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1 **Running head: Fungal contaminants of bambinella (Research paper)**

2
3 **Title: Characterisation of fungal contaminants affecting spoilage of the Small Maltese June Pear,**
4 ***Pyrus communis* var. *bambinella***

5
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Abstract

Fungal pathogens cause premature fruit spoilage of *Bambinella*, a fruit endemic to the Maltese islands, leading to the loss of fruit during the post-harvest phase. The objective of this study was to isolate, quantify and characterise fungal contaminants of the small Maltese June Pear and describe their growth kinetics. In total, 284 fungicide-free fruits were collected over three consecutive summers (2014, 2015, 2016). The isolated fungi were identified by using forward and reverse colonial morphology. Species identification was determined by PCR-based methods. The number of colony forming units of each species (CFU) per cm² of *Bambinella* outer skin was calculated. Mycelium diameter growth rate studies of the isolates were also carried out at seven different temperatures ranging from 5-35°C. Fungi isolated from *Bambinella* included *Cladosporium ramotenellum*, *Alternaria arborescens*, *Penicillium lanosum*, *Penicillium expansum* and *Aspergillus sydowii*, listed from the most abundant to the least abundant. The Rosso model was fitted to the growth kinetic data and showed that the optimal temperatures for growth of all the five fungi were in the range of 20-22°C, while growth was slower at temperatures below 10°C and above 30°C. As observed in the diameter studies, the order of highest to lowest germination rate was found to be: *P. expansum*, *A. sydowii*, *P. lanosum*, *C. ramotenellum* and *A. arborescens*. Germination studies showed that the highest germination rate was observed for *P. lanosum* followed by *A. arborescens*, *C. ramotenellum*, *P. expansum* and *A. sydowii*, in descending order. The highest germination lag time was observed by *A. arborescens*, followed by *C. ramotenellum*, *P. expansum*, *P. lanosum* and *A. sydowii*, in ascending order.

Highlights

- The most prevalent fungus found in harvested *Bambinella* was *C. ramotenellum*
- The highest germination rate on *Bambinella* contaminants was observed for *P. lanosum*
- Predictive mycology tools could be applied for the estimation of fungal cardinal values
- 3D Laser Scanner allowed the estimation of the surface area of *Bambinella* specimens.

Introduction

55 *Pyrus communis* var. *bambinella* L. belongs to the *Rosaceae* L. (Rose) family. It is termed as *Small*
56 *Maltese June Pear* and is commonly known as *Bambinella* in Maltese language. The small pear is
57 normally cultivated for agricultural and horticultural purposes, but is also found in the wild as an escape
58 (6). The *Bambinella* fruit grows on macro-Phanerophyte trees which are high trees and the flowering
59 time occurs between March-April. Harvest of fruit takes place during summer months of July-
60 September (26). Malta is still highly dependent on food imports, making the country's economy
61 extremely vulnerable to swings in the world economy (14). The Malta National Reform Programme
62 (published in April 2013) presented a number of research and development strategies under the section
63 'Progress towards National Europe 2020'. This measure seeks to support farm investment to improve
64 the economic performance through better use of the production factors. Agriculture in Malta is
65 important in shaping the rural landscape, but, as an economic activity, it is hindered by complex
66 structural issues such as the small size of fields, high labour costs and the opportunity cost of agricultural
67 land (28). Promoting value-added products such as *Bambinella* fruits and supporting innovation in
68 agriculture and rural development can help off-set the impact of these constraints.

69 Produce such as pear fruits are prone to post-harvest diseases caused by fungi or bacteria and can occur
70 at any stage from harvest to consumption (8). Such organisms may cause soft spots or light brown
71 lesions on fruit and this may lead to advanced fungal invasion, which is clearly seen by a variety of
72 coloured mouldy growths on fruit (38). By time, the fruit becomes dry and mummified or if in moist
73 conditions, it becomes a soft wet mass (33). Post-harvest disease may be initiated in the field and may
74 not show symptoms until stored. Such infections are termed 'quiescent' or 'latent' since the manifestation
75 of disease is followed by a period of inactivity or dormancy, until physiological changes occur in the
76 host tissue and the infection proceeds (8). Consequently, the ripening of the stored fruit may often be a
77 trigger for the reactivation of quiescent or latent infections. A typical example of a post-harvest disease
78 arising from quiescent infection includes grey mould caused by *Botrytis cinerea* (40). During storage,
79 the infection continues to develop in the surface or in the fruit. Other types of infections are initiated
80 during or after harvest. The most common entry points for fungi to penetrate the fruit are through
81 wounds, cuts or bruises caused by mechanical damage during harvesting or by insect damage (27).
82 Natural openings such as lenticels may also be a pathway through which such infectious agents enter.

83 Common post-harvest diseases resulting from wound infections include blue mould caused by
 84 *Penicillium* spp. and transit rot caused by *Rhizopus stolonifer* (43).

