Effects of Temperature, Humidity, and Wound Age on *Valsa mali* Infection of Apple Shoot Pruning Wounds

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Abstract

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Valsa canker, caused by *Valsa mali*, is a destructive disease of apple in China. The pathogen infects apple branches, mainly through pruning wounds, and causes branch and tree death. To determine the conditions required for *V. mali* infection through pruning wounds and growth within the xylem, pruning wounds on 1- to 4-year-old apple branches were inoculated with conidia in vitro under artificially controlled conditions and in vivo in the orchard. The effects of temperature, wetness duration, and wound age on conidial infection through pruning wounds as well as hyphal growth in the xylem were examined. The results showed that, after invading through pruning wounds, *V. mali* hyphae grew along xylem vessels, tracheids, and rays, expanding longitudinally and laterally. The hyphae could enter adjacent xylem vessels and tracheids through micropores to form a dense hyphal network. Wetness duration did not exhibit an essential effect on conidial infection from pruning wounds. Conidia spread to pruning wounds

with rainwater could infect the xylem without any other extra moisture. Temperature for *V. mali* conidia infection through pruning wounds and hyphal extension in the xylem ranged from 5 to 35°C, with the optimum at 20°C. Pruning wounds made in late March were susceptible to *V. mali* infection in March, April, and May; the susceptibility was markedly decreased by June, and the pathogen could barely infect through the pruning wounds in November. The infected pruning wounds began to show symptoms from the spring of the following year. More than half of the observed *Valsa* canker lesions emerged in the spring of the second year, and new canker twigs were also developed from the inoculations in the spring of the third year. March, April, and May are the critical periods for protecting pruning wounds against infection by *V. mali* in China, and coating pruning wounds with protective film immediately after pruning is an easy and effective measure to protect the pruning wounds.

Valsa canker, caused by *Valsa mali* var. *mali* Miyabe & G. Yamada (anamorph *Cytospora* sp.) (Wang et al. 2011), is a destructive disease of apple and mainly harms the trunk and main branches, leading to cortical rot followed by branch and tree death. At the present time, the disease still cannot be effectively controlled in China, although many studies have been conducted recent years (Ke et al. 2013; Li et al. 2015; Peng et al. 2016; Wang et al. 2014; Yin et al. 2015; Zhang et al. 2015). Outbreak of apple *Valsa* canker may ruin an orchard, posing a serious threat to the apple industry in China. A survey in 2008 showed that the overall incidence of cankered trees was 52.7% in 147 apple orchards in 10 major apple-producing provinces and cities in China (Cao et al. 2009). China has had four major outbreaks of *Valsa* canker in the last century. By the late 1980s, the disease epidemics had almost destroyed all of the apple orchards that had been established from the 1950s (Chen 1996). *Valsa* canker of apple was first reported in Hokkaido, Japan, in 1887 and later spread to the main island of Japan and North Korea. In 1916, *Valsa* canker spread to China due to the introduction of a new apple variety (Sakuma 1990; Uhm and Sohn 1995). *Valsa* canker of apple is mainly observed in East Asia, including South Korea (Suzaki 2008; Vasilyeva and Kim 2000). In China, *Valsa* canker is now widespread all over the apple-producing areas, and provinces in Northern China suffer more serious damage.

V. mali is a weakly parasitic fungus that mainly infects apple trees through pruning wounds, frostbite, sunburn, and other wounds (Biggs 1989; Chen et al. 1981; Kalkanci et al. 2006; Kempley and Jacobi 2000; Willison 1936). Among the infection sites, pruning wounds are the most susceptible to the pathogen (Chen et al. 1987) and primarily provide ingress for the fungal infection. Wang et al. (2012) examined

967 new disease scars in 2011, with 80.0% that developed from pruning wound infections. After infection through a pruning wound, the pathogen mainly grows near or below xylem vessels or is latent in the wound surface and survives in the xylem for at least 3 to 5 years (Ke et al. 2013; Liu et al. 1979; Mu and Liu 1994). Canker lesion occurrence on pruning wounds and reoccurrence on old lesions are both due to hyphal growth and expansion of the fungi within the xylem (Wang et al. 1985).

Currently, scraping off the canker lesions and smearing the tree with fungicide are important practices for preventing and controlling the canker disease on apple trees. However, after being scraped off, the canker lesions often relapse in the next year or successive years, due to growth and expansion of the pathogen in the xylem. Fungicides that exhibit excellent inhibitory effects on mycelia growth under laboratory conditions often exhibit poor control effects when applied in an orchard because it is difficult for traditional fungicides to reach the target points and prevent the fungal growth and expansion in branch tissues. In order to reduce the opportunity for the spores of the pathogen to spread to pruning wounds, it was suggested that the pruning time be postponed from winter to the following spring. However, the postponement did not produce effective control. To date, all apple cultivars and lines lack effective resistance against *V. mali* (Bessho et al. 1994; Liu et al. 2011). Therefore, the most effective strategy for preventing and controlling *Valsa* canker should be to prevent the pathogen from invading the host tissue, especially the xylem, through pruning wounds. Once the pathogen infects the host tissue, it is difficult to prevent disease progression using any other prevention measures and, when the canker occurs on the tree, it will recur in successive years (Mu and Liu 1994; Tamura and Saito 1982; Wang et al. 1985).

Although pruning wounds are important for providing ingress for *V. mali* infection, little is known about the conditions required for infection of pruning wounds, the process of colonization of xylem vessels, and the effect of pruning wound age on infection by the pathogen. In this study, inoculation experiments were conducted under artificially controlled conditions to address the following topics:

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(i) the colonization of xylem vessel elements after inoculation of pruning wounds, (ii) the temperature and moisture required for the pathogen infection of pruning wounds, (iii) the effect of temperature on mycelium growth rate within the xylem, and (iv) the effect of pruning wound age on the pathogen infection as well as the canker symptom development dynamics after infection. The results can be used to improve management of Valsa canker.

