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**Verrucarin A and roridin E produced on spinach by *Myrothecium verrucaria* under different temperatures and CO<sub>2</sub> levels**

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## 13 **Abstract**

14 The behaviour of *Myrothecium verrucaria*, artificially inoculated on spinach, was studied under  
15 seven different temperature conditions (from 5 to 35 °C), and under eight different combinations of  
16 temperature and CO<sub>2</sub> concentration (14 -30 °C and 775-870 or 1550-1650 mg/m<sup>3</sup>). The isolate used  
17 for this study was growing well on spinach, and the mycotoxins verrucarins A and roridin E were  
18 produced under all tested temperature and CO<sub>2</sub> conditions. The maximum levels of verrucarins A  
19 (18.59 ng/g) and roridin E (49.62 ng/g) were found at a temperature of 26-30 °C and a CO<sub>2</sub> level of  
20 1550-1650 mg/m<sup>3</sup>. Rises in temperature as well as in temperature and CO<sub>2</sub> concentrations had a  
21 significant effect by increasing *Myrothecium* leaf spots on spinach. The biosynthesis of verrucarins  
22 A was significantly increased at the highest temperature (35 °C), while roridin E was influenced by  
23 the CO<sub>2</sub> concentration. These results show that a positive correlation between climate condition and  
24 macrocyclic trichothecene production is possible. However, because of the ability of *M. verrucaria*  
25 to produce mycotoxins, an increase in temperature could induce the spread of *M. verrucaria* in  
26 temperate regions, this pathogen may gain importance in the future.

27 **Keywords:** *Spinacia oleracea*, phytotron, mycotoxins, HPLC-MS/MS, climate change.

## 28 **Introduction**

29 According to the “Intergovernmental Panel on Climate Change” (IPCC), the global mean  
30 temperature has increased by 0.74 °C over the last 100 years, and, since 1750, the CO<sub>2</sub>  
31 concentration in the atmosphere increased from 543 to 713 mg/m<sup>3</sup> in 2000 (IPCC 2001). Human  
32 activities during the mid-20<sup>th</sup> century may have caused these global temperature increase and  
33 changes in the Earth’s climate (IPCC 2007). For the next 10-20 years, several authors expect CO<sub>2</sub>  
34 concentrations to double and triple, and because of this, expect that the global temperature may  
35 increase by between 1.5 and 5 °C (Medina et al. 2015, IPCC 2014). Such climate changes could  
36 produce two different effects: higher temperatures favor the growth of many fungi, and thus lead to  
37 the occurrence of some mycotoxins in regions in which currently cooler temperatures prevent

38 fungal growth. Alternatively, temperatures may rise to a level in already hot regions that the  
39 incidence of fungal pathogens will decrease (Paterson and Lima 2011). Research that has focused  
40 on the study of the typical pathogens in warmer climate conditions has shown a good capability of  
41 adaptation of these pathogens under different environmental conditions, in part as a consequence of  
42 climate change (Gullino et al. 2014).

43 Leafy vegetables constitute a dynamic productive sector that is generally related to new cultivation  
44 techniques and affected by new diseases and, more recently, by global climate changes (Gullino et  
45 al. 2014). Leafy vegetables are increasingly being grown in many countries, in part because of the  
46 popularity of ready-to-eat products and in part because of the increasing attention towards healthy  
47 eating habits. Italy, together with Great Britain, is a leader producer of fresh vegetable and ready-to-  
48 eat products in Europe, with a total production volume of 2.15 million tons in 2013 and, 80  
49 processing plants. Spinach (*Spinacia oleracea* L.), with a production of 83,000 tons in 2013, is one  
50 of the most important commodities in Italy (FAOSTAT 2016), and is consumed extensively  
51 because of its importance in children's diets. Spinach is an important source of essential nutrients  
52 such as vitamins (K, C, A, E, B6), minerals (Mg, K) and antioxidant (phenolic compound, ascorbic  
53 acid).

