Intraspecific variability of growth and ochratoxin A production by *Aspergillus carbonarius* from different foods and geographical areas

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Abstract

Ochratoxin A (OTA) is a nephrotoxic mycotoxin naturally found in a wide range of food commodities throughout the world. *Aspergillus carbonarius* is the most important source of OTA in food commodities such as wine, grapes and dried vine fruits and is also responsible for the formation of OTA in coffee.

The aim of this study was to determine the simultaneous effect of three culture media (Czapek Yeast Extract Broth (CYB); Synthetic Grape Juice Medium (SGM) and White grape juice (WGJ)) at three water activity (a_w) levels (0.90; 0.95 and 0.98-0.99), and three incubation temperatures (15ºC, 25ºC and 35ºC) on the growth and OTA production by 16 strains of *A. carbonarius.* The strains were selected on the basis of the geographical origin of the substrate and included strains from different climatic zones of Spain as well as from other countries with different climatology. All the strains were confirmed for identity by sequencing of the calmodulin gene. The assay was performed in microtiter plates, determining the absorbance at 530 nm and the concentration of OTA after 1, 2, 4 and 10 days of incubation.

No significant differences were observed in absorbance values between the strains. The highest absorbance values were recorded in CYB at 0.99 a_w and at 0.95 a_w after 10 days of incubation at 25ºC and 35ºC. None of the strains were able to grow at 0.90 a_w and 15ºC in any culture media after 10 days of incubation. OTA concentration was statistically higher at 15ºC than at 25ºC or 35ºC. The highest significant OTA values were obtained at 0.98-0.99 a_w and the best culture media for OTA production was CYB, followed by WGJ and SGM. While strains isolated from Mediterranean climate foods had a similar behavior despite being isolated from different geographical areas, OTA

concentration produced by one Robusta coffee strain from Thailand was statistically higher at 25ºC than at 15ºC. This would suggest that the type of food matrices and consequently the adaptation of *A. carbonarius* strains to different climatic conditions would have a greater influence on the ecophysiology of the strains than only their geographical origin.

Keywords: Aspergillus carbonarius, coffee, ecophysiology, grapes, raisins, ochratoxin A

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several fungal species of the genera *Penicillium* and *Aspergillus*. 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). OTA contamination of food commodities, including cereals and cereal products, pulses, coffee, beer, grape juice, dry vine fruits and wine as well as cacao products, nuts and spices, has been reported from all over the world (EFSA 2006). In the European diet, wine was identified as the second contributor to the human OTA exposure after cereals. Dried vine fruit and grape juice contributed to a significant extent to the OTA-exposure for specific groups of vulnerable groups of consumers such as children. Coffee represented the third contributor to the mean European total dietary intake of OTA (European Commission, 2002). As a consequence of the possible health hazards related to ingestion of OTA, the European Union introduced maximum limits for OTA in a wide range of foodstuffs (Commission of the European Communities 2006; 2010; 2012; 2015).

A large number of studies have shown that *Aspergillus carbonarius* is the main responsible source of OTA in wine or dried vine fruits from main vinicultural regions worldwide (Cabañes and Bragulat 2018; Visconti et al.,2008). According to recent surveys, *A. carbonarius* is also the most important ochratoxin producer in Robusta coffee beans (Noonim et al., 2008).

Aspergillus carbonarius is very consistent in producing this mycotoxin, and non OTA-producing strains in this species are very rare (Cabañes et al., 2013).

72 Contamination of grapes by this species can occur since the beginning of maturation
73 stage but becomes more prominent near harvest time (Bau et al., 2005; Visconti et al.,
74 2008). With respect to coffee, OTA contamination can be regarded as a post-harvest
75 problem. The use of dry processing method, surface contact with dust and soil increase
76 the chance of *A. carbonarius* contamination (Noonim et al., 2008).
77 The knowledge of the influence of environmental parameters on OTA production
78 may contribute to prevention of OTA contamination in food commodities. In the last
79 years many studies have been published on the effect of some environmental factors
80 on *A. carbonarius* growth and in a lesser extent on its OTA production ability, as
81 reviewed by several authors (Amézqueta et al., 2012; Battilani and Camardo, 2015;
82 Magan et al., 2011). These kind of ecophysiological studies are routinely performed in
83 Petri dishes and the number of isolates included is usually low because they are
84 laborious and time-consuming methods.
85 In order to know the existence of intraspecific variability on growth and OTA
86 production, several strains have to be included in ecophysiological studies. Garcia et al.
87 (2011) assessed the impact of suboptimal environmental conditions on thirty isolates
88 of *A. carbonarius*. Although they included a large number of isolates, only one culture
89 medium and three water activity/temperature conditions (0.98 aw/25ºC; 0.90
90 aw/25ºC; 0.98 aw/37ºC) were tested. Using above data, García et al. (2012)
91 mathematically assessed the minimum number of isolates that would lead to
92 equivalent growth parameters estimates to those obtained with a high number of
93 strains. They concluded that 12-17 isolates of *A. carbonarius* led to the same growth
94 parameters as the total 30.

In this study we have adapted a previously described method using microtiter plates (Abarca et al., 2014; 2019) to determine the simultaneous effect of three culture media at three water activity levels and three incubation temperatures on the growth and OTA production by 16 strains of *A. carbonarius.*

2. Materials and Methods

2.1. Strains and molecular identification

Sixteen *A. carbonarius* strains, mainly isolated from grapes and raisins were studied (Table 1). All the strains were previously detected as OTA-producers in our laboratory and had been morphologically identified as *A. carbonarius*. The strains were selected on the basis of the geographical origin of the substrate and included strains from different climatic zones of Spain as well as from other countries with different climatology.

