Impact of some environmental factors on growth and ochratoxin A production by *Aspergillus niger* and *Aspergillus welwitschiae*

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Abstract

Ochratoxin A (OTA) is a nephrotoxic mycotoxin which may contaminate various foods and feed products worldwide. *Aspergillus niger* is one of the species responsible for OTA contamination in grapes and derived products. This species has recently been split into *A. niger* and *Aspergillus welwitschiae*. Both species can not be distinguished by phenotypic or extrolite profiles and to date there is no ecophysiological information of *A.welwitschiae*.

The aim of this study was to determine the effects of water activity (a_w) (0.90; 0.95 and 0.98-0.99), culture media (Yeast Extract Sucrose Broth (YESB); Synthetic Grape Juice Medium (SGM); White grape juice (WGJ)) and temperature (15ºC, 25ºC and 35ºC) on the growth and OTA production of four strains of *A. niger* and six strains of *A.welwitschiae.* The assay was performed in microtiter plates, determining the absorbance at 530 nm and the concentration of OTA at 1, 2, 4 and 10 days.

No significant differences were observed in absorbance and OTA values between the two species under study. The highest absorbance values were recorded in YESB, followed by SGM and WGJ. Absorbance values increased with increasing a_w and temperature. The highest OTA values were obtained at 0.98-0.99 a_w and the best culture media for OTA production was YESB, followed by WGJ and SGM. The studied strains of *A. niger* produced the highest mean OTA level at 25ºC whereas *A. welwitschiae* strains produced the highest mean OTA concentration at 15ºC, although not differing significantly from concentration produced at 25ºC.

To our knowledge, this is the first report on the impact of some environmental factors on growth and OTA production by *A. welwitschiae.*

Keywords: *Aspergillus niger; Aspergillus welwitschiae;* ecophysiology; grapes; ochratoxin A; raisins

1. Introduction

Among the different mycotoxins which merit special concern for the hazard they represent in food commodities, ochratoxin A (OTA) deserves particular attention. OTA is nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic in various animals *in vitro*, with renal toxicity and carcinogenesis being the key adverse effects (Heussner and Bingle, 2015). Cereals, pulses, coffee, beer, wine and grape juice as well as dried vine fruits, nuts, cacao products and spices have been found to be contaminated frequently with OTA (EFSA, 2006). To date the European Union has established maximum OTA levels for different food products (Commission of the European Communities 2006, 2010, 2012, 2015).

OTA is produced by some *Penicillium* and *Aspergillus* spp. In *Aspergillus* section *Nigri*, different studies have shown that *Aspergillus carbonarius* and *Aspergillus niger* are an important source of OTA in food commodities such as wine, grapes and dried vine fruits (Abarca et al., 2004; Cabañes and Bragulat 2018; Frisvad et al., 2007; Visconti et al., 2012). The taxonomy of strains in the *Aspergillus* section *Nigri* has been studied and debated for decades. Recently, the taxon *Aspergillus niger sensu stricto* has been split into *A. niger* and *A.welwitschiae* (Hong et al., 2013; Perrone et al., 2011). Both species can not be distinguished by phenotypic or ecological data including extrolite profiles (Perrone et al., 2011).

Temperature and water activity (a_w) are the key environmental factors that influence both the rate of fungal spoilage and the production of mycotoxins (Magan and Aldred, 2007). To date, there is no ecophysiological information of *A.welwitschiae* and there are only some studies on the impact of both environmental factors on the

73 growth and OTA production by *A. niger* isolates on semisynthetic media (Astoreca et
74 al., 2007; Barberis et al., 2009a,b; Lasram et al., 2016), on simulated grape juice
75 medium (Leong et al., 2006; Passamani et al., 2014; Selouane et al., 2009; Zouhair et
76 al., 2017) or on natural substrates (Alborch et al., 2011; Astoreca et al., 2009 a,b). In
77 most of those studies, only one or two *A. niger* strains were included.
78 In ecophysiological studies, growth is usually assessed by radial growth
79 measurement of fungal colonies developed at each culture media and incubation
80 conditions. The use of replicate plates for each condition studied and the subsequent
81 OTA extraction and quantification make these studies time-and labor intensive and not
82 suitable when a large number of isolates has to be evaluated. In our laboratory we
83 developed a new screening method to detect growth and OTA production by some
84 *Aspergillus* spp. and *Penicillium* spp. growing in a small quantity of culture media, using
85 microtiter plates (Abarca et al., 2014). The aim of this study was to adapt this method
86 to determine simultaneously the effects of three culture media at three water activity
87 levels and three incubation temperatures on the growth and OTA production by *A.*
88 *niger* and *A.welwitschiae.*
89
90 **2. Materials and Methods**
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92 *2.1. Strains and molecular identification*
93
94 Four *A. niger* strains and six *A.welwitschiae* strains, mainly isolated from grapes
95 and raisins were studied (Table 1). All the strains were previously detected as OTA-
96 producers in our laboratory and had been initially identified as *A. niger*.

All the strains were confirmed for identity by sequencing of the calmodulin gene. Briefly, DNA was extracted and purified from 48 h old cultures in malt extract broth according to the FastDNA Spin kit protocol with the FastPrep FP-24 instrument (MP Biomedicals, Biolink, Barcelona, Spain). The DNA was kept at -20 ºC until used as template for PCR amplification. Following the DNA extraction, the calmodulin gene was amplified and sequenced by using the fungal primers CL1/CL2A (O'Donnell et al., 2000). For the phylogenetic analyses, sequences obtained were aligned using Clustal X v2.0.12 (Larkin et al., 2007) and analyzed to generate a phylogenetic tree in Mega 6 software (Tamura et al. , 2013). The Neighbor-Joining method based on the Tamura-Nei model (Tamura and Nei, 1993) with 1,000 bootstrap replicates was used. *Aspergillus flavus* CBS 569.65^{T} was used as outgroup in this analysis.

