Running title: ***High CO_2 and temperature effects on powdery mildew***

EFFECTS OF ELEVATED CO_2 AND TEMPERATURE ON INTERACTIONS OF ZUCCHINI AND POWDERY MILDEW

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Summary. Effects of increased CO_2 and temperature on powdery mildew (*Podosphaera xanthii*) of zucchini (*Cucurbita pepo*), were evaluated under controlled conditions. Zucchini plants were grown in phytotrons under four different simulated climatic conditions: 450 ppm of CO_2 at standard (18°C night, 24°C day) and elevated temperatures (22°C night, 28°C day), elevated CO_2 (800 ppm) with standard temperature and elevated CO_2 (800 ppm) with elevated temperature (4°C higher than standard). Physiological responses of zucchini and pathogen development were studied. Under elevated CO_2 both healthy and infected zucchini plants grew better when temperature was lower. Elevated CO_2 generally caused no significant differences in pathogen development or disease severity, whereas elevated temperature

stimulated the development of the pathogen. A combination of elevated CO_2 and temperature always stimulated the development of the pathogen and disease severity compared to standard conditions.

Key words: climate change, *Cucurbita pepo, Podosphaera xanthii*, epidemiology, phytotrons.

Introduction

Climate has changed in the recent past and is predicted to change in the future (Smith *et al.*, 2002). Atmospheric CO_2 concentrationhas increased from 367 ppm to 379 ppm in the last six years (Le Treut *et al.*, 2007), and is predicted to reach 730 to 1020 ppm by 2100, due to increasing world population and economic activity (Meehl *et al.*, 2007). At the same time, the rising concentrations of CO_2 and other greenhouse gases will lead to an increase between 1.8 and 4°C in mean global temperature (Meehl *et al.*, 2007).

Since both CO_2 and temperature are key variables affecting plants and their diseases, potential influences of climate change on plant growth, global food supply and disease risk are attracting considerable research interest in many countries (Rosenzweig and Parry, 1994; Myneni *et al.*, 1997; Harvell *et al.*, 2002). Numerous studies have measured plant growth under conditions of elevated CO_2 and temperature. Despite the diversity of experimental approaches and study subjects, Morison and Lawlor (1999) concluded that increased CO_2 generally produced larger plants with more and/or larger organs, while warmer temperatures accelerated the rate of organ development and expansion but decreased organ life time. A meta-analysis, encompassing data from more than 120 peer-reviewed articles describing physiology and production in 12 large scale Free-Air CO_2 Enrichment (FACE) experiments

during the past 15 years, confirmed some results from previous chamber experiments, and also addressed general variances between species.

Increases in CO_2 and temperature are expected to induce complex effects on plant pathosystems. Although research on the effects of climate change continues to be limited, new phytotron facilities are permitting study of effects of climate variables on infection rates in some pathosystems (Runion, 2003; Chakraborty, 2005; Garrett *et al.*, 2006; Pugliese *et al.*, 2010). An increase in production of defensive compounds and/or other changes in host physiology, morphology, or anatomy under elevated CO_2 could lead to reductions in disease incidence or severity for pathosystems such as *Erysiphe graminis*-barley and *Colletotrichum gloeosporioides* - *Stylosanthes scabra* (Runion *et al.*, 1994; Hibberd *et al.*, 1996; Chakraborty *et al.*, 2000; Pangga *et al.*, 2004). Several model approaches, such as climate matching and climatic mapping, have also been used to simulate and predict plant diseases under changing environments (Bourgeois *et al.*, 2004). Increased downy mildew on grapevine in 2030, 2050 and 2080 was forecasted to occur in Acqui Terme, Italy, using a weather-disease combined model. Increased number of days during May and June with weather conditions favorable to downy mildew were predicted, due to the advance in the date of primary outbreaks (Salinari *et al.*, 2007). An outcome of many studies and observations is increased powdery mildew severity on different hosts, as a result of temperature increases in mild climates (Boland *et al.*, 2004).

