Botrytis squamosa: The Pathogen of Market Disease of Garlic Sprouts

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Summary

Garlic sprouts in Shanghai markets frequently suffer from bract rot attached with white molds and brown rot in stem. Isolation and pathogenic identification of the pathogen were conducted according to the Koch’s Rule, and the major pathogen named EXGL-19 was identified as Botrytis squamosa J.C. Walker via morphological characters and the internal transcribed spacer (ITS) sequence of the ribosomal DNA. The young aerial mycelium of EXGL-19 was white, and then turned to grey after maturation. This fungus could grow on potato dextrose agar (PDA) at the temperatures that ranged from 0–30°C, with the optimum at 15–20°C. Microconidia could form after 16 days of culture at 20°C, while macroconidia only formed after 60 days of culture at 0°C. The pathogen can significantly promote the ethylene release of the garlic sprouts, which accelerated aging of garlic tissue. In summary, these findings provided basic clues for controlling the market disease of garlic sprouts and reducing the losses.

Key words: Garlic sprouts, Market disease, Botrytis squamosa, Ethylene

Introduction

Garlic sprout is the scape of Allium sativum L. and is one of the year-round supply vegetables, which has the largest storage in low temperature and the longest storage period in China. Pathogen isolation and identification of diseased garlic sprouts in markets are important to develop controlling methods to reduce losses. Most researches about post-harvest diseases of garlic sprouts are storage diseases1–7, however, via market research, we discovered that the market diseases of garlic sprouts were more serious but less reported.

Garlic sprouts are popular in China, and Shanghai is one of the largest fresh vegetables logistics terminal markets where the sale of garlic sprouts is up to 25,000 kg per day. Market research of garlic sprouts diseases was conducted, and it was found that bract rot attached with white mold and brown rot in stem were more serious in the markets. The fungal pathogens were isolated and identified using morphological and molecular technologies, and its biological characteristics had also been analyzed for providing strategies to effectively control these market diseases.

Materials and Methods

Plant materials

The diseased garlic sprouts were collected from wholesale markets, supermarkets, and retail stores in Shanghai from January 2011 to October 2012.

Pathogen isolation and pathogenicity analysis

Pathogen isolation was carried out according to Xu et al.8). For the pathogenicity assay, 5 mm diameter mycelium plugs were excised from the edge of a purified colony using an aseptic hole puncher, and then were inoculated onto needle-wounded and non-wounded healthy garlic sprouts in the sterilized crispers. After 7 days of incubation at 25°C, pathogenicity was recorded as average decay diameters. To test the effect of different temperatures on the disease development, the wound-inoculated garlic sprouts were incubated...
at 0, 5, 10, 15, 20, 25, 30 and 35°C, respectively. Each treatment had 25 mycelium plates and the experiments were repeated 3 times. We also inoculated the pathogen on healthy strawberry, onion, cucumber and cayenne pepper to test the host range.

**Morphological identification of the pathogen**

According to Zhang’s description\(^\text{30}\), the morphological characteristics of the pathogen, including the color, morphology and growth rate of the mycelium were observed in the cultures on PDA medium at 25°C. In addition, the morphology, size, formation time and the status on mycelium of the conidia were measured under a microscope.

**Pathogen ribosomal DNA-ITS sequence analysis**

Fungal DNA was extracted by using the method described previously\(^\text{31}\). The internal transcribed spacer (ITS) region (ITS1, 5.8S and ITS2) of rDNA was amplified by PCR using universal primers ITS1 and ITS4\(^\text{32}\), and the PCR amplification program was conducted using Lin’s method\(^\text{33}\). Nucleotide sequence of the rDNA-ITS was deposited into GenBank (http://www.ncbi.nlm.nih.gov) as accession KC335151.

**Effects of temperature and pH on growth rate of pathogen**

Mycelium plugs (5 mm diameter) were inoculated on PDA and then cultured at different temperatures including 0, 5, 10, 15, 20, 25, 30 and 35°C, each with 6 plates as replicates. Colony diameters were recorded every day. 1 M HCl or 1 M NaOH was used to set the pH values of the PDA medium at a range from 2 to 9, mycelium plugs (5 mm diameter) were inoculated on these PDA, and then they were incubated for 5 days at 25°C. Each treatment had 6 replicates.

**Measurement of ethylene**

Ethylene levels in the pathogen-garlic sprouts system were determined by using a GC9800 gas chromatograph (GBPI Instruments, Guangdong, China) equipped with a flame ionization detector and a specific analysis column containing porous polymer beads with polydivinylbenzene, according to Zhu et al.\(^\text{34}\). Sterilized healthy garlic sprouts with similar thickness and length were randomly separated into 4 parts, including the bract, upper stem (30 cm from bract), middle stem (20 cm from bract), and bottom of stem (10 cm from bract). The inoculated and weighed garlic sprouts were placed in 55 ml sealed tubes at 25°C for 4 days. Non-inoculated garlic sprouts were used as controls. To prepare the gas samples, the tubes were first opened and placed on a sterilized bench for 30 min to refresh the inside air and then sealed to incubate for 2 hr. The ethylene production rates were calculated using milliliter ethylene per gram weight per hour. Each experiment was repeated twice.

**Statistical analysis**

The data were analyzed with ANOVA followed by Duncan’s multiple range tests for means comparison with the use of SPSS 17.0. Significant differences (\(p < 0.05\)) among treatments in each group were indicated by different letters.