2.2. Inoculum preparation and verification

Spore concentration was adjusted to around 10^6 conidia/ml. Briefly, the inoculum suspensions were prepared in sterile saline (0.85%) containing 0.05% Tween 80 from 7-day-old cultures on malt extract agar at 25ºC. After heavy particles were allowed to settle for 10-15 minutes, the upper homogenous suspensions were transferred to sterile tubes and adjusted to 0.6 McFarland turbidity standard (Abarca et al., 2014) by using a photometric method (Densimat, BioMérieux). The inoculum size was confirmed by haemocytometer counting and quantitative colony counts.

2.3. Culture media and microtiter inoculation

121 Sterile 96-well flat-bottom microtiter plates were used. Three liquid culture media
122 were assayed: Yeast Extract Sucrose broth (YESB), used as a control, synthetic grape
123 juice medium (SGM) representative of grape composition at mid-veraison (Mitchell et
124 al., 2004), and white grape juice (WGJ).
125 YESB contained per liter: yeast extract, 20 g; sucrose, 150 g; $FeSO_4 \cdot 7\ H_2O$, 0.5 g;
126 pH adjusted to 6.5 (Samson et al., 2000). SGM consisted of D(+) glucose, 70g; D(-)
127 fructose, 30g; L(-) tartaric acid, 7g; L(-) malic acid, 10g; $(NH_4)_2HPO_4$, 0.67g; KH_2PO_4,
128 0.67g; $MgSO_4 \cdot 7\ H_2O$, 1.5g; NaCl, 0.15g; $CaCl_2$, 0.15g; $CuCl_2$, 0.0015g; $FeSO_4\ 7\ H_2O$,
129 0.021g; $ZnSO_4 \cdot 7\ H_2O$, 0.0075g; (+) Catechin hydrate, 0.05g; distilled water, 1 L; pH
130 adjusted with 10M NaOH to pH 4.0-4.2 (Mitchell et al., 2004). WGJ was prepared with
131 200ml of commercially sold white grape juice made from ecological grapes and 800 ml
132 of distilled water; pH adjusted at 4.0.
133 The initial a_w was 0.98 for YESB and SGM media, and 0.99 for WGJ. These initial
134 values were modified to 0.95 a_w and 0.90 a_w by the addition of different amounts of
135 glycerol. Media were autoclaved and the final a_w values were checked with
136 LabMASTER-a_w (Novasina. Switzerland).
137 For each a_w level, the adjusted fungal suspensions were diluted 1:100 in the
138 culture medium assayed (YESB, SGM, WGJ). In each microplate column, five wells were
139 inoculated with 200 µl of the diluted suspension of each strain and one well, used as a
140 blank, was filled with 200 µl of un-inoculated culture media (YESB, SGM ,WGJ).
141 Growth assessment and OTA production at each a_w level were determined after 1,
142 2, 4, and 10 days of incubation at three different temperatures (15, 25, and 35ºC).
143 Thus, each strain-$a_w$level-temperature combination was repeated in 4 microplates,
144 one for each reading day. For each sampling occasion and temperature assayed,

145 microtiter plates with the same water activity level were enclosed in sealed
146 polyethylene bags. The entire experiment was repeated twice on different days.
147
148 *2.4. Growth measurement and OTA extraction procedure*
149
150 For each culture media, a_w level and temperature assayed, growth was monitored
151 by absorbance measurements at 530nm using the Multilabel-Reader Mithras LB 940
152 (Berthold Technologies, Bad Wildbad, Germany) after 1, 2, 4 and 10 days of incubation.
153 The absorbance of the corresponding uninoculated medium, used as blank was
154 subtracted to the absorbance values of the inoculated media. After each reading,
155 microplates were sealed and stored at -80ºC until they were analyzed for OTA content.
156 OTA production was detected using a previously described high-pressure liquid
157 chromatography (HPLC) screening method developed in our laboratory for fungi
158 growing in microtiter wells (Abarca et al., 2014). On each sampling occasion, one of the
159 five replicate wells inoculated for each strain, culture media, a_w level and incubation
160 temperature, were randomly selected and their content was removed and extracted
161 with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC. The
162 limit of quantification was 0.045 µg/ml for this mycotoxin.
163
164 *2.5. Statistical Analysis*
165
166 Data obtained from the different conditions tested were statistically analyzed by
167 means of one-way analysis of variance test and Student's test. The Pearson's
168 coefficient (r) was used to quantify the relationship between haemocytometer counts

and colony counts of inocula. All statistical analyses were performed using Minitab 17 statistical software (Minitab Inc, State College, Pennsylvania, USA).

3. Results

3.1. Molecular species identification

Based on the calmodulin sequences, six strains were identified as *A. welwitschiae* and four as *A. niger.* The phylogenetic tree was reconstructed showing that the isolates split into two distinct clades: one grouping with *A. niger* CBS 554.65^{T} and the other with the sibling species *A. welwitschiae* CBS 139.54^{T} (Fig. 1). The nucleotide sequences of the calmodulin gene determined in this study have been deposited in the GenBank and their accession numbers are given in Table 1. Sequence analysis revealed the existence of 2 sequence types in *A. niger* and 3 sequence types in *A. welwitschiae*. Sequence positions and differences of *A. welwitschiae* compared to *A. niger* JX500080 in calmodulin gene were: 146 (T), 169 (C), 190-191 (CT), 197-198 (TT), 221 (-), and 505 (T) (Fig. S1).