The pathosystem zucchini (*Cucurbita pepo*) - powdery mildew (*Podosphaera xanthii*) was chosen to study the effects of elevated atmospheric CO_2 concentrations and temperatures and their interaction. Zucchini is a typical vegetable crop in the Mediterranean area. Powdery mildew, caused by the biotrophic fungus *P. xanthii*, is one of the most important diseases of this crop in many areas of the world, and yield losses due to the disease can reach 50% (Sherf and Macnab, 1986; Zitter *et al.*, 1996). The disease is generally favoured by dry atmospheric conditions, moderate temperatures, reduced light intensity and succulent plant growth

(Sitterly, 1978). Optimum temperature for spore germination is 28°C (Sitterly, 1978) and 20-27°C for disease development (Sherf and Macnab, 1986). Powdery mildew reduces yields by decreasing the size and/or number of zucchini fruit (McGrath and Thomas, 1996).

The present study was undertaken in phytotrons, where temperature and CO_2 concentration were manipulated to simulate possible future climate scenarios. Host physiological parameters (chlorophyll content, gas exchange activity and plant growth), and phytopathological factors, including changes in *P. xanthii* growth and infection structures, were evaluated.

Materials and methods

Growth of plants

Seeds of *Cucurbita pepo* cv. Genovese were sown in greenhouse (18-26° C, RH = 70%, natural light). When the first seedling-leaves developed, about 10 days after sowing, plants were transplanted into pots (one plant/pot) containing 3:3:l of a peat-clay-perlite substrate. Pots were then moved into three controlled environment phytotrons (PGC 9.2, TECNO.EL, Italy) (44 plants/phytotron), maintained at different concentrations of carbon dioxide (CO_2) and different temperatures, and at the relative humidity and photoperiod conditions outlined in Table 1. Concerning temperature, five trials were performed with controlled temperature (considered standard) of 18°C minimum during night to 24°C (maximum during day). Temperatures, air relative humidity and light changed gradually from day to night, to better simulate natural situations. The tested variables were: 450 ppm of CO_2 (standard) with standard temperature (experimental control), elevated CO_2 (800 ppm) with standard temperature, standard CO_2) with elevated temperature (4°C higher than standard) and elevated CO_2 with elevated temperature. Phytotron settings are summarized in Table 1.

The phytotrons used allowed environmental parameters (temperature, relative humidity, air CO_2 concentration, air speed, leaf temperature, leaf wetness) to be accurately controlled. Soil temperature and soil water content (absolute volumetric moisture content) were also monitored in the pot containers. Lighting was from two different sources to obtain the best spectrum for plant growth. Environmental parameters were also measured and recorded in order to fully characterize the internal phytotron environments.

Pathogen and inoculation procedure

Podosphaera xanthii inoculum was prepared from diseased plants maintained under greenhouse conditions. Conidia were collected from infected leaves, counted and adjusted using an haeomyctometer (Bürker) in order to obtain conidial suspensions containing 5×10^5-1×10^6 conidia mL^{-1}. One drop of polisorbate 20 (Tween 20, Croda International Plc, Snaith, Goole, United Kingdom) was added to each suspension, and 2 mL was sprayed on the adaxial surface of each plant when the second true leaf was completely open (about 1 week after being moved into phytotrons).

At this time, plants to be inoculated were moved outside for a few minutes and sprayed with the inoculum. The plants were returned into the phytotrons. In each phytotron half of the plants were inoculated, while the others were left uninoculated as healthy experimental controls**.** During the experiments, inoculated and uninoculated plants inside each phytotron where not separated, to avoid influencing the microclimate in each phytotron unit. Assessments were carried out on primary infections and trials were terminated before the development of secondary infections.

Assessment of the influence on host physiological activity and growth

The physiological activity of host plants was monitored through measurement of gas exchange and chlorophyll content index (CCI) in 2nd leaves (four replicates/leaf) on three

infected plants and healthy plants in each phytotron. Gas exchange measurements were recorded, using a CO_2 and H_2O infra-red gas analyzer (ADC, Hoddesdon, UK) under open system. Intercellular concentration of CO_2 at 5 days after inoculation (dai), Ci (ppm), stomatal conductance at 5 dai, gs (mmol H_2O m^{-2} s^{-1}), transpiration rate, E (mmol H_2O m^{-2} s^{-1}) and assimilation A (μmol CO_2 m^{-2} s^{-1}) at 3, 5 and 10 dai, were measured both for healthy and diseased plants grown under all conditions. Water use efficiency (WUE, μmol CO_2 mmol H_2O^{-1}) was calculated as ratio of assimilation (A) to transpiration rate (E). Chlorophyll content index (CCI) was determined at 10 dai, using a chlorophyll meter (SPAD-502, Minolta).

