Effect of elevated atmospheric CO2 and temperature on the disease severity of rocket plants caused by Fusarium wilt under phytotron condition.

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22 November 2024

1 **Effect of elevated atmospheric CO_2 and temperature on**

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3 **wilt under phytotron conditions**

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15 Running title: Phytotrons to simulate climate changes on rocket Fusarium wilt

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Abstract

The severity of *F. oxysporum* f.sp. *conglutinans* on rocket plants grown under simulated climate change conditions has been studied. The rocket plants were cultivated on an infested substrate (4 log CFU g^{-1}) and a non-infested substrate over three cycles. Pots were placed in six phytotrons in order to simulate different environmental conditions: 1) 400-450 ppm CO_2, 18–22 °C; 2) 800-850 ppm CO_2, 18–22 °C; 3) 400-450 ppm CO_2, 22–26 °C, 4) 800-850 ppm CO_2, 22-26 °C, 5) 400-450 ppm CO_2, 26-30 °C; 6) 800-850 ppm CO_2, 26-30 °C. Substrates from the infested and control samples were collected from each phytotron at 0, 60 and 120 days after transplanting. The disease index, microbial abundance, leaf physiological performances, root exudates and variability in the fungal profiles were monitored. The disease index was found to be significantly influenced by higher levels of temperature and CO_2. Plate counts showed that fungal and bacterial development was not affected by the different CO_2 and temperature levels, but a significant decreasing trend was observed from 0 up to 120 days. Conversely, the *F. oxysporum* f.sp. *conglutinans* plate counts did not show any significantly decrease from 0 up to 120 days. The fungal profiles, evaluated by means of polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE), showed a relationship to temperature and CO_2 on fungal diversity profiles. Different exudation patterns were observed when the controls and infested plants were compared, and it was found that both CO_2 and temperature can influence the release of compounds from the roots of rocket plants. In short, the results show that global climate changes could influence disease incidence, probably through plant-mediated effects, caused by soilborne pathogens.

Introduction

Nowadays, how climate changes will influence plant-pathogen interactions and their impact on production is largely debated and represents a challenge for future programmes focused on disease management under global change conditions [1]. An increasing numbers of multidisciplinary approaches have reported the effects of rising temperature and CO_2 levels on crop productions and physiological changes [2]. On the other hand, researchers have only recently focused on plant disease prediction and management under climate changes [3]. Plant pathogens represents an important constraint for the security of our future food as a consequence of population increases, urbanization, globalization and changes in climate [4]. Moreover, several pathogens produce toxins and other compounds that are dangerous to human and animal health which could affect market and world trade. On these grounds, a multidisciplinary approach is needed to implement predictive models and include higher levels of ecological interactions [5]. As recently reported by an Intergovernmental Panel on Climate Changes [6], anthropogenic emissions of greenhouse gases have caused negative impacts on human and natural systems over the last three decades. In other words, changes in the atmospheric composition and temperature as well as humidity alterations can have an impact on host plant physiology and influence a plant's resistance against pathogens [7–9]. On one hand, rises in CO_2 and temperature can affect the sense and responses of a plant by increasing the photosynthesis rates, water and light-use efficiency, leaf surface properties, changes in anatomy, morphology, phenology and root exudates, thus profoundly modifying native plants and soil microbial communities [1,3,8,10,11]. On the other hand, elevated CO_2 influences the pathogenicity, host-pathogen interaction and epidemiology of fungal diseases [3,12-15,16]. An enlarged canopy coupled with a favourable microclimate offers more sites for infection and increases fungal pathogen fecundity, which has been shown to lead to twice the number of lesions for high CO_2 concentrations [17,18].

74 Furthermore, the mutation and selection of plant pathogens and the consequent development

75 of new strains have also been predicted [3,19].

76 Phytotron-based studies are optimized to study the interactions that occur between plants, soil

77 and soil microbiota and they combine environmental parameters such as light intensity, CO_2

78 concentrations, temperature, and relative humidity [20]. Phytotron chambers have in fact

79 been largely used to study the effects of global changes on several pathosystems [11, 14–

80 16,21]. Plants directly or indirectly affect the development of soil microbiota in the

81 rhizosphere and in bulk soil by means of root exudates [22]. However, most studies

82 conducted so far report contrasting results; such as a decrease [23], an increase [24] or no

83 changes [25] in microbial diversity and activities. The development of soil microbial

84 populations needs a multiphasic approach that can couple traditional microbial analysis with

85 molecular tools such as polymerase chain reaction denaturing gradient gel electrophoresis

86 (PCR-DGGE). This technique has been optimized to monitor soil microflora changes under

87 climate change conditions [16,26,27]. *Fusarium oxysporum* and the related *formae specialis*

88 cause greater economic damage to several crops than other plant pathogens [28]. Rocket

89 (*Eruca sativa*) is a high-value horticultural crop, mainly cultivated in the Mediterranean area,

90 up to 3-5 times in the same soil which is affected by emerging soilborne pathogens [20,29]

91 and *Fusarium oxysporum* f.sp. *conglutinans* has recently been detected in Italy on cultivated

92 (*Eruca sativa*) and wild rocket (*Diplotaxis tenuifolia*) [30].

93   The aim of this work was to study the effect of *F. oxysporum* f.sp. *conglutinans*,

94 artificially infested in a growing substrate, on rocket plants grown under simulated climate

95 change conditions with rising CO_2 concentrations and temperatures in phytotron chambers.

