

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1578663> since 2016-10-13T16:25:44Z

Published version:

DOI:10.1371/journal.pone.0140769.

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This is the author's final version of the contribution published as:

Chitarra, W.; Siciliano, I.; Ferrocino, I.; Gullino, M.L.; Garibaldi, A. Effect of elevated atmospheric CO₂ and temperature on the disease severity of rocket plants caused by Fusarium wilt under phytotron condition.. PLOS ONE. None pp: 1-16.

DOI: 10.1371/journal.pone.0140769.

When citing, please refer to the published version.

Link to this full text:

<http://hdl.handle.net/2318/1578663>

1 **Effect of elevated atmospheric CO₂ and temperature on**
2 **the disease severity of rocket plants caused by *Fusarium***
3 **wilt under phytotron conditions**

4
5 **Walter Chitarra^{1,3*}, Ilenia Siciliano¹, Ilario Ferrocino², Maria Lodovica Gullino^{1,2} and**
6 **Angelo Garibaldi¹**

7 ¹Centre for Innovation in the Agro-Environmental Sector, AGROINNOVA, University of
8 Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy

9 ²Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino,
10 Largo P. Braccini 2, 10095, Grugliasco (TO), Italy

11

12 Present address: ³Institute for Sustainable Plant Protection, National Research Council
13 (IPSP-CNR), Grugliasco unit, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy

14

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

16

17 *Corresponding author: Walter Chitarra

18 Tel.: +39 0116708666

19 Fax: +39 0116708658

20 E-mail address: walter.chitarra@ipsp.cnr.it

21

22 **Keywords:** Climate change, *Fusarium oxysporum* f.sp. *conglutinans*, PCR-DGGE, plant-
23 pathogen interaction, soilborne pathogen, fungal microbiota

24 **Abstract**

25 The severity of *F. oxysporum* f.sp. *conglutinans* on rocket plants grown under
26 simulated climate change conditions has been studied. The rocket plants were cultivated on
27 an infested substrate (4 log CFU g⁻¹) and a non-infested substrate over three cycles. Pots were
28 placed in six phytotrons in order to simulate different environmental conditions: 1) 400-450
29 ppm CO₂, 18–22 °C; 2) 800-850 ppm CO₂, 18–22 °C; 3) 400-450 ppm CO₂, 22–26 °C, 4) 800-
30 850 ppm CO₂, 22-26 °C, 5) 400-450 ppm CO₂, 26-30 °C; 6) 800-850 ppm CO₂, 26-30 °C.
31 Substrates from the infested and control samples were collected from each phytotron at 0, 60
32 and 120 days after transplanting. The disease index, microbial abundance, leaf physiological
33 performances, root exudates and variability in the fungal profiles were monitored. The
34 disease index was found to be significantly influenced by higher levels of temperature and
35 CO₂. Plate counts showed that fungal and bacterial development was not affected by the
36 different CO₂ and temperature levels, but a significant decreasing trend was observed from 0
37 up to 120 days. Conversely, the *F. oxysporum* f.sp. *conglutinans* plate counts did not show
38 any significantly decrease from 0 up to 120 days. The fungal profiles, evaluated by means
39 of polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE), showed a
40 relationship to temperature and CO₂ on fungal diversity profiles. Different exudation patterns
41 were observed when the controls and infested plants were compared, and it was found that
42 both CO₂ and temperature can influence the release of compounds from the roots of rocket
43 plants. In short, the results show that global climate changes could influence disease
44 incidence, probably through plant-mediated effects, caused by soilborne pathogens.