3.2. Inoculum standardization

Mean colony counts of *A. niger* and *A. welwitschiae* suspensions adjusted to 0.6 McFarland turbidity standard were 0.8 ± 0.2 x 10^6 cfu/ml and 0.7 ± 0.2 x 10^6 cfu/ml respectively. The inocula enumerated with a cell-counting haemocytometer provided suspensions of 1.8 ± 0.1 x 10^6 conidia/ml for *A. niger,* and 1.9 ± 0.2 x 10^6 conidia/ml for

193 *A. welwitschiae.* Pearson's coefficient between both systems of measurements was
194 0.912.
195
196 *3.3. Growth measurement*
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198 Results of one-way analysis of variance of absorbance values versus each of the
199 variables assayed are shown in Table 2. No significant differences were observed in
200 absorbance values neither between the species, nor between the assays, nor between
201 the replicates ($p > 0.05$). Temperature, culture media and water activity significantly
202 affected ($p<0.01$) growth of all the strains studied. The higher the a_w and temperature,
203 the higher absorbance values. The highest significant absorbance values ($p<0.001$)
204 were obtained at 35ºC and 0.99 a_w. Regarding culture media, the highest significant
205 absorbance values ($p<0.001$) were recorded in YESB, followed by SGM and WGJ.
206 Table 3 shows mean absorbance values of all the studied strains at each condition
207 assayed and incubation time. As no statistically significant differences were observed
208 between species, replicates or experiment, the results are expressed as a mean value.
209 The highest absorbance values were obtained at 35ºC in all culture media and a_w. At
210 this high temperature a statistically significant growth increase was recorded after 2
211 days (0.98-0.99 a_w and 0.95 a_w) or 4 days (0.90 a_w) of incubation, while at 25ºC this
212 increase was observed after 2-4 days (0.98-0.99 a_w and 0.95 a_w) or 10 days (0.90 a_w) of
213 incubation. At 15ºC, none of the strains grew in any culture media adjusted at 0.90 a_w
214 during the experimental period of this study. At this low temperature, a significant
215 increase in absorbance values was observed at 10 days of incubation at 0.95 a_w and
216 0.98-0.99 a_w, although a slight increase was observed after four days of incubation in

YESB and WGJ. In all culture media, initial growth in the microtiter wells could be visually detected at the naked eye, when absorbance value was greater than 0.1.

3.4. OTA production

No significant differences were observed in OTA concentration neither between the species, nor between the assays ($p > 0.05$) (Table 2). Temperature, culture media and water activity significantly affected ($p<0.01$) OTA production of all the strains studied. The highest significant OTA values ($p<0.001$) were obtained at 0.98-0.99 a_w. The best culture media ($p<0.001$) for OTA production was YESB, followed by WGJ and SGM, although no statistically significant differences were observed between both last culture media.

In order to see the individual behavior of strains, OTA concentration produced by *A. niger* and *A. welwitschiae* at each condition assayed and incubation time are shown in Tables 4 and 5 respectively. Results are expressed as mean value of both experiments as no statistically significant differences were observed ($p>0.05$). Interval Plot of OTA mean values in *A. niger* and *A. welwitschiae* strains at each a_w and temperature assayed is shown in Fig. 2. The studied strains of *A. niger* produced the highest mean OTA level at 25ºC whereas *A. welwitschiae* strains produced the highest mean OTA concentration at 15ºC, although not differing significantly from concentration produced at 25ºC. For both species, YESB was in general the most favorable medium for OTA production. In the remaining media, mean OTA levels were higher in WGJ than in SGM, although this difference was not statistically significant. In relation to the a_w, the greatest production of OTA was observed at the highest a_w.

None of the strains produced detectable levels of OTA in any culture media adjusted at 0.90 a_w.

In *A. niger* strains, the highest OTA concentration was recorded after 4 days of incubation at 25ºC in YESB-0.98a_w (strains A-75, A-136 and A-1609) and in SGM-0.98a_w (strain A-3919). In three of the four strains studied, OTA production could be detected after only two days in WGJ-0.99 a_w-25ºC and in YESB-0.98 a_w-35ºC.

In *A. welwitschiae* strains, the highest OTA concentration was recorded also after 4 or 10 days of incubation at 25ºC in YESB-0.98a_w (strains A-942, A-943, A-3204 and A-3694). Strains A-1899 and A-1944 instead, achieved their maximum OTA concentration at 15ºC in YESB-0.98a_w. Quantifiable levels of OTA were detected after only 2 days in WGJ in some conditions.

4. Discussion

For each strain and reading day (1, 2, 4 and 10 days), the total number of conditions studied were 27 (3 culture media at 3 water activities and 3 incubation temperatures). Only few studies have examined the combined effect of temperature and water activity on growth and OTA production by *A. niger* strains. These studies are usually carried out in only one solid culture medium and with a reduced number of strains. In previous studies on YES agar, the *A. niger* strains A-75 and A-136 produced the highest OTA level at 20-25ºC and 0.98-0.99 a_w (Esteban et al., 2004; 2006). So, similar results have been obtained by using microtiter plates.

