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## Controlling Anthracnose of Passion Fruit by Antagonistic Yeast

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**Abstract** The purpose of this study was to survey, isolate and collect of the yeast fungi from fruits and vegetable crops. The collected yeasts are used for controlling *Colletotrichum boninense* a causal agent of passion fruit anthracnose, in the laboratory and in the greenhouse. Seventeen isolates of *C. boninense* are isolated from various locations of passion fruit farms in Chiang Mai. Of these three isolate are carbendazim resistance. The results shown that 49 isolates of the yeasts are isolated from 28 samples of fruits and vegetable crops. Using dual culture agar plating technique for screening the antagonistic activity against the growth of *C. boninense* which resistance to carbendazim found that 10 isolate of the yeasts are able to inhibit the growth of this fungus, of these, isolate BMF1 give the maximum inhibiting at 67.69 percent. This BMF1 will be used for the further experiment to control the passion fruit anthracnose in the greenhouse. *C. boninense* was first described from *Crinum asiaticum* var. *sinicum* (*Amaryllidaceae*) collected in the Bonin Islands, Japan (Moriwaki *et al.* 2003). According to these authors, the species was associated with a variety of host plants in Japan, including *Clivia miniata* (*Amaryllidaceae*), *Cucumis melo* (*Cucurbitaceae*), *Cattleya* sp., *Cymbidium* sp. and *Dendrobium kingianum* (*Orchidaceae*), *Passiflora edulis* (*Passifloraceae*) and *Prunus mume* (*Rosaceae*).

**Keywords:** Passion Fruit, Anthracnose disease, *Colletotrichum boninense*, phylogen

### Introduction

Passion fruit is a fruit in New Thailand. Currently, the government and the private sector are promoting the cultivation of passion fruit. Passion fruit is a plant that is important. Economic, because the fruit is sour. And fragrant Water extracted from a high acidity. Therefore, to use in the beverage industry with carbonate by the diluted or mixed with any other fruit to taste more mellow.

*C. boninense* (in its wide sense prior to our research) has frequently been identified as a pathogen causing fruit and leaf anthracnose, as well as an endophyte of a range of host plants worldwide, especially belonging to *Amaryllidaceae*, *Orchidaceae*, *Proteaceae* and *Solanaceae*. For example, *C. boninense* was found to be associated with diseases of *Leucospermum* and

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*Protea cynaroides* Teleomorph developed on SNA. Ascomata ovoid to obpyriform, medium to dark brown,  $170\text{--}210 \times 110\text{--}140 \mu\text{m}$ , glabrous, ostiolate, neck hyaline to pale brown, wall  $5\text{--}10 \mu\text{m}$  thick, outer layer composed of flattened medium brown angular cells,  $5\text{--}10 \mu\text{m}$  diam. Interascal tissue composed of paraphyses, hyaline, septate, branched at the base, disintegrating quickly,  $38\text{--}60\mu\text{m}$  long, base  $3\text{--}4.5 \mu\text{m}$  diam, apically free, the apex rounded. Asci cylindrical to clavate,  $58\text{--}74 \times 11\text{--}16 \mu\text{m}$ , 8-spored. Ascospores arranged biserially, hyaline, smooth-walled, aseptate, cylindrical to narrowly fabiform, straight or rarely very slightly curved, both sides rounded,  $(13.5\text{--})15\text{--}17(\text{--}18.5) \times 5\text{--}6 \mu\text{m}$ , mean  $\pm$  SD =  $16.0 \pm 1.1 \times 5.6 \pm 0.4 \mu\text{m}$ , L/W ratio = 2.9.

The objective of this study was to explore the collection and isolation of yeasts subpoenaed from fruits and vegetables. Test enmity of yeast that collected the fungus *Colletotrichum boninense* causes anthracnose of passion fruit. And test the ability of yeast antagonistic to control anthracnose Track passion fruit in the laboratory.