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

118 software (Tamura et al., 2013). The Neighbor-Joining method based on the Tamura-
119 Nei model (Tamura and Nei 1993) with 1,000 bootstrap replicates was used.
120
121 *2.2. Inoculum preparation and verification*
122
123 Spore concentration was adjusted to around 10^6 conidia/ml. Briefly, the inoculum
124 suspensions were prepared in sterile saline (0.85%) containing 0.05% Tween 80 from
125 7-day-old cultures on malt extract agar at 25ºC. After heavy particles were allowed to
126 settle for 10-15 minutes, the upper homogenous suspensions were transferred to
127 sterile tubes and adjusted to 0.8 McFarland turbidity standard (Abarca et al., 2014) by
128 using a photometric method (Densimat, BioMérieux). The inoculum size was confirmed
129 by haemocytometer counting and quantitative colony counts.
130
131 *2.3. Culture media and microtiter inoculation*
132
133 Sterile 96-well flat-bottom microtiter plates were used. Three liquid culture media
134 were assayed: Czapek Yeast Extract broth (CYB), used as a control, synthetic grape
135 juice medium (SGM) representative of grape composition at mid-veraison (Mitchell et
136 al., 2004), and white grape juice (WGJ).
137 CYB contained per liter: K_2HPO_4, 1 g; Czapek concentrate, 10 ml; trace metal
138 solution, 1 ml; yeast extract, 5 g; sucrose, 30 g; pH adjusted to 6.3 ± 0.2 (Pitt and
139 Hocking, 2009). SGM contained per liter: D(+) glucose, 70g; D(-) fructose, 30g; L(-)
140 tartaric acid, 7g; L(-) malic acid, 10g; $(NH_4)_2HPO_4$, 0.67g; KH_2PO_4, 0.67g; $MgSO_4 \cdot 7\ H_2O$,
141 1.5g; NaCl, 0.15g; $CaCl_2$, 0.15g; $CuCl_2$, 0.0015g; $FeSO_4 \cdot 7\ H_2O$, 0.021g; $ZnSO_4 \cdot 7\ H_2O$,

142 0.0075g; (+) Catechin hydrate, 0.05g; pH adjusted with 10M NaOH to pH 4.0-4.2
143 (Mitchell et al., 2004). WGJ was prepared with 200ml of commercially sold white grape
144 juice made from ecological grapes and 800 ml of distilled water; pH adjusted at 4.0.
145 The initial a_w was 0.99 for CYB and WGJ media, and 0.98 for SGM. These initial
146 values were modified to 0.95 a_w and 0.90 a_w by the addition of different amounts of
147 glycerol. Media were autoclaved and the final a_w values were checked with
148 LabMASTER-a_w (Novasina. Switzerland).
149 For each a_w level the adjusted fungal suspensions were diluted 1:100 in the culture
150 medium assayed (CYB, SGM, WGJ). In each microplate column, five wells were
151 inoculated with 200 µl of the diluted suspension of each strain and one well, used as a
152 blank, was filled with 200 µl of un-inoculated culture media (CYB, SGM ,WGJ).
153 Growth assessment and OTA production at each a_w level were determined after 1,
154 2, 4, and 10 days of incubation at three different temperatures (15, 25, and 35ºC).
155 Thus, each strain-$a_w$level-temperature combination was repeated in 4 microplates, one
156 for each sampling day.
157 For each sampling occasion and temperature assayed, microtiter plates with the
158 same water activity level were enclosed in sealed polyethylene bags. The entire
159 experiment was repeated twice on different days.
160
161 *2.4. Growth measurement and OTA extraction procedure*
162
163 For each culture media, a_w level and temperature assayed, growth was monitored
164 by absorbance measurements at 530nm using the Multilabel-Reader Mithras LB 940
165 (Berthold Technologies, Bad Wildbad, Germany) after 1, 2, 4 and 10 days of

incubation. The absorbance of the corresponding uninoculated medium, used as blank was subtracted to the absorbance values of the inoculated media. After each reading, microplates were sealed and stored at -80ºC until they were analyzed for OTA content.

OTA production was detected using a previously described high-pressure liquid chromatography (HPLC) screening method developed in our laboratory for fungi growing in microtiter wells (Abarca et al., 2014). On each sampling occasion, one of the five replicate wells inoculated for each strain, culture media, a_w level and incubation temperature, were randomly selected and their content was removed and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC. The limit of quantification was 0.045 µg/ml for this mycotoxin.

2.5. Statistical Analysis

Data obtained from the different conditions tested were statistically analyzed by means of one-way analysis of variance test. 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, all the strains were identified as *A. carbonarius*. The phylogenetic tree was reconstructed showing that the isolates

190 grouped with *A. carbonarius* CBS 556.65^{T} (Fig. 1). Sequence analysis revealed that all
191 the strains had the same sequence. The nucleotide sequence of one representative
192 strain (A-1002) has been deposited in GenBank under accession number MK778845.
193
194 *3.2. Inoculum standardization*
195
196 Inocula adjusted to 0.8 McFarland turbidity standard provided suspensions of 1.1 ±
197 0.1 x 10^{6} conidia/ml by microscopic enumeration with a cell-counting
198 haemocytometer. Mean colony counts of the above *A. carbonarius* suspensions were
199 0.3 ± 0.1 x 10^{6} cfu/ml.
200
201 *3.3. Growth measurement*
202
203 Table 2 shows results of one-way analysis of variance for the effects of the
204 variables assayed (experiment, temperature, water activity, culture media and strain)
205 on absorbance values. No significant differences were observed in absorbance values
206 neither between the experiments, nor between the strains ($p > 0.05$). The remaining
207 variables had a significant effect on growth of all the strains studied. Regardless a_w
208 level or culture medium, the highest absorbance values were recorded at 35ºC,
209 although not statistically different from those obtained at 25ºC. Values recorded at
210 0.98-0.99 a_w and 0.95 a_w were significantly higher than at 0.90 a_w. In relation to culture
211 media, the highest significant absorbance values ($p<0.001$) were recorded in CYB,
212 followed by SGM and WGJ.

