Journal Pre-proof

Effects of water activity and temperature on fusaric and fusarinolic acid production by *Fusarium temperatum*

M. Veronica Fumero, Michael Sulyok, Maria L. Ramirez, John F. Leslie, Sofia N. Chulze

Please cite this article as: Fumero M.V., Sulyok M., Ramirez M.L., Leslie J.F. & Chulze S.N., Effects of water activity and temperature on fusaric and fusarinolic acid production by *Fusarium temperatum*, *Food Control* (2020), doi: https://doi.org/10.1016/j.foodcont.2020.107263.

Fumero: conceptualization, methodology, data analysis, writing: original draft preparation, reviewing and editing

Sulyok: methodology, data analysis, writing: reviewing and editing

Ramirez: data analysis, writing: reviewing and editing

Leslie: conceptualization, writing: reviewing and editing

Chulze: supervision, writing: reviewing and editing

1   **Effects of Water Activity and Temperature on Fusaric and Fusarinolic Acid Production by**

2   ***Fusarium temperatum***

3

4   M. Veronica Fumero[a], Michael Sulyok[b], Maria L. Ramirez[a], John F. Leslie[c] and Sofia N. Chulze[a]

5

6   [a] Research Institute on Mycology and Mycotoxicology (IMICO), CONICET-Universidad

7   Nacional de Río Cuarto. Route 36, Km 601, (5800) Río Cuarto, Córdoba, Argentina

8   (mariaveronicafumero@gmail.com; mramirez@exa.unrc.edu.ar; schulze@exa.unrc.edu.ar)

9

10   [b] Center for Analytical Chemistry, Department of Agrobiotechnology (IFA Tulln), University of

11   Natural Resources and Life Sciences, Vienna (BOKU). Konrad Lorenz Strasse 20, A-3430,

12   Tulln, Austria (michael.sulyok@boku.ac.at)

13

14   [c] Department of Plant Pathology, Throckmorton Plant Sciences Center, Kansas State University.

15   1712 Claflin Avenue, Manhattan, Kansas, 66506, USA (jfl@ksu.edu)

16

17   **Corresponding Authors:** M. Veronica Fumero (mariaveronicafumero@gmail.com) and Sofia

18   N. Chulze (schulze@exa.unrc.edu.ar). Research Institute on Mycology and Mycotoxicology

19   (IMICO), CONICET-Universidad Nacional de Río Cuarto. Route 36, Km 601, (5800) Río

20   Cuarto, Córdoba, Argentina.

21

22   **ORCIDs:**

23   M. Veronica Fumero – 0000-0001-9668-810X

1

24   Michael Sulyok – 0000-0002-3302-0732

25   M. Laura Ramirez – 0000-0002-6921-2656

26   John F. Leslie – 0000-0002-6486-6992

27   Sofia N. Chulze – 0000-0002-8818-1103

28

29   **Declarations of interest:** none

30

31   **Abstract**

32   Fusaric acid (FA) is a secondary metabolite produced by several *Fusarium* species that

33   commonly is isolated from maize and maize-based foods and feeds, and is toxic to some plants

34   and animals, most notably cotton. Fusarinolic acid (FnA) is closely related to FA and is

35   enzymatically derived from it, but much less is known about its toxicity to humans and other

36   animals. We determined the effects of water activity (a_W – 0.95, 0.98 and 0.995), temperature

37   (15º, 25º and 30°C), incubation time (7, 14, 21 and 28 days) and their interactions on FA and

38   FnA production by two strains of *F. temperatum* isolated from maize growing on sterile maize

39   grain. The amount of FA and FnA accumulated was measured by high-performance liquid

40   chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC/ESI-

41   MS/MS). Both compounds were accumulated by both strains of *F. temperatum* under all

42   evaluated conditions. The amount of FnA produced always exceeded the amount of FA produced

43   (max 50,000 ng/g and 4,500 ng/g, respectively). Temperature, a_W, incubation time, and the two-

44   and three- way interactions amongst them all significantly impacted FA and FnA accumulation.

45   Factors favouring fungal growth and mycotoxin production include insect damage, high

46   humidity, delays in harvest, and improper (wet) storage. Grain colonization by *F. temperatum*

47 begins in the field, but fungal growth and mycotoxin production can easily continue in storage if

48 conditions are right. Thus, from a toxicological point of view, *F. temperatum* represents a risk

49 for maize under both field and storage conditions. Our data enable better risk estimates and

50 strategies to reduce FA and FnA in the food and feed chains. The highest level of FA was

51 detected at $0.995a_W$ and was independent of temperature and length of incubation, suggesting

52 that there is a limit to the amount of FA that can be accumulated by *F. temperatum* growing

53 under laboratory conditions. Strikingly high amounts of FnA were observed under all incubation

54 conditions, often exceeding FA levels by 20× to 200×. This result suggests that FnA is more

55 important to the fungus than is FA, and that FA might be little more than an intermediate in a

56 pathway to FnA. The role of the accumulated FnA is unknown, but its role as a toxin may have

57 been discounted since studies to date report limited toxicity. However, if FnA is tested for

58 toxicity at higher levels, such as those identified in this study, then it could have significant

59 toxicological, or other effects that have not previously been considered.

60

61 **Key words:** abiotic stress, ecophysiology, food and feed contaminants, *Fusarium temperatum***,**

62 maize, mycotoxins

63

3

64   **1.   Introduction**

65

66   Fusaric acid (FA) is a secondary metabolite produced by multiple species in the *Fusarium*

67   *fujikuroi* species complex (FFSC), including *F. andiyazi, F. fujikuroi, F. proliferatum, F.*

68   *subglutinans*, *F. temperatum*, *F. thapsinum*, and *F. verticillioides* (Leslie and Summerell, 2006;

69   Munkvold et al., 2019; Scauflaire et al., 2011), and also by more distantly related species such as

70   *F. oxysporum* and *F. solani* (Bohni et al., 2016; López-Díaz et al., 2018). FA is a widespread

71   contaminant of maize and maize-based food and feeds and is frequently found in sorghum and

72   other cereal grains, from which *Fusarium* species are isolated (Desjardins, 2006; Leslie and

73   Summerell, 2006; Munkvold et al., 2019).

74   *F. temperatum* is a widely distributed maize pathogen predominantly isolated in

75   temperate to cold regions of the world (Boutigny et al., 2017; Fumero et al., 2015; Lanza et al.,

76   2016; Ridout et al., 2016). Although *F. temperatum* is not the dominant *Fusarium* species

77   present in maize, it can compose 20-50% of the population being the second or third in

78   frequency after *F. verticillioides* and/or *F. proliferatum* (Fumero et al., 2015; Scauflaire et al.,

79   2011). *Fusarium temperatum* can cause seed rot, seedling blight, stalk rot and ear rot of maize

80   (Ridout et al., 2016; Scauflaire et al., 2012). It also produces mycotoxins, such as FA (the focus

81   of this study) as well as moniliformin, fusaproliferin, beauvericin, and enniatins (Fumero et al.,

82   2015a, b; Lanza et al., 2016; Ridout et al., 2016; Scauflaire et al., 2012).