## **Materials and methods**

### ***Sample Collection and Isolation***

Bring plant samples showing symptoms of the two under a stereo microscope. The direct isolation. But without the spore samples to be incubated inside a box plant diseases that increase the humidity to stimulate sporulation. Leave at room temperature The drop of distilled water on a sterile agar PDA (Potato Dextrose Agar) 1-2 drops of sterile needles at the kickoff of the spores on the surface plant was put on drip water. then spread plate and check the germination of spores after 6 or 24 hours (depending on the type of fungus) isolated single spore.

### ***Morphological characteristics***

The study of fungi, such as spore, conidiophore, fruiting body and the germination of spores, etc., stored in lactic acid. These structures by bringing to light under the microscope. In addition, the study of colonies of fungi on agar PDA (Potato Dextrose Agar), such as skin color, the growth of the colony. To study the fungi collected the study is fungal in any group. (Crous *et al.*, 2007, 2009, 2011)

### ***Pathogenicity tests***

Pathogenicity tests were done by inoculating detached leaves and fruits of spore with 5 mm. mycelial agar discs of seven-day-old cultures of each of the five isolates. Inoculated leaves and fruits controls treated with

sterile agar discs. After inoculation leaves and fruits were kept in moistened plastic boxes at 25°C.

### ***DNA sequencing and sequence analyses***

The mycelia of 3 *Colletotrichum* isolates were grown in PDA broth at room temperature (24 °C) for one week.

#### **1. DNA extraction**

Genomic DNA was extracted from fresh mycelia using E.Z.N.A. Forensic DNA Isolation Kit (Omega Bio-Tek), following the manufacturer's manual.

#### **2. PCR: ITS**

The internal transcribed spacer (ITS) region was amplified in a 50-ml reaction volume containing 1X buffer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.2µM of each primer (ITS5 and ITS4), and 1 U Taq DNA polymerase. The PCR temperature profile began with an initial denaturation at 96°C for 2 min, followed by 35 cycles of 96°C for 1 min, 53°C for 1 min and 72°C for 1:30 min. The final extension was carried out for 10 min at 72°C.

#### **3. Gel Electrophoresis and Sequencing**

PCR product was checked by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet (UV) transilluminator. The PCR product was sent to be sequenced for both directions on an automated DNA sequencer (Macrogen Inc., Korea)

#### **4. Sequence analyses**

The nucleotide sequences obtained from all primers were assembled using Cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) Program (<http://www.mbio.ncsu.edu/BioEdit/BioEdit.html>). The sequences were compared with nucleotide sequences on Genbank, CBS or suitable database. Sequences were compared with *Colletotrichum* sequences available in the EMBL/GenBank database.

### ***Phylogenetic analyses***

Phylogenetic analysis of the ITS region was performed with the isolates of *C. boninense* and three additional *Colletotrichum* species in Clade 1 (obtained from Biotec, DNA data bank, Table 1) using *C. gloeosporioides* as out-group to determine the phylogenetic positions. Sequences were aligned using the Clustal-X program (Thompson *et al.*, 1997). A

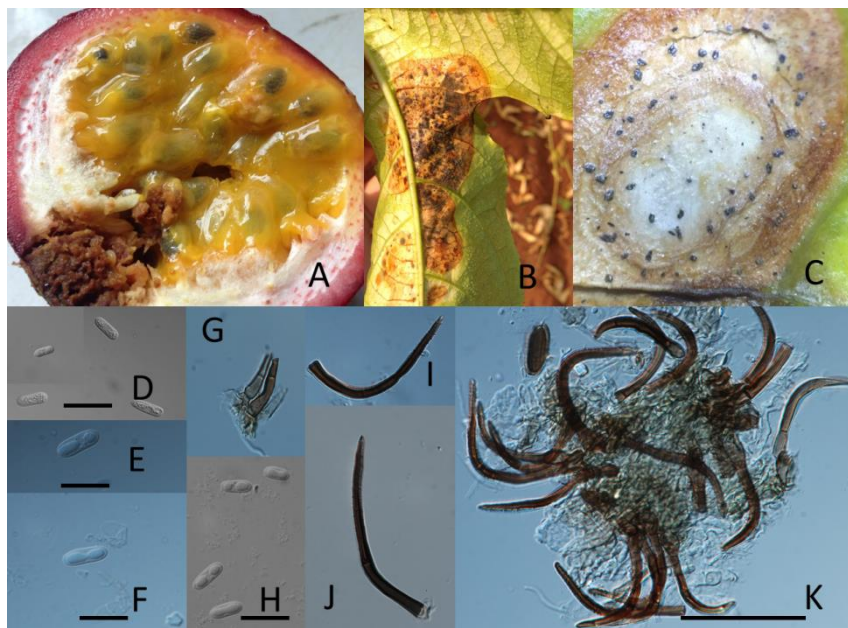
phylogenetic tree was constructed with the MEGA7, using the Neighbor-joining method with 1000 bootstrap replications.

