1 **CONTAMINATION OF FRESH AND DRIED TOMATO BY *ALTERNARIA* TOXINS**
2 **IN SOUTHERN ITALY**
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18 **ABSTRACT**

19 In the present investigation fresh and dried tomato samples from markets and packinghouses
20 located in Apulia (southern Italy) were analysed for *Alternaria* toxins. All samples proved to
21 be contaminated by tenuazonic acid (TeA). Dried tomatoes were contaminated in the range
22 425-81,592 µg/kg, whereas fresh tomatoes in the range 10.7-4,560 µg/kg. The second most
23 abundant toxin was alternariol monomethyl ether (AME), followed by tentoxin (TEN) and
24 alternariol (AOH). Overall dried tomatoes were more contaminated than the fresh ones,
25 although this seemed not directly due to the presence of sodium chloride. Five representative
26 *Alternaria* isolates within those collected from samples proved to be one *Alternaria*
27 *arborescens* (A215) and four *Alternaria alternata*. Within the latter species one strain
28 belonged to morphotype *tenuissima* (A216), and three to *alternata* (A214, A217 and A218).
29 They confirmed to produce TeA, AOH, and AME *in vitro*. This study demonstrates the
30 possible risk for consumers' health related to the consumption of contaminated fresh and
31 dried tomatoes, and thus the need perform suitable control strategies.

32

33 **KEYWORDS:** tomato, mycotoxins, *Alternaria*, tenuazonic acid.

34

35 **Introduction**

36 Tomatoes (*Solanum lycopersicum* L.) are very popular among consumers worldwide for
37 their organoleptic properties, and an important source of carotenes, lycopene, potassium, and
38 ascorbic acid (Soto-Zamora et al 2005; USDA 2012). They are not only eaten as fresh
39 product, but also processed into a variety of products, such as pulp, ketchup, sauces, paste,
40 juices, and dried tomatoes. These latter have several advantageous features, as increased
41 aroma and flavour due to the drying at moderate temperatures (Latapí and Barret 2006), as
42 well as a longer shelf life and reduced costs for transportation and storage (Doymaz 2007).
43 Typically, their preparation foresees the cutting of firm, ripe tomatoes in two pieces over the
44 length, and the drying in open-air at direct sunlight for about 7 days. Furthermore, just-cut
45 tomatoes are sprinkled with abundant salt to speed up the drying process. The drying is a
46 critical step, since microorganisms populating the environment might contaminate tomatoes.
47 Nowadays, there is an increasing concern about mould contamination of food derived
48 products. Indeed, since processing steps increase product value, the economic losses due to
49 the non marketability of tomato-derived products because of quality loss and presence of toxic
50 fungal secondary metabolites, are higher (Sanzani and Ippolito 2014).
51 Several hypotheses on the ecological reason for fungi to produce secondary metabolites have
52 been formulated. They might contribute to the survival of the producer contributing to the
53 adaptation to the environment (Roze et al 2011). This is supported by the fact that the
54 secondary metabolite profile of fungi can vary in response to changes in the external
55 environment, as substrate composition, temperature, water activity ($a_w$), and pH (Sanzani et al
56 2016). Moreover, their role as pathogenicity/virulence factors in the complex host/pathogen
57 interaction has been reported (Sanzani et al 2012; Snini et al 2016). Mycotoxin production in
58 food and feed represents a serious concern to human and animal health, since they are toxic at
59 various extents and not destroyed during most food processing operations, resulting in
60 contamination of finished products (Andersen and Frisvad 2004; Siegel et al 2010).

61 Tomatoes are highly susceptible to fungal infestation due to their soft epidermis and a high
62 $a_w$ of about 0.99 (Moss 1984). The most common fungi that infect tomato plant and fruit are
63 *Alternaria* species, among which *A. alternata* is the most frequently reported (Garganese et al
64 2018). The occurrence of related mycotoxins in derived products has been reported in
65 Argentina (Somma et al 2011), Belgium (Walravens et al 2016), Brazil (da Motta and Soares
66 2001), China (Zhao et al 2015), Germany (Ackermann et al 2011), Netherlands (Lopez et al
67 2016), and Switzerland (Noser et al 2011). Indeed, *Alternaria* spp. can produce a variety of
68 metabolites belonging to three different structural groups: (i) the dibenzopyrone derivatives
69 alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), and altenuene
70 (ALT); (ii) the perylene derivatives altertoxins (ATX-I and II); and (iii) the tetramic acid
71 derivative, tenuazonic acid (TeA) (Barkai-Golan and Paster 2008). TeA is reported to occur in
72 higher concentrations as compared to AOH and AME (Van de Perre et al 2014). These latter,
73 on the other hand, are known mutagenic (Brugger et al 2006; Pfeiffer et al 2007), and possibly
74 genotoxic (EFSA 2011); moreover, they are reported to be implicated in esophageal cancer
75 (Liu et al 1991). AOH and AME can reach concentrations of up to 1300 and 270 μg/kg,
76 respectively, in infected tomatoes (Logrieco et al 2009; Ostry 2008). A dietary exposure
77 assessment was performed with Belgian consumption data, and the obtained mean value for
78 TeA (4230 ng/kg bw/day) was higher than the threshold of toxicological concern of 1500
79 ng/kg bw/day set by the European Safety Authority (EFSA 2011).
80 This experimental study started following an alert of high levels of TeA in dried tomatoes
81 produced in Apulia (southern Italy) and destined to European markets. Therefore, samplings
82 were conducted to highlight the presence of *Alternaria* spp. and related toxins in both the final
83 product and the starting material.
84