83   The mode of action of FA is not well understood. Amongst plants, FA reduces

84   pigmentation in tomato, cucumber and banana leaves, which reduces photosynthesis and

85   contributes to plant wilt (Ding et al., 2018; López-Díaz et al., 2018), and is highly toxic to cotton

86   (Stipanovic et al., 2011). FA also inhibits quorum sensing by Gram negative bacteria, e.g.,

87   *Pseudomonas* (Tung et al., 2017). Amongst animals and humans, FA has potent cytotoxic effects

88   on lymphocytes and HeLa cell lines in *in vitro* assays (Dhani et al., 2017; Mamur et al., 2018). In

89   these cells, exposure to FA alters cell membrane activity, reduces dopamine β-hydroxylase,

90   peroxidase, polyphenol oxidase and mitochondrial activity, inhibits ATP synthesis, and chelates

91   divalent cations (Tung et al., 2017). FA also enhances the effects of other mycotoxins, such as

92   fumonisins, deoxynivalenol and diacetoxyscirpenol (Bacon et al., 1996; Fairchild et al., 2005;

93   May et al., 2000). Maize contaminated with one of these toxins and FA may be synergistically

94   more toxigenic than predicted from additive effects based on studies with single toxins. Although

95   FA could potentially have a large impact on the safety of human food and animal feed, there

96   currently are no regulations or advisory limits worldwide for FA in human foods or animal feeds.

97   Mycotoxin production by fungi is a complex process that is not yet fully understood.

98   Nonetheless, new genomic and transcriptomic approaches are quickly filling in many missing

99   details. A gene cluster that encodes the components of the FA biosynthetic pathway, *FUB*, is

100   found in *F. verticillioides*, *F. fujikuroi* and *F. oxysporum*. This cluster contains 12 genes

101   arranged in two blocks (Brown et al., 2015; Niehaus et al., 2014; Studt et al., 2016), and encodes

102   proteins for all of the steps in the FA biosynthetic pathway. The cluster also contains genes that

103   encode components required for two putative FA self-protection strategies (Crutcher et al., 2015,

104   Studt et al., 2016). One potential strategy is the enzymatic conversion of FA to the less-toxic

105   fusarinolic acid (FnA). The other strategy uses an MFS superfamily transporter to specifically

106   export FA, but not FnA, from the cell (Crutcher et al., 2015).

107   FnA is less toxic for plant vegetative tissues than FA (Crutcher et al., 2017; Stipanovic et

108   al., 2011), but its toxicity towards humans and animals has not been extensively evaluated. FnA

109   was weakly cytotoxic against several human cancer cell lines, and did not inhibit growth of

110   strains of *Staphylococcus aureus* or *Bacillus subtilis* (Wang et al., 2011). Conditions regulating

111   FA or FnA production in laboratory growth on synthetic culture media include pH, nitrogen level

112   and intracellular levels of FA (López-Díaz et al., 2018; Studt et al., 2016). Many abiotic factors,

113   *e.g.*, temperature, water activity (a_W), pH, and nutrient availability, can alter mycotoxin

114   production and toxigenic potential under field conditions. Temperature and a_W both can modify

115   expression of biosynthetic genes and the production of *Fusarium* mycotoxins such as fumonisins

116   and deoxynivalenol (Cendoya et al., 2017; Schmidt-Heydt et al., 2010). However, the effects of

117   these variables on FA and FnA biosynthesis are not known.

118   Our objective in this study was to determine the impact of strain, temperature, a_W and

119   incubation time on the production of FA and FnA by *F. temperatum*. Our working hypotheses

120   were: (i) that the range of conditions for FA and FnA production was narrower than the range of

121   conditions for *F. temperatum* growth, and (ii) that abiotic factors affect FA and FnA production

122   similarly. This study advances the field by identifying abiotic factors that affect the synthesis of

123   these toxins and enabling the development of strategies to reduce the accumulation of these

124   metabolites in maize food and feed chains.

125

126   **2. Material and Methods**

127

128   2.1 Fungal strains

129   *Fusarium temperatum* strains RC 2903 and RC 2914 were both isolated from maize in

130   Argentina (Fumero et al., 2015). Cultures were purified by subculturing single microconidia and

131   preserved in sterile 15% glycerol at -80°C (Leslie and Summerell, 2006). Preserved cultures are

6

132   maintained in the culture collection of the Research Institute on Mycology and Mycotoxicology

133   (IMICO), CONICET-UNRC.

134

135   2.2 Growth substrate

136   Maize grain was gamma irradiated (12 kGy) with a Cobalt radiation source (CNEA,

137   Ezeiza, Argentina) and stored aseptically at 4ºC. The irradiated grain contained no mycotoxins or

138   viable microbes. A moisture adsorption curve was prepared for the maize to determine the

139   amount of water to be added to adjust a_W levels to 0.95, 0.98 and 0.995. Five hundred grams of

140   irradiated maize (14% initial water content, $a_W = 0.72$) was weighed into sterile 1-L flasks and

141   hydrated to the test a_W by addition of sterile distilled water. Flasks were subsequently

142   refrigerated at 4ºC for 72 h with periodic shaking by hand to improve water absorption and

143   equilibration. The a_W levels were measured with an Aqualab Series 3 water activity meter

144   (Labcell Ltd., Basingstoke, Hants, UK).

145

146   2.3 Inoculation and growth conditions

147   A 3-mm-diameter agar disk from the margin of a 7-day-old colony growing on Spezieller-

148   Nährstoffarmer Agar (SNA) medium (Leslie and Summerell, 2006) was used to inoculate maize

149   grain in 100 mm diameter Petri dishes. Maize grain with different a_W (0.95, 0.98 and 0.995)

150   values were incubated at 15, 25 or 30°C for 7, 14, 21 and 28 days. There were three replicates

151   per treatment and the complete experiment was conducted twice.

152

153   2.4 Mycotoxin analysis

154   Three replicates per treatment were destructively sampled after each of 7, 14, 21 and 28

155   days, dried in a forced air oven at 60°C for 2 h, ground to pass through a 2 mm sieve, and stored

156   at 4°C until analyzed for FA or FnA. Mycotoxins were extracted from 5 g of ground substrate

157   with 20 ml of extraction solvent (acetonitrile/water/acetic acid 79:20:1 v/v/v). The solvent was

158   added to 5 g of ground substrate and the mixture homogenized for 30 min on a rotatory shaker at

159   150 rpm, at room temperature (24 ± 1°C). The substrate slurry was filtered through Whatman

160   No. 4 filter paper, and 2 ml of filtrate transferred to a glass vial before evaporation to dryness

161   under N_2. Dried samples were resuspended in 1 ml of methanol and analyzed by liquid

162   chromatography with electrospray ionization triple quadrupole mass spectrometry (LC/ESI-

163   MS/MS) as previously described by Malachová et al. (2014). This method has been validated for

164   seven different matrices (including maize) and is continuously checked by proficiency testing

165   (Sulyok et al., 2020). The LOD and LOQ are 5.3 and 17.6 µg/kg respectively (Malachová et al.,

166   2014). As no reference standard is available for FnA, it was semi-quantified with an external

167   calibration function obtained for the FA standard, assuming an identical response factor.

