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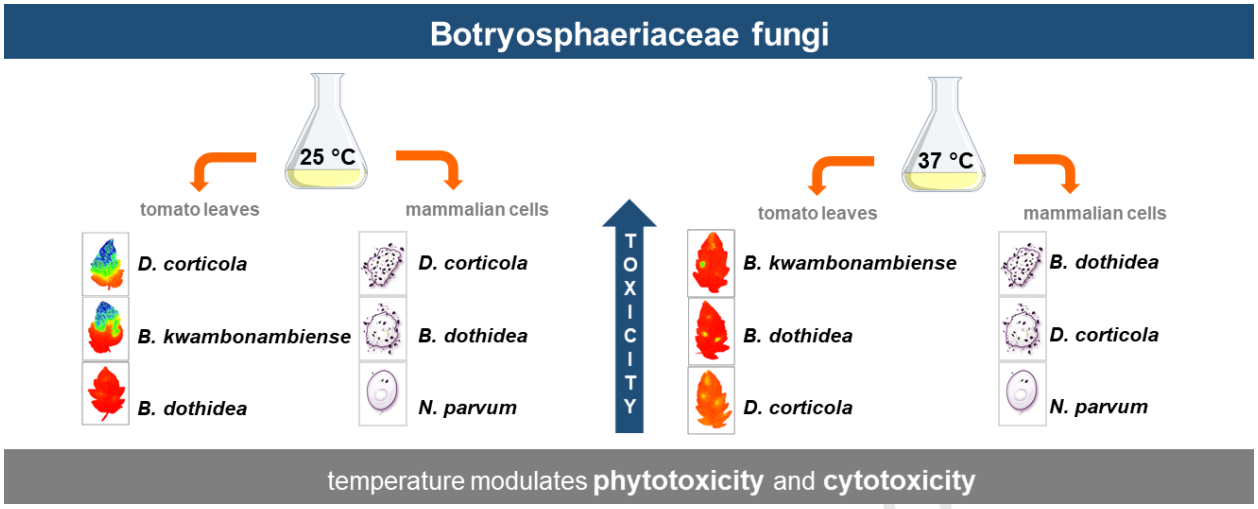

Effect of temperature on the phytotoxicity and cytotoxicity of Botryosphaeriaceae fungi

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Abstract

Botryosphaeriaceae fungi are phytopathogens mostly of woody hosts, causing numerous diseases, which ultimately may result in death of the host plant. Also, several Botryosphaeriaceae species have been associated with human infections. The number of available reports describing the effect of the expected increase on environmental temperature will have on Botryosphaeriaceae fungi are still scarce. In this study, the influence of temperature on the phytotoxicity and cytotoxicity of the culture filtrates of five Botryosphaeriaceae species — *Botryosphaeria dothidea* CAA642; *Diplodia corticola* CAA500; *Neofusicoccum parvum* CAA366 and CAA704; *N. eucalyptorum* CAA558 and *N. kwambonambiense* CAA755 — were evaluated on detached tomato leaves and on mammalian cell lines (Vero cells and 3T3 cells).

All culture filtrates of fungi grown at 25 °C were toxic to tomato leaves: symptoms were evaluated based on visual inspection of necrosis areas and on the maximum quantum yield of photosystem II (PSII), F_v/F_m. *D. corticola* and *N. kwambonambiense* culture filtrates were the most toxic, followed by *N. parvum* CAA704 and *B. dothidea*. On the contrary, *N. parvum* CAA366 and *N. eucalyptorum* were the least pathogenic. However, with the exception of *B. dothidea* culture filtrate, phytotoxicity dramatically decreased when strains were grown at 37 °C. All strains, except for *N. parvum* CAA366 and *N. eucalyptorum,* grown either at 25 °C or 37 °C, were cytotoxic to both animal cell lines (3T3 and Vero cells). *Neofusicoccum parvum* CAA366 and *N. eucalyptorum* were only cytotoxic to 3T3 cells. The culture filtrate of *D. corticola* grown at 25 °C was the most cytotoxic to mammalian cells, followed by the culture filtrate of *B. dothidea*. Also, we showed that *B. dothidea* was the most cytotoxic strain to both cell lines, at 37 °C,

followed by *D. corticola* and *N. parvum* CAA704. Although the toxicity of *B. dothidea* to both cell lines and of *N. kwambonambiense* to Vero cells increased with temperature, the opposite was found for *D. corticola*, *N. parvum* CAA366, and *N. eucalyptorum*. Phytotoxicity and cytotoxicity of Botryosphaeriaceae suggest that temperature modulates the expression of toxic compounds. In a scenario of a global increase of temperature, this modulation may contribute to new infections to plants by *B. dothidea* but also to humans specially in the case of *B. dothidea.*

Keywords: phytotoxicity; cytotoxicity; phytopathogenic fungi; Botryosphaeriaceae; climate change

Introduction

Earth is facing a global climate change, with a predicted continuous increase of temperature, CO_2 level and heavy precipitation (IPCC, 2014). Increasing temperature is altering microorganisms' biogeographical distribution (Bebber et al., 2013) and modifying the dynamics of microorganism-host interactions (Eastburn et al., 2011). Environmental modifications also may convert symbiotic or commensal relations into pathogenic interactions (Bliska and Casadevall, 2009). Exposure of phytopathogenic fungi to these alterations may reveal threats to human health and to several economically important crops. Nevertheless, little effort has been directed to the identification of the impact that increased temperature will have on microorganism-host interactions (Eastburn et al., 2011; Gallana et al., 2015).

The family Botryosphaeriaceae comprises phytopathogens, saprobes or endophytes, mostly on woody hosts (Barradas et al., 2016; Burgess et al., 2006; Gramaje et al., 2012; Linaldeddu et al., 2009; Mohali et al., 2007; Sakalidis et al., 2013). These fungi generally exist as hemibiotrophs in healthy plant tissues, which makes them particularly important in international trade, since they may spread undetected from one region to another, promoting potentially severe damage to hosts (Slippers and Wingfield, 2007). The interchange from an endophytic life style to a pathogenic one has been suggested to be triggered by stress such as drought, extreme temperature fluctuations, nutrient deficiencies and mechanical injuries (Slippers and Wingfield, 2007). Therefore, it is foreseen that these stress-related fungal pathogens will benefit from the current scenario of climate change. Infected plants can exhibit a multiplicity of disease symptoms such as dieback, canker, fruit rot, and ultimately death (Lawrence et al., 2017; Urbez-Torres and Gubler, 2009; Zlatkovic et al., 2016). Although foliar symptoms are observable, these pathogens have never been isolated from leaves, suggesting that extracellular molecules (metabolites and proteins) are the main drive for pathogenicity. It has been demonstrated that they are able to produce cell wall-degrading enzymes and phytotoxic metabolites whose synergistic action plays a role in the development of foliar symptoms (Andolfi et al*.,* 2011, Félix et al., 2019b, Gonçalves et al., 2019). In addition, a recent study also highlighted the role of secreted proteins for the cytotoxicity of *N. parvum* culture filtrates (Bénard-Gellon et al., 2015). Since extracellular molecules of these fungi are the main effectors for fungus-plant interactions, we centered our analysis on their extracellular molecules (present on the *in vitro* culture filtrate).

Barradas et al. (2016) characterised the phytopathogenicity/aggressiveness - based on the lesion length on *Eucalyptus* stem - of *Neofusicoccum parvum* (CAA704 and CAA366), *N. kwambonambiense*, *N. eucalyptorum*, *Diplodia corticola*, and *Botryosphaeria dothidea*. All six species were pathogenic to *Eucalyptus* but with distinct in aggressiveness: *D. corticola* and *N. kwambonambiense* were the most aggressive while *B. dothidea* was the least aggressive.

Botryosphaeriaceae fungi have occasionally been associated with human infections. This is the case of *L. theobromae* (Kindo et al., 2010; Saha et al., 2012a, 2012b; Summerbell et al., 2004; Thew and Todd, 2008; Woo et al., 2008) and *Macrophomina phaseolina* (Arora et al., 2012; Tan et al., 2008). Recently, *Botryosphaeria dothidea* a common plant pathogen has been associated with a case of ungual phaeohyphomycosis (Noguchi et al., 2017). A recent study revealed that the secretome of *L. theobromae* is cytotoxic to mammalian cells, supporting its ability to infect humans (Félix et al., 2016). In humans, fungal infections are difficult to treat due to the elevated toxicity of the fungicides available (Shalchian-Tabrizi et al., 2008).

Nonetheless, despite the relevance of these phytopathogens, only a few studies have highlighted the toxicity of the culture filtrates from Botryosphaeriaceae species to leaves of different plants (Bénard-Gellon et al., 2015; Guan et al., 2016; Martos et al., 2008; Ramírez -Suero et al., 2014; Reveglia et al., 2019). Furthermore, almost no studies have been carried out on the toxicity of Botryosphaeriaceae fungi culture filtrates towards animal cells. At last, there are only some studies that investigated the effect of temperature on the toxicity of the Botryosphaeriaceae fungus culture filtrate *Lasiodiplodia theobromae* (Félix et al., 2016, 2018, 2019).