85 The efficient spread of infection usually requires warm temperature and high moisture, however, some
 86 storage rots are able to grow normally even at low temperatures (8). *Penicillium* spp. causing blue mould
 87 is a typical example of a fungus which can grow slowly at a temperature close to 0°C (48). The disease
 88 may spread by contact from one fruit to another, if one fruit is infected (34). Insects may also be
 89 involved in spreading such diseases, by acting as vectors. A typical example is the fruit fly which may
 90 be a carrier of the ‘sour rot’, *Geotrichum* spp., passing on the fungal spores from infected to healthy
 91 product. Apart from causing fruit spoilage, a number of fungal genera are known to be mycotoxin
 92 producers. Mycotoxins are secondary metabolites of such fungi which are known to cause sickness or
 93 death in humans or animals, apart from causing economic loss (49). Mycotoxin producers include
 94 species of *Penicillium*, *Alternaria* and *Fusarium* (35). To date, no mycotoxin published assessments are
 95 available on the small Maltese June pear.

96 The identification and pathogenesis of the mould causing the post-harvest disease is the first step in
 97 selecting the ideal method for controlling fruit spoilage (40). The most common fungal pear (relevant
 98 family of fruits for the *Maltese June Pear*) diseases include Blue mould, Gray mould, Bitter rot, Black
 99 spot and Brown rot. Other responsible agents include Bull’s eye rot, *Phytophthora* rot, Pink mould rot,
 100 Powdery mildew, *Rhizopus* rot, Scab, Side rot, Sooty blotch and flyspeck. *Penicillium expansum*, *B.*
 101 *cinerea* and *Mucor piriformis* are the three most common aetiological factors causing pear rot.
 102 *Phialophora malorum*, *Alternaria* spp., *Cladosporium herbarum* and *Neofabraea* spp. are also known
 103 to cause fungal infections in pears (38, 43). Such species are environmental organisms and may be
 104 found in air, water and soil. They infect the fruit by gaining access through wounds or other pathways
 105 leading to fungal invasion and decay (25). There are no studies that describe the fungal post-harvest
 106 disease in *Bambinella* species.

107 The objective was to identify and characterise the main contaminants of *Pyrus communis* var.
 108 *bambinella* and identify the temperature dependence of these isolates *in vitro*. Additionally, the growth
 109 kinetics of spores under optimal temperature conditions are assessed in order to identify the fastest
 110 growing species.

111

112 **Mateirals and Methods**

113

114 **Collection of Fruit.***Bambinella* (*P. communis* var *Bambinella*) fruits were provided by Mġarr Farming
115 Limited (Malta) during three consecutive harvesting years (2014, 2015, and 2016). The fruits originated
116 from fields in Siġġiewi (35°83'84.5"N 14°42'83.8"E) and Rabat (35°52'44.2"N 14°23'43.5"E), Malta.
117 Only selected fields from these two localities were chosen for sampling as it was guaranteed by the
118 farmers that the fruits were not sprayed with fungicides during the last two months before their
119 harvesting time. All the healthy looking ripe fruits present on each tree in each field were sampled by
120 convenience and placed in sterile plastic bags, amounting to a total sample size of 134 fruits collected
121 during August 2014, another 150 fruits collected during August 2015 and 74 during June 2016 (earlier
122 harvesting period because of climatic conditions). The fruits collected in summer 2016 were only used
123 for germination studies.

124

125 **Determination of the fungal spores on the fruits.**The *Bambinella* fruit surface area was estimated by
126 Dr. PICZA3 3D Laser Scanner (Roland DG Corporation, Japan). The polygonal surface area and
127 volume calculations were performed with Autodesk inventor (Autodesk Incorporation, USA) with mesh
128 enabler add-on, which displayed the total 3D area of the fruit. An average of the surface area in cm²
129 was calculated from four *Bambinella* specimens.

130 The small fruits (approximately 3cm long and 2 cm wide) were placed in a sterile plastic bag (seven per
131 bag) containing 25 mL of 1:1 mix of sterile 1% Tween 80 (BDH, Middle East, LLC) solution with
132 Ringer's solution. Serial dilutions of the fruit washing suspension was spread on Sabouraud Dextrose
133 Agar plates with Chloramphenicol (SDC) (Oxoid, England and Abtek, Liverpool), incubated at 25°C
134 (±3). Four replicates per dilution per plate were prepared. After 48-72 hours of incubation, different
135 colonies were separated on a purity plate, incubated at 25°C (±3) for 72-90 hours, until a large fungal
136 colony was observed. Each different colony was identified using the colonial appearance and
137 microscopy by producing lactophenol cotton blue (Sigma-Aldrich Co. LLC., Germany) tape mounts. A
138 Mycology Atlas (11) was then used for the macroscopic identification. The CFU per *Bambinella* surface

139 area (in cm²) was calculated based on the calculation of the average skin area of the 7 *Bambinella* fruits
140 analysed per bag .

