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Description of a strain from an atypical population of *Aspergillus parasiticus* that produces aflatoxins B only, and the impact of temperature on fungal growth and mycotoxin production

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Abstract In this study, an atypical strain of *Aspergillus parasiticus* is described. This strain, reported from Portuguese almonds, was named *Aspergillus parasiticus* B strain. The strain is herein characterised at the morphological and physiological levels, and compared with the typical *A. parasiticus* strain and other similar species in section *Flavi*. Previously published morphological and molecular data support that the B strain is very closely related to the *A. parasiticus* type strain. However, while *A. parasiticus* typically produces aflatoxins B and G, B strain produces aflatoxins B only. Furthermore, this atypical strain showed to differ from the typical strain in the fact that higher growth (colony diameter) and aflatoxin B1 production were observed at 25ºC than at 30ºC, whereas the opposite was observed for the typical strain. This strain can become a major food safety concern in colder regions where the typical *A. parasiticus* strains are not well adapted.

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Aspergillus flavus and *Aspergillus parasiticus* remain the most important and representative species of *Aspergillus* section *Flavi* occurring naturally in food commodities. These species are responsible for the production of aflatoxins in a wide variety of foods all over the world. Aflatoxins are highly toxic secondary metabolites and aflatoxin B1 (AFB1) is regarded as the most potent naturally occurring carcinogen (Bennett and Klich 2003). Given the relevance of aflatoxins in food and feeds, considerable interest has been put in the intraspecific toxigenic variability of *A. flavus* and *A. parasiticus*. While *A. flavus* populations have generally been found to be extremely diverse in terms of toxigenicity, *A. parasiticus* strains are more uniform in their toxigenic abilities: they are usually strongly aflatoxigenic, producing both AFBs and AFGs, and do not produce cyclopiazonic acid (CPA), a mycotoxin that is commonly used to differentiate species belonging to section *Flavi*. Non-aflatoxigenic strains seem to be extremely rare (Blaney et al. 1989; Doster et al. 1996; Horn et al. 1996; McAlpin et al. 1998; Tran-Dinh et al. 1999; Vaamonde et al. 2003).

Aspergillus parasiticus is apparently less widespread in nature than *A. flavus*, and it seems to be more adapted to survival in the soil and less dependent on crop infection (Horn 2007). As a matter of fact, this species is generally isolated quite rarely from the majority of foods

and was reported to be important only in soils and underground foods like peanuts (Horn 2005; Klich 2002; Vaamonde et al. 2003). Also, it has been stated to be geographically restricted to USA, South America and Australia (Frisvad et al. 2006), but our group has reported unusually high proportions of *A. parasiticus* contaminating Portuguese almonds (48 % of all *Aspergillus* section *Flavi* isolates) (Rodrigues et al., 2009, 2011). For many years, researchers did not separate *A. flavus* from *A. parasiticus* in field studies, which led to some confusion regarding their distribution. Even today, as new species are constantly being described, some doubts remain on the true identification of some isolates that phenotypically resemble the most common species. As a consequence, biodiversity and biogeography of aflatoxigenic species need to be regarded with caution. In fact, with the development of more complete and sensitive schemes of identification, three new aflatoxigenic species closely related to *A. parasiticus* have been recently described from foods: *A. arachidicola* (Pildain et al. 2008), *A. transmontanensis* (Soares et al. 2012) and *A. sergii* (Soares et al. 2012). *Aspergillus nomius* is also strongly aflatoxigenic, having an aflatoxigenic profile similar to *A. parasiticus* (Kurtzman et al. 1987).

Geographical differences and differences in isolates from ecological niches have been largely described for *A. flavus* (Cotty and Cardwell 1999; Klich and Pitt 1988), but not for *A. parasiticus*. In a survey conducted by our group on *Aspergillus* section *Flavi* from Portuguese almonds, one group of nine isolates was obtained which morphologically resembled *A. parasiticus*, but showed an extrolite profile (in terms of AFs and CPA) different from both *A. parasiticus* and *A. flavus* (Rodrigues et al. 2011). While typical *A. parasiticus* isolates produced both AFBs and AFGs, these isolates produced AFBs only, thus resembling *A. flavus*. On the other hand, CPA production ability was not detected in these isolates, which resembles typical *A. parasiticus*. Despite this unusual mycotoxigenic pattern, the detailed comparison of these isolates with other section *Flavi* type-strains in terms of sequence analysis of partial calmodulin gene as well as protein spectral analysis by MALDI-TOF ICMS grouped these isolates with the typical *A. parasiticus* strains (Rodrigues et al. 2011).

