Effect of elevated atmospheric CO_2 and temperature on the disease severity of rocket plants caused by Fusarium wilt under phytotron conditions

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

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Abstract

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

Introduction

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

74 Furthermore, the mutation and selection of plant pathogens and the consequent development
75 of new strains have also been predicted [3,19].
76 Phytotron-based studies are optimized to study the interactions that occur between plants, soil
77 and soil microbiota and they combine environmental parameters such as light intensity, CO_2
78 concentrations, temperature, and relative humidity [20]. Phytotron chambers have in fact
79 been largely used to study the effects of global changes on several pathosystems [11, 14–
80 16,21]. Plants directly or indirectly affect the development of soil microbiota in the
81 rhizosphere and in bulk soil by means of root exudates [22]. However, most studies
82 conducted so far report contrasting results; such as a decrease [23], an increase [24] or no
83 changes [25] in microbial diversity and activities. The development of soil microbial
84 populations needs a multiphasic approach that can couple traditional microbial analysis with
85 molecular tools such as polymerase chain reaction denaturing gradient gel electrophoresis
86 (PCR-DGGE). This technique has been optimized to monitor soil microflora changes under
87 climate change conditions [16,26,27]. *Fusarium oxysporum* and the related *formae specialis*
88 cause greater economic damage to several crops than other plant pathogens [28]. Rocket
89 (*Eruca sativa*) is a high-value horticultural crop, mainly cultivated in the Mediterranean area,
90 up to 3-5 times in the same soil which is affected by emerging soilborne pathogens [20,29]
91 and *Fusarium oxysporum* f.sp. *conglutinans* has recently been detected in Italy on cultivated
92 (*Eruca sativa*) and wild rocket (*Diplotaxis tenuifolia*) [30].
93 The aim of this work was to study the effect of *F. oxysporum* f.sp. *conglutinans*,
94 artificially infested in a growing substrate, on rocket plants grown under simulated climate
95 change conditions with rising CO_2 concentrations and temperatures in phytotron chambers.
96 Six temperature/CO_2 combinations were studied. Disease incidence, the physiological
97 performances of leaves, microbial and fungal cultivable abundance and the main root exudate
98 components were monitored to evaluate the effects of climatic changes on disease

development over time. Furthermore, shifts in fungal communities were assessed using the DGGE technique on DNA directly extracted from an infested growing substrate.

Materials and methods

Inoculum preparation

F. oxysporum f.sp. *conglutinans* (ATCC16600RB, Agroinnova, Grugliasco, Italy), which is resistant to benomyl [31], was used and cultured in 1000-mL Erlenmeyer flasks containing 250 mL of hydrolized casein. The flasks were incubated on a platform shaker at 20-25 °C for 12 days. Chlamydospores were recovered by means of centrifugation for 20 min at 8000*g* at 20 °C, following the removal of mycelia fragments by sieving through cheesecloth. The chlamydospore suspension was dried and mixed with sterile talc powder (1:2 w/w), as described by Locke & Colhoun [32], and stored at room temperature for further use. The number of chlamydospores per gram of talc was assessed by serial plating on a Komada medium [33] containing 10 mg L^{-1} of benomyl (Benlate, 50% a.i.; DuPont de Nemours, Milan, Italy). The talc formulation was incorporated into the soil to achieve the desired inoculum of 4 Log colony forming units (CFU) g^{-1}.

Plant material and experimental set-up

Two experimental trials were carried out at Agroinnova (Grugliasco, Itay). Plastic tanks containing 100L of a mixture (1:1 v/v) of peat-perlite substrate (Tecno2, Turco Silvestro sphagnum peat moss, Albenga, SV, Italy) and sandy loam soil (pH, 7.3; organic matter content, 2.2 %; cation exchange capacity, 2.6 meq/100 g soil) were prepared. The final substrate was made up of: sand, 76 %; silt, 14 %; clay, 10%; pH, 7.51; organic matter content, 2,59 % and had a cation exchange capacity of 5.99 meq/100 g soil and subjected to