Materials and Methods

Inoculum origin and preparation. In August 2009, apple branches with lesions of Valsa canker were collected from a 'Fuji' apple orchard in Qixia, Shandong Province, China. The cortical tissue at the junction of the diseased and healthy sites was cut off, disinfected with 75% alcohol for 10 s, and placed onto potato dextrose agar (PDA) to isolate the pathogen of *V. mali*. The tip of a single hypha at the edge of a developing colony was cut off using a capillary tube of 0.8 mm in diameter and transferred onto fresh PDA to purify the pathogen. The purified strain, numbered as LX080601, was identified as *V. mali* var. *mali* according to the pathogenicity, morphology, and ribosomal DNA-internal transcribed spacer sequences (Chen et al. 2012; Wang et al. 2011; Wang et al. 2014) and preserved in a refrigerator of 4°C.

Prior to inoculation, strain LX080601 was activated on PDA for 3 to 5 days. Mycelia plugs were punched using a puncher of 5 mm in diameter from the edge of the colony to inoculate barley culture medium (70 g of barley seed with shell immersed in clean water for 2 h, combined with 20 ml of 6% honey water and 20 ml of 1% peptone solution, sterilized at 121°C for 1 h) (Zhao et al. 2012). The inoculated medium was incubated at 25°C for 15 days for the pathogen to produce a large amount of pycnidia. Barley seed bearing pycnidia were transferred into a 9-cm petri dish containing 1% water agar and incubated at 25°C for 3 days until plenty of orange conidial masses or horns extruded from the pycnidia (Ma et al. 2010; Zhao et al. 2012). Fresh conidia masses were used directly for inoculation or placed into pure water to prepare conidial suspensions. The fresh conidial suspension, containing 10^6 conidia/ml, was used for inoculation immediately after preparation (≤ 30 min of storage).

Apple branches of several varieties with fresh Valsa canker lesions were collected from four apple orchards in March 2016 in Laiyang, Shandong Province, China. The branches were washed with tap water and placed into a plastic box. The box was sealed to keep saturated relative humidity (RH) inside and transferred to an incubator at 25°C. Conidial horns extruded from the pycnidia on canker lesions after 24 to 72 h of incubation. The conidial horns were picked up from at least five different branches and a mixture of conidial suspension was prepared.

Shoot selection and handling. From February to April 2014, dormant apple twigs with a diameter of 1 to 2 cm at the base were cut from 5-year-old Fuji apple trees and cut into 20-cm segments using disinfected pruning shears. Twig segments with a diameter longer than 0.8 cm were selected and placed in groups of six into tissue culture jars. Distilled water was added to the jars and the base of the twig segments submerged by 2 cm. Twig segments were inoculated within 6 h, and the distilled water was changed once every 2 days during the experiment.

Hyphal colonization in xylem. Twelve newly cut twig segments, maintained in two jars in vitro, were used to examine the colonization process of xylem vessels by *V. mali* hyphae. The conidial suspension of strain LX080601 was inoculated onto the exposed pruning wounds of the twig segments using a hand sprayer until the suspension began to run off. After inoculation, the segments with the jars were immediately sealed in two plastic bags to keep the pruning wound moist and then moved into an incubator. The temperature of the incubator was set to 25°C in advance, and the RH ranged from 60 to 80%. The plastic bags were removed 48 h later and the segments were incubated for another 12 days.

Two inoculated twig segments were taken out every 2 days, starting from the fourth day after incubation, and approximately three to five sections of approximately 0.5 to 1 cm in length were cut from the inoculation end of each inoculated twig segment. After the cortex

was removed, xylem tissue sections of approximately 5 to 20 μ m in thickness were sliced along transections and longitudinal sections of the twig section with a cryostat microtome (Micro HM525; Thermo Fisher Scientific Inc.). The tissue sections were decolorized and clarified with 10% sodium hypochlorite for 5 to 10 min, then stained with 0.5% aniline blue for 1 to 2 min, followed by two washes with distilled water. Temporary slides, using 80% glycerol as a supporting agent, were examined under a fluorescence microscope (DM2500; Leica Instruments Ltd.). The structure, distribution, density, and colonization process of hyphae in xylem were examined under UV light of 365 nm. Typical fields of view were selected and photographed.

Effect of temperature and wetness duration on conidial infection through pruning wounds. The effects of temperature on conidial infection were examined at seven temperatures: 5, 10, 15, 20, 25, 30, and 35°C. Under each temperature, the effect of wetness durations of 0, 1, 3, 6, 12, 24, and 48 h of wetness after inoculation were investigated. In total, there were 49 temperature and wetness combinations, and 12 twig segments in two jars were inoculated for each combination. The temperature was controlled using seven incubators (MGC-250BP-2; Shanghai Bluepard Instruments Co. Ltd.).

In total, 84 twig segments in 14 jars (6 per jar) were placed in each incubator and pretreated at the corresponding temperature for 12 h. The twig segments were taken out and inoculated by spraying a fresh conidial suspension (10^6 conidia/ml) of strain LX080601 onto the exposed pruning wounds using a hand sprayer until the suspension began to run off. Each group of twig segments with the jar was immediately sealed in one plastic bag after inoculation to keep the

Fig. 1. Hyphae (h) and conidia (c) of *Valsa mali* were in xylem tissue of 1-year-old Fuji apple shoots, which were inoculated with *V. mali* conidial suspension at pruning wounds on detached twig segments maintained in jars, with the base soaked in distilled water at 25°C for 2 weeks. See text for details. **A**, Longitudinal section of xylem tissue showing *V. mali* hyphal expansion in the xylem vessels and tracheids; **B**, longitudinal section of xylem tissue showing germinated conidia within the xylem vessels; **C**, longitudinal section of xylem tissue showing *V. mali* hyphae passing through micropores on the sidewall of tracheids to enter an adjacent tracheid; and **D**, cross-section of xylem tissue showing *V. mali* hyphal growth and expansion in xylem vessels, tracheids, and rays.

pruning wounds wet and then placed back into the incubator. For the 0-h wetness duration treatment, the shoots were directly placed back into the corresponding incubator without sealing them in the bag. At the end of each wetness duration treatment, the plastic bag was removed and the twig segments were kept at the same incubator for the rest incubation time. The RH inside the incubator during the experimental period ranged from 50 to 70%. At 72 h after inoculation, all the shoots were taken out, and the inoculated end of the segments was immersed in 75% alcohol for 10 s to kill the remaining conidia at the wound surface. All the twig segments were then transferred to a chamber set at 25°C and 75% RH and incubated for another 7 days.