45

46

47

48

49 **Introduction**

50 Nowadays, how climate changes will influence plant-pathogen interactions and their
51 impact on production is largely debated and represents a challenge for future programmes
52 focused on disease management under global change conditions [1]. An increasing numbers
53 of multidisciplinary approaches have reported the effects of rising temperature and CO₂
54 levels on crop productions and physiological changes [2]. On the other hand, researchers
55 have only recently focused on plant disease prediction and management under climate
56 changes [3]. Plant pathogens represents an important constraint for the security of our future
57 food as a consequence of population increases, urbanization, globalization and changes in
58 climate [4]. Moreover, several pathogens produce toxins and other compounds that are
59 dangerous to human and animal health which could affect market and world trade. On these
60 grounds, a multidisciplinary approach is needed to implement predictive models and include
61 higher levels of ecological interactions [5]. As recently reported by an Intergovernmental
62 Panel on Climate Changes [6], anthropogenic emissions of greenhouse gases have caused
63 negative impacts on human and natural systems over the last three decades. In other words,
64 changes in the atmospheric composition and temperature as well as humidity alterations can
65 have an impact on host plant physiology and influence a plant's resistance against pathogens
66 [7–9]. On one hand, rises in CO₂ and temperature can affect the sense and responses of a
67 plant by increasing the photosynthesis rates, water and light-use efficiency, leaf surface
68 properties, changes in anatomy, morphology, phenology and root exudates, thus profoundly
69 modifying native plants and soil microbial communities [1,3,8,10,11]. On the other hand,
70 elevated CO₂ influences the pathogenicity, host-pathogen interaction and epidemiology of
71 fungal diseases [3,12-15,16]. An enlarged canopy coupled with a favourable microclimate
72 offers more sites for infection and increases fungal pathogen fecundity, which has been
73 shown to lead to twice the number of lesions for high CO₂ 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, CO₂
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 CO₂ concentrations and temperatures in phytotron chambers.
96 Six temperature/CO₂ 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

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

102 **Materials and methods**

103 **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 hydrolyzed 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 8000g 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

116 **Plant material and experimental set-up**

117 Two experimental trials were carried out at Agroinnova (Grugliasco, Italy). 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 hypochloride 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 CO₂ combinations according to the following ranges: 1) 400-450
137 ppm CO₂, 18-22 °C; 2) 800-850 ppm CO₂, 18-22 °C; 3) 400-450 ppm CO₂, 22-26 °C, 4) 800-
138 850 ppm CO₂, 22-26 °C, 5) 400-450 ppm CO₂, 26-30 °C; 6) 800-850 ppm CO₂, 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

148 **Disease incidence evaluation**

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

158

159 **Physiological measurements of the leaves**

160 In order to observe the effects of the climate change conditions on the leaf
161 physiological activity of the infected and control rocket plants, the photosynthetic efficiency
162 and chlorophyll content were monitored. Measurements were performed following the
163 experimental protocol reported by Pugliese et al. [35], with only minor modifications.
164 The chlorophyll content index (CCI) was measured with the SPAD 502 chlorophyll meter
165 (CCM-200, Opti-Sciences, Inc., Hudson, NH, USA), which determined the relative amount
166 of chlorophyll in the leaf by measuring the absorbance in the red and near-infrared regions
167 (650 and 940 nm, respectively). Chlorophyll meter readings were taken from each rocket
168 plant in the second or third leaves (fully developed) from the top on ten randomly selected
169 plants (one leaf/plant) at the end of each cultivation cycle.
170 The photosynthetic efficiency measurements were performed on five randomly selected
171 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

175 **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 CaSO₄ 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 ddH₂O. 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 K₂Cr₂O₇ (2 N) and 1 mL of H₂SO₄ 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 μ m, Phenomenex, Torrance, CA,
201 USA) in an isocratic condition, using buffer phosphate (50 mM) as the mobile phase. The
202 detector was set at $\lambda=214$ nm. A mixture of standard organic acids (Sigma-Aldrich) was used
203 as the reference.

204

205 **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

219 **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 extract according to the protocol

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

228 **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

243 **Results**

244 **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 CO₂ 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 CO₂ 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 CO₂ ranges (P
250 < 0.05) than the other conditions. Conversely, the DI significantly increased at 22-26 °C,
251 particularly for higher CO₂ levels ($P < 0.05$) where a DI of 54 was reached. At 26-30 °C, a
252 slightly decreasing trend was recorded for both CO₂ levels, although a higher DI (43,4) was
253 observed at 800-850 ppm CO₂, 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, CO₂ and disease
256 development (Table 1). Lower FW values were obtained at 18-22 °C for both CO₂ conditions
257 and in phytotrons 4 and 6 (800-850 ppm CO₂, 22-26 °C and 800-850 ppm CO₂, 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 CO₂, 18–22 °C and 800-850 ppm CO₂, 18–22 °C) in which FW was
261 significantly lower ($P < 0.05$) than the other tested.

