High Humidity and Age-Dependent Fruit Susceptibility Promote Development of *Trichothecium* Black Spot on Apple

Pengbo Dai, Xiaofei Liang, Yajing Wang, State Key Laboratory of Crop Stress Biology in Arid Areas and College of Plant Protection, Northwest A&F University, Yangling, Shaanxi, 712100, China; Mark L. Gleason, Department of Plant Pathology and Microbiology, Iowa State University, Ames, IA 50011, U.S.A; and Rong Zhang and Guangyu Sun,[†] State Key Laboratory of Crop Stress Biology in Arid Areas and College of Plant Protection, Northwest A&F University, Yangling, Shaanxi, 712100, China

Abstract

Fruit bagging is a widely used orchard practice in China. *Trichothecium* black spot (TBS) is a disease highly associated with the fruit bagging. In this study, we characterized in vitro factors affecting the causal agent, *Trichothecium roseum*, and TBS development and infection histology on field-bagged apple fruit in situ. Under in vitro conditions, conidial germination required exogenous nutrients, and the germination rate was significantly promoted by high humidity, a condition mimicking the bag microenvironment. Germ tubes penetrated fruit via natural openings including stomata, lenticels, and surface cracks. To determine the chronology of infection by *T. roseum*, ‘Fuji’ fruit were inoculated in the field at

different developmental stages. The earliest infection occurred 60 days after full bloom (dafb), and disease incidence increased as fruit maturity advanced. At harvest time (165 dafb), lesions on more recently inoculated fruit (105 dafb, 150 dafb) were larger than lesions from fruit inoculated on earlier dates. Histological observation showed that infection of younger fruit elicited stronger host lignification responses restricting lesion development. Taken together, our results support the hypothesis that high humidity in sealed bags and increased susceptibility associated with advancing fruit maturity are key factors promoting *T. roseum* infection and TBS symptom development on bagged apple fruit.

Fruit bagging, which is a common practice in China to produce blemish-free apple fruit, entails sealing young fruit (~2 to 3 cm in diameter) in paper or plastic bags until near maturity (Fig. 1). By providing physical protection, fruit bagging not only improves fruit appearance by promoting skin coloration but also protects fruit against damage from pesticide residues, insects, diseases, bird pecks, and mechanical abrasion from hailstones (Feng et al. 2014; Wen and Ma 2006). This practice was first introduced from Japan in the 1990s, and has become widespread in the last 20 years in China. It is now applied to over 70% of apple orchards in China. With the proliferation of fruit bagging, pests such as *Carposina niponensis* Walsingham, *Adoxophyes orana* Fischer von Roslerstamm, and infectious apple diseases such as ring rot, bitter rot, and apple scab, were controlled efficiently in China (Wang et al. 2006).

However, fruit bagging has resulted in new disease problems, including black spot (Wang et al. 2006). Black spot is caused by the fungi *Trichothecium roseum* (Pers.) Link (Guo et al. 2005), *Alternaria tenuissima* (Nees) Wiltshire, *Acremonium sclerotigenum* (Moreau & R. Moreau ex Valenta) W. Gams (Wang et al. 2014), and *Acremonium stritum* (Pers.) Link (Guo et al. 2005). Among these pathogens *T. roseum* is one of the most important, causing 1- to 6-mm-diameter circular black spots in the fruit surface, with most spots located on the epicarps surrounding sepals (Fig. 2) (Chen et al. 2002); this form of black spot is called *Trichothecium* black spot (TBS). A similar disease called pink rot caused by *T. roseum* was reported on bagged apple fruit in Japan (Yukita and Kuradate 1988).

Black spot occurs widely in Shaanxi, Hebei, and Shandong provinces (Li et al. 2015). The incidence ranged from 10 to 30% among orchards in the region of Yangtai and Shandong, and reached up to 90% in some orchards in 2011 (Wang et al. 2014). Black spot had

a higher severity in cv. Fuji fruit, and relative lower severity in early-maturing varieties, such as cvs. Pink Lady, Gala, and Jonagold in the field (Guo 2013; Liu et al. 2007). Black spot caused significant economic losses by degrading fruit appearance and reducing storage life (Li et al. 2015; Liu et al. 2007).