As no statistically significant differences were observed in absorbance and OTA values between the experiments, our method are suitable to assess the impact of a

265 great number of environmental conditions on growth and OTA production of a large
266 number of isolates.
267 In spectrophotometric methods inoculum size is a critical variable. In this paper we
268 have used the initial inoculum size (10^6 conidia/ml) and final inoculum concentration in
269 the wells (10^4 conidia/ml) recommended in the in vitro antifungal susceptibility testing
270 of filamentous fungi (CLSI, 2008*).* Using conidial suspensions adjusted to 0.6 Mc
271 Farland units, good correlation between haemocytometer counting of conidia and
272 colony counts were obtained. So, the adjustment of suspensions using the
273 densitometer Densimat (BioMérieux) can be a good alternative to adjust turbidity of
274 suspensions of *A. niger* and *A. welwitschiae* as we have previously recommended for
275 some *Aspergillus* spp. and *Penicillium* spp. (Abarca et al., 2014; Cabañas et al., 2009).
276 *Aspergillus welwitschiae* and *A. niger* cannot be separated from each other using
277 either morphological or extrolite data and only molecular approaches can be used
278 reliably to distinguish them (Hong et al., 2013; Perrone et al., 2011; Varga et al., 2011).
279 We found the fixed nucleotide differences between them suggested for their
280 identification by Hong et al. (2013) in the calmodulin sequences.
281 Our results show that the effect of water activity, culture media and temperature
282 on growth and ochratoxin A production by *A. niger* and *A. welwitschiae* have been very
283 similar and no significant differences between both species were observed neither in
284 absorbance values nor in OTA concentration. As we have previously reported for *A.*
285 *niger* strains, the range of a_w and temperature conditions for growth was wider than
286 that for OTA production and in some cases, the amounts of OTA detected decreased
287 when increasing incubation time (Esteban et al., 2004; 2006). Some authors suggested
288 that strains could remove and assimilate the phenylalanine moiety from the OTA

289 molecule, as other nitrogen sources of the culture medium become exhausted (Téren et
290 al., 1996).
291 Nevertheless, taking into account that *A. niger* and *A. welwitschiae* have been
292 distinguished only recently, the reported ecophysiological data are probably that of
293 both species. Perrone et al. (2011) reported that *A. niger* and *A. welwistchiae* had the
294 same ranges of growth rates in the culture media and temperatures recommended to
295 identify black aspergilli (Samson et al., 2007), but nothing is known about optimal
296 conditions for OTA production. To our knowledge, this is the first report on the impact
297 of some environmental factors on growth and OTA production by *A. welwitschiae.*
298 In an attempt to find other criteria to distinguish *A. niger* from *A. welwitschiae,* Varga
299 et al. (2011) reported some physiological differences in elastase activities and abilities
300 to utilize 2-deoxy-D-glucose as sole carbon source. In our study we found some
301 differences in the optimum temperature of OTA production: 25ºC for the studied *A.*
302 *niger* strains and 15-25ºC for *A. welwitschiae*, depending on the strains.
303 In view of the importance of these species in mycotoxin contamination of various
304 agricultural products, new studies including correctly identified strains are needed. The
305 method used here is simple, technically easy, and appropriate for ecophysiological
306 studies with a large number of isolates and conditions.
307

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309

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Figure captions

Fig. 1. Phylogenetic tree of *Aspergillus* section *Nigri* inferred from Neighbor-Joining analysis of partial calmodulin gene. Bootstrap values >70% in 1,000 replications are shown at nodes. Sequence of *Aspergillus flavus* CBS 569.65^{T} was selected as outgroup for the tree construction.

Fig. 2. Interval Plot of mean OTA production/values in *A. niger* (An) and *A. welwitschiae* (Aw) strains at each water activity (a_w) and temperature (T) values assayed.

Fig. S1. Alignment of calmodulin sequences of *A. welwitschiae* and *A. niger* genetic types and *A. niger* JX500080 sequence. Identical nucleotides are indicated by dots.

Table 1.

Strains, identification, source, location and calmodulin sequence type.

Strain number[a]	Identification	Source, location	Calmodulin sequence type (GenBank acc. no.)
A-75	*A. niger*	Feedstuffs, Spain	I (MH614646)
A-136	*A. niger*	Soya beans, Spain	I
A-942	*A. welwitschiae*	Raisins, Spain	III (MH614648)
A-943	*A. welwitschiae*	Grapes, Portugal	IV (MH614649)
A-1609	*A. niger*	Grapes, Spain	I
A-1899	*A. welwitschiae*	Grapes, Italy	V(MH614650)
A-1944	*A. welwitschiae*	Grapes, Portugal	IV
A-3204	*A. welwitschiae*	Popcorn kernel, Spain	IV
A-3694	*A. welwitschiae*	Grapes, Spain	IV
A-3919	*A. niger*	Raisins, Iran	II (MH614647)

[a] Culture Collection of the Veterinary Mycology Group, Universitat Autònoma de Barcelona, Spain.

Table 2.

One-way analysis of variance of Absorbance (ABS) and Ochratoxin A (OTA) values *versus (vs.)* each of the variables assayed.

Strains		p value					
		vs. EXP	*vs.* a_w	*vs.* T (ºC)	*vs.* culture media	*vs.* spp.	*vs.* strain
All (n=10)	ABS	0.463	0.000 ($0.99^a > 0.95^b > 0.90^c$)	0.000 ($35º\ ^a > 25º\ ^b > 15º\ ^c$)	0.000 ($YESB^a > SGM^b > WGJ^c$)	0.373	0.974
	OTA	0.422	0.000 ($0.99^a > 0.95^b > 0.90^b$)	0.000 ($25º\ ^a > 15º\ ^{a,b} > 35º\ ^b$)	0.000 ($YESB^a > WGJ^b > SGM^b$)	0.676	0.300
A. niger (n=4)	ABS	0.779	0.04 ($0.99^a > 0.95^{a,b} > 0.90^b$)	0.000 ($35º\ ^a > 25º\ ^b > 15º\ ^c$)	0.000 ($YESB^a > SGM^b > WGJ^c$)	-	0.787
	OTA	0.468	0.018 ($0.99^a > 0.95^b > 0.90^b$)	0.011 ($25º\ ^a > 15º\ ^b > 35º\ ^b$)	0.000 ($YESB^a > WGJ^b > SGM^b$)	-	0.634
A. welwitschiae (n=6)	ABS	0.478	0.000 ($0.99^a > 0.95^b > 0.90^b$)	0.000 ($35º\ ^a > 25º\ ^b > 15º\ ^c$)	0.000 ($YESB^a > SGM^b > WGJ^c$)	-	0.968
	OTA	0.691	0.018 ($0.99^a > 0.95^{a,b} > 0.90^b$)	0.010 ($15º\ ^a > 25º\ ^a > 35º\ ^b$)	0.000 ($YESB^a > WGJ^b > SGM^b$)	-	0.058

