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Black aspergilli in Brazilian onions: From field to market

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ABSTRACT

The occurrence of black aspergilli in onions has been reported as frequent, and this group of fungi harbors potentially toxigenic species. In addition, *Aspergillus niger* has been reported as the causative agent of black mold rot, an important postharvest disease that causes damage throughout the world. Brazil stands out as one of the world's largest onion producers. However, few studies have been conducted to investigate the mycobiota in Brazilian onions. For this reason, we investigated the mycobiota of 48 market ($n = 25$) and field ($n = 23$) onion bulb samples. Nineteen soil samples were collected from the same fields and evaluated. In field onions and soil samples, *Penicillium* spp. was the prevalent fungal group, whereas in market samples *A.* section *Nigri* was the most frequent group. Due to the taxonomic complexity of this group, species identification was supported by phylogenetic data (*CaM* gene). *A. welwitschiae* was the most prevalent species in market samples. Black aspergillus strains were evaluated for fumonisin B_2 (FB_2) and ochratoxin A (OTA) production. Overall, 53% and 2.2% of the strains produced FB_2 and OTA, respectively. The occurrence of FB_2 and OTA was also investigated in onion bulb samples but none showed contamination with these mycotoxins.

1. Introduction

Onion (*Allium cepa* L.) is one of the main vegetable crops in Brazil. The country is the second-largest producer of onions in the Americas and the ninth-largest in the world (FAOSTAT, 2020). The production of onions in Brazil amounted to 1.55 million metric tons in 2018, and over half this total was produced by small farmers (https://www.statista.com/statistics/1075735/onion-production-value-brazil/). Because the production of onion is limited to a specific season, storage is important to keep up with year-round regular consumer demand (Mohammed et al., 2015). However, some 35–40% of onions are lost due to damage caused by storage diseases (Yadav et al., 2015). There are a diverse of fungal pathogen species that attack the onion bulb, and *Aspergillus* section *Nigri* is one of the most important.

Some species of *A.* section *Nigri* (=black aspergilli) are mycotoxin producers, but the presence of this kind of metabolite in onions has been little studied (Gherbawy et al., 2015; Mohammed et al., 2015). *A. carbonarius*, *A. sclerotioniger*, *A. niger* and *A. welwitschiae*, all belonging to *A.* section *Nigri*, can produce ochratoxin A (OTA); moreover, *A. niger* and *A. welwitschiae* can also produce fumonisins B_2, B_4 and B_6. The taxonomy of *A.* section *Nigri* is constantly changing and further studies are needed to determine the risk that these group might pose to food security (Gil-Serna et al., 2019), including onions, in which *A. niger* has historically been recognized as the main cause of one of the most destructive diseases in onions, black mold rot (Hayden et al., 1994; Ko, 2002; Prajapati and Patil, 2015; Ranganathan and Murugavel, 2017; Wani and Taskeen, 2011). This disease can affect onions at different stages, from field to market, but it occurs mainly after harvesting and predominantly during storage. The main symptom of this disease is a black discoloration at the neck, shallow lesions on outer scales, streaks of black mycelium and conidia beneath the dry outer scales and a black discoloration in bruised areas (Toit and Schwartz, 2011). According to some authors, contaminated soil and seeds appear to be the main source of inoculum (Hayden and Maude, 1992; Köycü and Özer, 1997; Tyson and Fullerton, 2004).

As mentioned above, few studies have so far investigated the

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presence of toxigenic black aspergilli species in onions and their potential for producing ochratoxin A and/or fumonisin (Gherbawy et al., 2015; Sang et al., 2014; Varga et al., 2012). Therefore, the main aim of this research was to investigate the presence of *Aspergillus* section *Nigri* in samples of onion produced in Brazil and in onion crop soil, as well as the presence of ochratoxin A and fumonisins in the onion bulbs.

2. Material and methods

2.1. Samples

A total of 48 samples (approximately 1 kg each) of onion bulbs (yellow variety) were evaluated, including 23 collected in the field on small farms in São José do Rio Pardo (21° 35′ 45″ S 46° 53′ 20″ O), Casa Branca (21° 46′ 26″ S 47° 05′ 09″ O), and Vargem Grande do Sul (21° 49′ 55″ S 46° 53′ 38″ O) (São Paulo State, Brazil). The remaining samples (n = 25) were collected from markets in Campinas (22° 54′ 21″ S 47° 03′ 39″ O) (São Paulo State, Brazil). In addition, 19 soil samples were collected from the onion fields mentioned above.