168

169   2.5 Statistical analysis

170   GraphPad Prism version 8.1.0 (221) for OS X (GraphPad Software, San Diego, CA,

171   USA) was used to evaluate the significance of the individual and combined effects of strain, a_W,

172   temperature, and incubation times, and a three-way ANOVA followed by Tukey's multiple

173   comparisons tests. Contour map graphs were produced with Sigmaplot v.10.0 (Systat Software

174   Inc., Hounslow, London, UK) to identify conditions that enabled the highest levels of mycotoxin

175   production.

176

3. Results

Both metabolites, FA and FnA, were detected in cultures of both *F. temperatum* strains evaluated, and differences in the amount of toxin accumulated were observed. Culture conditions affected the production of both metabolites, with the strains accumulating different absolute and relative amounts of each toxin depending on the incubation time and the particular combination of abiotic factors (Figure 1).

3.1 Effects of incubation time on fusaric and fusarinolic acids production by *Fusarium temperatum*

To determine when the maximum accumulation of FA and FnA occurred, as well as to explore the profiles of temporal variation, the effect of incubation time was analyzed for each strain (Figure 1). Nine two-way ANOVAs were performed separately with all possible combinations of temperature $\times$ a_W. The ANOVAs identified statistically significant effects of incubation time, strain, and the interaction between the two variables (Table 1). For FA, both strains generally responded similarly, but the absolute levels of FA accumulated differed. Strain RC 2903 always accumulated more FA than did strain RC 2914. Both strains accumulated the most FA after 14 or 21 days of incubation (Figure 1). After 7 or 28 days of incubation, both strains accumulated FA at similar low levels (10-500 ng/g). Both strains showed a particular two-peaks trend at 30°C/0.995a_W after 14 and 28 days of incubation (3,500 ng/g in average) (Figure 1).

FnA accumulation levels varied widely. Strain RC 2903 (Figure 1a) generally, but not always, accumulated more FnA than did strain RC 2914 (Figure 1b). With a few exceptions,

200 FnA levels were always higher than FA levels, often by a factor of 10 or more. FnA

201 accumulations could be the highest on any day other than day 7. Relatively large differences in

202 production or degradation of FnA could occur in a seven day period.

203   When profiles of temporal variation were analyzed, the FnA levels generally "paralleled"

204 those of FA, *i.e.*, when an increase in FA occurs, a subsequent increase in FnA also usually

205 occurs. The FnA levels usually were at least $10\times$ those of FA, often with ppm (µg) for FnA

206 versus ppb (ng) for FA. For strain RC 2903, FA levels significantly exceeded FnA levels on day

207 7 at $0.95a_W/30ºC$ and $0.98a_W/15ºC$. For strain RC 2914, FA levels significantly exceeded FA

208 levels at 15ºC on day 7 at $0.995a_W$ and on day 14 at $0.98a_W$ (Figure 1b). The conditions under

209 which FA levels exceeded FnA levels were always sub-optimal for fungal growth.

210

211 3.2 Effects of temperature and water activity on fusaric and fusarinolic acids production by

212 *Fusarium temperatum*

213   The combined effects of strain, temperature and a_W were evaluated with a three-way

214 ANOVA (Table 2) at 21 days of incubation. All the factors under study and the double and triple

215 interactions significantly affected the levels of FA and FnA observed. a_W was the most important

216 factor for FA levels (39% of total variation) with both a_W and temperature (28% and 36% of total

217 variation, respectively), similarly affecting FnA levels.

218   Temperature affected the accumulation of FA and FnA differently. For FA, temperature

219 was the main factor only at $0.995a_W$. At this a_W, both strains accumulated the least FA at 25ºC

220 and the most FA at 15º and 30ºC. For $0.98a_W$ and $0.95a_W$, strain was more important than

221 temperature for determining the amount of FA produced. Water activity had the largest effect on

222 the variation in FA levels, accounting for 39% of the observed variation (Table 2). Minimum FA

10

223 accumulation always occurred at $0.95a_W$, but the maximum for strain RC 2914 occurred at

224 $0.98a_W$ and for strain RC 2903 at $0.995a_W$ (Figure 1).

225   For FnA, temperature had a strong effect on metabolite accumulation regardless of a_W.

226 FnA levels increased with temperature, reaching a maximum at 30°C. FnA levels also increased

227 with a_W, with the minimum always at $0.95a_W$ and the maximum always at $0.995a_W$. As with FA,

228 strain RC 2903 usually accumulated more FnA than did strain RC 2914 (Figure 1).

229   Contour maps (Figure 2) were developed to relate the effect of $a_W \times$ temperature

230 combinations to FA and FnA accumulation. Optimal and marginal conditions were identified for

231 the accumulation of each metabolite, enabling the identification of conditions with the highest

232 and lowest toxicological risks. Based on the contour maps, FA production was highest at

233 $0.995a_W$, regardless of temperature, and FnA production was highest at $0.995a_W/30°C$.

234

235 **4. Discussion**

236

237   Several species of *Fusarium* are capable of producing FA on both synthetic media and

238 natural substrates such as maize and rice grains, however, the biotic and abiotic conditions that

239 favour its accumulation in maize grains by *F. temperatum* have not yet been studied. This is the

240 first work concerning the influence of strain, incubation time, water activity and temperature on

241 the production of FA and the related metabolite, FnA, by *F. temperatum* growing on maize

242 grains. Our data confirms the ability of this species to produce both FA and FnA, and documents

243 the impact of different water activity (a_W) and temperature conditions on their synthesis.

244   FA levels observed in this study (20-4,500 ng/g) are comparable to, but slightly lower

245 than previous reports of the natural occurrence of this mycotoxin on maize, 70-13,000 ng/g

11

246 (Okeke et al., 2018; Porter et al., 1995). Based on our results, production of FA by *F.*

247 *temperatum*, should be expected at high a_W (wet) and cool (15ºC) or hot (30ºC) temperatures.

248 These conditions are common in maize fields throughout the growing season. *F. temperatum* is

249 the third most commonly isolated *Fusarium* species from maize in Argentina (Fumero et al.,

250 2015), so FA contamination of maize by this species should be expected. Although there are no

251 current studies focused on determining tolerance limits for FA in human food and animal feed, it

252 is important to consider the presence of FA due to its potential synergistic effects on other

253 mycotoxins, e.g., fumonisin and deoxynivalenol (Bacon et al., 1996; Fairchild et al., 2005; May

254 et al., 2000). Maize contaminated with either of these mycotoxins and FA could be more toxic

255 due to the synergistic interactions with FA than predicted from studies with a single toxin alone.

256 Further studies of the single and combined impact of FA on the safety of human food and animal

257 feed are needed to better understand this interaction and to inform the development of advisory

258 limits for FA in human foods and animal feeds.

259 The biosynthesis and regulation of secondary metabolites, such as mycotoxins, are

260 complex cellular processes which are not yet completely understood. Although it is difficult to

261 make broad generalizations, our results indicate that conditions similar to those in which we

262 observed FA maximums, are widely reported as favourable for the biosynthesis of other

263 *Fusarium* mycotoxins that occur on maize, *e.g.*, fumonisins, beauvericin, fusaproliferin and

264 moniliformin (Cendoya et al., 2017; Fumero et al., 2015b). In particular, more FA accumulates at

265 $0.98a_W$ than at $0.995a_W$, and at 15° or 30°C than at 25°C.