85 **Materials and methods**

86 ***Sample collection***

87 Fresh (n = 8) and dried (n = 10) tomato samples showing characteristic *Alternaria* black spots
88 on the surface were collected in markets and packinghouses located in Apulia region
89 (southern Italy) (Table 1). They were stored at 4°C for maximum 24 h before fungal isolation
90 and toxin analysis.
91
92 ***Isolate collection***
93 Both fresh and dried tomatoes were washed with a 2% sodium hypochlorite solution for 2 min
94 and sterile distilled water for 1 min. Once dried, tissue pieces at the edges of the lesions were
95 cut by a sterile razor blade and transferred to plates containing PDA (Potato Dextrose Agar,
96 Conda, Madrid, Spain) amended with streptomycin sulfate and ampicillin (250 mg/l each,
97 Sigma-Aldrich, Milan, Italy). Plates were incubated for 5 days in the dark at 24°C. For each
98 plate, the most prominent colony resembling *Alternaria* was purified and deposited in the
99 "Fungal Collection" at University of Bari Aldo Moro (Italy). They were divided into groups
100 according to macro-morphological features, and for each group a representative isolate was
101 selected, namely A214-A218.
102
103 ***Toxin extraction and analysis***
104 *Chemicals.* AOH, AME and TEN standards (with a purity ≥98%) were purchased from Vinci-
105 Biochem S.r.l. (Vinci, Italy). The salts, and the standards TeA and Dinoseb [(RS)-2,4-Dinitro-
106 6-sec-butylphenol] (with a purity ≥98%) were purchased from Sigma Aldrich. High
107 performance liquid chromatography (HPLC) grade water was obtained by a Milli-Q system
108 (Millipore, Bedford, MA). Solvents were of LC-MS grade (Merck, KGaA Darmstadt,
109 Germany).
110 *Extraction from fresh tomatoes*. An aliquot of 10 g of each sample, finely ground and
111 homogenized by an Omnimixer (Sorvall Instruments, Norwalk, USA), was added with 200 µl
112 of an internal standard solution (Dinoseb, 5 mg/L in acetone:hexane 1:1) and 10 ml of

113 acetonitrile, and stirred for 5 min at 130 rpm on a orbital shaker (ASAL srl, Milan, Italy).
114 Then, 6.5 g of a mixture of extraction salts (400 g $MgSO_4$, 100 g NaCl, 50 g $C_6H_6Na_2O_7 \cdot 1.5$
115 $H_2O$, 100 g $C_6H_5Na_3O_7 \cdot 2H_2O$) was added, and the sample was stirred for 5 min at 130 rpm
116 and centrifuged for 10 min at 4000 rpm (Rotofix 32A, Hettich Instruments, Germany).
117 *Extraction from dried tomatoes*. An aliquot of 2 g of sample, finely ground and homogenized
118 as reported above, were added with 200 µl of the internal standard solution and 8 ml of
119 distilled water. Then, the mixture was vortexed for 1 min, added with 10 ml acetonitrile, and
120 extracted as reported above for fresh samples.
121 *LC-MS/MS analysis*. A sample aliquot of 10 µl was injected in a Triple Quadrupole LC-
122 MS/MS Mass Spectrometer API 3200 AB Sciex Instruments (SCIEX Framingham, USA)
123 coupled with a Controller CBM20 A lite Integrated System equipped with a LC20AD Pump,
124 a SIL-20A /HT autosampler, and an LC-MS/MS software Analyst® 1.5.2 (SHIMADZU,
125 Kyoto, Japan). The separation was performed on a reversed phase column Luna C8 250×4.6
126 mm, 5µ (Phenomenex, Torrance, California, USA). The following gradient was set using
127 solvent A (ammonium bicarbonate 1 mM in water for LC-MS/methanol 95:5) and solvent B
128 (methanol): 5% B, after 2 min switch to 75% B, and then, after 2 min, to 95%; remain at 95%
129 B for 4 min and then switch to 5 % B, followed by column conditioning for 10 min. The flow
130 rate was 0.3 ml/min. The mass spectrometer operated in ESI negative mode. Nitrogen was
131 used as the curtain gas and collision gas, at flow rates of 25 and 5 ml/min, respectively. The
132 spray voltage was 4.5 kV and the capillary temperature 550°C.
133 Confirmation of mycotoxin residues in samples was performed by comparing retention times
134 with those of pure standards in solvent and by checking the ratio of monitored ions (at least
135 two) for each mycotoxin. Retention times, monitored ions, and ion ratios ± tolerance deemed
136 acceptable for identification are reported in Table 1. Examples of chromatograms related to
137 the transitions monitored for each toxin are reported in Figure 1.

138 The quantification for each mycotoxin was performed using 6-points calibration curves in the
139 range 10-1,000 μg/Kg, obtained by diluting a standard mixture in matrix extracts of fresh or
140 dried tomatoes, free from residues of the analyzed mycotoxins. Detection limits were
141 evaluated considering the concentration for which 10 repetitions of the chromatographic
142 analysis guaranteed a signal-to-noise ratio ≥3. For all toxins, the values were between 3 and 5
143 μg/kg. Quantification limit, *i.e.* the lowest point of the calibration curves on matrix, was equal
144 in all cases to 10 μg/kg. Method repeatability was evaluated on spiked contaminated samples.
145 Fresh and dried tomato samples without toxins residues were spiked at two concentrations
146 levels: 50-500 and 200-1000 μg/kg, respectively.
147
148 ***Isolate characterization***
149 *Morphological characterization*. *Alternaria* isolates were grown on potato carrot agar (PCA,
150 20 g/l potatoes; 20 g/l carrots; 20 g/l agar in distilled water) and PDA. Plates were single point
151 seeded by 20 μl of a $10^7$ conidia/ml suspension, prepared by flooding a 7-day-old colony by a
152 0.1% Tween-20 (Sigma-Aldrich) solution, and harvesting conidia by a sterile spatula. The
153 suspension was filtered through sterile gauze, counted by a Thoma chamber (HGB
154 Henneberg-Sander GmbH, Lutzellinden, Germany), and diluted in sterile distilled water.
155 Plates were incubated for 10 days at 22±1°C in the dark. The macroscopic characteristics
156 (colour, margin, diameter, and texture) were analyzed as reported by Pryor and Michailides
157 (2002). Furthermore, to observe the microscopic characteristics, during the incubation in the
158 dark, a rectangular block (10×20 mm) of agar and mycelium was removed aseptically from
159 the colony-expanding margin, daylight exposed and returned to incubation surface (Simmons,
160 2007). By the end of incubation, it was pressed gently with a glass slip on a slide. For each
161 isolate, observations were made at ×40 magnification and sub-stage illumination. The
162 sporulation characteristics were compared with those reported in the *Alternaria* identification
163 manual (Simmons 2007) and by Pryor and Michailides (2002). Strains of *A. alternata,*