In the present study, we aimed to further analyse this atypical group of isolates at the morphological and physiological levels, and compare its ability to grow and produce mycotoxins under different conditions with the typical *A. parasiticus* and other closely related species in section *Flavi*.

The isolates of *Aspergillus* section *Flavi* used in this study are listed in Table 1. Isolates were maintained in 20 % glycerol at −20ºC and grown on Malt Extract Agar (MEA: Malt 20 g l^{-1}, Glucose 20 g l^{-1}, Peptone 1 g l^{-1}, Agar 20 g l^{-1}) in the dark for 7 d at 25ºC whenever

Table 1 Isolates of *Aspergillus* section *Flavi* used in this study (^{T} refers to type-strains)

Strain number	Source	GenBank accession number Calmodulin	Species name
MUM 10.238^T (CBS 117610)	*Arachis glabrata* leaf; Argentina	EF202049	*A. arachidicola*
MUM 10.237^T (CBS 100927)	Cellophane; South pacific Islands	EF202063	*A. flavus*
MUM 10.241^T (CBS 100928)	Soy sauce; Japan	EF202041	*A. sojae*
MUM 92.02 (NRRL 3386)		HQ340099	*A. parasiticus*
MUM 10.214 ^{T} (09AAsp260)	*Prunus dulcis* nut; Portugal	HQ340092	*A. transmontanensis*
MUM 10.219 ^{T} (09AAsp494)	*Prunus dulcis* nut; Portugal	HQ340097	*A. sergii*
MUM 09.02 (NRRL 13137)	Wheat, USA	EF202028	*A. nomius*
08AAsp158	*Prunus dulcis* nut; Portugal		*A. parasiticus* B strain
MUM 10.212 (09AAsp187)	*Prunus dulcis* nut; Portugal	HQ340090	*A. parasiticus* B strain
09AAsp232	*Prunus dulcis* nut; Portugal		*A. parasiticus* B strain
MUM 10.224 (09AAsp235)	*Prunus dulcis* nut; Portugal		*A. parasiticus* B strain
MUM 10.256 (09Aasp241)	*Prunus dulcis* nut; Portugal	JF500459	*A. parasiticus* B strain
MUM 10.257 (09Aasp246)	*Prunus dulcis* nut; Portugal	JF500460	*A. parasiticus* B strain
09AAsp539	*Prunus dulcis* nut; Portugal		*A. parasiticus* B strain

needed for further studies. All isolates listed were submitted to morphological and physiological analysis. For each isolate, a loop full of spores of 7 d-old cultures on MEA was suspended in 500 μl of 0.2 % agar, and this suspension was used for three-point inoculations on 9 cm diameter Petri dishes containing 20 ml of: MEA, Czapek Yeast Autolysate (CYA) and CY20S (as described by Klich 2002), *Aspergillus flavus* and *A. parasiticus* agar (AFPA; Oxoid, Basingstoke, United Kingdom) and YES (Yeast Extract 20 g l^{-1}, Sucrose 150 g l^{-1}, Agar 15 g l^{-1}). Cultures were incubated for 7 d, in the dark, at 5, 15, 25, 30, 37 and 42ºC, and then analysed for colony colour and diameter on the various media, as well as presence and size of sclerotia. The following micromorphological features were analysed in MEA at 25ºC: conidia size and ornamentation, head seriation, vesicle diameter, and size of metulae and phialide. Morphological identification followed the taxonomic keys and guides available for the *Aspergillus* genus (Klich 2002; Samson et al. 2004).

All strains were tested for aflatoxin production in aflatoxin-inducing Yeast Extract Sucrose (YES) medium. Strains were inoculated on 9 cm diameter plates (in triplicate) and incubated at 25, 30 and 37ºC for 7 days, in the dark. Then the methodology of Bragulat et al. (2001) was employed: 3 agar plugs were removed from each plate, weighted and placed into a 4 ml vial, where 1 ml of methanol was added. After 60 min, the extract was filtered by 0.45 μm filters. Samples were analysed using a HPLC equiped with a Jasco FP-920 fluorescence detector (365 nm exCitation wavelength; 435 nm emission wavelength), using a photochemical post-column derivatization reactor (PHRED unit - Aura Industries, USA). Chromatographic separations were performed on a reverse phase C18 column (Waters Spherisorb ODS2, 4.6 mm×250 mm, 5 μm), fitted with a precolumn with the same stationary phase. The mobile phase used was pumped at 1.0 ml/min and consisted of an isocratic programme as follows: water: acetonitrile: methanol (3:1:1, v/v). The injection volume was 20 μl. Aflatoxins standard was supplied by Biopure (Austria). A mix of aflatoxins, containing 2 μg/ml each of AFB1 and AFG1, and 0.5 μg/ml each of AFB2 and AFG2 was used.

