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Comparison of growth and aflatoxin B_1 production profiles of *Aspergillus flavus* strains on conventional and isogenic GM-maize-based nutritional matrices

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ABSTRACT

Maize grown in both North and South America are now predominantly genetically modified (GM) cultivars with some resistance to herbicide, pesticide, or both. There is little information on the relative colonisation and aflatoxin B_1 (AFB_1) production with maize meal-based nutritional matrices based on kernels of non-GM maize and isogenic GM-ones by strains of *Aspergillus flavus*. The objectives were to examine the effect of interacting conditions of temperature (25-35 ^oC) and water availability (0.99-0.90 water activity, a_w) on (a) mycelial growth, (b) AFB_1 production and (c) develop contour maps of optimum and marginal conditions of these parameters for four strains of *A. flavus* on three different non-GM and isogenic GM-maize based nutritional media. The growth of the four strains of *A. flavus* (three aflatoxigenic; one non-aflatoxigenic) was relatively similar in relation to the temperature × a_w conditions examined on both non-GM and GM-based matrices. Optimum growth overall was at 30-35 ^oC and 0.99 a_w for all four strains. Under water stress (0.90 a_w) growth was optimum at 35 ^oC. Statistically: non-GM, GM cultivars, temperature and a_w all significantly affected growth rates. For AFB_1 production, all single and interacting factors were statistically significant except for non-GM × GM cultivar. In conclusion, colonisation of GM- and non-GM nutritional sources was similar for the different *A. flavus* strains examined. The contour maps will be very useful for understanding the ecological niches for both toxigenic and non-toxigenic strains in the context of the competitive exclusion of those producing aflatoxins.

Keywords: genetically modified (GM), non-GM, nutritional media, temperature, water activity, growth, mycotoxin, contour maps

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1. Introduction

Aspergillus flavus is an important opportunistic fungal pathogen able to infect developing maize cobs during silking, especially if the plants have been weakened by drought stress (Dolezal et al., 2014), or damaged by insects (Chulze, 2010). Once *A. flavus* becomes established in the ripening cobs, it produces aflatoxins (AFs). At harvest, the moisture content (m.c.) of the maize cobs can be 18-20% which allows continued colonisation by *A. flavus* unless they are effectively dried to around 15% m.c. promptly (Gasperini et al., 2021). Often a delay in drying will lead to increased post-harvest AFs contamination, especially in tropical and sub-tropical environments. Due to its xerophilic nature (Araújo et al., 2020), *A. flavus* and related species have less competition during this process and are able to aggressively dominate the maize cob ecosystem and cause characteristic storage rots and concomitant AFs contamination (Battilani et al., 2016; Bhatnagar et al., 2006a; Scheidegger and Payne, 2003). The prevailing environmental conditions play a critical role in disease development. Interactions between intrinsic and extrinsic factors have been shown to have a significant influence on growth and mycotoxin production by such mycotoxigenic fungi in stored commodities (Magan et al., 2004).

A. flavus is capable of surviving and overwintering in plant residues as mycelium or sclerotia (Battilani et al., 2012). Crop residue is consequently a primary source of the fungus to allow conidia to be transmitted by air or insects to serve as new inoculum for the host plants to initiate a new infection cycle (Abbas et al., 2009). Infection and subsequent colonization is most aggressive on maize plants that have been subjected to heat or drought stress (Scheidegger and Payne, 2003). *A. flavus* development is favoured by hot dry conditions. It is considered to have a relatively wide temperature tolerance range (19–35 °C) with about 28 °C being considered optimum for growth and 28–30 °C for AFs production in maize. This contrasts with some other commodities, e.g., pistachio nuts, where optimum

79 conditions for growth were around 35 ^{o}C and for AFB_1 production 30-35 ^{o}C (Baazeem et al.,
80 2021). Some strains of *A. flavus* have been shown to grow in very dry environments (e.g.
81 0.73 a_w) and produce aflatoxins *at* >0.85 a_w (Sanchis and Magan, 2004). However, several
82 other authors demonstrated that *A. flavus* requires >0.90 a_w for AFB_1 production and >0.85 a_w
83 for growth (Lv et al., 2019; Nesci et al., 2003; Northolt et al., 1977; Peromingo et al., 2016).
84 However, none of these previous studies compared growth and toxin production on
85 genetically modified (GM) or non-GM isogenic lines of maize as nutritional sources.

86 There has been interest in the development of improved resistance to pests which cause
87 significant damage and allows *A. flavus* to enter as a secondary coloniser. There has also been
88 interest in better resistance to herbicides to maximise yield (Munkvold, 2014). This has led to
89 the development of hybrids with thicker pericarp have also been utilised to try and increase
90 resistance to pests and fungal infection. This resulted in the introduction of genes from
91 *Bacillus thuringiensis* (especially related to the production of *Bt* toxin) to make the maize
92 plants more resistant to certain pest species (Qaim, 2014). Similarly, GM crops with
93 herbicide-tolerance (HT) have been successfully developed and are now commonly used by
94 farmers for weed control in both North and South America.

95 The development and use of GM-maize has resulted in lower production costs and
96 increased farmer incomes (Brookes and Barfoot, 2015). This has resulted in GM cultivars
97 such as Roundup Ready® (tolerance to the herbicide glyphosate; Monsanto, USA),
98 LibertyLink™ (Bayer CropScience, USA) which provide tolerance to both glyphosate and
99 Glufosinate ammonium. Today, most of the maize cultivars have stacked technologies to
100 provide insect resistance (IR) and herbicide tolerance (HT) at the same time. Some studies
101 have suggested that GM-IR has reduced fungal infections, and in some cases a reduction in
102 mycotoxin content (Alma et al., 2005; Schnepf et al., 1998; Windham et al., 1999; Wu,
103 2006). However, no detailed studies have been conducted to examine the relationship

104 between interacting abiotic factors such as water availability × temperature on growth and
105 AFs production by strains of *A. flavus* especially in relation to the nutritional content of
106 conventional and isogenic GM-cultivars.

107 The objectives of this study were to compare the effect of (a) interacting water
108 activity (a_w) × temperature conditions on growth profiles and (b) on aflatoxin B_1 (AFB_1)
109 production by three toxigenic and one non-toxigenic strains of *A. flavus* on maize-based
110 media using milled maize kernels of three conventional (non-GM) and isogenic GM cultivars
111 as nutritional sources. Those examined included three conventional cultivars, and their
112 associated isogenic pesticide resistant, or herbicide resistant or pesticide + herbicide stacked
113 resistance. In addition, contour maps were developed on the optimum and marginal
114 conditions for growth and AFB_1 production by the toxigenic and non-toxigenic strains.
115

116 **2. Materials and methods**

117 *2.1. Strains used in this study*

118 The *A. flavus* strains were isolated from maize and their identity confirmed using
119 morphological and molecular analyses, and based on their AFs production patterns
120 (Gasperini, 2019). Thus, a total of four strains were examined. Three were selected for these
121 studies based on their ability to produce AFB_1 (toxigenic strains: *AfLRG-a*, *AfASC-a*,
122 *AfP30C-a).* A fourth non-toxigenic strain (*AfPHx)* was also included as it was shown in the
123 screening phase to not produce any AFs.
124

125 *2.2. Maize-based media used in these studies*

126 Three cultivars of non-GM maize and their respective isogenic GM lines (Table 1) were
127 used in these studies. Kernels of each maize cultivar (250 g) were milled in a laboratory
128 stainless steel blender (Waring® Commercial, Stamford, USA) for 60 seconds. The blender