213 Mean absorbance values recorded in both experiments by all the studied
214 strains at each condition and incubation time tested are shown in Table 3. The highest
215 absorbance values were recorded after 10 days of incubation at 25ºC and 35ºC in CYB
216 at 0.99 a_w and at 0.95 a_w.
217 In all culture media, initial growth in the microtiter wells could be visually detected
218 at the naked eye, when absorbance value was greater than 0.1. According to this, none
219 of the strains were able to grow at 0.90 a_w and 15ºC, the most extreme conditions
220 tested, in any culture media after 10 days of incubation. At this low temperature, a
221 significant increase in absorbance values was detected after 4-10 days of incubation in
222 the three culture media adjusted at 0.95 and 0.98-0.99 a_w.
223 At 25ºC, absorbance values statistically increased after 2-4 days (0.98-0.99 a_w and
224 0.95 a_w) or 10 days (0.90 a_w) of incubation.
225 At 35ºC a statistically growth increase was observed after 2 days of incubation in
226 the three culture media adjusted at 0.98-0.99 a_w and 0.95 a_w. At 0.90 a_w, this increase
227 was recorded after 2-4 days in SGM and WGJ and after 10 days in CYB.
228
229 *3.4. OTA production*
230
231 No significant differences were found in OTA concentration neither between the
232 experiments, nor between the strains ($p > 0.05$) (Table 2). Temperature, culture media
233 and water activity had significant effect ($p<0.01$) on OTA production. OTA
234 concentration was statistically higher ($p<0.01$) at 15ºC than at 25ºC or 35ºC. The
235 highest significant OTA values were obtained at 0.98-0.99 a_w and the best culture
236 media ($p<0.01$) for OTA production was CYB, followed by WGJ and SGM.

237 Table 4 shows OTA concentration produced by all the strains at each condition
238 assayed and incubation time. Results are expressed as mean value of both experiments
239 as no statistically significant differences were observed ($p>0.05$). As a general trend,
240 OTA production increased over incubation time achieving the highest concentration
241 ($p<0.01$) after 10 days of incubation. None of the strains produced detectable levels of
242 OTA in any culture media at 0.90 a_w and 15ºC. The highest OTA concentration was
243 recorded at 15ºC after 10 days of incubation in CYB at 0.99 a_w. It is worth to highlight
244 that in those conditions of temperature and a_w, all the strains produced detectable
245 levels of OTA in the three culture media studied.
246 At 25ºC and 0.98-0.99 a_w , all the strains produced OTA after only 2 days (CYB,
247 WGJ) or 4 days (SGM) of incubation. As water activity decreases, optimum
248 temperature for OTA production moved to 25ºC, although not all the studied strains
249 were able to produce the mycotoxin.
250 At 35ºC, and 0.98-0.99 a_w some strains produced low levels of OTA after only 1 or
251 2 days of incubation. By decreasing the water activity, decreases the number of OTA-
252 producing strains. At 0.90 a_w, still some strains could produce detectable levels of OTA
253 after 10 days of incubation.
254 Although taking into account all the results no intraspecific differences were
255 observed, the strain A-884 isolated from Robusta coffee cherries from Thailand,
256 showed a different behavior to the remaining strains, mostly isolated from grapes.
257 Thus, OTA concentration produced by this strain was statistically higher ($p<0.05$) at
258 25ºC than at 15ºC or 35ºC. Fig. 2a shows OTA values recorded in CYB medium by this
259 coffee strain (A-884) in comparison to a high OTA- producing grape strain (A-1002) and
260 a low OTA-producing grape strain (A-2128), both isolated from Spain, in each condition

assayed. The monthly maximum and minimum average temperatures in Mediterranean areas and in Thailand are also shown (Fig. 2b).

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). The large number of isolates and conditions studied here will allow a more comprehensive overview of the ecophysiology of *A. carbonarius*, difficult when using laborious methods based on Petri dishes.

Inoculum size is one of the technical variables that can influence the spectrophotometric tests outcome. Conidial suspensions adjusted to 0.8 McFarland yielded in all cases haemocytometer counting around 10^6 conidia/ml. The use of the portable photometer Densimat (BioMérieux) provides a substantial reduction in time as we have previously reported (Abarca et al., 2014; 2019; Cabañas et al., 2009). As no statistically significant differences were observed in absorbance and OTA values between the experiments, our method has proven to be an easy way to monitor growth and OTA production of *A. carbonarius* in ecophysiological studies.

Results obtained in this study confirm that *A. carbonarius* strains can grow at a wide range of temperatures (15-35ºC) and water activities (0.90-0.99). The optimum conditions of a_w and temperature for growth were in the range 25-35ºC and 0.95-0.99 a_w. The highest amount of OTA was obtained at 15ºC and 0.98-0.99 a_w. In previous studies in solid culture media, *A. carbonarius* strains also showed optimal conditions range for OTA production narrower than that for growth when temperature, water activity or pH effects were evaluated (analyzed) (Esteban et al., 2004; 2005; 2006).