96 Six temperature/CO_2 combinations were studied. Disease incidence, the physiological

97 performances of leaves, microbial and fungal cultivable abundance and the main root exudate

98 components were monitored to evaluate the effects of climatic changes on disease

4

99   development over time. Furthermore, shifts in fungal communities were assessed using the

100   DGGE technique on DNA directly extracted from an infested growing substrate.

101

Materials and methods

Inoculum preparation

104   *F. oxysporum* f.sp. *conglutinans* (ATCC16600RB, Agroinnova, Grugliasco, Italy),

105   which is resistant to benomyl [31], was used and cultured in 1000-mL Erlenmeyer flasks

106   containing 250 mL of hydrolized casein. The flasks were incubated on a platform shaker at

107   20-25 °C for 12 days. Chlamydospores were recovered by means of centrifugation for 20 min

108   at 8000*g* at 20 °C, following the removal of mycelia fragments by sieving through

109   cheesecloth. The chlamydospore suspension was dried and mixed with sterile talc powder

110   (1:2 w/w), as described by Locke & Colhoun [32], and stored at room temperature for further

111   use. The number of chlamydospores per gram of talc was assessed by serial plating on a

112   Komada medium [33] containing 10 mg L^{-1} of benomyl (Benlate, 50% a.i.; DuPont de

113   Nemours, Milan, Italy). The talc formulation was incorporated into the soil to achieve the

114   desired inoculum of 4 Log colony forming units (CFU) g^{-1}.

115

Plant material and experimental set-up

117   Two experimental trials were carried out at Agroinnova (Grugliasco, Itay). Plastic

118   tanks containing 100L of a mixture (1:1 v/v) of peat-perlite substrate (Tecno2, Turco

119   Silvestro sphagnum peat moss, Albenga, SV, Italy) and sandy loam soil (pH, 7.3; organic

120   matter content, 2.2 %; cation exchange capacity, 2.6 meq/100 g soil) were prepared. The final

121   substrate was made up of: sand, 76 %; silt, 14 %; clay, 10%; pH, 7.51; organic matter

122   content, 2,59 % and had a cation exchange capacity of 5.99 meq/100 g soil and subjected to

123   steam sterilization before use. The substrate was artificially inoculated with *F. oxysporum* f.

124   sp. *conglutinans* (ATCC16600RB) to reach a final concentration of 4 Log CFU ml^{-1}. A non-

125   inoculated tank was used as the control.

126   *Eruca sativa* Mill seeds (cultivated rocket) were disinfected in a solution of 1%

127   sodium hypocloride plus 0.01% of Tween-20, and then rinsed twice in water for 1 min. The

128   seeds were air-dried at RT and stored at 4 °C until use. The seeds were sown in a greenhouse

129   in plug trays (20-26 °C, 70% RH and natural light condition). After 15-20 days, the first

130   seedling-leaves were developed. The rocket plants were left to grow under Phytotron

131   conditions for 7 days. Subsequently, 48 pots (2L each) were prepared from the inoculated

132   tank and another 48 pots were prepared from the non-inoculated tank and used as controls.

133   The rocket plants were then transplanted (4 plants/pot), and 8 inoculated and 8 non-

134   inoculated pots were placed in 6 different phytotrons (PGC 9.2, TECNO.EL, Italy). One

135   replicate consisted of two pots. The rocket plants were kept in the phytotrons under six

136   different temperature and CO_2 combinations according to the following ranges: 1) 400-450

137   ppm CO_2, 18-22 °C; 2) 800-850 ppm CO_2, 18-22 °C; 3) 400-450 ppm CO_2, 22-26 °C, 4) 800-

138   850 ppm CO_2, 22-26 °C, 5) 400-450 ppm CO_2, 26-30 °C;   6) 800-850 ppm CO_2, 26-30 °C.

139   The temperatures, light and humidity were changed gradually during the day in order to

140   simulate natural conditions. Three subsequent cultivation cycles were carried out in the same

141   pot. Each crop cycle lasted 35-37 days after transplanting. The plants were irrigated daily in

142   order to maintain the soil moisture at field capacity.

143   Substrate samples were taken at time 0 (immediately before plant transplanting) and

144   after 60 and 120 days for microbial enumeration and molecular analyses (PCR-DGGE), while

145   the physiological measurements of the plant leaves were conducted at the end of each rocket

146   cycle.

147

Disease incidence evaluation

The effectiveness of the different simulated climate change conditions on the severity of *F. oxysporum* f.sp. *conglutinans* on rocket was checked weekly by evaluating the pathogen development using a previously reported disease index [34]. Wilted plants were counted and removed and the final disease rating was made at the end of the experiment (35-37 days after transplanting). At the end of each cycle, re-isolation from infected plants on a Komada medium supplemented with 10 mg L^{-1} of benomyl was performed to confirm the presence of *F. oxysporum* as the causal agent of the observed symptoms. During the latter survey, the total fresh plant biomass was also rated using a technical balance (Orma SNC, Milano, Italy) to evaluate the effect of the treatments on plant growth.

Physiological measurements of the leaves

In order to observe the effects of the climate change conditions on the leaf physiological activity of the infected and control rocket plants, the photosynthetic efficiency and chlorophyll content were monitored. Measurements were performed following the experimental protocol reported by Pugliese et al. [35], with only minor modifications. The chlorophyll content index (CCI) was measured with the SPAD 502 chlorophyll meter (CCM-200, Opti-Sciences, Inc., Hudson, NH, USA), which determined the relative amount of chlorophyll in the leaf by measuring the absorbance in the red and near-infrared regions (650 and 940 nm, respectively). Chlorophyll meter readings were taken from each rocket plant in the second or third leaves (fully developed) from the top on ten randomly selected plants (one leaf/plant) at the end of each cultivation cycle. The photosynthetic efficiency measurements were performed on five randomly selected leaves using a portable continuous-excitation type fluorimeter (Handy-PEA, Hansatech

172 Instruments Ltd, Norfolk, UK), according to the manufacturer's instructions, at the end of

173 each cultivation cycle of rocket plants grown in infested and control substrates.

