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Running title: ***Candida pruni* sp. nov., a new biocontrol species**

Abstract

Brown rot caused by *Monilinia* spp. is among the most important postharvest diseases of commercially grown stone fruits, and application of antagonistic yeasts to control brown rot is one promising strategy alternative to chemical fungicides. In this research, new yeast strains were isolated and tested for their activity against peach brown rot caused by *Monilinia fructicola.* Three yeast strains were originally isolated from the surface of plums (cv Chinese Angelino) collected in different organic orchards in the North of China. In artificially-wounded inoculation tests, the yeast at 1×10^8 CFU mL^{-1} reduced the brown rot incidence to 20%. The population of the yeast within inoculated wounds on peaches increased at 25°C from an initial level of 5.0×10^6 CFU to 4.45×10^7 CFU per wound after 1 day. The maximum yeast population (9.5×10^7 CFU per wound) was observed 4 days after inoculation. The antagonistic strains were belonging to a new species of the genus *Candida* by sequence comparisons of 26 S rDNA D1/D2 domain and internal transcribed spacer (ITS) region. The strains are most closely related to *C. asparagi* (sequence similarity: 95.0%), *C. musae* (sequence similarity: 96.0%) and *C. fructus* (sequence similarity: 96.0%) on the basis of the phylogenetic trees based on the D1/D2 region of 26S rDNA. However, the strains are notably different from *C. asparagi*, *C. musae* and *C. fructus,* in morphological and physiological characteristics. Therefore, the name *Candida pruni* is proposed for the novel species, with sp-Quan (=CBS12814^T=KCTC 27526^T=GCMC 6582^T) as the type strain. Our study showed that *Candida pruni* is a novel yeast species with potential biocontrol against brown rot caused by *M. fructicola* on peaches.

Introduction

Brown rot caused by *Monilinia* spp. is among the most important postharvest diseases of commercially grown stone fruits. The most common species isolated from brown rot-infected peaches and nectarines in China, as well as in other countries, is *M. fructicola* (Tian and Bertolini, 1999; Pellegrino *et al*., 2009), which causes severe losses during the postharvest storage. When permitted, applying synthetic fungicides is the best method to control the post-harvest diseases on fruits. However, consumers are increasingly concerned about safety, and prefer food free of pesticide residues, toxins and harmful microorganisms (Spadaro and Gullino, 2004). Moreover, many fungal strains are developing resistance to widely-used synthetic fungicides (Conway *et al*., 2004) and more effective fungicides are no longer re-registered (Ragsdale, 2000). Therefore, new alternative strategies need to be developed. Biological control using microbial antagonists has emerged as one of the most promising alternatives to fungicides in controlling postharvest diseases (Zhang *et al*. 2010a). Some bacteria, actinomycetes and yeasts have shown to be effective against postharvest diseases of fruits and vegetables (Macagan *et al*., 2008; Zhang *et al*., 2010b). Moreover, some filamentous fungi have been identified as biocontrol agents of postharvest brown rot in stone fruit such as *Epicoccum nigrum* (Melgarejo *et al*., 1986; Larena *et al*., 2005; De Cal *et al*., 2009) and *Penicillium frequentans* (Guijarro *et al*., 2007a; 2007b). Among these microbial antagonists, yeasts that occur naturally on fruits and vegetables have drawn the attention as potential antagonists of postharvest diseases due to their fast colonization on fruit surface and their minimal negative effect on the environment (Droby *et al*., 1997; Ippolito *et al*., 2000; Kurtzman and Droby, 2001; Karabulut and Baykal, 2003). In pursuit of antagonistic yeasts capable of controlling postharvest pathogens on fruits, a new *Candida* species was discovered from the plums collected in orchards in North China. The objective of this study is to describe a new antagonistic yeast species belonging to the genus *Candida* and to evaluate the efficacy of

75 the yeast to control peach brown rot.

