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**Effect of elevated atmospheric CO<sub>2</sub> and temperature on the disease severity of rocket plants caused by Fusarium wilt under phytotron condition.**

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1 **Effect of elevated atmospheric CO<sub>2</sub> and temperature on**  
2 **the disease severity of rocket plants caused by *Fusarium***  
3 **wilt under phytotron conditions**

4  
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14

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

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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<sup>-1</sup>) 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 CO<sub>2</sub>, 18–22 °C; 2) 800-850 ppm CO<sub>2</sub>, 18–22 °C; 3) 400-450 ppm CO<sub>2</sub>, 22–26 °C, 4) 800-  
30 850 ppm CO<sub>2</sub>, 22-26 °C, 5) 400-450 ppm CO<sub>2</sub>, 26-30 °C; 6) 800-850 ppm CO<sub>2</sub>, 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 CO<sub>2</sub>. Plate counts showed that fungal and bacterial development was not affected by the  
36 different CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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.

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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 CO<sub>2</sub>  
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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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<sub>2</sub>  
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<sub>2</sub> concentrations and temperatures in phytotron chambers.  
96 Six temperature/CO<sub>2</sub> 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<sup>-1</sup> 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<sup>-1</sup>.

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<sup>-1</sup>. 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 CO<sub>2</sub> combinations according to the following ranges: 1) 400-450  
137 ppm CO<sub>2</sub>, 18-22 °C; 2) 800-850 ppm CO<sub>2</sub>, 18-22 °C; 3) 400-450 ppm CO<sub>2</sub>, 22-26 °C, 4) 800-  
138 850 ppm CO<sub>2</sub>, 22-26 °C, 5) 400-450 ppm CO<sub>2</sub>, 26-30 °C; 6) 800-850 ppm CO<sub>2</sub>, 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<sup>-1</sup> 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 CaSO<sub>4</sub> 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<sub>2</sub>O. 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<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> (2 N) and 1 mL of H<sub>2</sub>SO<sub>4</sub> 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  $\mu$ 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<sup>-1</sup> 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  $\mu\text{l}^{-1}$ . 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](http://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 CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> ranges ( $P$   
250  $< 0.05$ ) than the other conditions. Conversely, the DI significantly increased at 22-26 °C,  
251 particularly for higher CO<sub>2</sub> 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<sub>2</sub> levels, although a higher DI (43,4) was  
253 observed at 800-850 ppm CO<sub>2</sub>, 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<sub>2</sub> and disease  
256 development (Table 1). Lower FW values were obtained at 18-22 °C for both CO<sub>2</sub> conditions  
257 and in phytotrons 4 and 6 (800-850 ppm CO<sub>2</sub>, 22-26 °C and 800-850 ppm CO<sub>2</sub>, 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<sub>2</sub>, 18–22 °C and 800-850 ppm CO<sub>2</sub>, 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 CO<sub>2</sub>, 18–22 °C; 800-850 ppm CO<sub>2</sub>, 18–22 °C and 800-850 ppm CO<sub>2</sub>, 22-26 °C,

270 respectively). Moreover, the highest temperature provided the best evidence of impaired PI at  
271 high CO<sub>2</sub> 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<sub>2</sub>, 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<sub>2</sub>, 22–26 °C and 400-450 ppm CO<sub>2</sub>, 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 CO<sub>2</sub> levels ( $P < 0.05$ ).  
290 No significant differences were observed between the infected and non-infected plants for  
291 lower CO<sub>2</sub> concentrations. The root exudates from the infected samples did not show any  
292 significant differences in TOC concentration at 400-450 ppm of CO<sub>2</sub>; conversely, an  
293 increasing trend of TOC concentration was registered when the temperature and CO<sub>2</sub> level  
294 were increased ( $P < 0.05$ ).