174

Root exudate analyses

176   In order to investigate the relationship between climate changes and the turnover of

177 low molecular weight organic compounds in the rhizosphere, water-soluble root exudates

178 were collected and analyzed. A sterilized $CaSO_4$ 0.01M solution (collection media) was used

179 to collect the root exudates [36]. For each condition tested, three randomly selected rocket

180 plants were taken from infested and not infested pots and   the soil was carefully removed

181 using deionized water, without damaging the roots. The plants were then placed in a 50 mL

182 centrifuge tube with 15 mL of collection media for 2 hours in each phytotron. The collection

183 media was then filtered through a 0.45 µm membrane filter. pH was measured and the

184 samples were freeze dried and dissolved in 3 mL of ddH_2O. Three main groups of organic

185 substances were analyzed: total organic carbon (TOC), total organic acids and amino acid

186 compounds.

187 TOC was determined by means of the colorimetric method. An aliquot of 1 mL was mixed

188 with 2 mL of $K_2Cr_2O_7$ (2 N) and 1 mL of H_2SO_4 concentrate and kept at 150 °C for 10 min.

189 The samples were cooled to room temperature and then analyzed by using a Spectroquant

190 Pharo 300 spectrophotometer (Merck, Darmstadt, Germany) at λ=585 nm. Glucose was used

191 as the reference C substance. For the amino acids analysis, 1 mL of sample was mixed with 1

192 mL of borate buffer (0.4 M pH=9.5) and 1 mL of dansyl chloride solution (Sigma-Aldrich,

193 St. Louis, MO, USA) at 10 mg/mL. The samples were incubated at 65 °C for 30 min [37].

194 The analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies,

195 Palo Alto, CA). Zorbax Eclipse Plus C18 (4.6x100 mm, 3.5 µm, Agilent Technologies) was

196 used for the chromatographic separation of the amino acids with a linear gradient from 10 to

197 100% of acetonitrile (Merck) in 15 min. The derivatized amino acids were detected by means

198 of a fluorescent detector with excitation at 335 nm and emission at 524 nm. A mixture of 20

199 standard amino acids (Sigma-Aldrich) was used as the reference. The organic acids were

200 separated with a Synergi Hydro-RP column (4.6x250 mm, 4 μm, Phenomenex, Torrance, CA,

201 USA) in an isocratic condition, using buffer phosphate (50 mM) as the mobile phase. The

202 detector was set at λ=214 nm. A mixture of standard organic acids (Sigma-Aldrich) was used

203 as the reference.

204

Microbial count

206 Substrate samples (25 grams) were collected from 8 pots for both infested and control

207 treatments, at a depth of 2-5 cm, and were placed in sterilized polyethylene bags using a

208 sterilized spatula. The samples were passed through a sieve to remove any vegetation, and

209 mixed at room temperature for 30 min with 225 mL of quarter strength Ringer's solution

210 (Merck) in sterilized flasks on a rotary shaker (100 rpm). Decimal dilutions were prepared in

211 quarter strength Ringer's solution and aliquots of 1 ml of the appropriate dilutions were

212 poured, in triplicate, onto the following media: plate count agar (PCA, Oxoid, Milan)

213 incubated at 28°C for 48h for the total bacterial counts; potato dextrose agar (PDA, Merck)

214 supplemented with streptomycin (0,5 g/L) and a Komada medium supplemented with 10 mg

215 L^{-1} of benomyl (Benlate, 50% w.g., DuPont, USA), kept at 25°C for 7 days for the total

216 fungal and *F. oxysporum* f.sp. *conglutinans* enumerations, respectively. The results were

217 calculated as the mean of the Log counts of three independent determinations.

218

Substrate DNA extraction and DGGE analysis

220 Infested substrate samples (250 mg) were taken from 4 pots/phytotron at 0, 60 and

221 120 days after transplanting for DNA extraction. DNA exctract according to the protocol

9

222 described by the NucleoSpin® Soil manufacturer (Macherey-Nagel, Germany) and was

223 quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific) and standardized

224 at 10 ng μl^{-1}. Fungal DNA was amplified using fungal ITS primers according to Gao *et al*.

225 [38]. PCR products for the ITS region were analyzed by denaturing gradient gel

226 electrophoresis (DGGE) at 25-35% using a Bio-Rad Dcode, as previously described [16].

227

Statistical analysis

229 The data obtained from the plate counts, root exudates and leaf physiological

230 measurements were analyzed using one-way analysis of variance (ANOVA), with treatments

231 being the main factor. ANOVA analyses were performed with the SPSS 22.0 statistical

232 software package (SPSS Inc., Cary, NC, USA). The Duncan HSD test was applied when

233 ANOVA revealed significant differences ($P < 0.05$). A database of fingerprints was created

234 using the PyElph software. A combined data matrix that included all the fingerprints for the

235 ITS region was obtained, and dendrograms of similarity were retrieved using the Dice

236 coefficient and the Unweighted Pair Group Method with the Arithmetic Average (UPGMA)

237 clustering algorithm [39] utilizing PyElph software [40]. The similarity distance matrix

238 generated through PyElph was used to build a Projection on Latent Structures - Discriminant

239 Analysis (PLS-DA) utilizing "mixOmics" in the R environment (www.r-project.org). The

240 obtained binary band-matching tables were considered to calculate the Shannon-Wiener

241 diversity index (*H'*) [41] using PAST (PAleontological STatistics) software [42].

