Identification of Clonostachys and Trichoderma spp. from banana fruit surfaces by cultural, morphological and molecular methods

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Species of Clonostachys and Trichoderma isolated from the surface of bananas were highly antagonistic to crown rot–causing fungal pathogens of banana, such as Lasiodiplodia theobromae, Thielaviopsis paradoxa, Colletotrichum musae, and Fusarium verticillioides. Cultural and morphological examinations revealed that the fungal antagonists were similar to C. byssicola and T. harzianum, with some characters overlapping with closely related species. Molecular identification is recommended since the fungal isolates could not be differentiated adequately by cultural and morphological methods. Accurate taxonomy of these fungal antagonists was essential for the subsequent biological control studies. The \textit{tub2} region of \(\beta\)-tubulin genes of Clonostachys and 5.8S rDNA with the ITS regions of Trichoderma isolates were analyzed to determine their phylogenetic placement. Molecular and phylogenetic analyses revealed 100% homology of \textit{Clonostachys} (accession number AB308539) to \textit{C. byssicola} (accession number AF358154) and 99% identity of \textit{Trichoderma} (AB308540) to \textit{T. harzianum} (accession number AF625068, AF443927). The identity of the fungal isolates was confirmed as \textit{Clonostachys byssicola} Schroeus and \textit{Trichoderma harzianum} Rifai.$

\textbf{Keywords:} antagonist; biological control agent; \textit{Clonostachys byssicola}; \textit{Trichoderma harzianum}

\textbf{Introduction}

The best method of obtaining a biocontrol agent is to isolate potential candidates from areas of the plant where it is expected to function in disease control. Isolates collected in this manner can then be screened for biocontrol efficacy (Howell 2003). Utilization of natural epiphytes is the most practical approach, because natural resident biocontrol agents colonize food sources in plants without damaging the cells (Janisiewicz et al. 1994).

Species of Clonostachys and Trichoderma, such as \textit{C. rosea}, \textit{T. asperellum}, \textit{T. viride} and \textit{T. harzianum}, are well known biological control agents of various plant pathogens (Elad et al. 1980; Papavivas 1985; Howell 1987; Harman and Stasz 1989; Hjeljord and Tronsmo 1998; Krauss and Soberanis 2001; Howell 2002; Ten Hoopen et al. 2003; Batta 2007; Jensen et al. 2007; Verma 2007; Sant et al. 2010). \textit{Trichoderma} and \textit{Gliocladium} spp. isolated from green banana peel suppressed crown-rot disease complex pathogens, \textit{C. musae}, \textit{L. theobromae} and \textit{Fusarium moniliforme} (Kraus et al. 1998). We isolated \textit{Clonostachys} and \textit{Trichoderma} from the surface of diseased bananas imported into Japan from the Philippines (Alvindia et al. 2000). These fungi inhibited the growth of crown rot-causing fungal pathogens of banana, such as \textit{Lasiodiplodia theobromae}, \textit{Thielaviopsis paradoxa}, \textit{Colletotrichum musae} and \textit{Fusarium verticillioides} in vitro and, remarkably, controlled this disease when applied as postharvest treatment (Alvindia and Natsuaki 2008).

Crown rot, the most important postharvest disease of banana, is caused by several fungi, including \textit{L. theobromae} (Ogawa 1970; Johanson and Blazquez 1992), \textit{C. musae} (Finlay and Brown 1993), \textit{T. paradoxa} (Alvindia et al. 2002), and a complex of \textit{Fusarium} spp. (Knight et al. 1977; Jimenez et al. 1993; Alvindia et al. 2000; Hirata et al. 2001). The fungi infect the crown through flesh wounds created after trimming the crown of the banana hand into a crescent shape. The symptoms of crown rot are not visible at packing stations in banana-growing countries, but develop later, during shipment, ripening and storage in consumer countries. During the rainy season, losses of more than 10% have been recorded for Windward Islands bananas arriving in the UK (Krauss and Johanson 2000). Losses as high as 86% have been observed in the case of bananas from the Philippines, having undergone no chemical treatment (Alvindia et al. 2000).

Identification by cultural and morphological methods showed that \textit{Clonostachys} and \textit{Trichoderma} isolated from banana surfaces were similar to \textit{C. byssicola} and...
T. harzianum, respectively. However, overlapping of some cultural and morphological characteristics of our isolates with related Clonostachys and Trichoderma spp. was evident. Obviously, our Clonostachys and Trichoderma isolates could not be differentiated adequately by cultural and morphological methods. Correct identification of these biological materials is important for subsequent biological control studies. In this paper, we report the identification of Clonostachys and Trichoderma isolated from banana surfaces by cultural and morphological methods and confirm the identities by molecular technique. We discuss the cultural and morphological similarities of our isolates with closely related species of Clonostachys and Trichoderma, and construct a phylogenetic tree by neighbor-joining analysis of their DNA sequences.