Factors affecting black spot occurrence included climate conditions, fruit bag types, and humidity inside fruit bags. Li et al. (2015) reported that the amount of rainfall in August and September correlated positively with black spot incidence. Fruit in plastic bags, with presumably less air circulation and higher humidity than in paper bags, tended to have higher incidence (Chen et al. 2002; Guo et al. 2005; Liu et al. 2007). Furthermore, decreasing humidity in plastic bags by pricking some holes in them reduced black spot incidence (Chen et al. 2002).

Understanding TBS biology is critical for effective disease control. The objective of this study was to characterize the infection biology of *T. roseum* and to identify key host, pathogen, and environmental factors affecting TBS development.

Materials and Methods

Inoculum preparation. The *Trichothecium roseum* (Pers.) Link isolate TR45, obtained from symptomatic apple fruit in Yangling, Shaanxi Province, China, was identified by morphology and molecular phylogenetic analysis, and its pathogenicity was verified according to Koch’s postulate. Isolate TR45 was used to produce a GFP strain used in this study. GFP expression was achieved by protoplast transformation with the GFP expression vector pRM7, where GFP was driven by the constitutive RP27 promoter. This GFP strain was maintained on potato dextrose agar (PDA).

To harvest conidia, 6-day-old PDA cultures grown under 25°C in darkness were flooded with 10 ml of sterile water, scraped, and filtered through one layer of Miracloth (Calbiochem, USA). The conidial suspension was adjusted to a concentration of 5×10^6 CFU ml⁻¹ and supplemented with Tween-80 to a final concentration of 0.1% for inoculation purposes.

Effects of exogenous nutrients, humidity, and darkness on in vitro conidial germination. To test the effect of exogenous nutrients on conidial germination, a 20-μl drop of conidial suspension (10^6 CFU ml⁻¹) was placed on a glass slide using a micropipette, air-dried for 10 to 15 min under air flow, and amended with a 30-μl drop of sterile water or 1, 10, or 100% apple juice. For each treatment,

[†]Corresponding author: G. Y. Sun; E-mail: sgy@nwsuaf.edu.cn

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drops were placed separately on the two ends and the middle of a glass slide. After the glass slide containing solution drops was placed in a petri dish containing approximately 20 ml of water agar, the petri dish was sealed by paraffin film. To test the effect of humidity on germination, three 20- μ l drops of conidial suspension (10^6 CFU ml^{-1}) were pipetted separately onto the two ends and middle of glass slides, then 30- μ l drops of 10% apple juice were added to each droplet, and the mixtures were air-dried for 10 to 15 min under air flow, and sealed in petri dishes or chambers with relative humidity (RH) of 100, 99, 97, 95, 92, or 85%. Relative humidity levels of 100, 99, 97, or 95% were achieved by amending approximately 20 ml of aqueous agar with 0, 0.3, 0.9, or 1.5 mol NaCl, respectively, in sealed 85-mm-diameter dishes (Harris et al. 1970; Wang et al. 2015; Xu et al. 2001). The glass slides were placed in the lid of petri dishes that contained agar supplemented with NaCl. In contrast, RH levels of 92 and 85% were achieved by saturated KNO_3 and KCl solutions, respectively, in glass chambers (Kosuge and Hewitt 1964). Slides were incubated on platforms that were 2 cm above the surface of the salt solutions. To test the effect of illumination on conidial germination, 20- μ l droplets of conidial suspension (10^6 CFU ml^{-1}) in 10% apple juice were deposited on the ends and middle of glass slides by a micropipette, then placed in the lids of petri dishes containing approximately 20 ml of aqueous agar to maintain 100% RH. Petri dishes were then sealed immediately with paraffin film, and either placed in an illumination incubator with constant light (cold white light; illuminance = 15,000 Lx) or wrapped in black plastic bags to ensure darkness. All treatments were incubated at 25°C. Thirty-six glass slides were used per treatment. The tested glass slides were taken from the incubators and observed for conidial germination by fluorescent microscopy at 3, 6, 9, 12, 15, 18, 24, 36, 48, 72, 96, and 120 h postinoculation (Olympus BX51, Japan; X-Cite 120Q). A conidium was considered germinated when the germ tube was longer than half of the length of the conidium. Three slides were used for counts at each observation time. Germination rates were calculated based on examination of at least 250 conidia. The experiments were each performed three times.