123 steam sterilization before use. The substrate was artificially inoculated with *F. oxysporum* f.
124 sp. *conglutinans* (ATCC16600RB) to reach a final concentration of 4 Log CFU ml^{-1}. A non-
125 inoculated tank was used as the control.
126 *Eruca sativa* Mill seeds (cultivated rocket) were disinfected in a solution of 1%
127 sodium hypocloride plus 0.01% of Tween-20, and then rinsed twice in water for 1 min. The
128 seeds were air-dried at RT and stored at 4 °C until use. The seeds were sown in a greenhouse
129 in plug trays (20-26 °C, 70% RH and natural light condition). After 15-20 days, the first
130 seedling-leaves were developed. The rocket plants were left to grow under Phytotron
131 conditions for 7 days. Subsequently, 48 pots (2L each) were prepared from the inoculated
132 tank and another 48 pots were prepared from the non-inoculated tank and used as controls.
133 The rocket plants were then transplanted (4 plants/pot), and 8 inoculated and 8 non-
134 inoculated pots were placed in 6 different phytotrons (PGC 9.2, TECNO.EL, Italy). One
135 replicate consisted of two pots. The rocket plants were kept in the phytotrons under six
136 different temperature and CO_2 combinations according to the following ranges: 1) 400-450
137 ppm CO_2, 18-22 °C; 2) 800-850 ppm CO_2, 18-22 °C; 3) 400-450 ppm CO_2, 22-26 °C, 4) 800-
138 850 ppm CO_2, 22-26 °C, 5) 400-450 ppm CO_2, 26-30 °C; 6) 800-850 ppm CO_2, 26-30 °C.
139 The temperatures, light and humidity were changed gradually during the day in order to
140 simulate natural conditions. Three subsequent cultivation cycles were carried out in the same
141 pot. Each crop cycle lasted 35-37 days after transplanting. The plants were irrigated daily in
142 order to maintain the soil moisture at field capacity.
143 Substrate samples were taken at time 0 (immediately before plant transplanting) and
144 after 60 and 120 days for microbial enumeration and molecular analyses (PCR-DGGE), while
145 the physiological measurements of the plant leaves were conducted at the end of each rocket
146 cycle.
147

Disease incidence evaluation

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

Physiological measurements of the leaves

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

Instruments Ltd, Norfolk, UK), according to the manufacturer's instructions, at the end of each cultivation cycle of rocket plants grown in infested and control substrates.

Root exudate analyses

In order to investigate the relationship between climate changes and the turnover of low molecular weight organic compounds in the rhizosphere, water-soluble root exudates were collected and analyzed. A sterilized $CaSO_4$ 0.01M solution (collection media) was used to collect the root exudates [36]. For each condition tested, three randomly selected rocket plants were taken from infested and not infested pots and the soil was carefully removed using deionized water, without damaging the roots. The plants were then placed in a 50 mL centrifuge tube with 15 mL of collection media for 2 hours in each phytotron. The collection media was then filtered through a 0.45 μm membrane filter. pH was measured and the samples were freeze dried and dissolved in 3 mL of ddH_2O. Three main groups of organic substances were analyzed: total organic carbon (TOC), total organic acids and amino acid compounds.

TOC was determined by means of the colorimetric method. An aliquot of 1 mL was mixed with 2 mL of $K_2Cr_2O_7$ (2 N) and 1 mL of H_2SO_4 concentrate and kept at 150 °C for 10 min. The samples were cooled to room temperature and then analyzed by using a Spectroquant Pharo 300 spectrophotometer (Merck, Darmstadt, Germany) at λ=585 nm. Glucose was used as the reference C substance. For the amino acids analysis, 1 mL of sample was mixed with 1 mL of borate buffer (0.4 M pH=9.5) and 1 mL of dansyl chloride solution (Sigma-Aldrich, St. Louis, MO, USA) at 10 mg/mL. The samples were incubated at 65 °C for 30 min [37]. The analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). Zorbax Eclipse Plus C18 (4.6x100 mm, 3.5 μm, Agilent Technologies) was used for the chromatographic separation of the amino acids with a linear gradient from 10 to

100% of acetonitrile (Merck) in 15 min. The derivatized amino acids were detected by means of a fluorescent detector with excitation at 335 nm and emission at 524 nm. A mixture of 20 standard amino acids (Sigma-Aldrich) was used as the reference. The organic acids were separated with a Synergi Hydro-RP column (4.6x250 mm, 4 µm, Phenomenex, Torrance, CA, USA) in an isocratic condition, using buffer phosphate (50 mM) as the mobile phase. The detector was set at λ=214 nm. A mixture of standard organic acids (Sigma-Aldrich) was used as the reference.