Materials and methods

Source and maintenance of fungal isolates

Species of Clonostachys and Trichoderma isolated from the surface of diseased Cavendish bananas imported from the Philippines (Alvindia al. 2000) were kept in a store room of Food Protection Division, PhilMech at 25°C. The isolates were periodically maintained in test tube slants of potato dextrose agar (PDA) supplemented with 5% malt extract (ME) for good growth. Representative of the fungal isolates were also deposited in the Genebank National Institute of Agrobiological Sciences (Ibaraki, Japan).

Cultural and morphological observations

PDA and oat meal agar (OA) were used for cultural and morphological observations of Clonostachys, while PDA, OA and corn meal dextrose medium (CMD) were utilize for Trichoderma. Methuen’s Handbook of Color (Kornerup and Wanscher 1978) was our guide in determining colony colors of the isolates. The works of Chaverri and Samuels (2004), Gams and Bissette (1998), Schroers (2001) and Samuels (2006) were followed for cultural and morphological descriptions.

Molecular and phylogenetic analysis

Mycelia grown on PDA at 25°C were harvested after 1–2 weeks. Genomic DNA was extracted from lyophilized hyphae based on the method of O’Donnell et al. (1997) with some modifications or with DNeasy Plant Mini Kit (Qiagen, Dusseldorf, Germany). For Trichoderma, the nuclear ribosomal internal transcribed spacer (ITS) region was amplified with primer pairs ITS1 and ITS4 (White et al. 1990) and the tub2 region of β-tubulin genes was amplified with primer pairs T1 and T224 (O’Donnell and Cigelnik 1997) for Clonostachys. Polymerase chain reaction (PCR) amplification of ribosomal DNA (rDNA) was performed with 30 cycles of incubation for 1 min at 96°C, 1 min at 52°C, and 2 min at 72°C, while the tub2 genes were subject to denaturation for 2 min at 95°C followed by 40 cycles of incubation for 35 s at 94°C, 55 s at 52°C, and 2 min at 72°C. Gene amplifications were performed with the TaKaRa ExTaq system (TaKaRa, Otsu, Japan). Sequencing was conducted with the ABI-Prism 377 DNA sequencing system (Applied Biosystems, Foster City, CA, USA) and DNA sequencing kit (Perkin-Elmer, Waltham, MA, USA) following the ABI protocol.

Sequence alignment and homology analysis were carried out using AssemblyLIGNTM 1.0.9c (Accelrys, San Diego, CA, USA) and CLUSTAL W package with McVector 6.5.3 (Accelrys) (Thompson et al. 1994). The aligned sequences were analyzed by the neighbor-joining method (Saitou and Nei, 1987), using PAUP 4.0b. The distance matrix was calculated using DNADIST with the Kimura’s two-parameter method, and the topology was tested with 1000 bootstrap trials.

Results

Clonostachys

Pure cultures of Clonostachys have cottony colonies with a powdery surface on PDA and OA media, measuring 55–58 mm in 7 days at 28°C. The colony surface turned granular with a light orange coloration over time, due to production of sporodochia and conidial masses, and pale yellow in reverse. Morphological examinations showed that isolates had dimorphic conidiophores composed of primary and secondary. Primary verticillium-like conidiophores observed throughout the colony. In primary conidiophores, stipes measured 10–110 × 3.5 μm. Bi- to quinquiesverticilliate, adpressed to divergent secondary conidiophores. Conidia hyaline, smooth to finely roughened surface, broadly rounded, with lateral hilum, 4–12.5 × 1.5–3.5 μm. Comparison of cultural and morphological characteristics revealed that our Clonostachys isolate was identical to C. byssicola (Table 1).

The sequenced nucleotide of tub2 in the β-tubulin region was 568 bp. A rooted molecular phylogenetic tree was constructed by neighbor-joining analysis of the aligned sequences of tub2 (Figure 1.). In the phylogenetic study, C. byssicola and our Clonostachys isolate were examined with Cylindrocladium parasiticum as outgroup. The topology of Clonostachys banana isolate was identical to C. byssicola (accession number AF358173) with 100% bootstrap values (Figure 1).