164 morphotypes *alternata* (112249) and *tenuissima* (112252), and of *A. arborescens* (109730),
165 purchased from CBS-KNAW (The Netherlands), were included for comparison.
166 *Molecular characterization.* For DNA extraction, each isolate was grown on Potato Dextrose
167 Broth (PDB, Conda) for 5 days at 24°C in the dark in a stirred culture (100 rpm). Once
168 separated from substrate, mycelia were stored at -80°C. DNA extraction was performed as
169 described by Baroncelli et al (2014). Sample concentration was determined by a
170 spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA)
171 and diluted to 50 ng/µl by ultra-pure nuclease-free water. PCR amplifications of the SCAR
172 marker OPA1-3 were performed in a total reaction volume of 50 µl (Garganese *et al.,* 2016).
173 Each reaction mixture contained 100 ng of template DNA, 1 µl of forward primer (10 µM), 1
174 µl of reverse primer (10 µM), 25 µl of Emerald Amp Max PCR Master Mix (Takara,
175 Clontech, Mountain View, CA, USA) and 21 µl of ultra-pure water. The primer pairs were
176 synthetized by Thermo Fisher Scientific. PCR reactions were performed in i-Cycler thermal
177 cycler (Bio-Rad, Hercules, CA, USA). Amplification products were loaded on a 1% agarose
178 gel, and the run was visualized by Gel Doc EZ Imager (Bio-Rad) using ImageLab software.
179 The amplicons were excised, gel-purified by Isolate II PCR and Gel Kit (Bioline, London,
180 UK), and sequenced at both directions by Macrogen Europe (Amsterdam, The Netherlands).
181 The obtained sequences were deposited in GenBank (Table 2). Sequences were aligned using
182 MUSCLE and introduced to MEGA6 for phylogenetic analysis with the Maximum
183 Likelihood method using the Tamura-Nei model (Garganese *et al.,* 2016). Analyses were
184 performed with 1000 bootstrap replications.
185 *Toxigenicity.* To assess the presence and quantity of mycotoxins produced, all strains were
186 grown on PDB (Conda, 3 replicates/strain) for 10 days at 24°C and 150 rpm. By the end of
187 incubation, the growth medium was filtered (Whatman no.1, Maidstone, UK) and the
188 mycotoxin concentration evaluated by HPLC-MS/MS as reported above. Data were reported
189 as average of the three replicates ± standard error of the mean (SEM).

190 ***Sensibility to different NaCl concentrations***

191 *Alternaria* strains were grown on PDA enriched with increasing doses (0, 5, 10, 20, 40, 60,

192 and 80 g/l) of food grade NaCl. The plates were centrally inoculated with a conidial

193 suspension as reported above, three plates per strain, and incubated for 7 days in the dark at

194 24°C. By the end of incubation, the colonies features were recorded. The effect of 20 g/l NaCl

195 on toxin production by the selected *Alternaria* strains was evaluated by HPLC-MS/MS as

196 reported above, and expressed as ng toxin/mm colony diameter. All experiments were

197 repeated three times.

198

199 **Results**

200 ***Analysis of fresh/dried tomatoes***

201 The recovery percentage for each analysed mycotoxin and the intra-day precision data for the

202 method are reported in Table 3. All analysed tomato samples resulted contaminated by

203 *Alternaria* toxins, although at various extent (Table 4). In particular, all dried tomato samples

204 were contaminated by TeA in the range 425-81,592 μg/kg, followed by AME (40%

205 contaminated samples, 16-42 μg/kg). Similarly, fresh tomato samples resulted all

206 contaminated, although at lower extent, by TeA (10.7-4,560 μg/kg) followed by AME (30%

207 contaminated samples, 10.2-18.3 μg/kg). AOH and TEN were present only in samples DS10

208 (dried tomato) and FS4 (fresh tomato).

209

210 ***Characterization of selected Alternaria isolates***

211 Macro- and microscopic characteristics of the colonies of the five selected isolates were

212 evaluated in details. Three isolates (A214, A217 and A218) exhibited flat, woolly and brown-

213 black colonies, with a mean diameter of 65 mm. They showed a sporulation pattern with a

214 single sub-erect conidiophore and an apical cluster of branching chains of small dark brown

215 conidia, separated by short secondary conidiophores. Conidia appeared oval-ellipsoidal with

216 3–5 transverse septa. These features matched those of *A. alternata* morphotype *alternata*
217 (reference strain CBS 112249). Whereas, isolate A215 presented colonies from greenish-grey
218 to brown, with an average diameter of 45 mm. Conidia were borne by long, well-defined
219 primary conidiophores, with few terminal and sub-terminal branches. They were short-ovoid
220 or ellipsoid, with 1-4 transepta and rarely 1-2 longitudinal or oblique septa, brown-coloured
221 with darker walls and septa, resembling the characteristics of *A. arborescens* (reference strain
222 CBS 109730). Finally, isolate ALT216 was characterized by greenish colony with white
223 margins, and short chains of terminal sharp-beaked conidia, as observed for *A. alternata*
224 morphotype *tenuissima* (reference strain CBS 112252).
225 To confirm identification, a specific primer pair designed upon the SCAR marker barcoding
226 region OPA1-3 amplified a fragment of 883 bp, which was then sequenced in both directions.
227 Sequences were blasted against GenBank database confirmed identification. A phylogenetic
228 tree, built up using CBS reference strains plus strains A29 and A65 (Garganese et al., 2016)
229 for comparison, confirmed the species association (Fig. 2). These analyses endorsed A215 as
230 *A. arborescens*, A216 as *A. alternata* morphotype *tenuissima,* and A214, A217 and A218 as
231 *A*. *alternata* morphotype *alternata.*
232
233 ***Mycotoxins synthesis by* Alternaria *strains***
234 Growth medium samples were screened for *Alternaria* mycotoxin presence by HPLC-MS/MS
235 (Table 2). TeA, AOH and AME were recorded in the growth medium. High values were
236 recorded for TeA, from a minimum of 25 mg/kg for A215 (*A. arborescens*) to a maximum of
237 41.02 mg/kg for A218 (*A. alternata* morphotype *alternata*). Furthermore, A216 the best
238 producer of AOH (61.33 µg/kg) and its derivate AME (16.33 µg/kg) and A214 the worst one
239 (2.3 and 0 µg/kg for AOH and AM, respectively) belonged to the morphotype *alternata*.
240
241 ***Sensibility to different NaCl concentrations***