129 was carefully cleaned between the milling of each cultivar using isopropanol and dried before
130 subsequent use. The milled maize of each cultivar was kept in separate sterile Duran bottles
131 and kept closed. These were afterwards used to prepare the nutritional media. The maize
132 meal agar (MMA) was prepared by mixing 3% milled maize and 2% technical agar number 3
133 (Oxoid, Basingstoke, UK) in water: glycerol mixtures to modify the a_w from 0.99 (control) to
134 0.95, 0.90. Glycerol was used as the non-ionic solute due to its stability over the experimental
135 temperature range tested. The MMA was prepared using deionised water (15 Ωm) and
136 autoclaved at 121 °C (103 kPA) for 15 min. The molten cooled media (50 °C) were shaken
137 well before pouring into 9 cm Petri plates. The cooled a_w treatment media were stored
138 separately in closed plastic bags at 4 °C until being used. The accuracy of the modifications
139 in a_w was confirmed using an Aqualab 4TE instrument (Decagon, Pullman, USA) to be
140 within ±0.002 a_w
141
142 *2.3. Inoculation and mycelial growth measurements*
143 Spore suspensions (10^6 spores mL^{-1}) of each of the *A. flavus* strains were spread-
144 plated on MEA (Malt Extract Agar; Oxoid, Basingstoke, UK) and incubated at 25 °C for 24 h
145 to allow germination to occur (Gasperini, 2019). These Petri plate colonies were used to
146 provide the inoculum for the experiments. Agar plugs (3 mm Ø) were taken from these
147 inoculation plates with a surface sterilised cork-borer and transferred to the centre of the
148 treatment and replicate MMA plates (9 cm Ø). The replicate plates for each a_w level was
149 enclosed in separate polyethylene sealed boxes with a glycerol/water solution of the same a_w
150 as the plates to keep the equilibrium relative humidity at the target abiotic conditions. The
151 boxes were incubated at 25, 30 and 35 °C for 10 days.
152 The diameters of the colonies were measured in two perpendicular directions at right
153 angles to each other daily for each maize treatment and replicate for each *A. flavus* strain.

154 The growth rate of each strain under the set of nutritional treatments at the different
155 a_w × temperature conditions were calculated by plotting the temporal diametric growth rate
156 daily. The regression lines of the linear growth phase were used for each replicate to obtain
157 the relative growth rate (mm/day) for each strain and under the different interacting abiotic
158 conditions for each maize nutritional medium using Microsoft Excel®. Experiments were
159 carried out with three replicates per treatment and the whole experiment carried out twice.
160
161 *2.4. Quantification of aflatoxin B_1 production by the* A. flavus *strains on the different non-*
162 *GM and GM-based nutritional media*
163
164 *Extraction of aflatoxins from cultures:* After 10 days incubation, 6 agar plugs (approx.
165 0.3 g) were taken from the cultures across each plate with a 5 mm-diameter sterile cork-borer
166 in a sterile flow bench. These plugs were placed into pre-weighed 2-mL safe-lock tubes and
167 re-weighed. Aflatoxins were extracted from the agar plugs by adding 1 mL of chloroform and
168 shaking for 1h at room temperature in the dark. An 800 µL aliquot from the tube was
169 transferred to a new tube and the solvent evaporated to dryness overnight. The derivatization
170 was then performed as detailed below.
171
172 *Derivatization of aflatoxins:* Into the 2-ml safe-lock tube with the residue of each
173 sample, 200 µL of hexane were added, vortexed for 30 sec, followed by the addition of 50 µL
174 trifluoracetic acid (TFA, Fisher Scientific, UK). The mixture was vortexed for 30 sec and left
175 for 5 min. Thereafter, 950 µL water: acetonitrile (9:1, v/v) was added and the entire contents
176 of the tube vortexed for 30 sec. Then the mixture was left for 10 min to allow thorough
177 separation of the layers. The hexane (upper layer) was discarded, and the aqueous portion
178 filtered through Nylon filters (13 mm × 0.22 µm) using a 1 mL syringe directly into amber

179 silanized 2-mL vials (Agilent Technologies, USA) for HPLC analysis. All the analytical
180 reagents used were HPLC grade.

181 *HPLC analysis:* The quantification of the production of AFs by the four strains of *A.*
182 *flavus* was performed by reverse phase HPLC. The HPLC system used was an Agilent 1200
183 series with fluorescence detector (λ_{exc} 360 nm; λ_{em} 440 nm). A C_{18} column (Agilent
184 Poroshell® 120 EC-18, 4.6 x 100 mm, 2.7 µm particle size preceded by a Phenomenex®
185 Gemini C18 column, 3 mm, 3 µm guard cartridge; Phenomenex, CA, USA) was used for
186 separation. Isocratic elution was made with methanol:water:acetonitrile (30:60:10, v/v/v) and
187 a mobile phase flow rate of 1.0 mL/min. The injection volume was 10-50 µL depending on
188 each set of samples. The mixed aflatoxin standard solution used was obtained from Romer
189 Labs U.K Ltd and comprised 0.2 µL mL^{-1} of aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1),
190 and 0.06 µL mL^{-1} of aflatoxin B_2 (AFB_2) and aflatoxin G_2 (AFG_2). This was diluted, left to
191 evaporate to dryness overnight and, thereafter, derivatized as described previously. The set of
192 standards injected was 0.05–4 ng of aflatoxins per injection, and the curves generated by
193 plotting the peaks area against the amounts of aflatoxins. Only AFB_1 was detected in the
194 samples from three of the strains. The correlation coefficient value for the calibration curve
195 was $R^2 = 0.995$.
196
197 *2.5. Statistical analysis*
198 Statistical analysis was performed using the package JMP® Pro 14 (SAS Institute Inc.,
199 USA). Shapiro-Wilk tests was used to determine normality and Levene's test to assess the
200 equality of variance. Both data from growth rates and AFB_1 production failed the
201 requirements for analysis of variance therefore the nonparametric (Wilcoxon /Kruskal-
202 Wallis; p=0.05) were used for analyses (Chan and Walmsley, 1997). Where there was
203 significance after the Kruskal-Wallis test, median comparisons for each pair of the different

cultivars were made using the Wilcoxon – Each Pair test (p=0.05). Forward stepwise regression was used to obtain factorial to degree equations for AFB_1 (ng/g) for conditions of toxin production (a_w, T °C, a_w × T °C) for each strain and for the combined strains for each nutritional medium based on the maize cultivar. Contour maps were built in JMP® Pro 14 using 5000 simulation data from the predicted formula.

3. Results

3.1. Effects of maize cultivar nutritional source and environmental factors on growth of toxigenic and non-toxigenic A. flavus *strains*

The overall growth rate for all four strains (toxigenic: *AfLRG-a*, *AfASC-a* and *AfP30C-a;* non-toxigenic strain *AfPHx*) were optimum at 30-35 °C and 0.99 a_w on both non-GM and GM maize meal-based media (Figure 1). The slowest growth rate was observed at 0.90 a_w in all the temperatures tested. However, growth rates were higher at 35 °C and 0.90 a_w for the four strains regardless of whether grown on non-GM or GM-based maize media. Statistically, there was no significant difference in the growth rate at 30 and 35 °C at 0.95 and 0.99 a_w ($p = 0.05$). Overall, there was no significant difference ($p = 0.05$) in the colonisation rates of GM × non-GM nutritional media and between the *A. flavus* strains studied, although there was a statistically significant effect ($p = 0.05$) of cultivar on the growth of the *A. flavus* strains (Table 2). The detailed growth data for each strain on non-GM and GM nutritional media are shown in Suppl. Figure 1.

A contour map for interacting conditions of temperature × a_w for a toxigenic and non-toxigenic strain of *A. flavus* is provided in Figure 2. The data for the toxigenic strains are based on the pooled data for the growth on both non-GM and GM cultivars. The mycelial growth profiles for the toxigenic and non-toxigenic strains were similar. The optimal growth

229 conditions for both strains were at 0.99 a_w and ≥30 °C with a maximum of 14 mm and 12
230 mm day^{-1} (strains *Af*LRG-a and *Af*PHX, respectively) over 10 days incubation.
231
232 *3.2. Effect of non-GM and GM nutritional sources and environmental factors on aflatoxin B_1*
233 *production by the* A. flavus *strains*
234
235 The overall AFB_1 production by the three toxigenic *A. flavus* strains was integrated
236 across non-GM and GM cultivars as there was no significant difference (p = 0.05) between
237 them. This shows that optimum AFB_1 production was at 0.99 a_w, especially at 30 and 35 °C
238 on both non-GM and GM nutritional media for all three strains. The other strain did not
239 produce any AFB_1 or indeed other AFs. It was noticeable that at 0.95 and 0.90 a_w the
240 amounts of AFB_1 produced was at least ten times less than at 0.99 a_w, especially at 30 and 35
241 °C (Figure 3). Statistically, all single and interacting factors were significant, except for non-
242 GM × GM cultivars (Table 2). The detailed data on production of AFB1 on non-GM and GM
243 nutritional media for each of the three strains is shown in Suppl. Figure 2.
244 The contour maps for the two toxigenic strains which produced the highest amounts
245 of AFB_1 are shown in Figures 4 and 5 based on the different media made from non-GM and
246 GM maize. The strain AfLRG-a was the highest AFB_1 producer amongst all the toxigenic
247 strains when the cultivar P30F53 CON was used as a substrate (≥6000 ng g^{-1}; Figure 4A).
248 Interestingly, the toxin production on the cultivar P2530 CON was about 60-fold lower (≥100
249 ng. g-1; Figure 4C). In 5 out of 6 maize cultivars used, this strain had optimal conditions for
250 AFB_1 production at 30 °C and >0.98 a_w. However, the opposite occurred when the strain was
251 grown on media made with cultivar P2530 Hx®, where more toxin was produced under stress
252 conditions: <30 °C/0.90 a_w (800 ng g^{-1}; Figure 4F).