Linearity, limit of detection (LOD) and limit of quantification (LOQ) were determined by two series of analyses, using five standard solutions of AFB1 and AFG1 each at concentrations of 200, 100, 50, 20 and 1 ng ml^{-1}, and AFB2 and AFG2 each at concentrations of 60, 30, 15, 6 and 0.3 ng ml^{-1}. Each standard solution was analysed in triplicate. LOD and LOQ were calculated according to the following equations (Taverniers et al. 2004): LOD=3sa/b and LOQ=10sa/b, where *sa* is the standard deviation of the intercept of the regression line obtained from the calibration curve and *b* is the slope of the line.

Since CPA production ability is a distinctive feature in section *Flavi*, the strains were also tested for CPA in CYA medium. All strains were inoculated on 6 cm diameter plates and incubated at 25 °C for 14 days, in the dark (Gqaleni et al. 1997). Then the methodology of Bragulat et al. (2001) was employed, as already described for aflatoxin analysis. Samples were analysed using a HPLC equiped with a Varian 2050 UV detector (285 nm). Chromatographic separations were performed on a EuroSpher 100 NH_2 column (Knauer, 4.6 mm×250 mm, 5 μm), fitted with a precolumn with the same stationary phase. The mobile phase used was pumped at 1.0 ml min^{-1} and consisted of an isocratic programme as follows: acetonitrile: 50 mM ammonium acetate (3:1, v/v), pH 5. The injection volume was 20 μl. CPA standard was supplied by Sigma (St. Louis, MO, USA). Linearity, limit of detection (LOD) and limit of quantification (LOQ) were determined as for aflatoxins, using six standard solutions of 100, 50, 20, 10, 1 and 0.5 μg ml^{-1}.

For the comparison of means of quantitative variables, samples were first tested for normal distribution by Shapiro-Wilk test (for $n<30$) or Kolmogorov-Smirnov test (for $n\geq30$), and for homogeneity of variances by Levene's test. Whenever samples followed these criteria, variances were analysed by *one-way* ANOVA, and multiple comparisons between samples pairs were made by Bonferroni's test ($n<30$) or by Tukey's test ($n\geq30$). When samples failed both premises, normality and homogeneity of variances, samples were analysed pairwise by the non-parametric Mann–Whitney test (Maroco 2003). In all cases, the mean differences were significant at $P<0.05$.

Morphological data as well as mycotoxigenic ability of the isolates included in this analysis are summarised in Table 2. Results shown for *Aspergillus parasiticus* B strain are the mean of all isolates listed in Table 1. In general, *A. parasiticus* B strain does not markedly differ from the typical *A. parasiticus* strain or from the other *A. parasiticus*-like species (*A. transmontanensis* and *A. sergii*), at both macro- and micromorphological levels. The major difference is observed in YES medium where, contrary to the typical *A. parasiticus*, conidia are almost absent in B strain colonies. Also, B strain sclerotia on CYA are significantly smaller than those from

Table 2 Summary of characteristics of the analysed strains (all measurements in μm)

Characteristic	*A. parasiticus* B strain	*A. transmontanensis*	*A. sergii*	*A. arachidicola*	*A. sojae*	*A. parasiticus*	*A. flavus*	*A. nomius*
Conidia colour on CYA	Dark green	Dark green	Dark green	Olive green	Olive brown	Dark green	Light green	Olive green
Conidia colour on MEA	Yellow Green	Yellow Green	Yellow green	Yellow green	Light green	Yellow green	Light green	Yellow green
Reverse on AFPA	orange	orange	orange	orange	brown	orange	orange	cream
Sclerotia size on MEA	-	-	518–788	-	-	432–724	540–810	500–75×1, 100–2,000
Sclerotia size on CYA	346–702	400–756	540–864	-	-	540–972	486–885	520–70×1, 300–2,000
Conidia ornamentation	rough	rough	rough	rough	rough	rough	smooth	rough
Conidia size	3.6–5.5	4.1–5.1	3.3–4.3	4.5–5	5.5–7	3.5–6	3–6	4.5–6.5
Head seriation	predominantly uniseriate	predominantly biseriate	uniseriate	uni/biseriate	predominantly uniseriate	predominantly uniseriate	predominantly biseriate	uni/biseriate
Vesicle diameter	17–42	27.5–38.2	26.0–35.8	28–50	17–35	20–35	20–45	25–65
Metulae size	3.8×6.6	2.9–4.6×7.1–12.3	-	5–6.5×9.5–13.5	5–9×10–15	3–7×7–10	5–7×8–10	4.0–8.6×8.1–17.3
Phialide size	3.7×7.2	3.02–4.8×6.9–8.4	2.5–3.1×5.5–6.8	3–6.5×7–11	4–6×9–12	3–5×8–11	3–4×7–12	3.8–6.5×7.6–11.3
Cyclopiazonic acid production	-	-	+	-	-	-	+	-
Aflatoxins produced	B only	B and G	B and G	B and G	none	B and G	B only	B and G
Fluorescence on CAM	violet	blue	blue	blue	-	blue	violet	blue