Growth of host plants was monitored by cutting and recording fresh and dry weights of shoots of three infected and three healthy plants from each environmental condition at 0, 10 and 15 dai. Dry weights were measured after shoots had been kept in a forced air oven for 48 h at 105°C. Number of open leaves, and number and maximum length of fruits at 15 dai were recorded and used as indexes of plant development. At least three plants were randomly sampled for measurement of each parameter at each sampling time.

Pathogen and disease assessments

Fungal development was assessed using microscopy, in order to evaluate: 1) maximum and minimum diameters of colonies measured at 3 dai, and then calculated as area (mm^2), assuming each colony to be an ellipse; 2) number of hyphal tips (number of tips/colony) at 3 dai and number of conidiophores (number of conidiophores/colony) at 5 dai.

Three 1.5 cm diameter leaf disks, taken from the central part of the second leaves of three infected plants per phytotron at 3 and 5 dai, were detached and prepared for microscopy. The leaf disks were placed, adaxial surface up, into a Petri dish containing disks of absorbing paper soaked with a solution of ethanol-acetic acid (3:1). After complete chlorophyll extraction, usually about 20 h, leaf sections were moved to other Petri dishes containing

absorbing paper soaked with water. After 1 h, the disks were moved to other Petri dishes containing absorbing paper, soaked with lactoglycerol (lactic acid:glycerol:water, 1: 1: 1) for 24 h. Finally, leaf disks were stained with 0.1% bromophenol blue and transferred to microscope slides for the assessment.

At 8 dai, for each environmental condition, six individual powdery mildew colonies from the second leaves of infected plants were collected and transferred into a 2 mL Eppendorf tube containing 1 mL of water. Then the tube was placed on a vortex shaker under 3400 rad s^{-1} for 3 min to obtain a well mixed suspension. The concentration of conidia in the suspension was determined with a microscope slide haemocytometer (Bürker camera) and the average number of conidia produced by each colony was calculated.

The number of powdery mildew spots or percentage of leaf surface infected was determined for seven plants for each of the environmental conditions, and translated into indices (0-7) at 8, 10 and 15 dai, to show the disease incidence, according to Horsfall and Cowling (1978). Translation standards for these indices are shown in Table 2.

Statistical analyses

Data were analyzed with analysis of variance (ANOVA), using SPSS software for Windows (release 17.0; SPSS Inc., USA). Significance of differences between treatment means was evaluated using the Tukey test ($P \leq 0.05$).

Results

Photosynthetic gas exchange

CCI was recorded at 10 dai and gas exchange activity was monitored at 3, 5 and 10 dai in 2nd leaves (Tables 3, 4 and 5). With 800 ppm CO_2, CCI of healthy plants was significantly decreased at higher temperatures (22° to 28° C). Average intercellular carbon dioxide (Ci)

and stomatal conductance (gs) results of three trials at 5 dai are shown in Table 3. Elevated CO_2 greatly enhanced intercellular carbon dioxide (Ci) both in healthy and diseased plants. With higher temperature the stimulation was even greater in infected plants (Table 3). Stomatal conductance (gs) was increased by elevated CO_2 on healthy and infected plants at 5 dai (Table 3). Considering only the rise of temperature or CO_2, CCI was significantly less compared to standard conditions, while Ci was significantly greater (Table 4).

Average assimilation (A) and transpiration rate (E) results at 3, 5 and 10 dai in 5 trials are shown in Table 5. Elevated CO_2 greatly increased net photosynthesis by accelerating assimilation (A), but not at elevated temperatures (Table 5).

Transpiration rate (E) was not statistically different (Table 5) for the different regimes. No differences were observed in water use efficiency (WUE) among the environmental regimes (Table 5).

The slope of the regression lines calculated for assimilation and for the traspiration rates at 0, 3, 5 and 10 dai was always negative. Curves of regression with determined coefficients are outlined in Table 6. Data from healthy and infected plants are mixed. The analysis of variance showed that there were no significant differences among temporal trends calculated under the four different environmental regimes (Table 6).

