Verrucarin A and roridin E produced on spinach by Myrothecium verrucaria under different temperatures and CO2 levels

Verrucarin A and roridin E produced on spinach by *Myrothecium verrucaria* under different temperatures and CO_2 levels

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Abstract

The behaviour of *Myrothecium verrucaria,* artificially inoculated on spinach, was studied under seven different temperature conditions (from 5 to 35 °C), and under eight different combinations of temperature and CO_2 concentration (14 -30 °C and 775-870 or 1550-1650 mg/m^3). The isolate used for this study was growing well on spinach, and the mycotoxins verrucarin A and roridin E were produced under all tested temperature and CO_2 conditions. The maximum levels of verrucarin A (18.59 ng/g) and roridin E (49.62 ng/g) were found at a temperature of 26-30 °C and a CO_2 level of 1550-1650 mg/m^3. Rises in temperature as well as in temperature and CO_2 concentrations had a significant effect by increasing *Myrothecium* leaf spots on spinach. The biosynthesis of verrucarin A was significantly increased at the highest temperature (35 °C), while roridin E was influenced by the CO_2 concentration. These results show that a positive correlation between climate condition and macrocyclic trichothecene production is possible. However, because of the ability of *M. verrucaria* to produce mycotoxins, an increase in temperature could induce the spread of *M. verrucaria* in temperate regions, this pathogen may gain importance in the future.

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

Introduction

According to the "Intergovernmental Panel on Climate Change" (IPCC), the global mean temperature has increased by 0.74 °C over the last 100 years, and, since 1750, the CO_2 concentration in the atmosphere increased from 543 to 713 mg/m^3 in 2000 (IPCC 2001). Human activities during the mid-20th century may have caused these global temperature increase and changes in the Earth's climate (IPCC 2007). For the next 10-20 years, several authors expect CO_2 concentrations to double and triple, and because of this, expect that the global temperature may increase by between 1.5 and 5 °C (Medina et al. 2015, IPCC 2014). Such climate changes could produce two different effects: higher temperatures favor the growth of many fungi, and thus lead to the occurrence of some mycotoxins in regions in which currently cooler temperatures prevent

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

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

A new spinach pathogen, *Myrothecium verrucaria*, was reported for the first time in northern Italy in 2015 (Garibaldi et al. 2016a). *Myrothecium* diseases are generally associated with warmer environments and wet conditions (Fish et al. 2012). *M. verrucaria* is a highly virulent pathogen that is capable of infecting several important species, such as vegetables and ornamental plants. *Myrothecium* spp. were originally reported in warm temperatures, but the ongoing changes in climate have allowed this pathogen to develop in new areas, although its ability to be seed-transmitted (Belisario 1999) should also be considered. Various strains of *M. verrucaria* have been reported as trichothecene producers (Abbas et al. 2001), such as other fungi. In particular, *Stachybotrys chartarum* was capable to produce macrocyclic tricothechenes 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_8 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_7-C_8 epoxide. Type D trichothecenes, which are called macrocyclic trichothecenes,
73 have an additional ring that links the C_4 and C_{15} 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_2 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_2 concentration combinations (from 14 to 30 °C and at 775-870 or 1550-1650
92 mg/m^3).

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 $5x10^6$ 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_2
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: $DS = \lfloor \sum(n° \; leaves \times x_{0-5}) \; / \; (total \; of \; leaves \; recorded) \rfloor$ 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 ≥ 99.3%) and verrucarin A standards from *Myrothecium* spp. (Purity ≥ 98%)
138 were purchased from Sigma-Aldrich in crystallized form; roridin E (Purity ≥ 95%) was purchased
139 from Iris Biotech GmbH (Germany). A stock solution of 1000 µg/mL and a working solution of 10

140 μg/ml were prepared in methanol for each molecule and kept at -20 °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 μl 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 × 3 mm, 3 μm, Phenomenex, Torrance, CA, USA), under a flow rate
157 of 200 μl/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.