Microbial count

Substrate samples (25 grams) were collected from 8 pots for both infested and control treatments, at a depth of 2-5 cm, and were placed in sterilized polyethylene bags using a sterilized spatula. The samples were passed through a sieve to remove any vegetation, and mixed at room temperature for 30 min with 225 mL of quarter strength Ringer's solution (Merck) in sterilized flasks on a rotary shaker (100 rpm). Decimal dilutions were prepared in quarter strength Ringer's solution and aliquots of 1 ml of the appropriate dilutions were poured, in triplicate, onto the following media: plate count agar (PCA, Oxoid, Milan) incubated at 28°C for 48h for the total bacterial counts; potato dextrose agar (PDA, Merck) supplemented with streptomycin (0,5 g/L) and a Komada medium supplemented with 10 mg L^{-1} of benomyl (Benlate, 50% w.g., DuPont, USA), kept at 25°C for 7 days for the total fungal and *F. oxysporum* f.sp. *conglutinans* enumerations, respectively. The results were calculated as the mean of the Log counts of three independent determinations.

Substrate DNA extraction and DGGE analysis

Infested substrate samples (250 mg) were taken from 4 pots/phytotron at 0, 60 and 120 days after transplanting for DNA extraction. DNA exctract according to the protocol

described by the NucleoSpin® Soil manufacturer (Macherey-Nagel, Germany) and was quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific) and standardized at 10 ng μl^{-1}. Fungal DNA was amplified using fungal ITS primers according to Gao *et al*. [38]. PCR products for the ITS region were analyzed by denaturing gradient gel electrophoresis (DGGE) at 25-35% using a Bio-Rad Dcode, as previously described [16].

Statistical analysis

The data obtained from the plate counts, root exudates and leaf physiological measurements were analyzed using one-way analysis of variance (ANOVA), with treatments being the main factor. ANOVA analyses were performed with the SPSS 22.0 statistical software package (SPSS Inc., Cary, NC, USA). The Duncan HSD test was applied when ANOVA revealed significant differences ($P < 0.05$). A database of fingerprints was created using the PyElph software. A combined data matrix that included all the fingerprints for the ITS region was obtained, and dendrograms of similarity were retrieved using the Dice coefficient and the Unweighted Pair Group Method with the Arithmetic Average (UPGMA) clustering algorithm [39] utilizing PyElph software [40]. The similarity distance matrix generated through PyElph was used to build a Projection on Latent Structures - Discriminant Analysis (PLS-DA) utilizing "mixOmics" in the R environment (www.r-project.org). The obtained binary band-matching tables were considered to calculate the Shannon-Wiener diversity index (*H'*) [41] using PAST (PAleontological STatistics) software [42].

Results

Disease index assessment and fresh biomass production

245 The disease index (DI, 0-100) was found to be significantly influenced by the
246 temperature and CO_2 levels (Table 1). No symptoms were observed for the control plants in
247 any of the cycles or phytotron conditions that were considered. Generally, higher levels of
248 CO_2 and temperature were factors that significantly influenced the DI in the infected plants in
249 the simulated conditions. At 18-22 °C, the DI was significantly lower for both CO_2 ranges ($P < 0.05$) than the other conditions. Conversely, the DI significantly increased at 22-26 °C,
251 particularly for higher CO_2 levels ($P < 0.05$) where a DI of 54 was reached. At 26-30 °C, a
252 slightly decreasing trend was recorded for both CO_2 levels, although a higher DI (43,4) was
253 observed at 800-850 ppm CO_2, with a significantly different value than that of the 18-22 °C
254 conditions ($P < 0.05$). The fresh weight (FW) data pertaining to the infected plants showed
255 significant differences ($P < 0.05$) as a consequence of temperature, CO_2 and disease
256 development (Table 1). Lower FW values were obtained at 18-22 °C for both CO_2 conditions
257 and in phytotrons 4 and 6 (800-850 ppm CO_2, 22-26 °C and 800-850 ppm CO_2, 26-30 °C,
258 respectively) where disease development was higher (Table 1). The control plants showed a
259 higher FW than the diseased rocket plants, with the exception of 18-22 °C (phytotrons 1
260 and 2: 400-450 ppm CO_2, 18–22 °C and 800-850 ppm CO_2, 18–22 °C) in which FW was
261 significantly lower ($P < 0.05$) than the other tested.
262