2.2. Water activity

The water activity in onion bulbs and soil was determined using an Aqualab Series 3TE instrument (Decagon, Pullman, WA, USA) at 25 ± 1 °C, in triplicate.

2.3. Fungal isolation

Onion bulbs were plated using the direct plating technique, according to Pitt and Hocking (2009). The onions were peeled and cut into small pieces in order to plate a pool of pieces obtained from all layers of the onion. The surface was disinfected in a 0.4% sodium hypochlorite solution for 2 min, then 50 pieces were plated on DG18 medium (10 pieces/plate; five plates 90 × 15 mm).

After seven days of incubation at 25 °C, the fungal occurrence was determined based on morphological characteristics, and the percentage of infected pieces was counted according to Pitt and Hocking (2009).

Soil samples were analyzed by serial dilution plating. Each soil sample (25 g) was diluted in 225 mL of 0.1% peptone water. An aliquot of 0.1 mL was spread-plated onto Dichloran (18%) Glycerol Agar (DG18) (two plates, 90 × 15 mm) and incubated at 25 °C for five days. The number of colony forming units (CFU) was then counted and fungal incidence determined based on morphological characteristics (Pitt and Hocking, 2009).

All isolates identified by appearance as *A.* section *Nigri* were isolated on CYA (Czapek Yeast Extract Agar), MEA (Malt Extract Agar) and CREA (Creatine Sucrose Agar) for morphological characterization and further purification for molecular analysis.

All strains analyzed here are deposited in the Food Technology Institute (ITAL, Campinas) collection.

2.4. OTA and FB_2 production by *A.* section *Nigri* isolates

A total of 325 *A.* section *Nigri* isolates were analyzed for fumonisin B_2 production according to Ferranti et al. (2018). All isolates from *A.* section *Nigri* obtained from field onions (n = 31) and soil samples (n = 33) were analyzed. For black aspergilli species obtained from onions in the market samples around 30% of the isolates obtained, were sampled randomly (n = 261) and analyzed. This sampling was necessary given the high number of black aspergilli species obtained in this set of samples.

To test for fumonisin B_2 production, the strains were inoculated onto CY20S (Czapek Yeast Extract 20% Sucrose agar) and incubated at 25 °C for 7 days. Small plugs of mycelium were removed and the toxin extracted with methanol. The extract was filtered and FB_2 derivatized using ortho-phthaldialdehyde reagent (OPA). The extract was injected into a Shimadzu LC-10VP (Shimadzu, Japan) HPLC system, with a fluorescence detector set to 335 nm excitation and 440 nm emission. A YMC column - Pack ODS-A (5 μm, 4.6 × 150 mm) (YMC Co., Ltd., Japan) and mobile phase of acetonitrile: water: acetic acid (51:47:02 v/v/v) were used. The respective flow rate, oven temperature and injection volume were: 1 mL/1 min, 40 °C and 20 μL. The methodology is described in detail in Ferranti et al. (2018).

All isolates from *A.* section *Nigri* obtained from field onions (n = 31), soil samples (n = 33) and market onions (n = 900) were analyzed for OTA production using the agar plug technique and Thin Layer Chromatography (TLC), according to Filtenborg et al. (1983) with modifications. The strains were inoculated onto Yeast Extract Sucrose agar (YES agar) and incubated at 25 °C for 7 days. OTA was extracted with methanol:chloroform (1:1) and separated and detected by TLC under UV light at 256 and 365 nm, according to the OTA standard. The mobile phase was toluene:ethyl acetate: 90% formic acid:chloroform (7:5:2:5).

2.5. Molecular analysis

Part of the calmodulin gene (*CaM*) sequence was determined for isolates representative of *A.* section *Nigri* obtained from onion bulbs and soil samples (field onion samples = 25 isolates; market onion samples = 68 isolates; soil samples = 14 isolates). The *CaM* gene portion was amplified using the CMD5 and CMD6 primer pairs, as described in Hong et al. (2005). PCR products were purified using ExoProStar™ 1-Step (GE Healthcare Life Sciences, UK), according to the manufacturer's recommended protocol. Amplicons were sequenced in both directions (forward and reverse) using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in an ABI 3500XL Genetic Analyzer (Applied Biosystems, USA).