54 A new spinach pathogen, *Myrothecium verrucaria*, was reported for the first time in northern Italy  
55 in 2015 (Garibaldi et al. 2016a). *Myrothecium* diseases are generally associated with warmer  
56 environments and wet conditions (Fish et al. 2012). *M. verrucaria* is a highly virulent pathogen that  
57 is capable of infecting several important species, such as vegetables and ornamental plants.  
58 *Myrothecium* spp. were originally reported in warm temperatures, but the ongoing changes in  
59 climate have allowed this pathogen to develop in new areas, although its ability to be seed-  
60 transmitted (Belisario 1999) should also be considered. Various strains of *M. verrucaria* have been  
61 reported as trichothecene producers (Abbas et al. 2001), such as other fungi. In particular,  
62 *Stachybotrys chartarum* was capable to produce macrocyclic tricothecenes with possible

63 involvement in public health due to contamination of culinary herbs and spices (Biermaier et al.  
64 2015).

65 Trichothecenes are mycotoxins that can move passively across cell membranes, causing feed  
66 refusal, immunological problems, vomiting, skin dermatitis and haemorrhagic lesions in humans.  
67 Trichothecenes are a family of more than 200 toxins with a common tricyclic 12,13-  
68 epoxytrichothec-9-ene core structure, which is responsible for their cytotoxicity. On the basis of  
69 substitution, they have been classified into four groups: types A, B, C and D (Ueno 1980). Types A,  
70 B and C can be divided on the basis of the substituent at the C<sub>8</sub> position. Type A trichothecenes  
71 have a hydroxyl group or an ester function, type B trichothecenes have a carbonyl function, and  
72 type C have a C<sub>7</sub>-C<sub>8</sub> epoxide. Type D trichothecenes, which are called macrocyclic trichothecenes,  
73 have an additional ring that links the C<sub>4</sub> and C<sub>15</sub> positions (Degenkolb et al. 2008). Among the  
74 macrocyclic trichothecenes, at least 12 compounds have been classified as verrucarins, and 27 as  
75 roridins (Grove 1993). The production of macrocyclic trichothecene mycotoxins, such as roridin E  
76 and H, of verrucarin A, B and J, and of trichoverrin A and B, which are produced by *M. verrucaria*,  
77 causes significant safety concerns. These molecules are known to be toxic to cultured mammalian  
78 cells and phytotoxic (Jarvis et al. 1988). Moreover, they may play a role in the phytopathogenicity  
79 of *M. verrucaria* (Kuti et al. 1989). Type D trichothecenes inhibit the formation of the peptide bond,  
80 thus preventing protein synthesis (McLaughlin et al. 1977). In particular, roridin and verrucarin  
81 derivatives are able to cause an increase in cellular leakage, growth inhibition and chlorophyll loss,  
82 while verrucarin A has been reported to be the most toxic metabolite (Abbas et al. 2002).

83 Primarily, phytotrons are used to investigate how environment controls and modifies plant growth  
84 and development, but they are used also to complement and supplement field and greenhouse  
85 research. Phytotrons are an efficient method to manage controlled-environment condition. To  
86 simulate climate change scenario, with different temperatures and CO<sub>2</sub> levels on different  
87 pathosystems they are commonly used (Pugliese et al. 2012; Gullino et al. 2011; Chitarra et al.  
88 2015).

89 The aim of this work was to better understand the behaviour of *M. verrucaria*, artificially inoculated  
90 on spinach, under seven different temperatures (from 5 to 35 °C) and under eight different  
91 temperature and CO<sub>2</sub> concentration combinations (from 14 to 30 °C and at 775-870 or 1550-1650  
92 mg/m<sup>3</sup>).

## 93 **Materials and methods**

### 94 **Plant and pathogen material**

95 Spinach seeds (cv. Crocodile, Rijk Zwaan, the Netherlands) were sown in 2 L plastic pots filled  
96 with a steamed (90 °C for 30 minutes) white peat:perlite mix, 80:20 v/v (Turco Silvestro, Albenga,  
97 Italy). At least 20-25 spinach plants were present in each pot. The plants were kept for 20-25 days at  
98 22-23 °C, before being transferred to a growth chamber or phytotrons, as reported hereafter.