242 *Alternaria* strains were inoculated on PDA supplemented with different amounts of NaCl. All
243 of them showed a constant decrease of colony diameter with the increasing of salt
244 concentration. Substantial reductions (10-50%) in growth were already present at
245 concentrations of 10-20 g/l. A change in colour from dark brown-green to brown-light brown
246 at NaCl concentrations of 5-40 g/l was also observed, together with a red halo in the medium.
247 At 20 g/L NaCl, with the exception of TeA by strain A214, same or lower amount of toxins
248 were produced by the tested *Alternaria* strains (Fig. 3).
249

250 **Discussion**

251 Tomatoes are highly susceptible to fungal colonization due to their soft epidermis and high
252 water content (Moss 1984). The commonest fungi that infect tomato plants and fruit belong to
253 *Alternaria* genus, which can grow at a large variety of temperatures, humidity, and available
254 carbon sources (Vaquera et al 2017).
255 *Alternaria* species can produce about 70 toxic secondary metabolites with significance for
256 human and animal health, so that national organisms as the German Federal Institute of Risk
257 Assessment (2003) and the Czech Scientific Committee on Food (2007) pointed out the
258 urgent need for more information about their harmfulness (Ostry 2008). Furthermore, the
259 EFSA CONTAM panel, which provides scientific advice on contaminants in the food chain,
260 published its opinion on the risks for animal and public health related to the presence of
261 *Alternaria* toxins in feed and food (EFSA 2011). Although EFSA risk assessment was
262 inconclusive due to limited representative occurrence and toxicity data, the Standing
263 Committee on the Food Chain and Animal Health (PAFF Committee) identified AOH, AME,
264 TeA, TEN and ALT as toxins of relevance, recommending their monitoring in national
265 surveys. In our investigation, several samples of fresh and dried tomatoes produced in Apulia
266 region (southern Italy) were collected because of suspect of *Alternaria* contamination, and
267 analysed for *Alternaria* toxin presence and extent. TeA resulted the most present and

268 abundant toxin, followed by AME. Whereas AOH and TEN were present in only two
269 samples. Similarly, Van De Perre et al (2014) reported the occurrence of TeA in tomato-
270 derived products at higher concentration than other *Alternaria* mycotoxins. Overall, dried
271 tomatoes resulted much more contaminated than fresh ones. This finding might be ascribed to
272 the toxin concentration in tissues due to dehydration process, but a further colonization of
273 affected tissues during drying procedures cannot be excluded. Furthermore, we analysed the
274 effect of NaCl on the growth and toxigenicity of our selected *Alternaria* strains, observing
275 that the salt was able to reduce fungal growth proportionally with its increasing concentration.
276 We selected a concentration (20 g/L) at which a reduction but not a complete suppression of
277 growth was observed to assess the effect on toxin biosynthesis. Overall, the presence of NaCl
278 did not induce toxin production. Similarly, Graf et al. (2012) found that in artificial YES
279 medium supplemented with NaCl the production of AOH and AME was drastically reduced
280 already at concentrations >5 g/l. Wei et al (2017) analysed several dried fruits recording all
281 main *Alternaria* toxins except ALT: AOH was detected in 2.3% of the samples, AME in 8.2%
282 of the samples, and TEN in 20.5% of the samples. As observed in our study, and formerly
283 elsewhere reported (EFSA 2011; Zhao et al 2015), AME was found more frequently than
284 AOH. Furthermore, Wei et al (2017) found TeA as the most recurrent toxin in all dried fruits
285 (42.7%) with concentrations in the range of 6.9–5665.3 μg/kg. Similarly, Gambacorta et al
286 (2018) found that TeA was the mycotoxin occurring at highest level in 43/45 samples of a
287 landrace of sweet pepper widely cultivated in Basilicata (Italy). Even higher frequency and
288 contamination extent were recorded in the present study. Those results are interesting even
289 because most of the investigations on *Alternaria* toxins incidence in fruit-derived products
290 focused on purees, juices, and wines (Hickert et al 2016; López et al 2016; Rodríguez-
291 Carrasco et al 2016).
292 As expected, the collected isolates belonged to the *Alternaria* genus and mainly to the species
293 *alternata*, although even *A. arborescens* was present. Therefore, the analysed strains were of

294 relevant economic significance, since not only pathogens to tomato but also potentially
295 producers of mycotoxins. Indeed, we found that all the tested *Alternaria* strains from tomato
296 were able to produce high quantities of TeA and, at a lesser extent, AOH/AME. Our strains
297 did not produce tentoxin *in vitro*. This fact is not unusual since in a similar paper by Siciliano
298 et al. (2015) 86% of the *Alternaria* strains tested did not produce TEN *in vitro*.
299 Similarly, assessing fresh tomatoes with apparent fungal lesions from markets in Denmark
300 and Spain, Andersen and Frisvad (2004) observed that *Alternaria* was the predominant genus
301 present in 40% of the samples. Whereas at different climatic conditions pathogen populations
302 changed dramatically. For example, Muhammad et al. (2004) observed a higher frequency of
303 tomatoes infected by *Aspergillus* spp. in Nigeria. Indeed contamination by the thermophilic
304 fungus *Aspergillus* would be more common in dry and hot climates and those by *Alternaria* in
305 humid and temperate ones (Santos et al 2016). Although the samples analysed in the present
306 investigation came from producers located in Apulia region, since they are marketed in
307 several European countries, the results of this study may have a broader significance.
308 In conclusion, our data confirmed that *Alternaria* mycotoxins presence should be included as
309 an important aspect in Hazard Analysis and Critical Control Point (HACCP) plans of food
310 industries that manufacture fresh and dried tomatoes. Preventive and control strategies from
311 field-to-table are needed, and require the development of rapid, sensitive and specific
312 analytical methods that could be applied at preharvest stages, but also on raw materials and
313 processed foodstuffs.
314

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428 **CAPTION TO FIGURES**

429

430 **Fig. 1.** Phylogenetic tree based on SCAR marker OPA1-3 sequences of 5 isolates of
431 *Alternaria* spp. isolated from tomato fruit. *Alternaria* reference strains (CBS112252, Genbank
432 accession no. MG063728; CBS112249, accession no. MG063725; CBS109730, accession no.
433 MG063730) plus strains A29 (accession no. KU933219) and A65 (accession no. KU933229)
434 were included for comparison. Numbers on nodes represent the maximum likelihood
435 bootstrap percentages. Branch lengths are proportional to the numbers of nucleotide
436 substitutions and are measured using the bar scale (0.005).