Therefore, in this study the phytotoxic and cytotoxic effects of six strains from five species of Botryosphaeriaceae culture filtrates — *Neofusicoccum parvum* CAA704 and CAA366, *N. kwambonambiense* CAA755, *N. eucalyptorum* CAA558, *Diplodia corticola* CAA500, and *Botryosphaeria dothidea* CAA642, with different levels of aggressiveness — were evaluated on detached tomato leaves and two different mammalian cell lines (3T3 cells and Vero cells. The modulation by temperature of the phytotoxic and cytotoxic potential of these species was also assessed.

Results

1. Phytotoxicity of culture filtrates

Phytotoxicity of culture filtrates of six phytopathogens including *B. dothidea* CAA642, *D. corticola* CAA500, *N. eucalyptorum* CAA558, *N. kwambonambiense* CAA755, *N. parvum* CAA704 and *N. parvum* CAA366, (grown at 25 °C and 37 °C) was assessed via tomato leaf puncture assay (Fig. 1A, S1 and S2). No symptoms were observed when Potato Dextrose Broth (PDB) was used as a control over the experiment time (Fig.1A).

All culture filtrates from fungi grown at 25 °C were able to induce visible necrosis or chlorosis on detached tomato leaves. When the leaves of tomato plants were inoculated with the culture filtrate from *D. corticola* evident severe necrotic lesions developed within a day. At 2 days post inoculation (dpi), similar severe necrosis were also observed for leaves inoculated with *N. kwambonambiense* culture filtrate. Both culture filtrates from *D. corticola* and *N. kwambonambiense* grown at 25 °C displayed high phytotoxicity, and most of the leaves treated with these culture filtrates turned dark and necrotic at 6 dpi (Fig. 1A). The culture filtrates from *N. parvum* CAA704, *B. dothidea*, and *N. parvum* CAA366 grown at 25 °C started to cause small necrotic lesions on tomato leaves at 2 dpi. The development of symptoms was observed on leaves inoculated with *N. parvum* CAA704 culture filtrate until the end of the experiment. However, their phytotoxicity was lower compared with culture filtrates of *D. corticola* and *N. kwambonambiense* (Fig. 1A). The culture filtrate of *N. eucalyptorum* caused only a chlorotic halo around the inoculation sites in tomato leaves, without evident necrosis (Fig. 1A).

Culture filtrates of *D. corticola*, *N. kwambonambiense*, *N. parvum* CAA704 and *N. parvum* CAA366 grown at 37 °C visibly showed delayed and reduced severity

143 symptoms in detached tomato leaves comparing to the symptoms induced by culture
144 filtrates grown at 25 °C (Fig. 1A). *B. dothidea* and *N. eucalyptorum* culture filtrates
145 grown at 25 °C and 37 °C caused similar effects on tomato leaves (Fig. 1A). Symptoms
146 mostly appeared as chlorotic to pale green around inoculation sites or, in the case of *B.*
147 *dothidea*, as very small dark necrotic spots until 6 dpi (Fig. 1A).
148 Besides visually monitoring symptom development, we evaluated the phytotoxicity of
149 culture filtrates by measuring the maximum quantum efficiency of PSII (F_v/F_m) in control
150 and treated leaves for 6 days (Fig. 2). The results showed that F_v/F_m values of leaves
151 exposed to culture filtrates grown at 25 °C significantly decreased in a time-dependent
152 manner (Fig. 2). Changes in F_v/F_m were correlated with symptom development. As an
153 example, F_v/F_m values of leaves treated with the culture filtrate of *D. corticola* grown at
154 25 °C decreased rapidly at 1 dpi from 0.79 ± 0.007 to 0.58 ± 0.023, which is coincident
155 with onset of the visual necrosis symptoms (Fig. 1B and 2). Similarly, F_v/F_m values of
156 the remaining culture filtrates were reduced at 2 dpi, when visual necrosis symptoms
157 had been observed (Fig. 1B and 2).
158 As expected, the lowest F_v/F_m value was recorded in severely damaged leaves
159 inoculated with *D. corticola* and *N. kwambonambiense* culture filtrates grown at 25 °C
160 from 0.79 ± 0.007and 0.80 ± 0.009 to 0.32 ± 0.05 and 0.40 ± 0.07, respectively at 6 dpi
161 (Fig. 2). The leaves inoculated with *N. parvum* CAA704 culture filtrate grown at 25 °C
162 also showed a significant reduction in F_v/F_m values from 0.79 ± 0.005 at 1 dpi to 0.53 ±
163 0.08 at 6 dpi (Fig. 1B). There was only a slight reduction of F_v/F_m value in leaves
164 inoculated with *N. parvum* CAA366, *B. dothidea*, and *N. eucalyptorum* to 0.64 ± 0.05,
165 0.66 ± 0.07, and 0.71 ± 0.02, respectively at 6 dpi (Fig. 1B).

166 At 37 °C, culture filtrates of *B. dothidea* and *N. kwambonambiense* induced a reduction
167 of F_v/F_m values (Fig. 2, Table 1) that, at the end of the experiment, was significantly
168 different from the control (Fig. 2). Furthermore, the decline in F_v/F_m values was lower
169 comparing to that grown at 25 °C (Fig. 2, Table 1).
170 The rate of F_v/F_m decrease (determined as the slope of the linear phase of F_v/F_m
171 decrease) (Table 1) confirms the visual symptoms: at 25 °C, culture filtrates of *D.*
172 *corticola, N. kwambonambiense* and *N. parvum* CAA704 induced a significant reduction
173 of the F_v/F_m rate decrease of tomato leaves. Overall, fungus growing at higher
174 temperatures leads to lower phytotoxicity.
175

176 **2. Cytotoxicity of culture filtrates**

177 In addition to phytotoxicity, we investigated the cytotoxicity of the culture filtrates from
178 the strains *D. corticola* CAA500, *N. kwambonambiense* CAA755, *N. parvum* CAA704
179 and CAA366, *B. dothidea* CAA642, and *N. eucalyptorum* CAA558, grown at 25 °C and
180 37 °C, to Vero and 3T3 mammalian cell cultures (Fig. 3).
181 The culture filtrates of *D. corticola*, *N. parvum* CAA704, and *B. dothidea* grown either at
182 25 °C or 37 °C decreased significantly Vero cells' viability (Fig. 3A, B, and C). The
183 culture filtrate of *N. kwambonambiense* only caused significant reduction in Vero cells
184 viability, when the strain was grown at 37 °C (Fig. 3D). The culture filtrates of *N. parvum*
185 CAA366 and *N. eucalyptorum* grown either at 25 °C or 37 °C did not show any
186 significant effect on Vero cells' viability (Fig. 3E and F).

All strains grown either at 25 °C or 37 °C showed significant mortality of 3T3 cells comparing to control (Fig. 4A, B, C, D, and F), except for *N. parvum* (CAA366) grown at 37 °C, dilution 1:1 (Fig. 4E).

The cytotoxic effect of *B. dothidea* culture filtrate to both Vero and 3T3 cells increased with growth temperature, 25 and 37 °C, leading to the loss of about 47 % and 94 % cell viability, respectively (Fig. 3C and 4C). In contrast, increasing temperature had an opposite effect on *D. corticola*, as shown by a significant reduction of cytotoxicity on both cell lines (Fig. 3A and 4C): 3T3 cell mortality was over 90 % for dilutions 1:1 and 1:4 of *D. corticola* culture filtrate when grown at 25 °C, while at 37 °C, a lower reduction of cell survival was found (<50 %).

Neofusicoccum parvum CAA704 (grown either at 25 °C or 37 °C) induced a slight decrease on the viability of Vero cells and 3T3 cells that was not significantly affected by fungi growth temperature (Fig. 3B and 4B).

Neofusicoccum kwambonambiense culture filtrate was not cytotoxic to Vero cells, when the fungus was grown at 25 °C, and induced a slight, but significant toxic effect when the fungus was grown at 37 °C (Fig. 3D). 3T3 cells were susceptible to *N. kwambonambiense* regardless of growth temperature (Fig. 4D).

The cell viability for Vero cells exposed to culture filtrates of *N. parvum* CAA366 and *N. eucalyptorum* was 100 % at both temperatures (Fig. 3E and F). However, both culture filtrates grown either at 25 °C or 37 °C induced a reduction in cell viability of 3T3 cell line, although cytotoxic effects were more pronounced when *N. eucalyptorum* was grown at 25 °C (Fig. 4E and F).

Discussion

In this study, the culture filtrates produced by six strains from five species of Botryosphaeriaceae grown at 25 °C and 37 °C were evaluated for toxicity on tomato leaves and on mammalian cells. Healthy-unstressed leaves usually display an F_v/F_m ratio of approximately 0.8, whereas stress conditions cause photosynthetic inactivation or damage resulting in F_v/F_m reduction (Krause and Weis, 1991). F_v/F_m values of control and inoculated leaves indirectly indicate the degree of tissue damage (Murchie and Lawson, 2013) and showed to be excellent indicators of pathogenicity. In the detached leaves trial, all culture filtrates of fungi grown at 25 °C induced visual phytotoxicity symptoms accompanied by a reduction of the F_v/F_m values. F_v/F_m decreased following a biphasic pattern with a fast initial decrease and a slower (with a tendency for stabilization) second phase. The marked differences in aggressiveness between strains were quantified and are expressed by the rate of F_v/F_m decrease during the linear phase.