All the twig segments were taken out of the chamber 10 days after inoculation, and the cortex was removed with a knife. Five twig sections, each of 1 cm in length, were sequentially cut from the inoculated end of each twig segment using pruning shears and immersed

in 75% alcohol for 0.5 min for disinfection. The twig sections were sequentially coded from the inoculation end downward and inserted vertically into PDA with the basal end (distal to the inoculation end) in contact with the medium. The five twig sections derived from the same segment were placed in one petri dish and incubated at 25°C. Starting from the second day, mycelia of *V. mali* produced from each shoot segment were examined daily until the end of day 5. If hyphae had extended to the position from the inoculated point over the 10 days of incubation, mycelia of *V. mali* would grow out from the base of the twig section. After the code of the section was recorded, the twig section and the mycelia-bearing agar were removed from the petri dish to prevent spread of mycelia to the other sections. The twig sections that did not produce *V. mali* mycelia within 5 days were considered negative. At the end of the examination, the twig sections with *V. mali* were counted in the 12 samples at the same depth for

Fig. 2. Incidence and standard deviations of *Valsa mali* reisolated from the xylem of twig sections at different distances below the point of inoculation in 1-year-old detached twigs segments of Fuji apple. The twig segments, maintained in tissue culture jars with the base soaked in distilled water, were inoculated with conidia of *V. mali* strain LX080601 at pruning wounds, incubated at seven different temperatures for 72 h (36 shoots for each temperature and wetness combination), then transferred to a chamber maintained at 25°C and incubated for 7 days.

each temperature and wetness duration combination. The experiments were repeated three times at different times, and the incubators were randomly assigned to experimental temperatures for each repetition.

Effect of temperature on hyphal growth in xylem. The effect of temperature on hyphal growth was studied at 5, 10, 15, 20, 25, 30, and 35°C. Six twig segments in one jar were prepared for each temperature. The twig segments were inoculated by spraying a fresh conidial suspension of strain LX080601 using a hand sprayer onto the pruning wounds to run off. The inoculated segments with the jar were immediately sealed in a plastic bag to maintain 100% RH and moved into an incubator at 25°C. After 24 h of incubation, the twig segments were taken out and the inoculated ends were immersed in 75% alcohol for 10 s to kill the uninfected conidia. The twig segments were then transferred into seven different incubators, each set at one of the temperatures indicated above and incubated for 1 week.

Subsequently, the twig segments were moved out, and the cortex was removed with a knife. Two 0.5-cm sections were cut first from the inoculation point, and four 1-cm sections were cut off thereafter. All the sections were immersed in 75% alcohol for 0.5 min for disinfection. The twig sections were sequentially coded and then inserted into PDA with the basal end touching the medium. The sections were incubated in an incubator at 25°C for 5 days and mycelia of *V. mali* were examined daily, as described above. The code of the shoot segments carrying *V. mali* was recorded, and the number of twig sections infected with *V. mali* was counted for each sample at the same depth among the six segments of each temperature treatment. The experiments were repeated twice at different times, and the incubators were randomly assigned for the seven temperatures in each repetition.

Conidial infection of pruning wounds on attached branches. To verify the conidial infection in vivo, pruning wounds on attached branches on 7-year-old Fuji apple trees were inoculated with a mixture conidial suspension (conidia produced in more than five cankered lesions). In March 2016, 30 1-year-old twigs with diameters larger than 1 cm at the base were pruned at a 10- to 20-cm distance from the base and the pruning wounds were immediately inoculated by spraying a mixture conidial suspension to runoff using a hand sprayer. In all, 15 inoculated wounds were covered with a slightly damp cotton ball and wrapped with plastic film to maintain wetness for 48 h (treatment A) and the other 15 inoculated wounds did not maintain wetness after inoculation (treatment B). Fifteen overwintered pruning wounds on 1-year-old twigs, made in November 2015, were inoculated without maintaining wetness (treatment C). Fifteen 3- or 4-year-old branches were pruned at a 10- to 20-cm distance from the base and immediately inoculated without maintaining wetness (treatment D). As a control, 15 twig segments were inoculated with a mixture conidial suspension as described in the previous experiments, except maintaining wetness (treatment E). All attached pruning wounds were maintained outside and temperature ranged from 5 to 25°C during the experimental time.

Two weeks after inoculations, twig sections of 1 cm in length were cut from the points of inoculation of all twigs and branches, the

cortex was removed with a knife and immersed in 75% alcohol for 0.5 min for disinfection, and the segments were inserted into PDA to reisolate *V. mali*, as described in the previous experiments. If the pruning wound was infected, mycelia of *V. mali* would grow out from the twig section. The experiment was replicated three times in March and April 2016.