the other analysed species ($P<0.031$). In terms of mycotoxin profile (aflatoxins and CPA), the B strain differs from all the other tested species (Table 2). B strain produces AFBs but not AFGs, an AF profile typical of *A. flavus*, and is a CPA non-producer, like *A. parasiticus*.

Fungal growth on MEA and CYA at different temperatures is shown in Table 3. No growth occurred for any of the isolates at 5ºC. The B strain showed good growth at both 25 and 37ºC, but grew poorly at 42ºC, as did the remaining species. It grew better than *A. parasiticus* at 25ºC, and the opposite relation occurred at 37ºC, but with no significant difference ($P>0.05$). There was also no significant difference ($P>0.05$) between strain B and the other species for the three temperatures tested. At 42ºC, the atypical strain grew better than the typical *A. parasiticus* strain on MEA (but not on CYA), but not significantly ($P>0.05$). At 42ºC, the atypical strain also grew better than the *A. parasiticus*-like species *A. transmontanensis* and *A. sergii*.

The effect of temperature on AF and CPA production is shown in Table 4. The *A. parasiticus* B strain, *A. transmontanensis* and *A. sergii* produce significantly more AFBs at 25ºC than at 30ºC or 37ºC ($P<0.05$). On the other hand, *A. parasiticus*, *A. nomius* and *A. arachidicola* produce significantly more AFB1 at 30ºC than at 25ºC or 37ºC ($P=0$). *A. flavus* produces only residual levels of AFB1, only detectable at 30ºC. At 37ºC, only *A. arachidicola* and *A. nomius* produce detectable amounts of AFB1 and AFG1.

In this study, we describe a fungal strain which is morphologically and genetically very closely related to *A. parasiticus*, but shows the distinctive feature of producing AFBs only. We have designated this atypical *A. parasiticus* strain as *A. parasiticus* B strain. To our knowledge, no other *A. parasiticus* strain has been described as producing AFBs only. Typical *A. parasiticus* strains are consistently reported as strongly aflatoxigenic, producing both AFBs and AFGs, and only few studies have reported very low proportions (3–6 %) of non-aflatoxigenic isolates within the species (Blaney et al. 1989; Doster et al. 1996; Horn et al. 1996; McAlpin et al. 1998; Tran-Dinh et al. 1999; Vaamonde et al. 2003). Contrary to *A. flavus*, *A. parasiticus* strains are also CPA non-producers, as is *A. parasiticus* B strain. Recently, two species closely related to *A. parasiticus*, *A. transmontanensis* and *A. sergii*, have been described (Soares et al. 2012), but the B strain is not similar to any of these species in its extrolite profile. Additional molecular (multilocus) analysis is needed to clarify the relation between this population and the typical *A. parasiticus*. Also, the analysis of key genes within the AF gene cluster would help to determine the reason for the atypical AF production ability.

The B strain seems to be better adapted to lower temperatures than the typical *A. parasiticus* strain, not only in terms of growth, but mostly in terms of aflatoxin production. *A. parasiticus* B strain, *A. transmontanensis* and *A. sergii* produce higher amounts of AFBs at 25ºC than at 30ºC, whereas *A. parasiticus*, *A. arachidicola*, *A. nomius* and *A. flavus* produce higher amounts of AFs at 30ºC.