Fig. 1. Young apples are covered with fruit bags at 3–4 cm in diameter after bloom.

In vitro inoculation. To determine penetration sites of *T. roseum*, apple fruit (cv. Fuji) were harvested at 30, 45, 75, 120, and 150 days after full bloom (dafb) for laboratory inoculations. After harvested apples were sprayed with 75% ethanol and air-dried, about 1 ml of inoculum suspension (5×10^6 CFU ml^{-1}) was sprayed uniformly on the entire apple surface. For each developmental stage, 24 fruit of similar size were inoculated, and an additional five fruit were mock inoculated with sterile distilled water. Inoculated apples were placed in plastic boxes (eight fruit per $40 \times 28 \times 12$ cm box). The sterile gauze moistened with 50 ml sterile water was placed in the bottom of each plastic box to maintain high relative humidity (RH = $98 \pm 1\%$). Boxes with inoculated apples were kept at $25 \pm 1^\circ\text{C}$ in darkness. Apple peels ($1 \times 1 \text{ cm}^2$) were sampled for microscopic examination at 0, 6, 12, 18, 24, 36, 48, and 72 h postinoculation (hpi). For each sample point, three apples were taken from the box and examined using fluorescent microscopy (Olympus BX51, Japan; X-Cite 120Q). Five peel segments of each apple were examined. Each developmental stage had three replicates. The experiment was conducted in 2015 and repeated in 2016.

Scanning electron microscopy. Apple peels containing germinated conidia identified by fluorescent microscopy were further sampled for scanning electron microscope observation. The peels ($5 \times 5 \text{ mm}^2$) were fixed in 4% glutaraldehyde solution for 24 h. The samples were washed three times with a phosphate buffer, then dehydrated in a series of aqueous-ethanol solutions (50, 70, 85, 95, and 100%). Subsequently, samples were dried in a critical point dryer under CO_2 and mounted with double-adhesive tape onto aluminum stubs and coated with gold. Samples were examined using a scanning electron microscope (Model S-4800, Hitachi, Japan). For each developmental stage, peels from three apples were examined and five peel segments were sampled per fruit.

Field inoculation and microscopic observation. Field inoculation in 2015 and 2016 was carried out in a block of 7-year-old trees (cv. Fuji) on M.9 rootstock in an apple orchard located in Yangling. Inoculations were performed in situ on fruit at 30, 45, 60, 75, 105, or 150 dafb. Fungicides were not applied in the experimental plots. For each inoculation scheme, 30 asymptomatic apples were randomly selected from five trees and were inoculated in the same way as in vitro inoculation. In addition, 10 control fruit per developmental stage were mock-inoculated with 0.1% Tween-80. All inoculations were done near sunset. After inoculation, the apples were covered with wet two-layer paper bags (Jingguo, Jinggong Ltd., China). TBS incidence was investigated 15 days after inoculation; incidence of TBS was calculated as follows:

$$\text{Incidence \%} = \left(\frac{\text{number of diseased fruit}}{\text{total number of fruit}} \right) \times 100$$

Fruit showing TBS symptoms were harvested from the orchard; black spot tissues were excised. Slices were made perpendicular to the fruit surface by free hand, and examined under light and fluorescent microscopy.

Statistical analysis. Variance of incidence of TBS on apples for each fruit developmental stage and year was calculated using the “general linear model - univariate” function in IBM SPSS Statistics 19 at $P = 0.05$ level. Effect of apple age and year on diameter of TBS lesions was also analyzed as described above.