^{a,b,c} values of variables with the same superscript are not significantly different ($p>0.05$).

Abbreviations: EXP, experiment; a_w, water activity; T, temperature; spp., species; YESB, Yeast extract sucrose broth; SGM, Synthetic grape juice medium; WGJ, White grape juice.

Table 3.
Mean absorbance values recorded in both experiments by all the studied strains (*A. niger* and *A. welwitschiae*) at each condition and incubation time tested.

Culture media /days	15ºC			25ºC			35ºC		
	0.98-0.99	0.95	0.90	0.98-0.99	0.95	0.90	0.98-0.99	0.95	0.90
YESB / 1	0.0080[a]	0.0068[a]	0.0113[a]	0.0276[a]	0.0023[a]	0.0044[a]	0.0545[a]	0.0298[a]	0.0111[a]
/ 2	0.0081[a]	0.0057[a]	0.0041[b]	0.3862[b]	0.0242[a]	0.0066[a]	1.6657[b]	1.1940[b]	0.0289[a]
/ 4	0.0662[b]	0.0089[a]	0.0040[b]	1.7813[c]	1.4915[b]	0.0310[b]	2.1753[c]	2.0634[c]	1.3995[b]
/ 10	1.7138[c]	0.3164[b]	0.0054[b]	2.4674[d]	2.3731[c]	1.7550[c]	2.8329[d]	2.6408[d]	2.3439[c]
SGM / 1	0.0003[a]	0.0006[a]	0.0228[a]	0.0057[a]	0.0023[a]	0.0134[a]	0.0445[a]	0.0258[a]	0.0155[a]
/ 2	0.0013[a]	0.0010[a]	0.0103[b]	0.0413[a]	0.0177[a]	0.0111[a]	0.6125[b]	0.2068[b]	0.0251[a]
/ 4	0.0280[a]	0.0023[a]	0.0110[b]	0.8444[b]	0.3203[b]	0.0217[a]	1.1583[c]	0.5651[c]	0.2336[b]
/ 10	0.6844[b]	0.2215[b]	0.0084[b]	1.2521[c]	0.7638[c]	0.3105[b]	1.3923[d]	0.7060[d]	0.5238[c]
WGJ / 1	- 0.0007[a]	- 0.0005[a]	0.0074[a]	0.0358[a]	0.0060[a]	0.0051[a]	0.1386[a]	0.0494[a]	0.0202[a]
/ 2	0.0093[b]	- 0.0005[a]	0.0046[a]	0.1937[b]	0.0492[b]	0.0101[a]	0.4262[b]	0.3041[b]	0.0713[b]
/ 4	0.1422[c]	0.0252[b]	-0.0051[b]	0.3291[c]	0.2175[c]	0.0394[b]	0.5508[c]	0.3793[c]	0.1876[c]
/ 10	0.3365[d]	0.2447[c]	0.0191[c]	0.4302[d]	0.3152[d]	0.1472[c]	0.6890[d]	0.5209[d]	0.3166[d]

[a,b,c,d] In columns, values with the same superscript within each culture medium are not significantly different (P>0.05).

Table 4.

OTA concentration in µg/ml produced by *A. niger* strains at each condition assayed and incubation time.

ref. strain	days	15ºC		25ºC						35ºC
		0.98-0.99		0.98-0.99			0.95			0.98
		YESB	WGJ	YESB	SGM	WGJ	YESB	SGM	WGJ	YESB
A-75	2	- [a]	-	-	-	0.055	-	-	-	0.16
	4	-	-	4.68	0.057	0.05	0.18	-	0.052	0.14
	10	0.73	0.052	2.59	-	0.068	0.14	-	0.052	-
A-136	2	-	-	-	-	0,1	-	-	-	0.12
	4	-	-	8.83	0.052	0.094	0.15	0,095	0.055	0.091
	10	0.055	0.055	0.82	-	0.11	0.12	-	0.055	-
A-1609	2	-	-	-	-	0.06	-	-	-	0.14
	4	-	-	10.1	-	0.06	0.2	-	0.062	-
	10	0.15	0.052	1.15	-	0.068	0.075	-	0.055	-
A-3919	2	-	-	-	-	-	-	-	-	-
	4	-	-	-	1.65	-	-	-	-	-
	10	0.16	-	0.16	0.61	-	-	-	-	-

