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2 **INFLUENCE OF ENVIRONMENTAL FACTORS ON TENUAZONIC ACID**
3 **PRODUCTION BY *Epicoccum sorghinum*: AN INTEGRATIVE APPROACH OF**
4 **FIELD AND LABORATORY CONDITIONS**

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37 **Abstract**

38 Sorghum is the fifth most cultivated and consumed grain in the world. However, this grain
39 is frequently contaminated with toxins from fungi. The present study evaluated the effects
40 of environmental factors on tenuazonic acid (TeA) production by *Epicoccum sorghinum*
41 in the field and in controlled laboratory conditions. In this study, 50 sorghum grain
42 samples were collected from summer and autumn growing seasons and analyzed for TeA
43 contamination using LC-MS/MS. To further understand the ecophysiology of this fungus,
44 an isolated strain of *E. sorghinum* from the field was investigated for its development and
45 TeA production under controlled environmental conditions in the laboratory. In the
46 ecophysiological investigation, the effects of water activity (0.90, 0.95, 0.99) and
47 temperature (18, 22, 26 and 30 °C) were evaluated on the radial growth, enzymatic
48 production and expression of *TASI*, which is the gene involved in TeA production.
49 Results showed that in the field, the summer season presented the highest TeA average
50 level in the grains (587.8 µg/kg) compared to level found in the autumn (440.5 µg/kg).
51 The ecophysiological investigation confirmed that *E. sorghinum* produces more actively
52 TeA under environmental conditions simulating the summer season. Optimum growth,
53 maximum *TASI* gene expression, and higher extracellular enzymatic production were
54 observed at 26°C with a water activity of 0.99. Pearson correlation analyses showed that
55 the production of TeA highly correlates with fungal growth. The present study
56 demonstrates that abiotic factors in a combined approach of field and laboratory
57 conditions will assist in predicting the driving environmental factors that could affect
58 growth of *E. sorghinum* and TeA production in sorghum grains.

59
60 **Keywords:** Sorghum; Ecophysiology; Gene expression; *Phoma sorghina*; LC-MS/MS

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63 1. Introduction

64 Sorghum is a crop with increasing global importance in food, animal feed, energy
65 production and other diverse industrial uses [1]. According to the USDA [2], sorghum
66 production worldwide in 2016/2017 was estimated to be 62.36 million metric tons, which
67 represented an increase of 4.03 million tons compared to 2015/2016. However, one of the
68 major biotic constraint to sorghum improvement and productivity is fungal
69 contamination, especially in the field when grain development can coincide with
70 favorable weather conditions for fungal growth and subsequent mycotoxin formation [3].
71 Among the diverse fungi reported to contaminate sorghum grain, *Epicoccum sorghinum*
72 (also known as *Phoma sorghina*), is commonly described as one of the major fungal
73 contaminants at pre- and post-harvest stages [3,4,5,6]. This fungus is a producer of
74 tenuazonic acid (TeA), a mycotoxin that is considered a potent inhibitor of protein
75 biosynthesis and has been reported to cause acute toxicity to several animals [7,8,9].
76 Moreover, TeA is considered to be responsible for causing Onyalai, a human
77 hematological disorder [10].

78 In recent years, TeA has gained considerable attention in the scientific community due
79 to their frequent contamination in food and animal feed. However, most available data
80 about TeA occurrence has been linked to the presence of *Alternaria* spp., while little is
81 known about TeA production by *E. sorghinum*. In 2011, the European Food Safety
82 Authority [11] published a report on the risks of *Alternaria* toxins for animal and public
83 health. TeA was considered unlikely to be of human health concern. However, only a
84 few data on the occurrence of TeA in food commodities were available at that time and
85 the TeA uptake estimation was based on chronic dietary exposure of adult populations
86 [12]. More recently Rychlik et al. [13] determined that TeA contamination on