99 *M. verrucaria* (strain IT6) was grown on a potato dextrose agar (PDA, Merck, Darmstadt,  
100 Germany) amended with streptomycin sulphate for 7-10 days at 20-23 °C, with a 12 h photoperiod.  
101 The suspension, containing 5x10<sup>6</sup> conidia/ml, was used for the inoculation of the plants (five ml of  
102 inoculum/pot). The artificial inoculation was carried out 24 h after transferring the plants to the  
103 growth chamber, and seven days after moving them to the phytotrons.

104 Immediately after the artificial inoculation, the plants were enclosed in a clear polyethylene moist  
105 chamber (100x100x50 cm), which was covered by a transparent polyethylene film (50 µm thick), in  
106 order to create a dew chamber with 100% of relative humidity, and they were then left there for 6  
107 hours.

### 108 **Experimental set-up under growth chamber conditions**

109 The spinach plants were kept in the growth chambers under a 12 h/d fluorescent light regime, at  
110 temperatures of 5, 10, 15, 20, 25, 30 and 35 °C. The range of temperatures selected for the present  
111 study is representative of the ambient temperatures at which spinach is cultivated in temperate  
112 regions in different seasons. A randomised block design was used with four replicates. Each  
113 replicate was represented by one pot with 20 - 25 plants.

### 114 **Experimental set-up under phytotron conditions**

115 The spinach plants were kept in the phytotrons under eight different temperature and CO<sub>2</sub>  
116 combinations as show in Table 1. The photoperiod was changed gradually during the day, with 12  
117 hours of dark and 12 of light. Three types of lamp were used. These were turned on sequentially,  
118 from 1 to 3 degrees of intensity of light, and turned off from 3 to 1, in order to simulate natural light  
119 conditions. The lowest temperature of each range corresponded to the night period, and it was  
120 gradually increased during the day according to the light intensity. The experiments were repeated  
121 three times, under the eight tested environmental combinations, and under completely controlled  
122 environmental conditions. A total of six pots per experiment were used. The phytotrons were  
123 randomized by changing the environmental conditions and combinations during the first and second  
124 sets of experiments.

#### 125 **Disease assessment**

126 The inoculated spinach plants and controls were checked for disease development, starting from the  
127 appearance of the first symptoms. In particular, small, circular, sunken, grey-brown spots (1 to 2  
128 mm in diameter) with a well-defined border, developed in concentric rings, coalesced, with water-  
129 soaked tissues were evaluated. Myrothecium leaf spot severity was evaluated through a visual  
130 estimation of the symptoms on 50 leaves/pot, as the percentage of infected leaf area (disease  
131 severity). Disease severity was recorded by adopting a scale of 0 to 5 (0 = no symptoms; 1 = up to 5  
132 % of infected leaf area; 2 = 6 to 10% of infected leaf area; 3 = 11 to 25% of infected leaf area; 4 =  
133 26 to 50% of infected leaf area; 5 = 51 to 100 % of infected leaf area). Disease severity was  
134 calculated using the formula:  $DS = [\sum(n^{\circ} \text{ leaves} \times x_{0-5}) / (\text{total of leaves recorded})]$  with  
135  $x_{0-5} = (x_0 = 0; x_1 = 3\%; x_2 = 8\%; x_3 = 18\%; x_4 = 38\%; x_5 = 75.5\%)$ .

#### 136 **Chemicals**

137 Neosolaniol (Purity  $\geq 99.3\%$ ) and verrucarin A standards from *Myrothecium* spp. (Purity  $\geq 98\%$ )  
138 were purchased from Sigma-Aldrich in crystallized form; roridin E (Purity  $\geq 95\%$ ) was purchased  
139 from Iris Biotech GmbH (Germany). A stock solution of 1000  $\mu\text{g/mL}$  and a working solution of 10

140  $\mu\text{g/ml}$  were prepared in methanol for each molecule and kept at  $-20\text{ }^{\circ}\text{C}$ . Standards for HPLC  
141 calibration were prepared by diluting the working solution.