242

Results

Disease index assessment and fresh biomass production

245   The disease index (DI, 0-100) was found to be significantly influenced by the

246   temperature and CO_2 levels (Table 1). No symptoms were observed for the control plants in

247   any of the cycles or phytotron conditions that were considered. Generally, higher levels of

248   CO_2 and temperature were factors that significantly influenced the DI in the infected plants in

249   the simulated conditions. At 18-22 °C, the DI was significantly lower for both CO_2 ranges (P

250   < 0.05) than   the other conditions. Conversely, the DI significantly increased at 22-26 °C,

251   particularly for higher CO_2 levels (P < 0.05) where a DI of 54 was reached. At 26-30 °C, a

252   slightly decreasing trend was recorded for both CO_2 levels, although a higher DI (43,4) was

253   observed at 800-850 ppm CO_2, with a significantly different value than that of the 18-22 °C

254   conditions (P < 0.05). The fresh weight (FW) data pertaining to the infected plants showed

255   significant differences (P < 0.05) as a consequence of temperature, CO_2 and disease

256   development (Table 1). Lower FW values were obtained at 18-22 °C for both CO_2 conditions

257   and in phytotrons 4 and 6 (800-850 ppm CO_2, 22-26 °C and 800-850 ppm CO_2, 26-30 °C,

258   respectively) where disease development was higher (Table 1). The control plants showed a

259   higher FW   than the diseased rocket plants, with the exception of 18-22 °C (phytotrons 1

260   and 2: 400-450 ppm CO_2, 18–22 °C and 800-850 ppm CO_2, 18–22 °C) in which FW was

261   significantly lower (P < 0.05) than the other tested.

262

Effect on the physiological performances of the leaves

264   The photosynthetic efficiency index (PI) for the infected plants showed a particularly

265   pronounced and statistically significant (P < 0.05) decreasing trend for all the conditions

266   tested at the end of the cycles (Fig. 1). In other words, PI appeared to be particularly affected

267   by disease development (Table 1) coupled with the environmental conditions. Lower PI

268   values (P < 0.05) were recorded for the infected plants from   phytotrons 1, 2 and 4 (400-450

269   ppm CO_2, 18–22 °C; 800-850 ppm CO_2, 18–22 °C and 800-850 ppm CO_2, 22-26 °C,

270 respectively). Moreover, the highest temperature provided the best evidence of impaired PI at

271 high CO_2 in the infected plants.

272 In general, the non-inoculated plants showed higher photosynthetic efficiency than the

273 infected ones, with the exception of those from phytotron 4 (800-850 ppm CO_2, 22-26 °C)

274 where PI was lower than for the other conditions.

275 Similar trends were also observed for the CCI measurements in the infected samples

276 (Fig. 2) where no significant differences were observed between the phytotrons ($P < 0.05$).

277 The non-infected plants showed higher levels of chlorophyll content than the infected rocket

278 plants. Furthermore, significantly lower values were measured in the non-infected plants in

279 phytotrons 3 and 5 (400-450 ppm CO_2, 22–26 °C and 400-450 ppm CO_2, 26-30 °C,

280 respectively) ($P < 0.05$). Overall, the physiological measurements have confirmed that the

281 disease development led to a remarkable decrease in the physiological performances of the

282 leaves.

283

Effect on root exudates

285 The results pertaining to the root exudates (Table 2) showed a similar trend for TOC

286 and amino acids (AA), when the exudates from the infected roots and the control plants were

287 compared. In both cases, when the temperature was lower (18-22 °C), an increased

288 concentration was registered in the samples from the infected plants. At 22-26 °C and 26-30

289 °C, the TOC and AA concentrations significantly increased for higher CO_2 levels ($P < 0.05$).

290 No significant differences were observed between the infected and non-infected plants for

291 lower CO_2 concentrations. The root exudates from the infected samples did not show any

292 significant differences in TOC concentration at 400-450 ppm of CO_2; conversely, an

293 increasing trend of TOC concentration was registered when the temperature and CO_2 level

294 were increased ($P < 0.05$).

295   The amino acid levels from the infected plants were not different at lower CO_2 levels;

296   conversely, higher CO_2 levels only induced a significant increase in amino acids at high

297   temperatures ($P < 0.05$).

298   The organic acids, in general, showed a different trend. When CO_2 and temperature

299   were kept low, no significant differences were registered between the treatments or in the

300   phytotrons. However, increasing the concentration of CO_2 led to an increased production of

301   organic acids, but only in the control samples. Furthermore, at 22-26 °C, for both levels of

302   CO_2, the organic acid concentration increased for all the samples. In general, the organic acid

303   concentrations of all the samples were always significantly higher for higher CO_2 levels ($P <$

304   0.05).

305

Microbial analysis of substrate samples

307   The results of the bacterial and fungal plate counts on specific media are shown in

308   Tables 3, 4 and 5. The plate counts of mesophilic bacteria (TBC) (Table 3) from infested

309   substrate samples were not affected by the different CO_2 and temperature levels. A significant

310   decreasing trend was observed from time 0 up to 120 days for all the conditions considered (P

311   < 0.05). Furthermore, the samples at 120 days were not significantly different between the

312   phytotrons. The control substrate samples showed a similar trend to that observed in the

313   infested samples. However, the samples taken after 120 days in phytotrons 2 and 4 (800-850

314   ppm CO_2, 18–22 °C and 800-850 ppm CO_2, 22-26 °C) were significantly higher ($P < 0.05$),

315   that is, 7.17 and 6.87 Log CFU g^{-1}, respectively.