Trichoderma

Pure cultures were cottony colonies that overgrew the 90-mm PDA, OA and CMD plates after 2 days at 28°C. Colonies on PDA and OA were shaded light green with sparse conidiation; powdery to granular colonies on
Table 1. Comparison of the cultural and morphological characteristics of the *Clonostachys* banana isolate and three *Clonostachys* species.

| Character                          | *Clonostachys* banana isolate | *Clonostachys* byssicola | *Clonostachys* rosea agrawalii | *Clonostachys* agrawalii |
|-----------------------------------|-------------------------------|--------------------------|---------------------------------|--------------------------|
| Colony diameter (OA, 25°C, 7 days) | 55–58                         | 50                       | 40–50                           | 40                       |
| Colony color                      | Creamy white                  | Pale yellow              | Greyish green to olivaceous green | Orange hues             |
| Colony surface                    | Powdery, felty to cottony     | Finely to coarsely granular, felty to cottony | Granular due to conidial masses; felty to cottony | Felty to granulose |
| Conidial masses                   | White to pale yellow          | White to pale yellow     | Uncolored to greenish; to olivaceous green | Light yellow to light orange shades |
| Colony reverse                     | Pale yellow                   | Pale yellow              | Greyish-green to olivaceous green | Yellowish white to light yellow |
| Shape of primary conidiophores    | Verticillium-like             | Verticillium-like        | Verticillium-like                | Verticillium-like        |
| Stipe length in primary conidiophores (μm) | 10–110 × 3–5                 | 10–100 × 3–5             | 25–200 × 3.5–5.5                 | 10–60 × 4               |
| Size of conidium (μm)             | 4–12.5 × 1.5–3                | 3.2–10.8 × 1.8–2.8       | 4.2–6.6 × 2–3.4                  | 3.8–5.8 × 2.2–3         |
| Shape of secondary conidiophores  | Adpressed to divergent        | Adpressed to divergent   | Adpressed                        | Mostly divergent         |
| Habitat                           | Plant                         | Fungi, plant             | Soil, fungi, plant               | Animal                   |
| Teleomorph                        | Not produced                  | *Bionectria byssicola*   | *Bionectria ochroleuca*          | Unknown                  |

Note: Morphological descriptions of *C. byssicola, C. rosea*, and *C. agrawalii* based on Schroers (2001).

Figure 1. Phylogenetic tree for *Clonostachys* banana isolate by neighbor-joining analysis of the tub2 (β-tubulin) sequences.
CMA due to dense dark-green conidiation after 7 days. Reverse on all media colorless to dull yellow. Regular verticillate conidiophores forming a pyramidal structure. Phialides ampulliform, 5.0–8.0 × 2.5–3.5 μm; conidia sub-globose to ovoidal, smooth-walled, subhyaline to pale green, 2.5–4.0 × 2.5–3.0 μm; chlamydospores produced. 

Comparison of the morphological characteristics between our *Trichoderma* isolate and three related *Trichoderma* species showed that the banana isolate was identical to *T. harzianum* (Table 2). 

The sequenced nucleotide of the rDNA ITS region was 486 bp. A rooted molecular phylogenetic tree was constructed by neighbor-joining analysis of the aligned nucleotide sequences of the ITS and 5.8s regions (Figure 2.). In the phylogenetic study, *T. harzianum* and *Trichoderma* banana isolate were compared. Based on the results shown (Figure 2), the topology of *Trichoderma* banana isolate was identical to *T. harzianum* (accession numbers AY625068 and AF443927) with 99% bootstrap values. 

**Discussion**

The genus *Clonostachys* and *Trichoderma* are well reported and studied biocontrol agents. However, identification of species belonged to these genera are notoriously difficult via traditional method. Cultural and morphological characteristics, such as growth rate, colony color and surface, stipe length, and size of conidia, of the *C. byssicola* banana isolate were distinctly different with *C. rosea*, which made identification more difficult. Gams and Samuels (2004), with its rapid colony growth, sparse conidiation and non-production of soluble pigment on media. The size of conidia and phialides overlapped with *C. rosea*, which made identification more complicated, corroborating Schroers’ (2001) observation that *C. rosea* and *C. byssicola* can only be distinguished by molecular means. The confusing results achieved by classical methods lead to the molecular identification of our isolates. The 100% homology of our *Clonostachys* isolate with *C. byssicola* confirmed the identity of the fungus. 

Most published works on *Clonostachys* as a biocontrol agent has focused on *C. rosea*, which controls *Alternaria brassicicola* in broccoli seeds (Sivapalan 1993), *Bipolaris sorokiniana* and *F. culmorum* in seedlings of barley and wheat (Knudsen et al. 1995), *Botrytis* spp. in chick pea and onion (Burgess et al. 1997; Sutton et al. 1997), *Moniliophthora roreri,* *Phytophthora* palmivora in cacao pods (Krauss and Soberanis 2001), *Phomopsis sclerotioides* in cucumber (Moody and Gindrat 1977) and *Verticillium dahliae* in soil (Keinath et al. 1991). Conversely, the bio-control efficacy of *C. byssicola* is limited to the work of Martijn ten Hoopen et al. (2006). Hence, our isolate is added to the list, with *C. byssicola* as a biocontrol agent of crown rot-causing fungal pathogens in banana (Alvindia and Natsuki 2008).