Canker development on inoculated pruning wounds of different ages. On 24 March 2013, 200 shoots with diameters of approximately 0.8 to 2.0 cm at the base were randomly selected in 4-year-old Fuji apple trees in Jiaozhou Apple Orchard of Qingdao Agricultural University (Qingdao, China). The shoots were pruned at a distance of approximately 5 to 10 cm from the base, and the pruning wound was marked and maintained outside under natural orchard conditions. Starting from the end of March, 20 shoots were inoculated with conidia of *V. mali* strain LX080601 each month until November. To prevent the pathogen from contaminating the phloem, a sterile toothpick was used to pick up conidial masses produced on barley seed and to smear them onto the center of the xylem on each pruning wound without touching the phloem. After inoculation, a small, slightly damp cotton ball was placed on the center of each pruning wound and wrapped with plastic film to maintain wetness for 48 h. The inoculated wounds were examined for Valsa canker lesions every month for 10 months from the inoculation date and irregularly from the second year until the end of June 2015. The wound

Fig. 3. Percentages (circles) of *Valsa mali* reisolated from the xylem of the section 3 cm below the point of inoculation on 1-year-old detached twig segments of Fuji apple. The twig segments, maintained in tissue culture jars with the base soaked in distilled water, were inoculated with conidia of *V. mali* strain LX080601 at pruning wounds, incubated at seven different temperatures for 72 h (252 shoots for each temperature), transferred to a chamber maintained at 25°C, and incubated for 7 days. The solid line was the fitted model.

Table 1. Incidence of *Valsa mali* reisolated from the xylem of twig sections at 3 cm below the point of inoculation of 1-year-old detached twig segments of Fuji apple trees^a

WD (h)	Temperature (°C)							Means
	5	10	15	20	25	30	35	
0	0.0 ± 0.0	2.8 ± 4.8	5.6 ± 9.6	27.8 ± 48.1	11.1 ± 19.3	22.2 ± 17.4	0.0 ± 0.0	9.9 ± 20.5A
1	0.0 ± 0.0	5.6 ± 9.6	13.9 ± 24.1	30.6 ± 12.7	13.9 ± 24.1	8.3 ± 8.3	0.0 ± 0.0	10.3 ± 15.8A
3	2.8 ± 4.8	25.0 ± 36.3	33.3 ± 57.7	25.0 ± 8.3	2.8 ± 4.8	11.1 ± 19.3	0.0 ± 0.0	14.3 ± 26.0A
6	0.0 ± 0.0	8.3 ± 8.3	25.0 ± 22.1	11.1 ± 12.7	11.1 ± 9.6	8.3 ± 8.3	0.0 ± 0.0	9.1 ± 12.3A
12	0.0 ± 0.0	11.1 ± 12.7	19.4 ± 17.4	33.3 ± 30.1	5.6 ± 4.8	19.4 ± 9.6	0.0 ± 0.0	12.7 ± 16.8A
24	22.2 ± 31.6	0.0 ± 0.0	30.6 ± 45.9	44.4 ± 19.3	22.2 ± 12.7	5.6 ± 9.6	0.0 ± 0.0	17.9 ± 25.0A
48	2.8 ± 4.8	8.3 ± 14.4	16.7 ± 28.9	33.3 ± 50.7	25.0 ± 22.1	25.0 ± 30.1	0.0 ± 0.0	15.9 ± 25.4A
Means	4.0 ± 12.8d	8.7 ± 15.7cd	20.6 ± 29.3ab	29.4 ± 27.3 a	13.1 ± 15.3bc	14.3 ± 15.6bc	0.0 ± 0.0d	...

^a Twig segments, maintained in tissue culture jars with the base soaked in distilled water, were inoculated with conidia of *V. mali* strain LX080601 at pruning wounds, incubated at different combinations of temperatures and wetness duration (WD) for 72 h (36 shoots for each combination), then transferred to a chamber of 25°C and incubated for another 7 days. Different letters indicate significant differences at the 0.05 level.

was considered cankered and recorded when the Valsa canker lesion was greater than 1 cm in length below the pruning wound. There was no replication for the experiment.

Data analysis. Analysis of variance (ANOVA) was conducted using generalized linear models (GLM) to assess the effects of temperature, wetness duration, and different types of pruning wounds on conidial infection and hypha extension in the xylem. The replications were treated as block factors, the temperature of the five treatments was treated as a whole-plot factor, and the wetness duration was treated as a subplot factor for the ANOVA. The infected shoot segments at the same depth level in each combination or treatment, which were assumed to follow a binomial distribution in GLM, were applied in the data analysis and used to calculate the means and standard deviations. Regression analysis was used to fit the mean percentages of the infected twig sections at a 3-cm depth for each moisture treatment to the temperature. The temperature (T in $^{\circ}\text{C}$) was considered to be an independent variable and was divided by 10 to avoid small values of estimated parameters. The statistical analyses were performed using R (version 3.0.2).

Results

Hyphal colonization in xylem. Hyphae of *V. mali* were revealed under UV light of 365 nm on the tissue sections of xylem after being clarified and dyed. Under a fluorescent microscope, hyphae appeared yellow-green, while the host tissue was whitish (Fig. 1A). Most observed conidia inoculated onto the pruning wounds germinated inside vessel elements approximately 30 to 100 μm away from the inoculating point after 4 days of incubation (Fig. 1B). The result indicated that the conidia inoculated onto the pruning wounds were absorbed into the xylem. The germ tube extended along the vessel

elements and tracheids after the conidia germinated and formed a criss-cross hyphal network in the xylem. The hyphae grew and extended more rapidly along the longitudinal direction in xylem and often aggregated to form a hyphal strand or "rhizomorph" in vessels and filled the vessel lumen (Fig. 1A and D). The pathogen also grew and extended in the xylem tracheids with the hyphae in single strings. Hyphae could also pass through micropores on the side wall of xylem vessels and tracheids and, thereby, enter the adjacent vessels and tracheids (Fig. 1C). The fungi extending longitudinally along xylem vessels or tracheids often expanded into xylem rays through branching and laterally extended in tracheids. Hyphae growing laterally in xylem rays also returned to longitudinal xylem vessels and tracheids for longitudinal extension. Hyphal growth and expansion in xylem rays enlarged the pathogen's distribution area in the xylem. When reaching the cortex from the xylem, the pathogen induced canker lesions on the cortex, and the pathogen was able to grow back into xylem from cankered lesions.