**Results**

**Market disease symptoms and pathogen isolation**

Disease symptoms on garlic sprouts appeared during the sale period. The diseases were characterized by brown dry-decay of stem (Fig. 1A) and bract rot with white mold (Fig. 1B). 287 fungi were isolated from the diseased garlic sprouts. Based on the wound and non-wound inoculation assay for pathogenicity, we found the dominant pathogen was named EXGL-19 and caused the same disease symptoms to the original samples. In the early infection, brown dry-decay spot on the stems sags slightly (Fig. 1C and D), but tissues around the lesion turned yellow and unwatered over time, and then tissues became brown rot at the later stages of infection (Fig. 1E). The infected bracts decayed severely after inoculated with EXGL-19 for 7 days (Fig. 1F).

**Morphological and molecular identification of the pathogen**

A mycelial expansion assay of strain EXGL-19 indicated that its colony diameter could reach 77.6 mm after 7 days of culture on PDA at 25°C. The aerial mycelia appeared frequently and changed from white to gray (Fig. 1G and I). The edges of the colony were radial. Black sclerotiums were abundantly produced at 25°C (Fig. 1H), however, fewer were made at the low temperatures (Fig. 1J). Conidia were hardly visible at low temperatures except to the 60-day-old culture on PDA at 0°C. The conidia were ovate shaped, colourless, and (11.2–18.7) µm × (11.3–16) µm in size (Fig. 1K); they were gregarious on conidiophore like grapes (Fig. 1L). Furthermore, microconida which were produced in the
16-day culture on PDA at 20°C were spherosome, colourless and 3–4 μm (Fig. 1K); the status of microconidia was obviously different from that of macroconidia (Fig. 1M). The pathogenicity assay showed that EXGL-19 caused onion decay, but was not pathogenic to strawberries, cucumbers or cayenne peppers. The morphological characteristics of the isolate were consistent with the previously published descriptions of *Botrytis squamosa* Walker. The rDNA-ITS sequence of EXGL-19 was deposited into GenBank, and sequence analysis revealed that the isolate shared 98% nucleotide sequence identity with *B. squamosa* found in sequence databases, supporting the morphological identification of the isolate.

**Growth characteristics of EXGL-19**

The effect of temperature on mycelial growth of EXGL-19 was shown in Fig. 2. Mycelial growth of EXGL-19 ranged 0–30°C, and 35°C obviously restricted its growth, but it grew at 0°C; the optimal temperature was 20°C and the colony diameter was up to 82.95 mm when cultured on PDA for 5 days at 20°C. As for the medium acidity, the mycelia could grow from pH 3 to pH 6, while the optimum was at pH 4.

**Pathogenicity of EXGL-19**

The pathogenicity test was conducted at different temperatures as described above. EXGL-19 severely infected garlic sprouts at the temperatures between 15 and 20°C,
the lesion diameter was about 17.11 mm, which was significantly higher than those at other temperatures. The pathogenicity dropped when the temperatures increased or decreased. Under saturated humidity, mycelia of EXGL-19 withered and lost pathogenicity at 35°C. Mycelia could survive at 0°C, the lesions were invisible after 7 days inoculation (Fig. 3).

**EXGL-19 promoted ethylene release of garlic sprouts**

Ethylene is the plant senescence hormone. The bracts of diseased garlic sprouts in markets turned yellowed and decayed easier than stems. To study whether infection of EXGL-19 could accelerate ethylene release of garlic sprouts, we compared ethylene content from of different parts inoculated and non-inoculated garlic sprouts. Garlic sprouts were divided into 4 parts to compare their ethylene production. Fig. 4 shows the amount of ethylene production of the bract was more than the other parts, which indicates that bracts were the main part producing ethylene. In contrast, ethylene production had a sharp rise after inoculation with EXGL-19, the ethylene emission rate of bracts and the whole garlic sprouts were almost 2 times as much as control. We concluded, that EXGL-19 infection promoted ethylene emission and senescence of garlic sprouts.

**Discussion**

*Botrytis squamosa* Walker belongs to *Deuteromycotina, Botrytis*. This pathogen can directly induce diseases of *Allium* crops, such as onion^{15-20}, garlic and chives^{20}. There are many reports about this pathogen infecting garlic sprouts in storage periods^{12,5}, but fewer about the disease symptoms and damage in markets. A dominant pathogen named EXGL-19 was isolated from the diseased garlic sprouts in whole markets, super markets and retail stores.
in Shanghai. It could cause rot of bracts attached with white mold and brown rot of stem. EXGL-19 was identified as *B. squamosa* by morphological characters, rDNA-ITS and host ranges. The temperature for the mycelial growth of EXGL-19 ranged from 0–30°C, with the optimum at 20°C. The pathogen had higher pathogenicity between 15 and 20°C, and pathogenicity under wounded inoculation conditions was significantly higher than that of non-wounded inoculation. In conclusion, avoiding mechanical damage and using cold chain logistics are effective means to prevent occurrence of the disease during selling.

We never found conidia of *B. squamosa* on the diseased tissue of garlic sprouts. However, microconidia were produced on PDA after a 16-day incubation at 20°C. It is reported that microconidia in *B. cinerea* can act as spermatacia, but there are rarely reports about their germination. Whether microconidia of *B. squamosa* are capable of causing infection should be researched further. The artificial induction of macroconidial production is difficult for this fungus. To our surprise, the conidia were produced on PDA cultured for 60 days at 0°C. This pathogen might be a weak contagion in the markets for the short shelf life of garlic sprouts, whereas improving the detection methods of *B. squamosa* in the field and storage are important ways to control market diseases. It is worth concentrating on the epidemiology of *B. squamosa*.

Ethylene is a plant hormone which can induce and accelerate ripening and senescence of fruits and vegetables. Bracts produced more ethylene than stems of garlic sprouts, which indicated that the bract is the main part of physiological metabolism. The amount of ethylene produced by bracts had risen after inoculation with *B. squamosa*. In summary, inhibiting senescence and pathogen infection of bracts is very important to control market diseases and decrease economic loss.

**References**