Plant growth and development

Plant shoot weights at 15 dai are outlined in Table 7. No differences in fresh weights were detected for the different environmental regimes. The warmer temperatures caused a decrease in dry weight, especially on infected plants. Disease reduced plant growth under all conditions, and the reduction was highly significant under warmer temperature conditions.

At standard temperatures, more fruits were produced by healthy plants under elevated CO_2 than at 450 ppm CO_2; on the contrary, higher temperature reduced fruit development (Table

7). At 800 ppm of CO_2 and under higher temperatures, the plants had more fully opened leaves (Table 7), although the leaves were smaller than at lower temperature.

Development of powdery mildew and disease index

Results of microscopy observations are shown in Table 8.

Colony area, number of hyphal tips, conidiophores and conidia per colony were significantly greater at temperature ranging from 22° to 28° C compared to standard conditions. Disease indices (0-7) also varied in different trials due to the quantity of inoculum, but were never influenced only by increased CO_2. On the contrary, elevated temperature and CO_2 significantly stimulated disease index (Table 8).

There was a clear increment of growth of the pathogen, fecundity and severity of the disease observed at the higher temperature-higher CO_2 combination compared with control temperature combinations, in particular with higher CO_2 at standard temperatures.

Discussion

In our study, both healthy and powdery mildew-infected zucchini plants grew better under elevated CO_2. Similar results have been previously reported for several host-pathogen combinations (Wechsung *et al.*, 1995; Usuda and Shimogawara, 1998; Morison and Lawlor, 1999; Wand *et al.*, 1999; Kaddour and Fuller, 2004; Ainsworth and Long, 2005; Pugliese *et al.*, 2010).

With elevated CO_2 and temperature, the vegetative-reproductive balance of zucchini plants was in favour of vegetative development: plants produced more leaves and less fruit, especially in diseased plants. The same plants had lower dry weights and similar fresh weights to plants grown at standard temperature and CO_2 concentration, which corresponded to greater water content and, so, to greater susceptibility to pathogens.

In short, elevated CO_2 reduced stomatal conductance (gs), stimulated intercellular concentration of CO_2 (Ci), and assimilation (A). With higher temperature, the results were similar, except that there was a reduction of CCI and smaller increase of assimilation. The reduction of chlorophyll was caused by more rapid leaf senescence under higher temperature. Intercellular concentration of CO_2 in plants grown at elevated carbon dioxide was significantly increased, even compared to values observed in plants grown under elevated temperature. Under elevated CO_2 and temperature, plants developed much more rapidly and warmer temperature accelerated the rate of organ development and expansion but reduced the duration (Morison and Lawlor, 1999), but elevated CO_2 did not completely compensate for this negative effect. So, under elevated CO_2 and temperature, healthy zucchini plants aged rapidly with less chlorophyll in 2nd leaves at 10 dai, and produced more but smaller leaves, fewer and smaller fruits, resulting in lower dry weight at the end of the trials (15 dai).

Considering the assimilation data, zucchini plants probably belong to the group of plant which are able to cope well with excess carbohydrate, similarly to trees, which show large sink capacities (Davey *et al.*, 2006; Ainsworth and Rogers, 2007).

At standard temperatures, elevated CO_2 alone did not affect growth of *P. xanthii*; on the contrary, in combination with an increase of temperature pathogen development was always stimulated. These results suggest that the increase of CO_2 *per se* had no effect on disease development, while temperatures *per se* significantly increased pathogen development. When temperatures are favorable to powdery mildew, elevated CO_2 may favour growth of these pathogens by increasing sugar supply for plant growth (Hibberd *et al.*, 1996). Similar to the results of Jenkyn and Bainbridge (1978), infection of powdery mildew increased plant respiration and reduced chlorophyll content index and stomatal conductance, thus reducing the net photosynthesis and plant weight under all environmental conditions, though not always significantly. Increased respiration due to more rapid pathogen development under