87 sorghum/millet-based food represents a potential risk to infant health. These recent
88 findings raised more concerns about health and safety of the TeA mycotoxin.
89 As part of effective crop protection strategy, the understanding of the ecophysiology of
90 fungal pathogens as well as the occurrence of mycotoxins triggered by environmental
91 conditions, are essential to better understand and prevent mycotoxin contaminations. Our
92 recent study described the identification of the TeA biosynthetic gene (*TASI*) in
93 *Epicoccum sorghinum* [14]. This study enabled us to investigate the TeA biosynthesis in
94 more detail in the present study. Moreover, there are no data on the ecophysiology of *E.*
95 *sorghinum* as well as the effect of environmental factors linking the TeA occurrence in
96 the field.
97 Therefore, this study aims to integrate molecular and ecological approaches to better
98 understand the key abiotic factors in TeA production by *E. sorghinum*. For that, we
99 evaluated whether growing seasons (summer and autumn) affect the occurrence of TeA
100 mycotoxin in sorghum grains. In Brazil, these seasons have different temperatures and
101 humidity levels. Therefore, controlled laboratory experiments simulating field conditions
102 with a field isolate of *E. sorghinum* was also investigated under different temperatures
103 and water activities (a_w). These two environmental parameters were correlated with
104 fungal growth and gene expression of TeA as well as enzymatic activity.

105

106 **2. Material and methods**

107 **2.1. Field study**

108 Cultivation and sampling of sorghum grains occurred from November 2012 to May 2013
109 to determine the influence of growing seasons (summer and autumn) on the occurrence
110 of TeA in sorghum grains. A total of 50 sorghum grain samples (25 from each growing
111 season) were collected in a sorghum plantation owned by the São Paulo Agency for
112 Agribusiness Technology (APTA) (Votuporanga, the state of São Paulo, latitude 20° 28'

113 S, longitude 50° 04' W). APTA uses the best practice for sorghum cultivation in Brazil.
114 The cultivar DKB 550 (Dekalb seeds), commonly grown in Brazil, was used for both
115 growing seasons. The sowing of summer and autumn crops occurred in November of
116 2012 and February of 2013, respectively, with a distance of 0.5 m between rows and a
117 final population of 180 000 plants ha⁻¹.
118 For sampling, a stratified random sampling design was followed [15]. The experimental
119 area used for both growing seasons was divided into five uniform plots. The samples were
120 composed of five panicles randomly collected from each plot. The panicles were hand-
121 harvested and pooled, and 100g of each sample was immediately analyzed for fungal
122 contamination and water activity (a_w) and then stored at 4°C until mycotoxin analyses.
123 The water activity was determined by automatic analysis using an Aqualab CX-2
124 apparatus (Decagon Devices, Pullman, WA, USA).
125 The prevailing climatic factors during the sample collection periods, including
126 temperature (°C) and precipitation (mm), were monitored by the Climatological Station
127 (CIIAGRO), located at Votuporanga, SP, Brazil. The data were obtained daily during
128 both growing seasons, from sowing to harvest time.

129

130 **2.2 Frequency of *E. sorghinum***

131 From each sample, 30 sorghum grains were surface-disinfected with a sodium
132 hypochlorite solution (1%) and then plated (10 grains per Petri dishes) onto a potato
133 dextrose agar (Oxoid, Basingstoke, UK) supplemented with chloramphenicol (100 mg l⁻¹
134 ¹). The plates were incubated at 25°C in the dark for five days. Colonies of *Epicoccum*
135 spp. developing from the sorghum grains were then counted and morphologically
136 identified according to *Phoma* Identification Manual [16]. Colonies were identified using
137 morphological criteria, such as colony aspect and micromorphological features. The
138 identification at the species level was performed using molecular analysis of ITS region

139 of the rRNA gene, as described by Oliveira et al. [17]. The results are reported in
140 percentage of infected grains.

141

142 **2.3. Tenuazonic acid analysis**

143 The sorghum samples were analyzed for TeA mycotoxin contamination utilizing high-
144 performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) based
145 on the method described by Oliveira et al. 2017 [17].

146 Briefly, samples of 5 g of ground sorghum grain were homogenized for 90 min with 15
147 mL of acetonitrile:water (1:1, v/v), acidified with 110 μ L of formic acid. After
148 centrifugation (5 min at 4000 rpm), an aliquot of 100 μ L of supernatant was taken and
149 diluted with 900 μ l of water. TeA was determined by API 5000 LC-MS/MS system
150 (Applied Biosystems, Foster City, CA, USA) equipped with an Ion Electrospray
151 Ionization (ESI) source in the negative ionization mode. The column used was a 50 mm
152 x 4.6 mm x 1.8 μ m SB C-18 (Agilent). Multiple-reaction monitoring (MRM) was used
153 for TeA determination. The precursor peak of TeA (m/z 196.0) and two products peaks
154 m/z 139 (DP - 75, CE -26 V, CXP -19 V) m/z 112.0 (DP - 75, CE -34 V, CXP -15 V)
155 were monitored to accomplish both quantitation and qualification criteria. A binary
156 gradient at a flow rate of 0.4 mL/min was performed with solvent A (water) and solvent
157 B (methanol), following the conditions described by Oliveira et al. 2017 [17].