Results

Effects of exogenous nutrients, relative humidity, and darkness on in vitro conidial germination of *T. roseum*. At 12 hpi, germination rate of *T. roseum* in sterile distilled water was 2%, which increased to ~20% by 72 hpi. At 12 hpi, germination rates in 1, 10, and 100% apple juice were $5 \pm 0.8\%$, $68.8 \pm 8.8\%$, and $98.7 \pm 1.5\%$, respectively (Fig. 3A). At 9 hpi, nearly 100% of conidia had germinated at RH = 100, 99, or 97%. At RH = 95% and RH = 92%, 100% germination was observed at 18 hpi and 48 hpi, respectively (Fig. 3B). Conidial germination was completely inhibited at RH = 85%.

Constant light delayed germination by ~2 h compared with complete darkness (Fig. 3C).

Penetration of fruit in vitro by *T. roseum*. Conidial germination began at 9 hpi, and germination rate steadily increased. Conidia surrounding lenticels were more likely to germinate than those located further from lenticels. At 24 hpi, >80% of the conidia near lenticels (within a radius of 200 μ m) germinated, whereas the germination rate was 10% for those located at greater distances (Fig. 4 A–C).

Germinated conidia penetrated apple fruit via natural openings. In early stage fruit (75 dafb), penetration mainly occurred through stomata (Fig. 4E). For fruit at 120 dafb, germinated conidia mostly penetrated via lenticels (Fig. 4D). For fruit at 150 dafb, most conidia near lenticels did not germinate or penetrate the lenticels, presumably because the lenticels had become heavily lignified, as evidenced by strong autofluorescence (Fig. 4G). *T. roseum* conidia also penetrated the fruit surface via microcracks in the epicuticular wax layer (Fig. 4F).

Infection of fruit in field trials. The incidence of black spots at 15 days postinoculation (dpi) was calculated in 2015 and 2016. Apple age significantly affected incidence of black spots ($P = 0.003$) (Table 1). The effect of year was not significant ($P = 0.553$) (Table 1); hence, the data sets for the 2 years were pooled and analyzed (Fig. 5A). TBS symptoms did not show up on fruit that had been inoculated at 30 dafb or 45 dafb. For fruit inoculated at 60 dafb, disease incidence per fruit was about 40%, and the number of black spots per symptomatic fruit ranged from two to five. For fruit inoculated at 75 dafb, disease incidence was about 60%, and the number of black spots per symptomatic fruit ranged between 2 and 15 (P. Dai, unpublished data). At 150 dafb, disease incidence increased to 90%,

and the number of black spots per symptomatic fruit ranged from 2 to 11 (P. Dai, unpublished data).

At harvest maturity (165 dafb), we also quantified TBS lesion diameter on fruit. Apple age significantly affected diameter of black spots ($P = 0.010$) (Table 2). The effect of year was not significant ($P = 0.182$) (Table 2), and hence the 2 years of data were pooled together and analyzed (Fig. 5B and C). In general, fruit inoculated at later maturity dates produced larger TBS lesions. Lesion diameters for fruit inoculated at 60 and 75 dafb ranged from 500 μ m to 3 mm, but most were smaller than 2 mm. For fruit inoculated at 105 dafb, lesion diameter ranged from 1 to 6 mm, and >90% were between 1.5 and 4 mm (Fig. 5B). For fruit inoculated at 150 dafb, lesion diameter ranged from 1 to 7 mm, and most were larger than 2 mm (Fig. 5B and C). The average diameter of fruit inoculated at 105 or 150 dafb was significantly larger than for fruit inoculated at 60 to 75 dafb (Fig. 5B).

Histological changes in fruit response to infection at different fruit developmental stages. Inoculation at 75 dafb induced approximately 800- μ m-diameter TBS lesions at 15 dpi. At the lesion sites, epidermal, hypodermal, and parenchymal cells were observed to have changed color, which was interpreted as cell death. Hyphal colonization, however, was limited to the epidermal and hypodermal cell layers (Fig. 6A). At 30 to 40 dpi, lesion sizes had expanded to around 1,400 μ m in diameter, but hyphal colonization was still restricted mainly to the epidermal and hypodermal cell layers, and hyphal extension into dead parenchymal cell layers was observed rarely (Fig. 6B). At 50 to 70 dpi, infectious hyphae began to decline as evidenced by decreased green fluorescence. Hyphae in the inner tissue layer appeared to decline first, leaving remnant green fluorescence

Fig. 2. Apple with *Trichothecium* black spot after removing bag in field.

at the penetration sites (Fig. 6C). In certain sections, green fluorescence disappeared completely, and a yellow fluorescent zone surrounding lesions was observed, possibly due to lignification (Fig. 6D).