255 higher temperature may explain why infected plants had higher Ci than those grown under the
256 standard environmental condition.
257 Four different sets of environmental conditions were tested in the present study: 450 ppm of
258 CO_2 with standard and elevated temperatures; 800 ppm of CO_2 with standard and elevated
259 temperatures. Florides and Christodoulides (2009) reported that an increase in the CO_2 level
260 is directly correlated to an increase in temperatures, so our purpose was to consider a possible
261 future scenario in which temperatures and CO_2 will both be greater. Our results showed that
262 under the tested conditions, zucchini powdery mildew development is more influenced by the
263 combination of CO_2 air concentration and temperature than by CO_2 level alone. However,
264 increased CO_2 can induce some changes in host physiology and anatomy, for instance linked
265 to the observed increased assimilation under high concentration of CO_2, that can be involved
266 in the activation of plant defense mechanisms.
267 Considering that the rising concentrations of CO_2 and other greenhouse gases will lead to an
268 increase in global temperature, we can assume that the increasing levels of CO_2 are likely to
269 indirectly affect powdery mildew of zucchini, and may also similarly affect other powdery
270 mildew pathogens.
271

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Tables

Table 1. Different environmental conditions used in phytotrons for assessing the effects of elevated CO_2 and temperature on interactions of zucchini and powdery mildew.

Phytotron	Temperature(°C)		Carbon Dioxide (ppm)	Relative Humidity (%)		PAR* (µmol m^{-2} s^{-1}) (16 h photoperiod)	
	Max (Day)	Min (Night)		Min (Day)	Max (Night)	Min (Night)	Max (Day)
1	24	18	450	40	70	0	700
2	24	18	800	40	70	0	700
3	28	22	450	40	70	0	700
4	28	22	800	40	70	0	700

* PAR, Photosynthetically active radiation.

Table 2. Standard disease index of percentage of leaf area infected or number of powdery mildew spots per leaf.

Disease index	Percentage (%)	Number of spots
0	0	0
1	(0-2.5)	(0-30)
2	(2.5-5)	(30-65)
3	(5-10)	(65-100)
4	(10-25)	(100-150)
5	(25-50)	(150-250)
6	(50-75)	(250-400)
7	(75-100)	>400

Table 3. Mean chlorophyll content indices (CCI), intercellular carbon dioxide (Ci) and stomatal conductance (gs) in 2nd leaves of zucchini plants grown in different temperature and CO_2 regimes. Data are shown as means ± SE (n≥5). Average results of five trials.

Temperature (°C)	CO_2 (ppm)	CCI, 10 dai		Ci (ppm), 10 dai		Gs (mmol m^{-2} s^{-1}), 10 dai	
		healthy	infected	healthy	infected	healthy	infected
18-24	450	23.7±1.27 a	20.3±1.06 a	365.8±22.04 b[a]	341±10.59 d	0.19±0.042 b	0.16±0.042 ab
18-24	800	25.5±1.30 a	20.2±1.62 a	594.6±32.43 a	513±20.28 b	0.28±0.037 a	0.26±0.054 a
22-28	450	24.9±1.53 a	12.9±1.89 b	353.7±7.88 b	420±4.61 c	0.20±0.016 b	0.17±0.031 ab
22-28	800	19.4±1.55 b	9.7±1.74 b	595.4±8.14 a	604±23.38 a	0.16±0.028 b	0.13±0.032 b

[a] Means of each parameter accompanied by the same letter are not significantly different at *P*=0.05 (Tukey's test).

Table 4. Mean chlorophyll content indices (CCI) and intercellular carbon dioxide (Ci) in 2nd leaves of zucchini plants grown in different temperature and CO_2 regimes considering temperature or CO_2. Data are shown as means ± SE (n≥5). Average results of five trials.

Parameter	value	CCI	Ci (ppm)
CO_2 (ppm)	450	18.3±0.82 a[a]	391±7.9 b
	800	15.7±0.90 b	524±14.2 a
Temperature (°C)	18-24	21.7±0.67 a	418±13.1 b
	22-28	12.8±0.86 b	588±11.6 a

[a]Means of each parameter accompanied by the same letter are not significantly different at P =0.05 (Tukey's test).

Table 5. Mean assimilation (A), transpiration rate (E), and water use efficiency (WUE) in 2nd leaves of zucchini plants grown in different temperature and CO_2 regimes. Data are shown as means of three trials.