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_2, 18–22 °C; 800-850 ppm CO_2, 18–22 °C and 800-850 ppm CO_2, 22-26 °C,

respectively). Moreover, the highest temperature provided the best evidence of impaired PI at high CO_2 in the infected plants.

In general, the non-inoculated plants showed higher photosynthetic efficiency than the infected ones, with the exception of those from phytotron 4 (800-850 ppm CO_2, 22-26 $^{\circ}$C) where PI was lower than for the other conditions.

Similar trends were also observed for the CCI measurements in the infected samples (Fig. 2) where no significant differences were observed between the phytotrons ($P < 0.05$). The non-infected plants showed higher levels of chlorophyll content than the infected rocket plants. Furthermore, significantly lower values were measured in the non-infected plants in phytotrons 3 and 5 (400-450 ppm CO_2, 22–26 $^{\circ}$C and 400-450 ppm CO_2, 26-30 $^{\circ}$C, respectively) ($P < 0.05$). Overall, the physiological measurements have confirmed that the disease development led to a remarkable decrease in the physiological performances of the leaves.

Effect on root exudates

The results pertaining to the root exudates (Table 2) showed a similar trend for TOC and amino acids (AA), when the exudates from the infected roots and the control plants were compared. In both cases, when the temperature was lower (18-22 °C), an increased concentration was registered in the samples from the infected plants. At 22-26 °C and 26-30 °C, the TOC and AA concentrations significantly increased for higher CO_2 levels ($P < 0.05$). No significant differences were observed between the infected and non-infected plants for lower CO_2 concentrations. The root exudates from the infected samples did not show any significant differences in TOC concentration at 400-450 ppm of CO_2; conversely, an increasing trend of TOC concentration was registered when the temperature and CO_2 level were increased ($P < 0.05$).

295 The amino acid levels from the infected plants were not different at lower CO_2 levels;
296 conversely, higher CO_2 levels only induced a significant increase in amino acids at high
297 temperatures ($P < 0.05$).
298 The organic acids, in general, showed a different trend. When CO_2 and temperature
299 were kept low, no significant differences were registered between the treatments or in the
300 phytotrons. However, increasing the concentration of CO_2 led to an increased production of
301 organic acids, but only in the control samples. Furthermore, at 22-26 °C, for both levels of
302 CO_2, the organic acid concentration increased for all the samples. In general, the organic acid
303 concentrations of all the samples were always significantly higher for higher CO_2 levels ($P <$
304 0.05).
305

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_2 and temperature levels. A significant
310 decreasing trend was observed from time 0 up to 120 days for all the conditions considered (P
311 < 0.05). Furthermore, the samples at 120 days were not significantly different between the
312 phytotrons. The control substrate samples showed a similar trend to that observed in the
313 infested samples. However, the samples taken after 120 days in phytotrons 2 and 4 (800-850
314 ppm CO_2, 18–22 °C and 800-850 ppm CO_2, 22-26 °C) were significantly higher ($P < 0.05$),
315 that is, 7.17 and 6.87 Log CFU g^{-1}, respectively.
316 The total fungal community plate counts (TFC, Table 4) from the infested samples mirrored
317 the bacterial counts; a significant decreasing trend ($P < 0.05$) was observed over time from
318 time 0, without any significant differences between the phytotrons at 120 days. The control
319 substrate sample counts were similar to the infested ones, with the exception of those from