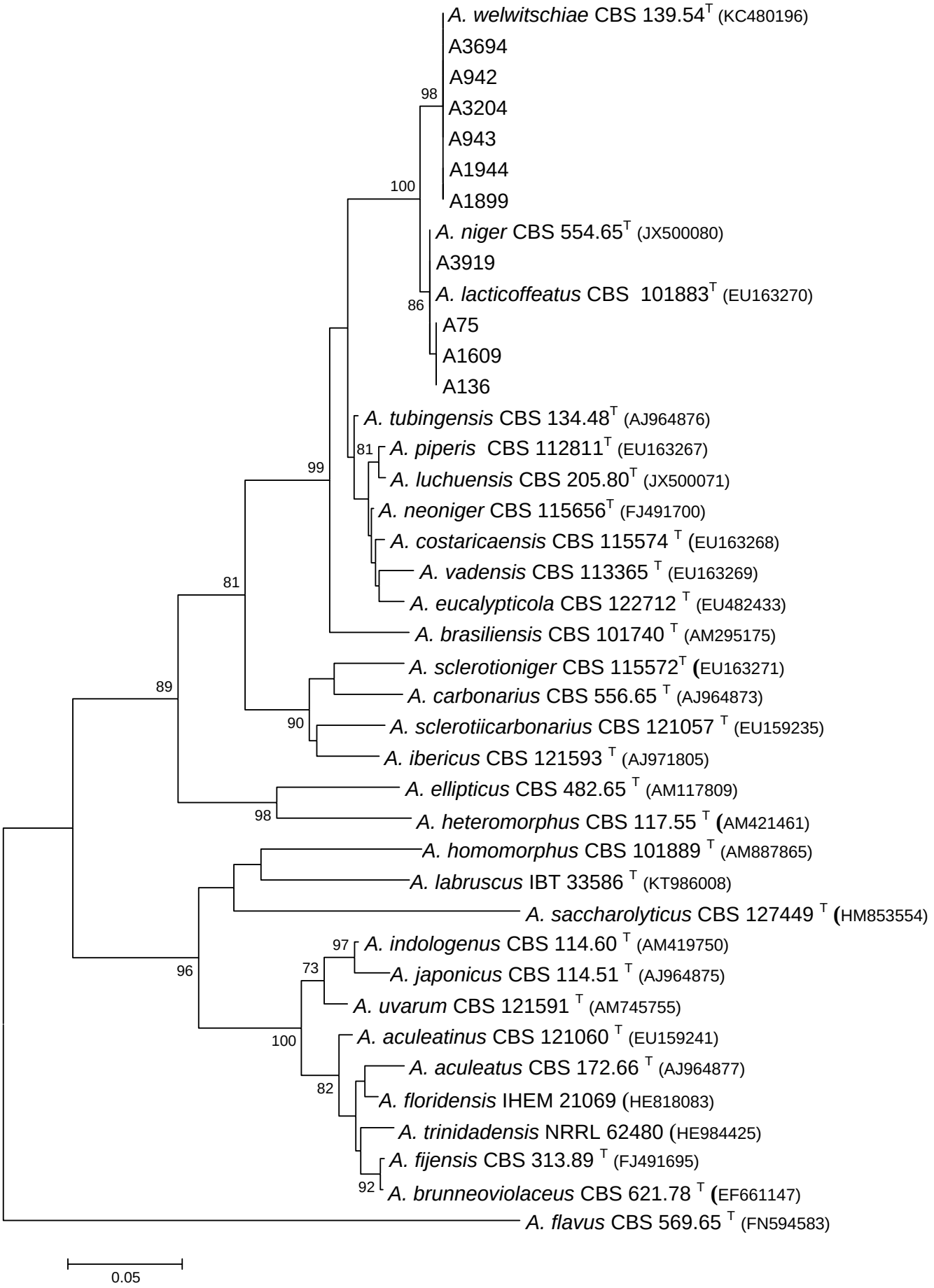
[a] -, denotes not detected

Table 5.

OTA concentration in µg/ml produced by *A. welwitschiae* strains at each condition assayed and incubation time.

ref. strain	days	15ºC					25ºC						35ºC
		0.98-0.99			0.95		0.98-0.99			0.95			0.98
		YESB	SGM	WGJ	SGM	WGJ	YESB	SGM	WGJ	YESB	SGM	WGJ	YESB
A-942	2	- [a]	-	-	-	-	-	-	0.11	-	-	0.052	-
	4	-	-	-	-	-	3.37	0.067	0.10	0.21	0.20	0.08	0.6
	10	1.21	0.084	0.052	0.062	0.062	5.94	0.052	0.075	2.20	0.070	0.063	-
A-943	2	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	0.19	-	0.052	-	-	-	-
	10	0.067	0.060	0.052	-	-	0.17	-	0.057	-	-	-	-
A-1899	2	-	-	-	-	-	-	-	0.062	-	-	-	-
	4	-	-	-	-	-	1.05	0.11	0.06	0.12	-	0.052	-
	10	5.76	0.12	0.06	-	-	0.68	-	0.06	1.47	-	0.052	-
A-1944	2	-	-	-	-	-	-	-	0.06	-	-	-	-
	4	-	-	-	-	-	2.28	0.078	0.06	0.44	-	0.055	-
	10	7.30	0.08	0.08	0.055	0.052	0.88	-	0.055	1.08	-	-	-
A-3204	2	-	-	-	-	-	-	-	0.055	-	-	-	-
	4	-	-	-	-	-	1.61	0.080	0.06	0.10	-	-	-
	10	0.37	0.07	0.055	-	-	0.25	-	0.052	0.12	-	-	-
A-3694	2	-	-	-	-	-	-	-	0.057	-	-	-	0.08
	4	-	-	-	-	-	1.62	-	0.074	0.072	-	-	-
	10	0.25	-	0.055	-	-	0.51	-	0.055	0.065	-	-	-