253 The toxigenic strain *Af*P30C-a also produced more AFB_1 when cultivated on the non-
254 GM P30F53 CON maize meal as a substrate (≥1000 ng/g; Figure 5A). However, lower levels
255 were synthesised by this species on the non-GM cultivar P2530 CON (≥80 ng g^{-1}; Figure
256 5C). The optimal conditions for this strain were also modified, when grown on the GM-maize
257 cultivar. P2530 Hx®, with more AFB_1 produced at 35°C/0.98 a_w (≥300 ng g^{-1}; Figure 5F).
258 The contour maps for the other toxigenic strain is provided in Suppl. Figure 3.
259
260 **4. Discussion**
261
262 Colonisation of nutritional media based on GM or non-GM isogenic maize kernels
263 under different aw x temperature conditions has no effect on the relative growth rates by
264 three toxigeic strains and a non-toxgenic strain of *A. flavus*. However, AFB_1 production was
265 significantly different for the toxigenic strains. Data was utilised to develop contour maps of
266 both the optimum and boundary conditions for colonisation and AFB_1 production by
267 individual and pooled data for the three toxigenic strains and for the non-toxigenic strains on
268 both GM and non-GM matrices.
269 The optimal conditions for growth and AFB_1 production found in this work were
270 distinct with slightly different optima depending on the a_w × temperature conditions.
271 Previously, Medina et al. (2014) also found differences in growth and toxin boundary
272 conditions depending on a_w × temperature interactions. Some studies have also suggested that
273 the biosynthesis of secondary metabolites can sometimes be increased under environmental
274 stress conditions, especially related to water availability, and elevated temperatures.
275 According to Bhatnagar et al. (2006, 2018), aflatoxin biosynthesis in *A. flavus* is optimal at
276 temperatures between 29 and 30 °C, but it is significantly decreased at temperatures <25 and
277 >37 °C. Unlike most fungi, *A. flavus* is favoured by hot dry conditions. Sanchis and Magan

278 (2004) described that some strains of *A. flavus* can grow in very dry environments (e.g., 0.73
279 a_w) and produce aflatoxins at ≥0.85 a_w. These were based on long incubation periods of up to
280 25-30 days. However, other studies demonstrated that *A. flavus* required >0.90 a_w for AFB_1
281 production and >0.85 a_w for growth, dependent on the nutritional medium used (Lv et al.,
282 2019; Nesci et al., 2003; Northolt et al., 1977; Peromingo et al., 2016). In addition, these
283 abiotic interactions have been demonstrated to influence the biosynthetic gene cluster
284 expression and thus AFB_1 biosynthesis (Abdel-Hadi et al., 2010; Medina et al., 2015).

285 It was noted that the interactions between environmental effects and the nutritional
286 status of different maize cultivars influenced the patterns of AFB_1 production. The same
287 strain produced different amounts of AFB_1 in each maize cultivar. This suggests that the
288 cultivars may perhaps have slightly different micro-nutritional components. This could have
289 influenced mycotoxin production, although no differences in colonisation were discernible.

290 The concentration of AFB_1 was not consistently higher in a specific type of maize
291 (non-Gm vs GM) indicating that each cultivar itself results in more or less toxin despite the
292 genetic modifications. For example, when the cultivar P30F53 CON was used, for example,
293 the AFB_1 accumulation was significantly higher than its isogenic GM line for 2 strains
294 (*Af*LRG-a and *Af*P30C-a). Nevertheless, the conditions for the maximum production were
295 modified by the interactions between the abiotic factors and cultivar. Thus, according to the
296 contour maps for the strain *Af*LRG-a: 0.90 a_w at 25 °C were best for AFB_1 production for the
297 GM cultivar (P2530 Hx), whereas 0.99 a_w at 25 °C were optimum for the equivalent non-GM
298 one (P2530 CON). One possibility for these differences could be the nutritional content of
299 each hybrid that may have influenced the toxin amount produced if the metabolism of the *A.*
300 *flavus* strain was influenced, despite there being no significant impacts on colonisation
301 patterns.

Bakan et al. (2002) suggested that the biochemical composition of GM maize (MON 810 *Bt*) and the near isogenic non-GM line could be responsible for the differences in fumonisins content found. However, it was reported that the content of proteins, soluble nitrogen, starch and sugars did not differ between GM hybrids and their near-isogenic non-GM lines (Masoero et al., 1999; Betz et al., 2000). Therefore, from a macro-nutritional assessment, the GM maize cultivars may have many similar amounts to the parental maize line (Reuter et al., 2002).

Another possibility is that there is a link between fatty acid (FAs) content in the non-GM and GM cultivars which may influence the AFB_1 production by the different strains. Levels of free FAs increase in response to various stresses and can play a pivotal role in plant–microbe interactions (Walley et al., 2013). Seed FAs composition has also been suggested to be a component of pathogen susceptibility and seed colonisation (Dall'Asta et al., 2012). As free FAs, linoleic acid levels partly regulate development, seed colonisation and mycotoxin production by *Aspergillus* spp. (Calvo et al., 1999; Scarpari et al., 2014).

Studies by Jiménez et al. (2009) found FA profiles in the non-GM and GM maize with insect-resistance (*Cry1*Ab gene) cultivars to be relatively similar. (Frank et al., 2012) suggested that most differences between non-GM and GM maize cultivars with insect or herbicide resistance (DKC78-15B, TXP 138F, DKC78-35R, respectively) were due to abiotic and not genetic factors. Thus, location, weather patterns and season predominantly influenced the FA profiles. Battilani et al. (2018) have suggested that oxylipins may be important, both in terms of defence reactions to fungal infection and potentially as virulence factors for mycotoxigenic fungi such as *Fusarium verticillioides.* However, no studies have examined comparisons of oxylipins in non-GM and cultivars and their effect on *A. flavus* development.

Manetti et al. (2004, 2006) and Piccioni et al. (2009) tried to identify the metabolomic fingerprint of non-GM and GM maize seeds using Nuclear Magnetic Resonance spectroscopy

(NMR). NMR was thus used to profile the metabolome of insect-resistant GM maize containing *cry1A(b)* gene. Amongst the 40 water-soluble metabolites identified in all samples, those which could be used for discrimination between non-GM and GM cultivars were ethanol, citric acid, trehalose, and glycine-betaine. These were all present in higher levels in the GM maize samples (Piccioni et al., 2009). However, the relationship between maize cultivar modifications, the content of these metabolites and the genetic modification has not been elucidated (Simó et al., 2014). However, none of these studies examined the effect that modification of such compounds may have on colonisation by *A. flavus* or AFs contamination.

The growth of the non-aflatoxigenic strain suggests similar growth patterns to the toxigenic strains on both non-GM and GM-based media as a nutritional source. This may be important that the resilience of such strains over the range of interacting abiotic factors over which toxigenic strains can grow and produce AFs (Medina et al., 2017; Gasperini et al., 2019). It has also been shown that the utilisation patterns of key C-sources in maize vary between toxigenic and non-toxigenic species (Mohale et al., 2013; Medina et al., 2017). It is critical for effective control of both pathogens and potential toxin contamination that the ecological niche profile of the biocontrol strain is similar to that of the toxigenic strains (Magan, 2020). The present study suggests that in both non-GM and GM cultivars the non-toxigenic strain can grow effectively over the key two-dimensional abiotic ranges similar to the toxigenic strains.