76

77 **Materials and methods**

78 **Microorganisms and culture conditions**

79 Ten plums cv were collected from three different organic orchards located in North China. The plums were

80 suspended in a 300 mL conical flask containing 100 mL of sterile deionized water and then incubated at

81 27°C for 20 min on a rotary shaker at 150 rpm. One hundred microliters of the suspension was aliquoted on

82 YPDA plate (20 g L^{-1} dextro-glucose, 20 g L^{-1} peptone casein, 10 g L^{-1} yeast extract, 20 g L^{-1} agar)

83 supplemented with 100 mg L^{-1} of streptomycin sulphate, and the plates were subsequently incubated at

84 30°C for 48 h. The cultures were purified twice by streaking on YPDA plates. The three strains, sp-Quan,

85 YG1, and GR1 were isolated respectively from plums coming from Beijing, Hebei, and Tianjing province.

86 Two strains (GenBank accession numbers: KJ131183 and KJ131184) of *Monilinia fructicola* (G.Winter)

87 Honey were isolated from rotted peaches and selected for their virulence by inoculation in artificially

88 wounded peaches. They were used as a mixture (each strain accounted for 1/2 of the total final

89 concentration) throughout this work, to ensure a high level of disease. Each strain was stored in slants on

90 Potato Dextrose Agar (39 g L^{-1}; PDA; Merck, Darmstadt, Germany) with 50 mg L^{-1} of streptomycin

91 (Merck) at 4°C. Spore suspensions were prepared by growing the isolates on Petri dishes at 25°C for 7 days

92 on Peach Agar [PA; 500 mL L^{-1} peach juice + 20 g L^{-1} agar (Merck); pH 7.0] medium. *M. fructicola* spores

93 were collected and suspended in sterile Ringer solution (pH 6.9±0.1; Merck). After filtering through 8

94 layers of sterile cheese-cloth, spores were quantified with a Bürker chamber and brought to the final

95 concentration required.

96

Antagonistic activity of yeast strains against peach brown rot

In preliminary experiments, we found that three strains (sp-Quan, YG1, and GR1) grew faster than other yeasts and could inhibit the growth of *M. fructicola* on PDA plates (data not shown). The strain sp-Quan was selected for further testing the biocontrol activity against peach brown rot. To evaluate the activity of the yeast in controlling brown rot on peaches, the experiments were conducted as described by Zhang et al. (2010b) with small modifications. Peaches (cv Springcrest) harvested at commercial maturity were surface-disinfected with 1% commercial sodium hypochlorite for 1 min, then rinsed with sterilized tap water, and three artificial wounds (about 3 mm wide × 3mm deep) were made along the equatorial zone of the peach. 30-µl aliquots of *M. fructicola* suspension at 1×10^5 conidia mL^{-1} were pipetted into each wound, and the fruits, after air-drying for 2 h at 25°C, were treated by pipetting 30 µl yeast suspension at 1×10^8 CFU mL^{-1} into each wound. Fruits inoculated with only the pathogen and only the yeast served as inoculated control and yeast control, respectively, and fruits treated with tebuconazole (Folicur, Bayer Crop Science; a.i.: 25.0 %) were used as chemical control. All fruits were incubated at 25°C and 95% RH. Six days after inoculation, the percentage of infected wounds was recorded and the diameter of lesions was measured. There were three replicates of fifteen fruits per treatment, and the experiment was repeated twice.

Population studies of antagonistic yeast

The experiments for population study were carried out according to the method by Viñas et al. (1998), with small modifications. The peaches were wounded as described above. Thirty µL yeast cell suspension (1×10^8 CFU mL^{-1}) was applied into each wound on fruits. All fruits were incubated at 25°C and 95% RH. The yeast was recovered from wounds after incubation at 25°C for 0 (1 h after treatment), 1, 2, 3, 4, 5 and 6

days, respectively. Wounded tissue was removed with sterilized 7 mm (internal diameter) cork borer and ground with a autoclaved mortar and pestle in 50 mL of sterile 0.85 sodium chloride. Serial tenfold dilutions were prepared and 0.1 mL each dilution was spread in NYDA. The plates were incubated at 28°C for 48 h, and the colonies were counted and recorded. Population densities of the antagonistic yeast were reported as $\log_{10}$ CFU per wound. Three single fruit replicates were prepared per treatment, and the experiments were repeated twice.