262

263 **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 CO₂, 18–22 °C; 800-850 ppm CO₂, 18–22 °C and 800-850 ppm CO₂, 22-26 °C,

270 respectively). Moreover, the highest temperature provided the best evidence of impaired PI at
271 high CO₂ 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 CO₂, 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 CO₂, 22–26 °C and 400-450 ppm CO₂, 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

284 **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 CO₂ levels ($P < 0.05$).
290 No significant differences were observed between the infected and non-infected plants for
291 lower CO₂ concentrations. The root exudates from the infected samples did not show any
292 significant differences in TOC concentration at 400-450 ppm of CO₂; conversely, an
293 increasing trend of TOC concentration was registered when the temperature and CO₂ level
294 were increased ($P < 0.05$).

295 The amino acid levels from the infected plants were not different at lower CO₂ levels;
296 conversely, higher CO₂ 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 CO₂ and temperature
299 were kept low, no significant differences were registered between the treatments or in the
300 phytotrons. However, increasing the concentration of CO₂ 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 CO₂, 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 CO₂ levels (P <
304 0.05).

305

306 **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 CO₂ 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 CO₂, 18–22 °C and 800-850 ppm CO₂, 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

320 phytotron 2 (18-22 °C and 850 ppm CO₂) 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 CO₂ 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 CO₂ were shown to positively affect the growth of *Fusarium oxysporum*
332 f.sp. *conglutinans*. No pathogen was detected in the control substrate samples.

333

334 **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 CO₂ concentration, and the results showed a clear
342 separation between samples at 850 and samples at 450 ppm CO₂ (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 CO₂), and the H'
356 index (2.83) and richness (17) were higher for Phytotron 1 (18-22 °C and 450 ppm CO₂).
357 Higher CO₂ and temperature were shown to be selective and to reduce fungal community
358 diversity.

359

360 **Discussion**

361 Plants that grow under elevated temperature and CO₂ concentrations often exhibit
362 positive responses by increasing photosynthesis and/or water and nutrient use efficiency [43].
363 However, how increases in CO₂ 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 CO₂ 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 CO₂ 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 CO₂
386 combinations in the six environmental conditions had a significant influence on the incidence
387 of *Fusarium* wilt. Higher CO₂ 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 CO₂
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 CO₂ 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 CO₂ 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 CO₂ 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

420 the combined temperature and high CO₂ 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 CO₂ 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 CO₂

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 CO₂ 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 CO₂. 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 CO₂ 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 CO₂ 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

474 **Acknowledgements**

475 This research was supported by the European Union's Horizon 2020 research and
476 innovation programme under grant agreement No. 634179 "Effective Management of Pests
477 and Harmful Alien Species - Integrated Solutions" (EMPHASIS). The authors are grateful to
478 Marguerite Jones for the English editing.

479

480 **References**

481

- 482 1. Chakraborty S, Pangga IB, Roper MM. Climate change and multitrophic interaction
483 in soil: the primacy of plants and functional domains. *Glob Chang Biol.* 2012;
484 18:2111-2125.
- 485 2. Lobell DB, Gourdji SM. The influence of climate change on global crop productivity.
486 *Plant Physiol.* 2012; 160(4):1686-1697.
- 487 3. Chakraborty S. Migrate or evolve: options for plant pathogens under climate change.
488 *Glob Chang Biol.* 2013; 19:1985-2000.
- 489 4. Gregory PJ, Jhonson SN, Newton AC, Ingram JSI. Integrating pests and pathogens
490 into the climate change/food security debate. *J Exp Bot.* 2009; 60:2827-2838.
- 491 5. Shabani F, Kumar L, Esmaeili A. Future distribution of *Fusarium oxysporum* f. spp.
492 in European, Middle Eastern and North African agricultural regions under climate
493 change. *Agric Ecosist Environ.* 2014; 197:96-105.
- 494 6. IPCC Intergovernmental Panel on Climate Change. IPCC. 2013. Fifth Assessment
495 Report: Summary for Policymakers. 36p.
- 496 7. Pangga IB, Chakraborty S, Yates D. Canopy Size and induced resistance in
497 *Stylosanthes scabra* determine anthracnose severity at High CO₂. *Phytopathol.* 2004;
498 94(3):221-227.
- 499 8. Ainsworth E, Rogers A. The response of photosynthesis and stomatal conductance to
500 rising [CO₂]: mechanisms and environmental interactions. *Plant Cell Environ.* 2007;
501 30:258-270.
- 502 9. Lake J, Wade R. Plant-pathogen interactions and elevated CO₂: morphological
503 changes in favour of pathogens. *J Exp Bot.* 2009; 60:3123-3131.
- 504 10. Harley SE, Jones CJ, Couper GC, Jones TH. Biosynthesis of plant phenolic
505 compounds in elevated atmospheric CO₂. *Glob Chang Biol.* 2000; 6:497-506.