316   The total fungal community plate counts (TFC, Table 4) from the infested samples mirrored

317   the bacterial counts; a significant decreasing trend ($P < 0.05$) was observed over time from

318   time 0, without any significant differences between the phytotrons at 120 days. The control

319   substrate sample counts were similar to the infested ones, with the exception of those from

13

320   phytotron 2 (18-22 °C and 850 ppm CO_2) at 120 days, when the statistical values were higher

321   (4.89 Log CFU g^{-1}) than in the other conditions. However, (among fungal community)

322   *Fusarium oxysporum* f.sp. *conglutinans* development was observed over time on the Komada

323   medium supplemented with benomyl in the infested substrate samples. In general, most of the

324   fungal community was represented by the pathogen, which developed over time and almost

325   completely covered the total fungal community values up to the last survey (120 days). In

326   particular, a general increasing trend was observed for all the conditions from time 0 in the

327   phytotrons compared to time 120. Although no significant differences between phytotrons

328   were observed at 120 days. A significant increasing trend from time 0 was observed for

329   phytotrons 2 and 4 for high CO_2 conditions in the last survey (from 3.80 to 4.38 and from

330   3.73 to 4.32 Log CFU g^{-1}, respectively) ($P < 0.05$). With the exception of 26-30 °C, high

331   concentrations of CO_2 were shown to positively affect the growth of *Fusarium oxysporum*

332   f.sp. *conglutinans*. No pathogen was detected in the control substrate samples.

333

DGGE analysis of the fungal microbiota

335   The PCR-DGGE fingerprints of the fungal community obtained from the DNA

336   extracted directly from the infested samples in all the adopted conditions are presented in

337   Figure 3. Repeated DNA extraction and PCR-DGGE analysis confirmed the results of the

338   fingerprinting.

339   The ITS based DGGE analysis showed an average result of 11.7 bands per sample (min 3,

340   max 17), (S1Fig.). The similarity matrix generated through the PyElph software was used to

341   build a PLS-DA, as a function of the CO_2 concentration, and the results showed a clear

342   separation between samples at 850 and samples at 450 ppm CO_2 (Fig. 3A). Regardless of the

343   temperature, the PLS models showed a clear separation of the samples (Fig. 3B). When the

344   samples were grouped on the basis of the sampling time, the samples at time 0 were clearly

345 separated from the other samples (Fig. 3C). The PLS-DA analysis, as a function of the

346 phytotrons (Fig. 3D), resulted in a certain degree of separation. The separation was

347 particularly important for the samples from phytotron 4, which appear to group together and

348 to be separated from the other phytotrons.

349   Furthermore, a binary band-matching table was analyzed in order to calculate the

350 index of diversity (Shannon-Wiener diversity index *H'*). The results, using only the data from

351 the ITS region, revealed, on the basis of their phytotron origin, that there was a biological

352 diversity in the samples. In particular, the *H'* index and richness of the fungal community

353 changed according to the environmental conditions that were set, and ranged from 1.61 to

354 2.83 and 5 to 17, respectively. At the end of the experiment, the *H'* index (1.79) and richness

355 (10) were lower for phytotrons 4 and 6 (22-26 and 26-30 °C at 850 ppm CO_2), and the H'

356 index (2.83) and richness (17) were higher for Phytotron 1 (18-22 °C and 450 ppm CO_2).

357 Higher CO_2 and temperature were shown to be selective and to reduce fungal community

358 diversity.

359

Discussion

361   Plants that grow under elevated temperature and CO_2 concentrations often exhibit

362 positive responses by increasing photosynthesis and/or water and nutrient use efficiency [43].

363 However, how increases in CO_2 might affect plant-pathogen interaction has not been

364 addressed or debated to any great extent. The aim of this work was to evaluate the effect of

365 simulated climate changes, by increasing CO_2 concentrations and temperature levels, on the

366 severity of *Fusarium oxysporum* f.sp. *conglutinans* on rocket plants. Phytotron chambers

367 have already been used in several studies, since they provide total control of the

368 environmental conditions and reproducible data in diverse pathosystems [20, 14-16]. Climate

369 change is an on-going phenomenon, which affects whole ecosystems and causes multitrophic

370   interactions that are difficult to understand [1]. The possibility of reproducing different

371   climatic conditions by coupling carbon dioxide and temperature levels can/has been

372   considered be very useful to obtain a better understanding of plant-pathogen interactions, as

373   has been done in the present work which deals with a soilborne pathogen. Focusing on plant-

374   soil systems, it is well known that plants directly or indirectly control and influence the

375   multitrophic interactions of the soil through root exudates [22]. The results reported in this

376   study refer to disease incidence, cultivable abundance, leaf physiological performances, root

377   exudate analyses, and variability in substrate fungal profiles by PCR-DGGE. Several studies

378   have reported that rising CO_2 concentrations can cause plants to modify their root

379   architecture and exudation compounds in the rhizosphere [44,45]. In addition, it has been

380   predicted that global warming will directly influence multitrophic interactions if the soil

381   temperature exceeds its buffering capacity and this could be followed by changes in the

382   quality, quantity and diversity of plant and soil microbial communities and therefore plant

383   pathogen development [1]. In the present study, a good disease level was reached under

384   artificial inoculation of *F. oxysporum* f.sp. *conglutinans*, thus making it possible to evaluate

385   the effect of the different climate change simulations. The temperatures and CO_2

386   combinations in the six environmental conditions had a significant influence on the incidence

387   of *Fusarium* wilt. Higher CO_2 concentrations and temperatures caused a significant increase

388   in DI, in particular in the 22-26 °C range (Phytotron 4, Table 1). The effect of increased CO_2

389   concentrations was still observed at 26-30°C on disease severity development, although

390   lower values were observed than in the 22-26 °C range, probably due to the suboptimal range

391   of temperature that limits pathogen development. Although elevated CO_2 had a significant

392   effect on *Fusarium* wilt incidence, the increase in temperature also had a significant effect on

393   disease development, particularly at 22-26 °C, which is a favourable range of temperatures

394   for disease development [30]. As previously reported, higher temperature or CO_2 levels

395   generally correspond to a greater severity of *Fusarium* wilt in other crops such as lettuce

396   [16,46] or wheat [47]. As reviewed by Ainsworth and Rogers [8], rising CO_2 concentrations

397   and temperatures could influence leaf physiology, morphology and indirectly crop production

398   by increasing or blocking their metabolic performances and photosynthetic efficiency.