Early colonization events (15 and 30 dpi) of *T. roseum* on fruit inoculated at 105 dafb were similar to those inoculated at 75 dafb, except that the colonized area was larger (Fig. 6E and F). At 50 to 60 dpi, significant hyphal colonization of parenchymal cells was observed (Fig. 6G), and no hyphal death or lignification zone formation was observed in >20 independent section samples.

Fig. 3. Effects of nutrition (A), humidity (B), and light conditions (C) on in vitro conidial germination of *Trichothecium roseum*. Apple juice solution (10%) was used as the nutrient source to assess the effects of humidity and light. For each treatment, germination rate was calculated based on at least 250 conidia.

For fruit inoculated at 150 dafb, intense colonization of parenchymal cells was obvious as early as 15 dpi (Fig. 6H).

At harvest maturity (165 dafb), a layer separating TBS lesions from healthy fruit tissue was obvious with fruit that had been inoculated at 60 dafb, but not when inoculation occurred on fruit that were nearly mature (150 dafb) (Fig. 7A and B). Phloroglucinol staining, indicating lignin accumulation, was observed in the separation layer on young fruit (60 dafb), but not on mature fruit (150 dafb) (Fig. 7C and D).

Discussion

TBS is an emerging disease in China that degrades the appearance and storage time of field-bagged apple fruit. Our results showed that a favorable microenvironment and fruit age-dependent susceptibility were key factors promoting TBS disease development.

On fruit surfaces, conidia near lenticels had much higher germination rates than conidia far away from lenticels. We demonstrated that the availability of exogenous nutrients is of critical importance for *T. roseum* conidial germination based on an in vitro assay. Many sugars and organic acids, including glucose, fructose, sucrose, malic acid, and citric acid, can be exuded around lenticels during fruit expansion (Belding et al. 2000; Liu and Xiao 2005; Wrona and Grabowski 2004). We speculate that *T. roseum* might rely on lenticel-derived nutrients for conidial germination and subsequent fruit infection.

The moist microenvironment inside fruit bags could be a factor promoting conidial germination of *T. roseum*. Li et al. (2008) measured the dynamic diurnal RH changes inside fruit bags from late June to late August, discovering that RH inside fruit bags stayed above 97% for more than 10 h at night. Furthermore, when a small amount of rainfall occurred (e.g., 0.2 mm during 1 h), RH inside fruit bags stayed above 97% for around 12 h. Li et al. (2008) proposed that RH inside fruit bags could reach 97% for 10 h or longer at night or in association with rainfall periods. In the present study, we characterized the effect of humidity on conidial germination of *T. roseum*. At 25°C, in free water or at RH >97%, most conidia germinated within 9 h, whereas at RH of 95 or 92%, 12 or 40 h, respectively, were needed for 50% of conidia to germinate. These results suggest that RH levels inside fruit bags often satisfy requirements for *T. roseum* conidial germination and infection. Several investigations showed that apple fruit enclosed in plastic bags had higher incidence of black spot than fruit in paper bags (Chen et al. 2002; Guo et al. 2005; Liu et al. 2007). We speculate that the reason for this difference is that the high levels of RH associated with *T. roseum* activity occur more commonly in plastic than in paper fruit bags.

