Elsevier Editorial System(tm) for International Journal of Food Microbiology
Manuscript Draft

Manuscript Number: FOOD-D-10-00544R1

Title: Detection of the sour-rot pathogen Geotrichum candidum in tomato fruit and juice by using a highly specific monoclonal antibody-based ELISA

Article Type: Full Length Article

Keywords: Geotrichum candidum, hybridoma, monoclonal antibody, enzyme-linked immunosorbent assay

Corresponding Author: Dr Christopher Thornton, PhD

Corresponding Author's Institution: University of Exeter

First Author: Christopher R Thornton, BSc, PhD

Order of Authors: Christopher R Thornton, BSc, PhD; David C Slaughter; Richard M Davis

Revised manuscript with highlighted changes
Click here to view linked References

1 # Detection of the sour-rot pathogen *Geotrichum candidum* in tomato fruit
2 # and juice by using a highly specific monoclonal antibody-based ELISA

3

4 **Christopher R. Thornton[a,]*, David C. Slaughter[b] and R. Michael Davis[b]**

5 [1]University of Exeter, Exeter, United Kingdom and [2]University of California, Davis, California

6

7 ***Corresponding author: Tel: +44 (0)1392 725172. Fax: +44 (0)1392 263434. *E-mail address*:**
8 **C.R.Thornton@ex.ac.uk**

9

10 **Suggestion for an abbreviated title:** *Geotrichum candidum* enzyme-linked immunosorbent assay

11
12
13
14
15
16
17
18
19
20
21
22

Abstract

Geotrichum candidum is a common soil-borne fungus that causes sour-rot of tomatoes, citrus fruits and vegetables, and is a major contaminant on tomato processing equipment. The aim of this work was to produce a monoclonal antibody and diagnostic assay for its detection in tomato fruit and juice. Using hybridoma technology, a cell line (FE10) was generated that produced a monoclonal antibody belonging to the immunoglobulin class M (IgM) that was specific to *G. candidum* and the closely related teleomorphic species *Galactomyces geotrichum* and anamorphic species *Geotrichum europaeum* and *Geotrichum pseudocandidum* in the *Galactomyces geotrichum/G. candidum* complex. The MAb did not cross-react with a wide range of unrelated fungi, including some likely to be encountered during crop production and processing. The MAb binds to an immunodominant high molecular mass (>200 kDa) extracellular polysaccharide antigen that is present on the surface of arthroconidia and hyphae of *G. candidum*. The MAb was used in a highly specific Enzyme-Linked Immunosorbent Assay (ELISA) to accurately detect the fungus in infected tomato fruit and juice. Specificity of the ELISA was confirmed by sequencing of the internally transcribed spacer (ITS) 1-5.8S-ITS2 rRNA-encoding regions of fungi isolated from naturally-infected tomatoes.

Keywords: Geotrichum candidum, hybridoma, monoclonal antibody, enzyme-linked immunosorbent assay

46 **1. Introduction**

47 *Geotrichum candidum* is a filamentous yeast-like fungus. It is a ubiquitous organism
48 found in a range of habitats including air, water, silage and soil (Butler and Eckert, 1962;
49 O'Brien et al., 2005) and also in foodstuffs such as milk, cheese and fermented milk products
50 (Boutrou and Guégen, 2005; Marcellino et al., 2001; Mistry, 2004; Pottier et al., 2008; Ruas-
51 Madiedo et al., 2006; Wouters et al., 2002). As a component of the normal microbiota of the
52 human digestive tract, it has been reported, albeit infrequently, as a cause of infection in
53 immuno-compromised patients (Farina et al., 1999; Vasei and Imanieh, 1999; Verghese and
54 Ravichandran, 2003; Andre et al., 2004; Sfakianakis et al., 2007; Henrich et al., 2009).

55 As a plant pathogen, *G. candidum* causes sour-rot of citrus fruit, tomatoes, carrot, and
56 other fruits and vegetables (Pitt and Hocking, 2009). Most susceptible to infection are ripe or
57 overripe fruits and vegetables, particularly those kept in moisture-holding packaging (Skavia et
58 al., 2004) and wounded tissue (Moline, 1984). Decay spreads rapidly, resulting in a sour-
59 smelling watery mass. While the fungus prefers high temperatures and humidity, it is active at
60 temperatures as low as 2°C. Consequently, the fungus is an important post-harvest storage
61 pathogen (Moline, 1984; Oladiran and Iwu, 1992; Skavia, 2004; Tournas, 2005). In addition, *G.*
62 *candidum* is the predominant contaminant on tomato processing equipment and is referred to as
63 "machinery mould" (Splittstoesser et al., 2006). As a consequence, the fungus is used by
64 regulatory inspectors as a microbiological criterion to assess sanitation of equipment in fruit and
65 vegetable processing plants and in bottling works (Xu and Hang, 1988).

66 While microscope and culture-based mould counts and a lectin-mediated chitin-binding
67 assay can be used to assess the total fungal burden in raw tomato juice (Potts et al., 2000, 2001;
68 AOAC, 2010), these tests are not sufficiently specific to discriminate between different

contaminating organisms. As an alternative to mould counts, an enzyme immunoassay using polyclonal antiserum was developed to quantify mould content of tomato paste, but the antibodies cross-reacted with all of the major tomato spoilage organisms (Robertson and Patel, 1989). Because of this, there is a pressing need for the development of a test that allows the specific detection of *G. candidum* during the production, harvesting, post-harvest handling and processing of tomatoes.

Hybridoma technology allows the production of monoclonal antibodies (MAbs) that are specific to individual genera, species or even isolates of fungi (Thornton et al., 2002; Thornton, 2008; Thornton, 2009). The aim of this paper is to describe the development of a MAb specific to *G. candidum* and its use to develop a highly specific and sensitive diagnostic assay (ELISA) for detection of the fungus in tomato fruit and juice.

92 **2. Materials and methods**

93 *2.1. Fungal culture*

94 All fungi (Table 1) were routinely cultured on malt yeast agar (MYA) slants under a 16 h
95 fluorescent light regime at 26 ^{o}C. MYA agar consisted of malt yeast (MY) medium
96 (bactopeptone, 0.5% w/v; malt extract, 1.0% w/v; glucose, 0.4% w/v; yeast extract, 0.4% w/v)
97 and agar, 2.0% w/v; and was adjusted to pH 7.3 with 1M NaOH prior to autoclaving at 121 ^{o}C
98 for 15 min.