399   However, no relevant effects linked to disease development or environment conditions have

400   been observed on PI and CCI data for infested and control plants, respectively. In fact, in

401   infected rocket plants, large reductions of both indices have been recorded compared to

402   controls, thus suggesting the high susceptibility of the photosynthetic machinery already at

403   lower disease severity values. Furthermore, photosynthetic efficiency and leaf chlorophyll

404   content are indicators of photosynthetic activity and chlorophyll stability. Fluorimeters and

405   SPAD chlorophyll meters are frequently used for the measurement of foliar damage provoked

406   by different biotic and abiotic stresses [35,48], although they were not considered useful for

407   the present investigation.

408   Recent research in plant biology has pointed out the importance and the role of root

409   exudates in mediating biological interactions in the rhizosphere. The chemical components of

410   root exudates could deter or attract an organism with different effects on the plants. This

411   would be particularly important during the pathogenesis of root-infecting fungal pathogens.

412   However, the signalling and composition of root exudates in plant-pathogen interactions have

413   not yet been elucidated [49-51]. To the best of the authors' knowledge, this is the first report

414   to have been made on the analysis of root exudates on wilted rocket plants under simulated

415   climate change conditions. The results have shown a different exudation pattern between the

416   controls and infected plants. Interestingly, higher levels of pH, organic acid and TOC (mainly

417   composed of sugars) were observed in the infected samples in phytotron 4 where the disease

418   index was higher. In addition, high values of organic acid, TOC and amino acids were also

419   recorded in phytotron 6, where a consistent level of DI was observed. These results suggest

17

420   the combined temperature and high CO_2 effect on root component release. In particular, the

421   TOC compounds underwent a significant increase, since they represent a carbon source for

422   microbial metabolism and energy that in turn could influence the attraction and severity of

423   the soilborne pathogen. Similarly, Kerks et al. [52] reported the activation of root exudate

424   chemotaxis and pathogenicity genes of *S. enterica* serovar Typhimurium in lettuce, which are

425   involved in root attachment and subsequent colonization. Although a great deal of

426   information is available concerning the relationships between symbionts and plants, limited

427   knowledge exists about the communication between plants and root pathogens mediated by

428   rhizodeposits and/or by pathogen metabolite production (e.g. volatile organic compounds,

429   VOCs) [53]. Further studies are needed to increase analytical skills and to analyse the

430   chemistry of root exudates, and thus to resolve the dialogue that takes place between

431   pathogens and plant roots. In addition, root exudates represent the main source of soil organic

432   carbon, defined as soluble low-molecular weight components that are mainly composed of

433   sugars, amino acids, organic acids and other secondary metabolites which vary from plant to

434   plant and which are able to shape the rhizosphere microbiome [22]. Conversely, no relevant

435   effects of CO_2 or temperature levels have been observed in the control plants to explain the

436   variation in the root exudate components in phytotron conditions, in line with what has been

437   reported by Uselman et al. [54] in *Robinia pseudoacacia*.

438   It is well known that DGGE fingerprints can be used to describe microbial

439   composition and diversity, but they do not provide any information on the abundance and

440   concentration of separate microbial species [55]. For this reason, this limitation has partially

441   been addressed here by conducting plate counts on several media. In the present experimental

442   conditions, the total bacteria and fungi plate counts in both the infested and control substrate

443   samples showed a decreasing trend over time up to the end of the experiment, without climate

444   simulation effects being observed. These results suggested that the forecast increase in CO_2

445 and temperature levels due to climate changes would have a limited effect on fungal and

446 bacterial development. Recent reports [16,26,56] have shown similar results, while other

447 studies have reported results that depend to a great extent upon the host, microorganisms and

448 environment [57]. Conversely, the *F. oxysporum* f.sp. *conglutinans* plate counts at the end of

449 the present experiment have shown a slight increase or no significant variation for time 0,

450 except for phytotron 4 at higher CO_2 concentrations, where the development and disease

451 incidence were significantly higher. In phytotron 6, where disease incidence was high, no

452 significant development of pathogen over time was detected. This behaviour supports the

453 hypothesis of a plant-mediated effect increasing disease incidence. The variability in the

454 fungal profiles was assessed by means of the PCR-DGGE approach, which has frequently

455 been used, with good results, in other simulated global change studies [16,26]. The samples

456 were clearly separated in relation to the temperature or CO_2. In addition, this result was

457 further confirmed from an examination of the Shannon-Wiener indices. Interestingly,

458 phytotrons 4 and 6 showed the least diversity and the highest DI. Thus, a low diversity might

459 have a higher impact on disease development efficiency than the species visualized from the

460 DGGE examination. The loss in diversity caused by higher temperature and CO_2 levels could

461 be due to a plant-mediated effect that causes the selection of a few dominant species and

462 which may exclude others species through a competition strategy.

463

464 # Conclusions

465   The disease incidence of pathogenic *Fusarium* species could increase due to the

466 effects of the global changes that have been predicted for the future. The present

467 experimental conditions have shown a coupled temperature and CO_2 effect on disease

468 severity, which is probably plant-mediated, as reported for other pathosystems. Although no

469 specific predictions can be made on field conditions, the data obtained from phytotron growth

470 chambers could help to unravel the complexity of plant-soilborne pathogen interactions that

471 take place under climate change conditions, in order to implement model prediction and

472 prevention strategies.