Occurrence of TBS is influenced by fruit developmental stage. According to our data on infection sites and hyphal extension at different fruit ages, we divided TBS occurrence into three stages. The first stage, at 30 to 45 dafb, resulted in no *T. roseum* infections, and microscopic examination showed that most conidia on the surface of these fruit did not germinate. Zhang et al. (2010) reported that there were no detectable soluble nutrients on apple fruit surfaces before 45 dafb. We speculate that a lack of diffusible nutrients limited conidial germination. At the second stage (60 to 75 dafb), hyphae of *T. roseum* penetrated apple epicarps via stomata on the fruit surface; most black spots were relatively small (<2 mm) and they did not expand. Microscopic examination showed that hyphae were restricted from spreading into the parenchymal cell layers, and the hyphae eventually died. Lesion borders were clearly confined by a separation zone containing heavily lignified cells. At this stage, low pH and high levels of antimicrobial substances such as phenolics and acids in apple fruit might retard hyphal extension (Hwang 1983; Kohn and Hendrix 1983; Sitterly and Shay 1960). With further fruit expansion, most stomata transformed into lenticels, and some microcracks appeared on fruit surfaces. The third stage (105 to 150 dafb) entails lenticel and microcrack infection. At these fruit ages, hyphae penetrated apple epicarps via lenticels or microcracks on fruit surfaces, and most black spots were relatively large (3 to 6 mm in diameter). Microscopic examination showed that fungal hyphae spread into the parenchymal cell layer more extensively than at earlier fruit ages, and there was no evidence of hyphal death or separation zones. At

these stages, ripening fruit undergo a series of changes such as pH and soluble nutrient increase and decline in concentration of antimicrobial substances (Hwang 1983; Kohn and Hendrix 1983; Sitterly and Shay 1960), which might reduce fruit defense reactions and stimulate hyphal extension.

T. roseum can cause apple fruit rot during storage (Hamid et al. 2014; Kwon et al. 2014). We inferred that hyphae of *T. roseum* in black spots might contribute to *T. roseum* fruit rot in storage. For

Table 1. Analysis of variance of incidence of *Trichothecium* black spot on apples at different apple ages and in different years

Factors	df ^a	F-value	P value
Age of apples ^b	5	74.517	0.003
Year ^c	1	0.492	0.533

^a Degrees of freedom. *P* value indicated significance at 0.05 level.

^b Inoculation was done at 30, 45, 60, 75, 105, 150 days after full bloom.

^c Experiment was conducted in 2015 and 2016.

Fig. 4. Conidia germination and entrance of *Trichothecium roseum* on apple fruit. **A, B, C, D,** Conidia on fruit surface tend to germinate in the vicinity of the lenticel site, but not on cuticle-coated area (white box in A, B). **E,** Stomata penetration of *T. roseum* germ tube on apple that was inoculated 75 days after full bloom. **F,** Penetration of microcrack in the epicuticular wax layer on an apple that was inoculated 150 days after full bloom. **G,** Lenticels of fruit that were inoculated 150 days after full bloom exhibited strong autofluorescence, and the inoculated conidia failed to germinate within 10 days. GC: germinated conidia; UC: ungerminated conidia; GT: germ tube; CC: cuticle-coated area; L: lenticel; LL: lignified lenticel; S: stoma; MC: microcrack. Bar (A and G) = 200 μ m, Bar (C) = 100 μ m, Bar (B and F) = 20 μ m, Bar (D and E) = 50 μ m.

the stomatal infection type, *T. roseum* hyphae were dead in black spots or constrained by the host's lignified zone. We speculate that these early-fruit-stage black spots might not extend or cause fruit rot at storage time. For lenticel and microcrack infections, hyphae remained viable in black spots. We speculate that these black spots could cause fruit rot during cold storage. Moreover, we also found that apple fruit with no black spot symptoms at harvest produced TBS during storage. Therefore, we speculate that *T. roseum* formed quiescent infections in microcracks or nonlignified lenticels on apple fruit surface that could lead to fruit rot after harvest.

Bagging protects apple fruit from pests and diseases, but also creates microenvironments that can favor development of TBS. As bagging blocks access by fungicide sprays, it poses challenges for

chemical management of TBS. Based on our results that RH inside fruit bags is important for conidial germination, we suggest reducing RH inside bags and in orchards to suppress TBS. A first step would

Table 2. Analysis of variance of diameter of *Trichothecium* black spot on apples at different apple ages and in different years

Factors	df ^a	F-value	P value
Age of apples ^b	3	30.460	0.010
Year ^c	1	2.994	0.182

^a Degrees of freedom. *P* value indicated significance at 0.05 level.

^b Inoculation was done at 30, 45, 60, 75, 105, 150 days after full bloom.