99

100 *2.2. Development of MAbs, preparation of immunogen, and immunization regime*

101 Balb/c mice were immunized with soluble antigens prepared from lyophilized mycelium of
102 *G. candidum* (CBS strain 115.23). Arthroconidia were suspended in water after 5-d-old MYA
103 slant cultures were flooded with 5 mL dH_2O and gently agitated with an inoculation loop.
104 Conidial suspensions were then filtered through Miracloth to remove mycelium and transferred to
105 1.5 mL micro-centrifuge tubes. The conidia were washed three times with dH_2O by repeated
106 vortexing and centrifugation at 14,462 *g* for 5 min and finally suspended in dH_2O to give a
107 concentration of 10^6 conidia/mL solution. Flasks containing 150 mL of MY medium were
108 inoculated with 200 mL of the conidial suspension and incubated with shaking (150 rpm) for 48 h
109 at 26 ^{o}C. The contents of the flasks were snap frozen in liquid N_2, and lyophilized. Lyophilized
110 material was suspended in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM
111 Na_2HPO_4, and 1.5 mM KH_2PO_4 [pH7.2]) and the resultant suspension centrifuged for 5 min at
112 14,462 *g*. The supernatant, containing solubilized antigens, was used as the immunogen and as a
113 source of antigens for hybridoma screening assays. For immunization, 6-wk-old BALB/c female
114 white mice were given four intraperitoneal injections (300 µL per injection) of antigen extract

115 containing 2.3 mg protein per mL PBS at 2-wk intervals and a single booster injection five days
116 before fusion.
117

118 *2.3. Production and screening of hybridomas and determination of antibody specificity*

119 Hybridoma cells were produced by the method described elsewhere (Thornton, 2001) and
120 the supernatants were screened by enzyme-linked immunosorbent assay (ELISA) against
121 antigens immobilized to the wells of Maxisorp microtitre plates (Nunc; 442404)(50 μL per
122 well). For antibody specificity tests, fungi were grown on MYA slopes and surface washings
123 prepared as described elsewhere (Thornton, 2009). Protein concentrations, determined
124 spectrophotometrically at 280 nm (Nanodrop, Agilent Technologies Limited, Berkshire, UK),
125 were adjusted to 60 μg/mL buffer. Fifty μL volumes were then used to coat the wells of
126 microtitre plates. After incubating overnight at 4 ^{o}C, wells were washed four times with PBST
127 (PBS containing Tween 20, 0.05% v/v) and once each with PBS and dH_2O and air-dried at 23
128 ^{o}C in a laminar flow hood. The plates were stored in sealed plastic bags at 4 ^{o}C in preparation
129 for screening of hybridoma supernatants by ELISA as described below.
130

131 *2.4. Enzyme-Linked Immunosorbent Assay*

132 Wells containing immobilized antigens were incubated successively with hybridoma
133 supernatant for 1 h, followed with goat anti-mouse polyvalent (immunoglobulin classes IgG,
134 IgA, and IgM) peroxidase conjugate (Sigma Chemical Company, Poole, United Kingdom; A-
135 0412) diluted 1 in 1000 in PBST for a further hour. Bound antibody was visualized by
136 incubating wells with tetramethyl benzidine (Sigma; T-2885) substrate solution (Thornton,
137 2001) for 30 min. The reactions were stopped by the addition of 3 M H_2SO_4. Absorbance values

138 were determined at 450 nm with an MRX automated microplate reader (Dynex Technologies,
139 Billingshurst, UK). Wells were given four 5-min rinses with PBST between incubations.
140 Working volumes were 50 μL per well, and control wells were incubated with tissue culture
141 medium (TCM) containing 10% (v/v) fetal bovine serum. All incubation steps were performed
142 at 23 ^{o}C in sealed plastic bags. The threshold for detection of the antigen in ELISA was
143 determined from control means (2 x TCM absorbance values)(Sutula et al., 1986). These values
144 were consistently in the range 0.050-0.100. Consequently, absorbance values >0.100 were
145 considered as positive for the detection of antigen.

146

147 *2.5. Determination of Ig subclass and cloning procedure*

148 The Ig class of MAbs was determined by using antigen-mediated ELISA. Wells of
149 microtitre plates coated with *G. candidum* antigens were incubated successively with hybridoma
150 supernatant for 1 h, followed with goat anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, or IgA-
151 specific antiserum (Sigma; ISO-2) diluted 1 in 3000 in PBST for 30 min and rabbit anti-goat
152 peroxidase conjugate diluted 1 in 1000 (Sigma; A-5420) for a further 30 min. Bound antibody
153 was visualized with TMB substrate as described above. Hybridoma cells lines were sub-cloned
154 three times by limiting dilution, and cell lines were grown in bulk in a non-selective medium
155 preserved by slowly freezing in fetal bovine serum/dimethyl sulfoxide (92:8 [v/v]), and stored in
156 liquid nitrogen.

157

158 *2.6. Epitope characterization by periodate oxidation*

159 Microtitre wells containing immobilized antigens were treated with sodium metaperiodate
160 (20 mM $NaIO_4$ in 50 mM sodium acetate buffer [pH 4.5]) whereas control wells received only

161 buffer. After incubation for the appropriate time period in darkness at 4 ^{o}C, wells were washed
162 three times with PBS and assayed by ELISA with MAb FE10 as described above. There were
163 four replicates for each treatment.
164
165 *2.7. Epitope characterization by protease digestion*
166 Immobilised antigens were incubated with pronase (Sigma, Protease XIV; P-5147)(0.25 U
167 per well) or trypsin (Sigma; T-7168) solution (1 mg/mL in PBS) at 37 ^{o}C or 4 ^{o}C for 5 h and
168 washed three times with PBS. Wells incubated with trypsin were treated for 10 min with a 0.1
169 mg/mL solution of trypsin inhibitor (Sigma) and given three more washes with PBS. Controls
170 received PBS without pronase or trypsin and inhibitor but were otherwise treated similarly. The
171 wells were assayed by ELISA with MAb FE10 as described above. There were six replicates for
172 each treatment.
173
174 *2.8. Immunofluorescence microscopy of arthroconidia and hyphae*
175 For IF studies, washed arthroconidia of *G. candidum* were suspended in sterile filtered
176 (0.2 μM) 1% (w/v) glucose solution and transferred to the wells of multi-well slides. After
177 incubation at 26 ºC for 16 h, slides were air-dried and fixed as described elsewhere (Thornton,
178 2001). Wells were incubated for 1 h with 50 μL of MAb FE10 or TCM only. Slides were
179 washed three times with PBS with gentle agitation and incubated for a further 30 min with goat
180 anti-mouse polyvalent FITC conjugate (Sigma; F-1010) diluted 1 in 40 in PBS. After the slides
181 were given three 5-min rinses with PBS, the wells were overlaid with coverslips mounted in
182 Fluoromount (Sigma; F-4680). Slides were examined with a Zeiss Axiophot microscope fitted
183 with epifluorescence, using a UV excitation filter of 365 nm and an absorption filter of 420 nm.

184 All incubation steps were performed at 23 °C in a moist environment and slides were stored at 4

185 °C in the dark in Petri dishes containing moistened Whatman filter paper no. 1.

186

187 *2.9. Polyacrylamide gel electrophoresis, Western blotting and ELISA of artificially infested*

188 *tomato juice*

189 Flasks containing 150 mL commercially processed tomato juice (containing lemon juice

190 and 7.2 g/L salt) were autoclaved for 15 min at 121 °C and were then inoculated with 10, 100 or

191 1000 washed arthroconidia of *G. candidum*/ml juice. Spore concentrations were quantified by

192 using a haemocytometer. Controls consisted of autoclaved tomato juice only. There were 3

193 replicate flasks per treatment and flasks were incubated at 26 °C with shaking. At 24 h and 48 h

194 post inoculation, 1 mL samples were removed, centrifuged at 14,462 *g* for 5 min and the

195 supernatants containing soluble antigens analysed by Western blotting and ELISA.