- 506 11. Chakraborty S. Potential impact of climate change on plant-pathogen interactions.
507 Australas Plant Pathol. 2005; 34:443-448.
- 508 12. Mithcell CE, Reich PB, Tilman D, Groth JV. Effects of elevated CO₂, nitrogen
509 deposition, and decreased species diversity on foliar fungal plant disease. Glob Chang
510 Biol. 2003; 9:438-451.
- 511 13. Eastburn DM, Degennaro MM, Delucia EH, Dermody O, Mcelrone AJ. Elevated
512 atmospheric carbon dioxide and ozone alter soybean diseases at SoyFACE. Glob
513 Chang Biol. 2010; 16:320-330.
- 514 14. Pugliese M, Cogliati E, Gullino ML, Garibaldi A. Effect of climate change on
515 Alternaria leaf spot of rocket salad and black spot of basil under controlled
516 environment. Commun Agric Appl Biol Sci. 2012a; 77: 241-4.
- 517 15. Pugliese M, Liu J, Titone P, Garibaldi A, Gullino ML. Effect of elevated CO₂ and
518 temperature on interactions of zucchini and powdery mildew. Phytopathol Mediterr.
519 2012b; 51: 480-487.
- 520 16. Ferrocino I, Chitarra W, Pugliese M, Gilardi G, Gulino ML, Garibaldi A. Effect of
521 elevated atmospheric CO₂ and temperature on disease severity of *Fusarium*
522 *oxysporum* f.sp. *lactucae* on lettuce plants. Appl Soil Ecol. 2013; 72:1-6.
- 523 17. Chakraborty S, Pangga IB, Lupton J, Hart L, Room PM, Yates D. Production and
524 dispersal of *Colletotrichum gleosporoides* spores on *Stylosanthes scabra* under
525 elevated CO₂. Environ Poll. 2000; 108:381-387.
- 526 18. Pangga IB. Effects of elevated CO₂ on plant architecture of *Stylosanthes scabra* and
527 epidemiology of anthracnose disease. PhD Thesis, University of Queensland. 2002.
- 528 19. Chakraborty S, Datta S. How will plant pathogens adapt to host plant resistance at
529 elevated CO₂ under a changing climate?. New Phytol. 2003; 159:733-742.
- 530 20. Gullino ML, Garibaldi A. Soil solarization under greenhouse conditions. In: Gamliel
531 A, Katan J. editors. Soil solarization: Theory and Practice. St. Paul, MN, USA, APS
532 Press; 2012. pp 187-191.
- 533 21. Ainsworth EA, Long SP. What have we learned from 15 years of free-air CO₂
534 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis,
535 canopy properties and plant production to rising CO₂. New Phytol. 2005; 165(2):351-
536 71.
- 537 22. Haichar FZ, Santaella C, Heulin T, Achouak W. Root exudates mediated interactions
538 belowground. Soil Biol Biochem. 2014; 77:69-80.
- 539 23. Ebersberger D, Niklaus PA, Kandeler E. Long term CO₂ enrichment stimulates N-
540 mineralisation and enzyme activities on calcareous grassland. Soil Biol Biochem.
541 2003; 35:965-972.
- 542 24. Cheng L, Booker FL, Burkey KO, Tu C, Shew HD. Soil microbial responses to
543 elevated CO₂ and O₃ in a nitrogen-aggrading agroecosystem. PLoS One. 2011;
544 6:e21377.
- 545 25. Zack DR, Pregitzer KS, Curtis PS, Holmes WE. Atmospheric CO₂ and the
546 composition and function of soil microbial communities. Ecol Appl. 2000; 10:47-59.
- 547 26. Guenet B, Lenhart K, Leloup J, Giusti-Miller S, Pouteau V, Mora P, et al. The impact
548 of long-term CO₂ enrichment and moisture levels on soil microbial community
549 structure and enzyme activities. Geoderma. 2012; 170:331-336.
- 550 27. Ferrocino I, Gilardi G, Pugliese M, Gullino ML, Garibaldi A. Shifts in ascomycete
551 community of biosolarized substrate infested with *Fusarium oxysporum* f.sp.
552 *conglutinans* and *F. oxysporum* f.sp. *basilici* by PCR-DGGE. Appl Soil Ecol. 2014;
553 81:12-21.
- 554 28. Correll J. The relationship between formae speciales, races, and vegetative
555 compatibility groups in *Fusarium oxysporum*. Phytopathology. 1991; 81:1061-1064.