^c Experiment was conducted in 2015 and 2016.

Fig. 5. Symptom development on apples that were inoculated with *Trichothecium roseum* at different ages after full bloom (dafb). **A**, Disease incidence (calculated as fruit infection rate) surveyed at 15 days postinoculation. Data points represent the mean incidence of black spots pooled from 2015 and 2016 field data. Bars indicate the standard error of the mean. Mean incidence for each year was based on examination of at least 30 independent fruit. Different lowercase letters on columns indicate significant difference ($P < 0.05$) based on Tukey's honest significant difference (HSD) mean separation test. **B**, Lesion diameters at harvest (165 day after full bloom). Each boxplot represents measurements from 40 independent *Trichothecium* black spot (TBS) lesions pooled over the 2015 and 2016 field experiments. Different lowercase letters on boxplots indicate significant difference ($P < 0.05$) based on Tukey's honest significant difference (HSD) mean separation test. **C**, Typical symptoms at harvest of TBS inoculated at 60 dafb, 75 dafb, 105 dafb, and 150 dafb. Bar = 5 mm.

be to select waterproof bags with better air exchange capabilities in order to shorten periods of high humidity inside the bags. A second step would be to reduce humidity, and improve ventilation and light conditions in orchards. Batzer et al. (2013) showed that pruning was an effective measure to reduce the risk of blemishing by sooty blotch and flyspeck fungi by decreasing orchard RH. In most orchards in

China, apple trees are overcrowded. We therefore suggest that increased pruning could improve air movement, hasten drying, and reduce periods of high RH. In our work, apple fruit were susceptible to *T. roseum* from fruit expansion to ripening stages, and fungicide access to fruit would not be possible due to presence of the fruit bags. We speculate that protectant fungicides or biological agents with

Fig. 6. Histological infection features of *Trichothecium roseum* conidia inoculated on apples at 75 days after full bloom (dafb), 105 dafb, and 150 dafb. **A, B, C, D,** 75 dafb. **A,** At 15 days postinoculation (dpi), hyphae colonized intensively under the stoma. **B,** At 30 dpi, most hyphae colonized hypodermal cell layers. **C,** At 60 dpi, green hyphae remained at openings, and hyphae in hypodermal cell layers were reduced. **D,** At 70 dpi, a complete lignification zone was formed around black spot tissues. **E, F, G,** 105 dafb. **E,** At 15 dpi, hyphae colonized dead tissues under lenticels. **F,** At 30 dpi, hyphae colonized hypodermal cell layers. **G,** At 60 dpi, hyphae extended to parenchymal cell layers. **H,** 150 dafb. At 15 dpi (around the harvest time), hyphae had intensively colonized parenchymal cells. Bar (A–G) = 200 μ m.

Fig. 7. Trichothecium black spot-induced host defense reactions observed on fruit inoculated at 60 (A, C) and 150 days after full bloom (dafb) (B, D). A, B, Separation layer that had formed in host tissue at harvest (165 dafb) (A, black arrow) was not evident in B. C, D, Differences in lignin deposition (based on phloroglucinol-hydrochloric acid staining) observed in C (purple staining indicated by black arrow) but not in D at harvest. Bar (A–D) = 100 μ m.

long residual efficacy could be applied within 1 day before fruit bagging in order to reduce *T. roseum*.

Fruit bagging can efficiently prevent infection by many pathogens, such as *Colletotrichum* spp. causing bitter rot and *Botryosphaeria* spp. causing ring rot. But *T. roseum* can exist on sepals and floral residues on young apple fruit (Chen et al. 2002). We speculated that *T. roseum* thalli, existing on sepals and floral residues before fruit bagging, could be inocula for TBS. This also could partly explain why most black spots occur around sepals.

T. roseum can cause many diseases on diverse hosts with different symptoms, such as apple core rot (Gao et al. 2013), pink mold rot on apple, orange, tomato (Hamid et al. 2014), avocado (Sharma et al. 2016), peach (Hong and Michailides 1997), and strawberry (Kwon et al. 2010), and leaf spot on *Mangifera indica* L. (He et al. 2011). Whether there are pathogenicity differences is still unknown.

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