266 Although a few general patterns could be discerned from the present results, none of the

267 temperature $\times$ a_W combinations systematically favoured the production of one of the metabolites

268 or the other. Rather, the effect of abiotic factors was both strain and metabolite-specific (Table

269   2). Low temperature (15°C) was better than 25°C or 30°C for FA production, consistent with the

270   results for other common maize contaminants, *e.g.*, fumonisins and trichothecenes, (Fumero et

271   al., 2015b; Kokkonen, et al., 2010). FnA variation, however, was totally different than FA, with

272   FnA levels generally increasing with temperature to a maximum at 30°C and with increased

273   water availability to a maximum at $0.995a_W$. However, considerable variation, often strain and

274   not species specific, is known and more work is needed to determine which conditions are

275   optimal for which strains (Cendoya et al., 2014; Fumero et al., 2015b; Kokkonen et al., 2010).

276   In the present study, both FA and FnA usually were produced at high water activity

277   ($0.98-0.995a_W$). These results are important because the water content levels we assayed are

278   similar to those occurring in the field at multiple stages of the maize plant's life cycle. For

279   example, between silking and ripening, maize kernels have an initial water content of about 40-

280   50% ($a_W = 0.995$ and 1.0, respectively). Subsequently, these levels are reduced to between 25

281   and 20% (a_W, = 0.95 and 0.90, respectively) as the kernels ripen (Sanchis and Magan, 2004). The

282   conditions of water availability that favour FA and FnA production by *F. temperatum* are those

283   that occur during silking. These conditions can persist in the field for a considerable portion of

284   the maize season and continue in storage for grain that is not dried properly. Thus, strains of *F.*

285   *temperatum* may have a relatively long time to produce both FA and FnA in the developing

286   kernels (Chulze, 2010).

287   Optimal growth conditions for *F. temperatum* range from $0.98a_W/25°C$ to $0.995a_W/30°C$

288   (Fumero et al., 2015b). The optimal conditions for FA production ($0.995a_W/15°C$ and

289    $0.995a_W/30°C$) overlap with but are not inclusive of the optimal growth conditions. This pattern

290   is consistent with previous studies of mycotoxin production profiles for fusaproliferin,

13

291 beauvericin and moniliformin by *F. temperatum* and fumonisins produced by *F. proliferatum*,

292 (Cendoya et al., 2017; Fumero et al., 2015b).

293 FA and FnA, are synthesized in a common pathway (Studt et al., 2016), and therefore, the

294 levels of the two metabolites are expected to be related. The profiles of temporal variation that

295 we observed suggest that FnA levels "parallel" those of FA, *i.e.*, when FA levels change FnA

296 levels change as well. The concentrations of both metabolites varied depending on the

297 environmental factors, but at most of temperature $\times$ a_W combinations less FA was accumulated

298 than FnA by both of the tested *F. temperatum* strains. Previous work with *F. oxysporum* and *F.*

299 *fujikuroi* also found that growth substrate influenced FA and FnA levels. *F. oxysporum* (López-

300 Díaz et al., 2018; Studt et al., 2016) always produced higher levels of FnA than FA in both

301 synthetic (Czapek-Dox agar) and semi-defined media (potato-dextrose agar). In *F. fujikuroi*

302 (Studt et al., 2016), FA levels were higher than FnA levels, but the growth media used are not

303 strictly comparable since media supplemented with nitrogen were used for the study.

304 Our study showed that in some cases, FA levels exceeded those of FnA, but these levels

305 did not persist temporally until the next evaluation. Increased levels of FA inhibit hyphal growth

306 of *F. fujikuroi* (Studt et al., 2016) and increase expression of *FUB12*, which encodes a

307 $Zn(II)_2Cys_6$ transcription factor (Brown et al., 2015) that is involved in the regulation of the

308 derivatization of FA to FnA. The conversion of FA to FnA is catalyzed by cluster-independent

309 cytochrome P450 monooxygenases (CYPs) which are regulated by *FUB12* (Studt et al., 2016).

310 Exceeding a threshold of FA could trigger *FUB12* expression and the conversion of FA to FnA.

311 We found that, regardless of strain, time or abiotic conditions, there was a maximum level of FA,

312 ~4,500 ng/g, that was never exceeded and could result in peculiar peak production patterns.

313 Thus, there may be a limit to the level of FA contamination in grains and other foods and feeds,

314 with further studies required to understand the mechanism(s) that limits *in situ* accumulation of

315 FA.

316   The relationship between FA and FnA is not clear. Studt et al. (2016) proposed that FnA

317 is a self-detoxification mechanism and demonstrated that increased levels of FA were toxic to the

318 fungal cell. In the present study, although FnA levels always paralleled those of FA, indirect

319 support was given for the detoxification since there was virtually always more FnA present than

320 FA. Another way of thinking about the relationship between FA and FnA is that FA is simply an

321 intermediate that can accumulate in the process of synthesizing FnA. If FA is an intermediate

322 rather than an endpoint, then the FA levels could indicate a rate-limiting step in the synthesis of

323 FnA. Levels of FA could parallel those of FnA while the entire pathway was operational, and FA

324 levels would eventually fall as the cell's demand for FnA decreased. FnA accumulation, rather

325 than FA accumulation, would be the goal of the process and the limit on FA accumulation could

326 reflect the efficiency of the overall pathway. An excess of the end product over an intermediate

327 in the pathway is expected. This intermediate hypothesis lacks one critical fact at the moment,

328 however – a role for FnA.

329   *F. temperatum* is a toxicological risk for maize under field and storage conditions since it

330 can grow and produce mycotoxins under a wide range of abiotic conditions. The data in this

331 study enable estimates of toxicological risks posed by FA and FnA and the development of

332 strategies to reduce their levels in food and feed chains. There were no obvious upper limits to

333 FnA accumulation, and the amount of FnA accumulated might more accurately reflect the

334 metabolic flux through the FnA pathway than the level of FA accumulated by a culture. As the

335 amount of FnA present in a sample could be $10\times$ more than the amount of FA present, however,

336 further studies of FnA are needed to better understand its physiological and toxicological

337   importance and its role in the metabolism of both the fungus and its host(s). A report of low

338   toxicity to the nematode *Meloidogyne incognita* (Bogner et al., 2016) is available as are reports

339   of low level plant toxigenicity (Crutcher et al., 2017; Stipanovic et al., 2011) and of low level

340   impacts on several bacteria and human cell lines (Wang et al., 2011). In light of our results, it is

341   now particularly important to determine if the higher levels of FnA that we observed are

342   problematic. Determining if FnA can increase cell membrane permeability, as does FA, and

343   thereby synergistically increase the toxicity of other metabolites, e.g., aflatoxins, fumonisins,

344   trichothecenes and zearalenone, also becomes an important question. Such studies could greatly

345   influence our view of dangers associated with FnA contaminated foods and feeds.