158

159 **2.3.1 Method validation**

160 Method performance characteristics such as recovery, linearity, limits of detection (LOD)
161 and limits of quantification (LOQ) were evaluated. For the recovery test, free TeA
162 samples of sorghum grain were spiked with a standard solution at two concentrations (100
163 μ g/kg and 200 μ g/kg), in triplicates. A calibration curve was obtained using six TeA
164 standard concentrations (20, 200, 400, 600, 800, and 1000 μ g/kg).

165 The mean recoveries for both concentrations were 96.3% and 99.7%, respectively. Good
166 linearity was achieved with a correlation coefficient higher than 0.999. LOD (signal to
167 noise ratio = 3) and LOQ (signal to noise ratio = 10) were 4 and 10 µg/kg, respectively
168 and showed the suitability of the method for the TeA determination in sorghum grains.

169

170 **2.4. Assessment of eco-physiology of *E. sorghinum* isolated**

171 **2.4.1. Fungal strain and growth media**

172 The TeA producer strain *Epicoccum sorghinum* P48 was used in this study. It was
173 previously isolated from the same sorghum field studied and the species identification
174 and ability to produce TeA was confirmed using molecular tools and LC-MS/MS [17].

175 The strain was grown on oatmeal agar (Difco, Detroit, Michigan) for 7 days at 25 °C and
176 maintained in 15% glycerol at -80 °C in the Department of Microbiology at the
177 University of Sao Paulo. The nucleotide sequence is available in the GenBank database
178 under the accession number: KT31009.

179 For the experimental conditions, such as growth rate, *TASI* gene expression and
180 enzymatic production a sorghum-base medium was used. This medium was prepared by
181 blending 4% (w/v) ground sorghum grains in sterile water and then adding 2% (w/v) agar
182 [18]. Except for enzymatic analysis, in which a sorghum-base broth was used instead of
183 agar, since the API-ZYM method cannot be used in agar plates. For the a_w , we used the
184 sorghum-base agar supplemented or not with glycerol to obtain a_w of 0.90 (280 ml/L);
185 0.95 (152 ml/L) and 0.99 (nothing added). Water activity was determined by an Aqualab
186 CX-2 apparatus (Decagon Devices, Pullman, USA).

187

188 **2.4.2. Growth rate**

189 A 5-mm-diameter mycelial disk from the margin of a 7-day-old growing colony of *E.*
190 *sorghinum* strain was used to centrally inoculate all treatments. The plates containing

191 medium with different a_w values (0.90, 0.95, 0.99) were incubated at 18, 22, 26 and 30°C
192 for 7 days; the experiments were performed in triplicate. Assessments of radial growth
193 were made daily by measuring two right-angled diameters of the colonies. The diameters
194 were plotted against time, and radial growth rates (mm/day) were obtained from the slope
195 of linear regressions [19].

196 197 **2.4.3. *TASI* gene expression**

198 **2.4.3.1. RNA extraction and cDNA synthesis**

199 Petri plates (diameter 90 mm) containing autoclaved media treatments were overlaid with
200 sterile cellophane membranes to facilitate removal of the mycelium for RNA extractions.
201 The total RNA was extracted from mycelium of each of the three replicates using the
202 RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's
203 instructions. First strand cDNA was synthesized using the High Capacity cDNA Reverse
204 Transcription kit (Applied Biosystems, Carlsbad, CA, USA), according to the
205 manufacturer. The synthesis was performed in a Veriti thermal cycler (Applied
206 Biosystems) using the following conditions: hybridization step of 10 min at 25 °C, reverse
207 transcriptase (RT) step of 120 min at 37 °C and 5 min at 85 °C. The cDNA samples were
208 stored at – 20 °C.

