (Bell et al., 2021; Ji et al.,

90 2021).

91 As called by the United Nations in the Sustainable Development Goals, agricultural

92 practices that increase organic farming and reduce the dependency on pesticides are needed to

93 ensure sustainable food production systems (United-Nations, 2015). Novel and sustainable

94 alternatives aimed to the preservation of blueberries during postharvest have also been claimed

95 (Bell et al., 2021). Mycofumigation consists in the exposure of fruits and vegetables to Volatile

96 Organic Compounds (VOCs) produced by fungi, which have antimicrobial activity against

97 different postharvest pathogens (Gomes et al., 2015). Mycofumigation has advantageous

98 properties that could be useful in postharvest conditions. Considering their volatile nature, VOCs

99 can diffuse and spread rapidly in closed environments, saturating the atmosphere and reaching

100 infected habitats in the fruits, without the necessity for a direct contact between VOCs producer

101 fungi and target pathogens (Gomes et al., 2015; Tilocca et al., 2020). Furthermore, VOCs are

102 highly effective at low concentrations and show bioactivity against different crop pathogens.

103 These compounds are produced in low amounts, which do not represent a harmful risk to human

104 health and the environment. In addition, these VOCs do not persist on treated commodities and

105 dissipate rapidly (Tilocca et al., 2020). These characteristics make mycofumigation a promising

106 alternative to the use of synthetic pesticides.

107 *Trichoderma* spp. are filamentous fungi, often plant symbionts, which are widely used in

108 agriculture to control different plant pathogens and to stimulate plant growth (Ferreira et al.,

109 2021). *Trichoderma* spp. produce different secondary metabolites with antifungal and

110 antibacterial activities such as harzianic acid, peptaboils and the lactone 6-Pentyl-2H-pyran-2-one

111 (6-pentyl-α-pyrone; abbreviated 6PP) (Vinale et al., 2020). The 6PP is a VOC with proved

112 antifungal activity against *B. cinerea*, *Rhizoctonia solani* and *Fusarium* spp. (Antonov et al.,

113 1997; El-Hasan et al., 2007; Scarselletti et al., 1994). Induction of defense responses has also

114 been attributed to 6PP (El-Hasan et al., 2007). Besides, 6PP is a GRAS substance used in the

115 food industry as natural flavoring agent due to its characteristic coconut aroma (Gomes et al.,

116 2020; Oser et al., 1984). In postharvest conditions, 6PP has been topically applied to control *B.*

117 *cinerea* in kiwi fruits (Poole et al., 1998). However, its role to control *B. cinerea* in postharvest

118 blueberries has not been studied.

119 Previous characterization of different *Trichoderma* strains suggested that *T. atroviride* IC-

120 11 could produce 6PP, because this strain elicited a characteristic coconut aroma (Ferreira et al.,

121 2020). However, the presence of 6PP and other VOCs produced by this strain were not identified.

122 The objective of this work was to evaluate the effectivity of VOCs produced by *T. atroviride* IC-

123 11 to inhibit gray mold decays on postharvest blueberries (*Vaccinium corymbosum*). This

124 mycofumigation approach combined with low temperature constitutes a natural and proficient

125 approach to control *B. cinerea* in stored blueberries.

126

127 **2.   Material and Methods**

128 **2.1.   Strains and culture conditions**. *Trichoderma atroviride* IC-11 was previously

129 isolated and identified by our group (Ferreira et al., 2020). *Botrytis cinerea* FB was isolated from

130 decayed harvested blueberries, which were stored in a packing facility and showed symptoms of

131 gray mold rot. A piece of mycelium was picked with a sterile loop and dissolved in 5 mL of

132 sterile 0.1% (v/v) Tween 20. Aliquots of 100 µL of serial dilutions were loaded on PDA plates

133 and seeded by using a Drigalski spatula. The plates were incubated at 28 ℃ until appearance of

134 colonies. Individual colonies were picked with a sterile toothpick and seeded in PDA plates,

135 which were incubated at 28 ℃ for 7 days. Identification of *Botrytis cinerea* was performed by

136 analysis of conidiophore structures with a microscope and comparison with those previously

137 reported (Pitt et al., 2009). To support Koch postulates, sterile and fresh blueberries fruits were

138 inoculated with 5 µL of 1×10^4 conidia/mL suspension (quantified with a Neubauer chamber) and

139   incubated at 25 °C and 90% relative humidity (RH) until visualization of symptoms of gray mold

140   disease. The fruits were sterilized by immersion in 10% (v/v) sodium hypochlorite for 10 min and

141   rinsed with 70% (v/v) ethanol. For long storage conditions, a colonized PDA plate was flooded

142   with 5 mL of sterile distilled water and conidia were dissolved by scrapping with a sterile glass

143   rod. Samples of 1.5 mL were extracted, loaded into 2 mL cryotubes containing 300 µL of

144   glycerol (final concentration 20% (v/v)) and stored at -80 ºC. For all the experiments, a plug of

145   culture (5 mm in diameter) was cut from the margin of stock culture using a cork borer and

146   placed on PDA plates, which were further incubated at 28 ºC.

147   **2.2.   Molecular identification of *B. cinerea* strain and phylogenetic analysis**.

148   Genomic DNA from *B. cinerea* FB was purified by the cetyltrimethylammonium bromide

149   (CTAB) method, following published procedures (Siddiquee, 2017). The purified genomic DNA

150   was used as template in a PCR reaction to amplify the ITS1-5.8s-ITS2 region of rRNA gene,

151   using   universal   primers   ITS1   (5'-TCCGTAGGTGAACCTGCGG-3')   and   ITS4   (5'-

152   TCCTCCGCTTATTGATATGC-3'). The obtained PCR product (around 500 bp) was purified

153   with EasyPure PCR purification kit (TransGen Biotech Co., Beijing, China) following the

154   manufacturer instructions and sequenced by Macrogen Inc. (Seoul, Korea). The obtained

155   nucleotide sequence was compared with sequences available in NCBI-Gen-Bank by Blast tool

156   (Altschul et al., 1990).

157   To perform phylogenetic analysis, the obtained ITS sequence was aligned to reference

158   sequences of *Botrytis* spp. with CLUSTALX (Larkin et al., 2007), using the sequences of

159   *Monilinia laxa* COY1B5 (AF150674) and *M. fructicola* (MH864497) as outgroup (Ferrada et al.,

160   2016). Well-established reference sequences of *Botrytis* spp. were obtained from previous studies

161   (Ferrada et al., 2016). Sequence alignments were inspected and trimmed with CLUSTALX.

7

162 Maximum likelihood phylogenetic trees were constructed in MEGA 7.0 using the substitution

163 model Kimura 2-parameter with gamma distribution (K2+G) and pairwise deletion (Kumar et al.,

164 2018). Bootstrapping was performed with 1,000 replications (Ferreira et al., 2020).

165 **2.3.** *In vitro* **inhibition of** *B. cinerea* **FB growth by** *T. atroviride* **IC-11 VOCs.** A

166 published procedure with some modifications was applied to assay the inhibitory effect of VOCs

167 released by *T. atroviride* IC-11 on the growth of *B. cinerea* FB *in vitro* (Li et al., 2018;

168 Ruangwong et al., 2021). *T. atroviride* IC-11 was grown in darkness on PDA in 9 cm Petri dishes

169 for 5 days at 28 ºC. A 9 cm diameter PDA plate was inoculated with *B. cinerea* FB in the center

170 and inverted, disposed as lid and facing to the grown *T. atroviride* IC-11 plate, which was the

171 base plate. The plates were sealed with parafilm and incubated at 28 ºC for 14 days (*T. atroviride*

172 IC-11 VOCs treatment). The same procedure was followed in control treatment except that the

173 PDA plate inoculated with *B. cinerea* FB was faced to an empty glass plate. Diameter of *B.*

174 *cinerea* FB colony was measured after 7, 8, 11 and 14 days. The experiment included three

175 replicates for each treatment and was repeated three times.

176 **2.4.** **GC-MS analyses.** *T. atroviride* IC-11 was seeded in glass vials containing 5 mL

177 slants of the following media: PDA (Potato Dextrose Agar), MEA (Maltose Dextrose Agar)

178 and/or SDA (Sabouraud Dextrose Agar). The vials were sealed with a rubber stopper and

179 incubated at 28 ºC during 5 days in darkness. VOCs were collected by headspace solid phase

180 microextraction (HS-SPME) during 1 h using a 100 μm polydimethylsiloxane (PDMS) fiber

181 (Supelco, Bellefonte, PA, USA). The VOCs bound to the fiber were desorbed for 2 min in a

182 split/splitless injector of a Hewlett Packard 6890 gas chromatograph coupled to HP 5973 mass

183 selective detector (MSD) and having a HP-5MS column (30 m length, 0.25 mm inner diameter,

184 0.25 µm film thickness). The injector was set in the splitless mode at 250 °C and the oven

185 program was 40 °C for 2 min, 10 °C/min to 200 °C, 25 °C/min to 260 °C (hold 5 min). The MSD

186 parameters were: electron ionization at 70 eV, source at 230 °C and quadrupole 150 °C, mass

187 range 45–400 amu. The VOCs were identified by comparison of their mass spectral

188 fragmentation with data deposited in ChemStation HP MS library NIST98 and with spectra of

189 VOCs from *Trichoderma* spp. previously reported (Guo et al., 2019; Moya et al., 2018;

190 Siddiquee, 2014; Stoppacher et al., 2010). The spectra of these VOCs were obtained from

191 PubChem database (Kim et al., 2021).

192 **2.5. Inhibition of *B. cinerea* FB growth by volatile 6PP.** A similar procedure

193 described in the bibliography was followed to quantify the inhibition of pure 6PP on *B. cinerea*

194 FB growth (Jin et al., 2020). A PDA plate was inoculated in the center with *B. cinerea* FB and

195 inverted to dispose the PDA plate on the top and the lid plate on the bottom. A sterilized filter

196 paper disc (20 mm diameter) was attached on the center of the inner surface of the lid, facing the

197 *B. cinerea* FB inoculum. Different volumes (0, 5, 10, 20 and 45 µL) of pure 6PP (Sigma-Aldrich,

198 Germany) were added onto the paper disc and the plates were sealed with parafilm and incubated

199 at 28 ºC. Diameters of *B. cinerea* FB colonies were measured after 7 days of incubation. The

200 experiment included three replicates for each treatment and was repeated three times.