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

Statistical analysis

ANOVA analyses were performed with the SPSS 22.0 statistical software package (SPSS Inc., Cary, NC, USA). Tukey's HSD test was applied when ANOVA revealed significant differences ($P < 0.05$). The statistical analysis of the results was carried out using the Levene test to check for the homogeneity of variance. One-way ANOVA was used to investigate the effect of temperature and CO_2, and their combinations, on disease severity caused by *M. verrucaria* on spinach grown in phytotrons.

Results

Growth chamber trials

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

Phytotron trials

One-way analysis of variance confirmed that temperature ($p<0.0001$), and the combination of CO_2 and temperature, were significant factors ($p<0.0001$) of influence on disease severity, while CO_2 concentrations from 775 to 870 mg/m^3 and from 1550 to 1650 mg/m^3 of CO_2 did not significantly influence the leaf spot caused by *M. verrucaria* ($p=0.089$) (Fig. 2). However, significant differences were found for the temperature and CO_2 combinations (Fig. 2). The highest levels of DS (74.3 and 76.4), were found for both CO_2 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_2 concentrations. In fact, at 775-870 mg/m^3
201 and 1550-1650 mg/m^3 of the CO_2 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_2 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_2 levels. The averaged
205 concentration of verrucarin A at 775-870 mg/m^3 ranged from 6.44 ng/g to 13.77 ng/g; at 1550-1650
206 mg/m^3 the concentration ranged from 7.38 ng/g to 18.59 ng/g. At a low CO_2 concentration (775-870
207 mg/m^3), 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^3 of CO_2 and between 14 °C and 22 °C. The CO_2 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

evaluate the pathogenicity and the mycotoxigenic potential of fungi belonging to the *M. verrucaria* species under a climate change scenario. Phytotrons, with different CO_2 concentrations and different temperature levels, were used to obtain a better understanding of different factors on mycotoxin production by *M. verrucaria* on artificially inoculated spinach plants.

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

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

242 and sesquiterpenoids. Trichoverroids are the precursors of type-D macrocyclic trichothecenes,
243 which are biosynthetized after the condensation of the C_4 and C_{15} polyketide chains and form
244 roridin E, from which verrucarins derive (McCormick et al. 2011). Roridin A and E and verrucarin
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 verrucarin A and roridin E, and these metabolites were produced at all the considered temperature
248 and CO_2 conditions. The biosynthesis of verrucarin A was significantly increased at the highest
249 temperature (35 °C) in our study. The conversion from roridin to verrucarin 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_2 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_2 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_2
261 concentration, the verrucarin 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_2 concentration are not a significant factor of influence on verrucarin A production. On the
264 contrary, the tests conducted with coupled variations of the temperature and the CO_2 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

7 times higher increment when the temperature was 26-30 °C and the CO_2 was 1550-1650 mg/m^3. The synthesis of fungal toxins is a very complex biochemical process that involves many different factors, and the relationship between climatic conditions and the production of verrucarin A and roridin E has not yet been clarified.

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

Conclusions

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

Acknowledgments

This research was supported by the European Union's Horizon 2020 research and innovation programme under grant agreement No. 634179 "Effective Management of Pests and Harmful Alien

293 Species-Integrated Solutions" (EMPHASIS). The authors are grateful to Federico Berta for his
294 technical support and to Marguerite Jones for the English editing.

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_2 concentration used in the experimental set-up under
389 phytotron conditions.
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Phytotron	Temperature [°C]	CO_2 concentration [mg/m^3]
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

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Figure caption

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

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

Fig. 3 Verrucarin A and roridin E production under different temperature conditions. The boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside the boxes represents the median (2nd quartile). The whiskers denote the lowest and the highest values within 1.56 IQR from the first and third quartiles, respectively. The circles represent outliers beyond the whiskers.

Fig. 4 Verrucarin A and roridin E production under different phytotron conditions. Boxes A and C show production at 870 $mg/m^3$of CO_2, and boxes B and D show production at 1650 $mg/m^3$of CO_2. The boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside the boxes represents the median (2nd quartile). The whiskers denote the lowest and the highest values within 1.56 IQR from the first and third quartiles, respectively. The circles represent outliers beyond the whiskers.