196 For SDS-PAGE, supernatants from flasks inoculated with 1000 conidia/ml juice were

197 mixed with Laemmli buffer and denatured by heating at 95 °C for 10 min in the presence of β-

198 mercaptoethanol prior to gel loading. Polyacrylamide gel electrophoresis (PAGE) was carried

199 under denaturing conditions, with 4-20% (w/v) gradient polyacrylamide gels (Bio-Rad

200 Laboratories Limited, Hemel Hempstead, UK). Proteins were separated for 1.5 h at 23 °C

201 (165V) and pre-stained, broad range markers (Bio-Rad) were used for molecular mass

202 determinations. For Westerns, separated proteins were transferred electrophoretically to a PVDF

203 membrane (Bio-Rad). The membranes were blocked for 16 h at 4 °C with PBS containing 1%

204 (w/v) bovine serum albumin (BSA) and incubated with MAb FE10 supernatant diluted 1 in 2

205 with PBS containing 0.5% (wt/vol) BSA (PBSA) for 2 h at 23 °C. After washing three times

206 with PBS, membranes were incubated for 1 h with goat anti-mouse IgM (μ-chain specific)

207 alkaline phosphatase conjugate (Sigma; A-9688), diluted 1 in 15,000 in PBSA. After
208 membranes were washed twice with PBS and once with PBST, the bound antibodies were
209 visualized by incubation in substrate solution (Thornton, 2008, 2009). Reactions were stopped
210 by immersion in dH_2O and air-dried between sheets of Whatman filter paper.

211 For ELISA, dilution series of soluble antigens were prepared by double diluting
212 supernatants into 50 μL volumes of PBS in the wells of microtitre plates. Thereafter, the plates
213 were washed and assayed by ELISA as described above. The threshold absorbance value for
214 antigen detection at each dilution was determined with absorbance values obtained from dilution
215 series of uninoculated tomato juice.

216

217 *2.10. Detection of Geotrichum candidum in naturally infected tomato fruit by ELISA and*
218 *identification of fungi by analysis of the internally transcribed (ITS) regions of the rRNA-*
219 *encoding gene unit*

220 Fully ripe packaged tomatoes were purchased from supermarkets (Tesco and Sainsbury's
221 stores, Exeter, UK). Eight rotting fruit and 4 control (uninfected) fruit with no visible fungal
222 growth, each weighing approximately 14 g, were placed in 10 mL PBS in sterile 50 mL Falcon
223 tubes and shaken vigorously for 1 h at 23 ºC. To isolate fungi, 300 μL samples of the fruit
224 suspensions were spread on the surface of MYA using a glass spreader. After 2 days of
225 incubation at 23 ºC, axenic cultures of individual species were prepared by sub-culture onto
226 fresh MYA plates. Fungi were identified by internally transcribed spacer (ITS) region
227 sequencing according to procedures described elsewhere (Thornton, 2009). For ELISA, fruit
228 suspensions were centrifuged at 14,462 *g* for 5 min and the supernatants containing solubilized

229 antigens transferred to the wells of Maxisorp microtitre plates (50 μL per well) for assay as

230 described.

231

232 *2.11. Nucleotide sequence accession numbers*

233 Newly determined ITS sequences of fungi recovered from infected tomatoes were

234 submitted to GenBank, and accession numbers HM210832 to HM210843 were obtained.

235

236

237

238

239

240

241

242

243

244

245

246

247

248

249

250

251 **3. Results**

252 *3.1. Production of hybridoma cell lines, isotyping of MAb and specificity*

253 A single fusion was performed and 403 hybridomas were screened for MAb production. A
254 single cell line, FE10, produced MAb belonging to the immunoglobulin class M (IgM), which
255 reacted strongly in ELISA tests with antigens from *G. candidum*. Monoclonal antibody FE10 was
256 tested further for specificity against a wide range of related and unrelated fungi. The MAb was
257 highly specific, reacting with antigens from *G. candidum*, *Geotrichum pseudocandidum* and
258 *Geotrichum europaeum* only. It did not react with antigens from a wide range of unrelated
259 species, including fungi and oomycetes commonly recovered from infected tomatoes. Results of
260 ELISA specificity tests with MAb FE10 are shown in Table 1.

261

262 *3.2. Characterization of antigen by using periodate and proteases*

263 The epitope bound by MAb FE10 was periodate-sensitive (Table 2), with almost complete
264 elimination of antibody binding in ELISA following periodate treatment of immobilized antigens.
265 This shows that the antibody recognizes carbohydrate residues containing vicinal hydroxyl
266 groups. There was no significant effect of pronase or trypsin on MAb binding, showing that the
267 epitope does not contain protein moieties (Table 3).

268

269 *3.3. Localization of antigen by immunofluorescence microscopy*

270 Immunofluorescence microscopy studies with MAb FE10, showed that the antigen is
271 present on the surface of ungerminated arthroconidia and on the hyphal surface of *G. candidum*
272 germlings (Fig. 1).

273

274 *3.4. Polyacrylamide gel electrophoresis, Western blotting, and ELISA of artificially infested*
275 *tomato juice*

276 Based on Western blotting analysis of 1-d-old and 2-d-old extracts from inoculated tomato
277 juice, MAb FE10 binds to an extracellular antigen(s) with a molecular mass >200 kDa (Fig. 2).
278 There was no reaction of MAb FE10 with extracts from control (uninoculated) tomato juice,
279 showing that the antibody is specific for the fungal antigen and does not exhibit false positive
280 reactivity with tomato antigens.

281 ELISA tests using extracts from tomato juice inoculated with the fungus showed strong
282 detection of the FE10 exo-polysaccharide antigen in cultures inoculated with 10, 100 and 1000
283 arthroconidia/mL juice, 24 h and 48 h post inoculation (Fig. 3). However, this required dilution of
284 the extracts into PBS. Absorbance values increased with successive dilution of extracts, with
285 optimum values obtained at approximately 1 in 256 dilutions. Absorbance values of control
286 (uninoculated) tomato juice extracts were consistently <0.050 at all dilutions on both days of
287 sampling (only results for 2-d-old control cultures are shown). These results showed that the
288 ELISA is specific for the fungal antigen and confirmed results of Western blotting tests that no
289 false positive reactivity is exhibited with tomato antigens.
290

291 *3.5. Immunoassay of tomato fruits*

292 Eight tomatoes with visible evidence of fungal colonization (fruits 1 to 8) and four control
293 tomatoes with no visible colonization (fruits 9 to 12) were obtained from supermarkets and were
294 used to test the specificity of MAb FE10. Only two tomatoes (1 and 2) were colonized with
295 *Geotrichum candidum*, the isolates being present as co-contaminants with other fungi, and whose
296 identity was determined by ITS sequencing (Table 4). These two tomatoes were positive for the

297 FE10 antigen (absorbance values >1.000), compared to the remaining six tomatoes (3 to 8), which
298 were colonised by unrelated fungi and which gave absorbance values <0.100. ELISA tests of
299 antigens from axenic cultures of the fungi (Table 1) showed that only the two *G. candidum*
300 isolates (GC1 and GC2) were positive for the FE10 antigen (absorbance values 1.182 and 1.207
301 respectively). None of the other fungi or control (uninfected) tomatoes reacted with MAb FE10;
302 thus, the positive ELISA results for tomatoes 1 and 2 were apparently due to colonization by *G.*
303 *candidum*.
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319

320 **4. Discussion**

321 The fungus *Geotrichum candidum* causes sour-rot of fruit and vegetables and is an
322 important post-harvest storage pathogen. The fungus is the predominant contaminant on tomato
323 processing equipment, but its presence is not restricted to the equipment within the processing
324 plant, being found on harvesting equipment, and in field to factory transport gondolas. In this
325 regard, a highly specific assay for *G. candidum* would be a useful management tool to help
326 tomato producers and processors to track the fungus and to distinguish it from other types of
327 fungal infections in order to determine the most appropriate course of action (equipment
328 sanitation versus tomato field fungicide applications).