#### 142 **Extraction of secondary metabolites from plant material**

143 Samples were blended in a food processor GM-200 (Retsch GmbH, Germany) until complete  
144 disruption. Three g of each homogenized sample was placed in a centrifuge tube, extracted twice  
145 with 10 ml of extraction solution (acetonitrile:water 1:1 v/v with 0.1% of acetic acid), and mixed for  
146 2 min on a wrist-action shaker (Vortex). Samples were centrifuged (6000 rpm for 5 min) and the  
147 liquid extracts were collected in a new centrifuge tube. The solid residues were extracted with 20 ml  
148 of ethyl acetate. The organic extract was mixed together with the aqueous one and centrifuged  
149 (6000 rpm for 2 min). The organic phase was collected and evaporated to dryness by rotary vacuum  
150 evaporation, and the residue was dissolved in methanol:water (1:1 v/v) for HPLC-MS/MS analysis.

#### 151 **HPLC-MS/MS analysis**

152 Analyses were carried out by using a 1260 Agilent Technologies system, consisting of a binary  
153 pump and a vacuum degasser, connected to a Varian autosampler, Model 410 Prostar (Hansen Way,  
154 CA, USA), equipped with a 20  $\mu\text{l}$  loop. Chromatographic system was coupled to a triple quadrupole  
155 (Varian 310-MS TQ Mass Spectrometer). The separation of the mycotoxins was performed using a  
156 Gemini-NX C18 column ( $150 \times 3\text{ mm}$ ,  $3\text{ }\mu\text{m}$ , Phenomenex, Torrance, CA, USA), under a flow rate  
157 of 200  $\mu\text{l/min}$ . Solvent A was water acidified with 0.05% of formic acid, while solvent B was  
158 acetonitrile. The HPLC analysis was performed using a linear gradient from 70% to 100% of  
159 solvent B in 10 min, and then 5 min at 100% of solvent B.

160 Samples were ionized using an electrospray (ESI) ion source, operating in positive ion mode. Two  
161 transitions were selected for each compound for the MRM experiments:  $m/z\ 400 > 305$  (CE: 12 eV)  
162 and  $m/z\ 400 > 215$  (CE: 18 eV) for neosolaniol;  $m/z\ 520 > 249$  (CE: 18 eV) and  $m/z\ 520 > 457$  (CE:  
163 12 eV) for verrucarin A;  $m/z\ 532 > 361$  (CE: 16 eV) and  $m/z\ 532 > 113$  (CE: 24 eV) for roridin E.  
164 The collision gas (Ar) pressure was set at 2 mbar for all of the experiments.

165 Quantification was performed using external calibration based on serial dilution, from 1 to 500  
166 ng/g, of a multi-analyte stock solution. Results were corrected by recoveries (109% for verrucarins A  
167 and 98% roridin E) that had been determined by spiking three different blank samples at two  
168 concentration levels.

### 169 **Statistical analysis**

170 ANOVA analyses were performed with the SPSS 22.0 statistical software package (SPSS Inc.,  
171 Cary, NC, USA). Tukey's HSD test was applied when ANOVA revealed significant differences ( $P$   
172  $< 0.05$ ). The statistical analysis of the results was carried out using the Levene test to check for the  
173 homogeneity of variance. One-way ANOVA was used to investigate the effect of temperature and  
174 CO<sub>2</sub>, and their combinations, on disease severity caused by *M. verrucaria* on spinach grown in  
175 phytotrons.

## 176 **Results**

### 177 **Growth chamber trials**

178 The artificial inoculation with *M. verrucaria* led to a high level of disease; at the final assessment,  
179 carried out 20-25 days after the inoculation, disease severity has been affected significantly by the  
180 temperatures (Fig. 1). The lowest disease severity was observed at 5 °C and 10 °C, with an average  
181 disease severity of 2.7 to 15.2, respectively. DS values of 41.6, 56.9 and 71.2 were reached at 20  
182 °C, 25 °C and 30 °C respectively, while the highest DS, of 85.9, was observed at 35 °C (Fig.1).