Differences in virulence and pathogenicity within these strains have been previously reported in artificial inoculation trials of *Eucalyptus globulus* (Barradas et al., 2016). The least virulent isolate to *E. globulus* was *B. dothidea* while *D. corticola* and *N. kwambonambiense* were the most aggressive. The remaining strains, including *N. parvum* CAA704, *N. parvum* CAA366, (previously reported as *N. algeriense*) and *N. eucalyptorum* CAA558 were considered as intermediate pathogens of *E*. *globulus* (Barradas et al., 2016). Similarly, in our study, the culture filtrate of *D. corticola* and *N. kwambonambiense* were the most toxic to detached tomato leaves (higher Fv/Fm initial decrease rates). We also investigated the effect of growth temperature on the

233 phytotoxicity of culture filtrates. The phytotoxicity of all strains decreased with the
234 increase of temperature. Only *B. dothidea* preserved its virulence at 37 °C, producing
235 similar sized lesions at the end of the experiment, corroborating the data by Qiu et al.
236 (2016) which suggested that *B. dothidea* is more virulent at higher temperatures (35
237 °C). Michailides and Owaga (2004) had also reported that infections on pistachio
238 caused by *B. dothidea* developed rapidly in the late spring and summer.
239 Interestingly, the same trend was observed for toxicity towards mammalian cells (Vero
240 and 3T3 cells): all strains promoted lower toxicity when grown at 37 °C, except *N.*
241 *parvum* CAA704 and *B. dothidea* CAA642. In fact, *B. dothidea* toxicity increased when
242 the fungus is grown at 37 °C, resulting in higher cell mortality rates, especially of 3T3
243 cells.
244 Phytotoxicity and cytotoxicity data suggest that the expression of toxic compounds
245 produced by Botryosphaeriaceae strains are modulated by temperature. Modulation of
246 virulence determinants by temperature was already suggested by Félix et al. (2016) that
247 showed that temperature modulates the expression of extracellular proteins and
248 metabolites by *Lasiodiplodia theobromae.* On the other hand, in our study, we observed
249 that temperature had no influence on the cytotoxic effect of the culture filtrate of *N.*
250 *parvum* CAA704, suggesting that the modulation of temperature on the cytotoxic effect
251 of the culture filtrate is strain dependent.
252 Optimal growth temperature for Botryosphaeriaceae species is usually between 25 °C
253 and 30 °C (Qiu et al., 2016). Thus, we can argue that the ability to infect humans may
254 result from an adaptation to increasing temperatures. Our data shows that *B. dothidea*
255 is highly cytotoxic to mammalian cells when grown at 37 °C, suggesting that it could be

able to adapt to human body temperature, and potentially become able to infect human hosts. In fact, *B. dothidea* has been reported on a (single, up to now) case of phaeohyphomycosis infection in a human patient (Noguchi et al., 2017).

Our data seem to suggest that plant cells are less sensitive than mammalian cells to the culture filtrates, which could be related to a higher defense competence towards these fungi, eventually related to the capacity of plant cells to degrade fungal toxic compounds, while mammalian cells cannot (De Gara et al., 2010). Among the cell lines studied, 3T3 cells are more sensitive than Vero cells, in agreement to the literature (Das and Devi, 2015; Félix et al., 2018, 2019).

Conclusion

We showed that temperature modulates phytotoxicity and cytotoxicity of Botryosphaeriaceae fungi. In general, phyto and cytotoxicity are higher when fungi are grown at 25 °C. Nonetheless, *B. dothidea, D. corticola* and *N. parvum* CAA704 induce high cell mortality when grown at 37 °C, in agreement with their human infection potential. A deeper study of the human/animal pathogenic potential of these fungi is still necessary, but our data indicates that higher environmental temperatures may lead to higher virulence.

We also showed that the well-known chlorophyll fluorescence index F_v/F_m can be used to quantify the damages to plant cells during pathogenicity assays, being more accurate than the traditional inspection of visual symptoms.

Material and methods

Fungal strains and plant material

Six fungal strains, from five Botryosphaeriaceae species isolated from *Eucalyptus globulus* in Portugal were used: *Botryosphaeria dothidea* CAA642, *Diplodia corticola* CAA500, *Neofusicoccum parvum* CAA366, *N. parvum* CAA704, *N. eucalyptorum* CAA558, and *N. kwambonambiense* CAA755. Cultures were maintained on half-strength Potato Dextrose Agar (PDA) (HIMEDIA, India) (Lopes et al., 2016).

Before assays, each strain was re-grown, by plating a mycelial plug on PDA and incubating at 25 °C for 5 days. Then, a plug of mycelium from the leading edge of the plate was inoculated into PDA plate and incubated at 25 °C for 7 days, except for CAA558 (10 days). Afterwards, two 5 mm plugs of mycelium were inoculated into a 250 mL Erlenmeyer flask containing 50 mL of PDB and each strain was incubated in triplicate at 25 °C and 37 °C for 10 (CAA755 and CAA366), 12 (CAA704, CAA500 and CAA642) or 20 days (CAA558), due to their different growth patterns. The cultures were filtered with filter paper and mycelium dry weight was determined after drying (50 °C, 48h, Félix et al., 2016). The extracellular media obtained were filtered (0.2 µm Whatman filter) and kept at -80 °C until phytotoxicity and cytotoxicity assays.

Tomato seeds (*Solanum lycopersicum* var. *cerasiforme*) were cultivated in plastic trays with vermiculite:peat [2:1 (w/w)] mixture and kept at 25-28 °C (16 h light period) in a growth chamber. All seedlings were equally well watered and fertilized weekly (5 ml/L Nutriquisa 5-8-10®) and grown for 90 days under the conditions described.

301 **Phytotoxicity assays on detached tomato leaves**

302 Phytotoxicity was assessed by a leaf puncture assay (Félix et al., 2019). 3-month old
303 tomato leaves were used. Three droplets (each drop 20 µL) of the culture filtrate were
304 applied on the leaves. The leaves were previously punctured in three places using a
305 sterilized needle and the stem placed in sterile water in a closed Petri dish. As control, a
306 droplet (20 µL) of PDB was applied on the leaves. Leaves were kept at room
307 temperature (22-25 °C). Symptoms' development was monitored daily for 6 dpi. All
308 assays were carried out in triplicate.

309 **Chlorophyll fluorescence imaging**

310 *In vivo* chlorophyll fluorescence was measured using a FluorCAM 800MF imaging
311 fluorometer (Photon System Instruments, Brno, Czech Republic), comprising a
312 computer operated control unit (SN-FC800-082; Photon System Instruments) and a
313 CCD camera (CCD381; PSI) with a f1.2 (2.8-6 mm) objective (Eneo, Rödermark,
314 Germany), as describe by Serôdio et al. (2013). Images of chlorophyll fluorescence
315 parameters F_o and F_m (dark-level and maximum fluorescence level, respectively),
316 before and after actinic illumination by applying modulated measuring light and
317 saturation pulses (<0.1 and >7,500 µmol photons m^{-2} s^{-1}, respectively), provided by red
318 LED panels (612 nm emission peak, 40-nm bandwidth). Images (512×512 pixels) were
319 processed by defining areas of interest (AOIs) matching the whole area of each leaf, by
320 excluding the non-fluorescent background signal using the FluorCam7 software (Photon
321 System Instruments). Images of F_o and F_m were captured on tomato leaves dark-
322 adapted for 20 min. The values of F_o and F_m were were calculated by averaging all pixel

values in each AOI (Serôdio et al., 2017). The maximum quantum yield of photosystem II (PSII) was calculated as $F_v/F_m = (F_m-F_o)/F_m$ (Schreiber et al., 1986). For the production of the images shown in Fig. 1, the scale of false colour of F_v/F_m values was normalized between 0.0 and 0.8 to ensure consistency between the different treatments. Fv/Fm decreased following a biphasic pattern with a fast initial decrease and a slower (with a tendency for stabilization) second phase. The differences between strains were quantified and are expressed by the rate of Fv/Fm decrease during the initial linear phase (Table 1).

Cytotoxicity assays on mammalian cell cultures

Mycelium dry weight was used to normalize the initial amount of culture filtrate. Culture filtrates were diluted [1:1, 1:4 and 1:16 (v:v)] with PBS (Phosphate Buffered Saline, Gibco). *In vitro* cytotoxicity assays were performed as described earlier (Duarte et al., 2015) with some modifications. A Vero cell line (ECACC 88020401, African Green Monkey Kidney cells, GMK clone) and a 3T3 cell line (DSMZ-ACC 173), Swiss Albino Mouse Embryonic Fibroblasts) were maintained and grown as described (Ammerman et al., 2008). 50 µl of a suspension of Vero and 3T3 cells in DMEM (Dulbecco's modified Eagle medium, Gibco) supplemented with 10 % heat-inactivated FBS (Fetal Bovine Serum, Gibco) and 1 % AA (Antibiotic Antimycotic Solution, Sigma-Aldrich) was distributed into a 96-well tissue culture plate (10^4 cells/well) and incubated at 37 °C in 5 % CO_2 for 24 h. After that, 50µl of each dilution prepared from culture filtrates [1:1, 1:4 and 1:16 (v:v)] was added to each well. The microtiter plates were incubated at 37 °C in 5 % CO_2 for 24 h. After exposure, the medium was removed by aspiration and 50 µL of

DMEM with 10 % resazurin (0.1 mg/mL in PBS) was added to each well to assess cell viability. The absorbance was measured at 570 and 600 nm wavelength in a microtiter plate spectrophotometer [Synergy™ HT Multi-Detection Microplate Reader (Biotek®)]. Wells containing only DMEM but no cells were used as negative control, and wells with cells exposed to PBS or PDB were used as positive control.