Relationships within *A.* section *Nigri* species were examined. All *CaM* gene sequences obtained herein were aligned with those from type or neotype strains (available in GenBank database) using ClustalW (Thompson et al., 1994). The Kimura-2-Parameter model (Kimura, 1980) with gamma distribution (+G) and invariant (+I) sites were selected for Maximum Likelihood (ML) and Bayesian inference (BI) analysis. MEGA 7 (Kumar et al., 2016) and MrBayes v. 3.2.6 (Ronquist et al., 2012) computational programs were used to obtain the phylogenetic inferences based on the respective ML and BI analyses.

To estimate the level of support for the ML topology, bootstrap analysis was performed on 1000 replicates. For BI analysis, the Markov chain Monte Carlo (MCMC) algorithm was run for 5,000,000 generations. Sample frequency was set to 100, with 25% of trees removed as burn-in. Convergence diagnostics were monitored based on an average standard deviation of split frequencies below 0.01, potential scale reduction factor (PSRF) values close to 1.0, and effective sample size (ESS) values above 200. The trees were visualized using FigTree version 1.4.2, developed by Andrew Rambaut (http://tree.bio.ed.ac.uk/software/figtree/).

Representatives of all intra and interspecific variability (based on the *CaM* gene) found in this study were deposited in GenBank and access numbers can be found in Supplementary Table 1.

2.6. Determination of mycotoxins in onion bulb samples

A total of 48 onion bulb samples, collected in the field (n = 23) and on the market (n = 25) were subjected to analysis by High-Performance Liquid Chromatography (HPLC) to determine the occurrence of OTA and FB_2.

For OTA analysis, 500 g of onion bulbs were ground in a blender (Oster classic); 25 g of this portion was used for OTA extraction using methanol:sodium bicarbonate 3% (1:1, v/v) homogenized in a shaker, and filtered using a quantitative paper filter (Nalgon, Germany) and glass fiber filter (Vicam, USA). The extract was diluted with phosphate buffer saline (PBS) and applied to an immunoaffinity column (OchraTest TM WB, Vicam, USA). OTA was eluted with HPLC grade methanol. The

dried extract was resuspended in mobile phase methanol:acetonitrile: water:acetic acid (35:35:29:01, v/v/v/v), with an isocratic flow rate of 0.8 mL/min. Detection and quantification were performed using the Shimadzu LC-10VP HPLC System (Shimadzu Corporation, Japan) with fluorescence detection (RF-10AxL) set to 333 nm excitation and 477 nm emission. Chromatographic separation was achieved using a Shimadzu Shimpack (5 μm, 4.6 × 250 mm). Positive daily control was performed, contaminating a sample with standard OTA (Sigma, St Louis, USA). This analysis was carried out according to Vargas et al. (2005).

For FB_2 analysis, 200 g of onion bulbs were ground in a blender (Oster classic) and 5 g of this portion was used for FB_2 extraction using methanol:acetonitrile:water (25:25:50, v/v/v), homogenized in a shaker and filtered through a quantitative paper filter (Nalgon, Germany). The extract was topped up with PBS, filtered through a glass fiber filter (Vicam, USA) and applied to the immunoaffinity column (FumoniTestWB, Vicam, USA). The FB_2 was then eluted with HPLC grade methanol. The dried extract was resuspended in acetonitrile:water (1:1, v/v), adding ortho-phthaldialdehyde reagent (OPA) in order to derivatize fumonisins. Detection and quantification were performed in a Shimadzu LC-10VP HPLC System (Shimadzu Corporation, Japan) with a fluorescence detector set to 335 nm excitation and 440 nm emission. Chromatographic separation was achieved using a Shimadzu YMC column (5 μm, 4.6 × 250 mm), and the mobile phase was acetonitrile: water:acetic acid (51:47:0.2, v/v/v) with a flow rate of 1 mL/min. Positive daily control was performed, contaminating a sample with standard FB_2 (0.5 μg/mL) (Sigma-Aldrich, USA). This analysis was carried out according to Vicam's protocol (FumoniTestWB).