209 210 **2.4.3.2. Quantitative real-time PCR (qRT-PCR)**

211 The transcription profiles of the TeA biosynthetic gene (*TASI*) and of β -tubulin gene
212 (*TUB2*), as a reference gene, were analyzed by using qRT-PCR. The primers were
213 designed using the NCBI tool for qRT-PCR Primer Design
214 (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and are listed in table 1. For the *TASI*
215 primer design, a search in the NCBI database was performed (www.ncbi.nlm.nih.gov) for
216 similar proteins of the *TASI* gene recently obtained from the sequenced genome of *E.*

217 *sorghinum* P48 strain [14]. Homolog proteins of *TASI* were found sharing over 83%
218 identity with domain structures of *TASI* gene (C–A–PCP–KS). Base on that, *TASI*
219 specific pairs of primer for *E. sorghinum* were designed from a sequence of KS domain
220 found in *E. sorghinum* genome, which was described as an indispensable domain for
221 *TASI* activity. The pair of primers used to amplify *TUB2* gene was designed from a
222 *TUB2* sequence of the *E. sorghinum* genome in a similar manner as the *TASI* primers.
223 The qRT-PCR assays were carried out using 2X Power SYBR Green PCR master-mix
224 (Invitrogen, U.S.A.), according to the manufacturer. Primer optimization was performed
225 following the manufacturer guidelines. The comparative $\Delta\Delta\text{Ct}$ method was used for the
226 analysis of the qRT-PCR including the melting curve (Step One Plus Real time PCR
227 system, Applied Biosystems). The PCR efficiencies for both genes were performed using
228 the suggested protocol from the manufacturer. Briefly, the cDNA of positive control
229 templates was diluted to the factor of 10 with 6 points and *TASI* and *TUB2* primers were
230 run at the same time to check for the qPCR validation and efficiency. The qPCR
231 parameters obtained in this study were acceptable to the conditions required to quantify
232 the relative gene expression using the $2^{-\Delta\Delta\text{CT}}$ method (Fig. S1) [20]. The ΔCt and $\Delta\Delta\text{Ct}$
233 were calculated from the Ct value obtained from the results of qRT-PCR following the
234 calculation described by the manufacturer of Applied Biosystems. Samples were
235 performed in triplicate at the same time with negative controls (without cDNA template)
236 and using the thermal cycle and the reaction mix described in Table 1. The results were
237 expressed in terms of fold change vs samples.

238

239 **2.4.4. Extracellular enzymatic production**

240 The effects of different temperatures on the extracellular enzymatic production of *E.*
241 *sorghinum* were evaluated by API-ZYM® system (Bio-Me'rieux, Marcy l'Etoile,
242 France). Five culture discs (5 mm) from each of *E. sorghinum* isolates were transferred

243 to flasks containing sorghum-base broth and were incubated at 22°C for 10 days [21,22].
244 The growth media were filtered with 0.22 µm Millex filters (Merck Millipore
245 Ltd., Tullagreen, Carrigtwohill, Ireland) and the filtrates were used to determine
246 production of 19 extracellular enzymes with the API-ZYM® system (Bio-Merieux,
247 Marcy l'Etoile, France). Briefly, 65 µL of each fungal culture broth were deposited in the
248 20 well-plates, and the plates were maintained at 30°C for 4 h in the dark. Thereafter, a
249 drop of ZYM A reagent (25 g of Tris–hydroxymethyl aminomethane + 11 mL of
250 hydrochloric acid 37% + 10 g of sodium lauryl sulphate + 100 mL of H₂O) and ZYM B
251 reagent (0.12 g 'Fast Blue BB' + 40 mL of methanol + 60 mL dimethyl sulphoxide) were
252 added to each well. The results were determined in nanomoles (nmol) of the hydrolyzed
253 substrate according to the intensity of the color reaction on a scale of 1–5, i.e., 1 = 5 nmol,
254 2 = 10 nmol, 3 = 20 nmol, 4 = 30 nmol and 5 = >40 nmol. All of the measurements were
255 taken in duplicate, and the sorghum-base broth was used as the negative control.