437

438 **Fig. 2.** Chromatograms related to the transitions monitored for each toxin: tenuazonic acid
439 (A), alternariol (B), alternariol monomethyl ether (C), and tentoxin (D). The first two
440 chromatograms are related to the highest point of calibration curves, the third and fourth
441 chromatograms to the positive sample DS10, the fifth and sixth chromatograms refer to the
442 blank sample used for recovery tests.

443

444 **Fig. 3.** Production of TeA (A), AOH (B) and AME (C) on artificial medium (PDA) amended
445 with 20 g/L NaCl (NaCl+). Non amended inoculated plates were used as a control (NaCl-).

**Table 1.** Retention times, monitored ions, ion ratios and tolerance in ion ratios which was deemed acceptable for identification.

| Q1 Mass | Q3 Mass | Dwell (msec) | DP | CEP | CE | CXP | Ion ratios (± tolerance)[a] | Identification | Retention time (min ± tolerance) |
|---|---|---|---|---|---|---|---|---|---|
| 196 | 138.9 | 50 | -70 | -28.17 | -28 | -7 | 1.6 ± 0.47 | Tenuazonic acid 1 | 7.72 ± 0.2 |
| 196 | 112.1 | 50 | -70 | -28.17 | -32 | -5 | | Tenuazonic acid 2 | 7.72 ± 0.2 |
| 257.1 | 215 | 50 | -45 | -29.82 | -20 | -3 | 4.8 ± 1.4 | Alternariol 1 | 9.07 ± 0.2 |
| 257.1 | 146.7 | 50 | -45 | -29.82 | -20 | -3 | | Alternariol 2 | 9.07 ± 0.2 |
| 271.1 | 256.2 | 50 | -45 | -30.2 | -20 | -3 | 3.2 ± 0.96 | Alternariol MME 1 | 10.09 ± 0.2 |
| 271.1 | 228.2 | 50 | -45 | -30.2 | -20 | -3 | | Alternariol MME 2 | 10.09 ± 0.2 |
| 413.5 | 271.2 | 50 | -45 | -34.04 | -20 | -3 | 1.0 ± 0.3 | Tentoxin 1 | 9.26 ± 0.2 |
| 413.5 | 214.8 | 50 | -45 | -34.04 | -20 | -3 | | Tentoxin 2 | 9.26 ± 0.2 |
| 239.1 | 132.1 | 50 | -50 | -29.33 | -33 | -4 | 0.02 ± 0.006 | Dinoseb 1 (internal standard) | 8.35 ± 0.2 |
| 239.1 | 193.1 | 50 | -50 | -29.33 | -33 | -4 | | Dinoseb 2 (internal standard) | 8.35 ± 0.2 |

[a] Ratios in all samples were compared with tolerance values deriving from average of calibration standards obtained from sample extracts in the same sequence; in all cases they were within ± 30% (relative).

7 **Table 2.** Selected *Alternaria* strains used in the study, Genbank accession numbers for the

8 sequences of SCAR marker OPA1-3, and production of tenuazonic acid (TeA), alternariol

9 (AOH), and alternariol monomethyleter (AME) after 10 days at 24°C in PDB agitated culture.

10 Data are the average of three replicates ± standard error of the mean (SEM).

11

| Isolate | Accession n. | TEA (mg/Kg) | AOH (μg/Kg) | AME (μg/Kg) |
|---|---|---|---|---|
| A214 | MK204937 | 33.0 ± 3.6 | 2.3 ± 2.1 | - |
| A215 | MK204938 | 25.0 ± 4.1 | 24.3 ± 3.3 | 5.7 ± 2.6 |
| A216 | MK204939 | 36.4 ± 3.3 | 61.3 ± 3.8 | 16.3 ± 3.3 |
| A217 | MK204940 | 38.5 ± 4.1 | 4.7 ± 1.2 | 0.3 ± 0.5 |
| A218 | MK204941 | 41.0 ± 4.5 | 14.7 ± 3.9 | 2.3 ± 1.2 |

12
13

14 **Table 3.** Method repeatability on spiked contaminated samples. Fresh and dried tomato
15 samples without toxins residues were spiked at two concentrations levels. The data shown in
16 the table refer to the obtained mean recovery values and to the relative standard deviation
17 obtained under intra-day repeatability conditions.