Overall, the growth profiles for the toxigenic and non-toxigenic strains were similar, regardless of whether the nutritional sources were based on non-GM or GM isogenic maize cultivar kernels. The contour maps show this clearly in relation to optimum and marginal conditions for colonisation. In addition, this also suggests that biocontrol strains may be effective on both non-GM and GM cultivars in terms of ecological windows for competition

with toxigenic strains. In terms of AFB_1 production, there were differences in terms of the amounts of toxin produced and the optimum and marginal quantities produced after a 10-day period. This does suggest that there may be some influence of cultivar type on AFB_1 production, whether they are non-GM or GM-based. More detailed studies are required of the potential impact of individual or groups of macro- and micro-nutrients which may be present in these two groups maize cultivars. The composition of the maize hybrids may have a bigger impact on AFs contamination. This is important as many countries where GM maize cultivars are grown have maximum legislative limits for food and feed chains.

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Figure legends

Figure 1. Comparison of the growth rate (mm day^{-1}) of the three toxigenic and one non-toxigenic strains of *A. flavus* on maize media based on non-GM and GM grain in relation to water availability (0.99-0.90 a_w) and temperature (25-35 ^oC). Bars indicate Average ± Standard Error.

Figure 2. Contour maps of the growth rate (mm day^{-1}) of *Aspergillus flavus* with pooled data from all the maize cultivars used as substrate (GM + non-GM) for (A) toxigenic strain (*Af*LRG-a) and (B) non-toxigenic strain (*Af*PHx) over 10 days incubation. The colour scale (lighter > darker tones) indicates higher growth rate (mm day^{-1}) in relation to the interacting abiotic factors.

Figure 3. Effect of colonisation of the maize-based media using non-GM and GM maize kernels on aflatoxin B_1 production (ng g^{-1}) under different water activity conditions at 25-35 ^oC. Bars indicate Average ± Standard Error. *Not the same scale.

Figure 4. Contour plots for aflatoxin B_1 (ng g^{-1}) production by a toxigenic strain of *Aspergillus flavus* (*Af*P30C) on milled maize agar using the non-GM maize cultivars (A) AS 1555 CON, (B) P30F53 CON and (C) P2530 CON and, their respective isogenic GM lines (D) AS 1555 PRO®, (E) P30F53 H® and (F) P2530 Hx® as substrates. The colour scale (lighter > darker tones) indicates higher concentration of AFB_1 (ng g^{-1}) in relation to the interacting abiotic factors.

Figure 5. Contour plots for aflatoxin B_1 (ng g^{-1}) production by the toxigenic strain of *Aspergillus flavus* (*Af*LRG-a) on milled maize agar using the non-GM maize cultivars (A) AS 1555 CON, (B) P30F53 CON and (C) P2530 CON and, their respective isogenic GM lines (D) AS 1555 PRO®, (E) P30F53 H® and (F) P2530 Hx® as substrates. The colour scale (lighter > darker tones) indicates higher concentration of AFB_1 (ng g^{-1}) in relation to the interacting abiotic factors.

Supplementary Figures

Suppl. Figure 1. Growth rate (mm day^{-1}) for the *A. flavus* strains AfLRG-a, AfASC-a,

AfP30C-a and AfPHx on milled maize agar using the non-GM maize cultivars (AS 1555 CON, P30F53 CON and P2530 CON) and their respective isogenic GM lines (AS 1555 PRO®, P30F53 H® and P2530 Hx®) as a substrate. Bars represent AV±SE.

Suppl. Figure 2. Aflatoxin production (AFB1 ng g^{-1}) for the *A. flavus* strains, AfASC-a, AfLRG-a and AfP30C-a on milled maize agar using the non-GM maize cultivars (AS 1555 CON, P30F53 CON and P2530 CON) and their respective isogenic GM lines (AS 1555 PRO®, P30F53 H® and P2530 Hx®) as a substrate. Bars represent AV±SE.

Suppl. Figure 3. Contour plots for aflatoxin B_1 (ng g-1) production by a toxigenic strain of Aspergillus flavus (AfASC-a) on milled maize agar using the non-GM maize cultivars (A) AS 1555 CON, (B) P30F53 CON and (C) P2530 CON and, their respective isogenic GM lines (D) AS 1555 PRO®, (E) P30F53 H® and (F) P2530 Hx® as substrates. The colour scale (lighter > darker tones) indicates higher concentration of AFB1 (ng g-1) over the variables.

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Table 1. Maize cultivars (non-GM and GM) selected for the ecophysiological studies as substrate for *A. flavus* growth. Key: IR- insect resistance; HT – herbicide tolerance.

Conventional cultivar (non-GM)	**Isogenic line (GM)**	**Tolerance traits present in the GM cvs**
AS 1555 CON	AS 1555 PRO®	IR - Lepidopteran Antibiotic resistance
P30F53 CON	P30F53 H®	HT - Glufosinate ammonium and glyphosate IR - Lepidopteran
P2530 CON	P2530 Hx®	IR - Lepidopteran

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Table 2. Statistical effect of abiotic and biotic factors on growth and aflatoxin B_1 production by *A. flavus* strains.

Effects	***p*-value (Prob>ChiSq)**	
	Growth (mm/day)	**AFB_1 (ng/g) ***
Fungal strains	0.4661	0.0013
Maize cvs	<.0001	0.0020
T°C	<.0001	0.0002
a_w	<.0001	<.0001
GM × non-GM	0.0616	0.6002
Maize cv × T°C	0.0683	0.0020
Maize cv × a_w	0.0683	<.0001
Maize cv × T°C × a_w	0.0683	0.0002

$P \geq 0.05$ was considered to be not statistically significant. Non-parametric Wilcoxon/Kruskal-Wallis Tests with 1-Way Test Chi-Square Approximation.

The *p*-value (Prob>ChiSq) was calculated from the values of ChiSquare and DF (degrees of freedom) of each effect.

* The effects on AFB_1 excluded the non-toxigenic *A. flavus* strain *A*fPHx.