Molecular identification, morphological and physiological characterization of strains

The strains were identified by sequencing the internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS2) according to White et al. (1990) and the D1/D2 domain at the 5' end of the LSU rRNA gene according to Kurtzman and Robnett (1998). The DNA, coming from the yeast strains grown in YPD for 48 h, was extracted as described by Lu *et al*. (2004). The sequences were analysed by using the software BLASTn (Basic Local Alignment Search Tool; Altschul *et al.*, 1990) for similarity.

Sequence data were first aligned with the program CLUSTAL_X (Thompson et al., 1997) and then subjected to the program MEGA 4.10. A phylogenetic tree was constructed from evolutionary distance data that were calculated with the neighbour-joining method (Saitou and Nei, 1987) using Kimura's two-parameter distance measure (Kimura, 1980). Confidence limits of phylogenetic trees were estimated from bootstrap analysis (1000 replications). Reference sequences were retrieved from GenBank under the accession numbers indicated in the tree.

To characterize the strains morphologically and physiologically, the fermentation and assimilation tests and procedures were performed as described by Yarrow (1998).

163

164 **Statistical analysis**

165 Data were subjected to analysis of variance (ANOVA) using SPSS (version 13.0) and statistical
166 significance was assessed at the level of $P<0.05$, and Duncan's multiple Range Test was used to separate
167 the means.

168

169 **Results and discussion**

170 **Antagonistic activity and population dynamics of the yeast**

171 At 6 days after inoculation, the percentage of infected wounds was recorded and the diameter of lesions
172 was measured. Compared with the inoculated control, the yeast sp-Quan reduced disease incidence from
173 100% to 27% (table 1). Moreover, compared with the inoculated control the yeast sp-Quan reduced brown
174 rot severity on peaches from 48 mm to 15 mm. The result indicated that, when applied at 1×10^8 CFU mL^{-1},
175 the strain sp-Quan not only significantly reduced disease incidence but also significantly inhibited the
176 development of brown rot caused by *M. fructicola*, suggesting that the strain sp-Quan has the potential to
177 control brown rot on peach fruits.

178 The number of yeast colonies greatly increased within inoculated fruits incubated at 25°C and 95% RH
179 from an initial level of 5.0×10^6 CFU per wound to 4.45×10^7 CFU per wound after 1 day (Fig. 1). Two days
180 after inoculation, the yeast population was increased to 6.8×10^7 CFU per wound. The maximum population
181 (9.5×10^7 CFU per wound) of the yeast was observed on 4^{th} day after inoculation. On 5^{th} day after
182 inoculation, the population of the yeast was a bit lower than the maximum population, however, it still
183 remained at a rather high level (9.4×10^7 CFU per wound). This result indicated that the yeast had a strong
184 capability of colonizing the fruit surfaces, suggesting that nutrition and space competition may play an

185 important role in the biocontrol mechanisms of the yeast against pathogens.

186 Antagonistic activity can act through multiple ways, the most common being production of antimicrobial
187 metabolites, competition for nutrients and direct parasitism (Saravanakumar et al., 2009), but other
188 mechanisms can also be involved, such as induced resistance, sometimes associated with reduction of
189 pathogen enzyme activity (Spadaro et al., 2004; Zhang et al., 2010b). Our experiments showed that the
190 novel yeast *Candida pruni* grew faster than other yeasts and could inhibit the growth of *M. fructicola* on
191 PDA plates (data not shown), also implying that nutrition and space competition may play an important
192 role in control.