### 183 **Phytotron trials**

184 One-way analysis of variance confirmed that temperature ( $p<0.0001$ ), and the combination of CO<sub>2</sub>  
185 and temperature, were significant factors ( $p<0.0001$ ) of influence on disease severity, while CO<sub>2</sub>  
186 concentrations from 775 to 870 mg/m<sup>3</sup> and from 1550 to 1650 mg/m<sup>3</sup> of CO<sub>2</sub> did not significantly  
187 influence the leaf spot caused by *M. verrucaria* ( $p=0.089$ ) (Fig. 2). However, significant differences  
188 were found for the temperature and CO<sub>2</sub> combinations (Fig. 2). The highest levels of DS (74.3 and  
189 76.4), were found for both CO<sub>2</sub> levels at 26-30 °C (Fig. 2), while, *Myrothecium* leaf spot severity

190 was significantly lower at 14-18 °C (DS from 18.3 to 31.3) and 18-22 °C (DS from 34.2 to 41.5)  
191 (Fig. 2).

### 192 **Mycotoxin production under growth chamber conditions**

193 The verrucarin A production was influenced by temperature, with a significant increase at the  
194 highest temperature (35 °C), at which an average concentration of 19.49 ng/g was produced (Fig.  
195 3). At temperature of 10 °C to 30 °C the isolates showed a lower average production of this  
196 metabolite with no significant differences (7.01 ng/g to 12.31 ng/g). Variations in the temperature  
197 did not influence roridin E production (Fig. 3), which was detected with an average concentration of  
198 7.20 ng/g, without any significant differences.

### 199 **Mycotoxin production under phytotron conditions**

200 Verrucarin A was not influenced by variations in the CO<sub>2</sub> concentrations. In fact, at 775-870 mg/m<sup>3</sup>  
201 and 1550-1650 mg/m<sup>3</sup> of the CO<sub>2</sub> concentration, the production of this metabolite showed the same  
202 trend and similar concentrations (Fig. 4). The maximum values were reached at 26-30 °C at both  
203 CO<sub>2</sub> levels (13.77 ng/g and 18.59 ng/g). The increase in temperature lead to an increase in  
204 verrucarin A production but without significant differences at both CO<sub>2</sub> levels. The averaged  
205 concentration of verrucarin A at 775-870 mg/m<sup>3</sup> ranged from 6.44 ng/g to 13.77 ng/g; at 1550-1650  
206 mg/m<sup>3</sup> the concentration ranged from 7.38 ng/g to 18.59 ng/g. At a low CO<sub>2</sub> concentration (775-870  
207 mg/m<sup>3</sup>), roridin E was produced significantly less at 14-18 °C (3.61 µg/g) than at other  
208 temperatures, at which the production was 17.32 ng/g, 20.12 ng/g and 21.74 ng/g at 18-22 °C, 22-  
209 26 °C and 26-30 °C, respectively (Fig. 4). An average concentration of 6.79 ng/g was detected for  
210 roridin E at 1550-1650 mg/m<sup>3</sup> of CO<sub>2</sub> and between 14 °C and 22 °C. The CO<sub>2</sub> concentration  
211 influenced roridin E production at a higher temperature, and a significant increase in concentration  
212 was detected, with 28.38 ng/g and 49.62 ng/g at 22-26 °C and 26-30 °C, respectively (Fig. 4).

### 213 **Discussion**

214 The understanding of the ability of mycotoxigenic fungi to respond to environmental changes is  
215 important because of the possible involvement in human health. The aim of the present work was to

216 evaluate the pathogenicity and the mycotoxigenic potential of fungi belonging to the *M. verrucaria*  
217 species under a climate change scenario. Phytotrons, with different CO<sub>2</sub> concentrations and different  
218 temperature levels, were used to obtain a better understanding of different factors on mycotoxin  
219 production by *M. verrucaria* on artificially inoculated spinach plants.