201 **2.6. *In vivo* inhibition of *B. cinerea* FB growth by *T. atroviride* IC-11 VOCs on**

202 **fresh blueberries.** In these experiments mycofumigation chambers were used, which were

203 constructed to avoid the direct contact between *T. atroviride* IC-11 and blueberries

204 (Supplementary Figure S1). A plastic PDA plate of 8.0 cm diameter was inoculated with *T.*

205 *atroviride* IC-11 and collocated inside a glass plate of 9.0 cm diameter having sterile wet filter

206 paper (base plate). An 8.5 cm diameter plastic mesh, previously sterilized by rinsing with 70%

207 (v/v) ethanol, was laid on the 8.0 cm plate and another 9.0 cm glass plate was collocated as lid on

208   the top. The plates were sealed with parafilm and incubated at 28 ℃ in darkness for 5 days to

209   promote *T. atroviride* IC-11 growth and VOCs production. In the control treatment an empty 8.0

210   cm plastic plate was used instead of the PDA plate inoculated with *T. atroviride* IC-11.

211   Blueberries fruits (*Vaccinium corymbosum*) of Emerald variety were harvested and the

212   next day were washed with distilled water, sterilized by immersion in 1.0% (v/v) sodium

213   hypochlorite for 10 min, rinsed in 70% (v/v) ethanol and dried at room temperature in a laminar

214   flow box. For the experiments, health fruits having similar characteristics of size, color and

215   quality were selected. Blueberries were wounded with a calibrated awl in the pedicel, producing

216   wounds of 1 mm diameter and 4 mm depth; and inoculated with 5 µL of a suspension 1×10^4

217   conidia/mL of *B. cinerea* FB, following volume and pathogenic concentration reported in the

218   bibliography (Poole et al., 1998). Groups of 15 inoculated fruits were accommodated on the mesh

219   of mycofumigation chambers (*T. atroviride* IC-11 VOCs treatment), with the inoculated pedicels

220   upwards. Similar procedure was followed in control treatment. The control and mycofumigation

221   chambers were sealed with parafilm and incubated at 20 ℃ during 14 days. The fruits were

222   observed after 7, 10 and 14 days and the incidence of gray mold was calculated as the percentage

223   of fruits showing symptoms of the gray mold disease. Each chamber having 15 inoculated fruits

224   was considered a replicate and six replicates were carried out in each treatment. The experiment

225   was repeated three times. To simulate situation of transport to distant markets and to know the

226   effect of temperature, the same procedure was repeated but the chambers were 4 ℃ during 45

227   days. Incidence of gray mold disease was determined after 31, 36 and 45 days of incubation.

228   The conidial suspension of *B. cinerea* FB was prepared by flooding a ten days old PDA

229   plate of *B. cinerea* FB grown at 28 ℃ with 8 mL of sterile distilled water, scrapping with a sterile

230   spatula and filtering the solution through sterile gauze. The obtained conidial suspension was

231 quantified with a Neubauer chamber and a suspension containing 1×10^4 conidia/mL was

232 prepared.

233   **2.7.**   **Determination of *T. atroviride* IC-11 presence on fruit surface.** Twenty health

234 blueberries (Emerald variety) were sterilized and placed within mycofumigation (*T. atroviride*

235 IC-11 VOCs treatment) and control chambers as described in section 2.6. The chambers were

236 sealed with parafilm and incubated at 20 ℃ for 14 days. Each chamber was considered a replicate

237 and three replicates were carried out in *T. atroviride* IC-11 VOCs and control treatments. The

238 same procedure was followed with different incubation conditions (4 ℃ for 30 days). After

239 incubation times, the lid was removed and the fruits were rinsed in 20 mL of sterile distilled

240 water with mild agitation (50 rpm) for 10 min. Serial dilutions were performed and 1 mL aliquots

241 were seeded in PDA having 100 µg/mL streptomycin and 100 µg/mL ampicillin by the pour plate

242 technique. The plates were incubated at 28 ℃ until appearance of fungal colonies (3-5 days).

243 Colonies were picked and seeded in PDA plates for identification of *T. atroviride* IC-11.

244   **2.8.**   **Measurement of fruit quality parameters.** Health blueberries of Emerald variety

245 having similar characteristics of size, color and quality were selected. Twenty blueberries were

246 sterilized and placed within mycofumigation (*T. atroviride* IC-11 VOCs treatment) and control

247 chambers as described in section 2.6. The chambers were sealed with parafilm and incubated at

248 20 ℃ for 14 days. The same procedure was followed with different incubation conditions (4 ℃

249 for 30 days).

250   After incubation times, the lid was removed and 20 g of blueberries exposed to control

251 and *T. atroviride* IC-11 VOCs treatments were homogenized with a blender and filtered through

252 1 mm mesh. Aliquots of the obtained juice were used to measure soluble solids content, pH, and

253 titratable acidity (Chiabrando et al., 2011). Total Soluble Solids (TSS) contents were determined

254 by a digital refractometer (ATAGO model DTM-1, Kyoto, Japan). The results were expressed as

255 °Brix. The pH of the juice was measured with an OAKTON pH-meter (Singapore). Titratable

256 acidity was determined by potentiometric titration of 1:10 diluted juice with 0.1 N Na(OH), using

257 the OAKTON pH-meter. The results were expressed as g of citric acid/100 g of blueberries juice

258 (%). Three measurements of the parameters were performed. Weight loss was determined by

259 weighing the samples of 20 blueberries with an AS 220R.2 Radwag electronic balance (Poland)

260 at the beginning of the experiment (initial weight) and after 14 days for blueberries incubated at

261 20 ºC and/or 30 days for the fruits incubated at 4 ºC (final weight). Values were determined by

262 the following expression: 100.(initial weight – final weight)/initial weight. Firmness was

263 measured using a texture analyzer (TX-TAplus, Stable Micro Systems Texture Technologies,

264 Scarsdale, NY) fitted with a 75 mm flat probe. Each fruit was compressed 10% at rate of 1 mm/s,

265 and the maximum force developed during the test was recorded in Newtons (N). Each chamber

266 was considered a replicate and six replicates were carried out in each treatment. The experiment

267 was repeated twice.

268   In sensory evaluation, twenty sterilized blueberries were placed within mycofumigation

269 (*T. atroviride* IC-11 VOCs treatment) and control chambers. The chambers were sealed with

270 parafilm and incubated at 20 ºC for 14 days. Sensory evaluation was performed with a trained

271 panel of ten participants aged between 30 and 50 years old, seven females and three males,

272 following published procedures (Vilela et al., 2016). For the evaluation of each panelist, five

273 blueberries randomly presented were extracted from the chambers and left at room temperature

274 for 1 h. Taste, sweetness and odor were evaluated on a 5-values scale, ranging from 1 (lowest

275 intensity) to 5 (highest intensity).

276   **2.9. Determination of *T. atroviride* IC-11 VOCs in blueberries.** The presence of

277   volatile compounds produced by *T. atroviride* IC-11 in blueberries was assayed by following

278   published procedures (Munitz et al., 2017). Twenty blueberries were sterilized and placed within

279   mycofumigation (*T. atroviride* IC-11 VOCs treatment) and/or control chambers as described in

280   section 2.6. The chambers (four for each treatment) were sealed with parafilm and incubated at

281   20 ºC for 7 days. The same procedure was followed with different incubation conditions (4 ºC for

282   30 days). After incubation times, the lid was removed and 100 g of blueberries exposed to control

283   and *T. atroviride* IC-11 VOCs treatments were homogenized with a blender; 5 g of pulp were

284   mixed with 10 mL of water and the pH was adjusted to 7.0. The extract was centrifuged for 15

285   min at 4,000 rpm. The supernatant was filtered and the presence of *T. atroviride* IC-11 VOCs

286   was determined through direct immersion-solid phase microextraction (DI-SPME), by exposing

287   the PDMS fiber to the extract for 30 min in an 8 mL amber glass vial. The extraction was

288   performed at 25 °C and stirring at 200 rpm. After extraction, the fiber was introduced into the GC

289   injector for thermal desorption and GC-MS analyses were performed as described in section 2.4.

290   The experiment was repeated twice.

291   **2.10.   Quantification of 6PP in blueberries.** Blueberries (100 g) were homogenized

292   with a blender; 5 g of pulp were mixed with 10 mL of water and the pH was adjusted to 7.0. The

293   extract was centrifuged for 15 min at 4,000 rpm. The supernatant was filtered and variable

294   amounts of 6PP were added to obtain final concentrations equal to 1, 5, 10, 20 and 30 µg/mL. A

295   control without 6PP was also processed. The PDMS fiber was immersed in the extracts for 30

296   min in an 8 mL amber glass vial (DI-SPME). The fiber was introduced into the GC injector for

297   thermal desorption and GC-MS analyses were performed as described in section 2.4. The heights

298   of 6PP peaks were graphed versus concentrations to obtain a calibration curve and linear

13

299 regression was applied to estimate the concentration of 6PP in samples of blueberries exposed to

300 *T. atroviride* IC-11 VOCs. Three replicates of the experiment were performed.

301 **2.11. Qualitative analysis of the effect of *T. atroviride* IC-11 VOCs on *B. cinerea* FB**

302 **mycelial growth.** Around 1.5 mL of molten PDA was loaded onto a sterile glass slide, to obtain a

303 2 mm thickness PDA block covering the width and length of the glass slide. Four 10 µL drops of

304 *B. cinerea* FB 1×10^4 conidia/mL were loaded on the PDA block with a separation of 1.8 cm

305 approximately. The slides were laid on the plastic mesh of mycofumigation (*T. atroviride* IC-11

306 VOCs treatment) and control chambers described in Supplementary Figure S1 and section 2.6.

307 The chambers were sealed with parafilm and incubated at 28 ºC for 48 and 72 h. The slides

308 having the PDA blocks were extracted, covered with sterile cover slips and visualized with a

309 microscope. Three replicates of each treatment were performed and the experiment was repeated

310 three times.

311 **2.12. Quantitative analysis of the effect of *T. atroviride* IC-11 VOCs on *B. cinerea***

312 **FB mycelial growth.** For this experiment, wet chambers described in Supplementary Figure S2

313 were used. These chambers were built by placing filter paper on a 9.0 cm diameter glass plate and

314 10 recipients made with taps extracted from 1.5 mL microtubes, which were collocated on the

315 filter paper with the raised locking rims upwards. This base plate was covered with a lid glass

316 plate and the obtained chamber was sterilized. For the experiments, the paper filter was wet with

317 sterile distilled water and the recipients were filled with 200 µL of a 1×10^5 conidia/mL

318 suspension of *B. cinerea* FB in PDB. In control treatment the base plate was covered with a 9.0

319 cm glass plate. Negative controls (no growth) were also carried out by filling the recipients with

320 200 µL of sterile PDB. In *T. atroviride* IC-11 VOCs treatment, the lid of the chamber was

321 replaced by a 9.0 cm diameter PDA plate colonized by *T. atroviride* IC-11 previously grown at

322   28 ℃ during 5 days in darkness. The plates were sealed with parafilm and the different chambers

323   were incubated at 28 ℃ during 16, 24, 36 and 48 h in darkness. After incubation, the contents of

324   the recipients were transferred to a microplate and the absorbance was recorded at 600 nm by

325   using a Rayto RT-2100C microplate reader (Rayto, China). Negative controls were considered as

326   blanks. Three replicates of each treatment were carried out and the experiment was repeated three

327   times.