- 556 29. Garibaldi A, Gullino ML. Emerging soilborne diseases of horticultural crops and new
557 trends in their management. *Acta Hortic.* 2010; 883:37-46.
- 558 30. Garibaldi A, Gilardi G, Gullino ML. Evidence for an expanded host range of
559 *Fusarium oxysporum* f.sp. *raphani*. *Phytoparasitica.* 2006; 34:115-121.
- 560 31. Lu P, Ricauda Aimonino D, Gilardi G, Gullino ML, Garibaldi A. Efficacy of different
561 steam distribution systems against five soilborne pathogens under controlled
562 laboratory conditions. *Phytoparasitica.* 2010; 38:175-189.
- 563 32. Locke T, Colhoun J. Contributions to a method of testing oil palm seedlings for
564 resistance to *Fusarium oxysporum* Schl. f. sp. *elaedis* Toovey. *Phytopathology.* 1974;
565 79:77-92.
- 566 33. Komada, H. Development of selective medium for quantitative isolation of *Fusarium*
567 *oxysporum* from natural soil. *Rev Plant Prot Res.* 1975; 8:114-125.
- 568 34. Gilardi G, Demarchi S, Gullino ML, Garibaldi A. Effect of simulated soil solarization
569 and organic amendments on *Fusarium* wilt of rocket and basil under controlled
570 conditions. *J Phytopathol.* 2013; 162: 557-566.
- 571 35. Pugliese M, Gullino ML, Garibaldi A. Effects of elevated CO₂ and temperature on
572 interactions of grapevine and powdery mildew: first results under phytotron
573 conditions. *J Plant Dis Prot.* 2010; 117: 9-14.
- 574 36. Aulakh MS, Wassmann R, Bueno C, Kreuzwieser J, Rennenberg H. Characterization
575 of root exudates at different growth stages of ten rice (*Oryza sativa* L.) cultivar. *Plant*
576 *Biol.* 2001; 3:139-148.
- 577 37. Mazzotti F, Benabdelkamel H, Di Donna L, Athanassopoulos CM, Napoli A, Sindona
578 G. Light and heavy dansyl reporter groups in food chemistry: amino acid assay in
579 beverages. *J Mass Spectrom.* 2012; 47:932-939.
- 580 38. Gao G, Yin D, Chen S, Xia F, Yang J, Li K, et al. Effect of biocontrol agent
581 *Pseudomonas fluorescens* 2P24 on soil fungal community in cucumber rhizosphere
582 using T-RFLP and DGGE. *PLoS One.* 2012; 7(2):e31806.
- 583 39. Vauterin L, Vauterin P. Computer-aided objective comparison of electrophoretic
584 patterns for grouping and identification of microorganisms. *Eur. Microbiol.* 1992;
585 1:37-41.
- 586 40. Pavel BA, Vasile IC. PyElph - a software tool for gel images analysis and
587 phylogenetics. *BMC Bioinformatics.* 2012; 13:9.
- 588 41. Shannon AE, Weaver W. (1949) *The Mathematical Theory of Communities.*
589 University Illinois Press: Urbana, IL, USA.
- 590 42. Hammer Ø, Harper DAT, Ryan PD. Past: paleontological statistics software package
591 for education and data analysis. *Palaeontologia Electron.* 2001;4:9-18.
- 592 43. Kimball BA, Kobayashi K, Bindi M. Responses of agricultural crops to free-air CO₂
593 enrichment. *Adv Agron.* 2002; 77:293-368.
- 594 44. Pritchard SG. Soil organisms and global climate change. *Plant Pathol.* 2011; 60:82-
595 99.
- 596 45. Pickles BJ, Genney DR, Anderson IC, Alexander IJ. Spatial analysis of
597 ectomycorrhizal fungi reveals that root tip communities are structured by competitive
598 interactions. *Mol Ecol.* 2012; 21(20):5510-5123.
- 599 46. Scott JC, Gordon TR. Effect of Temperature on Severity of *Fusarium* Wilt of Lettuce
600 Caused by *Fusarium oxysporum* f. sp. *lactucae*. *Plant Dis.* 2010; 94:13-17.
- 601 47. Melloy P, Hollaway G, Luck J, Norton R, Aitken E, Chakraborty S. Production and
602 fitness of *Fusarium pseudograminearum* inoculum at elevated carbon dioxide in
603 FACE. *Global Chang Biol.* 2010; 16(12): 3363–337.
- 604 48. Bijanzadeh E, Emam Y. Effect of defoliation and drought stress on yield components
605 and chlorophyll content of wheat. *Pak J Biol Sci.* 2010; 13:699-705.