To our knowledge, the isolation of the B strain from Portuguese almond orchards represents the first report of this atypical *A. parasiticus* strain not producing AFGs. The typical strain of *A. parasiticus* has been

Table 3 Colony diameter on MEA and CYA, at three different temperatures (in cm, mean ± SD, $n=3$)

	MEA				CYA			
	42ºC	37ºC	25ºC	15ºC	42ºC	37ºC	25ºC	15ºC
A. parasiticus. B strain	2.3±0.29	6.9±0.17	6.6±0.23	3.2±0.08	2.6±0.23	6.7±0.36	6.7±0.24	3.2±0.05
A. transmontanensis	1.6±0.12	5.2±0.06	5.7±0.21	3.1±0.06	2.2±0.29	6.6±0.17	6.7±0.06	3.2±0.06
A. sergii	1.4±0.35	6.2±0.25	6.3±0.58	2.8±0.12	2.1±0.23	> 7.0	6.6±0.15	2.9±0.25
A. arachidicola	2.0±0.15	6.1±0.31	5.8±0.12	2.6±0.1	2.5±0.21	> 7.0	6.7±0.17	2.5±0.06
A. sojae	2.1±0.15	> 7.0	5.6±0.31	3.0±0.15	2.4±0.45	6.1±0.15	6.4±0.15	2.6±0.35
A. parasiticus	1.8±0.21	> 7.0	6.3±0.06	3.0±0.1	3.2±0.23	6.9±0.12	6.4±0.15	3.0±0.23
A. flavus	2.5±0.26	> 7.0	6.7±0.17	1.1±0.1	3.5±0.26	> 7.0	6.9±0.17	1.2±0.1
A. nomius	0.6±0.06	> 7.0	> 7.0	2.2±0.21	0.3±0.1	> 7.0	> 7.0	2.3±0.25

Table 4 Production of cyclopiazonic acid (CPA) and aflatoxins B1, B2, G1 and G2 by the strains under study, in μg of mycotoxin per gram of agar used for extraction. (mean ± SD, $n=3$)

	25ºC					30ºC					37ºC				
	B1 (μg/g)	B2 (μg/g)	G1 (μg/g)	G2 (μg/g)	CPA (μg/g)	B1 (μg/g)	B2 (μg/g)	G1 (μg/g)	G2 (μg/g)	CPA (μg/g)	B1 (μg/g)	B2 (μg/g)	G1 (μg/g)	G2 (μg/g)	CPA (μg/g)
A. parasiticus B strain	27.7±12.01	0.9±0.34	< LOD	< LOD	< LOD	0.6±0.05	< LOQ	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
A. transmontanensis	1.6±0.46	0.4±0.12	18.9±5.04	6.3±2.45	44.25±2.92	0.9±0.07	0.3±0.02	7.5±0.74	2.6±0.26	72.25±19.09	< LOD	< LOD	< LOD	< LOD	32.20±11.44
A. sergii	120.2±15.3	5.3±1.0	180.9±28.80	10.6±3.10	149.99±8.03	2.5±0.12	< LOD	1.5±0.84	< LOD	118.50±10.96	< LOD	< LOD	< LOD	< LOD	67.03±10.44
A. arachidicola	56.4±11.4	2.2±0.40	215.7±46.02	13.4±3.12	57.17±2.61	396.6±41.32	16.2±1.7	331.9±82.27	20.3±1.01	78.39±14.71	2.3±0.58	< LOD	1.3±0.21	< LOD	37.65±11.51
A. sojae	< LOD	< LOD	< LOD	< LOD	27.46±0.43	< LOD	< LOD	< LOD	< LOD	23.97±0.51	< LOD	< LOD	< LOD	< LOD	17.79±1.57
A. parasiticus	4.4±0.36	0.2±0.05	36.7±4.92	1.9±0.69	< LOD	23.0±3.16	0.5±0.1	41.3±3.84	1.3±0.27	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
A. flavus	< LOQ	< LOD	< LOQ	< LOD	82.91±9.46	0.2±0.11	< LOD	< LOD	< LOD	97.98±16.53	< LOD	< LOD	< LOD	< LOD	91.30±11.40
A. nomius	176.83±60.70	8.96±3.23	546.59±191.86	12.65±3.81	< LOD	207.03±11.73	9.70±0.59	625.84±33.98	18.30±0.55	< LOD	34.25±12.84	1.74±0.66	25.07±9.46	0.80±0.18	< LOD

found to be the prevalent species among *Aspergillus* section *Flavi* in Portuguese almonds (Rodrigues et al. 2011), whereas studies from other regions of the world report only residual figures for this species in almonds and other nuts (e.g. Bayman et al. 2002; Baquião et al. 2013).

Fungal growth and mycotoxin production only occur under favourable conditions, which vary for each species depending on adaptability. Food intrinsic parameters associated to extrinsic factors are responsible for the spectrum of contaminating and dominating mycobiota. This is mostly related to the physiology of fungi and their adaptation to the different matrices and environmental conditions. The atypical *A. parasiticus* B strain herein described is of major interest due to the distinguishing physiological characteristic of producing larger amounts of AFB1 at 25ºC than the typical strain. This strain can become a major food safety concern in colder regions where the typical *A. parasiticus* strains are not well adapted.

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