328   **2.13.   Conidia germination test.** Recipients of wet chambers were filled with 200 µL of

329   *B. cinerea* FB 1×10^5 conidia/mL following the same procedures described in section 2.12 for

330   control and *T. atroviride* IC-11 VOCs treatments. The chambers were incubated at 28 ℃ during

331   16, 24, 36 and 48 h in the darkness. After different incubation times, the volume of the recipients

332   was transferred to a 1.5 mL microtube and centrifuged at 6,000 rpm for 10 min. The supernatant

333   was discarded and the conidia were resuspended in 50 µL of sterile distilled water. Aliquots of 10

334   µL were loaded on a glass slide, covered with glass cover slips and the spores were observed with

335   a microscope. The percentage of germination was calculated by counting the number of

336   germinated conidia from 200 total conidia. Conidia were considered germinated when the germ

337   tube length was twice the size of the conidia (Olmedo et al., 2017; Torres-Ossandón et al., 2019).

338   Three replicates were carried out for each treatment and the experiment was repeated three times.

339   **2.14.   Analysis of morphological alterations of *B. cinerea* FB produced by *T.**

340   *atroviride* IC-11 VOCs.** Wet chambers consisting in Petri plates having filter paper and a glass

341   slide supported on a "V" shaped glass rod on the base plate were sterilized. Approximately 1.5

342   mL of molten PDA was loaded onto the glass slide, to obtain a 2 mm thickness PDA block

343   covering the width and length of the glass slide. *B. cinerea* FB was seeded by scraping the

344   margin of a stock culture in a PDA plate with a sterile toothpick and displacing it horizontally

15

345 over the PDA block. The filter paper was embedded with sterile distilled water and the base plate

346 was covered with a lid glass plate of similar diameter, sealed with parafilm and incubated at 28

347 ºC for 7 days. After incubation, the slides colonized by *B. cinerea* FB were extracted and loaded

348 on the plastic mesh of mycofumigation (*T. atroviride* IC-11 VOCs treatment) and control

349 treatment chambers described in Supplementary Figure S1 and section 2.6. The chambers were

350 sealed with parafilm and incubated at 28 ºC during 5 days. The PDA slides colonized by *B.*

351 *cinerea* FB were extracted, covered with sterile cover slips and the structures of *B. cinerea* FB

352 were visualized with a microscope. Two replicates of each treatment were performed and the

353 experiment was repeated three times.

354   **2.15. MTT reduction analysis.** Cytotoxicity of *T. atroviride* IC-11 VOCs on *B. cinerea*

355 FB was estimated by the MTT (Methylthiazolyldiphenyl-tetrazolium bromide) assay, following

356 the experiments described by (Zarai et al., 2011) and (Sayari et al., 2016), with some

357 modifications. In this assay, the yellow compound MTT is reduced by the mitochondrial enzyme

358 succinate dehydrogenase of viable cells to the water-insoluble violet compound formazan, which

359 is further extracted and quantified at 570 nm. Recipients containing 200 µL of *B cinerea* FB

360 1×10^5 conidia/mL were grown following the same procedures described in section 2.12 for

361 control and *T. atroviride* IC-11 VOCs treatments. Negative controls consisting in 200 µL of

362 sterile PDB were also performed. The chambers were incubated at 28 ºC during 16, 24, 36 and 48

363 h in darkness. After different incubation times, 20 µL of sterile 5 mg/mL MTT in PBS were

364 added to each recipient. The chambers were incubated at 37 ºC for 3 h. The content of the

365 recipients was extracted and transferred to 1.5 mL tubes. The tubes were centrifuged at 10,000

366 rpm for 5 min and the supernatant was discarded. The violet tetrazolium crystals were extracted

367 by adding 1 mL of methanol/DMSO (50:50) and vortexing during 30 sec. The suspension was

368   centrifuged at 10,000 rpm for 5 min to separate cell dendrites and insoluble aggregates. The

369   supernatant was extracted and absorbance was recorded at 570 nm using a UV-1800 Shimadzu

370   spectrophotometer (Shimadzu, Tokyo, Japan). Negative controls were considered as blanks.

371   Three replicates of each treatment were carried out and the experiment was repeated twice.

372   **2.16. Cytotoxicity of 6PP to human cell culture.** The cytotoxic effect of 6PP on

373   human colon carcinoma Caco-2 cell line was evaluated following the methodology described in

374   (Montironi et al., 2022). Caco-2 cell line was maintained in Dulbecco's Modified Eagle Medium

375   (DMEM; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal

376   bovine serum (FBS) (Natocor, Villa Carlos Paz, Córdoba, Argentina) and Penicillin-

377   Streptomycin (1%) (Gibco, Life Technologies, Grand Island, NY, USA). Caco-2 cells (5×10^4

378   cells/well) were cultured in 96-well culture microplates and after monolayer formation were

379   exposed to different concentrations of 6PP (0, 23.53, 47.06, 94.12, 188.25, 376.50, 470.63,

380   564.75, 658.88, 753.00, 1129.50, 1506.00, 1882.50, 2635.50 and 3012.00 µg/mL) and incubated

381   at 37 ºC with 5% CO_2 and 95% humidity for 24 h. Cells with complete DMEM medium alone

382   were included as a control group and dimethylsulfoxide (DMSO) (0.05%) in complete DMEM

383   was added as a vehicle control in all assays. Caco-2 cells viability was evaluated by the

384   colorimetric reduction method with (MTT) (Sigma, Burlington, USA) described by (Mosmann,

385   1983). For each concentration, triplicate wells in two independent assays were performed. The

386   Caco-2 cells were also exposed to 100 µL of *T. atroviride* IC-11 VOCs, which were obtained by

387   steam distillation of 10 PDA cultures of *T. atroviride* IC-11 (9.0 cm diameter, extracted from

388   Petri plates) at 80 °C during 4 h. The VOCs were collected in 0.05 % DMSO.

389   **2.17. Cytotoxicity of 6PP to *B. cinerea* FB.** The toxicity of 6PP against *B. cinerea* FB

390   was assayed by following the broth microdilution test described previously, with some

391 modifications (Finos et al., 2021). Microplate wells were filled with 100 µL of malt extract broth

392 (MEB) and 1, 2, 3, 4 and 5 µL of diluted stocks of 6PP (at 0.1%, 1.0% and 10% (w/v)) and/or 0.7

393 and 1.0 µL of the concentrated stock (100% (w/v)) were added in different wells, obtaining 17

394 different concentrations of 6PP in the range 5.0 – 5,000 µg/mL. Controls with 0 µL of 6PP were

395 also included. The wells were inoculated with 100 µL of *B. cinerea* FB 1×10^6 conidia/mL. The

396 blank consisting in 100 µL of non-inoculated MEB and 100 µL of sterile distilled water was

397 included. Three replicates of the experiments were performed, using 96-wells microplates. The

398 microplates were incubated at 28 ℃ during 72 h. Fungal growth was estimated by visualizing the

399 microplates with a stereo microscope and by reading the absorbance at 600 nm with a microplate

400 reader. The presence of viable cells of *B. cinerea* FB was determined by spilling the content of

401 the wells in Petri plates and pouring over it 15 mL of molten MEA (Malt Extract Agar). The

402 plates were incubated at 28 ℃ during four days to favor the growing of *B. cinerea* FB. The plates

403 were observed periodically to detect and counting fungal colonies. Percentage viability of *B.*

404 *cinerea* FB was calculated considering 100% viability the number of colonies obtained in

405 controls with 0 µL of 6PP.

406 **2.18. Statistical analysis.** One-way analysis of variation was performed using

407 Statgraphics Centurion 18 (Statpoint Technologies Inc., The Plains, VA, USA). Significance of

408 each treatment was determined in according to the F-value. When a significant difference was

409 observed, separation of means was carried out using Tukey's test. Statistical significance was

410 determined at $P = 0.05$. The half maximal inhibitory concentration (IC_{50}) of 6PP was calculated

411 by adjusting the viability percentage values obtained at different concentrations of 6PP to a

412 sigmoid function with Statgraphics Centurion 18.

413

414   **3.   Results and Discussion**

415   **3.1.   *In vitro* inhibition of *B. cinerea* FB by *T. atroviride* IC-11 VOCs**

416   *B. cinerea* FB was isolated from decayed blueberries, which were stored in a postharvest

417   packing facility and showing symptoms of the gray mold disease. This strain was unequivocally

418   identified through microscopic observation of conidiophores, sequencing of ITS region and

419   phylogenetic analysis (Supplementary Figure S3).

420   The capability of VOCs produced by *T. atroviride* IC-11 to inhibit the growth of *B.*

421   *cinerea* FB was assayed *in vitro* by the sandwiched Petri plates method (Li et al., 2018). The

422   strain *T. atroviride* IC-11 was used because it produces a characteristic coconut aroma, which is

423   attributed to 6PP, a compound identified as potential inhibitor of *B. cinerea* growth (Antonov et

424   al., 1997; Poole et al., 1998). After 7 days of incubation at 28 ℃, *B. cinerea* FB colonies covered

425   the PDA plate in control treatment (9.0 cm diameter); whereas no growth was observed in *T.*

426   *atroviride* IC-11 VOCs treatment. Incipient growth of *B. cinerea* FB was observed after 14 days

427   of incubation in *T. atroviride* IC-11 VOCs treatment, with colonies showing 0.8 cm diameter

428   average. These results suggested that VOCs released from *T. atroviride* IC-11 have antifungal

429   activity against *B. cinerea* FB. In a similar procedure, Savas et al. reported that the volatile

430   metabolites of *T. atroviride* EGE-K-71 produced 71.8% inhibition of *B. cinerea* growth (Savas et

431   al., 2021). Moreover, these volatile metabolites showed the highest growth inhibition percentage

432   of *B. cinerea* growth *in vitro* compared with different assayed fungicides (Savas et al., 2021).

433   However, the VOCs produced by *T. atroviride* EGE-K-71 were not characterized.

434   **3.2.   GC-MS analyses of VOCs produced by *T. atroviride* IC-11 and antifungal**

435   **activity of pure 6PP**

436   The volatile compounds produced by *T. atroviride* IC-11 were identified through GC-MS

437   analysis (Figure 1 and Table 1). The results confirmed the presence of 6PP, which was the most

438   abundant compound of the VOCs produced by *T. atroviride* IC-11. The other identified

439   compounds were 2-pentylfuran, 2-undecanone, γ-cadinene and α- and β-bergamotene, which was

440   the second most abundant VOC (Figure 1 and Table 1). A compound with 281.3 g/mol molecular

441   weight was tentatively identified as diterpenoid. Except for 6PP, antifungal activity of the

442   identified VOCs has not been reported to date.