329 This paper describes the generation of an immunoglobulin M (IgM) monoclonal antibody
330 (MAb) against an extracellular antigen from the sour-rot pathogen *Geotrichum candidum* and its
331 use in the development of a *Geotrichum*-specific immuno-diagnostic assay. The MAb displays a
332 high degree of specificity, reacting with *G. candidum* and the closely related teleomorphic species
333 *Galactomyces geotrichum* and anamorphic species *Geotrichum europaeum* and *Geotrichum*
334 *pseudocandidum* in the *Galactomyces geotrichum/G. candidum* complex (De Hoog and Smith,
335 2004; Pottier et al., 2008). The MAb does not cross-react with a wide range of unrelated fungi,
336 some of which are likely to be encountered during tomato production and processing, or with the
337 tomato bacterial pathogens *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae*
338 pv. tomato (results not presented).

339 The MAb was used to develop a highly specific enzyme-linked immunosorbent assay
340 (ELISA) for detection of the fungus in tomato juice and fruit. Analytical sensitivity of the assay
341 was determined in tomato juice artificially infested with washed arthroconidia of the fungus,
342 which allowed the fungus to proliferate and produce extracellular antigen for detection by

343 Western blotting and ELISA. Western blotting tests of tomato juice extracts showed that the MAb
344 bound to an extracellular antigen with a molecular weight of >200 kDa. The smeared appearance
345 of the antigen in Western blots is characteristic of an exo-polysaccharide, and the carbohydrate
346 nature of the antigen was confirmed in ELISA tests, using periodate treated antigens, which
347 showed that the MAb binds to a carbohydrate epitope containing vicinal hydroxyl groups.
348 Optimum detection of the antigen by ELISA required dilution of juice extracts. The increases in
349 absorbance values in response to extract dilutions was most likely due to steric hindrance of
350 antibody-epitope binding at high concentrations of the antigen (Harlow and Lane, 1999). Despite
351 the need to dilute samples to optimize antigen detection, the ELISA was able to detect production
352 of the antigen within 24 h of inoculation of tomato juice with 10 arthroconidia/ml juice. No
353 antigen was detected in control (uninoculated) tomato juice extracts, showing that the MAb FE10
354 is specific for the fungus and does not exhibit false positive reactivity with tomato antigens. A
355 limitation of the assay for detecting the pathogen in tomato juice is the requirement for fungal
356 proliferation to allow biological amplification of the antigen for detection. While such a process of
357 biological amplification ensures that only viable fungal propagules are detected, detection of the
358 antigen in juice prior to the biological amplification step, would not guarantee differentiation of
359 viable versus dead propagules. However, it would indicate contamination by the pathogen during
360 juice production.

361 The accuracy of the ELISA in detecting *G. candidum* was confirmed using antigen extracts
362 from tomato fruits purchased from supermarkets that were naturally infected with fungi, and
363 subsequent identification of recovered isolates using ITS sequencing. The ELISA detected *G.*
364 *candidum* only. No reaction was found with other contaminant fungi including species that are
365 known pathogens of tomatoes, including *Alternaria alternata*, *Alternaria tenuissima*, *Aspergillus*

366 *niger*, *Botryotinia fuckelinia* (*Botrytis cinerea*), and *Penicillium* species (Oladiran and Iwu, 1993;

367 Abdel-Mallek et al., 1995).

368 While microscope and culture-based mould counts, a lectin-mediated chitin-binding assay

369 and polyclonal antibody immunoassay have been developed for the detection of fungi in

370 processed tomatoes, their lack of specificity means that they are unable to differentiate between

371 the major tomato spoilage organisms (Robertson and Patel, 1989; Potts et al., 2001, 2002;

372 AOAC, 2010). We have used hybridoma technology to develop a highly specific monoclonal

373 antibody-based diagnostic assay that can be used to detect a single pathogen of tomato,

374 *Geotrichum candidum*, in fruit and juice, and to discriminate its presence from other spoilage

375 organisms. This is the first time, to our knowledge, that a *Geotrichum*-specific assay has been

376 reported. Our objective is to incorporate the MAb into a lateral-flow device format (Thornton,

377 2008) to allow rapid on-site detection of the pathogen during the production, harvesting, post-

378 harvest handling and processing of tomatoes.

379

380

381 **Acknowledgements**

382 This work was funded by the Californian Processing Tomato Advisory Board (PTAB), to

383 whom we are grateful.

384

385

386

387

388

389 **References**

390 Abdel-Mallek, A.Y., Hemida, S.K., Bagy, M.M.K., 1995. Studies on fungi associated with tomato
391 fruits and effectiveness of some commercial fungicides against three pathogens.
392 Mycopathologia 130, 109-116.

393 Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped
394 BLAST and PSI-BLAST: A new generation of protein database programs. Nucleic Acids
395 Research 25, 3389-3402.

396 Andre, N., Coze, C., Gentet, J.-C., Perez, R., Bernard, J.-L., 2004. *Geotrichum candidum*
397 septicemia in a child with hepatoblastoma. Pediatric Infectious Diseases Journal 23, 86.

398 AOAC. 2010. Mold and rot fragments. Official Methods of Analysis 18th Ed Revision 3. AOAC
399 International. Gaithersburg, Maryland, USA.

400 Butler, E.E., Eckert, J.W., 1962. A sensitive method for the isolation of *Geotrichum candidum*
401 from soil. Mycologia 54, 106-109.

402 Boutrou, R., Guégen, M., 2005. Interests in *Geotrichum candidum* for cheese technology.
403 International Journal of Food Microbiology 102, 1-20.

404 De Hoog, G.S., Smith, M.T., 2004. Ribosomal gene phylogeny and species delimitation in
405 *Geotrichum* and its teleomorphs. Studies in Mycology 50, 489-515.

406 Farina, C., Vailati, F., Manisco, A., Goglio, A., 1999. Fungaemia survey: a 10-year experience in
407 Bergamo, Italy. Mycoses 42, 543-548.

408 Harlow, E., Lane, D., 1999. Antibody-Antigen Interactions. In: Using Antibodies: A Laboratory
409 Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

410 Henrich, T.J., Marty, F.M., Milner, D.A., Thorner, A.R., 2009. Disseminated *Geotrichum*
411 *candidum* infection in a patient with relapsed acute myelogenous leukemia following

412 allogeneic stem cell transplantation and review of the literature. Transplant Infectious
413 Diseases 11, 458-462.
414 Marcellino, N., Beuvier, E. Grappin, R., Guégen, M., Benson, D.R., 2001. Diversity of
415 *Geotrichum candidum* strains isolated from traditional cheese making fabrications in
416 France. Applied and Environmental Microbiology 67, 4752-4759.
417 Mistry, V.V., 2004. Fermented liquid milk products, In: Hui, Meunier-Goddik, Hansen,
418 Josephsen, Nip, Stanfield & Toldrá (Eds.), Handbook of food and beverage fermentation
419 technology, Marcel Dekker Inc., New York.
420 Moline, H.E., 1984. Comparative studies with two *Geotrichum* species inciting post harvest
421 decays of tomato fruit. Plant Disease 68, 46-48.
422 O'Brien, M., O'Kiely, P., Forristal, P.D., Fuller, H.T., 2005. Fungi isolated from contaminated
423 bailed grass silage on farms in the Irish Midlands. FEMS Microbiology Letters 247, 131-
424 135.
425 Oladiran, A.O., L.N. Iwu., 1993. Studies on the fungi associated with tomato fruit rots and effects
426 of environment on storage. Mycopathologia 121, 157-161.
427 Pitt, J.I., Hocking, A.D., 2009. Fungi and Food Spoilage. Springer, London, United Kingdom.
428 Pottier, I., Gente, S., Vernoux, J.-P., Gueguen, M., 2008. Safety assessment of dairy
429 microorganisms: *Geotrichum candidum*. International Journal of Food Microbiology 126,
430 327-332.
431 Potts, S.J., Slaughter, D.C., Thompson, J.F., 2000. A fluorescent lectin assay for the rapid
432 quantification of mold in tomato products. Journal of Food Science 65, 346-350.
433 Potts, S.J., Slaughter, D.C., Thompson, J.F., 2001. Measuring mold infestation in raw tomato
434 juice. Journal of Food Science 67, 321-325.