**Table 1.** Identity of the ITS sequences available in GenBank used in phylogenetic analysis.

| <i>Colletotrichum</i> species | Accession No. | ITS      | Country     |
|-------------------------------|---------------|----------|-------------|
| <i>C.karstii</i>              | CBS125388     | JQ005185 | Panama      |
| <i>C.karstii</i>              | CBS861.72     | JQ005184 | Brazil      |
| <i>C.karstii</i>              | CBS129927     | JQ005206 | Thailand    |
| <i>C.phyllanthi</i>           | CBS 175.67    | JQ005221 | India       |
| <i>C.annellatum</i>           | CBS129826     | JQ005222 | Colombia    |
| <i>C.petchii</i>              | CBS378.94     | JQ005223 | Italy       |
| <i>C.petchii</i>              | CBS379.94     | JQ005224 | Italy       |
| <i>C.petchii</i>              | CBS118774     | JQ005225 | China       |
| <i>C.petchii</i>              | CBS118193     | JQ005227 | China       |
| <i>C.petchii</i>              | CBS125957     | JQ005226 | Netherlands |
| <i>C.novae-zelandiae</i>      | CBS128505     | JQ005228 | New Zealand |
| <i>C.novae-zelandiae</i>      | CBS130240     | JQ005229 | New Zealand |
| <i>C.boninense</i>            | CBS123755     | JQ005155 | Japan       |
| <i>C.boninense</i>            | CBS123756     | JQ005154 | Japan       |
| <i>C.boninense</i>            | MAFF306162    | JQ005155 | Japan       |
| <i>Colletotrichum</i> species | Accession No. | ITS      | Country     |
| <i>C.boninense</i>            | CBS128549     | JQ005156 | New Zealand |
| <i>C.boninense</i>            | CBS128506     | JQ005157 | New Zealand |
| <i>C.boninense</i>            | CBS128546     | JQ005158 | New Zealand |
| <i>C.boninense</i>            | CBS128547     | JQ005159 | New Zealand |
| <i>C.boninense</i>            | CBS112115     | JQ005160 | Australia   |
| <i>C.boninense</i>            | CBS129831     | JQ005161 | Australia   |
| <i>C.boninense</i>            | CBS128526     | JQ005162 | New Zealand |
| <i>C.ondidii</i>              | CBS129828     | JQ005169 | Germany     |
| <i>C.ondidii</i>              | CBS130242     | JQ005170 | Germany     |
| <i>C.beeveri</i>              | CBS128527     | JQ005171 | New Zealand |
| <i>C.colombiense</i>          | CBS129817     | JQ005173 | Colombia    |
| <i>C.colombiense</i>          | CBS129818     | JQ005174 | Colombia    |
| <i>C.brassicicola</i>         | CBS101059     | JQ005172 | New Zealand |
| <i>C.hippeastri</i>           | CBS125377     | JQ005230 | China       |
| <i>C.hippeastri</i>           | CBS125376     | JQ005231 | China       |
| <i>C.hippeastri</i>           | CBS241.78     | JQ005232 | Netherlands |
| <i>C.parsoniae</i>            | CBS128525     | JQ005233 | New Zealand |
| <i>C.brasiliense</i>          | CBS128528     | JQ005234 | Brazil      |
| <i>C.brasiliense</i>          | CBS128501     | JQ005235 | Brazil      |
| <i>C.dacrycarpi</i>           | CBS130241     | JQ005236 | New Zealand |
| <i>C.constrictum</i>          | CBS128503     | JQ005237 | New Zealand |
| <i>C.constrictum</i>          | CBS128504     | JQ005238 | New Zealand |
| <i>C.gloeosporioides</i>      | CBS112999     | JQ005152 | Italy       |