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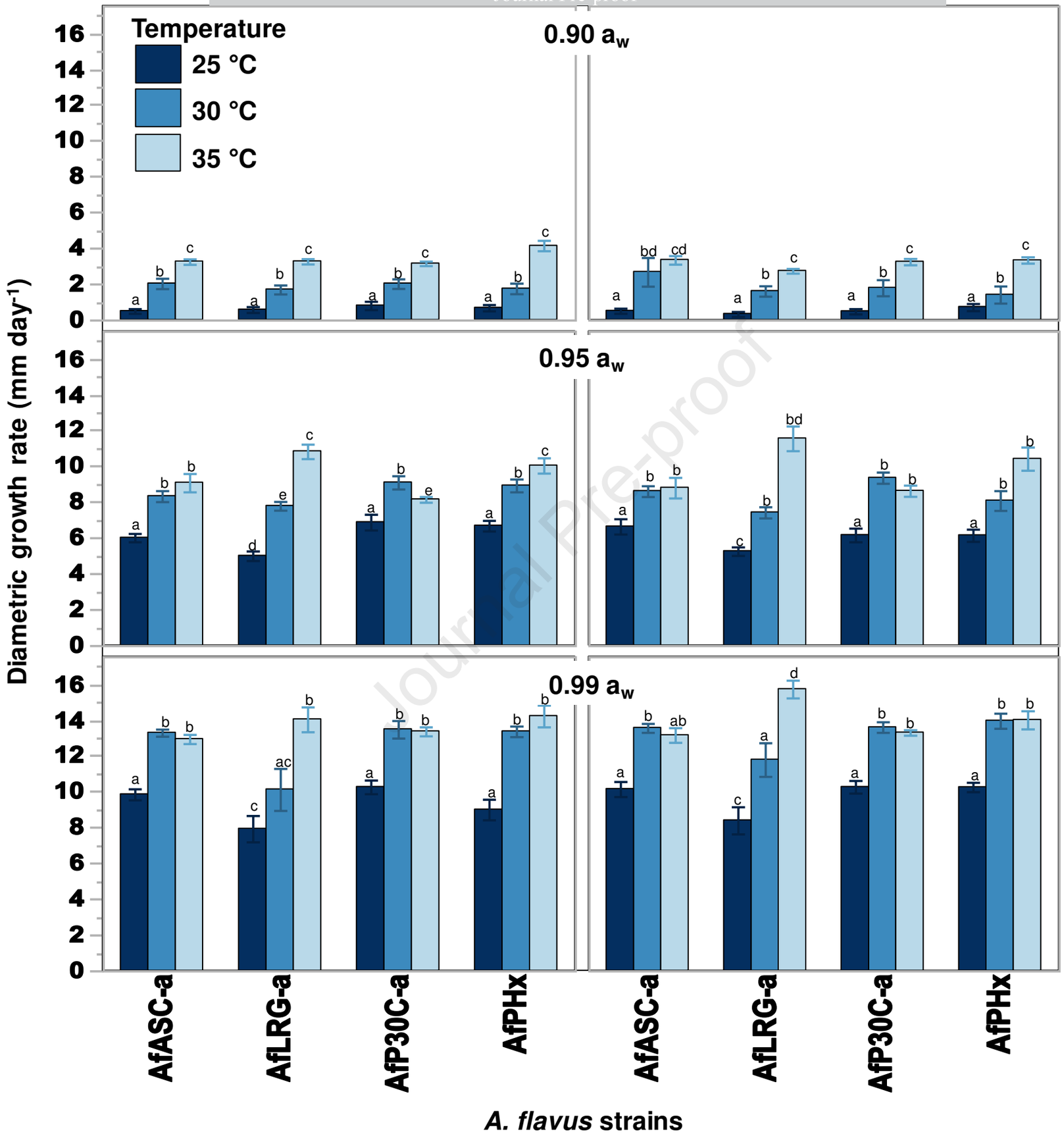

Figure 1.

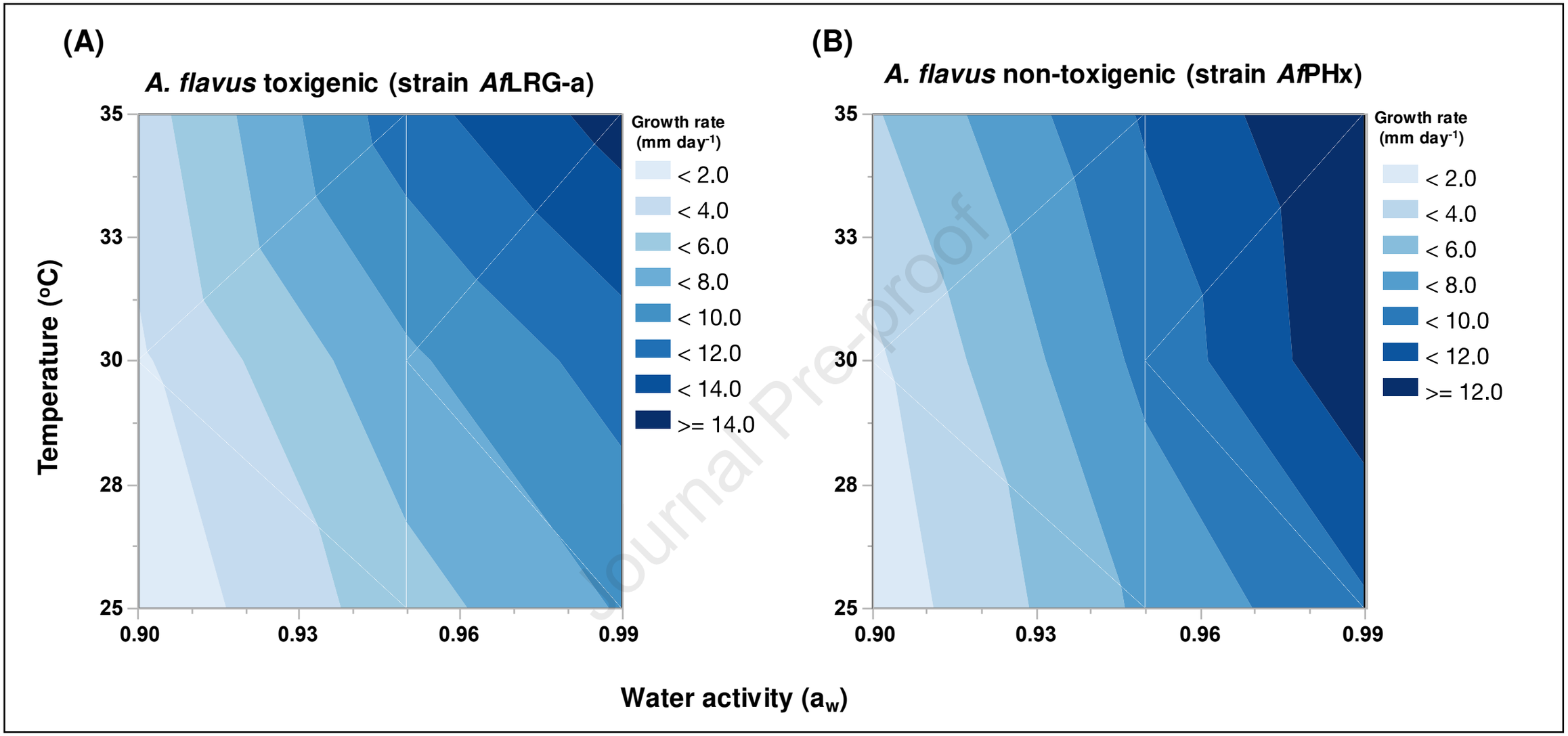
(A)
A. flavus toxigenic (strain AfLRG-a)
(B)
A. flavus non-toxigenic (strain AfPHx)
Growth rate (mm day-1)
< 2.0
< 4.0
< 6.0
< 8.0
< 10.0
< 12.0
< 14.0
>= 14.0
>= 12.0
Temperature (°C)
35
33
30
28
25
0.90
0.93
0.96
0.99
Water activity (aw)

Figure 2.

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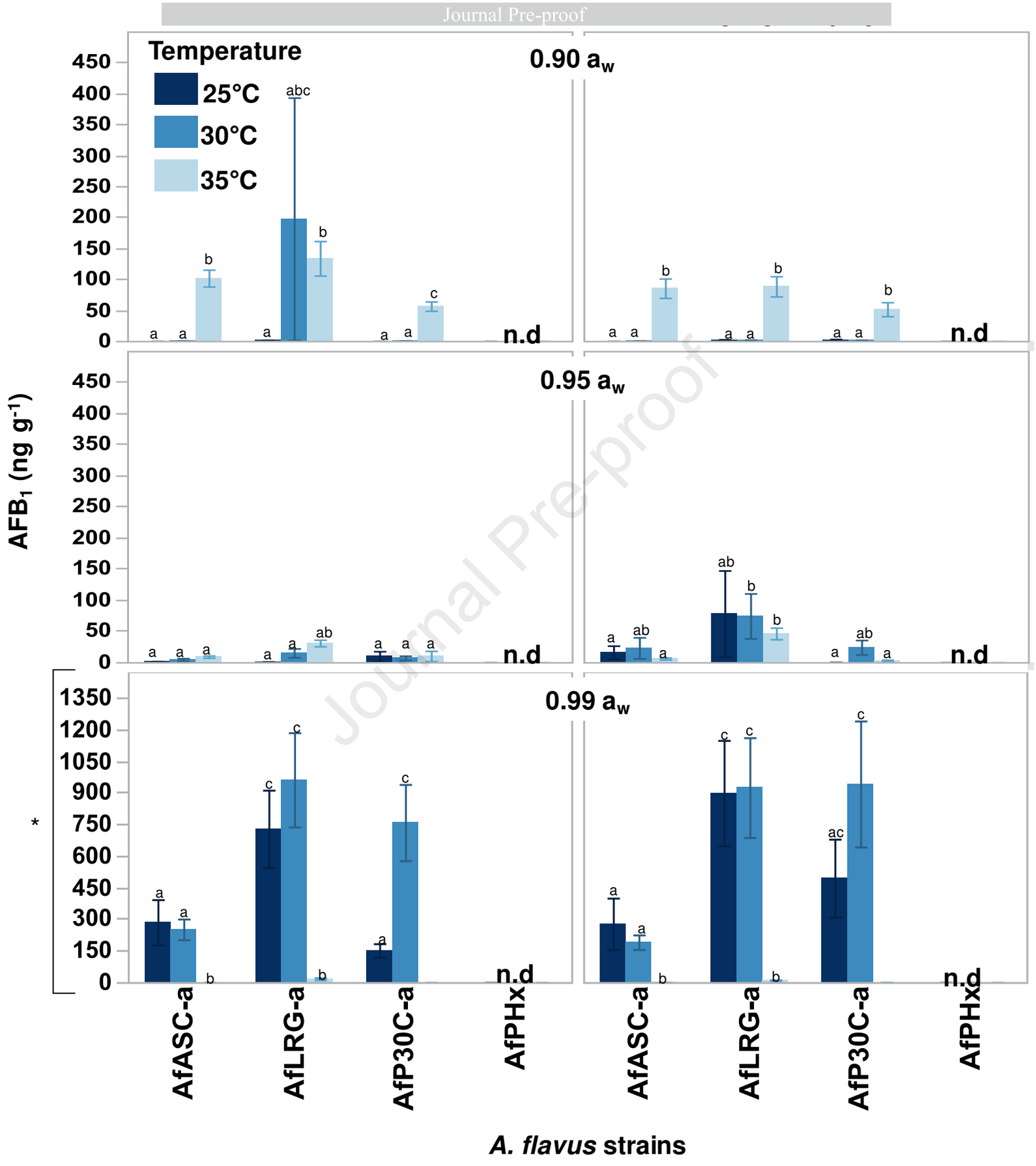