220 *M. verrucaria* is considered a facultative parasite of several plants. In the last few decades, a large  
221 number of new hosts of *Myrothecium* spp. have been studied, because new species or varieties have  
222 become favorable for the proliferation of this pathogen in new regions (Belisario et al. 1999;  
223 Garibaldi et al. 2016b; Kim et al. 2003; Mmbaga et al. 2010; Worapong et al. 2009; Zaho et al.  
224 2010). Plant disease is the result of an interaction between a host plant, a virulent pathogen and the  
225 environment. Our results show that a rise in temperature corresponds to an increase in disease  
226 severity. Yang and Jong (1995) tested *M. verrucaria*, isolated from *Euphorbia esula*, on 54 plant  
227 species belonging to 11 genera in 8 families, that is rice, cotton, sunflower, tomato, cucumber,  
228 soybean and peanut. They reported that, in an experiment in a dew chamber, conducted for 18 h at  
229 30 °C, the pathogen infected several plants under artificial inoculation with a high concentration of  
230 conidia. This study reported that each increasing step of temperature caused a visible increase in  
231 disease severity. It has also been demonstrated that a variation of 3 °C could alter the development  
232 of *Myrothecium* leaf spot on *aphelandra*, *peperomia* and *dieffenbachia* (Chase 1983). In our  
233 experiment, the maximum disease severity was obtained in a growth chamber at 35 °C, while in a  
234 previous study, the most favorable temperature for disease development on *Dieffenbachia maculata*  
235 was from 21 to 27 °C, and a severe reduction in leaf spot was observed at 32 °C (Chase and Poole  
236 1984).

237 Bioactive secondary metabolites are largely produced by the *Myrothecium* genus, and more than 20  
238 and 30 compounds have been reported to be produced by *M. roridum* and *M. verrucaria*,  
239 respectively (Wang et al. 2007). Both of these fungi have been reported to produce macrocyclic  
240 trichothecenes, potent cytotoxic compounds (Bean et al. 1984). The first studies on *M. verrucaria*  
241 revealed that this pathogen is able to synthesize highly functionalized trichoverroids, trichothecenes

242 and sesquiterpenoids. Trichoverroids are the precursors of type-D macrocyclic trichothecenes,  
243 which are biosynthesized after the condensation of the C<sub>4</sub> and C<sub>15</sub> polyketide chains and form  
244 roridin E, from which verrucarins derive (McCormick et al. 2011). Roridin A and E and verrucarins  
245 A and J are the principal trichothecene metabolites produced by *M. verrucaria* and *M. roridum*  
246 (Bean et al. 1984). The IT6 isolate used for this work could be considered a high producer of  
247 verrucarins A and roridin E, and these metabolites were produced at all the considered temperature  
248 and CO<sub>2</sub> conditions. The biosynthesis of verrucarins A was significantly increased at the highest  
249 temperature (35 °C) in our study. The conversion from roridin to verrucarins skeleton is induced by  
250 the oxidative removal of the two-carbon side chain at position 6', which could be favored at high  
251 temperatures.

252 Several other pathosystems have also been studied to understand how climate change could  
253 influence fungal behavior and their secondary metabolite production. Our experiments conducted in  
254 phytotrons, where temperature and CO<sub>2</sub> concentrations were modified, have shown that a higher  
255 disease index was observed for spinach plants inoculated with the *M. verrucaria* strain when the  
256 temperature was increased. Variation in the CO<sub>2</sub> concentration did not produce any significant  
257 alterations in disease index, with the only exception being at the lowest temperature (14-18 °C).  
258 Similar results were obtained in a previous work, where different *Alternaria alternata* strains  
259 inoculated on cultivated rocket showed the same behavior (Siciliano et al. 2016).

260 In the present study, which has been conducted by varying both the temperature and the CO<sub>2</sub>  
261 concentration, the verrucarins A production has been found to be comparable with data obtained by  
262 only varying the temperature, at the same range (14-30 °C). This result has shown that variations in  
263 the CO<sub>2</sub> concentration are not a significant factor of influence on verrucarins A production. On the  
264 contrary, the tests conducted with coupled variations of the temperature and the CO<sub>2</sub> concentration  
265 have shown a significant increase in roridin E production, a result that is very different from the  
266 results of the tests in which only the temperature was varied. In fact, the average roridin E  
267 production was 7.20 ng/g in the growth chamber, while the experiments in the phytotrons showed a

268 7 times higher increment when the temperature was 26-30 °C and the CO<sub>2</sub> was 1550-1650 mg/m<sup>3</sup>.  
269 The synthesis of fungal toxins is a very complex biochemical process that involves many different  
270 factors, and the relationship between climatic conditions and the production of verrucarins A and  
271 roridin E has not yet been clarified.