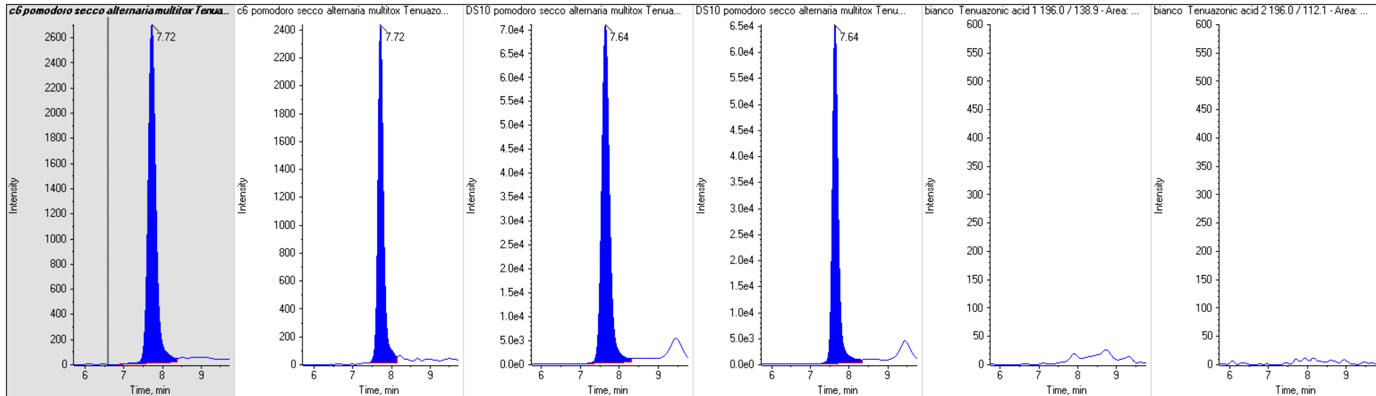
| **Toxin** | **Matrix** | **Spike Level (µg/kg)** | **Results** | **Mean (µg/kg)** | **Mean Recovery (%)** | **RSDr (%)** |
|---|---|---|---|---|---|---|
| Alternariol | Fresh tomato | 50 | 5 | 55.8 | 111.6 | 4.63 |
| Alternariol | Fresh tomato | 500 | 5 | 490 | 98.0 | 11.5 |
| Alternariol | Dried tomato | 200 | 5 | 180.5 | 90.3 | 7.69 |
| Alternariol | Dried tomato | 1000 | 5 | 1113 | 111.3 | 15.2 |
| Alternariol MME | Fresh tomato | 50 | 5 | 52.2 | 104.4 | 3.80 |
| Alternariol MME | Fresh tomato | 500 | 5 | 445 | 89.0 | 7.73 |
| Alternariol MME | Dried tomato | 200 | 5 | 188.5 | 94.3 | 9.00 |
| Alternariol MME | Dried tomato | 1000 | 5 | 1106 | 110.6 | 16.9 |
| Tentoxin | Fresh tomato | 50 | 5 | 56.9 | 113.8 | 4.60 |
| Tentoxin | Fresh tomato | 500 | 5 | 424 | 84.8 | 16.4 |
| Tentoxin | Dried tomato | 200 | 5 | 168 | 84.0 | 6.98 |
| Tentoxin | Dried tomato | 1000 | 5 | 958 | 95.8 | 14.9 |
| Tenuazonic acid | Fresh tomato | 50 | 5 | 40.4 | 80.9 | 7.24 |
| Tenuazonic acid | Fresh tomato | 500 | 5 | 411 | 82.2 | 8.7 |
| Tenuazonic acid | Dried tomato | 200 | 5 | 188 | 94.1 | 6.33 |
| Tenuazonic acid | Dried tomato | 1000 | 5 | 871 | 87.1 | 10.4 |

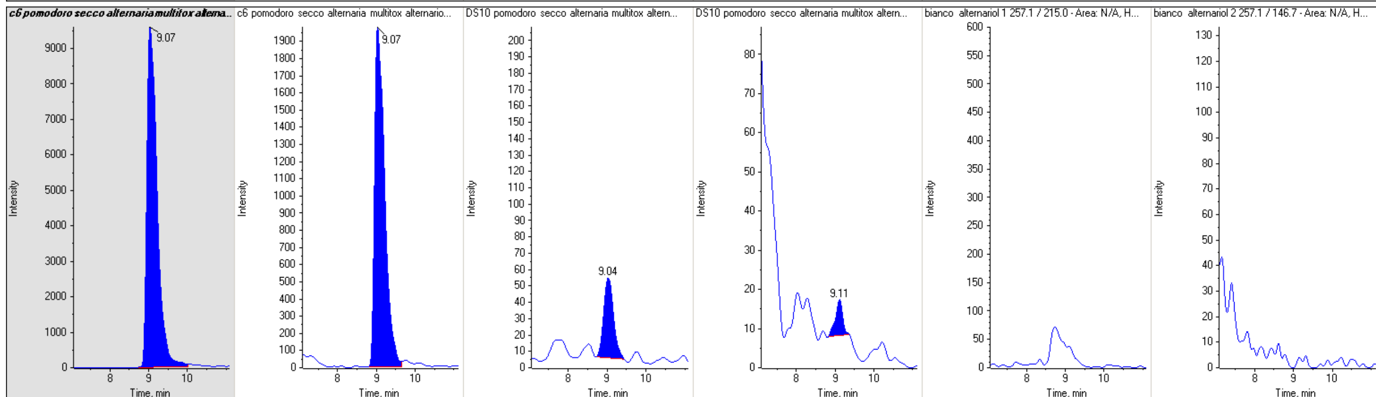
18

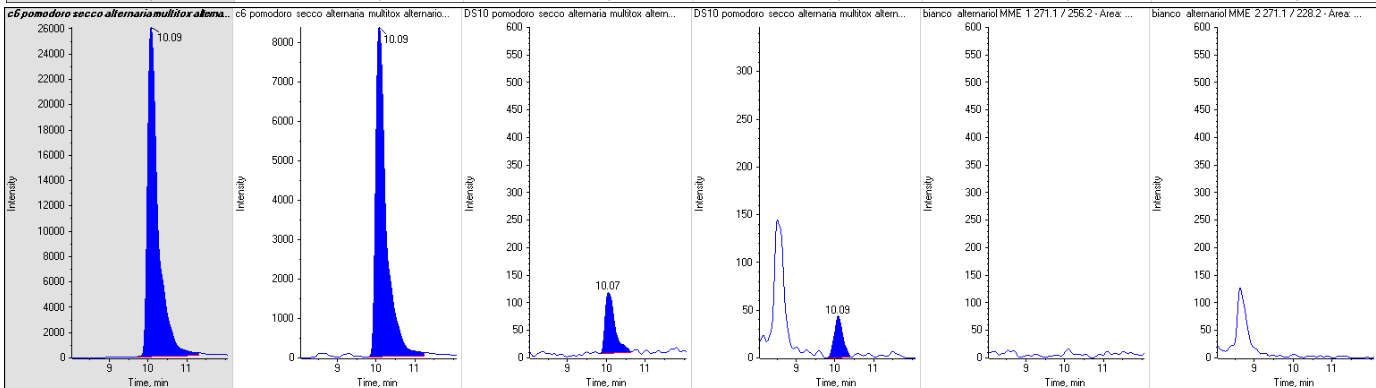
19 **Table 4.** Contamination (µg/Kg) by *Alternaria* toxins of dried (DS) and fresh (FS) tomato
20 collected in markets and packinghouses of Apulia (southern Italy) by tenuazonic acid (TeA),
21 alternariol (AOH), alternariol monomethyl ether (AME), and tentoxin (TEN).
22
23