320 phytotron 2 (18-22 °C and 850 ppm CO_2) at 120 days, when the statistical values were higher
321 (4.89 Log CFU g^{-1}) than in the other conditions. However, (among fungal community)
322 *Fusarium oxysporum* f.sp. *conglutinans* development was observed over time on the Komada
323 medium supplemented with benomyl in the infested substrate samples. In general, most of the
324 fungal community was represented by the pathogen, which developed over time and almost
325 completely covered the total fungal community values up to the last survey (120 days). In
326 particular, a general increasing trend was observed for all the conditions from time 0 in the
327 phytotrons compared to time 120. Although no significant differences between phytotrons
328 were observed at 120 days. A significant increasing trend from time 0 was observed for
329 phytotrons 2 and 4 for high CO_2 conditions in the last survey (from 3.80 to 4.38 and from
330 3.73 to 4.32 Log CFU g^{-1}, respectively) ($P < 0.05$). With the exception of 26-30 °C, high
331 concentrations of CO_2 were shown to positively affect the growth of *Fusarium oxysporum*
332 f.sp. *conglutinans*. No pathogen was detected in the control substrate samples.
333

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_2 concentration, and the results showed a clear
342 separation between samples at 850 and samples at 450 ppm CO_2 (Fig. 3A). Regardless of the
343 temperature, the PLS models showed a clear separation of the samples (Fig. 3B). When the
344 samples were grouped on the basis of the sampling time, the samples at time 0 were clearly

345 separated from the other samples (Fig. 3C). The PLS-DA analysis, as a function of the
346 phytotrons (Fig. 3D), resulted in a certain degree of separation. The separation was
347 particularly important for the samples from phytotron 4, which appear to group together and
348 to be separated from the other phytotrons.

349 Furthermore, a binary band-matching table was analyzed in order to calculate the
350 index of diversity (Shannon-Wiener diversity index *H'*). The results, using only the data from
351 the ITS region, revealed, on the basis of their phytotron origin, that there was a biological
352 diversity in the samples. In particular, the *H'* index and richness of the fungal community
353 changed according to the environmental conditions that were set, and ranged from 1.61 to
354 2.83 and 5 to 17, respectively. At the end of the experiment, the *H'* index (1.79) and richness
355 (10) were lower for phytotrons 4 and 6 (22-26 and 26-30 °C at 850 ppm CO_2), and the H'
356 index (2.83) and richness (17) were higher for Phytotron 1 (18-22 °C and 450 ppm CO_2).
357 Higher CO_2 and temperature were shown to be selective and to reduce fungal community
358 diversity.
359

360 # Discussion

361 Plants that grow under elevated temperature and CO_2 concentrations often exhibit
362 positive responses by increasing photosynthesis and/or water and nutrient use efficiency [43].
363 However, how increases in CO_2 might affect plant-pathogen interaction has not been
364 addressed or debated to any great extent. The aim of this work was to evaluate the effect of
365 simulated climate changes, by increasing CO_2 concentrations and temperature levels, on the
366 severity of *Fusarium oxysporum* f.sp. *conglutinans* on rocket plants. Phytotron chambers
367 have already been used in several studies, since they provide total control of the
368 environmental conditions and reproducible data in diverse pathosystems [20, 14-16]. Climate
369 change is an on-going phenomenon, which affects whole ecosystems and causes multitrophic

370 interactions that are difficult to understand [1]. The possibility of reproducing different
371 climatic conditions by coupling carbon dioxide and temperature levels can/has been
372 considered be very useful to obtain a better understanding of plant-pathogen interactions, as
373 has been done in the present work which deals with a soilborne pathogen. Focusing on plant-
374 soil systems, it is well known that plants directly or indirectly control and influence the
375 multitrophic interactions of the soil through root exudates [22]. The results reported in this
376 study refer to disease incidence, cultivable abundance, leaf physiological performances, root
377 exudate analyses, and variability in substrate fungal profiles by PCR-DGGE. Several studies
378 have reported that rising CO_2 concentrations can cause plants to modify their root
379 architecture and exudation compounds in the rhizosphere [44,45]. In addition, it has been
380 predicted that global warming will directly influence multitrophic interactions if the soil
381 temperature exceeds its buffering capacity and this could be followed by changes in the
382 quality, quantity and diversity of plant and soil microbial communities and therefore plant
383 pathogen development [1]. In the present study, a good disease level was reached under
384 artificial inoculation of *F. oxysporum* f.sp. *conglutinans*, thus making it possible to evaluate
385 the effect of the different climate change simulations. The temperatures and CO_2
386 combinations in the six environmental conditions had a significant influence on the incidence
387 of *Fusarium* wilt. Higher CO_2 concentrations and temperatures caused a significant increase
388 in DI, in particular in the 22-26 °C range (Phytotron 4, Table 1). The effect of increased CO_2
389 concentrations was still observed at 26-30°C on disease severity development, although
390 lower values were observed than in the 22-26 °C range, probably due to the suboptimal range
391 of temperature that limits pathogen development. Although elevated CO_2 had a significant
392 effect on *Fusarium* wilt incidence, the increase in temperature also had a significant effect on
393 disease development, particularly at 22-26 °C, which is a favourable range of temperatures
394 for disease development [30]. As previously reported, higher temperature or CO_2 levels