3. Results

3.1. Occurrence of fungi in onion bulbs and onion soil samples

Onion bulbs showed high fungal contamination with average infection of 57%. *A.* section *Nigri* was the most frequent group in the samples (37%), followed by *Penicillium* spp. (22%) (Table 1). Other fungal groups such as *Cladosporium* spp. and *Fusarium* spp. were also found but at low levels.

After sectioning the results, both market and field onion bulbs exhibited the same fungal genera, with the exception of *Cladosporium* spp. which was not found in the market samples. A higher infection was verified in onions from market, where average total infection was around 81%, with 100% of samples contaminated. *A.* section *Nigri* was the prevalent fungal group with average infection of 69% and 96%. *Penicillium* spp. was the second (28%) (Table 1). *A.* section *Nigri* was present in 24 of the 25 samples and the contamination range varied from 0 to 100%. In field onion samples, mean total infection was 30% and the main fungal groups found were *Penicillium* spp. (15%), followed by *Cladosporium* spp. (12%), *Fusarium* spp. (6%) and *A.* section *Nigri* (3%). *A.* section *Nigri* was present in only 10 of 23 the samples and the contamination range was 0 to 7%.

In soil samples ($n = 19$), the average fungal count was 3.3×10^4 CFU/g. The most frequent genera were *Penicillium* spp. (23.5%), *Trichoderma* spp. (17%) and *Aspergillus* spp. (16.2%) (Fig. 1). In *Aspergillus*, the highest incidence was found for *A.* section *Nigri* (5.5%) (Fig. 1), which was present in 6 of the 19 samples analyzed. As shown in Fig. 1, dematiaceous fungi, *Fusarium* spp., *Cladosporium* spp., *Absidia corymbifera*, and *Talaromyces* spp. were also found.

3.2. Molecular identification

A total of 107 *A.* section *Nigri* isolates were identified using a region of the calmodulin-encoding gene. Sequences were compared using BLAST against accessions in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and at the Westerdijk Fungal Biodiversity Institute (http://www.westerdijkinstitute.nl/aspergillus/), and were subjected to phylogenetic analysis. The maximum likelihood tree based on calmodulin (*CaM*) sequence data is shown in Fig. 2.

Five species were identified: *A. brasiliensis* ($n = 4$), *A. japonicus* ($n = 3$), *A. tubingensis* ($n = 1$), *A. niger* ($n = 16$) and *A. welwitschiae* ($n = 83$) (Fig. 2). Approximately 97% (66/68) of isolates from market onions were identified as *A. welwitschiae*. This species was also found in field onions and in onion soil samples. In contrast, *A. niger* was frequently found in field onions but not in market onions (only two strains) (Fig. 2).

3.3. Potential for mycotoxins production in black aspergillus strains

A total of 325 black aspergilli strains were tested for FB_2 production. Overall 53% of the strains produced FB_2 at levels ranging from 0.23 to 3.23 μg/g. Of the 964 strains analyzed, only 21 (2.2%) were OTA producers.

3.4. Ochratoxin A and fumonisin B_2 detection in onion samples

Of the 48 onion bulb samples analyzed, none exhibited OTA and/or FB_2 contamination. The limits of detection (LOD) for the OTA and FB_2 methods used were 0.1 μg/kg (OTA) and 0.04 μg/g (FB_2). The limits of quantification (LOQ) were 0.3 μg/kg (OTA) and 0.14 μg/g (FB_2).

4. Discussion

So far, only a few studies have investigated the occurrence of toxigenic fungi and mycotoxins in onions (Gherbawy et al., 2015; Sang et al., 2014; Varga et al., 2012). Most studies investigating onion mycobiota are focused on identifying phytopathogens (especially black mold rot) (Adongo et al., 2015; Onuorah and Obika, 2015; Orpin et al., 2018; Sang et al., 2014). To our knowledge, this is the first study investigating the occurrence of toxigenic fungi in onion crop soil, field onion bulbs, and onion bulbs purchased commercially.