Figure 3.

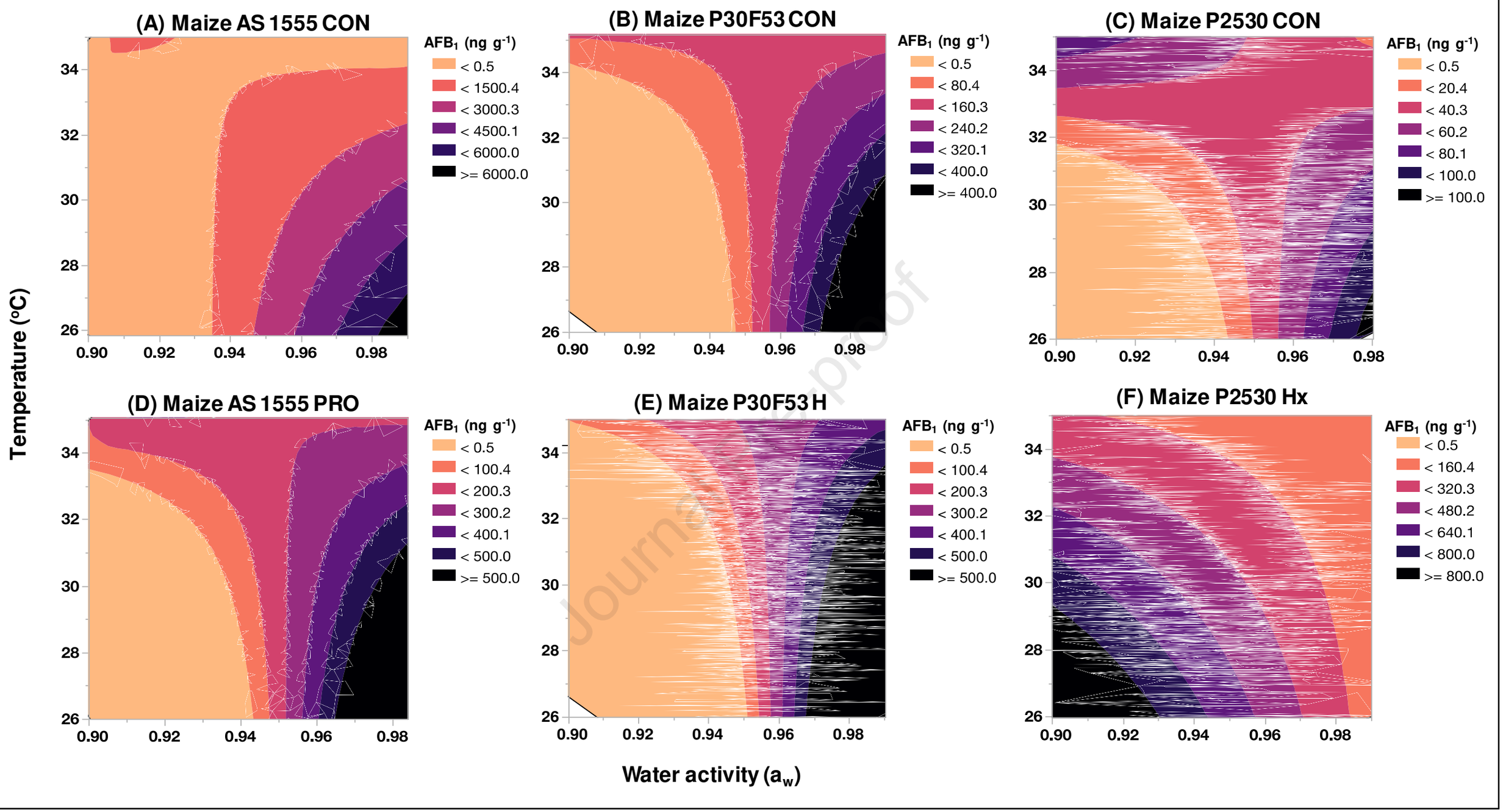
(A) Maize AS 1555 CON
(B) Maize P30F53 CON
(C) Maize P2530 CON
(D) Maize AS 1555 PRO
(E) Maize P30F53 H
(F) Maize P2530 Hx
AFB1 (ng g-1)
< 0.5
< 1500.4
< 3000.3
< 4500.1
< 6000.0
>= 6000.0
< 80.4
< 160.3
< 240.2
< 320.1
< 400.0
>= 400.0
< 20.4
< 40.3
< 60.2
< 80.1
< 100.0
>= 100.0
< 100.4
< 200.3
< 300.2
< 400.1
< 500.0
>= 500.0
< 160.4
< 320.3
< 480.2
< 640.1
< 800.0
>= 800.0
Temperature (°C)
Water activity (aw)
26
28
30
32
34
0.90
0.92
0.94
0.96
0.98

Figure 4.