193

194 **Phylogeny, Morphology and physiology**

195 The GenBank/EMBL/DDBJ accession numbers for the 26S rRNA gene D1/D2 and ITS region sequences
196 of strain *Candida* sp-Quan in this study are JQ917720 and JX257177, respectively. Phylogenetic analysis
197 of the D1/D2 domain of 26S rDNA sequence from the strain "sp-Quan" in a dataset of D1/D2 domain
198 sequences from all currently recognized ascomycetous yeasts included in the database of NCBI indicated
199 that this strain was placed in the clade of *Candida asparagi* and *C. fructus* and was most close to the
200 known species "*C. musae* CECT 11882^{T} (Genbank accession number: AJ539365.1)" and "*C. fructus* CECT
201 11884^{T} (Genbank accession number: AJ539366.1)" (De Llanos et al., 2004) (Fig. 2). However, the strain
202 had a very low identity to *C. asparagi* (sequence similarity: 95.0%), *C. musae* (sequence similarity: 96.0%)
203 and *C. fructus* (sequence similarity: 96.0%) . Moreover, the strain "sp-Quan" differs from the strains
204 "CECT1182^{T}" and "CECT1184^{T}" by 3.6% substitutions (18 out of 499 nucleotide positions). Previous
205 studies showed that ascomycetous yeasts strains differing by more than 1% substitutions in the D1/D2
206 domain of 26S rDNA sequence represent separate species (Kurtzman and Robnett, 1998). Based on the

phylogenetic analysis, the strain “sp-Quan” is described as a new species of genus *Candida*.

Morphological culture showed that the cells of the stain are subglobose to ovoid single, in pairs, and in short chains when grown in yeast-extract-peptone-D-glucose (YPD) broth for 3 days at 25°C, and the colonies appear white in colour when grown on YPD agar plates. These are typical characteristics of the *Candida* genus. No sexual state was observed in cultures of the strain sp-Quan, when grown either on 5% malt extract agar at 25°C for 1-3 weeks, or on corn-meal agar at 25°C for 1-4 weeks, or on YPD agar for 1-3 weeks, or on PDA for 1-3 weeks.

Phylogenetic analysis above demonstrated that *C. asparagi*, *C. musae* and *C. fructus* are the closest known relatives of sp-Quan. However, in terms of fermentation, assimilation and other physiological tests, the three strains are remarkably different from three known species. The strains are different from *C. asparagi* by not fermenting galactose, by their ability to assimilate ribose or citric acid and to grow at 37℃, or to grow on vitamin-free medium. They are also different from *C. musae* by their ability to assimilate galactose, ribose and cellobiose, to grow at 37℃ and on vitamin-free medium, and they also obviously differed from *C. fructus* in the assimilation reactions of galactose, sucrose, maltose and cellobiose, as well as their ability to grow at 37℃ and on vitamin-free medium (Kurtzman, 1998) (Table 2).

Description of *Candida pruni* sp. nov.

Candida pruni (pru'ni. L. gen. n. pruni, of a plum-tree). Growth in YM broth: 3 days after incubation at 25°C, the cells are globose to ovoidal, (2.4-4.8) × (2.4-4.8) μm in shape and occur singly, in pairs, short chains or groups (Fig. 3). In addition, the sediment is formed and budding is multilateral. Growth on YM agar: 1 month after incubation at 25°C, the colonies of the streak culture are butyrous in texture, off-white in colour, smooth and opaque, with an entire to flat border. Dalmau plate culture on corn-meal agar: 7 days

after incubation at 25°C, no pseudohyphae or ascospores are observed. Glucose is fermented; galactose, sucrose, maltose, lactose and raffinose are not. Glucose, galactose, sucrose, maltose, cellobiose, trehalose, D-xylose, D-ribose, ribitol, D-mannitol, succinic acid and citric acid are assimilated; lactose, raffinose, soluble starch, L-arabinose, L-rhamnose, erythritol and inositol are not. Nitrate, L-lysine and cadaverine dihydrochloride are assimilated; Growth in vitamin-free medium is positive. Maximum growth temperature is 37°C. The type strain, *Candida* sp-Quan, isolated from plums collected in organic orchard in North of China has been deposited in China General Microbiological Culture Collection Centre (CGMCC), Academia Sinica, Beijing, China as GCMCC 6582^{T}, in the CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands as CBS 12814^{T}, and in Korean Collection for Type Cultures designated as KCTC 27526^{T}. The MycoBank number is MB804750.