Statistics

Two-way analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison test was used to determine the statistical significance of cytotoxicity of each strain within the same temperature against the control (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Differences between Fv/Fm among the different experiences and the rate of Fv/Fm decrease of tomato leaves were tested using a two-way ANOVA, followed by the Dunnett's multiple comparison and Bonferroni's multiple comparison test, respectively, to determine the statistical significance of phytotoxicity of each strain within the same temperature against the control (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). All the analyses were performed with GraphPad Prism v.7 (GraphPad Software, Inc., La Jolla, CA, USA). Data are shown as the average of three independent replicates of each condition.

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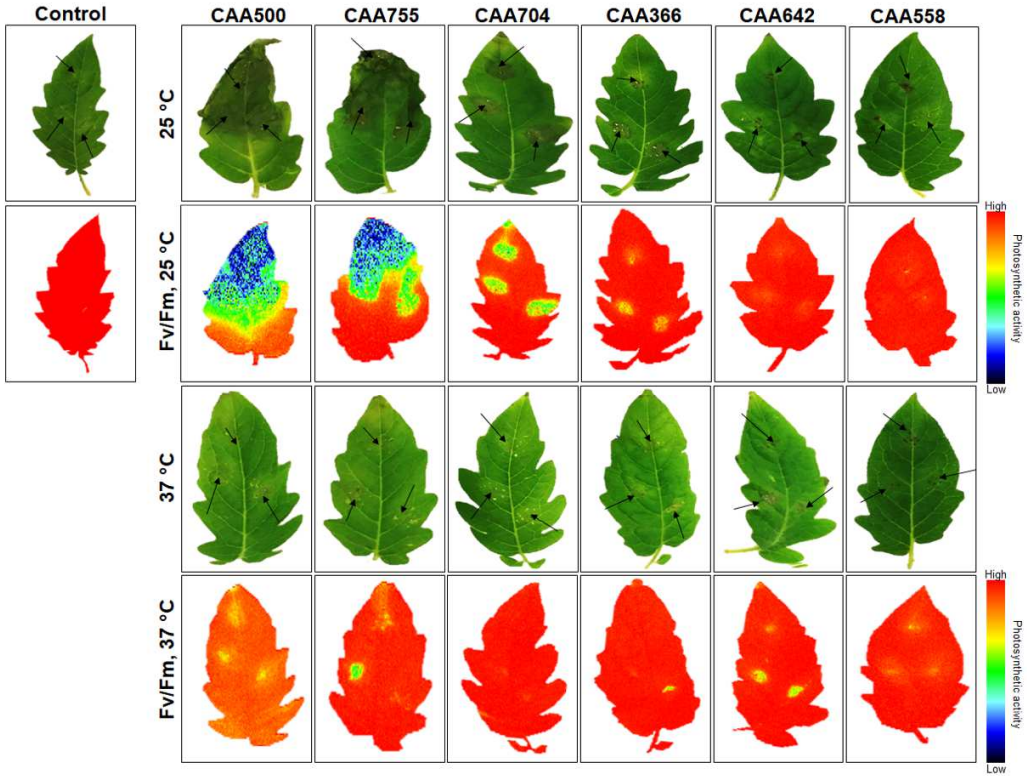

Fig. 1. Effect of culture filtrate from Botryosphaeriaceae species grown at 25 °C and 37 °C on detached tomato leaves after 6 dpi. Effect of culture filtrate of *D. corticola* CAA500, *N. kwambonambiense* CAA755, *N. parvum* CAA704 and CAA366, *B. dothidea* CAA642, and *N. eucalyptorum* CAA558 on symptoms development and chlorophyll fluorescence. PDB[a)] was used as control. The colour scale bar indicates the F_v/F_m intensity in false colours from high (red) to low (black) values. All measurements were performed in biological triplicates. [[a)]PDB: Potato Dextrose Broth]

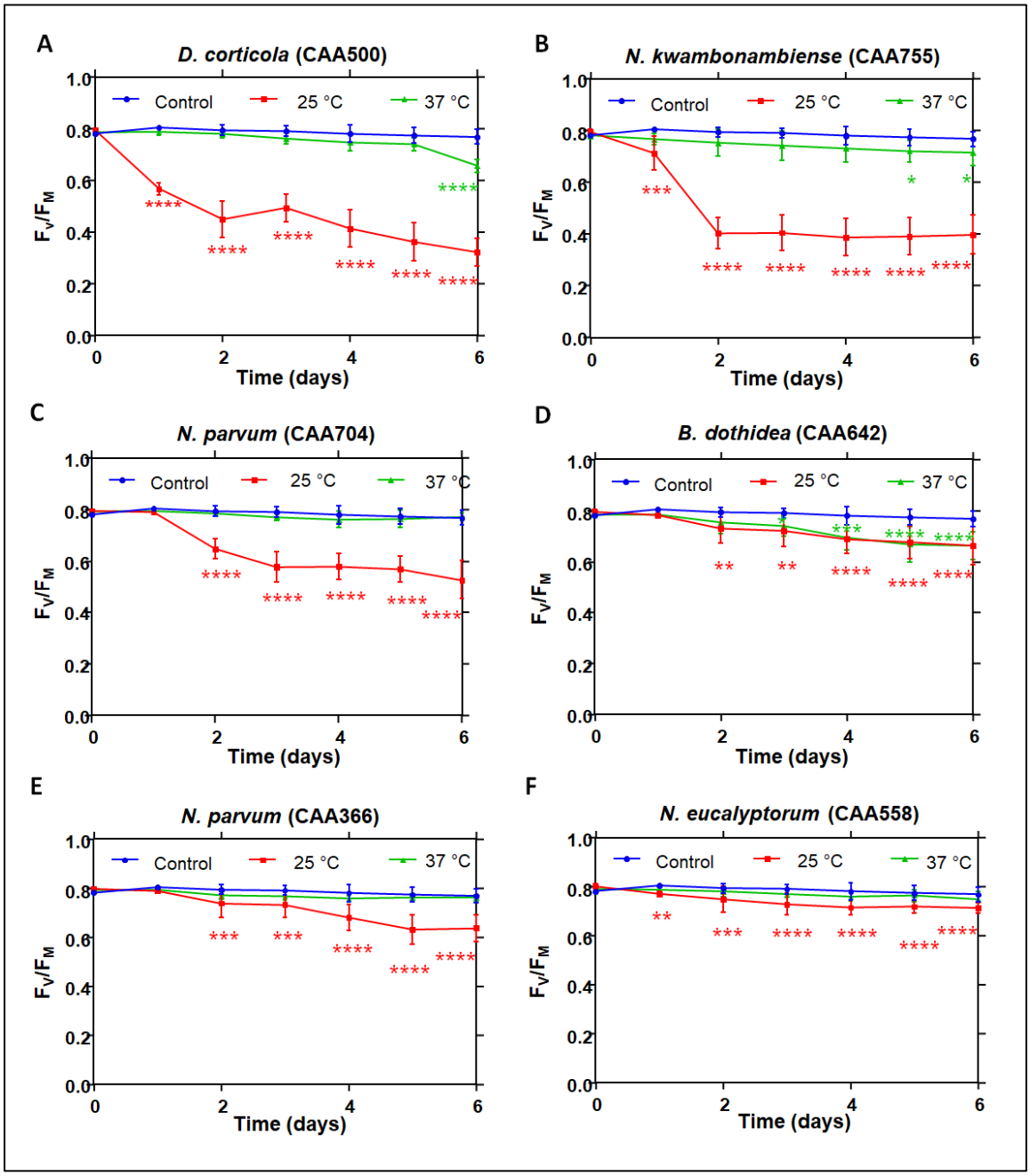

Fig. 2. Evaluation of phytotoxic effect of culture filtrates of Botryosphaeriaceae species on F_v/F_m value of tomato leaves. Culture filtrates were obtained from *D. corticola* CAA500 (**A**), *N. kwambonambiense* CAA755 (**B**), *N. parvum* CAA704 (**C**), *B. dothidea* CAA642 (**D**), *N. parvum* CAA366 (**E**), and *N. eucalyptorum* CAA558 (**F**). PDB[a)] was used as control. Each curve represents the mean ± SD of three independent leaves.