435 Robertson, A., Patel, N., 1989. Development of an enzyme immunoassay for the qunatification of
436 mould in tomato paste – an alternative to the Howard mould count. Food and Agricultural
437 Immunology 1, 73-81.
438 Ruas-Madiedo, P., Gueimonde, M., de los Reyes-Gavilán, C.G., Salminen, S., 2006. Short
439 communication: Effect of exopolysaccharide isolated from "viili" on the adhesion of
440 probiotics and pathogens to intestinal mucus. Journal of Dairy Science 89, 2355-2358.
441 Sfakianakis, A., Krasagakis, K., Stefanidou, M., Maraki, S., Koutsopoulos, A., Kofteridis, D.,
442 Samonis, G., Tosca, A., 2007. Invasive cutaneous infection with *Geotrichum candidum*:
443 sequential treatment with amphotericin B and voriconazole. Medical Mycology 45, 81-84.
444 Skavia, M., 2004. Spread of post harvest diseases, In: Naqvi (Ed.), Diseases of fruit and
445 vegetables, diagnosis and management, p. 328. Kluwer Academic Publishers, The
446 Netherlands.
447 Splittstoesser, D.F., Bowers, J., Kerschner, L., Wilkinson, M., 2006. Detection and incidence of
448 *Geotrichum candidum* in frozen blanched vegetables. Journal of Food Science 45, 511-513.
449 Sutula, C.L., Gillett, J.M., Morrisey, S.M., Ramsdell, D.C., 1986. Interpreting ELISA data and
450 establishing the positive-negative threshold. Plant Disease 70, 722-726.
451 Thornton, C.R., 2001. Immunological methods for fungi, In: Talbot (Ed.), Molecular and cellular
452 biology of filamentous fungi, a practical approach, pp. 227-257. University Press, Oxford,
453 United Kingdom.
454 Thornton, C.R., 2008. Development of an immunochromatographic lateral-flow device for rapid
455 serodiagnosis of invasive aspergillosis. Clinical and Vaccine Immunology 15, 1095-1105.
456 Thornton, C.R., 2009. Tracking the emerging human pathogen *Pseudallescheria boydii* by using
457 highly specific monoclonal antibodies. Clinical and Vaccine Immunology 15, 1095-1105.

458 Thornton, C.R., Pitt, D., Wakley, G.E., Talbot, N.J., 2002. Production of a monoclonal antibody
459 specific to the genus *Trichoderma* and closely related fungi, and its use to detect
460 *Trichoderma* spp. in naturally infested composts. Microbiology 148, 1263-1279.
461 Tournas, V.H., 2005. Spoilage of vegetable crops by bacteria and fungi and related health hazards.
462 Critical Reviews in Microbiology 31, 33-44.
463 Vasei, M., Imanieh, M.H., 1999. Duodenal colonization by *Geotrichum candidum* in a child with
464 transient low serum levels of IgA and IgM. APMIS 107, 681-684.
465 Verghese, S., Ravichandran, P., 2003. *Geotrichum candidum* infection in a renal transplant
466 recipient. Indian Journal of Nephrology 13, 72-74.
467 Wouters, J., Ayad, E., Hugenholtz, J., Smit, G., 2002. Microbes from raw milk for fermented dairy
468 products. International Dairy Journal 12, 91-109.
469 Xu, W.-Q., Hang, Y.D., 1989. Inhibition of *Geotrichum candidum* by bicarbonate. World Journal
470 of Microbiology and Biotechnology 5, 109-113.
471
472
473
474
475
476
477

478 Table 1. Details of fungi and results of ELISA specificity tests using MAb FE10

479

Organism	Isolate no.	Source[a]	Absorbance (450nm)[b]
Alternaria alternata	117143	CBS	0.061
Alternaria alternata[c]	AA1 (HM210840)	CRT	0.043
Alternaria arborescens	102605	CBS	0.029
Alternaria tenuissima	966.95	CBS	0.049
Arxula adeninivorans	L7C (FJ713087)	CRT	0.034
Aspergillus flavus	91856iii	IMI	0.007
Aspergillus fumigatus	AF293	SK	0.059
Aspergillus niger	102.40	CBS	0.027
Aspergillus niger[c]	AN1 (HM210842)	CRT	0.016
Aspergillus niger[c]	AN2 (HM210843)	CRT	0.016
Aspergillus terreus	601.65	CBS	0.063
Botryotinia fuckelinia[c]	BF1 (HM210833)	CRT	0.019
Botryotinia fuckelinia[c]	BF2 (HM210841)	CRT	0.034
Botrytis cinerea	121.39	CBS	0.000
Candida albicans	SC5314	SB	0.033
Candida catenulata	L1D (FJ713083)	CRT	0.035
Candida dubliniensis	8500	CBS	0.032

501

502 Table 1 - *Continued*

503

504	Organism	Isolate no.	Source[a]	Absorbance (450nm)[b]
505				
506	*Candida glabrata*	4692	CBS	0.031
507	*Cladosporium cladosporioides*	113.29	CBS	0.047
508	*Cladosporium cladosporioides*[c]	CC1 (HM210839)	CRT	0.022
509	*Cladosporium herbarum*	159.59	CBS	0.046
510	*Collectotrichum coccodes*	CCP	CRT	0.034
511	*Collectotrichum fructigenum*	490.92	CBS	0.027
512	*Corynespora cassiicola*	296.80	CBS	0.068
513	*Emericella nidulans*	A4	FGSC	0.000
514	*Filobasidium neoformans*	7779	CBS	0.044
515	*Fusarium oxysporum* f. sp. *melonis*	422.90	CBS	0.024
516	*Fusarium oxysporum* f. sp. *pisi*	260.50	CBS	0.023
517	*Fusarium solani*	FS1	CRT	0.033
518	*Geotrichum candidum*[c]	GC1 (HM210832)	CRT	1.182
519	*Geotrichum candidum*[c]	GC2 (HM210837)	CRT	1.207
520	*Geotrichum candidum*	115.23	CBS	1.369
521	*Geotrichum candidum*	298.74	CBS	1.286
522	*Geotrichum europaeum*	866.68	CBS	1.182
523	*Geotrichum pseudocandidum*	820.71	CBS	1.218