443   Concerns related to the safety of these compounds such as hazard classification and safe

444   concentrations were inquired by searching chemical information and toxicological data in

445   different databases (Tamburlin et al., 2021) (Table 1). The 6PP is classified as irritant; whereas 2-

446   pentylfuran is flammable and harmful if swallowed and 2-undecanone is toxic to aquatic life.

447   This classification corresponds to liquid and pure solutions of the compounds (100%). However,

448   these compounds are used in products applied in the skin such as cosmetics and insect repellents;

449   besides some of them are present in essential oils with therapeutic properties and also in foods

450   either naturally or as additives.

451   The 6PP is classified as GRAS substance by the Federal Drug Administration (FDA) and

452   is used in the food and cosmetics industries as natural flavoring agent or adjuvant (Gomes et al.,

453   2020; Oser et al., 1984). Besides, 6PP is naturally present in a wide variety of peaches

454   (Mohammed et al., 2021). However, high concentrations can produce adverse effects as liquid

455   solutions of 6PP in the range 5-10% produced severe irritation and moderate damage in slugs

456   (Polizzi et al., 2011).

457   The 2-pentylfuran is present in different thermally processed foods and drinks and has

458   been found in a wide range of vegetables, including artichoke, asparagus, avocado, beans,

459   cauliflower (Hu et al., 2016; Younes et al., 2021). The 2-pentylfuran does not induce gene

460   mutations and concerns for genotoxicity were ruled out but hematological effects were observed

461   in rats and cytotoxicity was noted above 86.3 µg/mL. The maximum allowed daily intake is 290

462   µg/person per day (Younes et al., 2021).

463   The 2-undecanone is a non-toxic ketone produced by the glandular trichomes of some

464   plants such as the wild tomato (*Lycopersicon hirsutumused*). This compound presents repellant

465   properties against mosquitoes and ticks and is used in commercial repellents (Bissinger et al.,

466   2009). The 2-undecanone does not present genotoxicity and does not have concerns for skin

467   sensitization (Api et al., 2019). Studies in mice have shown that 2-undecanone presents anti-

468   inflammatory properties and may play a protective role against renal inflammation (Wu et al.,

469   2021). However, the median lethal (LD_{50}) dose in rats is 5 g/kg of body weight (O'Neil, 2001).

470   The α- and β- bergamotene are sesquiterpenes produced by glandular trichomes of some

471   tomato plants and have toxic activity against herbivore insects predators (Wang et al., 2020).

472   These compounds are present in essential oils of different vegetal sources with therapeutic

473   properties (Boniface et al., 2017; Zhang et al., 2021). The γ-cadinene is a sesquiterpene found in

474   essential oil of the tree *Chamecyparis formosensis* at 9.9%. This essential oil stimulates a

475   pleasant mood status in humans by inhalation and has been proposed as suitable agent for the

476   development of regulators of sympathetic nervous system dysfunctions (Chen et al., 2015).

477   These studies suggested that *T. atroviride* IC-11 VOCs identified by GC-MS analysis do

478   not pose a hazard to the human health under controlled exposure conditions.

21

479   To know if different substrates influence the rate and spectrum of VOCs, *T. atroviride* IC-

480   11 was grown in SDA, MEA and PDA and the produced VOCs were analyzed by GC-MS. 6PP

481   was the more abundant compound in all assayed growth media (Supplementary Figure S4). The

482   highest 6PP yield was obtained in SDA, whereas the lowest 6PP production was observed in

483   MEA. Narrow variability in the types of VOCs was observed in compounds with low abundance.

484   Besides, the differences in VOCs profiles were eclipsed by the high intensity of 6PP signal

485   (Supplementary Figure S4). In agreement with our results, a *T. atroviride* strain showed low

486   variability in the composition of VOCs when was grown in different culture media (González-

487   Pérez et al., 2018). These results showed that the composition of the growing media influences on

488   the profile and concentrations of VOCs produced by *T. atroviride* IC-11. Similar results were

489   reported by Wheatly et al., who determined that the production of VOCs by *T. pseudokoningii*

490   was dependent on growing media (Wheatley et al., 1997). The type of amino acids but not the

491   range or the overall content present in the growing medium is determinant in the range of VOCs

492   produced by *Trichoderma* (Bruce et al., 2000). The nitrogen source also influenced on the yield

493   of 6PP produced by *T. harzianum* (Serrano-Carreon et al., 1992). Homogeneity of the growing

494   medium is relevant to obtain uniform VOCs preparations in terms of composition and thus

495   confirm safety in mycofumigation approaches.

496   Considering that the most abundant volatile compound produced by *T. atroviride* IC-11

497   was 6PP, we assayed *in vitro* the capability of vapors of the pure compound to inhibit the growth

498   of *B. cinerea* FB (Figure 2). Significant differences were observed between the treatments

499   containing pure 6PP and the control with 0 µL of 6PP, showing an inhibitory effect that depended

500   on the volume of pure 6PP (Figure 2). These results suggested that the 6PP produced by *T.*

501   *atroviride* IC-11 plays a relevant role in the inhibition of *B. cinerea* FB growth. In concordance, a

502 correlation between the concentration of 6PP produced by different *Trichoderma* strains and the

503 efficiency of these strains to inhibit mycelial growth and conidia germination of *B. cinerea* has

504 been reported (Pezet et al., 1999). Besides, 6PP purified from *T. harzianum* T23 showed

505 antifungal activity against *Fusarium moniliforme* growth (El-Hasan et al., 2007).

506 However, it has been demonstrated that 6PP and other VOCs produced by *Trichoderma*

507 could function synergistically to inhibit pathogens growth (Wonglom et al., 2020). Therefore, the

508 other VOCs produced by *T. atroviride* IC-11 could potentiate the antifungal activity of 6PP.

509 **3.3.   Determination of incidence of gray mold disease in postharvest blueberries**

510 **exposed to *T. atroviride* IC-11 VOCs**

511 The capability of VOCs produced by *T. atroviride* IC-11 to inhibit the gray mold was also

512 assayed upon *in vivo* conditions (Figure 3). Blueberries were artificially infected with 5 µL of *B.*

513 *cinerea* FB 1×10^4 conidia/mL and incubated in mycofumigation chambers containing a 5 days

514 old PDA plate colonized by *T. atroviride* IC-11 (VOCs treatment), and/or an empty plate (control

515 treatment). These chambers were specifically designed to avoid direct contact between *T.*

516 *atroviride* IC-11 and blueberries (Supplementary Figure S1). The chambers were incubated at 20

517 ℃ during 14 days and 4 ℃ during 45 days. Scarce development of gray mold was observed in

518 fruits exposed to *T. atroviride* IC-11 VOCs after 14 days at 20 ℃ (Figure 3A) as well as after 31

519 days at 4 ℃ (Figure 3B).

520 Incidence (percentage of infected fruits) of the gray mold was calculated at 20 ℃ (Figure

521 3C) and 4 ℃ (Figure 3D) after different incubation days. Significant differences were observed

522 between control and *T. atroviride* IC-11 VOCs treatments (P = 0.05). Rot percentage around 17%

523 was obtained in *T. atroviride* IC-11 VOCs treatment after 14 days of incubation at 20 ℃, whereas

524   100% of rot percentage was obtained in control treatment in the same conditions (Figure 3C). Rot

525   percentages around 24% and 82% were obtained in *T. atroviride* IC-11 VOCs and control

526   treatments respectively after 45 days of incubation at 4 ℃ (Figure 3D). In *T. atroviride* IC-11

527   VOCs treatment 11% rot percentage was observed after 31 days incubation at 4 ℃, lower to that

528   observed at 20 ℃ after shorter incubation time (17%, 14 days). These results suggested that

529   VOCs produced by *T. atroviride* IC-11 inhibited the decays of gray mold in postharvest

530   blueberries and this inhibition could be improved by combination with low temperatures.

531   PDA plates were inoculated with *T. atroviride* IC-11 and incubated at 4 ℃ during 15

532   days. No fungal growth was observed in these conditions. This result suggested that *T. atroviride*

533   IC-11 does not produce VOCs at 4 ℃; therefore the inhibition of the gray mold at 4 ℃ was

534   produced by the VOCs that were already present in the mycofumigation chambers.

535   To know if the exposure of blueberries to *T. atroviride* IC-11 VOCs harms the quality of

536   the fruits, different standard quality parameters such as pH, weight loss, titratable acidity, total

537   soluble content and texture were determined (Supplementary Table 1 and Supplementary Table

538   2). Organoleptic analyses including odor and taste were also performed (Supplementary Table 3).

539   No significant differences (P = 0.05) were observed between *T. atroviride* IC-11 VOCs and

540   control treatments, with exception of weight loss, which was higher in the control treatment. In

541   addition, the color and consistency of the fruits were similar in both treatments. These results

542   indicated that *T. atroviride* IC-11 VOCs did not modify the quality of blueberries.

543   The presence of *T. atroviride* IC-11conidia on the surface of the fruit was assayed after

544   incubation of healthy blueberries in mycofumigation chambers during 14 days at 20 ℃ and 30

545   days at 4 ℃. The fruits were rinsed in sterile water and fungal colonies were identified by plating

546 aliquots in PDA plates. *Trichoderma* spp. were not detected in PDA plates. This result

547 determined that there was no contact between *T. atroviride* IC-11 and blueberries; confirming the

548 absence of direct biological control mechanisms such as competition and/or mycoparasitism.

549   The efficiency of *Trichoderma* VOCs to inhibit the growth of different pathogens in

550 postharvest conditions has been reported. However, 6PP was not found as the main compound

551 with antifungal activity. VOCs from *T. asperellum* reduced significantly the incidence and

552 severity of rot caused by *B. cinerea* in strawberries (Jalali et al., 2016). GC-MS analysis

553 determined that isobutyric acid, 1,3,5,7-cyclooctatetraene, dimethyl sulfide, para-meta-6,8,dien-

554 2-ol-acetate, phenyl ethyl alcohol and 1-butanol, 3-methyl- acetate were the most abundant

555 compounds. *In vitro* inhibition assays with these pure compounds showed that isobutyric acid and

556 dimethyl sulfide were the most effective compounds to inhibit the mycelial growth and conidial

557 germination of *B. cinerea* (Jalali et al., 2016).

558   A complete inhibition of rot caused by *Fusarium incarnatum* was observed in

559 muskmelons exposed to VOCs emitted by *T. asperellum* T76-14 during seven days. The

560 dominant volatile compound released by *T. asperellum* T16 was phenyl ethyl alcohol, which

561 displayed antifungal activity *in vitro* against *F. incarnatum* (Intana et al., 2021).