Effect of temperature and wetness duration on conidial infection through pruning wounds. The conidia of *V. mali* infected the xylem and extended in the xylem at all seven examined temperatures after inoculation onto the pruning wounds of Fuji apple shoots. However, the percentages of *V. mali* reisolated from the xylem of twig sections below the point of inoculation, which were essentially the ratios of the infected twig sections and the high percentage from the twig sections at the same depth that indicated a high infection ratio of conidia through the pruning wounds, differed for the seven temperatures. For the inoculations at 15, 20, 25, and 30 $^{\circ}\text{C}$, *V. mali* was reisolated from 5 cm below the point of inoculation, and the average percentages of the pathogen reisolated from 252 shoot segments in the seven wetness duration treatments were 9.9, 25.4, 9.5, and 5.2%, respectively. The results indicated that the pathogen infected at the four temperatures extended more than 5 cm in xylem after 7 days of incubation at 25 $^{\circ}\text{C}$. For the inoculation at 10 $^{\circ}\text{C}$, *V. mali* could not be reisolated from the twig sections at 5 cm below the point of inoculation. However, the pathogen was reisolated from the sections at 4 cm, with a percentage of 0.4%. For the inoculation at 5 $^{\circ}\text{C}$, *V. mali* was reisolated from 3 cm, with an incidence of 4.0%. For the inoculation of 35 $^{\circ}\text{C}$, the pathogen was only isolated at 1 cm, with an incidence of only 2.0% (Fig. 2).

The percentage of *V. mali* reisolated from the xylem of the twig sections at 3 cm below the point of inoculation, the only complete

Fig. 4. Incidence of *Valsa mali* reisolated from the xylem of twig sections at different distances below the point of inoculation in 1-year-old detached twig segments of Fuji apple. The twig segments, maintained in tissue culture jars with the base soaked in distilled water, were inoculated with conidia of *V. mali* strain LX080601 at pruning wounds, incubated at 25 $^{\circ}\text{C}$ for 48 h, and then incubated at seven different temperatures for 7 days (18 shoots for each temperature).

Fig. 5. Incidence of *Valsa mali* reisolated from the xylem of twig section at 1 cm below the point of inoculation in twigs or branches of Fuji apple. The twigs or branches were inoculated with a mixture conidial suspension of *V. mali* and incubated outside or at 25 $^{\circ}\text{C}$ for 2 weeks (45 twigs or branches for each treatment). **A** and **B**, Fresh pruning wounds on attached 1-year-old twigs inoculated with (A) and without (B) maintaining wetness; **C**, overwintered pruning wounds on attached 1-year-old twigs inoculated without maintaining wetness; **D**, fresh pruning wounds on attached 3- or 4-year-old branches without maintaining wetness; **E**, fresh pruning wounds on detached 1-year-old twigs inoculated without maintaining wetness.

data for all the temperature and wetness treatments in the experiment, was significantly different ($P < 0.05$) among the inoculations at seven temperatures. The pathogen was reisolated from the twig sections at 3 cm below the point of inoculation in all six inoculation temperatures, except 35°C (Table 1). The highest isolation percentage was obtained on the twig segments inoculated at 20°C. The pathogen was reisolated from 74 of 252 (29.4%) twig segments inoculated in the seven wetness duration treatments. The value did not significantly differ ($P > 0.05$) from the percentage of inoculation at 15°C, which was 20.6%. However, this value was significantly higher ($P < 0.05$) than the percentages for the inoculations at the other five temperatures. *V. mali* was isolated at a rate of 4.0% from twigs inoculated at 5°C, which was comparable ($P > 0.05$) with the isolation rate of 8.7% from twigs inoculated at 10°C but was markedly lower ($P < 0.05$) than the percentage for twig segments inoculated at the other four temperatures.

The relationship between the percentage (P) of *V. mali* reisolated from the xylem of twig sections at 3 cm below the point of inoculation and inoculation T (the unit of T was °C/10, using 1/10 of the actual temperature to avoid small estimation of the model parameters) can be well described using the following polynomial model: $P = -(14.342 \pm 8.657) + (37.273 \pm 9.922)T - (9.467 \pm 2.424)T^2$, with $R^2 = 0.7931$.

This model explained 79.31% of the *V. mali* isolation percentage variance. Based on the model, the temperature for conidial infection of the pruning wounds ($P > 1\%$) ranged from 5 to 35°C (when $P = 1\%$, $T = 0.47$ and 3.47), with the optimum at 20°C ($T = 1.969$) (Fig. 3).

The wetness duration did not exhibit a significant effect on conidial infection of *V. mali* through pruning wounds of apple shoots. The percentage of *V. mali* reisolated from the xylem of the twig section at 3 cm below the point of inoculation was 9.9, 10, 14.3, 9.1, 12.7, 17.9, and 15.9% for the treatments of 0, 1, 3, 6, 12, 24, and 48 h of wetness duration, respectively (Table 1). These percentages did not differ significantly ($P > 0.05$) from each other.

Effect of temperature on hyphal growth in xylem. After infection through pruning wounds, *V. mali* grew and extended in the xylem; however, the hyphal growth rate varied with incubation temperatures. The pathogen was reisolated from the xylem of twig sections at 5 cm below the point of inoculation incubated at all six ion temperatures, except 35°C. The incidence of the pathogen isolated from the xylem of the twig sections at 4 cm below the point of inoculation was 11.1, 18.6, 11.1, 9.7, 15.3, 18.1, and 5.6% for 5, 10, 15, 20, 25, 30, and 35°C, respectively (Fig. 4), which did not differ significantly ($P > 0.05$) among the seven temperatures. The results indicated that the hyphae of *V. mali* extended rapidly, at least 0.5 cm/day, in xylem of apple branches at temperatures ranging from 5 to 35°C. Temperature did not show a significant effect on

the hyphal growth in xylem of apple twigs, except for the slight slowing of the pathogen extension at 35°C.