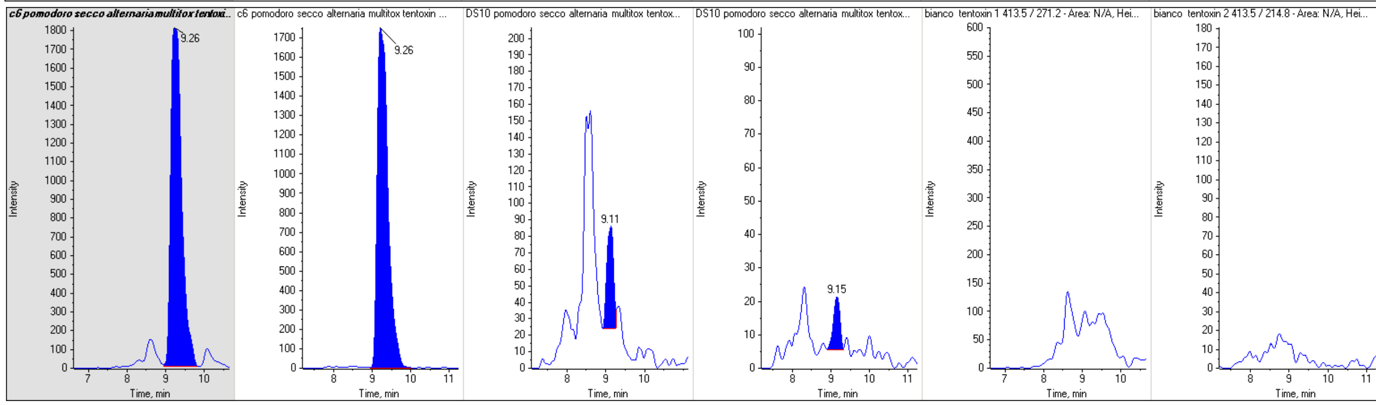
| | TEA | AOH | AME | TEN |
|---|---|---|---|---|
| DS1 | 9,698 | - | - | <10 |
| DS2 | 81,592 | - | - | <10 |
| DS3 | 12,568 | - | - | <10 |
| DS4 | 2,529 | - | - | <10 |
| DS5 | 1,240 | - | 42 | <10 |
| DS6 | 3,377 | - | 18 | <10 |
| DS7 | 558.5 | - | <10 | <10 |
| DS8 | 1,463 | - | <10 | <10 |
| DS9 | 425 | - | 16 | <10 |
| DS10 | 4,9462 | 22 | 24 | 38 |
| | | | | |
| FS1 | 2,715 | <10 | 18.3 | <10 |
| FS2 | 25.6 | <10 | 10.2 | <10 |
| FS3 | 16.6 | <10 | <10 | <10 |
| FS4 | 4,560 | 16.4 | 12.4 | 36 |
| FS5 | 34.8 | <10 | <10 | <10 |
| FS6 | 140 | <10 | <10 | <10 |
| FS7 | 10.7 | <10 | <10 | <10 |
| FS8 | 577 | <10 | <10 | <10 |