272 In last few decades, several works have focused on discovering the relationship between climate  
273 change, mycotoxin production and disease spread. Some authors tried to explain the response of  
274 trichothecenes to the different factors involved in climate change. Van der Fels-Klerx et al. (2012)  
275 demonstrated a positive correlation between high temperature and deoxynivalenol, T-2 toxin and  
276 HT-2 toxin production on four cereal grain types, and a negative correlation between high  
277 temperature and nivalenol production. Another study conducted on durum wheat, incubated at  
278 different temperature levels (5-40 °C), showed that T-2 toxin and HT-2 toxin production was higher  
279 at 15 and 10 °C, respectively (Nazari et al. 2014). Only a few studies have been published about the  
280 correlation between trichothecene production and pathogenicity, and none is available on  
281 macrocyclic trichothecenes. Landschoot et al. (2012) have found that the ratio between the disease  
282 index and deoxynivalenol presence was different every year from 2002 to 2010, thus pointing out  
283 the difficulties with interpretation of such observations.

## 284 **Conclusions**

285 The results presented here show that an increase in temperature can influence *M. verrucaria* disease  
286 severity, on spinach, to a great extent, and that the expected increase in temperature in the next few  
287 decades could foster the expansion of *M. verrucaria* in temperate regions. This aspect should be  
288 considered as a possible threat to spinach production, due to the ability of this pathogen to produce  
289 mycotoxins at levels which are a health concern.

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295 **Conflict of interest**

296 Authors declare that there is no conflict of interest for this manuscript.

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388 **Tab. 1** Conditions of temperature and CO<sub>2</sub> concentration used in the experimental set-up under  
389 phytotron conditions.  
390

Phytotron	Temperature [°C]	CO <sub>2</sub> concentration [mg/m <sup>3</sup> ]
1	14 - 18	775 - 870
2	14 - 18	1550 - 1650
3	18 - 22	775 - 870
4	18 - 22	1550 - 1650
5	22 - 26	775 - 870
6	22 - 26	1550 - 1650
7	26 - 30	775 - 870
8	26 - 30	1550 - 1650

391

392 **Figure caption**

393 **Fig. 1** Disease severity under seven different temperature conditions. The boxes represent the interquartile  
394 range (IQR) between the first and third quartiles, and the line inside the boxes represents the median (2nd  
395 quartile). The whiskers denote the lowest and the highest values within 1.56 IQR from the first and third  
396 quartiles, respectively. The circles represent outliers beyond the whiskers.

397 **Fig. 2** Disease severity under eight different phytotron conditions. The boxes represent the interquartile  
398 range (IQR) between the first and third quartiles, and the line inside the boxes represents the median (2nd  
399 quartile). The whiskers denote the lowest and the highest values within 1.56 IQR from the first and third  
400 quartiles, respectively. The circles represent outliers beyond the whiskers.

401 **Fig. 3** Verrucaric acid and rosidin D production under different temperature conditions. The boxes represent the  
402 interquartile range (IQR) between the first and third quartiles, and the line inside the boxes represents the  
403 median (2nd quartile). The whiskers denote the lowest and the highest values within 1.56 IQR from the first  
404 and third quartiles, respectively. The circles represent outliers beyond the whiskers.

405 **Fig. 4** Verrucaric acid and rosidin D production under different phytotron conditions. Boxes A and C show  
406 production at 870 mg/m<sup>3</sup> of CO<sub>2</sub>, and boxes B and D show production at 1650 mg/m<sup>3</sup> of CO<sub>2</sub>. The boxes  
407 represent the interquartile range (IQR) between the first and third quartiles, and the line inside the boxes  
408 represents the median (2nd quartile). The whiskers denote the lowest and the highest values within 1.56 IQR  
409 from the first and third quartiles, respectively. The circles represent outliers beyond the whiskers.