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257

2.5. Statistical analysis

258 To compare the frequency of *E. sorghinum* and TeA content between summer and autumn
259 growing seasons, t-test was used ($P < 0.05$). The effects of temperature, a_w and their
260 interaction were evaluated by the two-way ANOVA for growth rate and *TAS1* gene
261 expression. The differences among mean values were performed by Tukey's Multiple
262 Comparisons Test at a 99% confidence level. To better correlate the temperature and a_w
263 with growth and TeA production we used Pearson correlations. All the analysis was
264 performed in Origin™ software (OriginLab, Inc.).

265 3. Results

266 3.1. *E. sorghinum* and TeA occurrence in the field

267 Mycological and mycotoxin analysis of the sorghum grains grown during the two-
268 growing season (summer and autumn) are shown in Table 2. In both growing seasons *E.*
269 *sorghinum* was the major fungal species isolated, however, the natural variation of
270 environmental factors of each season affected the frequency of *E. sorghinum*. For
271 instance, 74% and 87.4% of the sorghum grains samples from summer and autumn
272 season, respectively, were contaminated with *E. sorghinum* ($P = 0.00005224$). The
273 highest average level of TeA was found in sorghum grains cultivated during the summer
274 (587.8 $\mu\text{g}/\text{kg}$), with contamination levels ranging from 165.4 to 1647.5 $\mu\text{g}/\text{kg}$; while in
275 autumn, the average of TeA levels was 440.5 $\mu\text{g}/\text{kg}$ (198.6 - 1154.9 $\mu\text{g}/\text{kg}$) ($P = 0.5680$).
276 These growing seasons present very distinct environmental conditions (Table 2). For
277 instance, the summer temperature average (26.3°C) was 4 °C higher than in autumn (22.4
278 °C). The total rainfall was similar in both seasons (10.5 mm - summer; 9.2 mm - autumn).
279 However, the percentage of rainy days observed during the summer (38.3%) was higher
280 than in autumn (16.6%). Precipitation is known to have a direct effect on the a_w levels in
281 sorghum grain. This was confirmed by the samples collected during the two seasons. For
282 instance, in the summer the a_w was around 0.98, as opposed to autumn that was 0.55.

283

284 **3.2 Ecophysiology of *E. sorghinum***

285 To better understand the impacts of a_w and temperature in the development and
286 production of TeA by this fungus, laboratory controlled experiments were performed with
287 different conditions of a_w and temperatures. These conditions were selected based on the
288 climatological data collected in the field during summer and autumn seasons.

289

290 **3.2.1. Mycelium growth**

291 Fig. 1 shows the radial growth rate of the *E. sorghinum* strain on sorghum media in
292 response to different a_w (0.90-0.99) and temperatures (18-30°C). The results indicated

293 that mycelium growth occurred over the temperature and a_w ranges investigated. The
294 optimum growth condition occurred at 0.99 a_w and 26°C (5.7 mm/day). The results also
295 confirmed that the combination of the lowest temperature and driest conditions (0.90 a_w ;
296 18°C) tested were less favorable to *E. sorghinum* growth compared to higher values (0.99
297 a_w ; 30°C). Statistical analysis of variance (ANOVA) showed that all single factors (a_w ,
298 temperature, and their interaction) were significant on the radial growth rate ($P < 0.01$),
299 except for 22°C and 30 °C at 0.95 a_w . The correlations of temperature and a_w with growth
300 and TeA production was also confirmed by the Pearson correlation (Fig. 2). In agreement
301 with the high TeA level found in sorghum grain grown on summer, a high and positive
302 correlation was found between growth and *TASI* gene expression in a combination of hot
303 and humid conditions (26 - 30 °C and 0.99 a_w) (Fig. 2; Table S1).

304

305 **3.2.2. *TASI* gene expression**

306 The results of the relative gene expression of the *TASI* in *E. sorghinum* strain grown on
307 sorghum medium under different combination of temperature and a_w is shown in Fig. 3.
308 The highest expression of *TASI* was observed at 26 °C and at 0.99 a_w , which was also the
309 same optimum condition found for mycelium growth and agree with the highest TeA
310 levels found in sorghum grains grown on summer season. These parameters also
311 correlated well in the Pearson correlation with 0.87 and 1.00 for the 0.99 a_w and 26 °C,
312 respectively (Fig. 2; Table S.1.). At lower temperatures (18°and 22°C), the transcriptional
313 level of the *TASI* was noticeably lower compared to the 26°C, except for 0.90 a_w . At
314 30°C, a considerable decrease of transcription was observed at 0.90 a_w . However, at 0.99
315 a_w , the expression followed the same pattern of 18° and 22°C. Moreover, it is interesting
316 to note that the influence of a_w in *TASI* gene expression was more pronounced at higher
317 than at lower temperatures. Statistical analysis of variance (ANOVA) showed that all

318 single factors (a_w , temperature, and their interaction) were significant ($p < 0.01$) on the
319 *TASI* gene expression, except for 0.90 a_w at 18, 22 and 26 °C.