As expected, the diversity of fungal biota in onion crop soil was higher than in onion bulbs. *Penicillium*, *Trichoderma*, *Fusarium*, *Aspergillus*, *Cladosporium*, *Absidia*, *Talaromyces* and dematiaceous fungi were found in soil samples. Members of the *Aspergillus* genus found in soil samples included *A.* section *Nigri*, *A.* section *Terrei*, *A.* section *Flavi*, *A.* section *Usti* and *A.* section *Cremei*. *Aspergillus* comprised 16.2% of total

Table 1
Mean percentage of fungal infection in market and field onion bulb samples.

Origin	Average infection (frequency of occurrence)					Water activity	
	Total fungal infection	*Penicillium* spp.	*Fusarium* spp.	*Cladosporium* spp.	*Aspergillus* section *Nigri*	Average	Range
Field ($n = 23$)	30.0(87)	15.0(69)	6.0(48)	12.0(52)	3.0(43)	0.994	0.984–1.00
Market ($n = 25$)	81.0(100)	28.0(72)	0.2(8)	0.0(0)	69.0(96)	0.993	0.978–1.00
Total ($n = 48$)	57.0(94)	22.0(73)	3.0(27)	6.0(25)	37.0(71)	0.990	0.978–1.00

Average infection (%) = sum of percentage infection of samples/total number of samples.
Frequency of occurrence (%) = number of samples infected with fungi.

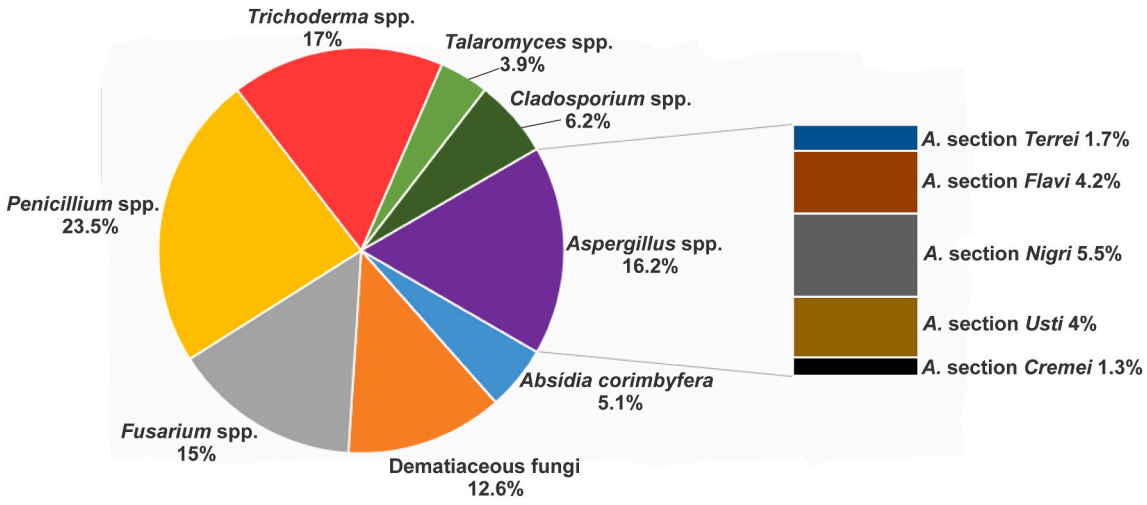

Fig. 1. Fungal groups in onion soil samples.

fungi isolated from the soil. Members of *A.* section *Nigri* were the most frequent (5.5%) (Fig. 1). These results are similar to the findings of Abdel-Gawad et al. (2017) researching fungi associated with onion crop soil in Egypt, where *Aspergillus* was represented by 9 species comprising around 40% of total fungi isolated. The soil has been pointed out as one of the main sources of inoculum of black aspergilli in foods, including onions (Varga et al., 2011). Hayden et al. (1994) reported that most of the bulbs grown in Sudan were contaminated with *A. niger* when harvested because of the high incidence of this species in the soil.

Among the *Aspergillus* isolated from onion bulbs (from field and market) only *A.* section *Nigri* were found. As revised by Gherbawy et al. (2015) many researchers previously reported *Aspergillus niger* as the predominant species in onion bulb samples. However, the taxonomy of *A.* section *Nigri*, especially those of the *A. niger* aggregate, has been very dynamic and species renaming has been constant.