473

Acknowledgements

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631

632 **Fig 1. Photosynthetic efficiency measurements.** Effect of different CO_2 and temperature
633 combinations on the photosynthetic efficiency of the leaves (PI) of rocket plants grown in a
634 substrate artificially infested with *F. oxysporum* f.sp. *conglutinans* and the control. Tukey's
635 HSD test ($P < 0.05$).
636
637 **Fig 2. Assessment of chlorophyll content.** Effect of different CO_2 and temperature
638 combinations on the chlorophyll content of the leaves (CCI, °SPAD) of rocket plants grown
639 in a substrate artificially infested with *F. oxysporum* f.sp. *conglutinans* and the control.
640 Tukey's HSD test ($P < 0.05$).
641
642 **Fig 3. PLS-DA models based on DGGE similarity distance matrix.** Plot A, PLS-DA
643 models based on the DGGE similarity matrix as a function of CO_2: 400-450 ppm (blue),
644 800-850 ppm (yellow); Plot B, PLS-DA models based on the DGGE similarity matrix as a
645 function of the temperature: 26°C (blue), 22°C (yellow) ,and 30°C (red): Plot C, PLS-DA
646 models based on the DGGE similarity matrix as a function of the time: time 0 (red), time 60
647 (yellow) and time120 (blue); Plot D; PLS-DA models based on the DGGE similarity matrix
648 as a function of the phytotrons: 1 (red), 2 (yellow), 3 (blue), 4 (black), 5 (green), 6 (pink).
649

650 **Table 1.** Disease index (0–100) and total fresh weight of the plants at the end of the replicates (FW).

651

	Parameters	Phytotron [a]					
		1 (400-450ppm CO_2 18-22°C)	2 (800-850ppm CO_2 18-22°C)	3 (400-450ppm CO_2 22-26°C)	4 (800-850ppm CO_2 22-26°C)	5 (400-450ppm CO_2 26-30°C)	6 (800-850ppm CO_2 26-30°C)
Infected plants	Disease Index (0-100)	17.2 ± 9.4 a	18.80 ± 11.40 a	25.00 ± 12.5 ab	54.00 ± 12.20 c	22.90 ± 3.60 ab	43.40 ± 11.90 bc
	Fresh Weight (g plants^{-1})	3.43 ± 0.21 a	2.73 ± 0.29 a	12.33 ± 0.21 c	3.87 ± 0.06 a	14.45 ± 0.13 d	9.70 ± 0.18 b
Control plants	Disease Index (0-100)	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a	0.00 a
	Fresh Weight (g plants^{-1})	3.80 ± 0.44 a	3.88 ± 0.21 a	13.33 ± 0.41 b	14.50 ± 0.63 b	16.27 ± 0.86 c	13.03 ± 0.46 b

[a]The disease index (0-100) and total plant fresh weight were expressed on the basis of three replicates. Values with different superscripts in the same row differ significantly according to Tukey's HSD test (P<0.05)

652

653

654 **Table 2.** Main root exudate components analyzed on rocket plants cultivated in infested and control substrates collected at the end of the cycle.

655

	Parameters	Phytotron[a]					
		1 (400-450ppm CO_2 18-22°C)	2 (800-850ppm CO_2 18-22°C)	3 (400-450ppm CO_2 22-26°C)	4 (800-850ppm CO_2 22-26°C)	5 (400-450ppm CO_2 26-30°C)	6 (800-850ppm CO_2 26-30°C)
Infested samples	pH	7.23 ± 0.15 ab	7.38 ± 0.32 ab	7.10 ± 0.09 ab	7.65 ± 0.55 b	7.36 ± 0.19 ab	7.35± 0.23 ab
	Amino acids (mM g^{-1})	1.57 ±0.04 ab	1.92 ± 0.18 a-c	1.92 ± 0.28 a-c	2.78 ± 0.18 a-c	2.00 ± 0.17 a-c	4.97 ±0.66 e
	Organic acids (mM g^{-1})	1.90 ± 0.14 a	2.35 ± 0.12 a-c	5.86 ± 0.14 e	7.39 ± 0.42 f	1.54 ± 0.18 a	3.01 ± 0.33 cd
	TOC (mg g^{-1})	57.2 ± 1.09 a-c	50.6 ± 2.97 a	67.1 ± 3.17 de	77.8 ± 5.80 ef	57.2 ± 1.09 ab	98.7 ± 3.06 g
Controls	pH	6.83 ± 0.10 a	7.08 ± 0.13 ab	7.01 ± 0.12 ab	7.29 ± 0.07 ab	7.26 ± 0.11 ab	7.22 ± 0.14 ab
	Amino acids (mM g^{-1})	2.27 ±0.24 a-c	3.20 ± 3.14 a-d	2.19 ± 0.26 a-c	1.54 ± 0.03 a	2.08 ± 0.35 a-c	2.43 ±0.46 b-d
	Organic acids (mM g^{-1})	1.99 ± 0.07 ab	5.72 ± 0.27 e	8.07 ± 0.68 f	2.90 ± 0.22 b-d	3.57 ± 0.41 d	2.37 ± 0.38 a-c
	TOC (mg g^{-1})	74.0 ± 3.89 de	89.3 ± 5.58 fg	58.0 ± 2.69 a-c	46.0 ± 3.70 a	56.1 ± 3.82 a-c	65.1 ± 6.22 b-d

[a] The root exudate parameters were expressed as the mean values of three plants for three cultivation cycles. Values with different superscripts in the same row differ significantly according to Tukey's HSD test (P<0.05)

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657

658 **Table 3.** Total bacterial counts (TBC) of mesophilic bacteria from the infested and control substrate samples following incubation in phytotrons

659 for 120 days.

