Introduction

It is important to develop human health-promoting agents in fruit and vegetables. Broccoli is the most well-known vegetable with anti-cancer properties\(^6,22\); it contains isothiocyanates, which are proven to fight several kinds of cancer\(^3\). Furthermore, broccoli is a major screening target of our laboratory for components that may lead to the development of new anti-cancer drugs. However, broccoli is vulnerable to various pathogens\(^17\), especially black spot pathogens. The diseased broccoli florets rapidly senesce and have reduced commercial value. According to our market survey, rapid yellowing of broccoli is caused by infection with black spot pathogen(s). In the present study, experiments were designed to identify the pathogen(s) causing black spot disease in postharvest broccoli obtained from markets in Shanghai, and disease expression was examined under different conditions in relation to yellowing and ethylene production.

Materials and Methods

Plant material

Broccoli (Brassica oleracea L. cv., Italica) was purchased from the Yinlong vegetable factory, Shanghai Cao’an Wholesale Market, and Cangyuan Farming Supermarket in Shanghai.

Isolation of the causal pathogen

Diseased portions of infected broccoli were detached from the head using sterilized scissors and tweezers, cut into small segments, and immersed in 70% (v/v) ethanol for 30 s and then 2% (v/v) sodium hypochlorite for 1 min for surface sterilization. The segments were placed on potato dextrose agar (PDA) and incubated at 25°C until fungal or bacterial colonies appeared. In fungal colonies, incubation was prolonged until spores were produced (5–7 days). Bacteria were purified through five repeated single-colony isolations. Isolation of fungal spores was performed according to methods described previously\(^20\). After several repeat single-spore purifications, spores were suspended in 0.05% (v/v) Triton buffer to prepare an inoculum at a

Postharvest Black Spot Disease in Broccoli Caused by *Alternaria brassicicola*

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Summary

Broccoli in markets frequently suffers from black spot disease and rapidly turns yellow, thus losing commercial value. To clarify the causal pathogen, we isolated micro-organisms from infected broccoli and examined them for their pathogenicity using an inoculation assay. The pathogenic isolates obtained were further examined and identified using morphological and molecular characteristics. For molecular analyses, the nucleotide sequences of ribosomal DNA internal transcribed spacer regions were determined, and specific gene sequences were amplified using PCR. The highly pathogenic isolate obtained was identified as *Alternaria brassicicola* HLYB-4. Vegetative hyphae of the isolate slowly elongate and produce spores, even during cold storage (5°C). Optimal hyphal growth was observed at 25°C. Both ethylene production and chlorophyll degradation of diseased broccoli increased after infection with this isolate, promoting senescence of florets. However, higher temperatures (35°C) severely restricted growth of the pathogen.
defined density.

To examine pathogenicity of the isolated microbes, we evenly sprayed spore or bacterial suspensions of $10^6$ cells/ml on the florets of uninfected broccoli. Inoculated broccoli was placed in a growth cabinet controlled at 25°C, under continuous illumination with 4000 lux with fluorescent lamps, until symptoms appeared (7–10 days). Experiments were repeated three times using five broccoli samples in each experiment.

The pathogenic fungal isolates obtained were transferred to PCA (20 g potato, 20 g carrot, 20 g agar in 1 l water) and incubated at 25°C for 7 days with 12 h illumination with near-ultraviolet light. The spores produced were suspended in sterilized water, and the suspension was dropped onto the edge of a water-soaked filter paper. After 2 to 3 days of incubation, the length, width, septum number, and rostrum length of spores were examined according to methods previously described\(^{18}\). In each experiment, 50 spores of the test fungi were measured.

**Assay for ribosomal DNA-ITS and species-specific sequences**

Fungal DNA was extracted using methods described previously\(^{10}\). For two steps of PCR, a universal primer set (ITS1, 5’-TCCGTAGGTGAACCTGCGG-3’; ITS4, 5’-TCCGCTTATTGATATGC-3’) and a species-specific primer set\(^{19}\) (Abra1, 5’-ACCTCAGCAGCATCTGCTTTTG-3’; Abra2, 5’-GGCTTTATGGATGCTGACC TTG-3’) were used to amplify internal transcribed spacer (ITS) regions and to detect the sequences typical for \textit{A. brassicicola}, respectively. PCR amplification was carried out under the following conditions: pre-heating at 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s and extension at 72°C for 1 min, and final extension at 72°C for 20 min, using the Taq-PCR reaction mixture (2.0 μM 10× buffer, 2.0 μM MgCl₂, 2.0 μM dNTP, 0.4 μM ITS1, 0.4 μM ITS4, template DNA 1.0 μM, 2.5 units Taq enzyme in 11.8 μL water).

**Estimation of chlorophyll content**

The spore suspension was diluted to $1 \times 10^6$ spores/mL and used to inoculate broccoli. Both inoculated and non-inoculated broccoli florets were kept in moist desiccators of the same size at 25±1°C for a defined number of days. Extraction of chlorophyll was conducted using a standard method\(^{10}\). Florets were homogenized in acetone, and the homogenates were clarified by centrifugation at 4000 rpm. Chlorophyll content in the supernatant was spectrophotometrically determined by measuring absorbance at 645 and 663 nm according to the Lambert–Beer law. Experiments were repeated three times.

**Estimation of ethylene and CO₂ production**

Spore suspension (1×10⁶ spores/mL) was used to inoculate healthy heads of broccoli. Both inoculated and non-inoculated broccoli was placed in desiccators and incubated at 25±1°C. The desiccators were sealed for 1 h before measurement to estimate levels of ethylene and CO₂ produced. The assay temperatures for ethylene detection were column 80°C, vaporizer 100°C, FID 120°C, 0.1 M Pa for N₂ carrier gas; and for carbon dioxide were column 80°C, vaporizer 100°C, TCD 100°C, 0.1 M Pa. AH bridge current of 115 mA was used with a gas chromatograph, GC9800. Each experiment was repeated three times.