- 606 49. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. The role of root exudates in
607 rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol.* 2006;
608 57:233-266.
- 609 50. Berendsen RL, Pieterse CMJ, Bakker PAHM. The rhizosphere microbiome and plant
610 health. *Trends Plant Sci.* 2012; 17:478-486.
- 611 51. Piattoni F, Roberti R, Servidio G, D'Aulerio AZ. Studies on the potential role of root
612 exudates in the interaction between musk melon roots and *Fusarium oxysporum* f.sp.
613 *melonis*. *J Plant Dis Prot.* 2014; 121(2):64-70.
- 614 52. Kerks MM, Franz E, Gent-Pelzer M, Zijlstra C, Bruggen AHC. Differential
615 interaction of *Salmonella enterica* serovars with lettuce cultivars and plant-microbe
616 factors influencing the colonization efficiency. *ISME J.* 2007; 1:620-631.
- 617 53. Mendes R, Garbeva P, Raaijmakers JM. The rhizosphere microbiome: significance of
618 plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS*
619 *Microbiol Rev.* 2013; 37:634-663.
- 620 54. Uselman SM, Qualls RG, Thomas RB. Effects of increased atmospheric CO₂,
621 temperature, and soil N availability on root exudation of dissolved organic carbon by
622 a N-fixing tree (*Robinia pseudoacacia* L.). *Plant Soil.* 2000; 222:191-202.
- 623 55. Gafan G, Lucas V, Roberts G. Statistical analyses of complex denaturing gradient gel
624 electrophoresis profiles. *J Clin Microbiol.* 2005; 43:3971-3978.
- 625 56. Runion GB, Prior SA, Price AJ, McElroy JS, Torbert HA. Effects of elevated CO₂ on
626 biomass and fungi associated with two ecotypes of ragweed (*Ambrosia artemisiifolia*
627 L.). *Front Plant Sci.* 2014; 5:500.
- 628 57. Ziska LH, Runion GB. Future weed, pest, and disease problems for plants. In: Carran
629 RA, Edwards GR, Niklaus PS, editors. *Agroecosystem in a Changing Climate.*
630 Newton PCD, Boca Raton, FL: CRC Press; 2007. pp. 261-287.
631

632 **Fig 1. Photosynthetic efficiency measurements.** Effect of different CO₂ 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 CO₂ 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 CO₂: 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 time 120 (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

^aThe 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)

656

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 (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	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 ^aThe 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)

663

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 (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	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 ^aThe 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)

669

670

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 ^aThe 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

677 **Supporting Information**

678

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

681