524

525 Table 1 - *Continued*

526

527	Organism	Isolate no.	Source[a]	Absorbance (450nm)[b]
528				
529	*Kluyveromyces nonfermentans*	L10A (FJ713088)	CRT	0.037
530	*Monilinia fructigena*	MF1	CRT	0.037
531	*Mucor circinelloides*	T13A (FJ713074)	CRT	0.030
532	*Mucor racemosus*	93	CRT	0.060
533	*Mycocladus corymbiferus*	T14A (FJ713070)	CRT	0.050
534	*Mycovellosiella fulva*	119.46	CBS	0.033
535	*Penicillium biourgeianum*[c]	PB1 (HM210835)	CRT	0.041
536	*Penicillium brevicompactum*[c]	PBC1 (HM210834)	CRT	0.009
537	*Penicillium brevicompactum*[c]	PBC2 (HM210836)	CRT	0.027
538	*Penicillium citrinum*	139.45	CBS	0.021
539	*Penicillium digitatum*	319.48	CBS	0.044
540	*Penicillium islandicum*	338.48	CBS	0.035
541	*Penicillium italicum*	490.75	CBS	0.028
542	*Penicillium olsonii*[c]	PO1 (HM210832)	CRT	0.023
543	*Penicillium roqueforti*	221.30	CBS	0.000
544	*Penicillium variabile*	385.48	CBS	0.070
545	*Phoma exigua* var. *exigua*	119.94	CBS	0.044
546	*Phoma destructiva*	378.73	CBS	0.033

547

548 Table 1 - *Continued*

549

550	Organism	Isolate no.	Source[a]	Absorbance (450nm)[b]
551				
552	*Phytophthora nicotianae*	294.35	CBS	0.015
553	*Pichia fermentans*	L1B (FJ713081)	CRT	0.027
554	*Pichia spartinae*	L12A (FJ713090)	CRT	0.021
555	*Rhizoctonia solani*	277.38	CBS	0.002
556	*Rhizomucor miehei*	MG4(2)(FJ713069)	CRT	0.080
557	*Rhizomucor pusillus*	T3B (FJ713079)	CRT	0.057
558	*Rhizomucor tauricus*	LB1A (FJ713094)	CRT	0.061
559	*Rhizopus oryzae*	395.54	CBS	0.015
560	*Rhizopus stolonifer*	G1	CRT	0.008
561	*Saccharomyces cerevisiae*	1240	CBS	0.050
562	*Saccharomyces paradoxus*	T7B (FJ713072)	CRT	0.036
563	*Sclerotinia sclerotiorum*	SS1 (FJ984493)	CRT	0.000
564	*Stemphylium lycopersici*	321.87	CBS	0.016
565	*Trichoderma hamatum*	GD12 (AY247559)	CRT	0.029
566	*Trichoderma harzianum*	102174	CBS	0.011
567	*Ulocladium consortiale*	105.31	CBS	0.008
568	*Ulocladium tuberculatum*	122327	CBS	0.049

569

570 [a] CBS = Centraalbureau voor Schimmelcultures, PO Box 85167, 3508 AD Utrecht, The
571 Netherlands; CRT = C.R. Thornton; IMI = International Mycological Institute, Egham, England; SB
572 = S. Bates, School of Biosciences, University of Exeter; SV = S. Krappman, Institute of
573 Microbiology and Genetics, Department of Molecular Microbiology and Genetics, Georg-August-
574 University, Gottingen, Germany.

575 [b] Each value represents the mean of replicated samples. The threshold absorbance value for
576 detection of antigen in ELISA is ≥0.100.

577 [c] Fungi recovered in this study from naturally infested tomatoes and identified by internal
578 transcribed spacer sequencing of rDNA. GenBank accession numbers are shown in parentheses.

579

580

581

582

583

584

585

586

587

588

589

590

591

592

593
Table 2. Absorbance values from ELISA tests with periodate-treated antigens by using MAb FE10

594
595
596
597
598
599
600
601
602
603
604

MAb	Time (h)	Absorbance (450 nm)[a]	
		Periodate	Control
FE10	24	0.184±0.002	1.147±0.018
	4	0.179±0.003	1.128±0.014
	3	0.176±0.002	1.162±0.011
	2	0.179±0.003	1.186±0.009
	1	0.228±0.005	1.192±0.014

605
606
607
[a] Each value represents the mean of four replicate values ± standard errors. The differences in absorbance values between periodate and control treatments at each time point are significant at $p \leq 0.05$ (Student's t-test).

608
609
610
611
612
613
614
615
616

617 Table 3. Absorbance values from ELISA tests with protease-treated antigens by using MAb FE10

618

MAb	Temp (^{o}C)	Absorbance (450 nm)[a]			
		Pronase	Pronase control	Trypsin	Trypsin control
FE10	4	1.049±0.008	1.276±0.014	1.084±0.014	1.099±0.017
	37	1.002±0.011	1.229±0.016	1.122±0.011	1.193±0.017

619
620
621
622
623
624
625
626

627 [a] Each value represents the mean of six replicate values ± standard errors. No significant difference

628 between treatments at p≤0.05 (Student's t-test).

629
630
631
632
633
634
635
636
637
638
639
640

641 Table 4. ELISA absorbance values of extracts from infected tomato fruits and species designation of
642 recovered fungi as determined by ITS sequence analysis

643

644 645 646	Tomato	ELISA absorbance (450nm)[a]	Species[b]	Isolate	Genbank accession no.
647	1	1.146	*Geotrichum candidum*	GC1	HM210838
648			*Aspergillus niger*	AN1	HM210842
649			*Penicillium olsonii*	PO1	HM210832
650	2	1.008	*Geotrichum candidum*	GC2	HM210837
651			*Botryotinia fuckelinia*	BF1	HM210833
652	3	0.021	*Alternaria alternata*	AA1	HM210840
653	4	0.033	*Penicillium brevicompactum*	PBC1	HM210834
654	5	0.067	*Cladosporium cladosporioides*	CC1	HM210839
655	6	0.047	*Aspergillus niger*	AN2	HM210843
656	7	0.022	*Penicillium biourgeianum*	PB1	HM210835
657	8	0.031	*Penicillium brevicompactum*	PBC2	HM210836
658			*Botryotinia fuckelinia*	BF2	HM210841
659	9[c]	0.021	No fungi recovered	-	-
660	10[c]	0.027	No fungi recovered	-	-
661	11[c]	0.031	No fungi recovered	-	-
662	12[c]	0.014	No fungi recovered	-	-

663

664 [a] The threshold absorbance value for detection of antigen in ELISA is ≥0.100.

665 [b] Species identity was predicted based on >99% sequence identity of the internal transcribed spacer

666 (ITS)1–5.8S–ITS2 sequence of fungi (E-value = 0.0)(Altschul et al., 1997) to species recorded in

667 GenBank.

668 [c] Tomatoes 9 to 12 were control tomatoes that did not display visible evidence of fungal colonization.

669

670

671

672

673

674

675

676

677

678

679

680

681

682

683

684

685

686

687

688 **Figure legends**

689 Fig. 1. Photomicrographs of *G. candidum* cells immunostained with MAb FE10 or tissue culture
690 medium (as a control) and anti-mouse polyvalent Ig fluorescein isothiocyanate. (A). Germinating
691 arthroconidium of *G. candidum* examined under a bright-field microscope. (B) Same slide shown
692 in panel A but examined under epifluorescence with MAb FE10. Note intense staining of cell wall
693 of the arthroconidium and the hyphal cell wall and septa. (C) Un-germinated arthroconidia of *G.*
694 *candidum* examined under a bright-field microscope. (D) Same slide shown in panel C but
695 examined under epifluorescence with MAb FE10. (E) Tissue culture medium control slide
696 showing germinated arthroconidium of *G. candium*. (F) Same slide shown in panel G but
697 examined under epifluoresence. Bar, 18 μm.