[a] -, denotes not detected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A3204
A943
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A1899
A. niger CBS 554.65T (JX500080)
A3919
A. lacticoffeatus CBS 101883T (EU163270)
A75
A1609
A136
A. tubingensis CBS 134.48T (AJ964876)
A. piperis CBS 112811T (EU163267)
A. luchuensis CBS 205.80T (JX500071)
A. neoniger CBS 115656T (FJ491700)
A. costaricaensis CBS 115574 T (EU163268)
A. vadensis CBS 113365 T (EU163269)
A. eucalypticola CBS 122712 T (EU482433)
A. brasiliensis CBS 101740 T (AM295175)
A. sclerotioniger CBS 115572T (EU163271)
A. carbonarius CBS 556.65 T (AJ964873)
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A. ibericus CBS 121593 T (AJ971805)
A. ellipticus CBS 482.65 T (AM117809)
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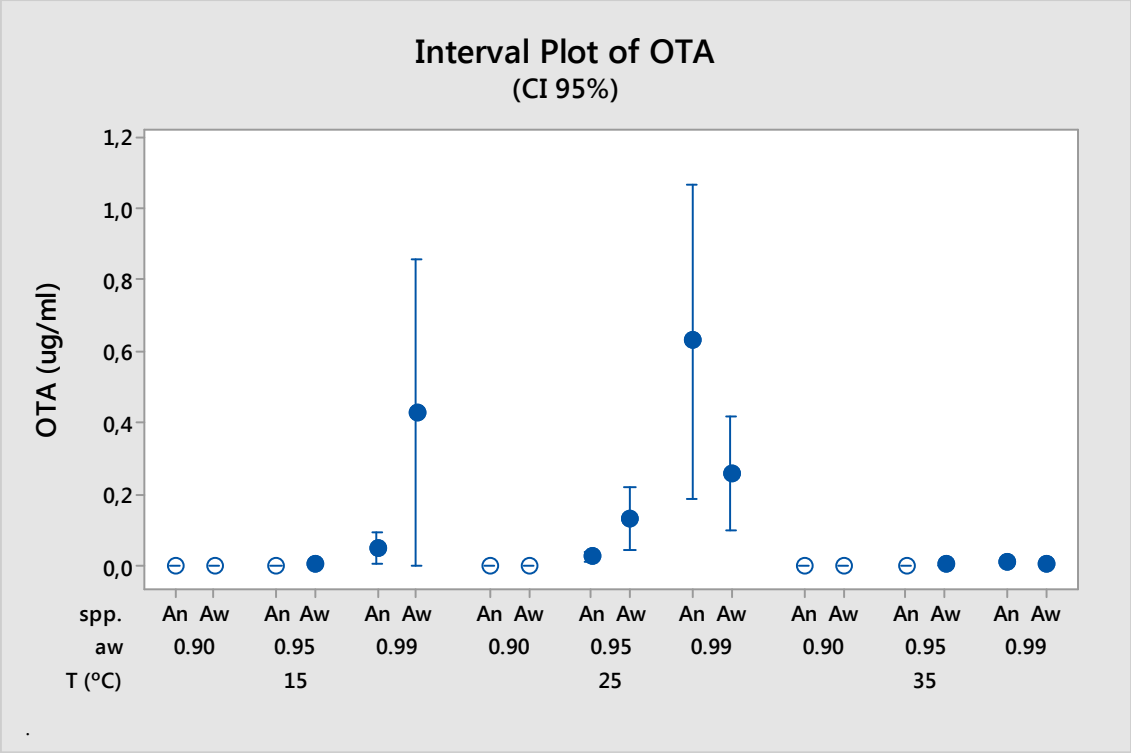
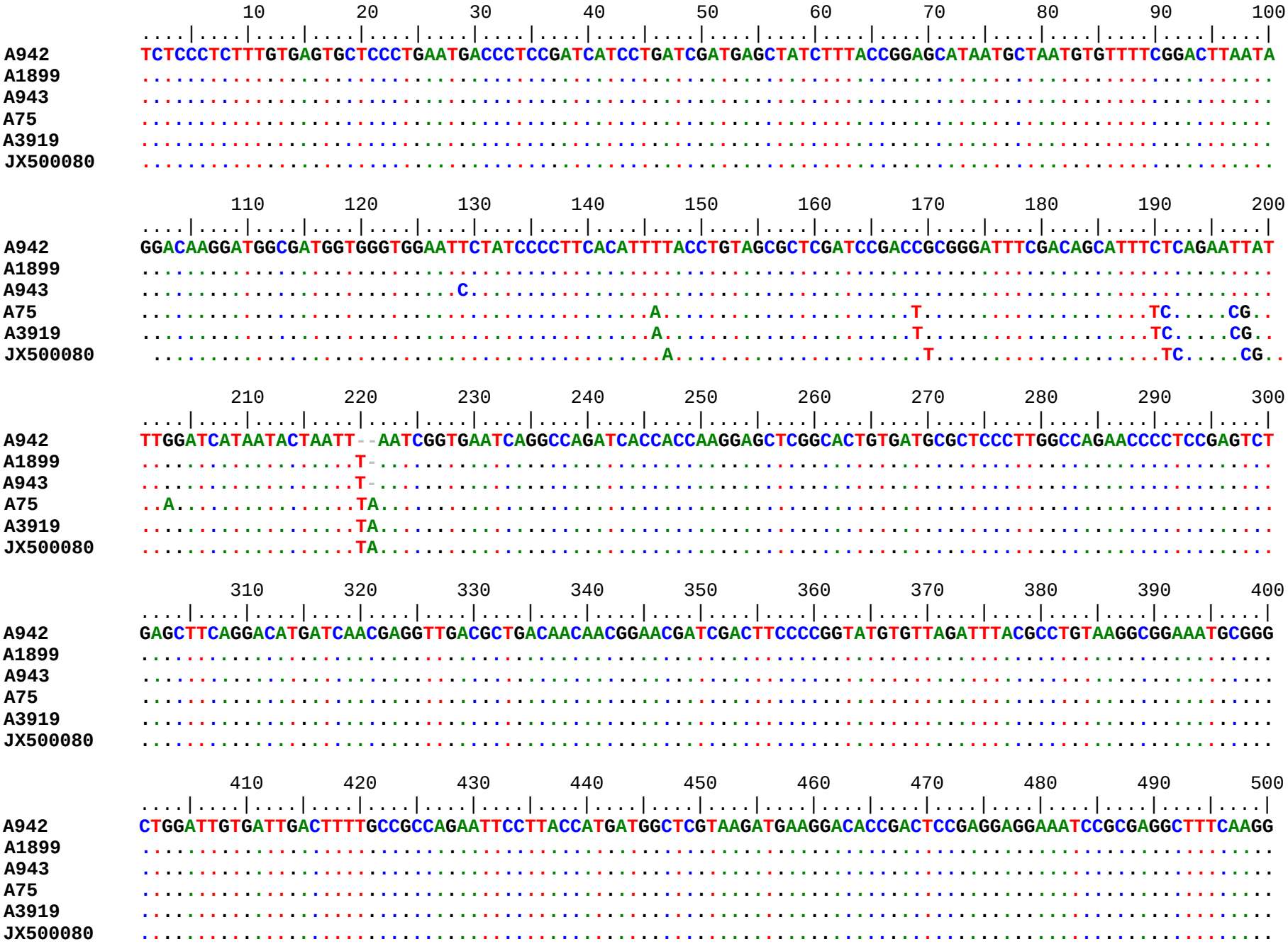
A. homomorphus CBS 101889 T (AM887865)
A. labruscus IBT 33586 T (KT986008)
A. saccharolyticus CBS 127449 T (HM853554)
A. indologenus CBS 114.60 T (AM419750)
A. japonicus CBS 114.51 T (AJ964875)
A. uvarum CBS 121591 T (AM745755)
A. aculeatinus CBS 121060 T (EU159241)
A. aculeatus CBS 172.66 T (AJ964877)
A. floridensis IHEM 21069 (HE818083)
A. trinidadensis NRRL 62480 (HE984425)
A. fijensis CBS 313.89 T (FJ491695)
A. brunneoviolaceus CBS 621.78 T (EF661147)
A. flavus CBS 569.65 T (FN594583)
98
100
86
99
81
81
89
90
98
97
73
96
100
82
92
0.05