141

142 **Molecular characterization of isolates.** Fungi of distinct characteristics as observed based on the
143 macroscopic identification (refer to Determination of the fungal spores on the fruits) isolated from the
144 *Bambinella* fruit were further identified molecularly. DNA of five isolates (after their regrowth on SDC)
145 was isolated using the Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany). The DNA was
146 eluted in 50 µL of nuclease free water and its concentration and purity were estimated using the Nano
147 Drop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The stock DNA from
148 each culture was then diluted to a working solution of 25 ng/µL using nuclease
149 free water as diluent. In order to amplify the region of interest in the fungal DNA, the internal
150 transcribed spacer 5 (ITS-5) forward primer and the ITS-4 reverse primer were used. Additionally, the
151 D1/D2 fragment was sequenced, although it did not vary the identification outcome of the previous
152 ones.

153 A 50 µL reaction mixture was prepared for each polymerase chain reaction (PCR) consisting of the
154 following components: 2µL of DNA at a concentration of 25 ng/µL, 10mM Tris-HCl buffer (pH 8.3),
155 50 mM KCl, 1.5 mM MgCl₂ (for optimal Taq specificity), 200 mM of each Deoxyribonucleotide
156 triphosphate (dNTP), 400 nM of the forward and reverse primers and 1.25 units of Dream Taq DNA
157 polymerase (ThermoScientific, Wilmington, DE, USA). The reaction mixture was topped up to 50 µL
158 using nuclease free water. A negative control was also included in the run whereby the DNA template
159 was replaced with an equal volume of water. The thermocycler program for each PCR reaction began
160 with an initial denaturation step at 94°C for 5 minutes followed by 50 cycles of a 30 seconds
161 denaturation step at 94°C, a 30 seconds annealing step at 56°C, and a 60 seconds elongation step at
162 72°C, then one final 7-minute elongation step at 72°C. Post-PCR products were stored at 4°C until
163 analysis could be performed. Agarose gel electrophoresis was performed to examine the amplified
164 products and to determine whether any potential size differences were present among the PCR products.
165 4 µL of each PCR product were run on a 2% agarose gel containing GelRedTM nucleic acid gel stain
166 for visualisation under ultraviolet light. PCR products were first purified by the NucleoSpinExtract II

167 kit (Machery-Nagel, Düren, 151 Germany) and then sequenced by Sanger method using ITS4 primer
168 and the kit BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in a automatic capilar
169 sequencer ABI 3130xl (Applied Biosystems) according to the manufacturer's instructions. The results
170 were viewed using the Chromas software (Technelysium Pty Ltd, Tewantin QLD, Australia) against
171 known sequences in the NCBI (National Centre for Biotechnology Information) blast database to
172 provide species identification.

173

174 **Effect of temperature on mycelial growth rate.** The spore suspensions of the above isolated strains
175 were maintained on sterile 10% glycerol (AnalaR, BDH Chemicals, Radnor) at -80°C. The five isolates
176 were routinely grown at 25°C on Sabouraud Dextrose Agar (SDA: Oxoid Limited, Basingstoke, UK)
177 for 7 days. Spores were then suspended in sterile distilled water containing 1% (v/v) of Tween 80 by
178 gently scraping the surface of the medium. After filtering the spores' suspensions through four layers
179 of sterile medical tissue (Medline Industries, Inc) in order to remove any debris (mostly mycelial
180 fragments), their final concentration was determined using a Neubauer counting chamber (Precicolor,
181 HBG, Germany). Immediately after preparation, the spore suspension was diluted in Ringer's solution
182 (Oxoid Limited, Basingstoke, UK) to yield an inoculum count of approximately 10⁵ spores/mL and
183 used as quickly as possible. Ten µL of the conidial suspension was pipetted gently on the centre of a
184 SDA plate. The point of inoculation was marked by drawing 4 intersecting lines on petri dish. Each
185 fungal isolate was studied at seven different temperatures, namely 5, 10, 15, 20, 25, 30 and 35°C.
186 Temperatures during the experiments were measured by using a digital thermometer. Three replicates
187 per temperature were monitored. Growth diameter was recorded till it reached not more than 4 cm. Four
188 diameters were measured at each time interval for each mycelium colony and their average calculated.

189

190 **Modelling the fungal dynamics - Primary modeling.** A linear model with breakpoint has been used
191 to estimate the diameter growth rate and the lag time (9).