Two-way ANOVA, followed by a Dunnet's multiple comparison test was used to determine the statistical significance of phytotoxicity of each strain within the same temperature against the control (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). [a)PDB: Potato Dextrose Broth]

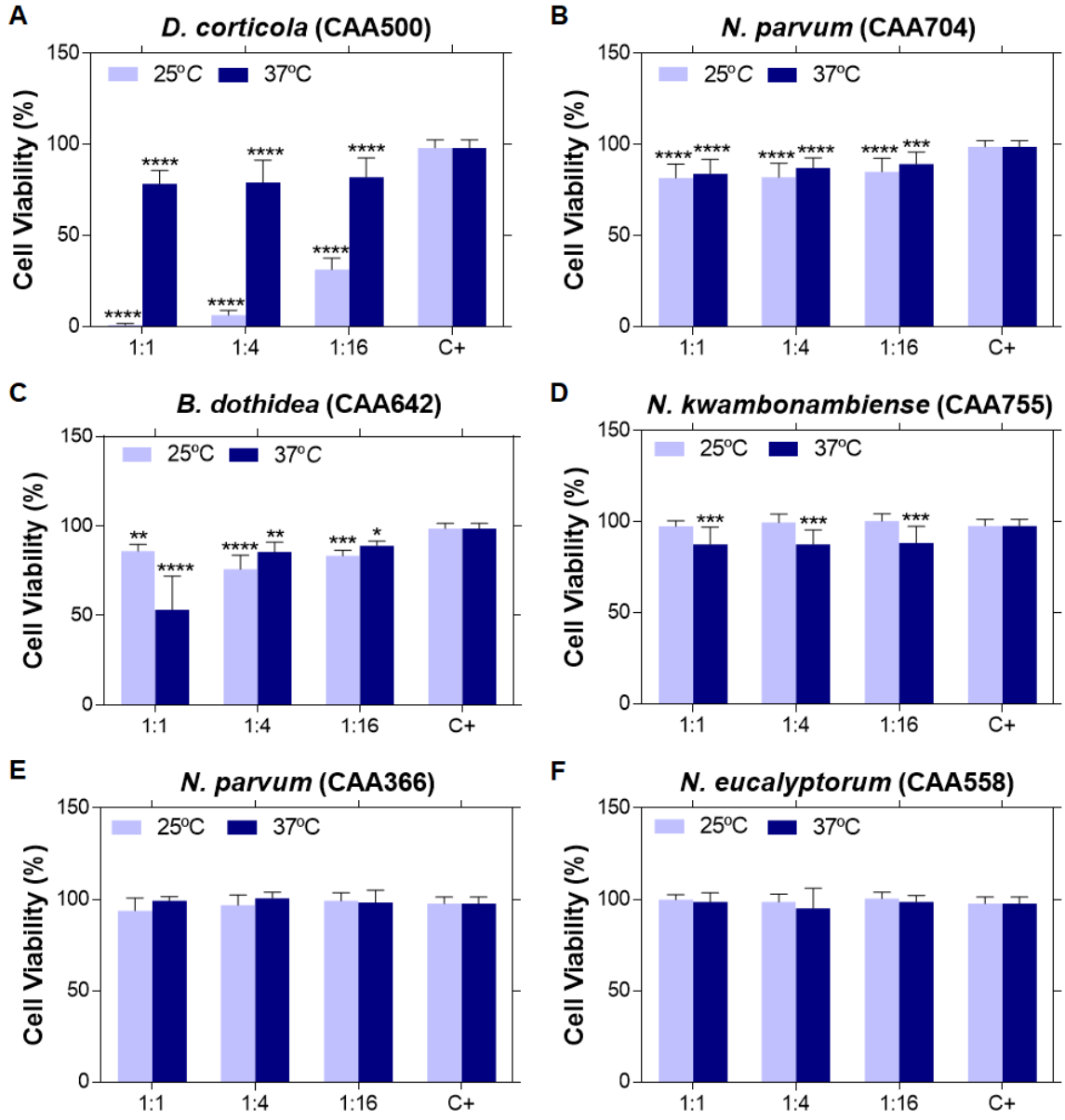

Fig. 3. Evaluation of Vero cells' viability (%) after exposure to the culture filtrates [1:1, 1:4, and 1:16 (v/v)] of Botryosphaeriaceae species grown at 25 °C and 37 °C. Vero cell line was exposed to *D. corticola* CAA500 (**A**), *N. parvum* CAA704 (**B**), *B. dothidea* CAA642 (**C**), *N. kwambonambiense* CAA755 (**D**), *N. parvum* CAA366 (**E**), and *N. eucalyptorum* CAA558 (**F**) culture filtrates. Wells containing only medium, but no cells were used as negative control. Cells exposed to PBS[a)] or PDB[b)] were used as positive

control. Data is presented as average ± standard error. Two-way ANOVA, followed by a Bonferroni multiple comparisons test, was used to determine the statistical significance of cytotoxicity to the control (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). [[a)]PBS: Phosphate Buffered Saline; [b)]PDB: Potato Dextrose Broth]

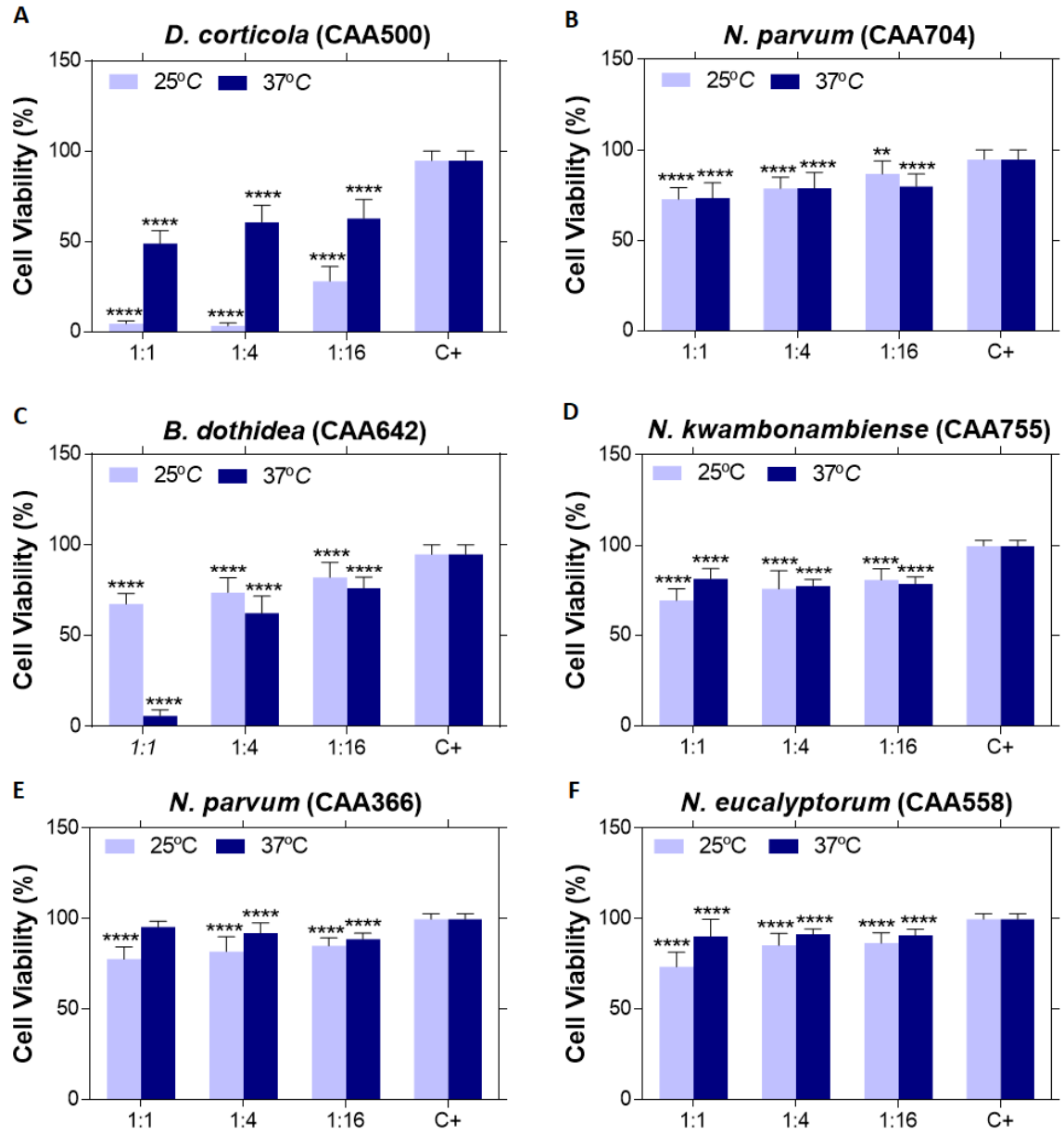

Fig. 4. Evaluation of 3T3 cells' viability (%) after exposure to the culture filtrates [1:1, 1:4, and 1:16 (v/v)] of Botryosphaeriaceae species grown at 25 °C and 37 °C. 3T3 cell line was exposed to *D. corticola* CAA500 (**A**), *N. parvum* CAA704 (**B**), *B. dothidea* CAA642 (**C**), *N. kwambonambiense* CAA755 (**D**), *N. parvum* CAA366 (**E**), and *N.*

eucalyptorum CAA558 (**F**) culture filtrates. Wells containing only medium, but no cells were used as negative control. Cells exposed to PBS[a)] or PDB[b)] were used as positive control. Data is presented as average ± standard error. Two-way ANOVA, followed by a Bonferroni multiple comparisons test, was used to determine the statistical significance of cytotoxicity to the control (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). [[a)]PBS: Phosphate Buffered Saline; [b)]PDB: Potato Dextrose Broth]