One of the most important taxonomic events relating to black aspergilli occurred in 2011, when a new cryptic species was described (Perrone et al., 2011). *A. awamori sensu* Perrone was initially considered a phylogenetic species and was later morphologically characterized and formally described as a new species, renamed as *A. welwitschiae* by Hong et al. (2013). As already mentioned, most studies investigating onion mycobiota have focused on postharvest conditions and phytopathogen identification (Adongo et al., 2015; Onuorah and Obika, 2015; Orpin et al., 2018; Sang et al., 2014), moreover, most did not use molecular data, which can be problematic when it comes to identifying evolutionarily close species, *e.g.*, species of the *A. niger* aggregate, this group currently comprises 10 species morphologically indistinguishable. The most recent taxonomic organization of *A.* section *Nigri* may lead to new understandings of the species responsible for black mold rot, since they are closely related species, and their optimal growth parameters: *aw*, temperature, nutritional conditions, and pH are similar.

In our study, molecular identification was based on part of the calmodulin gene, an alternative *A.* section *Nigri* barcode (Samson et al., 2014), since the official fungal barcode (ITS region) is not a good marker for this fungal group. We identified *A. welwitschiae* as the major species in market onion bulbs (Fig. 2), corroborating the work of Gherbawy et al. (2015), Sang et al. (2014), and Varga et al. (2012) who identified *A. welwitschiae* as the most common species in market onions in Taif city (Saudi Arabia), Korea and Hungary, respectively.

Varga et al. (2012) suggested that *A. welwitschiae* and not *A. niger* could be responsible for the black mold rot in onions in Hungary. *A. niger* and *A. welwitschiae* are morphologically indistinguishable cryptic species, dismembered only in 2011–2013 (Perrone et al., 2011; Hong et al., 2013). Studies prior to that date need to be reevaluated and this could result in new insights, for example: recently, using molecular analysis, Duarte et al. (2018) showed that *Aspergillus welwitschiae* (and not *A. niger*) is the causal agent of sisal bole rot disease.

As already highlighted, most of the studies on onion mycobiota were not based on molecular data, which is essential for discriminating *A. niger* and *A. welwitschiae*. All studies that examined postharvest onion mycobiota in light of molecular data (including the present study) corroborate *A. welwitschiae*, not *A. niger*, as the prevalent species. The greatest losses from black mold rot occur in the postharvest phase, so the idea that *A. welwitschiae* is the true etiologic agent of this disease is plausible, but further studies are needed to confirm this.

In the onion bulb samples collected under field conditions, black aspergilli species were present at low levels, but more diverse than those found in market onion bulbs. Five species were identified (*A. niger*, *A. welwitschiae*, *A. japonicus*, *A. tubingensis* and *A. brasiliensis*). *A. niger* and *A. welwitschiae* co-occurred in field onion bulbs, but not in market onions. Further studies are needed in order to understand why *A. welwitschiae* is favored under postharvest conditions and could open up new avenues for black mold rot control.

Fungal strains belonging to *A. niger* and *A. welwitschiae* have been reported to produce OTA and FB_2. However, this does not apply to all the strains of *A. niger* and *A. welwitschiae* and our knowledge concerning the frequency of isolates with potential for OTA and FB_2 production is limited. We found that 53% of the strains analyzed herein ($n = 325$) were FB_2 producers and 2.2% of the 964 strains analyzed were OTA producers.

Regarding onion bulb contamination by OTA and FB_2, Gherbawy et al. (2015) found FB_2 contamination in stored onions collected from markets in Saudi Arabia, but not OTA. Varga et al. (2012) also found fumonisin-contaminated onion bulbs in Hungary. Despite the high incidence of *A. welwitschiae* in Brazilian onion bulbs, none of the onion samples analyzed in this study exhibited contamination by any of the above-mentioned toxins.

5. Conclusions

There are considerable differences between the mycobiota in field and soil samples compared to market onions. Black aspergilli are present throughout the production chain, but especially in market onions. *A. niger* and *A. welwitschiae* co-occurred in field onion bulbs, but *Aspergillus welwitschiae* is found almost exclusively in the market onions. Further studies are needed in order to elucidate why *A. welwitschiae* is prevalent over its sister species, *A. niger*, in market onions. Although some isolates of black aspergilli recovered from onions were able to produce FB_2 and/or OTA, the onion bulbs were not contaminated by either of these mycotoxins.