192

193
$$d(t) = d_o + \mu(t - \lambda_m)$$

194

Where $d(t)$ is the diameter of the mycelium at time t ([h]), d_o is the diameter of the inoculation suspension drop ([mm]), μ is the diameter growth rate ([mm/h]), λ_m is the lag phase ([h]). The lag phase was obtained from the extrapolation of the straight line and the estimation of the time at which $d(t) = d_o$. The d_o of the inoculum was standardised at 6 mm. The linear model was selected (over Baranyi or Gompertz model) based on previous reports by Gougouli, and Koutsoumanis (18) in which diameter increase follows a linear behavior up to a certain level. Regression analysis was performed based on the minimization of the mean squared errors.

Secondary modeling. The primary model parameters were then fitted with a secondary model - the Rosso model (Rosso, Lobry, Bajard, & Flandrois, 1995). In this model, the diameter growth rate (μ) is described as a function of temperature. The original model contains four parameters, three cardinal temperature parameters (theoretical temperature below which no growth occurs, temperature above which no growth occurs and temperature at which the μ is optimal) and the diameter growth rate at the optimum temperature.

$$\mu(T) = \mu_{opt} \cdot \frac{(T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)]}$$

Where T_{opt} , T_{min} and T_{max} ([°C]) are the theoretical optimum, minimum and maximum temperatures for growth, respectively, and μ_{opt} are the optimum diameter growth rate ([mm/h]).

Germination kinetics of single spores. The germination kinetics of the previous isolates were also assessed on both SDA and bambinella medium. The method used for the preparation of bambinella medium was adapted from the in-house preparation of potato dextrose agar (PDA) (34). Fifteen g of agar-agar dehydrated medium was reconstituted in 500 mL of distilled water placed in a 1 L glass bottle. This was well shaken and autoclaved at 121°C for 15 minutes. Two hundred fifty g of peeled and diced fruits (collected during harvesting periods of 2014, 2015, 2016) were placed in an autoclaved glass beaker (1 L volume) and sterile water was added to the 700 mL mark. The beaker was placed on a hot

222 plate and the contents were allowed to gently boil for 30-45 minutes, making sure that the contents did
223 not dry. The infusion then was filtered through autoclaved cheesecloth and the filtrate was reserved.
224 The autoclaved agar was placed in a water bath set at 55°C for 15 minutes in order for it to cool down.
225 The filtrate was added to the agar solution and the contents of the bottle were well shaken. The resulting
226 liquid medium reached a volume of 1 L. The *Bambinella* medium was then poured in plates and was
227 allowed to solidify. Quality control checks on the prepared medium were performed. A sterility check
228 was performed made by incubating 5% non-inoculated media at 20°C, while for the positive control,
229 the fungal isolates of *Bambinella* were inoculated on the medium and incubated at 20°C, i.e., close to
230 optimum for all the isolated fungi. The positive control ensured that the medium produced supported
231 the growth of the species studied.

232 The fungal isolates were grown as described above (see section: Effect of temperature on mycelial
233 growth rate) Three SDA and three *Bambinella* medium plates were inoculated by surface plating with
234 100 µL of the 10⁶ /mL spore suspension. This method was adapted from Judet et al., 2008. A 1 cm²
235 section of the agar that was just inoculated with the diluted conidial suspension was cut using a sterile
236 blade and this was placed on a glass slide (22). The agar was then covered with a glass cover slip and
237 was placed under high power microscopy set at x 400 (Olympus-CX31, Tokyo, Japan). The spores were
238 observed to be non-germinated. The six plates were then incubated at 20°C which is a temperature very
239 close to the optimum, based on the findings from the first part of the study. After 1 hour, the degree of
240 germination was determined by cutting a 1 cm² piece of agar from the plate and placing it on a glass
241 slide. The agar square was then covered with a glass slide and the spores were observed under high
242 power microscopy. To make sure that areas of the agar are not screened for multiple times, a screening
243 procedure was used. Screening was started at the left corner of the agar square, moving towards the
244 right, then going slightly down and moving to the left direction, and so on. During each observation, at
245 least 100 spores from each plate (from the three replicates) were observed and the number of
246 germinating spores was calculated as the percentage of the total spores. By a rule of thumb, between 20
247 to 50 spores per microscope field were examined. It was made sure that each field did not contain less
248 than 10 spores or a high number of spores that overlapped each other.

249

250 **Modeling the germination kinetics.** The percentage of germinated spores was calculated as follows
251 (10):

252
253
$$P(\%) = \frac{(\text{No. of germinated spores})}{\text{Total no. of spores}} \times 100$$

254
255 The data (percentage over time) of each fungal strain and storage temperature was fitted to the modified
256 Gompertz equation (50) for the estimation of the germination kinetic parameters (μ_g and λ_g):

257
258
$$P_t = P_{max} \cdot \exp\left(-\exp\left[\frac{\mu_g \cdot e(1)}{P_{max}} \cdot (\lambda_g - t)\right] + 1\right) \quad (8)$$

259
260 Where t is the time([h]), P_t is the percentage of germinated spores at time t (%), P_{max} is the asymptotic
261 P_t value at $t \rightarrow +\infty$ (%), μ_g is the slope term of tangent line through the inflection point (1/h), λ_g is the
262 geometrical lag time (t-axis intercept of the tangent through the inflection point (h). P_{max} was a degree
263 of freedom when the regression analysis was performed.