Table 1

Rate of F_v/F_m decrease of tomato leaves, induced by culture filtrates of Botryosphaeriaceae fungi. Fungi were grown at 25 ºC or at 37 ºC. Two-way ANOVA, followed by a Bonferroni's multiple comparison test was used to determine the statistical significance of phytotoxicity of each strain within the same temperature against the control (**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

Condition/strain	**25 °C**	**37 °C**
Control	0.006 ± 0.003	0.006 ± 0.003
D. corticola CAA500	0.172 ± 0.011****	0.003 ± 0.003
N. kwambonambiense CAA755	0.196 ± 0.018****	0.014 ± 0.008
B. dothidea CAA642	0.032 ± 0.008	0.015 ± 0.006
N. parvum CAA704	0.073 ± 0.010****	0.004 ± 0.002
N. parvum CAA366	0.030 ± 0.008	0.012 ± 0.003
N. eucalyptorum CAA558	0.026 ± 0.007	0.005 ± 0.003

Supplementary files

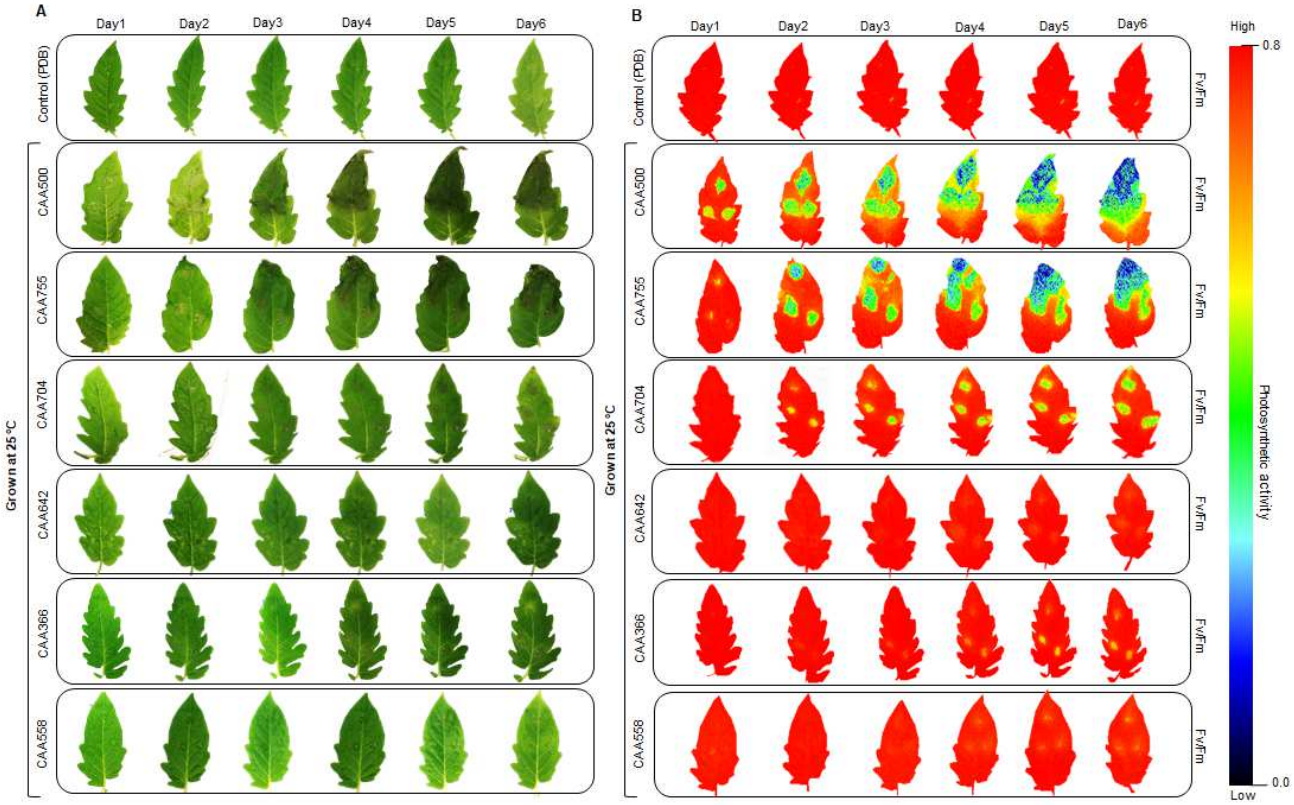

Fig. S1. Effect of culture filtrate of Botryosphaeriaceae fungi grown at 25 °C on detached tomato leaves after 6 days. Effect of culture filtrate of *D. corticola* CAA500, *N. kwambonambiense* CAA755, *N. parvum* CAA704, *B. dothidea* CAA642, *N. parvum* CAA366, and *N. eucalyptorum* CAA558 on symptoms development (**A**) and chlorophyll fluorescence (**B**). Leaves inoculated with PDB were used as control. The colour scale bar indicates the F_v/F_m intensity of the leaf pixels given in false colours from high (red) to low (black) values.

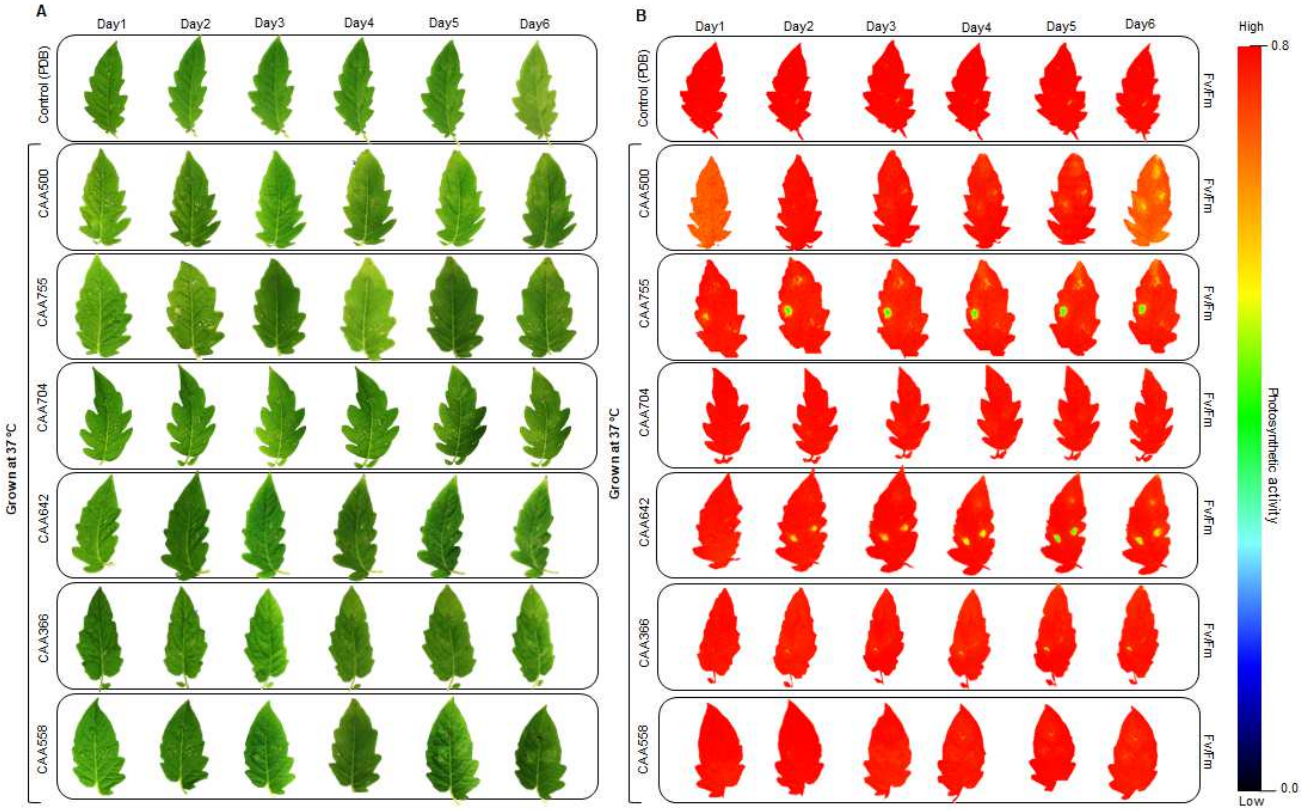

Fig. S2. Effect of culture filtrate of Botryosphaeriaceae fungi grown at 37 °C on detached tomato leaves after 6 days. Effect of culture filtrate of *D. corticola* CAA500, *N. kwambonambiense* CAA755, *N. parvum* CAA704, *B. dothidea* CAA642, *N. parvum* CAA366, and *N. eucalyptorum* CAA558 on symptoms development (**A**) and chlorophyll fluorescence (**B**). Leaves inoculated with PDB were used as control. The colour scale bar indicates the F_v/F_m intensity of the leaf pixels given in false colours from high (red) to low (black) values.

