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Short communication

A new disease of mung bean caused by *Botrytis cinerea*

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ABSTRACT

Gray mold caused by *Botrytis cinerea*, a ubiquitous and destructive plant pathogen, is an important disease of various crops including orchard crops, vegetables and ornamental plants. In June 2012, symptoms similar to gray mold were observed on mung bean (*Vigna radiata*) plants in Yongchuan, Chongqing, China. To confirm the causal agent, the pathogen was isolated and six fungal isolates were identified based on morphological and molecular characterization. The six isolates showed similar morphology to *Botrytis cinerea*. The rDNA-ITS sequences of the six isolates showed 99% identity to reported *B. cinerea* strains. The two specific primer pairs for *B. cinerea*, C_{729}^{+}/C_{729}^{-} and $Bc_{108}^{+}/Bc_{563}^{-}$, produced target fragments of 700-bp and 450-bp, respectively, in all six isolates, and their sequences displayed 99–100% identity to known *B. cinerea* strains. All of the isolates harbored two transposable elements of *B. cinerea*, *Flipper* and *Boty*, which indicated that they all belonged to the *transposa* group. Subsequently, pathogenicity tests demonstrated the pathogen was virulent not only on mung bean but also on other legume crops, including *Phaseolus vulgaris*, *Vigna unguiculata*, *Vigna angularis*, *Pisum sativum*, *Vicia faba*, and *Glycine max*. The results of morphological and molecular identification combined with pathogenicity tests confirmed that the pathogen isolated from mung bean was *B. cinerea*. To our knowledge, this is the first report of *B. cinerea* causing gray mold on mung bean in the world.

1. Introduction

Mung bean (*Vigna radiata* (L.) R. Wilczek) is an important legume crop that has attracted attention because of its nutritional profile and promising medical benefits (Nair et al., 2013; Tang et al., 2014). Mung bean is mainly distributed in tropical and subtropical regions in Asia, including China, India, Pakistan, Thailand, Indonesia and the Philippines (Jansen et al., 1996; Nair et al., 2013). China is the world's second largest mung bean producer with a 2000-year history of cultivation (Lin et al., 2002). Mung bean is affected by biotic and abiotic stresses throughout the whole growing season, which results in low productivity (Selvi et al., 2006; Wongpiyasatid et al., 1999). Mung bean can be attacked by 15 pathogens and more than 30 pests in China (Zhu and Duan, 2012). Diseases caused by fungal pathogens are a major limitation for mung bean production in China. Because of global climate change, new diseases continue to occur on legume crops (Vadez et al., 2012). *Botrytis cinerea* Pers. Fr., a ubiquitous and destructive pathogen, can cause gray mold in a wide range of hosts (over 200 species) around the world (Bézier et al., 2002; Durán-Patrón et al., 1999; Elad and Shtienberg, 1995; Rueda et al., 2014; Yuan et al., 1999), leading to serious yield losses especially on senescing or otherwise weakened or wounded plants (Köhl et al., 1995; Lorenzini and Zapparoli, 2014; O'neill et al., 1996). *Botrytis cinerea* is being continuously reported on new hosts including legume crops (Johnston et al., 2014).

The identification of pathogens in the *Botrytis* genus was traditionally based on the morphological characteristics of the conidia, conidiophores, and sclerotia, as well as host specificity (Lorenzini and Zapparoli, 2014). However, delimiting species with extremely similar or even identical morphological characteristics is difficult with traditional identification (Beever and Weeds, 2007; Nielsen et al., 2002). Moreover, some fungi show variable morphology when they are cultured in different growth conditions. Recently, *B. cinerea* was recognized as a highly variable species (including phenotypic and genetic variability) when incubated under unstable growth conditions (Hahn et al., 2014; Lorbeer,

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1980). In this context, molecular techniques provide powerful approaches to identify *Botrytis* species (Lorenzini and Zapparoli, 2014; Zhang et al., 2010a, 2010b). Analysis of sequences in the ribosomal internal transcribed spacer (ITS) regions is a common method for the identification of filamentous fungi and yeasts (Abd-Elmagid et al., 2013), and has been regularly used to identify *B. cinerea* (Özer and Bayraktar, 2014; Rigotti et al., 2006). To easily distinguish *B. cinerea* from other fungi *in planta*, two species-specific primer pairs, C^{+}_{729}/C^{-}_{729} and $BC^{+}_{108}/BC^{-}_{563}$, have been successfully developed and used to detect *B. cinerea* in various plant hosts (Gindro et al., 2015; Huang et al., 2012; Rigotti et al., 2002, 2006).

The genetic diversity of *B. cinerea* populations has been investigated in previous studies using molecular techniques (Fournier et al., 2003; Johnston et al., 2014; Tanović et al., 2014). The populations of *B. cinerea* were clustered into two main genetic groups, Group I and Group II, based on the presence or absence of two transposable elements (*Flipper* and *Boty*) and different responses to fenhexamid. Group I (*vacuma*) includes isolates that lack both transposons and are resistant to fenhexamid. By contrast, Group II contains four types, including *vacuma* (no transposon), *transposa* (both transposons), *Flipper*-only, and *Boty*-only, that are sensitive to fenhexamid (Fournier et al., 2005; Isenegger et al., 2008; Walker et al., 2011). In addition, phenotypic differences, aggressiveness and host preference vary among different groups and types (Johnston et al., 2014; Martinez et al., 2008; Tanović et al., 2014).