320

321 **3.2.3. Enzymatic production**

322 The effect of temperature in the extracellular enzymatic production of *E. sorghinum* is
323 shown in Fig. 4. The following five extracellular enzymes were produced: alkaline
324 phosphatase, acid phosphatase; naphthol-AS-BI phosphohydrolase; α -glucosidase and N-
325 acetyl- β -glucosaminidase. These five enzymes were only simultaneously produced at
326 22°C and 26°C. A thermal stress was observed at the lowest (18°C) and highest (30°C)
327 temperatures tested. Two out of the five enzymes were inhibited, alkaline phosphatase
328 and N-acetyl-b-glucosaminidase at 18°C and naphthol-AS-BI phosphohydrolase and α -
329 glucosidase at 30°C. Moreover, production of acid phosphatase and naphthol-AS-BI
330 phosphohydrolase increased at 18° C, when compared to the other temperatures. We also
331 observed a pigment production when the colonies were cultivated in lower temperatures,
332 with the highest production at 18 °C.

333 These results suggested that intermediate temperatures (22 and 26 °C) could lead to a
334 more effective grain colonization by this species, and may explain the high frequency of
335 *E. sorghinum* found in sorghum grains grown under the two-growing seasons (22 °C
336 autumn and 26 °C summer).

337

338 **4. Discussion**

339 It is well known that environmental factors modulate fungal development and mycotoxin
340 production, especially those related to climate change, which directly cause an impact on
341 food security and quality [23]. In Brazil, sorghum cultivation occurs mainly as succession
342 planting, especially in succession to summer crops, from September to March. In a recent
343 study, based on the zoning of climatic conditions, it was determined that 53% of the

344 national territory are able to produce this crop, with the potential to increase the
345 production up to 54 times [24]. However, there is no data on the influence of sowing
346 period concerning food safety aspects, such as fungal and mycotoxin contamination.

347 In our study, natural TeA contamination was found in sorghum grain grown in both
348 growing seasons (summer and autumn). However, environmental conditions prevailing
349 in the summer showed to be more favorable to TeA contamination. During this season,
350 the sorghum grains were exposed to warm and rainy weather with a mean temperature 4
351 °C higher than autumn and precipitation percentage more than double the value of the
352 autumn season (Table 2). The precipitation clearly influenced the a_w in the sorghum
353 grains, keeping high a_w levels (0.91) during the summer season. According to Srivastava
354 et al. [25] changing planting or harvest dates of sorghum crops can be an effective and
355 low-cost option to reduce pests and diseases in crops,

356 Currently, there are no regulations worldwide for TeA in food; however, the average of
357 TeA level found in sorghum grains grown in summer (587.8 µg/kg) was higher than the
358 regulatory limit proposed by Bavarian Health and Food Safety Authority (500 µg/kg the
359 TeA content in sorghum-based infant food) [13]. Therefore, a correct choice of sowing
360 date, especially during a cooler and dry season, can play an important role in reducing the
361 TeA contamination in sorghum crop.

362 To our knowledge, very little information on the ecophysiology of *E. sorghinum* is
363 available. The only previous work that evaluated the effects of temperature and pH on
364 growth of *P. sorghina* strains demonstrated that the optimum temperature for mycelium
365 growth was 28°C [26]. On the other hand, *Alternaria* spp., an important TeA producer,
366 has been studied extensively over the last decade. Several studies have examined the
367 response of *Alternaria* spp. under different environmental conditions [19,27] and
368 interestingly, comparing to our results, *E. sorghinum* have a similar pattern of growth and

369 TeA production, especially at elevated a_w and temperature conditions (≥ 0.95 ; 26°C). For
370 instance, the optimum condition for mycelia growth of *Alternaria* has been observed in a
371 range of $25\sim 30^\circ\text{C}$ and $0.98\sim 1 a_w$ [27]. In terms of TeA production, Megan and Baxter
372 [28] demonstrated that 0.99 was the optimum a_w level for TeA production by *A. alternata*
373 on sorghum grain. Similar results were found by Oviedo et al. [29], who reported
374 maximum TeA production at 0.98 a_w and between 25 and 30°C for strains of *A. alternata*
375 on soybean-based agar.