generally correspond to a greater severity of *Fusarium* wilt in other crops such as lettuce [16,46] or wheat [47]. As reviewed by Ainsworth and Rogers [8], rising CO_2 concentrations and temperatures could influence leaf physiology, morphology and indirectly crop production by increasing or blocking their metabolic performances and photosynthetic efficiency. However, no relevant effects linked to disease development or environment conditions have been observed on PI and CCI data for infested and control plants, respectively. In fact, in infected rocket plants, large reductions of both indices have been recorded compared to controls, thus suggesting the high susceptibility of the photosynthetic machinery already at lower disease severity values. Furthermore, photosynthetic efficiency and leaf chlorophyll content are indicators of photosynthetic activity and chlorophyll stability. Fluorimeters and SPAD chlorophyll meters are frequently used for the measurement of foliar damage provoked by different biotic and abiotic stresses [35,48], although they were not considered useful for the present investigation.

Recent research in plant biology has pointed out the importance and the role of root exudates in mediating biological interactions in the rhizosphere. The chemical components of root exudates could deter or attract an organism with different effects on the plants. This would be particularly important during the pathogenesis of root-infecting fungal pathogens. However, the signalling and composition of root exudates in plant-pathogen interactions have not yet been elucidated [49-51]. To the best of the authors' knowledge, this is the first report to have been made on the analysis of root exudates on wilted rocket plants under simulated climate change conditions. The results have shown a different exudation pattern between the controls and infected plants. Interestingly, higher levels of pH, organic acid and TOC (mainly composed of sugars) were observed in the infected samples in phytotron 4 where the disease index was higher. In addition, high values of organic acid, TOC and amino acids were also recorded in phytotron 6, where a consistent level of DI was observed. These results suggest

420 the combined temperature and high CO_2 effect on root component release. In particular, the
421 TOC compounds underwent a significant increase, since they represent a carbon source for
422 microbial metabolism and energy that in turn could influence the attraction and severity of
423 the soilborne pathogen. Similarly, Kerks et al. [52] reported the activation of root exudate
424 chemotaxis and pathogenicity genes of *S. enterica* serovar Typhimurium in lettuce, which are
425 involved in root attachment and subsequent colonization. Although a great deal of
426 information is available concerning the relationships between symbionts and plants, limited
427 knowledge exists about the communication between plants and root pathogens mediated by
428 rhizodeposits and/or by pathogen metabolite production (e.g. volatile organic compounds,
429 VOCs) [53]. Further studies are needed to increase analytical skills and to analyse the
430 chemistry of root exudates, and thus to resolve the dialogue that takes place between
431 pathogens and plant roots. In addition, root exudates represent the main source of soil organic
432 carbon, defined as soluble low-molecular weight components that are mainly composed of
433 sugars, amino acids, organic acids and other secondary metabolites which vary from plant to
434 plant and which are able to shape the rhizosphere microbiome [22]. Conversely, no relevant
435 effects of CO_2 or temperature levels have been observed in the control plants to explain the
436 variation in the root exudate components in phytotron conditions, in line with what has been
437 reported by Uselman et al. [54] in *Robinia pseudoacacia*.
438 It is well known that DGGE fingerprints can be used to describe microbial
439 composition and diversity, but they do not provide any information on the abundance and
440 concentration of separate microbial species [55]. For this reason, this limitation has partially
441 been addressed here by conducting plate counts on several media. In the present experimental
442 conditions, the total bacteria and fungi plate counts in both the infested and control substrate
443 samples showed a decreasing trend over time up to the end of the experiment, without climate
444 simulation effects being observed. These results suggested that the forecast increase in CO_2