In June 2012, symptoms similar to gray mold were observed on leaves and young pods of mung bean plants in fields in Yongchuan, Chongqing, China. At the early stage of the symptoms, small spot lesions were scattered over the leaves (Fig. 1a). As the disease progressed, the small spots enlarged and later gray masses of conidia were rapidly produced on necrotic tissues in the high-humidity climate (Fig. 1b). Flowers and pods were also infected on established crops (Fig. 1c, d). Diseased pods produced discolored, shriveled seeds without viability (Fig. 1c). Infected flower debris fell on the lower leaves causing rapid re-infection (Fig. 1d). This study was conducted to confirm the causal agent of the disease observed on mung bean using morphological and molecular characterization, as well as pathogenicity tests. The genetic structure of the *B. cinerea* isolates was also studied, because such knowledge may eventually be beneficial in controlling this disease.

2. Materials and methods

2.1. Sample collection, and fungal isolation

In June 2012, infected leaves and pods showing symptoms similar to gray mold were collected from diseased mung bean plants growing in fields in Yongchuan, Chongqing, China. The diseased tissues were cut into 3-mm pieces from the edge of the lesion, disinfected in 2% NaClO for 2 min, and rinsed in sterilized distilled water three times. The pieces were put in petri dishes containing potato dextrose agar (PDA) medium. The plates were incubated at 20 °C for 12 days. Single spore cultures were performed using the dilution method. All the isolates obtained were stored in 30% glycerol at −70 °C for long-term preservation. Six typical isolates were selected and used in this study.

2.2. Morphological identification

Morphological identification was performed according to Ellis (1971) and Jarvis (1980). To carry out the identification, a mycelial plug (5-mm diameter) from each isolate was cultured on a PDA plate with three replications at 20 °C in the dark. After 12 days of incubation, colony characteristics and conidial morphology were observed. Fifty sclerotia of each isolate were randomly selected to measure their size in length and width.

2.3. DNA extraction and molecular identification

To determine the molecular characteristics of the pathogen, genomic DNA of all six isolates was extracted using the CTAB method with minor modifications (Allen et al., 2006). PCR amplifications of the rDNA-ITS regions of the isolates were performed using the universal fungal primers ITS1/ITS4 in accordance with White et al. (1990). The amplification of specific fragments was carried out using two species-specific primer pairs, C^{+}_{729}/C^{-}_{729} and $Bc^{+}_{108}/Bc^{-}_{563}$, following Rigotti et al. (2002, 2006). The PCR products were separated on 1% agarose gels and sent to BGI-Tech (Shenzhen, China) for purification and sequencing. The resulting sequences were analyzed and BLAST (Basic Local Alignment Search Tool) searched in the NCBI (National Center for Biotechnology Information) database.

To detect the transposable elements of *B. cinerea*, *Flipper* and *Boty*, two specific primer pairs, F300/F1500 and Bot1/Bot2, respectively, were used for PCR amplifications according to previous studies by Martinez et al. (2005, 2008).

2.4. Pathogenicity tests

Two trials were conducted to determine the virulence of the six isolates by pathogenicity tests on the original host mung bean (cultivars Jilv 7 and Bailv 8) and six other legume hosts, including adzuki bean (*Vigna angularis* cv. Tonghong 2), cowpea (*Vigna unguiculata* cv. Zhongjiang 3), common bean (*Phaseolus vulgaris* cv. Yingguohong), pea (*Pisum sativum* cv. Longwan 1), broad bean (*Vicia faba* cv. Chenghu 15), and soybean (*Glycine max* cv. Zhonghuang 13), because *B. cinerea* has a wide host range. All inoculated materials were sown in 10-cm pots (five seeds per pot) filled with vermiculite, and randomly distributed on a greenhouse bench under a temperature of 25 ± 2 °C.

The first trial was performed *in vitro* using detached leaves inoculated with mycelial plugs. Leaves were collected from 15-day-old plants, washed with sterile distilled water (SDW), and then placed in Petri dishes containing filter paper moistened with sterile water. The petioles of the detached leaves were sealed with a small piece of moist cotton. Mycelial plugs of the six isolates were taken from the margins of 2-day-old colonies and inoculated onto the detached leaves, which were cultured face down. Leaves inoculated with sterile PDA plugs were used as a control. All treated leaves were incubated in a growth chamber at 20 °C with a 12 h photoperiod.

The second trial was performed *in vivo* on seedlings inoculated with a conidial suspension of isolate SCB7-3. First, to make the conidial suspension, the isolate SCB7-3 was inoculated onto detached leaves that were surface-sterilized as in the first trial, and incubated at 20 °C with a 12 h photoperiod for 7–10 days until sporulation. Young conidia were collected, filtered with two layers of cheesecloth, and diluted in SDW by shaking the infected leaves. The conidial suspension was adjusted to 1×10^6 conidia/mL with SDW and was sprayed onto 15-day-old seedlings until runoff over the whole plants using a hand-operated nebulizer. All plants were covered with plastic bags to maintain humidity and incubated at 20 °C. For the control plants, the seedlings were treated in the same way but with SDW only. After 3 days of incubation, the plastic bags were removed and the plants were grown under the same conditions for another 7 days. All experiments were repeated twice with three replicates. Re-isolation of the pathogen from inoculated plants was conducted to ensure Koch's rules were met.