698

699 Fig. 2. Analysis of *G. candidum* extracellular antigen by using denaturing SDS-PAGE and
700 Western blotting. Lanes M_r, molecular mass markers; lane A, Western immunoblot with MAb
701 FE10 of tomato juice extract (negative control); lane B, Western immunoblot with MAb FE10 of
702 antigen extract from a 1-d-old tomato juice culture of the fungus. lane C, Western immunoblot
703 with MAb FE10 of antigen extract from a 2-d-old tomato juice culture of the fungus. All wells
704 were loaded with 1.6 μg of protein. Note strong reaction of the MAb with extracts from inoculated
705 tomato juice (lanes B and C), but no reaction with tomato juice only (lane A).

706

707 Fig. 3. Enzyme-linked immunosorbent assay detection of the *G. candidum* extracellular antigen in
708 doubling dilutions of extracts from 1-d-old shake cultures of tomato juice inoculated with 10
709 arthroconidia/ml (●), 100 arthroconidia/ml (◆), or 1000 arthroconidia/ml (■) and from 2-d-old
710 cultures inoculated with 10 arthroconidia/ml (○), 100 arthroconidia/ml (◇), or 1000

711 arthroconidia/ml (☐). Controls consisted of doubling dilutions of extracts from flasks containing

712 uninoculated tomato juice only (△). Each point is the mean of the absorbance values from

713 replicate flasks ± standard error. Extract dilutions are shown as logarithms of the reciprocal

714 dilutions.

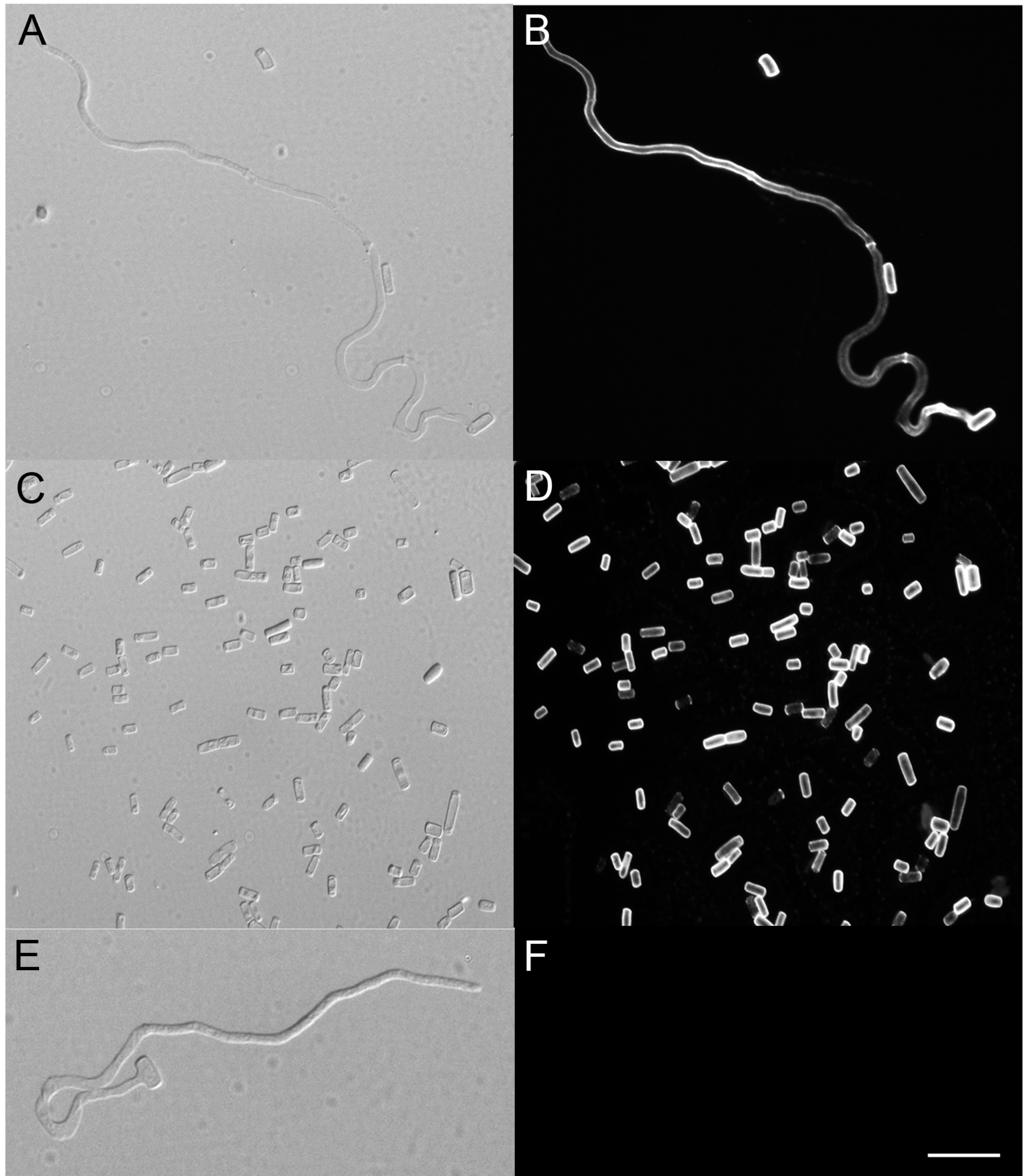

Figure 1. Thornton *et al*.