346   In conclusion, accumulation of both FA and FnA is influenced by environmental

347   conditions. Optimal accumulation usually occurs at very low or very high temperatures or water

348   activities, which are not optimal for vegetative growth of *F. temperatum*, whose maximum

349   growth occurs at 25ºC and 0.995a_W (Fumero et al., 2015b). Drying grain is the best single

350   strategy for reducing contamination of grain with FA and FnA, since both fungal growth and the

351   amount of toxin accumulated usually are lower at lower values of a_W. Sensitivity of the FA/FnA

352   biosynthetic pathway to temperature is not as clear, with high levels of contamination possible at

353   each of the three tested temperatures. The excess of FnA to FA under most of the conditions

354   tested suggests that FnA has a metabolic role beyond simply being less toxigenic than FA that is

355   not yet understood. Studies of strains in which *FUB12* has been knocked out and conversion of

356   FA to FnA is blocked might help identify what these roles might be.

357

358   **5. Acknowledgments**

359

364

6. References

366

367 Bacon, C.W., Porter, J.K., Norred, W.P., Leslie, J.F. (1996). Production of fusaric acid by

368 *Fusarium* species. *Applied and Environmental Microbiology,* **62,** 4039-4043.

369

370 Bogner, C.W., Ramsay, S.T., Kamdem, G., Sichtermann, C.M., Dirk, H., Jurgen Popp, P.P.,

371 Florian M.W., Grundler, A.S. (2017). Bioactive secondary metabolites with multiple activities

372 from a fungal endophyte. *Microbial Biotechnology,* **10,** 175-188. DOI: 10.1111/1751-

373 7915.12467.

374

375 Bohni, N., Hofstetter, V., Gindro, K., Buyck, B., Schumpp, O., Bertrand, S., Monod M.,

376 Wolfender, J. (2016). Production of fusaric acid by *Fusarium* spp. in pure culture and in solid

377 medium co-cultures. *Molecules,* **21,** 370. DOI: 10.3390/molecules21030370.

378

379 Boutigny, A.L., Scauflaire, J., Ballois, N., Ioos, R. (2017). *Fusarium temperatum* isolated from

380 maize in France. *European Journal of Plant Pathology*, **148,** 997-1001. DOI: 10.1007/s10658-

381 016-1137-x.

382

17

383   Brown, D.W., Butchko, R.A., Busman, M., Proctor, R.H. (2012). Identification of gene clusters

384   associated with fusaric acid, fusarin, and perithecial pigment production in *Fusarium*

385   *verticillioides*. *Fungal Genetics and Biology,* **49,** 521-532. DOI: 10.1016/j.fgb.2012.05.010.

386

387   Brown, D.W., Seung-Ho, L., Lee-Han, K., Jae-Gee, R., Soohyung, L., Yunhee, S., Ho, Y. K.,

388   Busman, M., Yun, S.-H., Proctor, R.H., Lee, T. (2015). Identification of a 12-gene fusaric acid

389   biosynthetic gene cluster in *Fusarium* species through comparative and functional genomics.

390   *Molecular Plant-Microbe Interactions,* **28,** 319-332. DOI: 10.1094/MPMI-09-14-0264-R.

391

392   Cendoya, E., Farnochi, M.C., Chulze, S.N., Ramirez, M.L. (2014). Two-dimensional

393   environmental profiles of growth and fumonisin production by *Fusarium proliferatum* on a

394   wheat-based substrate. *International Journal of Food Microbiology,* **182,** 9-17. DOI:

395   10.1016/j.ijfoodmicro.2014.04.028.

396

397   Cendoya, E., Pinson-Gadais, L., Farnochi, M.C., Ramirez, M.L., Chéreau, S., Marchegu, G.,

398   Ducos, C., Barreau, C., Richard-Forget, F. (2017). Abiotic conditions leading to *FUM* gene

399   expression and fumonisin accumulation by *Fusarium proliferatum* strains grown on a wheat-

400   based substrate. *International Journal of Food Microbiology,* **253,** 12-19. DOI:

401   10.1016/j.ijfoodmicro.2017.04.017.

402

403   Chulze, S.N. (2010). Strategies to reduce mycotoxin levels in maize during storage: a review.

404   *Food Additives and Contaminants*, **27**, 651-657. DOI: 10.1080/19440040903573032.

405

406   Crutcher, F.K., Liu, J., Puckhaber, L.S., Stipanovic, R.D., Bell, A.A., Nichols, R.L. (2015).

407   FUBT, a putative MFS transporter, promotes secretion of fusaric acid in the cotton pathogen

408   *Fusarium oxysporum* f. sp. *vasinfectum. Microbiology,* **161,** 875-883. DOI:

409   10.1099/mic.0.000043.

410

411   Crutcher, F.K., Puckhaber, L.S., Bell, A.A., Liu, J., Duke, S.E., Stipanovic, R.D., Nichols, R.L.

412   (2017). Detoxification of fusaric acid by the soil microbe *Mucor rouxii. Journal of Agricultural*

413   *and Food Chemistry,* **65,** 4989-4992. DOI: 10.1021/acs.jafc.7b01655.

414

415   Desjardins, A.E. (2006). *Fusarium* Mycotoxins: Chemistry, Genetics and Biology. APS Press,

416   St. Paul, Minnesota.

417

418   Dhani, S., Nagiah, S., Naidoo, D.B., Chuturgoon, A.A. (2017). Fusaric acid immunotoxicity and

419   MAPK activation in normal peripheral blood mononuclear cells and Thp-1 cells. *Scientific*

420   *Reports,* **7**(1), 3051. DOI: 10.1038/s41598-017-03183-0.

421

422   Ding, Z., Yang, L., Wang, G., Guo, L., Liu, L., Wang, J., Huang, J. (2018). Fusaric acid is a

423   virulence factor of *Fusarium oxysporum* f. sp. *cubense* on banana plantlets. *Tropical Plant*

424   *Pathology,* **43,** 297-305. DOI: 10.1007/s40858-018-0230-4.

425

426   Fairchild, A.S., Grimes, J.L., Porter, J.K., Croom Jr, W.J., Daniel, L.R., Hagler Jr, W.M. (2005).

427   Effects of diacetoxyscirpenol and fusaric acid on poults: Individual and combined effects of

19

428   dietary diacetoxyscirpenol and fusaric acid on turkey poult performance. *International Journal of*

429   *Poultry Sciences,* **4**(3). DOI: 10.3923/ijps.2005.350.355.

430

431   Fumero, M.V., Reynoso, M.M., Chulze, S. (2015a). *Fusarium temperatum* and *Fusarium*

432   *subglutinans* isolated from maize in Argentina. *International Journal of Food Microbiology,*

433   **199,** 86-92. DOI: 10.1016/j.ijfoodmicro.2015.01.011.

434

435   Fumero, M.V., Sulyok, M., Chulze, S. (2015b). Ecophysiology of *Fusarium temperatum* isolated

436   from maize in Argentina. *Food Additives and Contaminants Part A,* **33,** 147-156. DOI:

437   10.1080/19440049.2015.1107917.

438

439   Kokkonen, M., Ojala, L., Parikka, P., Jestoi, M. (2010). Mycotoxin production of selected

440   *Fusarium* species at different culture conditions. *International Journal of Food Microbiology,*

441   **143,** 17-25. DOI: 10.1016/j.ijfoodmicro.2010.07.015.