376 *Epicoccum sorghinum* is an important mold fungus infecting sorghum grains. To
377 overcome the barrier of the grain tissue, an array of extracellular hydrolytic enzymes are
378 produced to facilitate the penetration and subsequent infection on the host tissue [30].
379 However, thermal stress experienced by the microbial pathogen may impact the dynamics
380 of host/pathogen interactions and ultimately resulting in changes in virulence [31].
381 Results showed that temperature plays an important role on the enzymatic expression of
382 *E. sorghinum* (Fig. 4). Alkaline phosphatase and naphthol-AS-BI-phosphohydrolase,
383 important enzymes for the degradation of organic phosphorus compounds found in the
384 lipid layer of the plant cell plasma membrane, were inhibited at 18°C and 30°C
385 respectively, as well as N-acetyl- β -glucosaminidase at 18°C [32]. Moreover, α -
386 glucosidase, which hydrolyzes disaccharides and is usually involved in the plant
387 polysaccharides degradation, was inhibited at 30°C [33]. The results suggested that
388 intermediate temperatures (22 and 26°C) led to maximum enzymatic production, which
389 could lead to more effective grain colonization by this species.

390 In the case of the TeA biosynthetic gene, this is the first study evaluating the effects of
391 environmental factors on *TASI* gene expression. *TASI* was recently described in
392 *Magnaporthe oryzae*, a pathogen of rice, and consists of an NRPS (non-ribosomal peptide

393 synthetase) with an unique type PKS-KS domain (polyketide synthase) that
394 biosynthesizes TeA from isoleucine and acetoacetyl-CoA [34].

395 In the recently sequenced *E. sorghinum* genome, *TASI* was determined to be highly
396 conserved and had an identical domain described in *M. oryzae*, which is in agreement
397 with the capability of this species to produce TeA [14]. In the current work, temperature
398 and a_w showed a significant effect on *TASI* expression. It is noticeable that the best
399 condition of the *TASI* gene expression (26 °C and 0.99 a_w) matches the higher level of
400 TeA detected in the grains grown under the warm and humid conditions found in the
401 summer season. Moreover, the highest *TASI* expression range includes the optimal
402 conditions for growth and extracellular enzymatic production, suggesting that TeA
403 production by *E. sorghinum* also occur within the range of environmental conditions most
404 favorable for sorghum grain colonization.

405 Additional studies are needed to better understand the occurrence of TeA in grains in
406 relation to the ecophysiology of TeA fungal producers (*E. sorghinum* and *Alternaria* spp.)
407 with worldwide distribution, and to create an integrated management of field and
408 laboratory data to minimizing the risk of this mycotoxin contamination.

409
410

411 **5. Conclusion**

412 This study demonstrated that environmental conditions observed during different
413 growing seasons (summer and autumn) have a direct effect on the TeA contamination of
414 sorghum grains. The average of TeA levels detected in the sorghum samples grown
415 during the summer season was 25 % higher than those detected in autumn. Moreover, the
416 ecophysiological response of *E. sorghinum*, such as mycelium growth, *TASI* gene
417 expression and enzymatic production indicated a good agreement with the environmental
418 conditions observed in the field. Hot and humid days seems to play a role in *E. sorghinum*

419 development and consequently, in TeA accumulation in the grains. The results of the
420 combined approach of the environmental factors under field and laboratory conditions
421 has contributed to create an optimum risk assessment for mycotoxin production in
422 sorghum grains and added fundamental knowledge on the environmental conditions
423 inducing TeA biosynthesis.

424

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431

432 **Appendix A: Supplementary data**

433 Additional Supporting Information may be found in the online version of this article:

434 **Fig. S.1** Reference gene validation using serial dilutions with reference gene (*TUB2*) and
435 target gene (*TASI*) and checking for the slope of each line.

436 **Table S.1** Person Correlations of the growth versus gene expression at different
437 temperature and water activity levels

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