660

	Sampling day	Phytotron (Log CFU g^{-1} ± SD[a])					
		1	2	3	4	5	6
		(400-450ppm CO_2 18-22°C)	(800-850ppm CO_2 18-22°C)	(400-450ppm CO_2 22-26°C)	(800-850ppm CO_2 22-26°C)	(400-450ppm CO_2 26-30°C)	(800-850ppm CO_2 26-30°C)
Infested samples	0	7.38 ± 0.07 e	7.08 ± 0.11 c-e	7.19 ± 0.02 de	6.86 ± 0.03 b-e	7.05 ± 0.02 c-e	7.09 ± 0.02 c-e
	60	6.36 ± 0.10 ab	7.01 ± 0.05 c-e	6.80 ± 0.17 a-d	6.77 ± 0.07 a-d	6.56 ± 0.17 a-c	6.32 ± 0.28 ab
	120	6.45 ± 0.35 ab	6.43 ± 0.13 ab	6.54 ± 0.09 a-c	6.30 ± 0.52 a	6.34 ± 0.09 ab	6.26 ± 0.10 a
Controls	0	7.15 ± 0.08 c-e	7.39 ± 0.09 de	7.60 ± 0.02 e	7.19 ± 0.11 c-e	7.46 ± 0.02 de	7.22 ± 0.04 c-e
	60	6.40 ± 0.35 a	7.19 ± 0.18 c-e	6.62 ± 0.28 ab	7.13 ± 0.09 cd	6.91 ± 0.28 bc	7.57 ± 0.23 de
	120	6.26 ± 0.15 a	7.17 ± 0.07 c-e	6.42 ± 0.07 a	6.87 ± 0.10 c	6.57 ± 0.07 ab	6.17 ± 0.06 a

661 [a]The plate counts were calculated as the mean Log counts of the three replicates. Values with different superscripts differ significantly according to Tukey's HSD test

662 (P<0.05)

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664

665 **Table 4.** Total fungi community counts (TFC) from the infested and control substrate samples following incubation in phytotrons for 120 days.

666

	Sampling day	Phytotron (Log CFU g^{-1} ± SD[a])					
		1	2	3	4	5	6
		(400-450ppm CO_2 18-22°C)	(800-850ppm CO_2 18-22°C)	(400-450ppm CO_2 22-26°C)	(800-850ppm CO_2 22-26°C)	(400-450ppm CO_2 26-30°C)	(800-850ppm CO_2 26-30°C)
Infested samples	0	6.59 ± 0.36 g-i	6.19 ± 0.23 f-h	7.13 ± 0.06 i	6.43 ± 0.11 g-i	5.92 ± 0.11 e-g	6.88 ± 0.08 hi
	60	5.12 ± 0.20 a-d	5.30 ± 0.24 b-e	5.47 ± 0.05 c-e	5.56 ± 0.14 d-f	5.06 ± 0.72 a-d	5.35 ± 0.15 b-e
	120	4.65 ± 0.07 ab	4.77 ± 0.10 a-c	4.81 ± 0.04 a-c	4.42 ± 0.16 a	4.84 ± 0.13 a-c	4.80 ± 0.06 a-c
Controls	0	6.52 ± 0.09 f	6.69 ± 0.40 f	6.30 ± 0.13 f	6.23 ± 0.24 ef	6.30 ± 0.00 f	6.55 ± 0.30 f
	60	5.12 ± 0.18 bc	5.75 ± 0.07 de	5.08 ± 0.16 ab	5.45 ± 0.04 cd	5.08 ± 0.07 bc	5.08 ± 0.11 bc
	120	4.53 ± 0.07 a	4.89 ± 0.16 c-e	4.75 ± 0.13 ab	4.93 ± 0.21 a-c	5.00 ± 0.08 a-c	4.74 ± 0.14 ab

667 [a]The plate counts were calculated as the mean Log counts of the three replicates. Values with different superscripts differ significantly according to Tukey's HSD test

668 ($p < 0.05$)

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671 **Table 5.** Plate counts of *Fusarium oxysporum* f.sp. *conglutinans* from the infested substrate samples following incubation in phytotrons for 120

672 days.

673

| Sampling day | | Phytotron (Log CFU g^{-1} ± SD[a]) | | | | | |
		1 (400-450ppm CO_2 18-22°C)	2 (800-850ppm CO_2 18-22°C)	3 (400-450ppm CO_2 22-26°C)	4 (800-850ppm CO_2 22-26°C)	5 (400-450ppm CO_2 26-30°C)	6 (800-850ppm CO_2 26-30°C)
Infested samples	0	3.77 ± 0.04 ab	3.80 ± 0.05 ab	4.24 ± 0.00 a-d	3.73 ± 0.12 a	3.78 ± 0.08 ab	4.00 ± 0.18 a-c
	60	4.00 ± 0.10 a-c	4.89 ± 0.02 e	4.46 ± 0.04 de	4.91 ± 0.15 e	4.06 ± 0.04 a-c	4.81 ± 0.10 de
	120	4.11 ± 0.04 a-c	4.38 ± 0.19 b-e	4.34 ± 0.74 a-e	4.32 ± 0.10 bc	4.18 ± 0.19 a-c	3.87 ± 0.11 a-c

674 [a]The plate counts were calculated as the mean Log counts of the three replicates. Values with different superscripts differ significantly according to Tukey's HSD test

675 ($P<0.05$)

676

Supporting Information

S1 Fig. Similarity dendrogram generated from the digitized PCR-DGGE fingerprints of DNA directly extracted from the substrate infested with *F. oxysporum* f.sp. *conglutinans*.