Conidial infection of pruning wounds on attached branches. Conidia of *V. mali* were infected through newly made and overwintered pruning wounds on 1- to 4-year-old attached branches. The percentages of *V. mali* reisolated from the xylem of twig or branch sections at 1 cm below the point of inoculation did not show significant differences among the four treatments of fresh pruning wounds, which were 77, 75, 73, and 73% for treatment A, B, D, and E, respectively. The wetness duration did not affect significantly for the conidial infection through pruning wounds (A and B). The percentages of *V. mali* reisolated from fresh pruning wounds were significantly higher (45%) than that from overwintered ones (treatment C). The results indicated that newly made pruning wounds were more susceptible to conidia of *V. mali* than overwintered ones (Fig. 5).

Canker development on the inoculated pruning wounds of different ages. The pruning wounds were more susceptible in March, April, and May and became less susceptible in June, and only a few conidia were infected through the pruning wound in November. In total, 160 pruning wounds of different ages on 1-year-old Fuji apple twigs were inoculated with fresh conidia on eight occasions from 31 March to 4 November 2013. Except for 3 pruning wounds that were not examined the last three times due to tree death, Valsa canker was observed on 54 pruning wounds as of June 2015, which indicated a 33.8% disease incidence (Table 2). Among the eight inoculations, the highest canker incidence was observed for the pruning wounds inoculated in May 2013 (75%), and the second highest was in April (55%). Cankered incidence was decreased to 25% on twigs inoculated in June and only one twig was cankered on the pruning wounds inoculated in November (Table 2).

When infected from the pruning wounds, *V. mali* did not induce any canker lesions in the year following inoculation. The canker lesions began to appear from March 2014, and most lesions developed in spring and fall of the next year. By 19 April 2014, canker lesions were observed on 29 pruning wounds, with an incidence of 18.1%. From 6 July to 4 September 2014, 10 new canker lesions were formed on pruning wounds, with an incidence of 6.8%. In spring 2015, new canker lesions were observed on 9 uncankered pruning wounds (Table 2).

Discussion

Conidia of *V. mali* were able to infect xylem through pruning wounds on apple branches. The pathogen grew and extended in xylem vessels and tracheids after infection, expanding longitudinally and laterally and forming a dense hyphal network. The pathogen growing in the xylem cannot induce Valsa canker during the year following inoculation. This is in contrast to the infection of *Cytospora leucostoma*, causing Valsa canker on peach, resulting in secretion of

Table 2. Number of pruning wounds with Valsa canker on 1-year-old Fuji apple twigs at the observation dates after inoculation with conidia of *Valsa mali* strain of LX080601 in eight batches in 2013^y

	Inoculation date (2013)								
Obs ^z	31 Mar	27 Apr	25 May	22 Jun	27 Jul	25 Aug	23 Sep	4 Nov	Total
2014									
16 Mar	3	4	4	1	0	3	1	0	16
19 Apr	3	2	3	0	2	3	0	0	13
11 Jun	1	1	0	1	0	0	0	0	3
6 Jul	0	0	0	0	0	0	1	0	1
4 Sep	1	2	2	1	0	0	3	1	10
2015									
15 Mar	0	0	0	2	1	0	0	—	3
23 Apr	0	2	4	0	0	0	0	—	6
14 Jun	0	0	2	0	0	0	0	—	2
Total	8	11	15	5	3	6	5	1	54

^y Pruning wounds were made on 24 March 2013 and 20 shoots were inoculated at the pruning wounds in each batch. Abbreviations: Mar = March, Apr = April, Jun = June, Jul = July, Aug = August, Sep = September, Nov = November; – indicates that the tree died and Valsa canker on pruning wounds cannot be determined in such cases.

^z Observation dates. Valsa canker was examined on previously inoculated twigs for each inoculated batch in 2013 but no symptoms were found.

gum during the growing season and plugging the vessels of branches (Biggs et al. 1984; Hampson and Sinclair 1973). Growth of *V. mali* in the xylem did not show any influence on the function of the xylem. When the pathogen grew and reached the phloem by the next spring, it killed living cells in the phloem and caused Valsa canker (i.e., cortical rot). As a result, most Valsa cankers developed in the following spring and rarely occurred during the same year following infection. This result was consistent with the Valsa canker development under natural condition; approximately 70% of Valsa lesions appeared in spring and more than 20% developed in fall (Du et al. 2013). According to the present experimentation, a large number of Valsa lesions were formed in the spring, most probably due to the changes in the resistance of the apple tree rather than changes in temperature.

Once *V. mali* invades the xylem, especially deep into the xylem, it is difficult to eradicate. Research shows that *V. mali* can invade the xylem from the diseased cortex and grow in the xylem tissues whenever Valsa canker occurred on apple branches (Ke et al. 2013; Mu and Liu 1994; Wang et al. 1985). The pathogen exhibits cyclical growth in the xylem and can cause reoccurrence of Valsa canker in successive years (Chen et al. 1980, 1987; Du et al. 2013). Scraping canker lesions is the main method to cure Valsa canker. However, scraping canker lesions cannot completely eradicate the pathogen in branches (Mu and Liu 1994; Wang et al. 1985) according to the present experimental results. To completely eradicate the pathogen and prevent reoccurrence of Valsa canker, the cankered branches should be totally cut off, instead of just scraping off canker lesions, if the branch does not constitute a crucial part of the tree, especially in new orchards and young trees.