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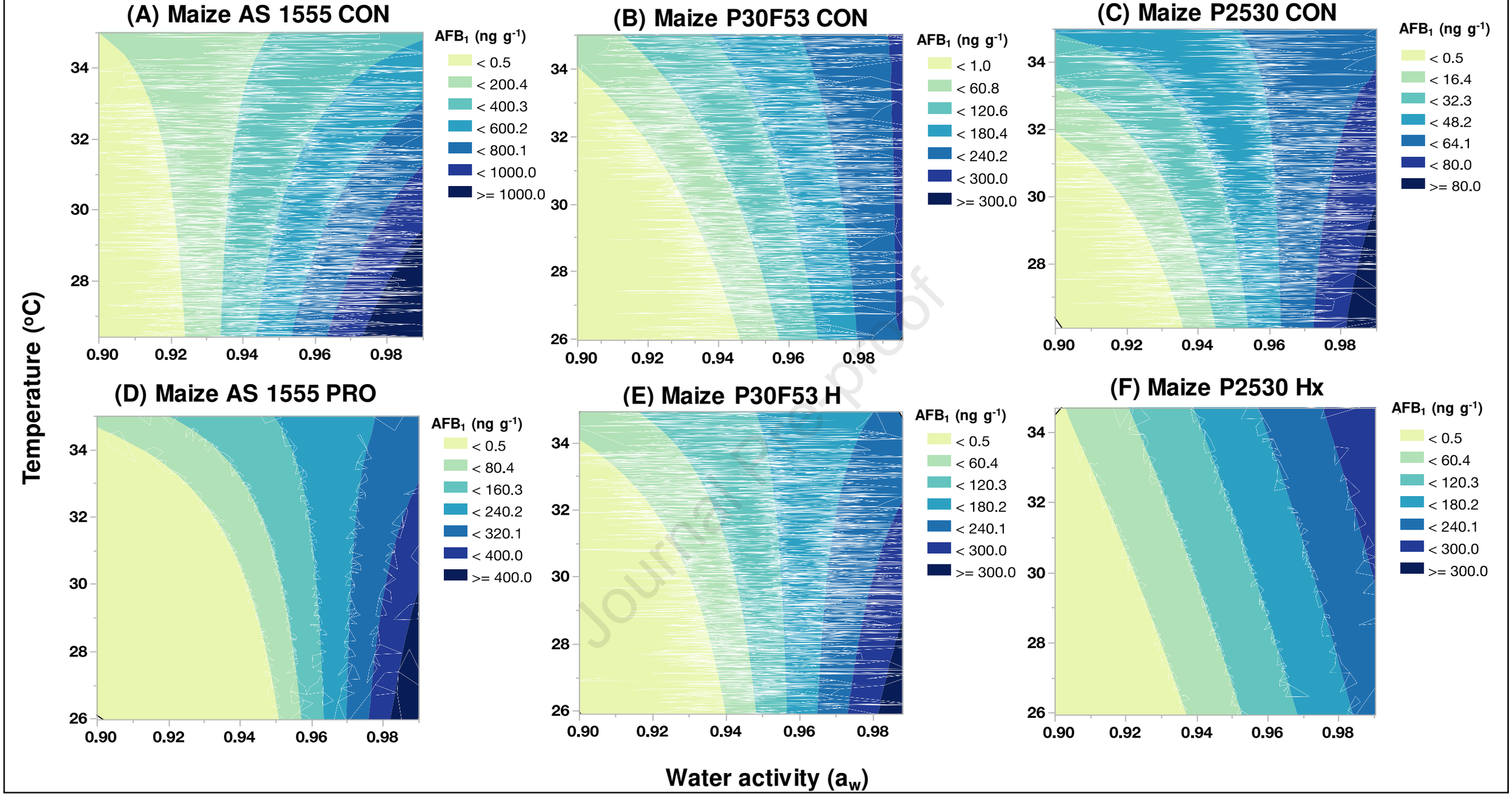

Figure 5.

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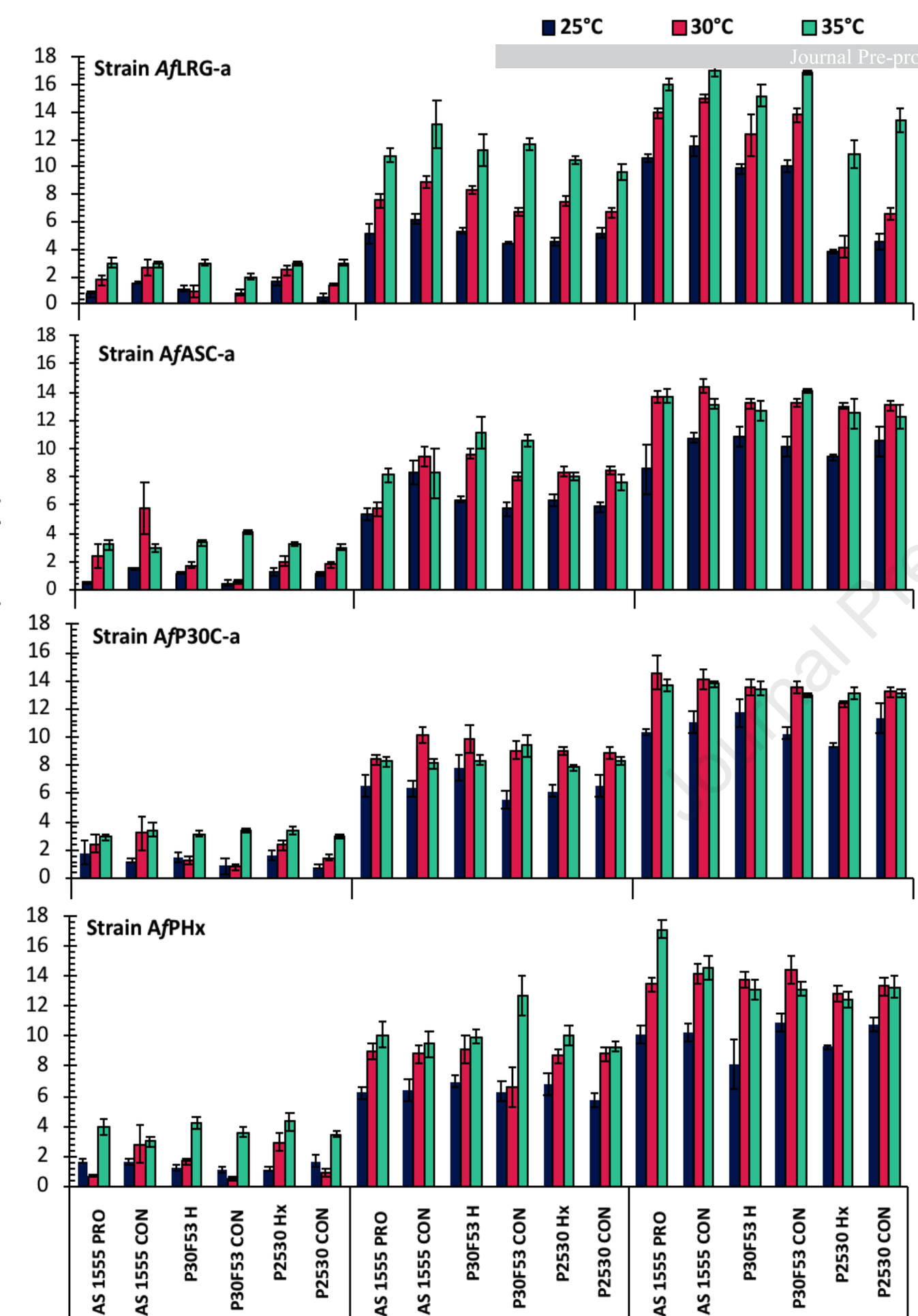

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Supplementary Figure 1.

Growth rate (mm day^{-1}) for the *A. flavus* strains *Af*LRG-a, *Af*ASC-a, *Af*P30C-a and *Af*PHx on milled maize agar using the non-GM maize cultivars (AS 1555 CON, P30F53 CON and P2530 CON) and their respective isogenic GM lines (AS 1555 PRO®, P30F53 H® and P2530 Hx®) as substrate. Bars represent AV±SE.