Table 1

Rate of F_v/F_m decrease of tomato leaves, induced by culture filtrates of Botryosphaeriaceae fungi. Fungi were grown at 25 ºC or at 37 ºC. Two-way ANOVA, followed by a Bonferroni's multiple comparison test was used to determine the statistical significance of phytotoxicity of each strain within the same temperature against the control (**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

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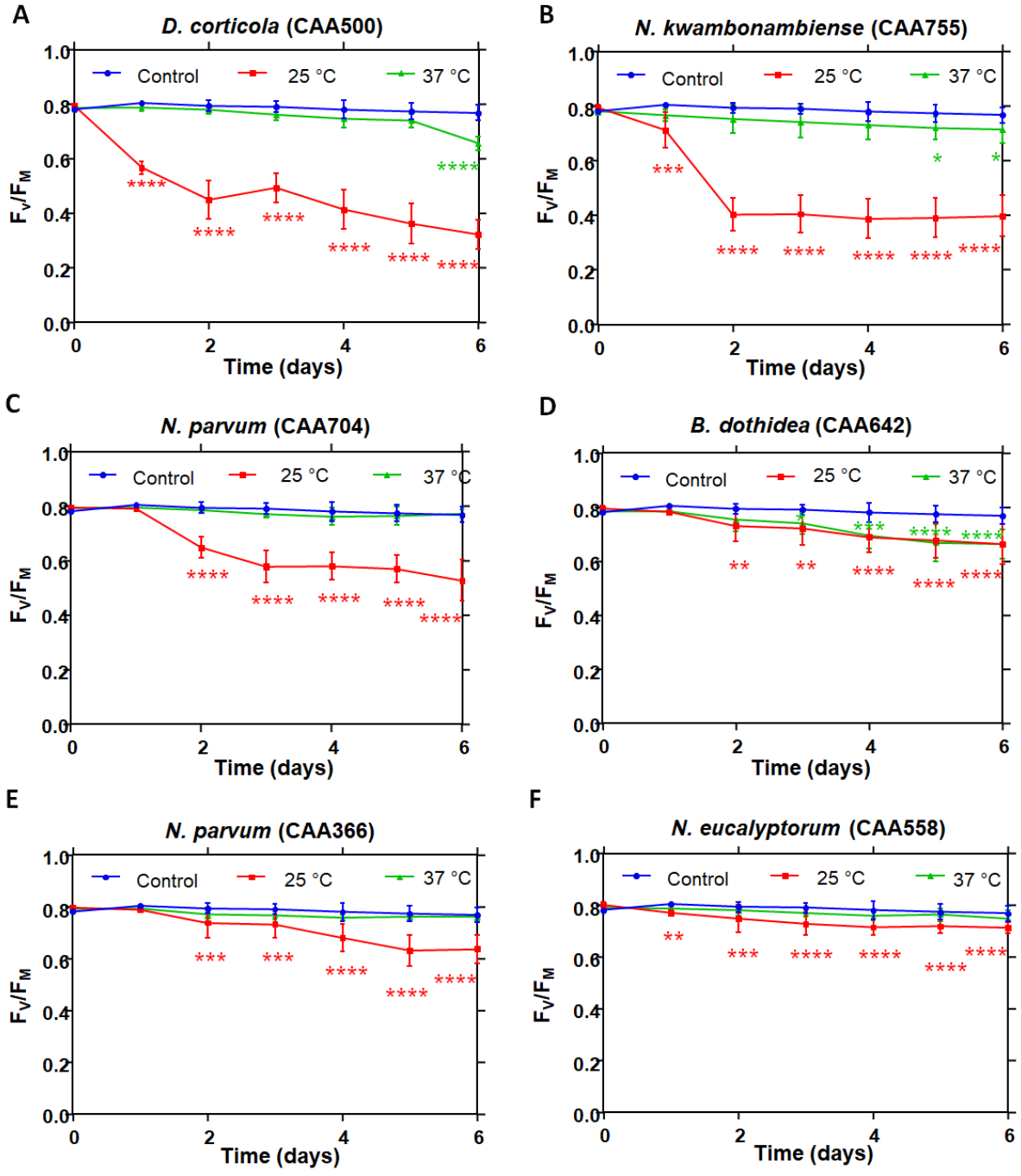
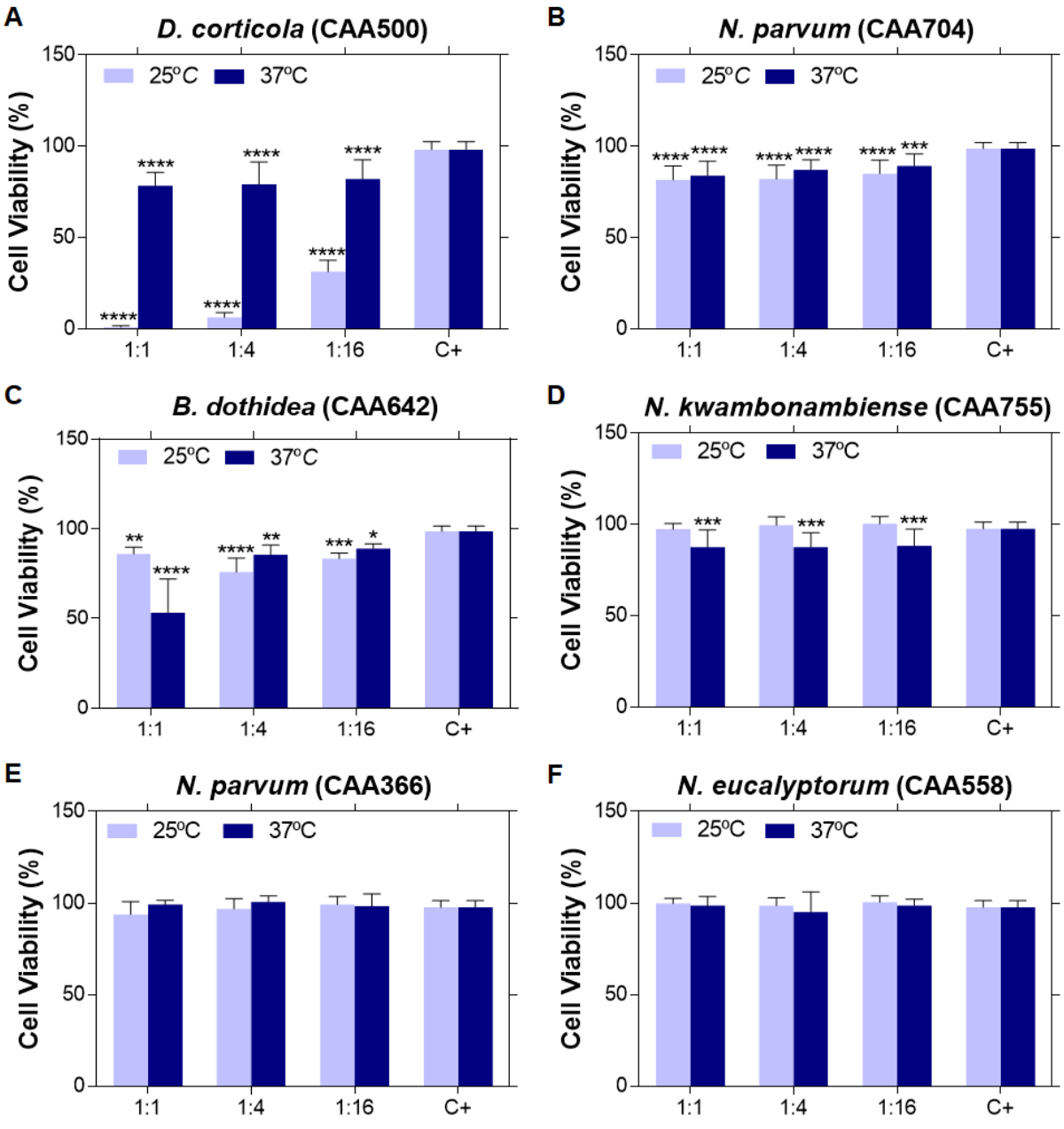
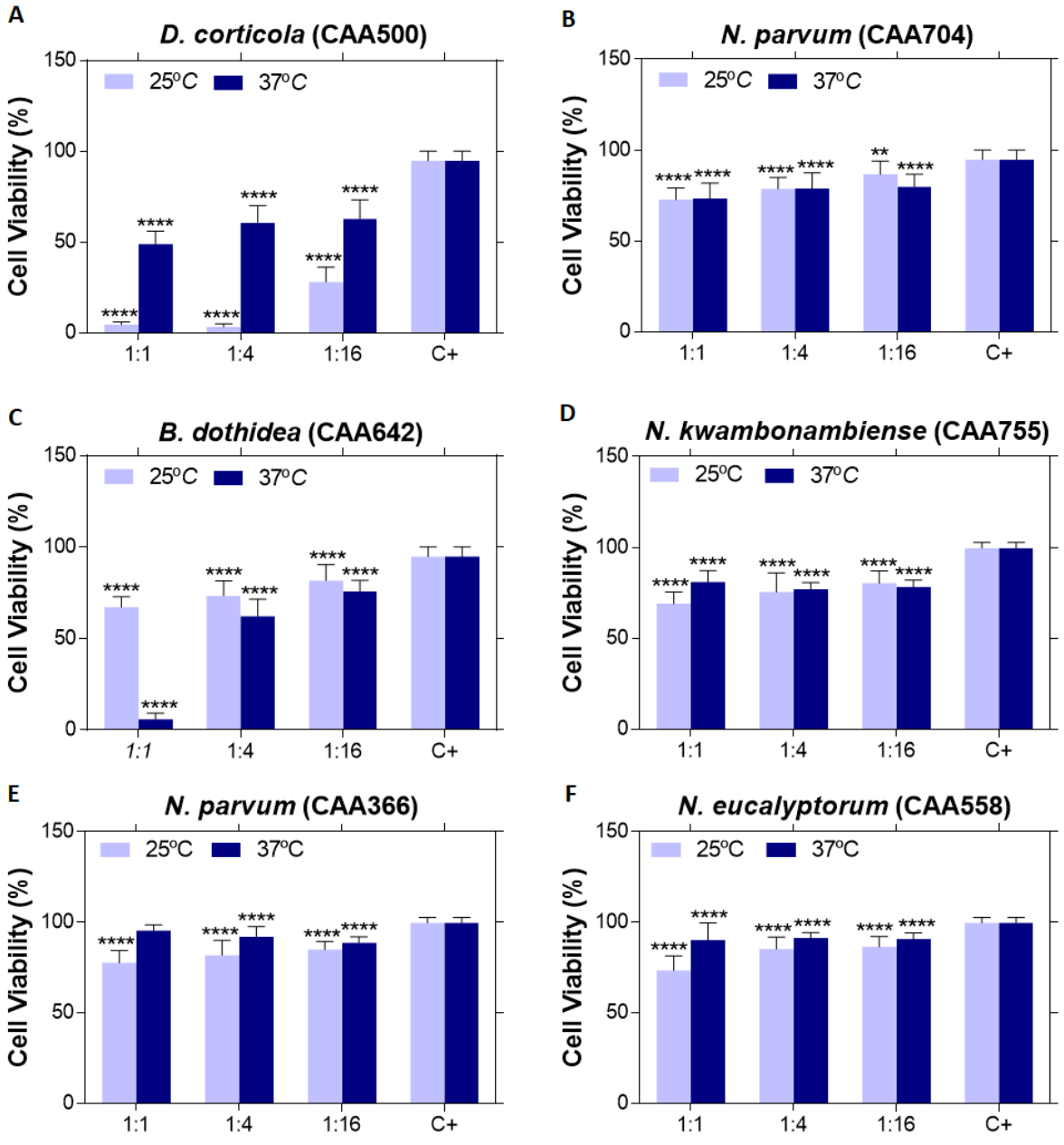
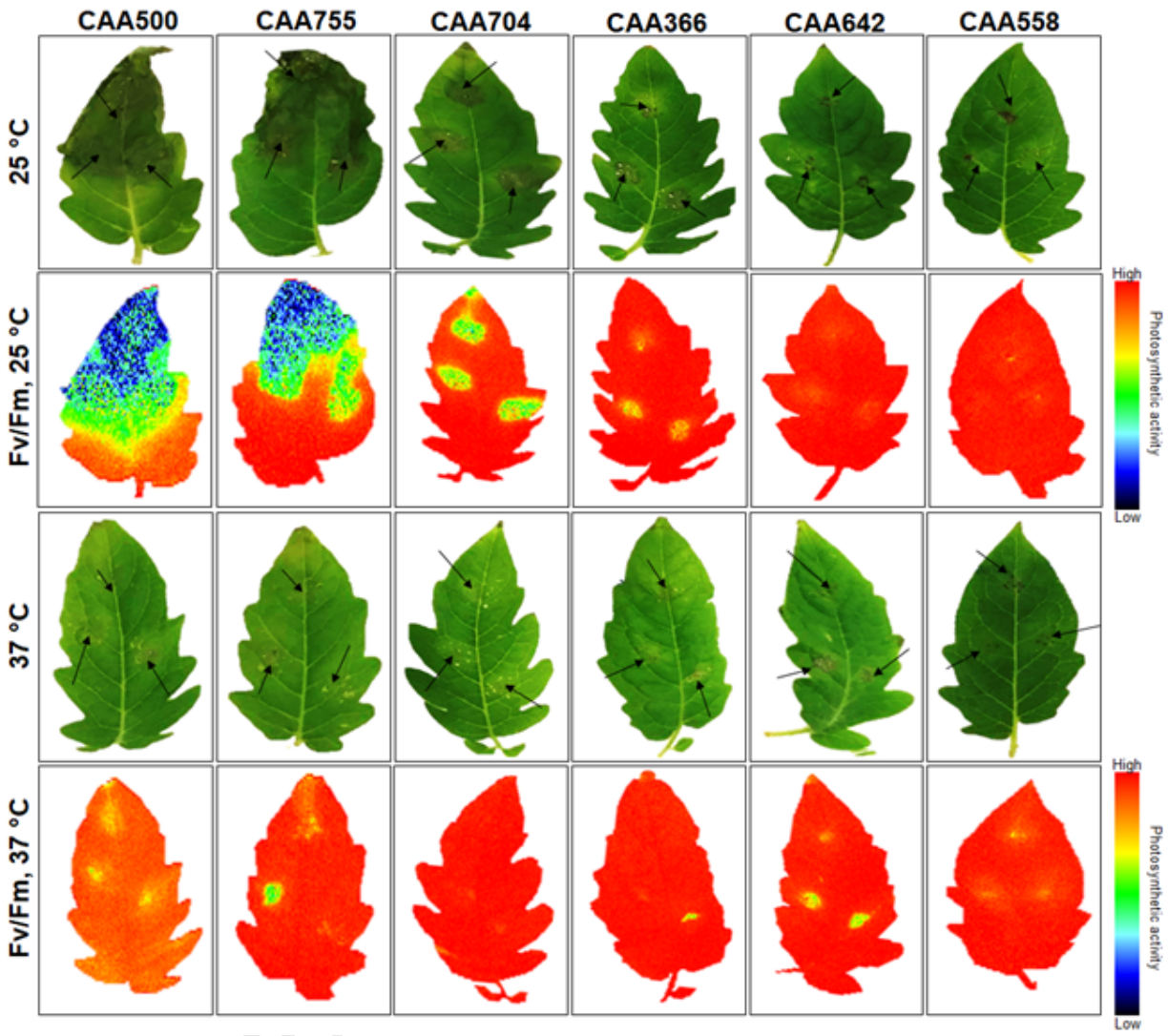
B. dothidea CAA642	0.032 ± 0.008	0.015 ± 0.006
N. parvum CAA704	0.073 ± 0.010****	0.004 ± 0.002
N. parvum CAA366	0.030 ± 0.008	0.012 ± 0.003
N. eucalyptorum CAA558	0.026 ± 0.007	0.005 ± 0.003