445 and temperature levels due to climate changes would have a limited effect on fungal and
446 bacterial development. Recent reports [16,26,56] have shown similar results, while other
447 studies have reported results that depend to a great extent upon the host, microorganisms and
448 environment [57]. Conversely, the *F. oxysporum* f.sp. *conglutinans* plate counts at the end of
449 the present experiment have shown a slight increase or no significant variation for time 0,
450 except for phytotron 4 at higher CO_2 concentrations, where the development and disease
451 incidence were significantly higher. In phytotron 6, where disease incidence was high, no
452 significant development of pathogen over time was detected. This behaviour supports the
453 hypothesis of a plant-mediated effect increasing disease incidence. The variability in the
454 fungal profiles was assessed by means of the PCR-DGGE approach, which has frequently
455 been used, with good results, in other simulated global change studies [16,26]. The samples
456 were clearly separated in relation to the temperature or CO_2. In addition, this result was
457 further confirmed from an examination of the Shannon-Wiener indices. Interestingly,
458 phytotrons 4 and 6 showed the least diversity and the highest DI. Thus, a low diversity might
459 have a higher impact on disease development efficiency than the species visualized from the
460 DGGE examination. The loss in diversity caused by higher temperature and CO_2 levels could
461 be due to a plant-mediated effect that causes the selection of a few dominant species and
462 which may exclude others species through a competition strategy.
463

464 # Conclusions

465 The disease incidence of pathogenic *Fusarium* species could increase due to the
466 effects of the global changes that have been predicted for the future. The present
467 experimental conditions have shown a coupled temperature and CO_2 effect on disease
468 severity, which is probably plant-mediated, as reported for other pathosystems. Although no
469 specific predictions can be made on field conditions, the data obtained from phytotron growth

chambers could help to unravel the complexity of plant-soilborne pathogen interactions that take place under climate change conditions, in order to implement model prediction and prevention strategies.

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631

Fig 1. Photosynthetic efficiency measurements. Effect of different CO_2 and temperature combinations on the photosynthetic efficiency of the leaves (PI) of rocket plants grown in a substrate artificially infested with *F. oxysporum* f.sp. *conglutinans* and the control. Tukey's HSD test ($P < 0.05$).

Fig 2. Assessment of chlorophyll content. Effect of different CO_2 and temperature combinations on the chlorophyll content of the leaves (CCI, °SPAD) of rocket plants grown in a substrate artificially infested with *F. oxysporum* f.sp. *conglutinans* and the control. Tukey's HSD test ($P < 0.05$).

Fig 3. PLS-DA models based on DGGE similarity distance matrix. Plot A, PLS-DA models based on the DGGE similarity matrix as a function of CO_2: 400-450 ppm (blue), 800-850 ppm (yellow); Plot B, PLS-DA models based on the DGGE similarity matrix as a function of the temperature: 26°C (blue), 22°C (yellow) ,and 30°C (red): Plot C, PLS-DA models based on the DGGE similarity matrix as a function of the time: time 0 (red), time 60 (yellow) and time120 (blue); Plot D; PLS-DA models based on the DGGE similarity matrix as a function of the phytotrons: 1 (red), 2 (yellow), 3 (blue), 4 (black), 5 (green), 6 (pink).

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

651

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

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

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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

Table 3. Total bacterial counts (TBC) of mesophilic bacteria from the infested and control substrate samples following incubation in phytotrons for 120 days.

	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

[a]The plate counts were calculated as the mean Log counts of the three replicates. Values with different superscripts differ significantly according to Tukey's HSD test ($P<0.05$)

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

668 ($P<0.05$)

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670

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

	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

[a]The plate counts were calculated as the mean Log counts of the three replicates. Values with different superscripts differ significantly according to Tukey's HSD test ($P<0.05$)

Supporting Information

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