## Results and Discussion

Anthraco disease can occur in all areas are planted with fruit high humidity. (Cedeno *et al.*, 1993; Lutchmeah, 1993; de Goes, 1998; Wolcan and Larran, 2000) Symptoms on the leaves of is small incision is about 2-3 ml larger turning brown in the center of the lesion. Symptoms on the branch found is dark brown approximately 4-6 mm enlarged to show signs of canker. Flowers and fruits were infected. It is seen as brown spots. The wound is gray to muddy brown lesions if there is a large drop of water on the surface of a muscular build acervulus wound. When humidity is high, a group of orange conidia is created in which the top dieback symptoms acervulus will not prosper. The rot and die. (de Goes, 1998) The spread of infection relies on rain or irrigation to the seeds and seedlings. The optimum temperature for germination of conidia is between 30-33 ° C at night and 22-25 C in the daytime. (Francisco Neto *et al.*, 1994)



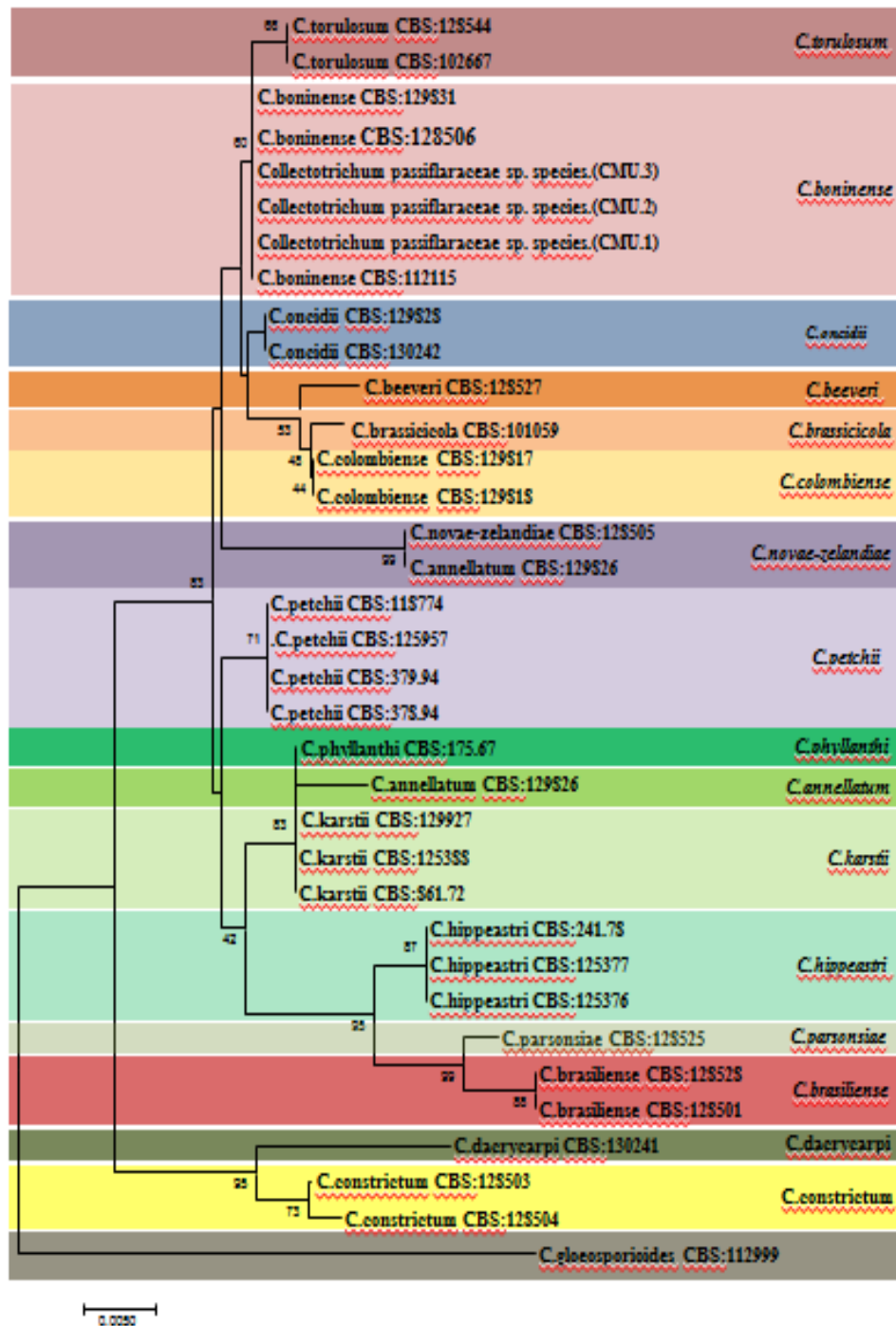
**Figure 1.** Anthracnose diseases on passion fruit (A- B), characteristic lesions on fruit(C), Group conidia on passion fruit (D, E, F, H: Scale bars = 5  $\mu$ m), seta of the fungus 40X (G, I, J, K: Scale bars = 17  $\mu$ m)