285 In agreement with our results, 15ºC or 15-20ºC have been reported as the
286 optimum temperatures for OTA production by *A. carbonarius* strains in different
287 culture media (Esteban et al., 2004; Leong et al., 2006; Marin et al., 2006; Mitchell et
288 al., 2004; Passamani et al., 2014; Romero et al., 2010; Tassou et al., 2007).
289 Although all the strains were able to produce OTA in the three culture media
290 studied, the highest OTA concentration was achieved in CYB (final pH 6.3 ± 0.2). Czapek
291 yeast extract agar (CYA) has been reported as a suitable medium to detect OTA-
292 producing ability of *A. carbonarius* strains (Abarca et al., 2014; Bragulat et al., 2001;
293 Esteban et al., 2004). In SGM and WGJ media, with a final pH of about 4, a decrease in
294 both growth and OTA production was obtained. The acidity of the growth medium
295 could exert some influence on the growth and OTA production by *A. carbonarius.* In
296 previous studies, *A. carbonarius* isolates were able to produce OTA at a wide range of
297 pH values (2 to 10) on CYA medium. When CYA plates were incubated at 15ºC, the
298 highest OTA levels were obtained generally at a higher pH range (5 to 7) (Esteban et
299 al., 2005). In a similar way, the greatest OTA concentration was reported in
300 semisynthetic grape culture medium at 15ºC, with pH above 6.0 and a_w of 0.99
301 (Passamani et al., 2014). In other studies, low pH level seemed optimal for maximum
302 OTA production (Spadaro et al., 2010), while Kapetanakou et al. (2009) reported that
303 pH (3.9-6.8) had no particular effect on OTA production by *A. carbonarius.*
304 In accordance with our results some studies have shown that *A. carbonarius*
305 strains isolated from grapes from different geographical origins have a common
306 pattern in growth and OTA production under variable conditions of temperature and
307 a_w. Strains of *A. carbonarius* isolated from Tunisian grapes behave as those from
308 Spanish grapes (Marin et al., 2006) and as those from Australian and European grapes

309 as reported in the literature (Lasram et al., 2010). Leong et al. (2006) reported that
310 trends for growth and OTA production were similar among Australian isolates and
311 those from European grapes. Using a cocktail inocula of strains isolated from
312 Argentinean dried vine fruits, similar results were obtained to those reported for single
313 *A. carbonarius* strains from European countries, Israel, Australia and South America
314 (Romero et al., 2010). Therefore, strains isolated from grapes seem to have a similar
315 behavior despite being isolated from different geographical areas.
316 In our study, OTA concentration produced by the strain A-884 isolated from
317 Robusta coffee cherries from Thailand was statistically higher at 25ºC than at 15ºC or
318 35ºC. Most, if not all, current ecophysiological studies have been carried out with *A.*
319 *carbonarius* strains isolated from grapes and derived products. Up to date, there are
320 few data available regarding the effect of environmental factors on *A. carbonarius*
321 strains isolated from coffee. In a previous study, including strains from different
322 substrates, *A. carbonarius* CBS 127.49 isolated from a seed of coffee Arabica produced
323 more OTA at 20ºC than at 15ºC (Esteban et al., 2004). Kouadio et al. (2007) reported
324 maximum OTA production at 0.99 a_w at a temperature range of 15-30ºC in one strain
325 of *A. carbonarius* isolated from Robusta coffee beans. On irradiated coffee cherries,
326 optimal conditions for OTA production was also reported at 25ºC and 0.99 a_w (Joosten
327 et al., 2001). In the same way, on a green coffee-based medium the highest OTA
328 production was obtained at 0.99a_w and 25ºC by the *A. carbonarius* strain studied,
329 isolated from Arabica coffee beans (Akbar, 2015).
330 The strain A-884 was isolated from the South of Thailand (Joosten et al., 2001). In
331 this tropical area the climate is hot all year round, with maximum and minimum
332 temperature ranges of 35-40ºC and 17-27ºC respectively (Fig. 2b). The remaining *A.*

carbonarius strains studied were isolated from areas with Mediterranean climate, with relatively mild winters and warm summers but reaching minimum temperatures of 4-18ºC, lower than those observed in tropical areas (Fig. 2b). This would explain that 15ºC be the optimum temperature for these strains, as OTA can be continuously produced in the field, in spite of there are large temperature variations between day and night.

Although very few coffee strains have been studied so far, this different pattern in relation to optimal temperature for OTA production could suggest that the type of food matrices and consequently the adaptation of *A. carbonarius* strains to different climatic conditions would have a greater influence on the ecophysiology of the strains than only their geographical origin. Further studies are needed including a great number of coffee isolates to corroborate it.

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497 **Figure captions**

498

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.

499

500 **Fig. 2.** OTA production of several *A. carbonarius* assayed strains: A-884 (Robusta coffee
501 cherries, Thailand), A-1002 (grapes, Spain) and A-2128 (grapes, Spain) at each
502 condition assayed (a) and monthly maximum and minimum average temperatures in
503 Mediterranean areas and in Thailand (b).

Table 1.

List of *A. carbonarius*[a] strains studied and their source and location.

Strain number [b]	Source, location
A-882	tomatoes, Morocco
A-884	coffee cherries, Thailand
A-1002	grapes, Spain
A-1004	grapes, Spain
A-1021	grapes, Spain
A-1625	grapes, Italy
A-1653	grapes, France
A-1687	grapes, Portugal
A-1749	grapes, Israel
A-1841	grapes, Greece
A-1995	grapes, Australia
A-2128	grapes, Spain
A-2275	grapes, Spain
A-2520	grapes, Spain
A-3615	raisins, Turkey
A-3905	raisins, Iran

[a] Identification confirmed by sequencing of the calmodulin gene.
[b] 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.

	p value				
	vs. EXP	*vs.* T (ºC)	*vs.* a_w	*vs.* culture media	*vs.* strain
ABS	0.078	0.000 (35º [a] > 25º [a] >15º [b])	0.000 (0.99 [a] > 0.95 [a] > 0.90 [b])	0.000 (CYB [a]>SGM [b]>WGJ [c])	0.999
OTA	0.257	0.000 (15º [a] >25º [b] >35º [b])	0.000 (0.99 [a] > 0.95 [b] > 0.90 [b])	0.000 (CYB [a]>WGJ [b]>SGM [b])	0.265

[a,b,c] values of variables with the same superscript are not significantly different ($p > 0.05$).
Abbreviations: EXP, experiment; T, temperature in ºC; a_w , water activity; YESB, Yeast extract sucrose broth; SGM, Synthetic grape juice medium; WGJ, White grape juice.