264 265 266 **Results and discussion**

267
268 **Isolation, identification and quantification of fungi.** Out of 284 *Bambinella* fruits (134 in 2014 and
269 150 in 2015), five fungal species were identified. The genus name was identified using macroscopic,
270 microscopic appearance and molecular identification. Results of isolated fungi and their quantification
271 in terms of colony forming units per cm² of *Bambinella* skin are presented in Table 1. The average
272 surface area of 1 *Bambinella* fruit was estimated to be 42.68 cm² (as estimated by using the Dr. PICZA3
273 3D Laser Scanner).

274 The most prevalent of the isolated fungi were *Cladosporium ramotenellum* which belongs to the same
275 genus of *C. herbarum* followed by *Alternaria arborescens*. *Cladosporium* species are ubiquitous
276 worldwide, and commonly isolated from soil and organic matter and represent the most frequently

277 isolated airborne fungi (5). Similarly, *Alternaria* species are ubiquitous organisms and are present as
278 saprophytes in soil matter and in air (32). The chance of these environmental organisms (both *A.*
279 *arborescens* and *C. ramotenellum*) to become fruit spoilers is low, as compared to other primary fruit
280 spoilers, and has to be aided by many intrinsic and extrinsic factors including over ripe fruits, injury by
281 insects and pesticide-free fruits (42). Such organisms do not normally invade healthy skin and require
282 very ripe injured fruits, where the spores or mycelia are able to penetrate under the skin and into the
283 tissue of the fruit, causing decay and eventually loss of the fruit product. On the other hand, the presence
284 of the *P. expansum*, although in small quantities, may still pose a threat to healthy fruits, as it is very
285 likely to cause pome fruit disease (41). *P. expansum* may attack the fruit on the tree, in the packing
286 house, in transit, in storage and in the market (7, 45, 47).

287 *P. lanosum* and *A. sydowii* were found in very low concentrations. To date, no literature suggests that
288 these organisms can cause disease in pome fruits; however, *P. lanosum* may be considered as a higher
289 threat than *A. sydowii*, as blue mould is caused by a vast array of *Penicillium* species, one of which may
290 be *P. lanosum* (41). The pathogenicity of *P. lanosum* on *Bambinella* fruits has to be further investigated
291 by isolating fungi from diseased fruit to check if this species of fungus is present. *Aspergillus sydowii*,
292 which occurred at very low concentrations, has never been associated with fungal disease of pome
293 fruits; however, it has been found in air and soil and was also recorded to cause disease in sea corals
294 (44). No *Botrytis* species were found on *Bambinellas*. This could either be since *Botrytis* was not present
295 in Malta in the specific harvesting areas during the period of the study.

296
297 The most prevalent fungus found in harvested *Bambinella* was *C. ramotenellum* followed by *A.*
298 *arborescens*, both of which are known environmental organisms, however, may still cause post-harvest
299 disease if conditions are favourable. *P. expansum*, *P. lanosum* and *A. sydowii* were found in lower
300 concentrations in 2014 only. *P. expansum* is the most likely fungus to cause disease in *Bambinellas* as
301 it is responsible for causing blue mould which is the most popular post-harvest disease in pome fruits.

302
303 **Modeling fungal dynamics.** The mycelium diameter increased with respect to time for all the five
304 fungal isolates and at all temperatures which was determined with good accuracy by using the linear

305 model. The growth rate data generated from the five studied fungi, at the seven temperatures were fitted
306 to the Rosso Model. The optimal growth rate (μ_{opt}), the minimum and maximum temperature at which
307 growth is possible (T_{min} and T_{max} , respectively) and the optimum temperature (T_{opt}) were estimated
308 (Table 2).

309
310 At temperatures close to the optimum (T_{opt} : *P. expansum* = 22.71°C ± 1.83, *A. sydowii* = 22.88°C ± 1.53,
311 *P. lanosum* = 22.76°C ± 1.63, *A. arborescens* = 22.10°C ± 1.05 and *C. ramotenellum* = 20.93°C ± 0.78),
312 *P. expansum* seems to have the highest growth rate followed by *A. sydowii* and *P. lanosum* and by *A.*
313 *arborescens*. *C. ramotenellum* has the lowest growth rate at optimum temperature. On the other hand,
314 *C. ramotenellum* seems to present the lowest theoretical minimum temperature for growth (T_{min} = -
315 0.81°C), while for the rest of the fungi the minimum theoretical temperature for growth ranges between
316 5.9°C to 6.3°C. Nevertheless, *C. ramotenellum* is not able to grow at temperatures above 30.1°C, unlike
317 the other four fungi, which can still grow at temperatures as high as 34.7°C – 35.2°C.