A
D. corticola (CAA500)
B
N. kwambonambiense (CAA755)
C
N. parvum (CAA704)
D
B. dothidea (CAA642)
E
N. parvum (CAA366)
F
N. eucalyptorum (CAA558)
Control
25 °C
37 °C
Fv/FM
Time (days)


A
D. corticola (CAA500)
B
N. parvum (CAA704)
C
B. dothidea (CAA642)
D
N. kwambonambiense (CAA755)
E
N. parvum (CAA366)
F
N. eucalyptorum (CAA558)
25ºC
37ºC
Cell Viability (%)
0
50
100
150
1:1
1:4
1:16
C+
****
***
**
*


A
D. corticola (CAA500)
B
N. parvum (CAA704)
C
B. dothidea (CAA642)
D
N. kwambonambiense (CAA755)
E
N. parvum (CAA366)
F
N. eucalyptorum (CAA558)
25ºC
37ºC
Cell Viability (%)
0
50
100
150
1:1
1:4
1:16
C+
****
**


CAA500
CAA755
CAA704
CAA366
CAA642
CAA558
25 °C
Fv/Fm, 25 °C
37 °C
Fv/Fm, 37 °C
High
Photosynthetic activity
Low
High
Photosynthetic activity
Low

Effect of temperature on the phytotoxicity and cytotoxicity of Botryosphaeriaceae fungi

Forough Nazar Pour, Vanessa Ferreira, Carina Félix, João Serôdio, Artur Alves, Ana Sofia Duarte, Ana Cristina Esteves

Highlights

- Botryosphaeriaceae fungi are important phyto- and human opportunist pathogens
- The forecast temperature increase will lead to unknown effects on these pathogens
- Temperature modulates the cyto- and phytotoxicity of Botryosphaeriaceae
- 25ºC benefits phytotoxicity while 37ºC facilitates animal cells' cytotoxicity
- Toxicity modulation by temperature may contribute to new infections