Table 3.

Mean absorbance (ABS) and standard deviation values in both experiments by all the studied strains of *Aspergillus carbonarius* at each condition and incubation time tested.

culture media	days	ABS ± δ								
		T	15ºC			25ºC			35ºC	
		a_w	0.99-0.98	0.95	0.90	0.99-0.98	0.95	0.90	0.99-0.98	0.95	0.90
CYB	1		0.0050 ± 0.0047 [a]	0.0009 ± 0.0084 [a]	- 0.00005 ± 0.0069 [a]	0.0467 ± 0.0203 [a]	0.0035 ± 0.0054 [a]	0.0051 ± 0.0064 [a]	0.0577 ± 0.0323 [a]	0.0506 ± 0.0357 [a]	0.0185 ± 0.0280 [a]
	2		0.0106 ± 0.0075 [a]	0.0010 ± 0.0121 [a]	0.0016 ± 0.0056 [b]	0.7721 ± 0.3356 [b]	0.0455 ± 0.0181 [b]	0.0034 ± 0.0060 [a]	1.0636 ± 0.7917 [b]	0.372 ± 0.2816 [b]	0.0384 ± 0.0400 [a]
	4		0.1478 ± 0.0597 [b]	0.0104 ± 0.0115 [a]	0.0036 ± 0.0034 [c]	2.8224 ± 0.1081 [c]	1.9008 ± 0.3453 [c]	0.0368 ± 0.0132 [a]	2.5320 ± 0.4117 [c]	2.5535 ± 0.3375 [c]	0.0842 ± 0.0673 [a]
	10		2.1620 ± 0.336 [c]	0.8526 ± 0.5574 [b]	0.0045 ± 0.0051 [c]	2.9535 ± 0.1252 [d]	2.9999 ± 0.0543 [d]	2.7043 ± 0.3234 [b]	2.7725 ± 0.4672 [d]	3.0476 ± 0.0878 [d]	1.5364 ± 0.4573 [b]
SGM	1		- 0.0048 ± 0.0064 [a]	0.0017 ± 0.0093 [a]	0.0071 ± 0.0108 [a]	0.0028 ± 0.0175 [a]	0.0013 ± 0.0090 [a]	0.0143 ± 0.0140 [a]	0.0200 ± 0.0198 [a]	0.0080 ± 0.0109 [a]	0.0243 ± 0.0160 [a]
	2		- 0.0035 ± 0.0081 [a]	0.0013 ± 0.0082 [a]	0.0069 ± 0.0105 [a]	0.0451 ± 0.0287 [a]	0.0183 ± 0.0120 [a]	0.0388 ± 0.0298 [a]	0.2812 ± 0.2362 [b]	0.1553 ± 0.1297 [b]	0.2409 ± 0.2440 [b]
	4		0.0349 ± 0.0186 [b]	0.0070 ± 0.0075 [a]	0.0124 ± 0.0092 [b]	0.5936 ± 0.3639 [b]	0.1290 ± 0.1043 [b]	0.0304 ± 0.0104 [a]	0.9074 ± 0.5209 [c]	0.7608 ± 0.5171 [c]	0.1571 ± 0.1582 [c]
	10		0.7549 ± 0.3014 [c]	0.2533 ± 0.1680 [b]	0.0105 ± 0.0084 [b]	1.0018 ± 0.4218 [c]	0.5128 ± 0.4123 [c]	0.2198 ± 0.0264 [b]	1.3746 ± 0.6066 [d]	1.1593 ± 0.5971 [d]	0.5938 ± 0.4964 [d]
WGJ	1		0.0022 ± 0.0065 [a]	0.0023 ± 0.0058 [a]	0.0064 ± 0.0057 [a]	0.0491 ± 0.0150 [a]	0.0118 ± 0.0076 [a]	0.0088 ± 0.0090 [a]	0.0803 ± 0.0365 [a]	0.0687 ± 0.0362 [a]	0.0109 ± 0.0073 [a]
	2		0.0191 ± 0.0110 [b]	0.0077 ± 0.0081 [a]	0.0054 ± 0.0053 [a]	0.1948 ± 0.0485 [b]	0.0933 ± 0.0313 [b]	0.0151 ± 0.0056 [a]	0.1591 ± 0.0710 [b]	0.3237 ± 0.1053 [b]	0.0511 ± 0.0251 [b]
	4		0.1242 ± 0.0279 [c]	0.0573 ± 0.0158 [b]	0.0065 ± 0.0067 [a]	0.3319 ± 0.0885 [c]	0.2099 ± 0.0661 [c]	0.0730 ± 0.0259 [b]	0.2182 ± 0.1050 [c]	0.5222 ± 0.1616 [c]	0.2142 ± 0.0934 [c]
	10		0.3095 ± 0.0628 [d]	0.1928 ± 0.0404 [c]	0.0265 ± 0.0089 [b]	0.4019 ± 0.1273 [d]	0.2714 ± 0.1036 [d]	0.2189 ± 0.1195 [c]	0.3469 ± 0.1842 [d]	0.6301 ± 0.1572 [d]	0.3845 ± 0.1406 [d]

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

Abbreviations: T, temperature in ºC; a_w , water activity; YESB, Yeast extract sucrose broth; SGM, Synthetic grape juice medium; WGJ, White grape juice.