318
319 Estimation of fungal growth parameters was also investigated in other studies. Baert et al. (2007) studied
320 *P. expansum* at various temperatures. T_{min} was found to be as low as -2°C. *Aspergillus niger* and
321 *Aspergillus carbonarius* which belong to the same genus as *A. sydowii* (of the current study) were
322 successfully grown at temperatures ranging from 10-37°C with a T_{opt} falling in the range of 30-37°C
323 while the minimum growth rates were at temperatures of 10°C (4). In another study, the same *Aspergilli*
324 were shown to give the highest growth rate at 30-35°C (24). The T_{opt} of *A. ochraceous* was recorded to
325 be in the range of 20-30°C (31). Gougouli, and Koutsoumanis also studied the growth parameters of *P.*
326 *expansum*, which were estimated as follows: T_{min} = -5.74°C, T_{max} = 30.97°C, T_{opt} = 22.08°C and μ_{opt} =
327 0.22 mm/h (18). The combined effect of temperature and water activity on the growth rate of *P.*
328 *expansum* was also studied by Lahlali, Serrhini, and Jijakli (23). The growth rate was seen to decrease,
329 as the temperature and water activity of the medium was lowered. The optimum conditions for mould
330 growth were concluded to be 25°C at a_w = 0.96 to 0.98. The T_{opt} of *P. expansum* estimated from the
331 current study is close to the T_{opt} published by the aforementioned authors. Most probably, the observed
332 differences in the minimum, optimum or optimum temperature for growth between resercheas for the

333 same species is due to the strain variability. Until very recently, it was thought that strain variability
334 was not related with fungi since there was no evidence supporting the opposite. However, some studies
335 (15,16) have shown significant differences in the phenotypic responses among identically treated strains
336 of *Aspergillus carbonarius* (30 strains) and *Penicillium expansum* (79 strains), in terms of growth and
337 mycotoxin production, suggesting that strain variability is also valid for fungi.

338

339 **Germination Studies.** The modified Gompertz equation was fitted to the obtained germination data,
340 expressed as percentage of germination (% *P*) versus time for estimating the germination kinetic
341 parameters (lag time, λ_g , and germination rate, μ_g). The ability of this equation to describe spore
342 germination has been previously demonstrated for various fungi (1,19, 21, 22; 30, 39).

343

344 Figure 2 represents % germination versus time fitted to the Modified Gompertz Model, showing an
345 exponential germination rate after a lag time. Germination stops at 100% which represents the time that
346 all spores have germinated.

347 Table 3 shows the parameters as estimated by the models. The shortest lag time is recorded for *A.*
348 *arborescens* followed by *C. ramotenellum* and closely by *P. expansum*. The longest lag times are
349 recorded for *P. lanosum* and *A. sydowii*. The slope of the graphs represents the germination rate, which
350 is recorded in the following order, from fastest to lowest: *P. lanosum*, *A. arborescens*, *C. ramotenellum*,
351 *P. expansum*, and *A. sydowii*.

352 The highest germination rate was observed in *P. lanosum* and *A. arborescens* (in both media), followed
353 by *P. expansum*, *C. ramotenellum* and *A. sydowii* in order of decreasing germination rate. On the other
354 hand, the lag time of *A. sydowii* seems to be one of the longest (10.96 hours for *Bambinella* media,
355 11.05 hours for SDA media), as compared to that of *A. arborescens* (1.17, 0.72 hours, respectively).

356 The shorter the lag time, the sooner the spores pass into the germination phase, which starts the process
357 of forming a visible mycelium and resulting in spoilage. The parameters of *P. expansum* and *C.*
358 *ramotenellum* seem to be very close in terms of germination. Both parameters of *A. sydowii* suggest
359 that it has the lowest germination ability, by having the highest germination lag time and the slowest
360 germination rate at the same storage conditions.

Gougouli, and Koutsoumanis studied the effect of storage temperature on the kinetic parameters of germination μ_g and λ_g of the *P. expansum* and *A. niger* spores on malt extract agar (20). Data was fitted with the Cardinal Model with Inflection which describes the maximum specific growth rate of microorganisms as a function of temperature. The predicted values at 20°C were approximately: *Penicillium expansum* $\lambda_g = 8.6$ h and $\mu_g = 26$ h⁻¹; *Aspergillus niger* $\lambda_g = 10.1$ h and $\mu_g = 26$ h⁻¹. The germination rate of *P. expansum* isolated from *Bambinella*, in the current study, was observed to be 33 h⁻¹, which is close to the aforementioned study. At the same temperature, germination rate of *A. sydowii* was 19.15 h⁻¹, as compared to the value of 23.5 h⁻¹ of *A. niger*. The lag time for germination for *P. expansum* in this study was found to be 6.89 hours, which is close to the value of 8.6 hours found by Gougouli, and Koutsoumanis, while that of *A. sydowii* was 11.07 hours. Considering the difference on the germination kinetics between the two studies it can be said that is the result of strain variability or/and the composition of the germination medium. In another study, it was observed that when temperature varied from optimum to marginal values a decrease in germination rates was observed for *Penicillium digitatum*, *P. italicum* and *Geotrichum candidum*. Maximum germination rates occurred at 25°C for *P. digitatum* and *P. italicum* and at 25–30°C for *G. candidum*. These values were around 30 h⁻¹ for the three studied fungi. Maximum percentage of germination was reached in 3–4 hours after the start of the process (36).