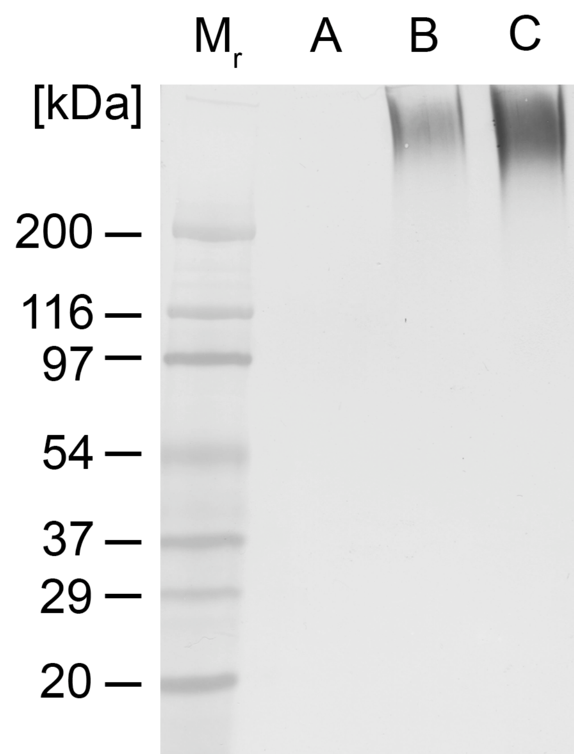

Figure 2. Thornton *et al.*

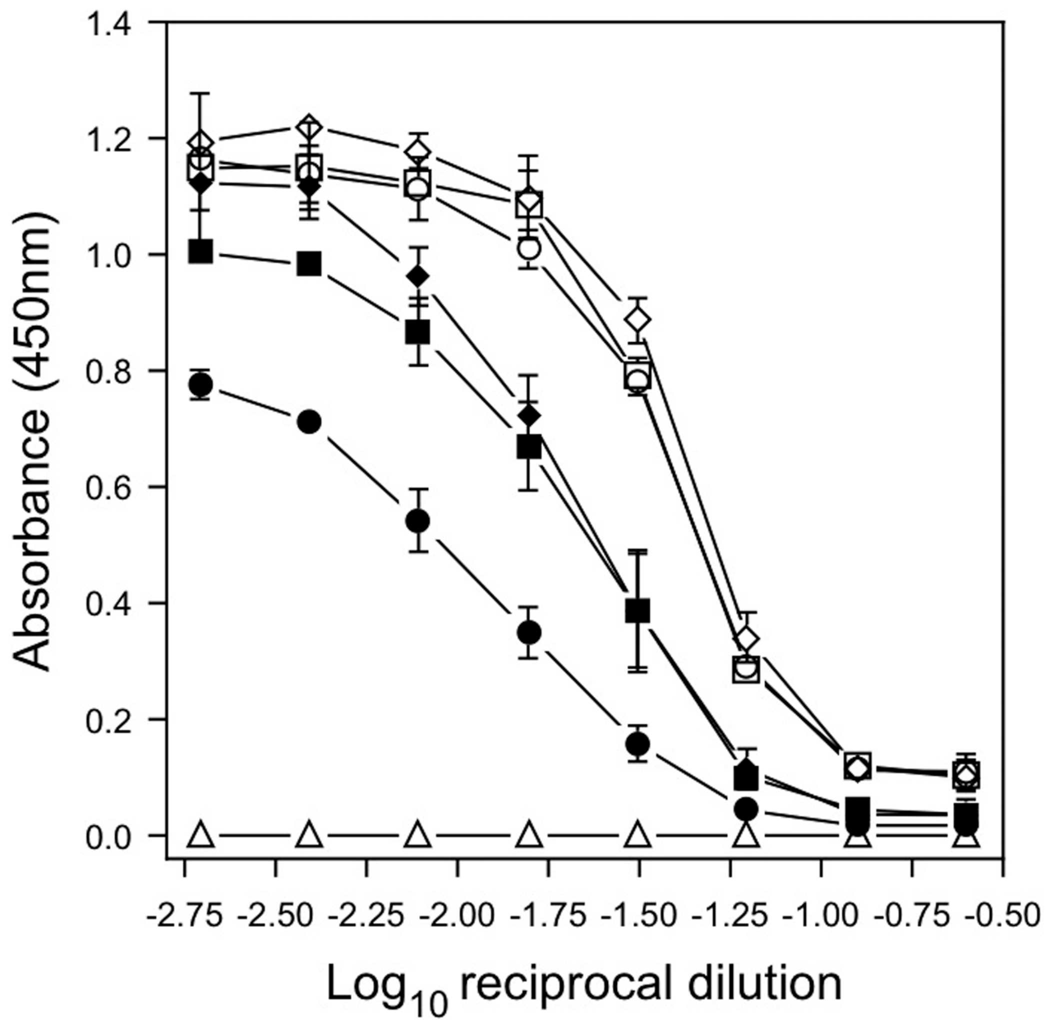

Figure 3. Thornton *et al*.

***Detailed Response to Reviewers**

Reviewer #1: Manuscript Number FOOF-D-10-0054

The manuscript is well written. It describes simply and logically the raising of a monoclonal antibody that specifically detects, by ELISA, Geotrichum candidum in tomato fruit in the presence of mixed fungal infections. An immunodiagnositic assay such as this is needed in the tomato processing industry. It is recommended that this manuscript be accepted for publication with the following minor additions, changes and/ or insertions:

1.The reason for not screening the specific monoclonal antibody against bacteria that may be involved in the sour rot complex should be addressed.

During specificity tests, MAb FE10 was tested for cross-reactivity with the tomato bacterial pathogens *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. tomato. I have added this information to the discussion (lines 334-336), but since MAb FE10 is fungus specific, I felt this information was not necessary in the original manuscript.

2.The insertion of the product numbers of all the Sigma anti- mouse antibodies used in the study should be included- see lines 133, 151, 152, 166, 167,180, 182, 207 and 209.

These insertions have now been made.

3.The source of tetramethyl benzidine, line 136, should be included.

This insertion has now been made.

4.The source and product number of the MaxiSorp 96-well microtitre plates line 122 should be included.

This has now been included.

5.Line 274 Fig. 2 should read Fig.1.

This has been corrected.

6.References:

a.no mention of the 2nd reference Altschul et al could be found in the text

This reference is in the footnote to Table 4. I have highlighted it.

b.references to Potts et al in the text lines 67 and 358 are dated 2001 and 2002 but in the reference section are dated 2000 and 2001

c.line 354 reference to Oladiran and Iwu is dated 1993 but in the reference section it is dated 1992.

These have now been corrected.

Reviewer #2: This manuscript describes the development of a monoclonal antibody-based ELISA technique for detecting Geotrichum species in tomatoes. Some discussion concerning practical application in tomato industry is encouraged. Additional comments are as follows:

Lines 94-98: I suggest that reference to Table 1 be made in this section. The reader will then know which fungi were included in the study.

Reference to Table 1 is now included (line 94).

Lines 106, 108, 112, 194, 227: State the gravity (g) used rather than rpm.

Rpm has been converted to *g*.

Line 207: Should be MY medium.

This has been corrected (line 107 though, not 207).

Line 190: Describe the tomato juice. Was it commercially processed juice from a can or was it fresh juice made in the authors' laboratory? If commercially processed juice was used, did it contain any added ingredients, e.g., NaCl?

Further details have now been included (please see lines 189-190).

Lines 220-222: I assume that tomatoes were fully ripe? On what basis were tomatoes judged "uninfected"? No visual fungal growth? What weight of fruit was placed in 10 ml of PBS? Shaking for 1 h seems excessive.

Further details have now been provided (please see lines 220-222).

Lines 217-229: This method does not quantitate Geotrichum or any other fungi. Nor does it reveal viable versus dead propagules. These limitations should be discussed later in the text when presenting detection data using the antibody-based ELISA method.

As requested by the reviewer, some discussion concerning practical applications in the tomato industry is now provided at the start of the discussion (lines 321-328) and also at the end of the discussion (lines 376-378).

The purpose of the test is to detect the fungus in tomato juice, not to quantify it. The test is specific to the target fungus *G. candidum*, and so will not detect any other fungi. This is a major benefit of the immunoassay.

The limitation of the test in discriminating viable versus dead propagules is now discussed on lines 354-360 of the discussion.

Line 233: Tables should be introduced in sequence. Change table numbers accordingly.

I have taken the reference to the table out at this point, so the table numbers now occur in sequence.

Line 274: Should this be Fig. 1?

Yes, I have now corrected this.

Lines 343-345: The procedure for quantitating arthroconidia should be described in the Materials and Methods section.

Details of quantification are now provided (please see lines 191-192).

Reviewer #3: Reasoning of work is clearle stated. An IgM was generated against extracellular antigen of G. candidum and two closely related species. Monoclonal antibody was used to develop a specific ELISA through which detection could be made in 24 h. Specificity was confirmed by testing naturally contaminated tomato samples.