Table 4.

Mean OTA concentration values (in µg/ml) produced by *Aspergillus carbonarius* strains at each condition assayed and incubation time.

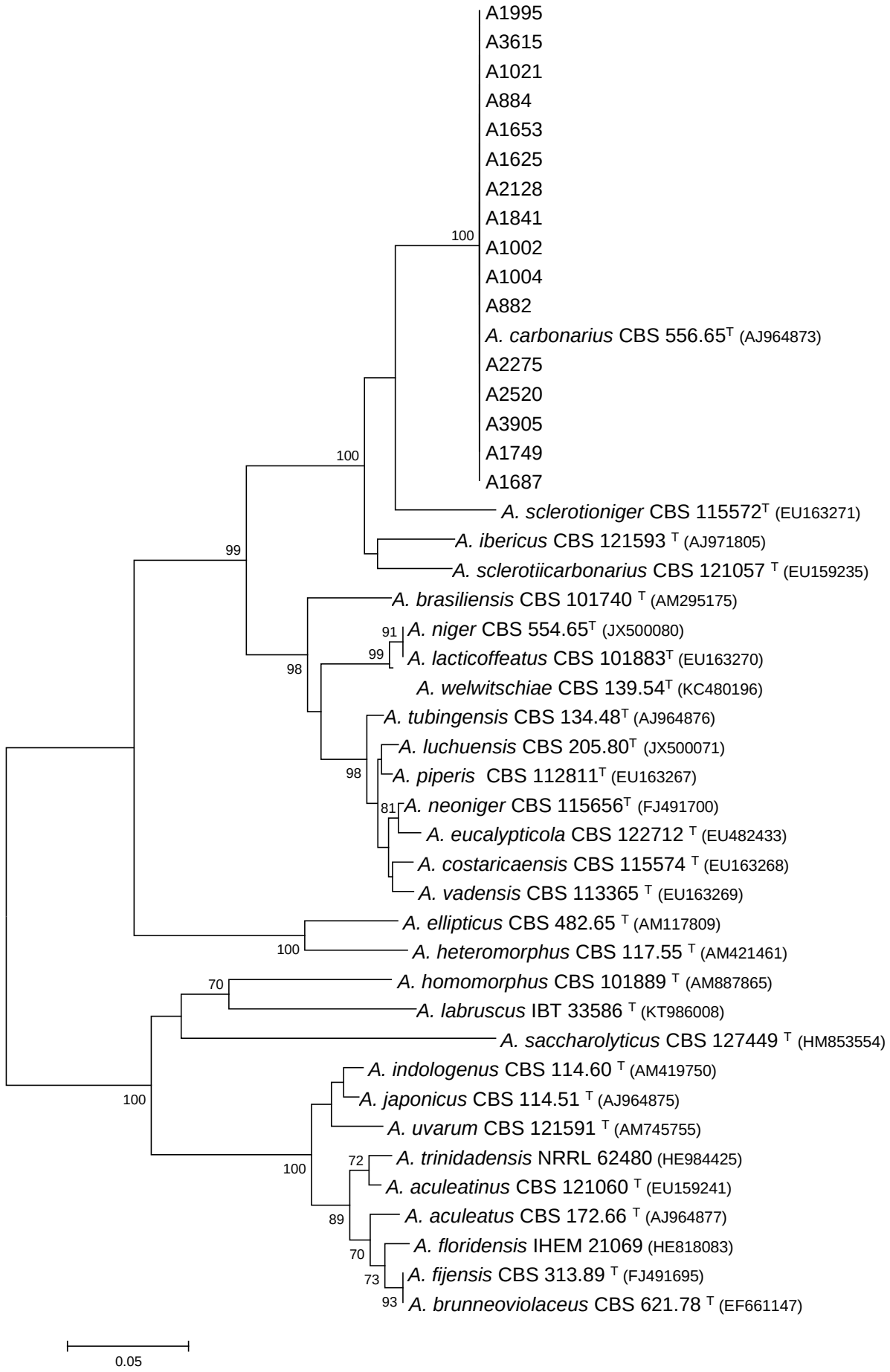
culture media	days	OTA (µg / ml) mean value ± δ (no. of OTA positive strains)								
		T	15ºC			25ºC			35ºC	
		a_w	0.99-0.98	0.95	0.90	0.99-0.98	0.95	0.90	0.99-0.98	0.95	0.90
CYB	1		ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	0.052 ± 0 [a] (1)	ND [a]	ND [a]
	2		ND [a]	ND [a]	ND [a]	0.1566 ± 0.2189 [b] (16)	ND [a]	ND [a]	0.1965 ± 0.2893 [a,b] (8)	ND [a]	ND [a]
	4		ND [a]	ND [a]	ND [a]	0.9742 ± 1.0972 [c] (16)	0.3022 ± 0.5395 [a,b] (12)	ND [a]	0.2828 ± 0.5487 [c] (15)	0.1145 ± 0.0439 [a] (2)	ND [a]
	10		8.4784 ± 7.361 [b] (16)	0.6995 ± 1.30 [b] (14)	ND [a]	0.9958 ± 1.0135 [c] (16)	1.0181 ± 1.6597 [b] (10)	0.3883 ± 0.572 [b] (7)	0.2039 ± 0.2894 [b,c] (16)	0.1083 ± 0.1607 [b] (15)	0.069 ± 0.011 [a] (1)
SGM	1		ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
	2		ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	0.0762 ± 0.0532 [b,c] (7)	ND [a]	ND [a]
	4		ND [a]	ND [a]	ND [a]	0.1783 ± 0.1459 [b] (16)	0.0907 ± 0.069 [a,b] (7)	ND [a]	0.0731 ± 0.0398 [b,c] (10)	0.061 ± 0.0057 [a,b] (3)	ND [a]
	10		0.1329 ± 0.0902 [b] (16)	0.0865 ± 0.0934 [b] (13)	ND [a]	0.1756 ± 0.2119 [b] (16)	0.122 ± 0.1276 [b] (13)	0.0694 ± 0.0304 [a] (4)	0.0775 ± 0.0285 [c] (15)	0.0613 ± 0.0126 [b] (6)	0.0586 ± 0.008 [a] (3)
WGJ	1		ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
	2		ND [a]	ND [a]	ND [a]	0.0919 ± 0.0334 [b] (16)	ND [a]	ND [a]	0.0724 ± 0.0315 [b] (15)	0.0596 ± 0.0069 [a] (4)	ND [a]
	4		ND [a]	ND [a]	ND [a]	0.1175 ± 0.0489 [c] (16)	0.0626 ± 0.0159 [b] (11)	ND [a]	0.0727 ± 0.027 [b] (15)	0.0696 ± 0.0123 [a] (3)	ND [a]
	10		0.098 ± 0.0416 [b] (16)	0.0799 ± 0.0255 [b] (16)	ND [a]	0.0884 ± 0.0334 [b] (16)	0.058 ± 0.0048 [b] (8)	0.0576 ± 0.0037 [a] (2)	0.0735 ± 0.0343 [b] (16)	0.0613 ± 0.0117 [b] (15)	0.0546 ± 0.0008 [b] (4)