The process of fungal spore germination can be split into four stages: (i) the breaking of the spore dormancy where the spore is activated, (ii) isotropic swelling which is dependent on the presence of oxygen, (iii) establishment of cell polarity and (iv) formation of the germ tube and maintaining polar growth (12). *Penicillium* and *Aspergillus* species usually need 2 hours for the second step to occur and an extra 5–6 hours for cell bulging to occur, under optimal conditions. After 8 hours at 25°C almost all cells would have produced a germ tube (46). This process is speeded up or slowed down by the effect of temperature, which does not express a linear relationship. In the current study, following 8 hours of incubation, only 10% of *P. lanosum* were fully germinated, however, 100% germination was observed 4 hours later (after 12 hours incubation at 20°C). 30% of *P. expansum* spores were germinated after 8 hours and all spores germinated after 10 hours incubation. *A. sydowii* spores, which had the longest lag time and slowest germination rate, showed 100% germination after 18 hours of incubation.

389 Moreover, the study of single spores may be a more realistic approach to the contamination of fruits
390 with spores, which, as seen in the first section of this study (isolation of fungi from healthy looking
391 fruits), occurs at very low concentrations. It is only in situations when the fruit is touching with an
392 advanced rotten fruit that it is contaminated with a high population of spores. Given the fact that single
393 spores behave in a different manner than spores occurring in population, it can be argued that
394 germination studies on single spores offer a more realistic picture of the study of fungal infections
395 related to post-harvest diseases (18).

396 *P. expansum* showed the highest μ_{opt} , followed by *A. sydowii*, *P. lanosum*, *A. arborescens* and *C.*
397 *ramotenellum*. Germination studies on single spores of the five fungi at 20°C on SDA and *Bambinella*
398 medium showed very similar results on both media. λ_g and μ_g were found for each fungus. *A.*
399 *arborescens* was found to have the lowest λ_g and the second fastest germination rate. *C. ramotenellum*
400 and *P. expansum* showed very similar germination parameters, while *A. sydowii* had the highest lag
401 time and lowest germination rate. *P. lanosum* showed the highest germination rate but had a relatively
402 low lag time.

403 The quantitative approach and the models developed in this work can be used as useful tools which can
404 predict rapidly the fruit spoilage by fungi. The next step of this study is to validate the models described
405 herein on *Bambinella* and other fruit products by taking into consideration fruit respiration in
406 conjunction with temperature and gas atmosphere in order to prevent fungal growth in terms of visible
407 growth on fruits during storage throughout the food supply chain. Furthermore, inoculation assays under
408 commercial conditions would further increase the consistency of conclusions. With regards to
409 molecular analysis of fungal species, analyzing all fungal isolates would greatly develop a more
410 powerful model that addresses strain variability.

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423

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Figure legends

Figure 1. Regression analysis of the secondary Rosso model describing the effect of temperature on mycelial growth rate for all the studied fungi. The points represent the observed data and the lines the prediction of the models of the following moulds: *A. arborescens* (---), *A. sydowii* (—), *P. exapnsum* (- -), *P. lanosum* (---), *C. ramotenellum* (—).

Figure 2: Regression analysis of the Gompertz model describing the percentage germination of the studied fungi versus time on (a) *Bambinella* medium of *A. arborescens* (---), *A. sydowii* (- -), *P. exapnsum* (—), *P. lanosum* (...), *C. ramotenellum* (—), (b) SDA at 20°C of *A. arborescens* (---), *A. sydowii* (- -), *P. exapnsum* (—), *P. lanosum* (...), *C. ramotenellum* (—). The points represent the raw germination data.

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588 Table 1: Levels of fungal species isolated from *Bambinella* fruit on two consecutive years

589

Identified isolate	¹ CFU/cm ²	
	2014	2015
<i>Cladosporium</i>	6	11
<i>ramotenellum</i>		
<i>Alternaria arborescens</i>	2	<1
<i>Penicillium lanosum</i>	<1	0
<i>Penicillium expansum</i>	<1	0
<i>Aspergillus sydowii</i>	<1	0

590

591 ¹Estimated average surface area of 1 *Bambinella* fruit: 42.68 cm²

592

593 Table 2. Growth parameters of the five isolates as estimated by the Rosso model describing the effect
594 of temperature on mycelial growth rate.