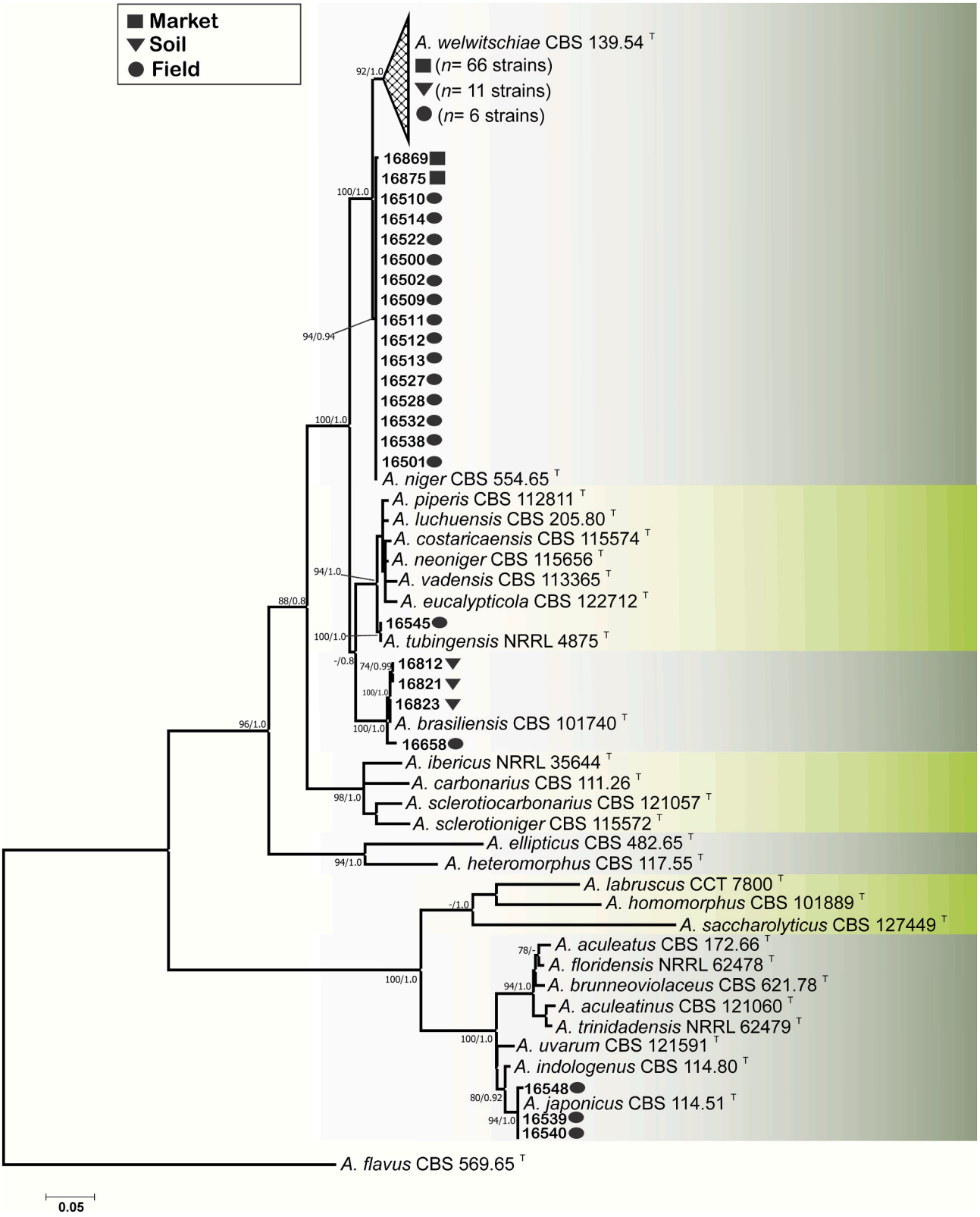

Fig. 2. Maximum likelihood tree based on calmodulin (*CaM*) sequence data. Sequences obtained in the present study (strains isolated from onion bulbs and onion soil) were aligned with corresponding sequences of type strains (T) of *Aspergillus* section *Nigri* deposited in the NCBI database. The bootstrap values (≥70%) and/or posterior probabilities (≥0.8) are indicated above each node. *Aspergillus flavus* was the outgroup.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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