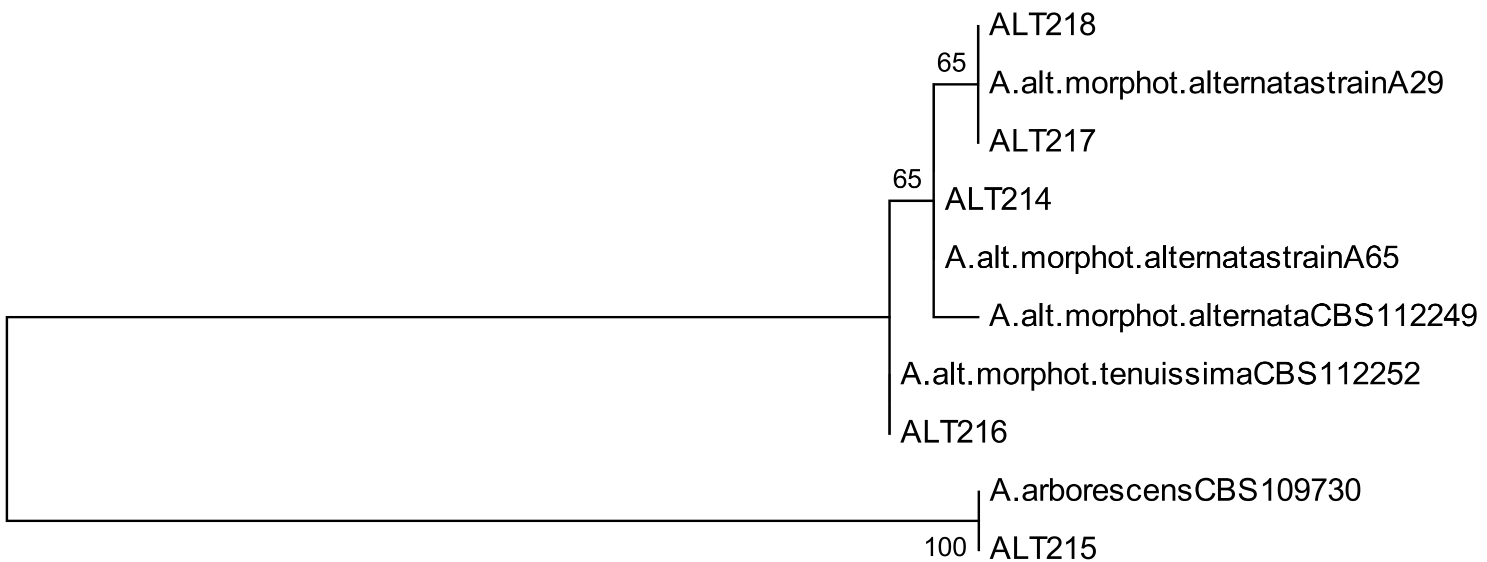
24

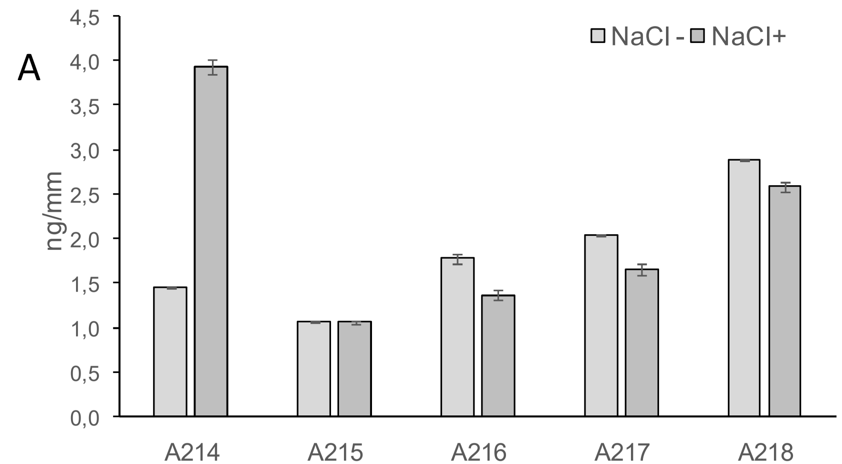

A
c6 pomodoro secco alternaria multitox Tenua...
c6 pomodoro secco alternaria multitox Tenuazo...
DS10 pomodoro secco alternaria multitox Tenua...
DS10 pomodoro secco alternaria multitox Tenu...
bianco Tenuazonic acid 1 196.0 / 138.9 - Area: ...
bianco Tenuazonic acid 2 196.0 / 112.1 - Area: ...
7.72
7.72
7.64
7.64
Intensity
Time, min

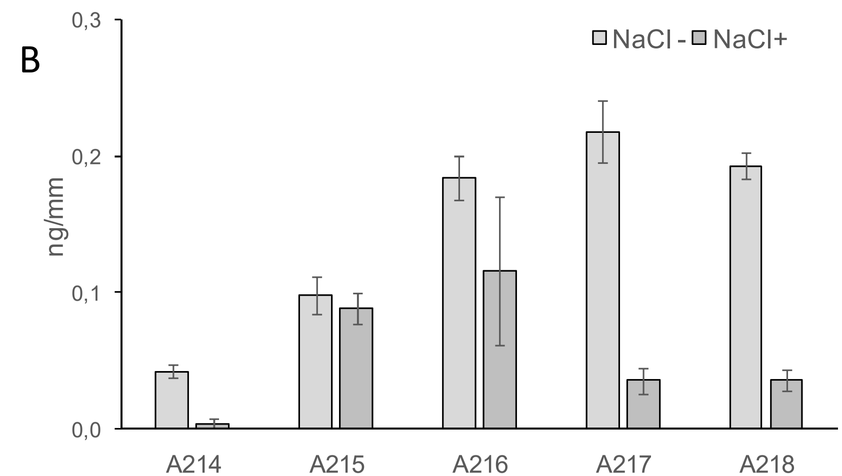

B
c6 pomodoro secco alternaria multitox alterna...
c6 pomodoro secco alternaria multitox alternario...
DS10 pomodoro secco alternaria multitox altern...
DS10 pomodoro secco alternaria multitox altern...
bianco alternariol 1 257.1 / 215.0 - Area: N/A, H...
bianco alternariol 2 257.1 / 146.7 - Area: N/A, H...
9.07
9.07
9.04
9.11
Intensity
Time, min

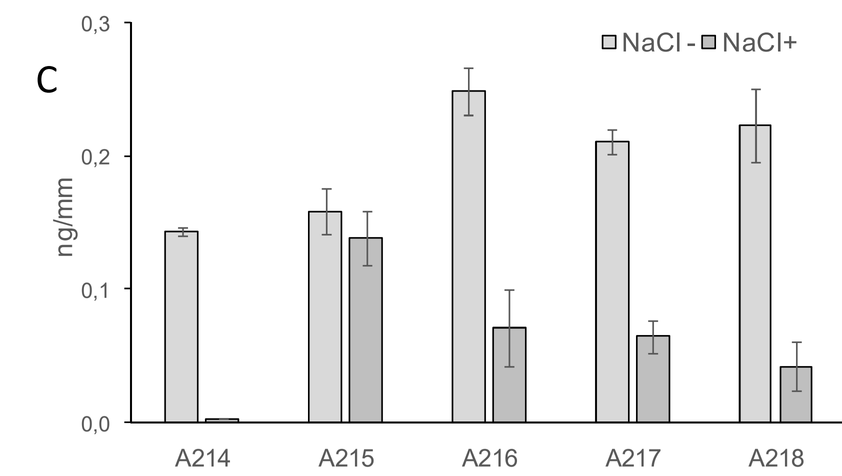

C
c6 pomodoro secco alternaria multitox alterna...
c6 pomodoro secco alternaria multitox alternario...
DS10 pomodoro secco alternaria multitox altern...
DS10 pomodoro secco alternaria multitox altern...
bianco alternariol MME 1 271.1 / 256.2 - Area: ...
bianco alternariol MME 2 271.1 / 228.2 - Area: ...
10.09
10.09
10.07
10.09
Intensity
Time, min


D
c6 pomodoro secco alternaria multitox tentoxi...
c6 pomodoro secco alternaria multitox tentoxin ...
DS10 pomodoro secco alternaria multitox tentox...
DS10 pomodoro secco alternaria multitox tentox...
bianco tentoxin 1 413.5 / 271.2 - Area: N/A, Hei...
bianco tentoxin 2 413.5 / 214.8 - Area: N/A, Hei...
9.26
9.26
9.11
9.15
Intensity
Time, min


ALT218
65
A.alt.morphot.alternatastrainA29
ALT217
65
ALT214
A.alt.morphot.alternatastrainA65
A.alt.morphot.alternataCBS112249
A.alt.morphot.tenuissimaCBS112252
ALT216
A.arborescensCBS109730
100
ALT215
0.005


A
NaCl - NaCl+
ng/mm
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
A214
A215
A216
A217
A218


B
NaCl - NaCl+
ng/mm
0,3
0,2
0,1
0,0
A214
A215
A216
A217
A218


C
NaCl - NaCl+
ng/mm
0,3
0,2
0,1
0,0
A214
A215
A216
A217
A218