**Results**

A total of 51 bacterial and fungal isolates were obtained from diseased broccoli heads from the markets (Table 1). None of the bacteria obtained were infectious to broccoli, and no symptoms occurred during incubation after inoculation. Of the fungal isolates, 21 were \textit{Alternaria} spp., which cause typical symptoms of black spot disease after inoculation. Some isolates were \textit{Fusaria} fungi, which cause rot and produce a white layer of mycelia on the broccoli heads but no yellowing or black spot disease.

During the survey of market broccoli, we found that black spot disease frequently caused rapid yellowing of the whole
broccoli head (Fig. 1A). Of the present isolates, a strain (HLYB-4) of *Alternaria* sp. produced visible black florets with spores within 48 h of inoculation, and, at the same time, the whole head rapidly turned yellow (Fig. 1B). These symptoms were very similar to the original symptoms observed in diseased broccoli in the market. Strain HLYB-4 produced a green-brown colony on PCA, with numerous spores on the surface (Fig. 1C). Spores formed chains of 24 to 51.5×7.5 to 23 μm, with two to six transverse septa and zero to three mediastinal septa (Fig. 1D). Based on the *Alternaria* morphology monograph 18), the present isolate was confirmed as *A. brassicicola* HLYB-4. Molecular analysis revealed that the rDNA-ITS sequence (FJ465174) of the present isolate was highly homologous to the 18S ribosomal RNA gene of *A. brassicicola* AY154707 (homology, 99%) from Blast analysis in the GenBank database (http://www.ncbi.nlm.nih.gov). The species-specific sequence (457-bp fragment) was obtained by PCR amplification of HLYB-4 chromosomal DNA (data not shown) and supported the present identification.

**Fig. 2** shows mycelial growth of *A. brassicicola* HLYB-4 on PDA at different temperatures. Numbers represent incubation temperature (°C) suppressed mycelial growth of HLYB-4. Under the same temperature conditions, spore production by HLYB-4 was tested (Fig. 3). HYL-4 initiated spore production 2 days after incubation and reached a maximum in 6 days when incubated at the optimal temperature for mycelial growth (25°C). However, lower temperatures delayed production of spores by the isolate, and at the lowest temperature (5°C), sporulation occurred almost 2 weeks after the start of incubation. Incubation at 35°C significantly inhibited spore formation, and any spores formed were unable to germinate.

Four days after inoculation with *A. brassicicola* at 25°C,
the total chlorophyll content of the broccoli was less than 50% of the control, indicating that chlorophyll of the inoculated broccoli degraded much faster than in the control (Fig. 4).

Fig. 5 shows changes in ethylene and CO\textsubscript{2} production in the non-inoculated and HYLB-4-inoculated broccoli. The inoculated broccoli produced ethylene within 48 h of inoculation, which was 24 h before the control. Peak ethylene production was observed after 96 h in both broccoli samples, but the level in the inoculated broccoli was much higher than in the non-inoculated broccoli. Also, respiration, assessed as CO\textsubscript{2} production of the inoculated broccoli was much higher than in the non-inoculated broccoli, although peak CO\textsubscript{2} production was detected after 96 h in both samples. The result indicates that HYLB-4 promotes both ethylene production and respiration of inoculated broccoli, leading to promotion of yellowing.

Discussion

Broccoli is a major export vegetable in China, and, at the same time, its domestic consumption increases every year. However, immature heads remain fresh for less than 3 days at ambient temperatures because of fungal infection and metabolism\textsuperscript{13}. Therefore, postharvest loss has become a critical problem for sales and exports\textsuperscript{4}. Effective methods for controlling postharvest decay of broccoli have therefore become increasingly important.

Broccoli black spot disease is a common postharvest disease in Shanghai. This infection is always accompanied by rapid plant senescence. In this research, we isolated the causal pathogen and analyzed its involvement in the yellowing process. On the basis of morphology and rDNA-ITS analysis, the pathogen was identified as \textit{A. brassicicola}. This is the first report on its pathogenicity in postharvest broccoli in China. Our results show that this pathogen can grow and form spores over a wide range of temperatures. Even low temperatures do not totally restrict its spread\textsuperscript{8}, although growth was obviously restricted at 5°C and 35°C, which may provide a basal approach for controlling postharvest black spot disease.

According to previous research\textsuperscript{7,21}, the rapid senescence of postharvest broccoli is associated with climacteric degradation of chlorophyll and/or oxidation of lipids. Many physical and chemical methods, such as controlled temperature, controlled atmosphere\textsuperscript{12}, ultraviolet...
radiation\textsuperscript{11}, alcohol\textsuperscript{15}, 1-MCP\textsuperscript{1}, and cytokinin\textsuperscript{16}, have been utilized to regulate senescence. However, the relationship between fungal infection and senescence of broccoli has been ignored. There are few reports on the control of broccoli postharvest disease and its pathogenic mechanism. Our analysis shows that in infection by \textit{A. brassicicola}, the degradation of chlorophyll around infected parts is remarkably accelerated, resulting in rapid yellowing of the whole head. Moreover, ethylene production and respiration intensity increase significantly after infection with \textit{A. brassicicola}, which may be the direct causes of yellowing. Therefore, restricting ethylene production after harvesting may be essential in fungal infection. Many fungi produce ethylene \textit{in vitro}\textsuperscript{9}, and a certain concentration of ethylene may affect pathogen growth\textsuperscript{2,5}. Therefore, ethylene may also be an important signal in fungal infection. Further work is needed to clarify the function of ethylene and how it affects vegetable shelf-life.

References