In conclusion, the three yeast strains (sp-Quan, YG1, and GR1) showed a great potential to control peach brown rot caused by *M. fructicola,* and the strains demonstrated to be a novel species of the genus *Candida,* by colony morphology, physiological features, and molecular analysis. Better understanding of the modes of action is essential for developing appropriate commercial formulation and application methods to maximize the potential of biocontrol agents (Spadaro and Gullino, 2004; De Cal et al., 2009). Future research will target the elucidation of the mechanisms of biocontrol against brown rot on peaches.

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320 **Table 1.** Antagonistic activity of the yeast strain sp-Quan to control brown rot caused by *M. fructicola* on

321 peaches

Treatment	Disease incidence (%)	Diameters of brown rot lesions (mm)**
sp-Quan	20 ± 2 c	12 ± 3 b
Tebuconazole*	5 ± 1 b	8 ± 2 b
Inoculated control	100 ± 0.0 d	47 ± 3 c
Yeast control	0 ± 0.0 a	0 ± 0.0 a
Blank control	0 ± 0.0 a	0 ± 0.0 a

322

323 * Peaches were treated with 2.5 mL L^{-1} of Folicur (Bayer Crop Science; tebuconazole: 25.0 %).

324 ** The yeast strain sp-Quan was applied at 10^8 cells mL^{-1}. Fruits (cv Springcrest) were stored at 25°C for 6

325 days. The results are the mean of two independent experiments. "±" stands for standard deviation of the

326 means. Values followed by the same letter are not statistically different by Duncan's Multiple Range Test

327 ($p < 0.05$).

328

Table 2. Comparison of the physiological characteristics of *Candida pruni* sp. nov. with three closely related species.*

Characteristics	*C. pruni*	*C. asparagi*	*C. fructus*	*C. musae*
Fermentation:				
Galactose	-	+	-	-
Assimilation:				
Galactose	+	+	-	-
D-arabinose	-	+	-	-
D-ribose	+	*w*	+	-
Maltose	+	+	-	+
Cellobiose	+	+	-	-
Citric acid	+	*w*	+	+
Sucrose	+	+	-	+
Nitrate	+	+	+	+
Other tests:				
Growth at 37℃	+	-	-	-
Vitamin-free medium	+	-	-	-

*The lists of carbon sources and nitrates and other tests were prepared using standard methods described by Yarrow (1998). In the reactions of fermentation, assimilation and other tests, only different characteristics were listed, and the rest characteristics are the same.

+ positive; - negative; *w* weak. Data of reference species were taken from Lu et al. (2004).

Figure captions

Figure 1

Population dynamics of *C. pruni* sp-quan in wounds of peaches. Thirty μL washed yeast cell suspension at 1×10^8 CFU mL^{-1} was applied into each wound on fruits, and the yeast colonies were recovered from the wounds after incubation at 25°C and 95% RH. Data are from one trial with each point representing the mean colony counts from replicate fruits.

Figure 2

Phylogenetic tree made from neighbour-joining analysis of 26S rRNA gene D1/D2 region sequences reveals the relationship of the novel species, *Candida* sp-Quan, with closely related taxa. Bootstrap values (%) based on 1000 replications are given on each node. Bar means 5% sequence divergence. Reference sequences were retrieved from GenBank under the accession numbers indicated in parentheses.

Figure 3

Cells of *Candida pruni* sp. nov.: after 3 days of growth in YM broth at 25℃, Bar=20 μm.