562   VOCs produced by *T. atroviride* T41 and T45 inhibited the mycelial growth of

563 *Phytophthora infestans* by 93.1 and 94.1% on potato tubers. The VOCs emitted by these strains

564 were analyzed and the most abundant compounds were 3-methyl-1-butanol, 6PP, 2-methyl-1-

565 propanol, and acetoin. However, when these four pure compounds were tested separately, the

566 most active VOC against *P. infestans* were 3-methyl-1-butanol and 2-methyl-1-propanol

567 (Elsherbiny et al., 2020).

25

568   The antifungal property of 6PP against *B. cinerea* upon *in vivo* conditions has been

569   demonstrated in postharvest kiwi fruits (Poole et al., 1998). Topical applications of commercially

570   available pure 6PP in the infected pedicel of fruits produced a nearly complete inhibition of gray

571   mold. In these experiments 6PP was dissolved in different solvents before the topical application

572   (Poole et al., 1998). However, this approach was effective when the compound was applied

573   specifically in the place where the pathogen is located. Our results suggested that the volatile 6PP

574   produced by *T. atroviride* IC-11 can diffuse in the chambers, reaching the sites where the

575   pathogen is located and inhibiting effectively *B. cinerea* growth in postharvest blueberries.

576   **3.4.   Effect of *T. atroviride* IC-11 VOCs on *B. cinerea* FB hyphal growth and**

577   **conidia germination**

578   The effect of *T. atroviride* IC-11 VOCs on *B. cinerea* FB growth was investigated by

579   qualitative and quantitative experiments (Figure 4). PDA slides containing separated drops of a

580   1×10^4 conidia/mL *B. cinerea* FB conidial suspension were incubated in *T. atroviride* IC-11 VOCs

581   mycofumigation chambers during 48 and 72 h at 28 ºC, observed with a microscope and

582   compared with a control without treatment. Inhibition of *B. cinerea* FB growth was evident in *T.*

583   *atroviride* IC-11 VOCs treatment (Figure 4A). Microscopic observations revealed less

584   development of *B. cinerea* FB hypahe in *T. atroviride* IC-11 VOCs treatment (Figure 4B). This

585   effect was also analyzed in liquid growth medium, by incubating aliquots of 200 µL of *B. cinerea*

586   FB 1×10^5 conidia/mL suspended in PDB in control and *T. atroviride* IC-11 VOCs chambers

587   (Figure 4C). The growth of *B. cinerea* FB was estimated by measuring the absorbance at 600 nm

588   after different incubation times at 28 ºC (Figure 4D). *T. atroviride* IC-11 VOCs inhibited almost

589   completely the growing of *B. cinerea* FB hyphae after 48 h of incubation at 28 ºC.

590   The effect of *T. atroviride* IC-11 VOCs on germination of *B. cinerea* FB conidia was also

591   analyzed, following a procedure similar to that described in Figure 4C (Figure 5). Lower

592   germination of *B. cinerea* FB conidia was observed in *T. atroviride* IC-11 VOCs treatment

593   (Figure 5A), showing 20% of germination after 48 h of incubation at 28 ºC; whereas 90% of

594   germination was obtained in control treatment (Figure 5B).

595   These results suggested that *T. atroviride* IC-11 VOCs inhibited both the hyphal growth

596   and conidia germination of *B. cinerea* FB.

597   **3.5.   Deterioration of *B. cinerea* FB structures by *T. atroviride* IC-11 VOCs**

598   To know how *T. atroviride* IC-11 VOCs affect different structures of *B. cinerea* FB,

599   microscopic observations of hyphae and conidiophores were carried out. A 7 days old culture of

600   *B. cinerea* FB grown in PDA slides was incubated in *T. atroviride* IC-11 VOCs mycofumigation

601   chamber and after 5 days was observed with a microscope and compared with a control without

602   treatment (Figure 6).

603   Bubbles on the surface of *B. cinerea* FB hyphae were observed in *T. atroviride* IC-11

604   VOCs chambers (Figure 6A). In control treatment, bubbles were not observed on hyphae of *B.*

605   *cinerea* FB, which were uniform and smooth (Figure 6A). These results suggested that the

606   volatile molecules emitted by *T. atroviride* IC-11 adsorb on hyphae of *B. cinerea* FB. In

607   concordance, it has been proposed that 6PP adsorbs on the hydrophobic cell membranes, forming

608   a hydrorepellent film that avoids the water absorption (Scarselletti & Faull, 1994). However,

609   disruption of cell wall and dissolution of cytoplasm were also associated to the antifungal activity

610   of 6PP (Ismaiel et al., 2017). Lysed hyphae with flat ribbon-like structure were observed when *B.*

611   *cinerea* FB was exposed to *T. atroviride* IC-11 VOCs (Figure 6B). The microscopic observations

612   also demonstrated increase in the number of vacuoles in hyphae of *B. cinerea* FB exposed to *T.*

613   *atroviride* IC-11 VOCs (Figure 6C). This effect could contribute to the inhibition of *B. cinerea*

614   FB growth, because the increase in the number of vacuoles weakens the integrity of the

615   mycelium (Richards et al., 2010). Similar morphological alterations were reported when hyphae

616   of *Aspergillus flavus*, *Penicillium expansum* and *Fusarium acuminatum* were treated with 6PP.

617   Scanning Electron Microscope observations of these fungi showed severe damage after treatment

618   with 6PP such as collapse, surface depression, roughness and linearity loosing of hyphae.

619   Cytoplasm dissolution, deterioration of mitochondria and vacuolation increase were other

620   noticeable effects attributed to 6PP (Ismaiel & Ali, 2017). However, other *Trichoderma* VOCs

621   with fitting hydrophobicity also bind to fungal cell wall and membranes of *B. cinerea*, such as

622   sesquiterpenes, disturbing their integrity and modifying the permeability (El Hawary et al., 2013).

623   Therefore, sesquiterpenes produced by *T. atroviride* IC-11 such as α- and β-bergamotene could

624   complement the antifungal activity of 6PP against *B. cinerea* FB.

3.6.   Inhibition of mitochondrial respiration in *B. cinerea* FB

626   It has been proposed that the mitochondrial deterioration caused by 6PP produces

627   inhibition of cell respiration (Ismaiel & Ali, 2017). Therefore, the activity of viable mitochondria

628   was measured through MTT reduction, which is catalyzed by the mitochondrial succinate

629   dehydrogenase (Sayari et al., 2016; Zarai et al., 2011). Aliquots of 200 µL of PDB containing

630   1×10^5 conidia/mL of *B. cinerea* FB were incubated in control and *T. atroviride* IC-11 VOCs

631   chambers during different times at 28 ºC, following a similar procedure to that described in

632   Figure 4C. After incubation, MTT was added and the reduction was evident in the control

633   treatment (violet, reduced state), whereas scarce MTT reduction was observed in *T. atroviride*

634   IC-11 VOCs treatment (yellow, oxidized state) (Figure 7A). The formazan crystals formed by the

635   reduction of MTT were extracted (Figure 7B) and quantified by measuring the absorbance at 600

636   nm (Figure 7C). *T. atroviride* IC-11 VOCs inhibited the reduction of MTT and therefore activity

637   of the mitochondrial succinate dehydrogenase.

638   **3.7.   Determination of *T. atroviride* IC-11 VOCs in blueberries**

639   To know if *T. atroviride* IC-11 VOCs persist on blueberries, samples of 20 fruits were

640   stored in mycofumigation chambers during 30 days at 4 ℃ and 7 days at 20 ℃. Fruit extracts

641   were prepared and the presence of *T. atroviride* IC-11 VOCs was assayed by GC-MS analyses.

642   The only detected VOC in blueberries was 6PP after 30 days of incubation at 4 ℃. *T. atroviride*

643   IC-11 VOCs, including 6PP, were not identified in fruits after 7 days incubation at 20 ℃. The

644   amount of 6PP in blueberries was quantified and the concentration in fruits was 2.24 µg/kg.

645   Further analyses were performed to know if this concentration of 6PP in blueberries

646   represents a risk for the human health, following published procedures (Tamburlin et al., 2021).

647   The Cramer classification of 6PP estimated by Toxtree (v3.1.0-1851-1525442531402, available

648   at: http://toxtree.sourceforge.net/) was Class III, which corresponds to high toxic hazard. 6PP was

649   classified as non-mutagenic, non-carcinogen and inactive for chromosomal aberration through

650   further *in silico* toxicological analyses performed with Vega (version 1.1.5 48, available at:

651   https://www.vegahub.eu/download/vega-qsar-download/).

652   The threshold of concern for a class III compound is 90 µg/person/day (EFSA Panel,

653   2011). According to EFSA database the hazard reference value for 6PP is 1.5 µg/kg bw/day

654   (Table 1). Considering this value, safety ingestions of 6PP would be 90 µg/day for adults (60 kg

655   body weight average), 57 µg/day for 12-year-old children (38 kg average) and 30 µg/day for 6-

656   year-old children (20 kg average); employing well-established reference values of body weight

657 (Tamburlin et al., 2021). These values are higher than the concertation of 6PP detected in

658 blueberries exposed to *T. atroviride* IC-11 VOCs (2.24 µg/kg). Therefore, the considered

659 population would not exceed safe amounts of 6PP after eating 1 kg per day of blueberries

660 exposed to *T. atroviride* IC-11 VOCs treatment, considering a 100% corporal absorption of 6PP.

661 In addition, the amount of 6PP detected in blueberries is lower than those present naturally in

662 peaches, which are in the range 6-134 ng/g, depending on the cultivar (Mohammed et al., 2021).

663 These results suggested that 6PP only persist on blueberries after long exposure time to *T.*

664 *atroviride* IC-11 VOCs; however, the concentration of this compound in the fruits would be safe

665 for the human consumption.

666 **3.8. Cytotoxicity of 6PP on human cells**

667 6PP was the only VOC produced by *T. atroviride* IC-11 found in blueberries after 30 days

668 exposure. Although 6PP is classified as GRAS compound, there are not evidences about its

669 toxicity on human tissues. To know if this compound causes injuries to human cells, its

670 cytotoxicity to intestinal human colon carcinoma cells (Caco-2) was assayed.

671 Low toxicity of these human cells was observed in the range 0 - 188.25 µg/mL of 6PP,

672 with viability percentages varying between 95% and 84%; whereas moderate toxicity was

673 observed at 376.5 - 470.63 µg/mL (77% - 69%) and high cytotoxicity was obtained at 564.75

674 µg/mL of 6PP and higher concentrations, showing 10% of cell viability (Figure 8A). No

675 cytotoxic effect of Caco-2 cells was observed at the concentration of 6PP detected in blueberries

676 (2.24 µg/mL). To compare the cytotoxicity of 6PP on these human cells with the cytotoxicity on

677 *B. cinerea* FB, similar experiments were carried out using conidial suspensions of the fungus.