Moisture had little effect on *V. mali* conidial infection of xylem through pruning wounds. This was similar to the infection by *Nectria galligena* of pruning wounds (Xu and Ridout 1998). When sprayed onto the pruning wound with water, *V. mali* conidia invaded the xylem without a need for other extra moisture. The xylem of a branch is the transmission channel for nutrients and moisture within the tree body. Nutrients and moisture are transferred as “sap” within a tree’s xylem during the growing season, and a portion of the sap thereof is exuded from the pruning wound, which leads to bleeding. The bleeding sap provides ample moisture and abundant nutrients for *V. mali* conidial germination and infection. The release and spread of conidia depend on rainwater (Wang et al. in press). In pruning wounds, the xylem has water retention capacity, and conidia are spread mainly with rainwater, which allows the xylem to absorb conidia with enough rainwater supporting the conidia germination and infection. Based on our results, *V. mali* conidia infect the xylem without extra moisture when spread to pruning wounds with rainwater under natural conditions.

To a certain extent, temperature affected the conidial infection of pruning wounds and hyphal growth in the xylem. Conidia of *V. mali* infected the xylem through pruning wounds and grew in xylem tissue under a temperature range of 5 to 35°C, with the optimum at 20°C. Nutrients exuded from fresh pruning wounds supply the necessary nutrients for conidial germination and enhance conidial adaptability to temperature stress. Based on the results, the ambient temperature from apple germination in March to apple defoliation in November meets the requirements for *V. mali* infection of pruning wounds and growth in xylem. In fact, a large number of spores infects and lurks in branches during the growing season in the cankered orchards (Zang et al. 2012).

The age of the pruning wound significantly affects *V. mali* conidial infection. Fresh pruning wounds were more susceptible to conidial infection and led to higher incidences of Valsa canker after inoculation. The results are consistent with the infection by *N. galligena* of pruning wounds (Xu and Ridout 1998) and the infection of peach bark by *C. leucostoma* (Biggs 1986). The pruning wounds formed in late March 2013 resulted in higher Valsa canker incidences on shoots inoculated in May and April. The incidence was slightly lower in March and markedly lower after June. In November, it was difficult for conidia to infect through pruning wounds due to pruning wound aging and lower temperature. These results are consistent with findings by Chen et al. (1987). The low infection rate in March

may be associated with a low temperature during inoculation. The decrease of infection ratio in June may be due to the wet season. Heavy rainfall washes away the bleeding sap from the pruning wounds and, thus, reduces supplementary nutrients for *V. mali* conidial germination and infection. In addition, the high humidity in the wet season causes colonization and growth of other fungi and bacteria on the pruning wounds, which suppresses *V. mali* infection (Chen et al. 1987).

In summary, pruning wounds are important ingresses for *V. mali* infection, and 80% of Valsa canker lesions are developed from pruning wounds (Wang et al. 2012). Most Valsa cankers were developed in March and April and released conidia in April and May, and pruning wounds were most susceptible at that time. Therefore, April and May was the key time for *V. mali* conidia to infect pruning wounds. Scraping off the canker lesions can prevent the pathogen from producing conidia but cannot prevent the canker lesions from reoccurring. Postponing the pruning time was not effective in controlling cankers. Critical measures for disease management are to protect pruning wounds and inhibit *V. mali* from infecting pruning wounds. Measures protecting pruning wounds should be applied immediately after pruning because the pathogen grows quickly and the pathogen can be transmitted with pruning tools. Coating pruning wounds with protective film such as paint mixed with carbendazim immediately after pruning can be used as the main measure to control Valsa canker of apple, according to the present study.

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Literature Cited

- Bessho, H., Soejima, J., and Tsuchiya, S. 1994. Screening methods of apple trees for resistance to Valsa canker. Pages 49-52 in: Progress in Temperate Fruit Breeding: Proceedings of the Eucarpia Fruit Breeding Section Meeting, Wädenswil/Einsiedeln, Switzerland, 30 August to 3 September, 1993. H. Schmidt and M. Kellerhals, eds. Springer, Dordrecht, Netherlands.
- Biggs, A. 1986. Wound age and infection of peach bark by *Cytospora leucostoma*. Can. J. Bot. 64:2319-2321.
- Biggs, A. 1989. Temporal changes in the infection court after wounding of peach bark and their association with cultivar variation in infection by *Leucostoma persoonii*. Phytopathology 79:627-630.
- Biggs, A., Merrill, W., and Davis, D. 1984. Discussion: Response of bark tissues to injury and infection. Can. J. For. Res. 14:351-356.
- Cao, K., Guo, L., Li, B., Sun, G., and Chen, H. 2009. Investigations on the occurrence and control of apple canker in China. Plant Prot. 35:114-116.
- Chen, C. 1996. The occurrence and management of apple diseases in China. Pesticides 7:6-9.
- Chen, C., Li, M., Shi, X., and Wang, J. 1987. Studies on the infection period of *Valsa mali* Miyabe et Yamada, the causal agent of apple tree canker. Acta Phytopathol. Sin. 17:3-6.
- Chen, C., Wang, J., Shi, X., and Li, M. 1980. Observation of Valsa canker development on apple tree in western Liaoning Province and chemical control. China Fruits 4:39-41.
- Chen, C., Wang, J., Shi, X., and Li, M. 1981. Colonized position of *Valsa mali* on bark apple branch and infection entrance. Plant Prot. 7:19-20.
- Chen, X., Niu, C., Li, B., Li, G., and Wang, C. 2012. The kinds and activities of cell wall-degrading enzymes produced by *Valsa ceratosperma*. Acta Agric. Borealia Sin. 27:207-212.
- Du, Z., Li, Z., Gao, X., Huang, L., and Han, Q. 2013. Study on the conidia dispersal and the disease dynamics of apple tree canker caused by *Valsa mali* var. *mali* in Shaanxi. J. Fruit Sci. 30:819-822.
- Hampson, M., and Sinclair, W. 1973. Xylem dysfunction in peach caused by *Cytospora leucostoma*. Phytopathology 63:676-681.
- Kalkanci, A., Kustimur, S., Turkoz Sucak, G., Senol, E., Sugita, T., Adams, G., Verkley, G., and Summerbell, R. 2006. Fulminating fungal sinusitis caused by *Valsa sordida*, a plant pathogen, in a patient immunocompromised by acute myeloid leukemia. Med. Mycol. 44:531-539.
- Ke, X., Huang, L., Han, Q., Gao, X., and Kang, Z. 2013. Histological and cytological investigations of the infection and colonization of apple bark by *Valsa mali* var. *mali*. Australas. Plant Pathol. 42:85-93.
- Kepley, J., and Jacobi, W. 2000. Pathogenicity of *Cytospora* fungi on six hardwood species. J. Arboricult. 26:326-331.
- Li, Z., Yin, Z., Fan, Y., Xu, M., Kang, Z., and Huang, L. 2015. Candidate effector proteins of the necrotrophic apple canker pathogen *Valsa mali* can suppress BAX-induced PCD. Front. Plant Sci. 6:579.