442

443   Lanza, F.E., Mayfield, D.A., Munkvold, G.P. (2016). First report of *Fusarium temperatum*

444   causing maize seedling blight and seed rot in North America. *Plant Disease,* **100,** 1019-1019.

445   DOI: 10.1094/PDIS-11-15-1301-PDN.

446

447   Leslie, J.F., Summerell, B.A. (2006). The *Fusarium* Laboratory Manual. Blackwell Professional,

448   Ames, Iowa, USA.

449

450   López-Díaz, C., Rahjoo, V., Sulyok, M., Ghionna, V., Martín-Vicente, A., Capilla, J., Di Pietro,

451   A., López-Berges, M.S. (2018). Fusaric acid contributes to virulence of *Fusarium oxysporum* on

452   plant and mammalian hosts. *Molecular Plant Pathology,* **19,** 440-453. DOI: 10.1111/mpp.12536.

453

454   Malachová, A., Sulyok, M., Beltrán, E., Berthiller, F., Krska, R. (2014). Optimization and

455   validation of a quantitative liquid chromatography–tandem mass spectrometric method covering

456   295 bacterial and fungal metabolites including all regulated mycotoxins in four model food

457   matrices. *Journal of Chromatography A,* **1362,** 145-156. DOI: 10.1016/j.chroma.2014.08.037.

458

459   Mamur, S., Unal, F., Yilmaz, S., Erikel, E., Yuzbasioglu, D. (2018). Evaluation of the cytotoxic

460   and genotoxic effects of mycotoxin fusaric acid. *Drug and Chemical Toxicology,* 1-9. DOI:

461   10.1080/01480545.2018.1499772.

462

463   May, H.D., Wu, Q., Blake, C.K. (2000). Effects of the *Fusarium* spp. mycotoxins fusaric acid

464   and deoxynivalenol on the growth of *Ruminococcus albus* and *Methanobrevibacter*

465   *ruminantium*. *Canadian Journal of Microbiology,* **46,** 692-699. DOI: 10.1139/w00-045.

466

467   Munkvold, G.P., Arias, S., Taschl, I., Gruber-Dorninger, C. (2019). Mycotoxins in corn:

468   Occurrence, impacts, and management. In Serna-Salvidar, S.O. (Ed.), Corn: Chemistry and

469   Technology, 3rd ed. Woodhead Publishing, Cambridge, MA, USA, pp. 235-287. DOI:

470   10.1016/B978-0-12-811971-6.00009-7.

471

472   Niehaus, E.M., von Bargen, K.W., Espino, J.J., Pfannmüller, A., Humpf, H.U., Tudzynski, B.

473   (2014). Characterization of the fusaric acid gene cluster in *Fusarium fujikuroi*. *Applied*

474   *Microbiology and Biotechnology,* **98,** 1749-1762. DOI: 10.1007/s00253-013-5453-1.

475

476   Okeke, C.A., Ezekiel, C.N., Sulyok, M., Ogunremi, O.R., Ezeamagu, C.O., Sarkanj, B., Krska,

477   R. (2018). Traditional processing impacts mycotoxin levels and nutritional value of ogi – a

478   maize-based complementary food. *Food Control,* **86,** 224-233. DOI:

479   10.1016/j.foodcont.2017.11.021.

480

481   Porter, J.K., Bacon, C.W., Wray, E.M., Hagler Jr, W.M. (1995). Fusaric acid in *Fusarium*

482   *moniliforme* cultures, corn, and feeds toxic to livestock and the neurochemical effects in the

483   brain and pineal gland of rats. *Natural Toxins,* **3,** 91-100.

484

485   Ridout, M.E., Newcombe, G., Godfrey, B. (2016). First report of *Fusarium temperatum* in

486   diseased sweet corn ears in the western United States. *Plant Disease,* **100,** 2527-2527. DOI:

487   10.1094/PDIS-04-16-0518-PDN.

488

489   Sanchis, V., Magan, N. (2004). Environmental conditions affecting mycotoxins. In Magan, N.,

490   and Olsen, M. (Eds.), Mycotoxins in Food: Detection and Control. Woodhead Publishing,

491   Cambridge, MA, USA, pp. 174-189

492

493   Scauflaire, J., Gourgue, M., Munaut, F. (2011). *Fusarium temperatum* sp. nov. from maize, an

494   emergent species closely related to *Fusarium subglutinans*. *Mycologia,* **103,** 586-597. DOI:

495   10.3852/10-135.

496

497   Scauflaire, J., Gourgue, M., Callebaut, A., Munaut, F. (2012). *Fusarium temperatum*, a

498   mycotoxin-producing pathogen of maize. *European Journal of Plant Pathology,* **133,** 911-922.

499   DOI: 10.1007/s10658-012-9958-8.

500

501   Schmidt-Heydt, M., Parra, R., Geisen, R., Magan, N. (2010). Modelling the relationship between

502   environmental factors, transcriptional genes and deoxynivalenol mycotoxin production by strains

503   of two *Fusarium* species. *Journal of the Royal Society Interface,* **8,** 117-126. DOI:

504   10.1098/rsif.2010.0131.

505

506   Stipanovic, R.D., Puckhaber, L.S., Liu, J., Bell, A.A. (2011). Phytotoxicity of fusaric acid and

507   analogs to cotton. *Toxicon,* **57,** 176-178. DOI: 10.1016/j.toxicon.2010.10.006.

508

509   Studt, L., Janevska, S., Niehaus, E.M., Burkhardt, I., Arndt, B., Sieber, C.M.K., Humpf, H.,

510   Dickschat, J.S., Tudzynski, B. (2016). Two separate key enzymes and two pathway-specific

511   transcription factors are involved in fusaric acid biosynthesis in *Fusarium fujikuroi*.

512   *Environmental Microbiology,* **18,** 936-956. DOI: 10.1111/1462-2920.13150.

513

514   Sulyok, M., Stadler, D., Steiner, D., Krska, R. (2020). Validation of an LC-MS/MS-based dilute-

515   and-shoot approach for the quantification of > 500 mycotoxins and other secondary metabolites

516   in food crops: Challenges and solutions. *Analytical and Bioanalytical Chemistry,* 1-14. DOI:

517   https://doi.org/10.1007/s00216-020-02489-9.

518

519   Tung, T.T., Jakobsen, T.H., Dao, T.T., Fuglsang, A.T., Givskov, M., Christensen, S.B., Nielsen,

520   J. (2017). Fusaric acid and analogues as Gram-negative bacterial quorum sensing inhibitors.

521   *European Journal of Medicinal Chemistry,* **126,** 1011-1020. DOI:

522   10.1016/j.ejmech.2016.11.044.

523

524   Wang, Q.-X., Li, S.-F., Zhao, F., Dai, H.-Q., Bao, L., Ding, R., Gao, H., Zhang, L.-X., Wen, H.-

525   A., and Liu, H.-W. (2011). Chemical constituents from endophytic fungus *Fusarium oxysporum.*

526   *Fitoterapia,* **82,** 777-781. DOI: 10.1016/j.fitote.2011.04.002.