678 Low cytotoxicity of *B. cinerea* FB was observed in the range 0 - 50 µg/mL of 6PP (100% - 85%

679   viability); however the viability percentage decreased abruptly at 100 µg/mL of 6PP (38%),

680   declining to 8.6% at 150 µg/mL and no fungal growth was observed at 250 µg/mL (Figure 8B).

681   The half maximal inhibitory concentration (IC$_{50}$) of 6PP for the human and fungal cells were

682   calculated from data showed in Figure 8 and were 501.1 µg/mL for Caco-2 cells and 92.8 µg/mL

683   for *B. cinerea* FB. These results showed that 6PP is more toxic to *B. cinerea* FB and conditions

684   that were greatly inhibitory to this fungus (150 µg/mL 6PP, 8.0% viability) presented low

685   cytotoxicity on Caco-2 cells (188.25 µg/mL 6PP, 84% viability). Our results are in the range of

686   reported toxicity values of 6PP for different fungi. The germination percentage of *B. cinerea* was

687   18% at 126 µg/mL and nil at 210 µg/mL of 6PP (Poole et al., 1998); whereas in *Fusarium*

688   *moniliforme*, 93.5% inhibition of mycelial growth was observed at 250 µg/mL (El-Hasan et al.,

689   2007).

690   These results suggested that if the concentration of 6PP in the volatile atmosphere of *T.*

691   *atroviride* IC-11 would be adjusted around 190 µg/mL, then selective cytotoxicity against *B.*

692   *cinerea* could be achieved, avoiding adverse effects on human cells.

693   The Caco-2 cells were also exposed to *T. atroviride* IC-11 VOCs and no cytotoxicity was

694   observed. However, adverse effects on the human health induced by long term exposure to *T.*

695   *atroviride* IC-11 VOCs should not be discarded (Polizzi et al., 2011). This concern would be

696   relevant in contexts involving recurrent exposure to VOCs such as occupational exposure by

697   workers. Applications of *T. atroviride* IC-11 VOCs in closed boxes along with standard

698   procedures promulgated by regulatory agencies to manipulate substances with fungicide activity

699   could minimize worker exposure (Woodrow et al., 2018).

700

31

701   **4.   Conclusions**

702   Our results suggest that VOCs produced by *T. atroviride* IC-11 are effective to inhibit the

703   *B. cinerea* FB growth upon *in vitro* and *in vivo* conditions. The antifungal activity of *T. atroviride*

704   IC-11 VOCs could be attributed to 6PP, which was the most abundant volatile compound and it

705   showed antifungal activity against *B. cinerea* FB. This compound binds to the membrane of

706   hyphae, causing morphological changes in *B. cinerea* FB structures, weakening hyphae and

707   promoting cell wall deterioration. However, other VOCs could play a secondary role in the

708   antifungal activity, such as sesquiterpenes, perhaps acting synergistically with 6PP against *B.*

709   *cinerea* FB.

710   The exposure of postharvest blueberries to VOCs produced by *T. atroviride* IC-11 was

711   effective to control gray mold decays in closed environments. This mycofumigation approach can

712   be combined with low temperature to extend the shelf life of fresh blueberries. This strategy does

713   not require the adaptation period needed in preventive treatments for the establishment of the

714   antagonist in the biocontrol environment and overcomes eventual restrictions of foreign markets

715   concerning to the presence of living microorganisms on fresh fruits. In addition, it avoids the

716   proliferation of resistant strains encountered with the intensive use of synthetic fungicides. To our

717   knowledge, this is the first report describing the ability of *Trichoderma* VOCs to suppress gray

718   mold on postharvest blueberries. This natural approach could be applied in other postharvest

719   fruits to control gray mold disease.

720   6PP was the only VOC produced by *T. atroviride* IC-11 that persisted in blueberries after

721   long exposure periods, in concentration *a priori* harmless for the human health. Furthermore, this

722   concentration was not toxic for intestinal human cells. However, further studies are needed to

723 determine the impact of 6PP and the other *T. atroviride* IC-11 VOCs on human health and non-

724 target organisms, as well as the potential to produce environmental pollution. These studies

725 should be focused on metabolism patterns, the effects of short-term and long-term exposure to

726 different doses of *T. atroviride* IC-11 VOCs (acute and chronic toxicity), and also irritancy trials

727 using rat as mammal model (Damalas et al., 2011). The fate of these VOCs after its release must

728 be also studied to evaluate environmental pollution. These studies are necessary for registration

729 and to support commercially feasible and safe applications of *T. atroviride* IC-11 VOCs in

730 mycofumigation approaches. However, *T. atroviride* IC-11 VOCs could be used beneficially by

731 minimizing exposure risks. In this aspect, a major challenge is to adjust precisely the

732 concentration of 6PP emitted from *T. atroviride* IC-11 in mycofumigation chambers to specific

733 doses that inhibit *B. cinerea* but do not harm human or environmental health. In addition,

734 minimal exposure could be achieved by applying VOCs in boxes, airtight conditions, employing

735 safety measures required to handle pesticides by workers, and in stages prior to consumption,

736 such as during transportation or storage.

737

738 **Figure legends**

739 **Figure 1.** GC-MS chromatogram of VOCs produced by *T. atroviride* IC-11.

740 **Figure 2.** *In vitro* assay of the capability of pure 6PP to inhibit the growth of *B. cinerea* FB. A

741 PDA plate was inoculated with *B. cinerea* FB, inverted and exposed to 0, 5, 10, 20 and 45 µL of

742 commercial pure 6PP. The plates were incubated at 28 ℃ for 7 days. Letters indicate

743 significantly different groups (P = 0.05).

744 **Figure 3.** Control of gray mold in blueberries by *T. atroviride* IC-11 VOCs. A) Blueberries were

745 wounded, inoculated with 5 µL of *B. cinerea* FB 1×10^4 conidia/mL and incubated in *T. atroviride*

746 IC-11 VOCs mycofumigation chambers (left) and control (right) chambers at 20 ℃ during 14

747 days. The pictures show the results of a single experiment of six repetitions. B) Results obtained

748 after applying the same procedure at 4 ℃ during 31 days. C) Incidence of gray mold

749 corresponding to *T. atroviride* IC-11 VOCs (gray bars) and control (black bars) treatments after

750 different incubation days at 20 ℃. D) Incidence of gray mold in *T. atroviride* IC-11 VOCs (gray

751 bars) and control (black bars) treatments at 4 ℃. The letters a and b indicate significantly

752 different groups (P = 0.05).

753 **Figure 4.** Qualitative and quantitative analyses of the inhibition of *B. cinerea* FB hyphal growth

754 caused by *T. atroviride* IC-11 VOCs. A) Macroscopic observations of 5 µL drops of *B. cinerea*

755 FB 1×10^4 conidia/mL seeded on PDA slides and incubated in control (left) and *T. atroviride* IC-

756 11 VOCs (right) chambers after 48 h and 72 h at 28 ℃. B) Microscopic observations of *B.*

757 *cinerea* FB drops seeded on PDA slides (microscope at 40×). C) Procedure applied to measure *B.*

758 *cinerea* FB hyphal growth. Ten recipients containing 20 µL of *B. cinerea* FB 1×10^5 conidia/mL

759 were incubated in a Petri plate having wet filter paper and taped with a glass plate (left, control

760 treatment) or a 5 days old PDA plate colonized by *T. atroviride* IC-11 (*T. atroviride* IC-11VOCs

761 treatment, right). The plates were incubated at 28 ℃ for 16, 24, 36 and 48 h. D) Absorbance at

762 600 nm of *B. cinerea* FB growth in control (black circles) and *T. atroviride* IC-11 VOCs (white

763 circles) treatments after different incubation times at 28 ℃.

764 **Figure 5.** Germination assay of conidia from *B. cinerea* FB. A) Microscopic pictures (63×)

765 showing fully germinated *B. cinerea* FB conidia in control treatment (left) and non-germinated

766 conidia in *T. atroviride* IC-11 VOCs treatment (right) after 48 h at 28 ℃. B) Germination

767 percentages of conidia from *B. cinerea* FB in control (black circles) and *T. atroviride* IC-11

768 VOCs (white circles) treatments after different incubation times at 28 ℃.

769 **Figure 6.** Morphological alterations of *B. cinerea* FB produced by *T. atroviride* IC-11 VOCs. A)

770 Hyphae of *B. cinerea* FB exposed to VOCs emitted by *T. atroviride* IC-11 compared with a

771 control without treatment. B) Lysis, roughness, linearity loosing and flat-ribbon like alterations

772 produced in hyphae and conidiophores of *B. cinerea* FB exposed to *T. atroviride* IC-11 VOCs. C)

773 Vacuolation in hyphae of *B. cinerea* FB exposed to *T. atroviride* IC-11 VOCs. Pictures were

774 taken with microscope at 63×.

775 **Figure 7.** Inhibition of cell respiration of *B. cinerea* FB caused by *T. atroviride* IC-11 VOCs. A)

776 Qualitative analysis showing violet crystals of formazan produced by reduction of MTT in

777 control treatment and scarce reduction of MTT, which is yellow in the oxidized state, in *T.*

778 *atroviride* IC-11 VOCs treatment. B) Formazan crystals extracted from one recipient of Figure

779 7A in control (violet) and *T. atroviride* IC-11 VOCs treatments. C) Quantitative analysis of

780 inhibition of cell respiration. The absorbance of formazan was measured at 570 nm after different

35

781 incubation times at 28 ºC in control (black circles) and *T. atroviride* IC-11 VOCs (white circles)

782 treatments.

783 **Figure 8.** Cytotoxicity of liquid 6PP to human intestinal cells and *B. cinerea* FB. A) Viability

784 percentage of Caco-2 human cells in different concentrations of 6PP. B) Viability percentage of

785 *B. cinerea* FB conidia in growing concentrations of pure 6PP.

786

787 **Table 1.** Volatile organic compounds produced by *T. atroviride* IC-11 identified by headspace –

788 gas chromatography-mass spectrometry

Compound	Retention Time (min)	Abundance × 10^6 (cps)	Safety and hazard classifications	Concentration issues
2-pentylfuran	8.06	0.28	H226 (11.36%): Flammable [b] H302 (100%): Harmful if swallowed [b] Class III [c]	LD_{50} 1.2 g/kg bw in mouse [d] (Moran et al., 1980)
2-undecanone	12.74	0.08	H400 (99.38%): toxic to aquatic life [b] Class II [c]	LD_{50} 5 g/kg bw in rats [d] (O'Neil, 2001)
α-bergamotene	14.71	0.28	No available information [b] Class I [c]	No available information
6-pentyl-α-pyrone (6PP)	15.06	9.5	H315 (100%): skin irritant [b] H319 (100%): eye irritant [b] H335 (100%): may cause respiratory irritation [b] Class III [c]	Hazard reference value [e]: 1.5 µg/kg bw/day (Dorne et al., 2017) Threshold of concern: 90 µg/person/day (EFSA Panel, 2011)
β-bergamotene	15.44	3.2	No available information [b] Class I [c]	No available information
γ-cadinene	17.09	0.31	No available information [b] Class I [c]	No available information
Diterpenoid [a]	20.26	0.26		

789 [a] Tentatively identified

790 [b] Globally Harmonized System (GHS) classification

791 [c] Cramer toxic hazard classification estimated by using the software Toxtree v3.1.0. Class I: low.
792 Class II: intermediate. Class III: high.