*Ascomata* perithecia, variable in shape but usually subglobose to pyriform, glabrous, medium brown, 100–300  $\times$  100–200  $\mu$ m, ostiolate, periphysate, neck hyaline to pale brown, to 100  $\mu$ m in length, outer wall composed of flattened angular cells 4–15  $\mu$ m diam. Interascal tissue composed of rather irregular thin-walled hyaline septate paraphyses. Asci in a basal fascicle, cylindrical-clavate, 45–60  $\times$  12.5–17  $\mu$ m, 8-spored, with a  $\pm$

truncate apex and a small refractive apical ring. Ascospores initially hyaline and aseptate, becoming 1–3-septate, septation sometimes occurring inside the ascus, light to medium brown-pigmented, sometimes verruculose prior to the start of germination, allantoid,  $(12.5\text{--}14\text{--}17\text{--}18) \times (4\text{--}5\text{--}6\text{--}6.5)$   $\mu\text{m}$ , mean  $\pm$  SD =  $15.6 \pm 1.4 \times 5.4 \pm 0.5$   $\mu\text{m}$ , L/W ratio = 2.9.

The ITS region of all five isolates (coll004, coll011 and coll013) was amplified using primers ITS5 and ITS4, Sequence analyses by BLAST indicated that the isolates were most similar to *C. boninense* isolate C24 and isolate 33-P1 (GenBank Accession No. AB688389 and KJ865230) with sequence identity values of 100 % and the sequence obtained in this study were aligned using the Clustal-X program. Phylogenetic analysis based on ITS5, ITS4 and 5.8S rDNA sequences clearly confirmed that the isolated fungus was *C. boninense*. A phylogenetic tree was constructed with the MAGA7 program, using the Neighbor-joining method with 1000 bootstrap replications (Figure 2). The MAGA7 program analysis confirmed that the fungus found on *Passiflora edulis* belongs to the genus *Colletotrichum* and can be grouped in the same clade as other taxa of the family *Glomerellaceae*, according to the new classification adopted by Martin *et al.* (2014). Morphological examination and MAGA7 program analysis revealed that the fungus found on *P. edulis* is *C. boninense*.

A phylogenetic tree was constructed using the MAGA7 program and phylogenetic distances were calculated using the neighbor-joining method. Bar = 0.005 genetic distance between samples. To our knowledge this is the first report of *C. gloeosporioides* causing anthracnose of passion fruit in Thailand.



**Figure 2.** Phylogenetic relationships of *C. boninense*, based on internal transcribed spacer rDNA sequences. Numerical values on branches are the bootstrap values as percentage of bootstrap replication from 1,000 replicate analyses.

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