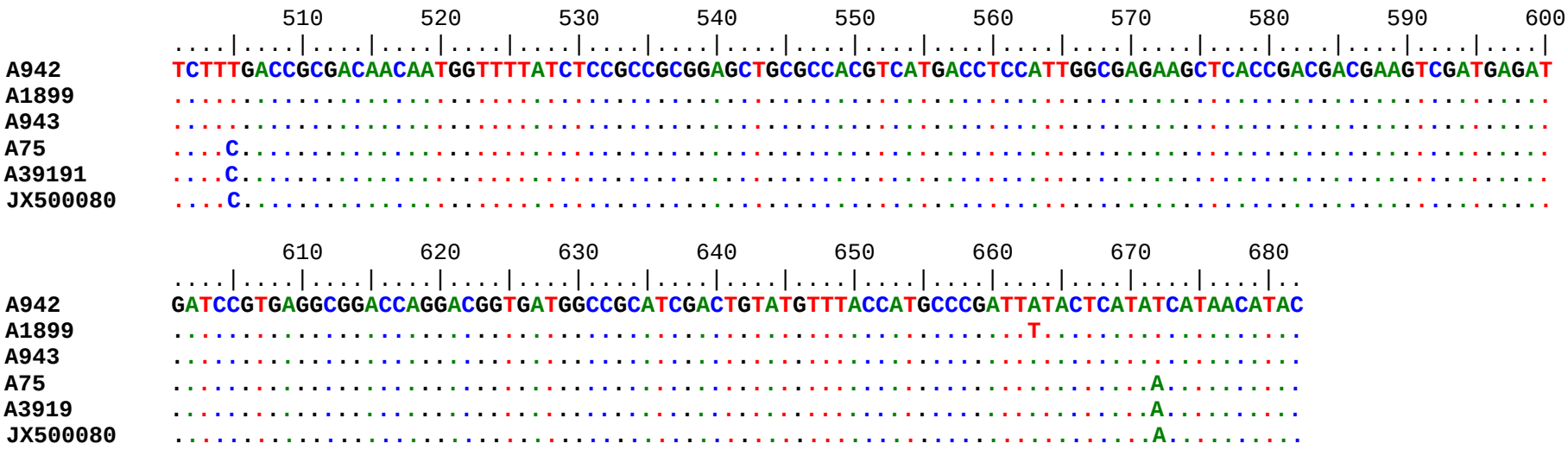
Fig. 2. Interval Plot of mean OTA values in *A. niger* (An) and *A. welwistchiae* (Aw) strains at each water activity (a_w) and temperature (T) values assayed.



ɵ, denotes OTA not detected.

Fig. S1. Alignment of calmodulin sequences of *A. welwitschiae* and *A. niger* genetic types and *A. niger* JX500080 sequence. Identical nucleotides are indicated by dots.

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A1899 .....
A943 .....
A75 ....C.....
A39191 ....C.....
JX500080 ....C.....
610 620 630 640 650 660 670 680
A942 GATCCGTGAGGCGGACCAGGACGGTGATGGCCGCATCGACTGTATGTTTACCATGCCCGATTATACTCATATCATAACATAC
A1899 .....T.....
A943 .....
A75 .....A.....
A3919 .....A.....
JX500080 .....A.....