793 [d] Values obtained from PubChem database

794 [e] The value was obtained from EFSA database, by entering the CAS number of 6PP (27593-23-3)
795 in the substance browser available at https://www.efsa.europa.eu/en/microstrategy/openfoodtox
796 (EFSA home page > resources > data reports > Chemical Hazards Database (OpenFoodTox) >
797 Chemical hazards).

798 LD_{50}: Lethal Doses media

37

799   bw/day: body weight per day

800

801 **Acknowledgments**

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809

810   5. References

811

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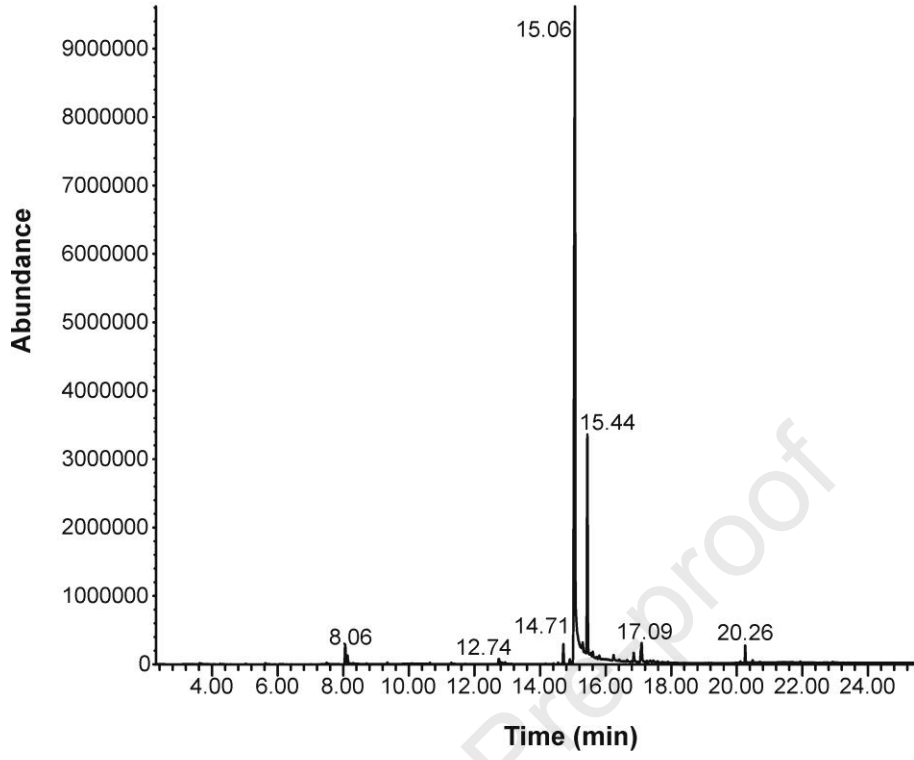931   *Advances in Nutrition, 11*(2), 224-236. doi.org/10.1093/advances/nmz065

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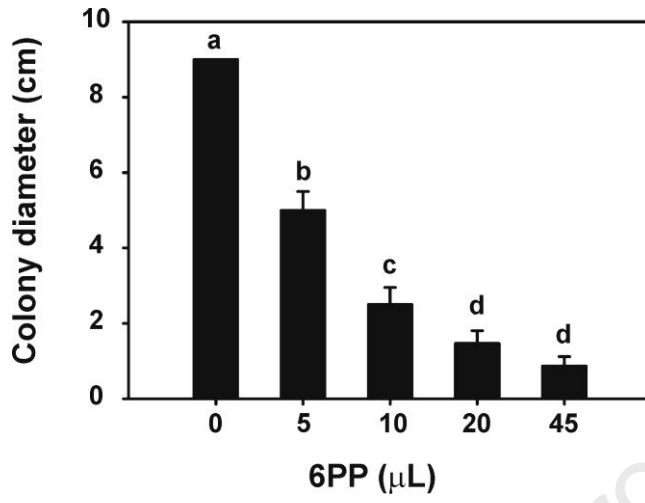
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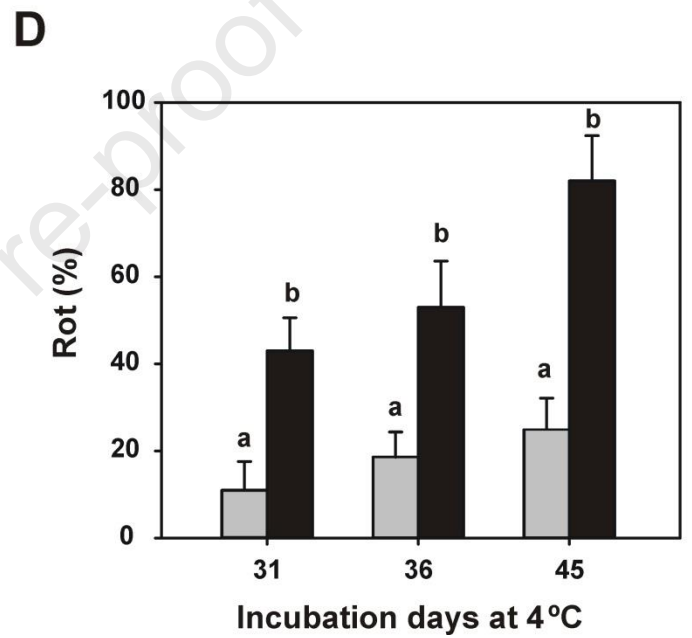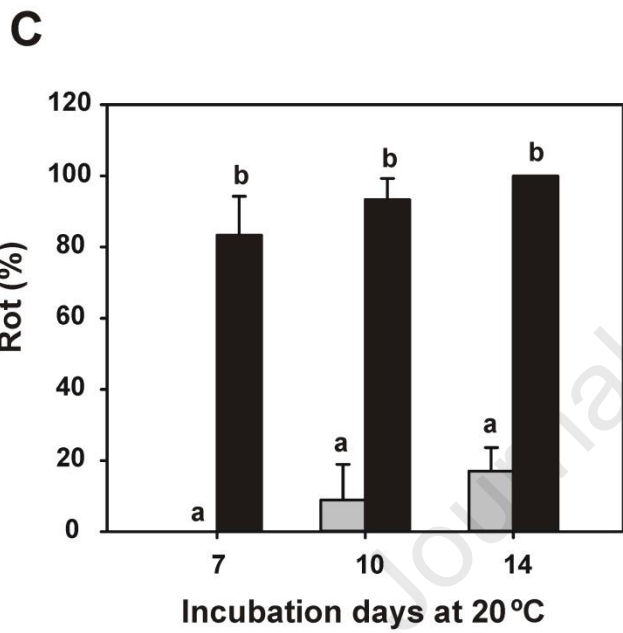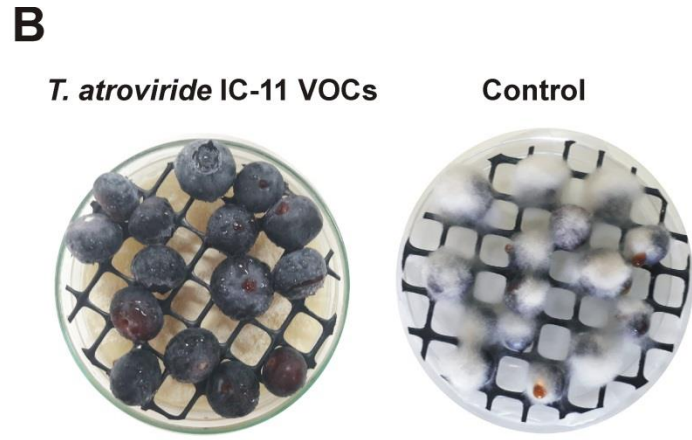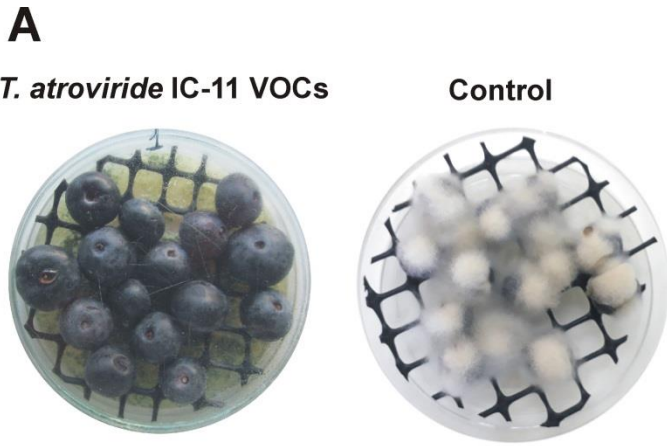
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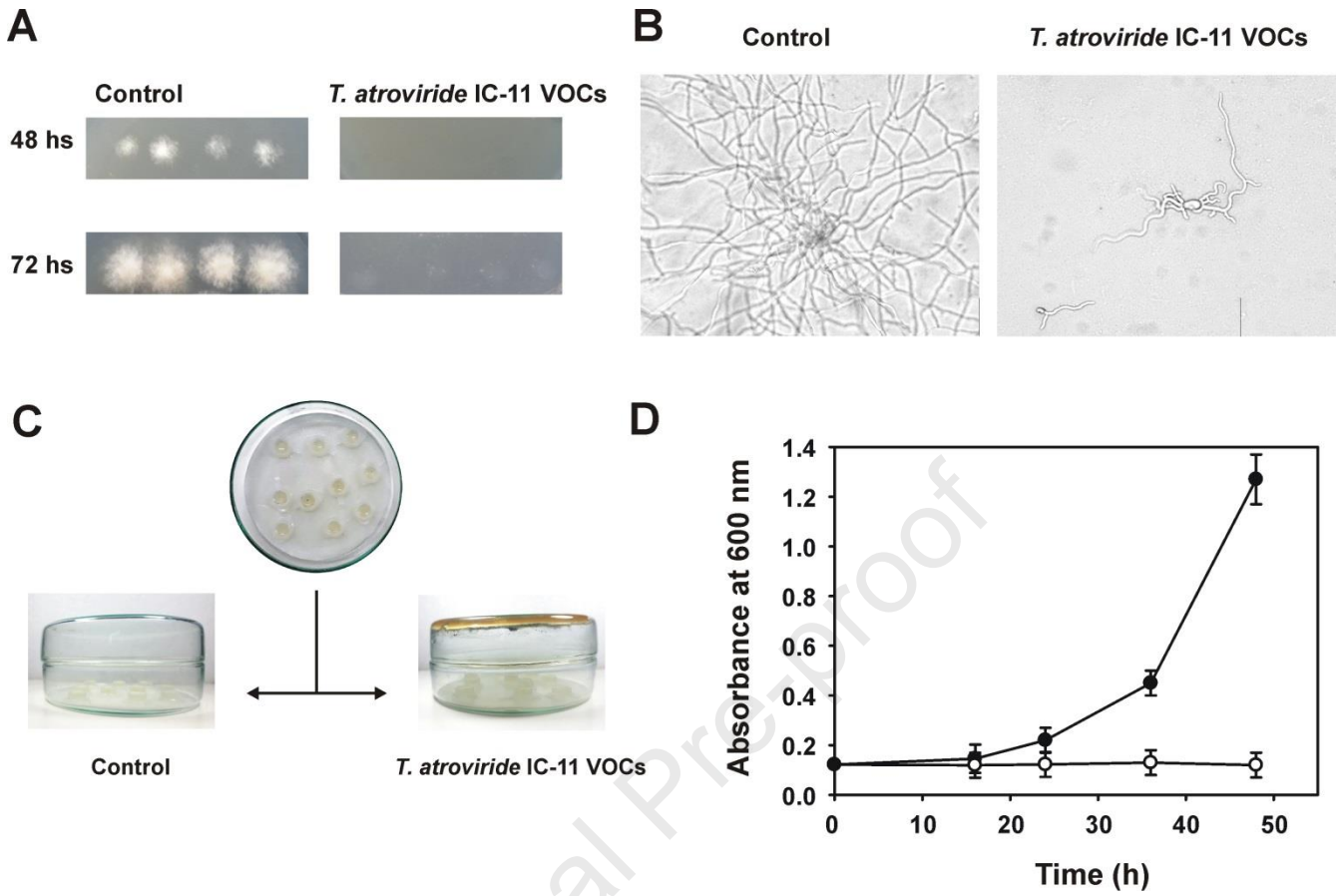1054   cundinamarcensis*) latex inhibit cell growth and cell wall integrity in *Botrytis cinerea*.