Temperature (°C)	CO_2 (ppm)	A+ (μmol CO_2 /m^2 /s)	E (mmol/m^2/s)	WUE (μmolCO_2/mmol H_2O)
18-24	450	20.52± 2.47 a[a]	4.93±0.87 a	4.2±1.45 a
18-24	800	30.72±6.76 b	4.46±0.54 a	6.9±1.33 a
22-28	450	17.49±3.52 a	3.84±0.39 a	4.6±1.26 a
22-28	800	22.66±2.61 a	4.13±0.68 a	5.5±0.69 a

[a]Means of each parameter accompanied by the same letter are not significantly different at P=0.05 (Tukey's test).

Table 6. Linear regression analyses of assimilation (A) and transpiration rate (E) for zucchini plants grown in different temperature and CO_2 regimes.

Phytotron	A+ (µmol CO_2 /m^2 /s)	Regression curve	R^2	E (mmol/m^2/s)	Regression curve	R^2
1 (18-24 °C, 450 ppm CO_2)	- 0.73 a	y = -0.73 x + 1.94	0.867	- 0.44 a	y = -0.44 x + 3.56	0.801
2 (18-24 °C, 800 ppm CO_2)	- 1.71 a	y = -1.71 x + 8.22	0.825	- 0.37 a	y = -0.37 x + 0.68	0.793
3 (22-28 °C, 450 ppm CO_2)	- 1.02 a	y = -1.02 x + 3.86	0.874	- 0.31 a	y = -0.31 x + 4.62	0.849
4 (22-28 °C, 800 ppm CO_2)	- 1.95 a	y = -1.95 x + 1.92	0.893	- 0.28 a	y = -0.28 x + 3.31	0.906

Table 7. Mean fresh and dry weights of plant shoots, numbers and maximum lengths of fruits, and numbers of open leaves at 15 days after inoculation, for zucchini plants grown in different temperature and CO_2 regimes. Data are shown as means ± SE (n≥5).

Temperature (°C)	CO_2 (ppm)	Shoot weight (g)				Zucchini fruits				Open leaves	
		Fresh		Dry		Number		max length (cm)		(number)	
		healthy	infected	healthy	infected	healthy	infected	healthy	infected	healthy	infected
18-24	450	115±16.86 a[a]	82.0±2.71 a	9.3±0.783 ab	9.0±0.319 a	1.0±0.19 b	1.7±0.36 a	2.8±0.42 ab	2.5±0.57 a	5.2±0.12 b	5.2±0.23 b
18-24	800	111±11.34 a	85.6±2.81 a	11.8±1.582 a	9.4±0.565 a	1.8±0.27 a	1.8±0.20 a	3.2±0.64 a	2.4±0.34 a	4.7±0.35 b	5.2±0.15 b
22-28	450	101±5.36 a	78.1±4.32 a	9.4±0.852 ab	6.4±0.649 b	0.7±0.21 b	0.3±0.18 b	2.2±0.38 ab	1.9±0.26 a	5.4±0.36 b	5.7±0.48 ab
22-28	800	116±13.67 a	75.9±6.76 a	8.8±0.314 b	5.3±0.632 b	0.5±0.24 b	0.1±0.15 b	0.3±0.13 b	0.11±0.11 b	6.7±0.13 a	6.3±0.21 a

[a]Means of each parameter accompanied by the same letter are not significantly different at P=0.05 (Tukey's test).

Table 8. Mean pathogen parameters and disease indices for powdery mildew on zucchini plants grown in different temperature and CO_2 regimes. Data are shown as means ± SE (n≥5).

Temperature (°C)	CO_2 (ppm)	Colony area (mm^2)	Hyphal tips (number/colony)	Conidiophores (number/colony)	Spores/colony (number)	Disease Index (0-7)[b]
18-24	450	0.35±0.033 a[a]	10.3±0.83 a	0.4±0.35 a	5315±840 a	2.8±0.33 a
18-24	800	0.31±0.031 a	9.5±0.71 a	2.0±0.89 a	4199±645 a	2.9±0.35 a
22-28	450	0.52±0.058 b	13.4±0.69 b	4.1±0.37 b	8491±684 b	3.4±0.41 ab
22-28	800	0.68±0.133 b	17.3±1.81 c	11.4±2.27 c	9871±721 b	4.0±0.36 b

[a]Means of each parameter accompanied by the same letter are not significantly different at *P*=0.05 (Tukey's test).

[b] The disease index refers to combined assessments at 8, 10 and 15 dai.