- Liu, F., Chen, C., Shi, X., Guo, J., Xing, Z., Zhang, X., and Chen, Y. 1979. Studies on the latent infection of the causal organism of Valsa canker of apple. *J. Plant Prot.* 6:1-8.
- Liu, X., Lü, S., Wang, Y., Wang, K., Li, T., Han, Z., and Zhang, X. 2011. Evaluation of resistance of malus germplasm to apple canker (*Valsa ceratosperma*). *J. Fruit Sci.* 28:843-848.
- Ma, J., Hill, C. B., and Hartman, G. L. 2010. Production of *Macrophomina phaseolina* conidia by multiple soybean isolates in culture. *Plant Dis.* 94:1088-1092.
- Mu, H., and Liu, K. 1994. Mechanism reoccurrence of Valsa canker lesions on apple branch and technology eradicating the pathogen. *Deciduous Fruits* 27:8-10.
- Peng, H., Wei, X., Xiao, Y., Sun, Y., Biggs, A., Gleason, M., Shang, S., Zhu, M., Guo, Y., and Sun, G. 2016. Management of Valsa canker on apple with adjustments to potassium nutrition. *Plant Dis.* 100:884-889.
- Sakuma, T. 1990. Canker diseases of deciduous fruit trees in Japan. *Jpn. Pestic. Inf.* 57:12-14.
- Suzaki, K. 2008. Population structure of *Valsa ceratosperma*, causal fungus of Valsa canker, in apple and pear orchards. *J. Gen. Plant Pathol.* 74:128-132.
- Tamura, O., and Saito, I. 1982. Histopathological changes of apple bark infected by *Valsa ceratosperma* (Tode ex Fr.) Maire during dormant and growing periods. *Ann. Phytopathol. Soc. Jpn.* 48:490-498.
- Uhm, J., and Sohn, H. 1995. Control of apple Valsa canker by localized spraying with neoasozin solution, an arsenic fungicide. *Korean J. Plant Pathol.* 11:23-25.
- Vasilyeva, L., and Kim, W. 2000. *Valsa mali* Miyabe et Yamada, the causal fungus of apple tree canker in East Asia. *Mycobiology* 28:153-157.
- Wang, C., Dong, X., Zhang, Z., Li, G., and Li, B. 2012. Outbreak and the reasons of apple Valsa canker in Yantai apple production area in 2011. *Plant Prot.* 38:136-138.
- Wang, C., Li, C., Li, B., Li, G., Dong, X., Wang, G., and Zhang, Q. 2014. Toxins produced by *Valsa mali* var. *mali* and their relationship with pathogenicity. *Toxins (Basel)* 6:1139-1154.
- Wang, J., Li, M., Shi, X., and Chen, C. 1985. Reason of reoccurrence of Valsa canker lesion on apple tree. *China Fruits* 9:37-42.
- Wang, S., Hu, T., Wang, Y., Luo, Y., Michailides, T. J., and Cao, K. New understanding on infection processes of Valsa canker of apple in China. *Eur. J. Plant Pathol.* In press. doi:10.1007/s10658-016-0937-3
- Wang, X., Wei, J., Huang, L., and Kang, Z. 2011. Re-evaluation of pathogens causing Valsa canker on apple in China. *Mycologia* 103:317-324.
- Willison, R. 1936. Peach canker investigations: II. Infection studies. *Can. J. Res.* 14c:27-44.
- Xu, X.-M., and Ridout, M. 1998. The effects of inoculum dose, duration of wet period, temperature and wound age on infection by *Nectria galligena* of pruning wounds on apple. *Eur. J. Plant Pathol.* 104:511-519.
- Yin, Z., Liu, H., Li, Z., Ke, X., Dou, D., Gao, X., Song, N., Dai, Q., Wu, Y., and Xu, J. 2015. Genome sequence of Valsa canker pathogens uncovers a potential adaptation of colonization of woody bark. *New Phytol.* 208:1202-1216.
- Zang, R., Yin, Z., Ke, X., Wang, X., Li, Z., Kang, Z., and Huang, L. 2012. A Nested PCR Assay for Detecting *Valsa mali* var. *mali* in Different Tissues of Apple Trees. *Plant Dis.* 96:1645-1652.
- Zhang, J., Gu, Y., Chi, F., Ji, Z., Wu, J., Dong, Q., and Zhou, Z. 2015. *Bacillus amyloliquefaciens* GB1 can effectively control apple valsa canker. *Biol. Control* 87:1-7.
- Zhao, H., Wang, C., Chen, X., Wang, H., and Li, B. 2012. Methods of promoting sporulation of *Valsa ceratosperma*. *Chin. Agric. Sci. Bull.* 10:029.