*Trichoderma* from banana surfaces differs from *T. harzianum* described in Schroers (2001) and Chaverri and Samuels (2004), with its rapid colony growth, sparse conidiation and non-production of soluble pigment on media. The size of conidia and phialides overlapped with *T. catoptron* and *T. stramenium*. The absence of a teleomorphic state made identification more difficult. Gams and Bissett (1998) showed that variations among *Trichoderma* spp. could not be differentiated satisfactorily via classical methods, thus making nomenclature placement uncertain. Hence, we only confirmed the taxonomy of our *Trichoderma* isolate by molecular means. The development of molecular techniques and the use of DNA sequence analysis became the new paradigm in fungal systematics for *Trichoderma* (Samuels 2006) and determined the taxonomical placement of this genus (Gams and Meyer 1998; Hermosa et al. 2000). The development of molecular tools has also enabled the positive identification of any strain.

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**Table 2.** Comparison of the morphological characteristics of the *Trichoderma* banana isolate and three *Trichoderma* species.

| Character                        | *Trichoderma* banana isolate | *Trichoderma harzianum* | *Trichoderma catoptron* | *Trichoderma stramenium* |
|----------------------------------|-------------------------------|-------------------------|-------------------------|-------------------------|
| Colony diameter (PDA, 90 in 2 days | 33–72                         | 36–49                   | 55–67                   |
| Colony color                     | Cottony                        | Cottony                 | Cottony                 | Cottony                 |
| Pigmentation on agar             | None                           | Yellow to brown         | None                    | None                    |
| Conidiation and color            | Sparse, light green            | Abundant, green         | Green, around the point of inoculum | Abundant, wet green droplets |
| Colony odor                      | No distinctive odor            | No distinctive odor     | No distinctive odor     | No distinctive odor     |
| Shape of conidium                | Sub-globose to ovoidal         | Sub-globose to ovoidal  | Ellipsoidal to oblong   | Broad ellipsoidal, sometimes oblong |
| Size of conidium (μm)            | 2.5–4.0 × 2.5–3.0              | 2.7–3.5 × 2.5–3.0       | 3.5–4.0 × 2.3–2.7       | 3.0–3.2 × 2.0–2.2       |
| Shape of conidiophores           | Pyramidal fashion              | Pyramidal fashion       | Verticillium-like       | Verticillium-like       |
| Size of phialides (μm)           | 5.0–8.0 × 2.5–3.5              | 4.8–8.5 × 2.5–3.5       | 5.5–7.2 × 3.2–4.2       | 4.7–5.0 × 3.0–3.2       |
| Chlamydospores                   | Present                        | Present                 | Absent                  | Absent                  |
| Teleomorph                       | Not produced                   | Hypocrea lxxii          | Hypocrea catoptron      | Hypocrea straminea      |

Note: Morphological descriptions of *T. harzianum*, *T. catoptron*, and *T. stramenium* based on Chaverri and Samuels (2002).
and the development of a phylogenetic tree. Identification of *Trichoderma* species and species in other economically important and species-rich genera will rely on DNA sequence data as the limits of phenotype to distinguish species are reached (Samuels 2006), as in the present study. The identification *T. harzianum* from banana as a biocontrol agent of crown rot-causing fungal pathogens added to the long lists of published articles on the antagonistic character of this fungus.

Being a highly perishable fruit, banana suffers severe postharvest losses both in terms of quality and quantity. Anthracnose, crown rot and cigar-end rot are common and serious postharvest diseases of banana. Although chemical control is a feasible option to control postharvest diseases, environmental and health risks are high. Therefore, consumer demand is increasing for fruit which has been treated non-chemically for postharvest pathogens, such as biological control.

The effective and safe application of biocontrol agents depend on a host of environmental conditions, target species and the accurate and reliable identification of potential isolates. In addition, the accurate identification of *Clonostachys* and *Trichoderma* isolated from banana peel is essentially important in pursuing our researches on the utilization of these fungi as biocontrol agents of banana diseases. We fulfilled one important step in our biological control research by correctly identifying the fungal antagonists. Correct identification will provide information on understanding the interparasitic relationship with target pathogens and the subsequent environmental fate of the antagonist needed for effective application.

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