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

ND, denotes not detected.

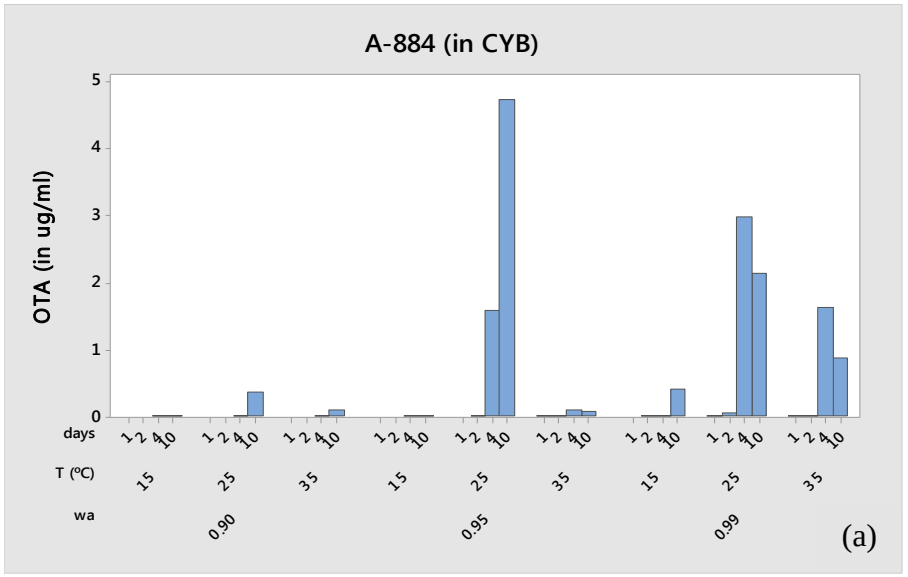
Abbreviations: T, temperature in ºC; a_w , water activity; YESB, Yeast extract sucrose broth; SGM, Synthetic grape juice medium; WGJ, White grape juice.