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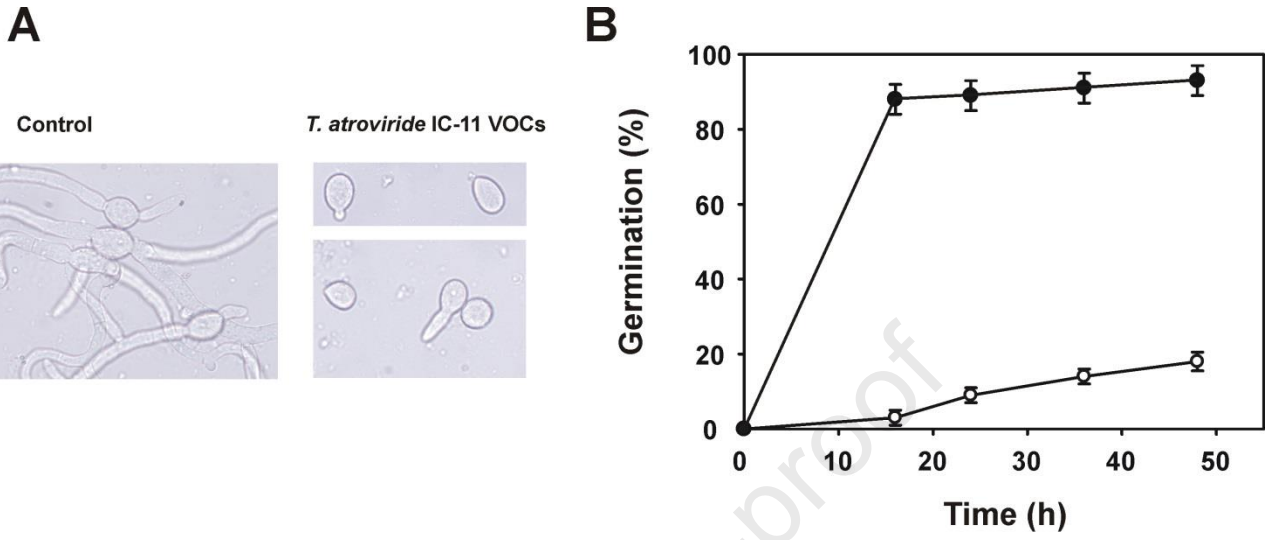
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A

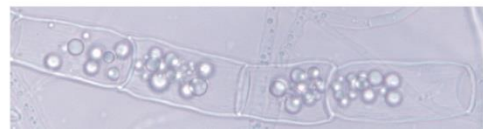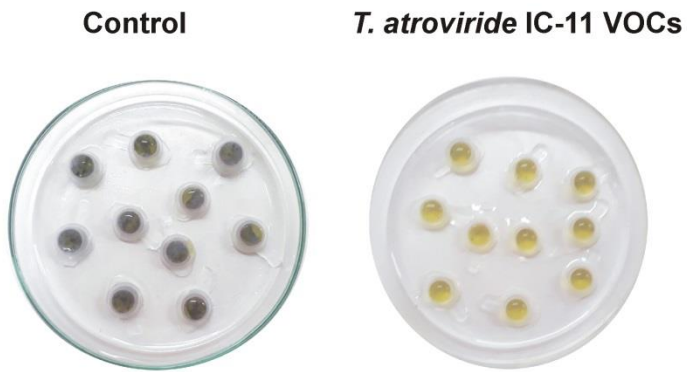*T. atroviride* IC-11 VOCs   Control

B

T. atroviride IC-11 VOCs   Control

C

Rot (%)

Incubation days at 20 °C

D

Rot (%)

Incubation days at 4 °C

A

Control   *T. atroviride* IC-11 VOCs

48 hs

72 hs

B   Control   *T. atroviride* IC-11 VOCs

C

Control   *T. atroviride* IC-11 VOCs

D

A

Control

T. atroviride IC-11 VOCs

B

A

T. atroviride IC-11 VOCs

Control

B

T. atroviride IC-11 VOCs

Control

C

A

Control   *T. atroviride* IC-11 VOCs
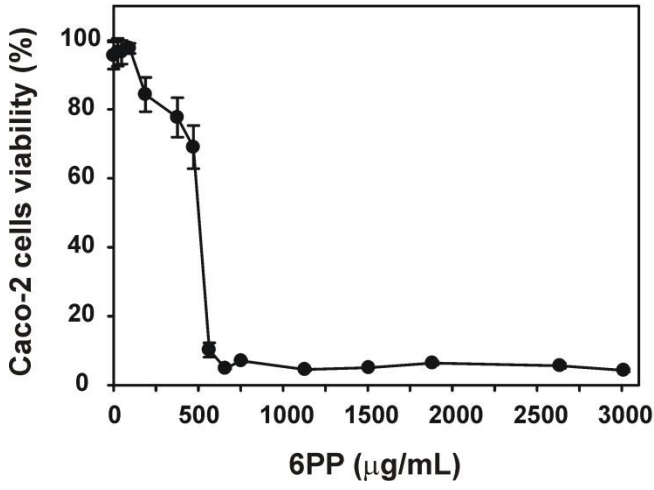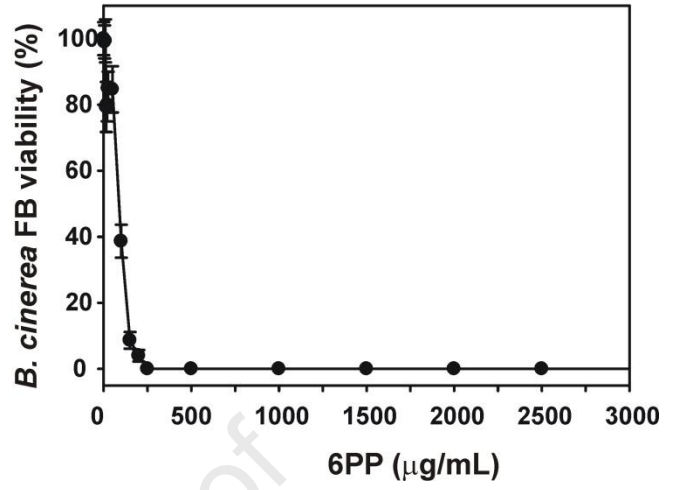
B

Control   VOCs
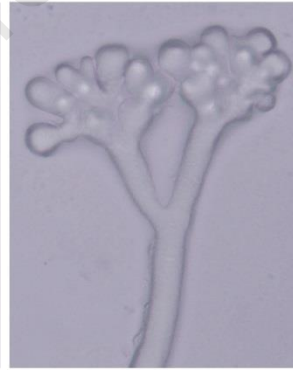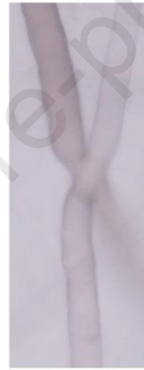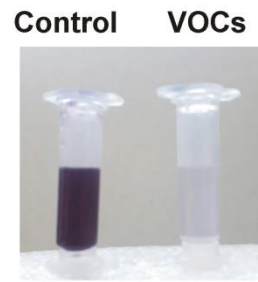
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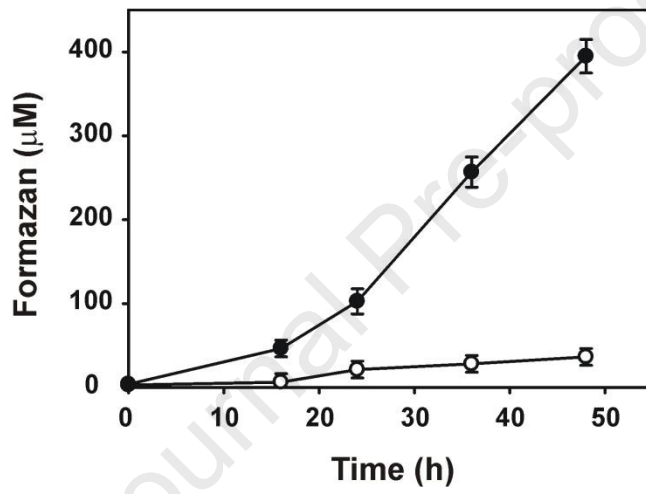
A

B

- Volatile compounds of *Trichoderma atroviride* IC-11 controlled *Botrytis cinerea* growth

- This control functioned optimally in blueberries after harvest at 4 ℃ and 20 ℃

- The volatile compounds inhibited hyphal growth and conidial germination

- The most abundant volatile compound was 6-pentyl-α-pyrone

- This compound controlled *B. cinerea* and presented low cytotoxicity on human cells

Declarations of interest: none