295 The amino acid levels from the infected plants were not different at lower CO<sub>2</sub> levels;  
296 conversely, higher CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>, 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<sub>2</sub> 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 CO<sub>2</sub> 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<sub>2</sub>, 18–22 °C and 800-850 ppm CO<sub>2</sub>, 22-26 °C) were significantly higher (P < 0.05),  
315 that is, 7.17 and 6.87 Log CFU g<sup>-1</sup>, 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 CO<sub>2</sub>) at 120 days, when the statistical values were higher  
321 (4.89 Log CFU g<sup>-1</sup>) 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<sub>2</sub> conditions in the last survey (from 3.80 to 4.38 and from  
330 3.73 to 4.32 Log CFU g<sup>-1</sup>, respectively) (P < 0.05). With the exception of 26-30 °C, high  
331 concentrations of CO<sub>2</sub> 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 CO<sub>2</sub> concentration, and the results showed a clear  
342 separation between samples at 850 and samples at 450 ppm CO<sub>2</sub> (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<sub>2</sub>), and the  $H'$   
356 index (2.83) and richness (17) were higher for Phytotron 1 (18-22 °C and 450 ppm CO<sub>2</sub>).  
357 Higher CO<sub>2</sub> 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 CO<sub>2</sub> concentrations often exhibit  
362 positive responses by increasing photosynthesis and/or water and nutrient use efficiency [43].  
363 However, how increases in CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>  
386 combinations in the six environmental conditions had a significant influence on the incidence  
387 of *Fusarium* wilt. Higher CO<sub>2</sub> 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<sub>2</sub>  
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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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 CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>

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<sub>2</sub> 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<sub>2</sub>. 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<sub>2</sub> 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<sub>2</sub> 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

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479

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631

632 **Fig 1. Photosynthetic efficiency measurements.** Effect of different CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>: 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 <sup>a</sup>					
		1 (400-450ppm CO <sub>2</sub> 18-22°C)	2 (800-850ppm CO <sub>2</sub> 18-22°C)	3 (400-450ppm CO <sub>2</sub> 22-26°C)	4 (800-850ppm CO <sub>2</sub> 22-26°C)	5 (400-450ppm CO <sub>2</sub> 26-30°C)	6 (800-850ppm CO <sub>2</sub> 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 <sup>-1</sup> )	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 <sup>-1</sup> )	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

<sup>a</sup>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 <sup>a</sup>					
		1 (400-450ppm CO <sub>2</sub> 18-22°C)	2 (800-850ppm CO <sub>2</sub> 18-22°C)	3 (400-450ppm CO <sub>2</sub> 22-26°C)	4 (800-850ppm CO <sub>2</sub> 22-26°C)	5 (400-450ppm CO <sub>2</sub> 26-30°C)	6 (800-850ppm CO <sub>2</sub> 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 <sup>-1</sup> )	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 <sup>-1</sup> )	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 <sup>-1</sup> )	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 <sup>-1</sup> )	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 <sup>-1</sup> )	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 <sup>-1</sup> )	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

<sup>a</sup> 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 <sup>-1</sup> ± SD <sup>a</sup> )					
		1 (400-450ppm CO <sub>2</sub> 18-22°C)	2 (800-850ppm CO <sub>2</sub> 18-22°C)	3 (400-450ppm CO <sub>2</sub> 22-26°C)	4 (800-850ppm CO <sub>2</sub> 22-26°C)	5 (400-450ppm CO <sub>2</sub> 26-30°C)	6 (800-850ppm CO <sub>2</sub> 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 <sup>a</sup>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 <sup>-1</sup> ± SD <sup>a</sup> )					
		1 (400-450ppm CO <sub>2</sub> 18-22°C)	2 (800-850ppm CO <sub>2</sub> 18-22°C)	3 (400-450ppm CO <sub>2</sub> 22-26°C)	4 (800-850ppm CO <sub>2</sub> 22-26°C)	5 (400-450ppm CO <sub>2</sub> 26-30°C)	6 (800-850ppm CO <sub>2</sub> 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 <sup>a</sup>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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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 <sup>-1</sup> ± SD <sup>a</sup> )					
		1 (400-450ppm CO <sub>2</sub> 18-22°C)	2 (800-850ppm CO <sub>2</sub> 18-22°C)	3 (400-450ppm CO <sub>2</sub> 22-26°C)	4 (800-850ppm CO <sub>2</sub> 22-26°C)	5 (400-450ppm CO <sub>2</sub> 26-30°C)	6 (800-850ppm CO <sub>2</sub> 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 <sup>a</sup>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

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*.

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