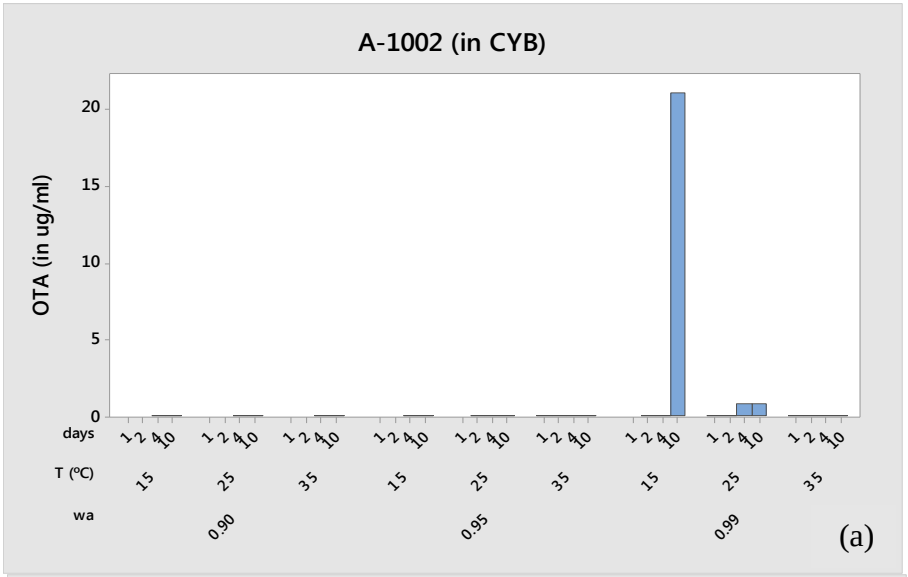
Fig.1

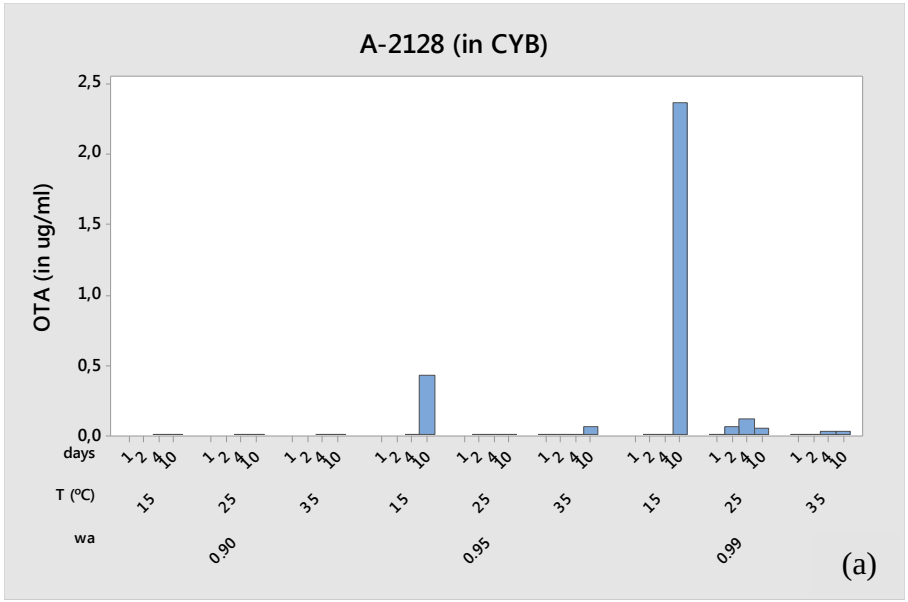

A1995
A3615
A1021
A884
A1653
A1625
A2128
A1841
A1002
A1004
A882
A. carbonarius CBS 556.65T (AJ964873)
A2275
A2520
A3905
A1749
A1687
A. sclerotioniger CBS 115572T (EU163271)
A. ibericus CBS 121593 T (AJ971805)
A. sclerotiicarbonarius CBS 121057 T (EU159235)
A. brasiliensis CBS 101740 T (AM295175)
A. niger CBS 554.65T (JX500080)
A. lacticoffeatus CBS 101883T (EU163270)
A. welwitschiae CBS 139.54T (KC480196)
A. tubingensis CBS 134.48T (AJ964876)
A. luchuensis CBS 205.80T (JX500071)
A. piperis CBS 112811T (EU163267)
A. neoniger CBS 115656T (FJ491700)
A. eucalypticola CBS 122712 T (EU482433)
A. costaricaensis CBS 115574 T (EU163268)
A. vadensis CBS 113365 T (EU163269)
A. ellipticus CBS 482.65 T (AM117809)
A. heteromorphus CBS 117.55 T (AM421461)
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. trinidadensis NRRL 62480 (HE984425)
A. aculeatinus CBS 121060 T (EU159241)
A. aculeatus CBS 172.66 T (AJ964877)
A. floridensis IHEM 21069 (HE818083)
A. fijensis CBS 313.89 T (FJ491695)
A. brunneoviolaceus CBS 621.78 T (EF661147)
100
100
99
91
99
98
98
81
100
70
100
72
100
89
70
73
93
0.05

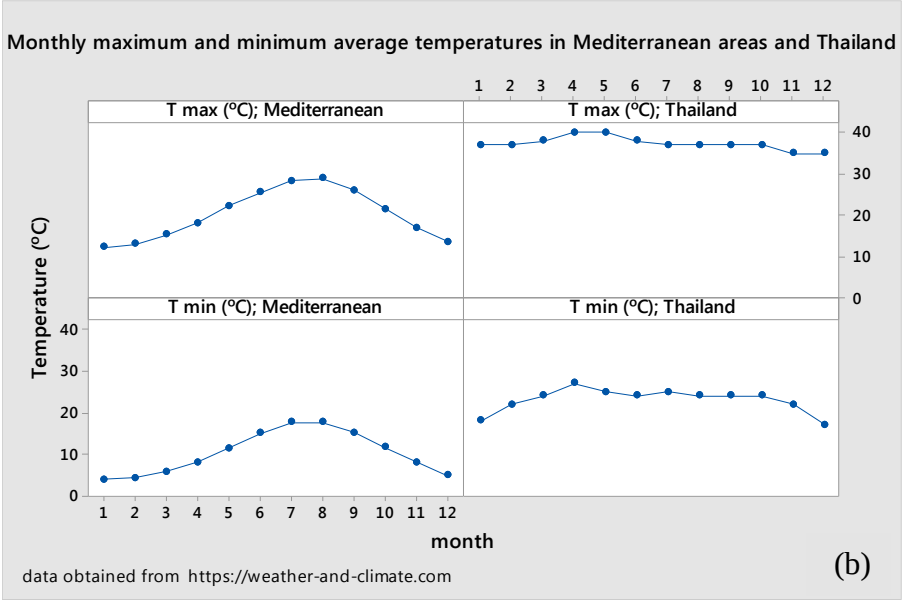
504
505

Fig. 2


A-884 (in CYB)
OTA (in ug/ml)
days
T (°C)
wa
(a)


A-1002 (in CYB)
OTA (in ug/ml)
days
T (°C)
wa
(a)


A-2128 (in CYB)
OTA (in ug/ml)
days
T (°C)
wa
(a)


Monthly maximum and minimum average temperatures in Mediterranean areas and Thailand
T max (°C); Mediterranean
T max (°C); Thailand
T min (°C); Mediterranean
T min (°C); Thailand
Temperature (°C)
month
data obtained from https://weather-and-climate.com
(b)