Fungus	Parameter	Estimated Value	Standard Error	RMSE
<i>Cladosporium</i>	μ_{opt} (mm/h)	0.24	±0.01	0.03
<i>ramotenellum</i>	T_{min} (°C)	-0.81	±2.38	
	T_{opt} (°C)	20.93	±0.78	
	T_{max} (°C)	30.09	±0.51	
<i>Penicillium lanosum</i>	μ_{opt} (mm/hr)	0.44	±0.06	0.17
	T_{min} (°C)	6.22	±1.43	
	T_{opt} (°C)	22.76	±1.63	
	T_{max} (°C)	34.66	±1.28	
<i>Penicillium expansum</i>	μ_{opt} (mm/hr)	0.46	±0.07	0.19
	T_{min} (°C)	5.98	±1.68	
	T_{opt} (°C)	22.71	±1.83	
	T_{max} (°C)	35.21	±1.65	
<i>Aspergillus sydowii</i>	μ_{opt} (mm/hr)	0.44	±0.05	0.16
	T_{min} (°C)	6.26	±1.36	
	T_{opt} (°C)	22.88	±1.53	
	T_{max} (°C)	34.76	±1.26	
<i>Aspergillus arborescens</i>	μ_{opt} (mm/hr)	0.38	±0.03	0.10
	T_{min} (°C)	6.16	±0.73	
	T_{opt} (°C)	22.10	±1.05	
	T_{max} (°C)	34.89	±1.05	

Table 3: Estimated average germination rate (μ_g) and average lag time (λ_g) on (a) *Bambinella* medium and (b) SDA through fitting of the Modified Gompertz model to the germination data of all studied fungi.

Fungus	μ_g (1/h)		μ_g (1/h)		λ_g (h)		λ_g (h)	
	(Bambinella medium)	Standard error	(control-SDA)	Standard error	(Bambinella medium)	Standard error	(control-SDA)	Standard error
<i>Aspergillus arborescens</i>	47.69	3.27	40.08	2.45	1.17	0.08	0.72	0.08
<i>Cladosporium ramotenellum</i>	26.60	0.57	32.48	2.66	6.92	0.07	6.76	0.17
<i>Penicillium expansum</i>	29.86	0.74	33.92	1.00	7.10	0.10	6.91	0.03
<i>Penicillium lanosum</i>	31.00	0.59	40.09	2.25	9.18	0.01	9.53	0.01
<i>Aspergillus sydowii</i>	19.32	1.22	19.37	0.31	10.96	0.01	11.05	0.03

The figures should be improved and the symbols that are associated with the adjustment lines should be removed from the legend.

line 218 - the authors must mention how they consolidated the model from the data they obtained.

lines 347-349 - the authors should review and clarify the model setting, the data presented in the figures do not seem to follow a sigmoidal behavior, so Pmax would be 100 in most cases. Explain how the adjustment was made.

♣ Experimental design described in this manuscript is also not adequate. It is not clear how the fungal analyses were done particularly in isolation from the fruits.

♣ All tables and figures should stand alone with full descriptions of any acronyms used and units.

Specific comments

625 Highlights It was NOT the fungal "pathogenesis" but general/physiological characteristics presented in
 626 this study. In addition, it is not clear how this study can help in reducing agricultural fungicides' use.
 627
 628
 629 L 134 Is this (3 cm x 2 cm) the whole fruit? Are they diseased or sound? Surface sanitized? If their
 630 surface was not sanitized, how the fungal isolates recovered from the fruit can be related to
 631 spoilage/disease? In other words, is there any evidence of common environmental/ubiquitous fungi
 632 causing spoilage/disease? Otherwise, this study cannot be justified. Even if only "diseased" fruits
 633 were used for fungal isolation, unless entire fruit was rotten, enumerating fungi from the whole fruit
 634 including common contaminants will negate the concept of this study.
 635
 636 L 154 Please justify why D1/D2 fragment was sequenced if it didn't affect the results of ITS-5.
 637
 638 L 201 Radial (r) growth or radius representing linear growth would be more appropriate (for the
 639 modeling) since the diameter (d) is bi-directional (or 2x of r).
 640
 641 L 222 Provide a reference or justification for bambinella medium, i.e. why this was necessary? Why
 642 not autoclave the juice/extract?
 643
 644 Discussion Growth characteristics of the fungi of interest obtained this study should be discussed in
 645 comparison with appropriate references since most of them are well established.
 646
 647 Regarding the above, the authors must clarify how many fruits were collected each year and how
 648 many of them were analyzed. It is also mentioned that crops of 3 consecutive years were analyzed
 649 and in the results, only crops from 2 years appear.