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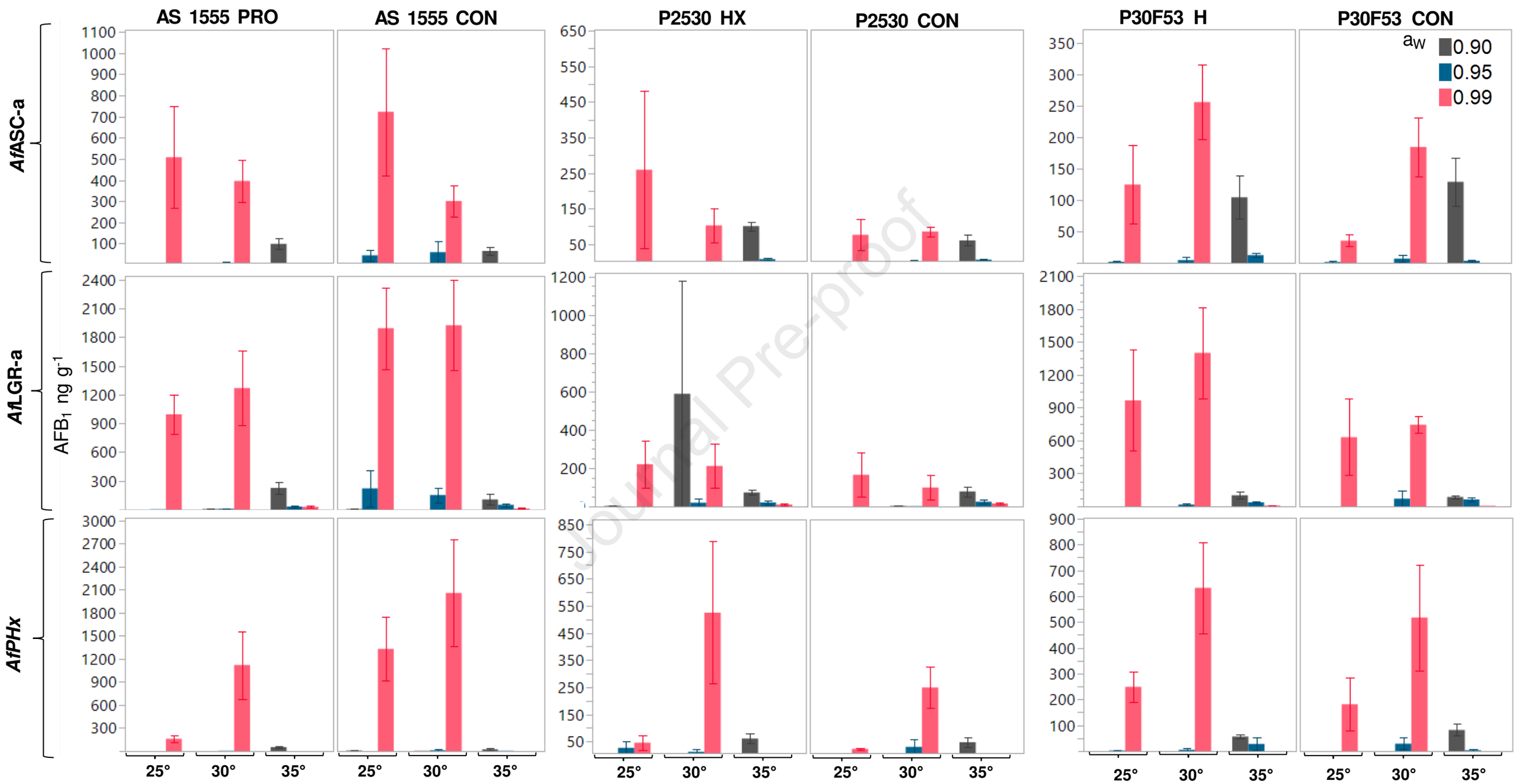
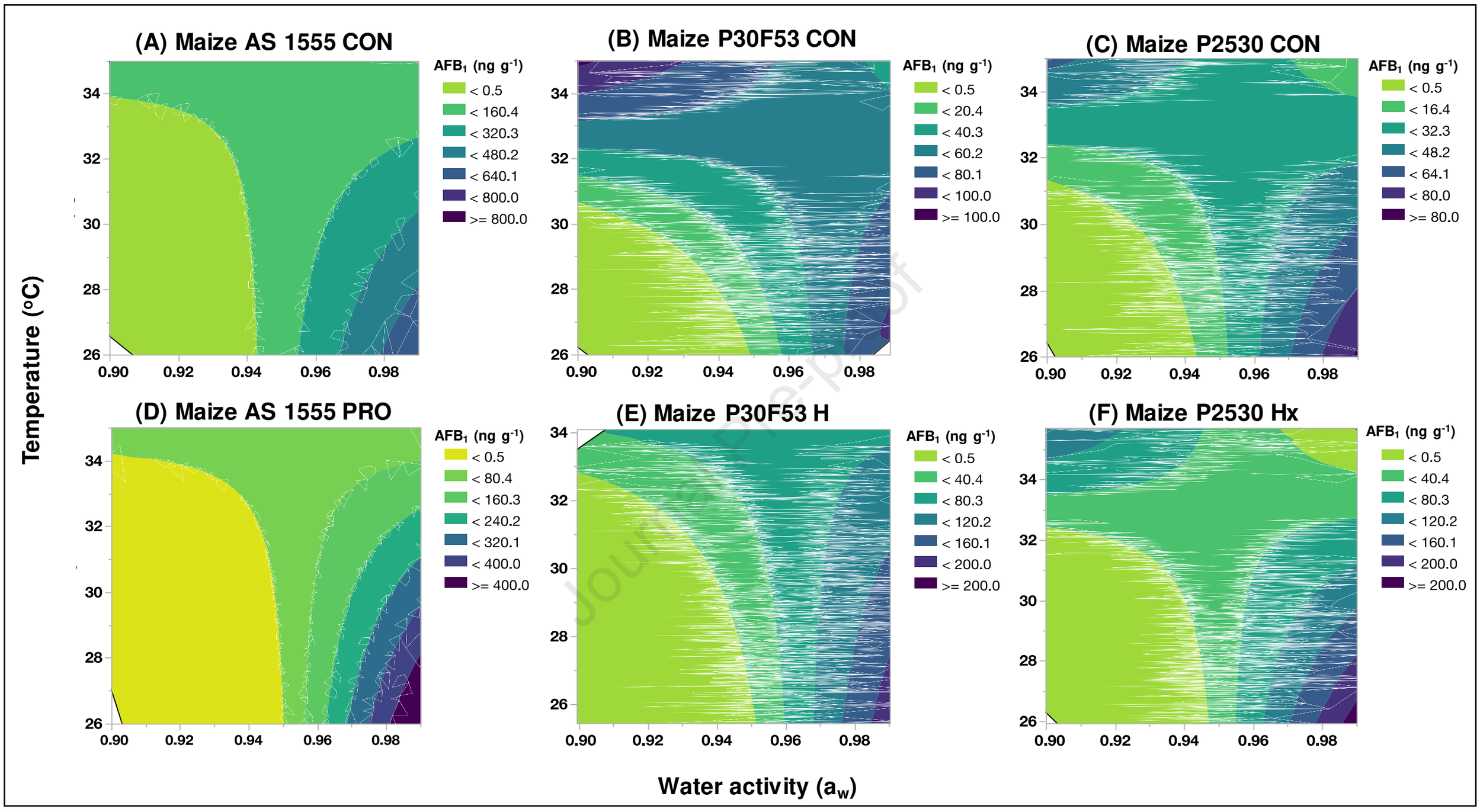


Supplementary Figure 2.

Aflatoxin production (AFB_1 ng g^{-1}) for the *A. flavus* strains, *Af*ASC-a, *Af*LRG-a and *Af*P30C-a on milled maize agar using the non-GM maize cultivars (AS 1555 CON, P30F53 CON and P2530 CON) and their respective isogenic GM lines (AS 1555 PRO®, P30F53 H® and P2530 Hx®) as substrate. Bars represent AV±SE.



Supplementary Figure 3.

Contour plots for aflatoxin B_1 (ng g^{-1}) production by a toxigenic strain of *Aspergillus flavus* (*Af*ASC-a) on milled maize agar using the non-GM maize cultivars (A) AS 1555 CON, (B) P30F53 CON and (C) P2530 CON and, their respective isogenic GM lines (D) AS 1555 PRO®, (E) P30F53 H® and (F) P2530 Hx® as substrates. The colour scale (lighter > darker tones) indicates higher concentration of AFB_1 (ng g^{-1}) over the variables.

Highlights

- *A. flavus* strains grew similarly on GM/non-GM nutritional media under interacting abiotic factors
- Contour maps of optimum and marginal conditions for growth were developed for 4 strains of *A. flavus*
- Aflatoxin B_1 production was optimal at 0.99 water activity and 30 ^{o}C, regardless of strain.
- Optimum and marginal toxin production contour maps were determined for toxigenic strains

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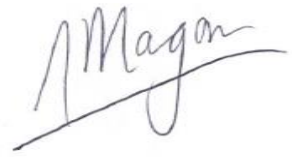
Fungal Biology

Re: Conflict of Interests Statement

We can confirm that there are no conflict of interests for the authors of this manuscript submitted to the Journal.

This was part of a PhD thesis which the Senior Author completed at Cranfield under the 'Science without Borders' scholarship scheme.

With best wishes,
Yours sincerely,



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Comparison of growth and aflatoxin B1 production profiles of Aspergillus flavus strains on conventional and isogenic GM-maize-based nutritional matrices

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