527

528   **Figure legends**

529

530   Figure 1. Fusaric acid (FA) and fusarinolic acid (FnA) levels (ng/g) accumulated by (a)

531   *Fusarium temperatum* RC 2903 and (b) *Fusarium temperatum* RC 2914 growing on maize

532   grains adjusted to different water activity (0.95a_W, 0.98a_W and 0.995a_W), temperature (15º, 25º or

533   30ºC) and incubation time (7, 14, 21 and 28 days).

534

535   Figure 2. Two-dimensional contour maps of fusaric acid and fusarinolic acid production profiles

536   by *Fusarium temperatum* in relation to temperature and water activity variations. The numbers

537   on the isopleths are the toxin concentration (ng/g).

538

Table 1. Two- way ANOVA for the effect of time of incubation on the levels of fusaric acid (FA) and fusarinolic acid (FnA; ng/g) produced by *F. temperatum*, under stable temperature $\times$ a$_W$ conditions (* $p < 0.001$; [NS] Not significant)

	Source of variation	F-value / % of total variation								
		15ºC			25ºC			30ºC		
		0.95a$_W$	0.98a$_W$	0.995a$_W$	0.95a$_W$	0.98a$_W$	0.995a$_W$	0.95a$_W$	0.98a$_W$	0.995a$_W$
FA	Strain	898/43 *	10/1.1 [NS]	47/16 *	26/7.7 *	77/34 *	24/31 *	35/11 *	15/1.9 [NS]	32/21*
	Time	230/33 *	246/81 *	73/71 *	84/75 *	22/29 *	6.3/25 [NS]	76/75 *	224/83 *	34/67 *
	Strain $\times$ time	162/23 *	47/16 *	8/7.8 [NS]	15/13 *	23/30 *	5.8/23 [NS]	8.1/8.1 [NS]	34/13 *	0.89/1.8 [NS]
FnA	Strain	692/35 *	16/5.4 *	20/7.3 *	137/33 *	234/34 *	95/25 *	96/10 *	282/14 *	8.4/4.8 [NS]
	Time	226/34 *	84/83 *	71/78 *	56/40 *	69/30 *	44/34 *	255/80 *	528/78 *	49/84 *
	Strain $\times$ time	205/31 *	6.8/6.6 [NS]	8.4/9.2 [NS]	32/23 *	76/33 *	47/37 *	25/7.7 *	46/6.9 *	1.1/1.9 [NS]

28

Table 2. Three-way ANOVA for the combined effects of temperature, water activity, strain and their interaction, on the accumulation (ng/g) of fusaric acid (FA) and fusarinolic acid (FnA) produced by *F. temperatum* at 21 days of incubation (*, $p < 0.01$, [NS], Not significant).

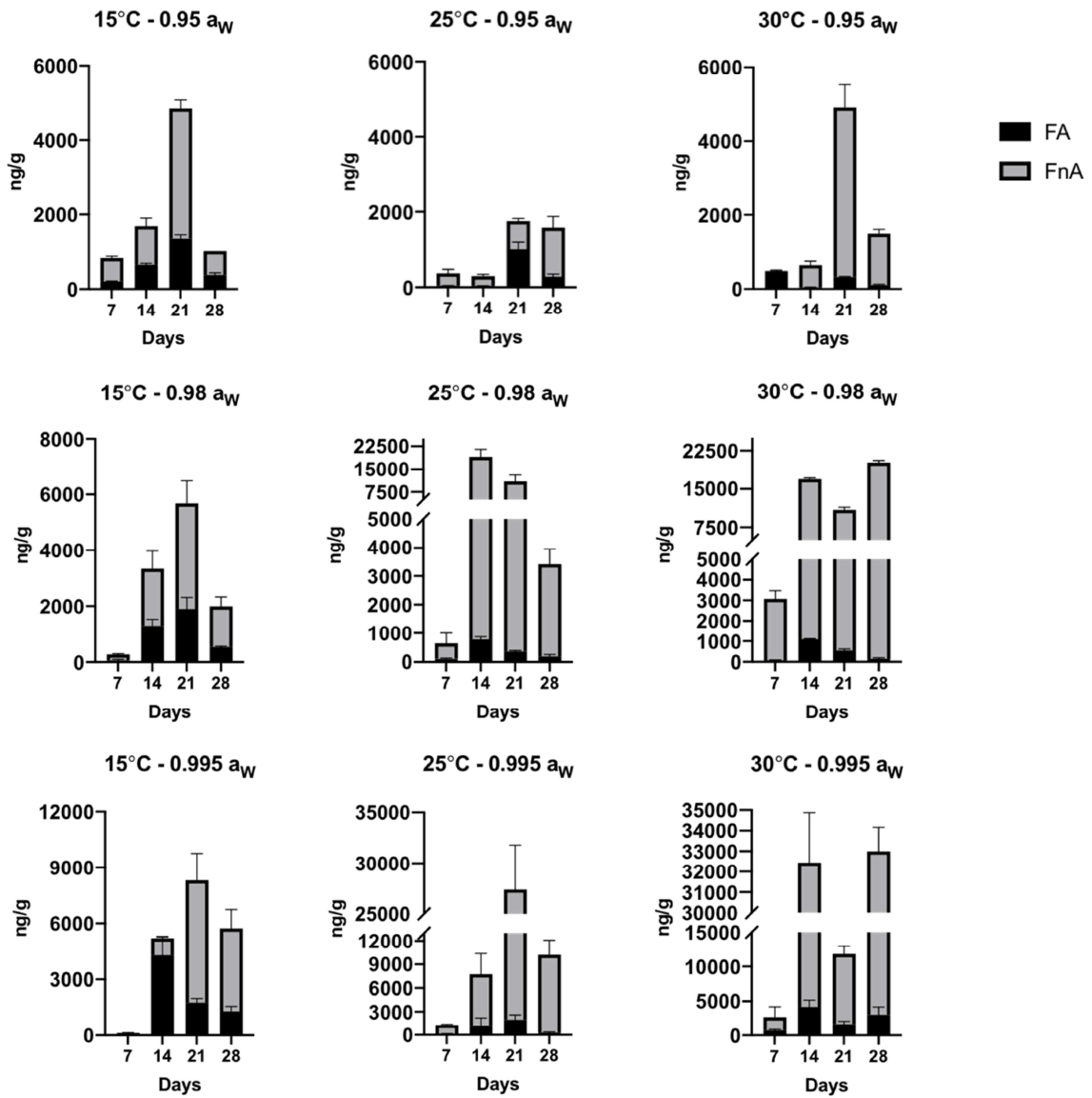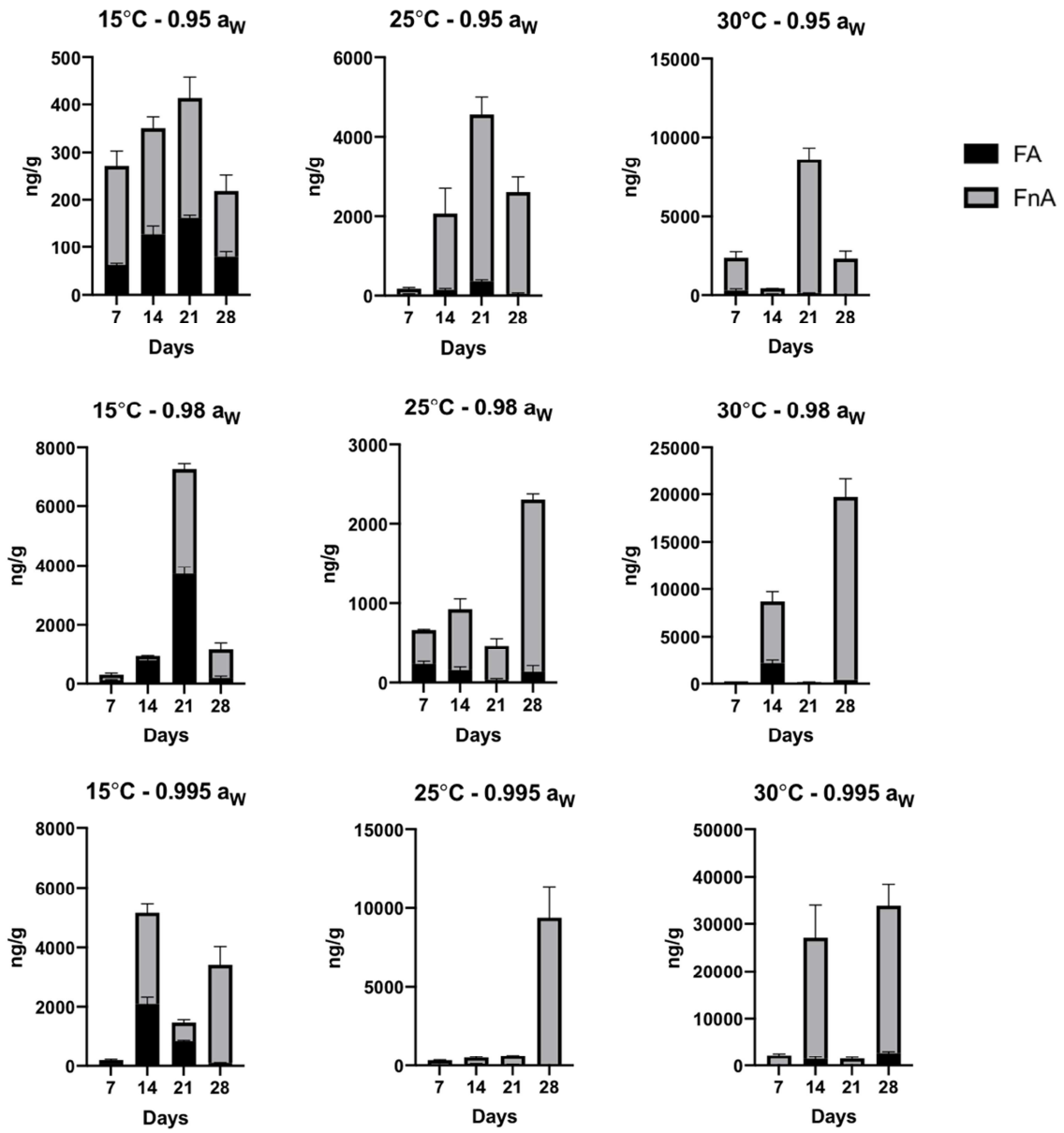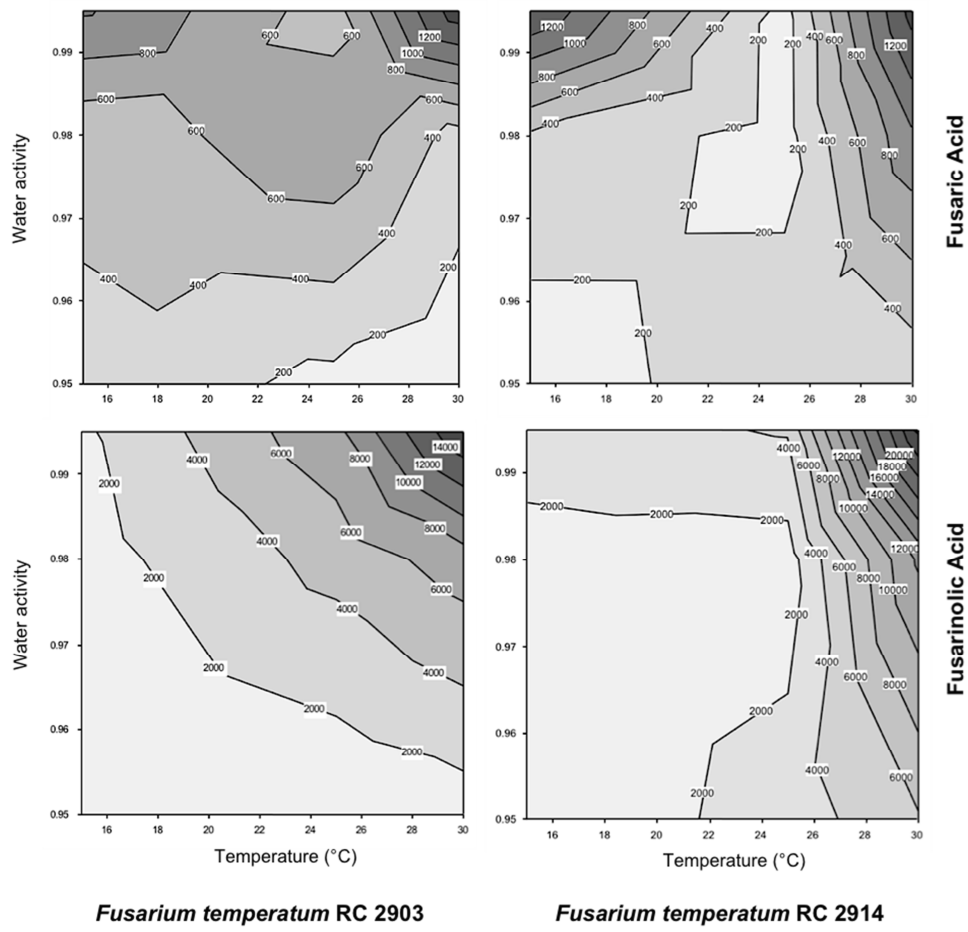|	Fusaric Acid		Fusarinolic Acid	
Source of variation	% of total variation	F	% of total variation	F
Temperature	6.6 [NS]	47	36.2 *	330
Strain	3.1 [NS]	44	2.5 [NS]	46
a_W	39.3 *	531	27.6 *	504
Temperature × strain	12.9 *	155	5.6 [NS]	51
Temperature × a_W	2.5 [NS]	17.6	10.7 *	97
Strain × a_W	0.7 [NS]	9.9	5.8 *	106
Temperature × strain × a_W	26.0 *	183	10.3 *	94

Figure 1 (a)

Figure 1 (b)

Figure 2.

32

Highlights

- Fusaric and fusarinolic acids are produced by *Fusarium temperatum*

- High water activity favored the production of both metabolites

- Temperature affected the production of both metabolites in different way

5