

## Effects of Interrupted Wetness Periods on Conidial Germination, Germ Tube Elongation and Infection Periods of *Botryosphaeria dothidea* Causing Apple White Rot

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Responses of *Botryosphaeria dothidea* to interrupted wetness periods were investigated under *in vivo* and *in vitro* conditions. Conidia of *B. dothidea* were allowed to germinate on apple fruits under wetting condition at 25°C for 5 hr. They were air-dried for 0, 1, 2 or 4 hr, and then rewetted at 25°C for 5 hr. Following an initial wetness period of 5 hr, 83% of the conidia germinated. The percent conidial germination increased to 96% when wetting was extended continuously another 5 hr. However, no further conidial germination was observed when wetting was interrupted by dry periods of 1, 2 and 4 hr, resulting in 83, 81 and 82%, respectively. The mean length of the germ tubes was 37 µm after 5 hr of wetting and elongated to 157 µm after 10 hr of continuous wetting. On the other hand, interruption of wetting by a dry period of 1 hr or longer after the 5 hr of initial wetting arrested the germ tube elongation at approximately 42 µm long. Prolonged rewetting up to 40 hr did not restore germ tube elongation on slide glasses under substrate treatments. Model simulation using weather data sets revealed that ending infection periods by a dry period of at least 1 hr decreased the daily infection periods, avoiding the overestimation of infection warning. This information can be incorporated into infection models for scheduling fungicide sprays to control apple white rot with fewer fungicide

applications.

**Keywords :** apple white rot, disease forecast model, infection period, interrupted wetting

Plant surface wetting is a complex phenomenon that involves multiple factors such as weather conditions, surface wettability, plant architecture and plantation structure (Huber and Gillespie, 1992). Wetness conditions are often established in the field by rainfall, dew formation, overhead irrigation, water evaporation or guttation water (Friesland and Schrodter, 1988; Huber and Gillespie, 1992). The presence of free water, especially on the host surface, triggers a number of biological activities of fungal pathogens, including spore discharge, spore germination and host infection in many host-parasite interactions. Either wetness period or relative humidity are therefore commonly employed in disease warning systems as a critical determinant of infection risk (Arauz et al., 2010; Grove, 2002; Huber and Gillespie, 1992; Kim and Yun, 2013; Lee et al., 2015; Xu et al., 2007).

However, wetness periods long enough for infection and disease development are not always continuous under field conditions. The interruption of wetness periods occurs in cases of nocturnal wetting and diurnal drying cycles, and intermittent rain showers. Thus, fungal spores or germlings are often subjected to alternating wet and dry periods before host infection. Several reports on the effects of interrupted wetness periods (IWP) on fungal growth and disease development have shown that fungal response to IWP differed depending on the fungal species. Reduction of conidial germination and germ tube elongation due to IWP were observed in *Botryosphaeria obtusa* (Arauz and Sutton, 1990). An interruption in wetness periods led to reduced

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disease severity as shown in apple scab caused by *Venturia inaequalis* (Schwabe, 1980) and in wheat rust caused by *Puccinia recondita* f. sp. *tritici* and *Puccinia striiformis* (De Vallavieille-Pope et al., 1995). On the other hand, germ tubes resistant to dry periods were noted for such fungi as *Alternaria porri* f. sp. *solani* (Bashi and Rotem, 1974), *Cercospora musae* (Good et al., 1967) and *Stemphylium botryosum* f. sp. *lycopersici* (Bashi and Rotem, 1974). Air drying significantly reduced the conidial germination of *Botryosphaeria dothidea* under *in vitro* condition (Sutton and Arauz, 1991). However, no information is available on the effect of IWP on germ tube elongation of *B. dothidea* causing apple white rot.

An infection model of apple white rot was developed to determine the infection periods of *B. dothidea* on apple fruits based on the effects of temperature and wetness period on conidial germination and appressorium formation (Kim et al., 2005). However, it may be necessary to consider the effect of IWP on viability of germ tubes after conidial germination in order to improve the infection model in the disease warning system for white rot management in apple orchards. In the case of *B. obtusa*, the causal organism of apple black rot, Arauz and Sutton (1990) constructed the infection model to end infection periods by IWP of at least 1 hr long. Confirmation is thus required regarding whether IWP is detrimental to the growth of *B. dothidea* or not. Here we report the inhibitory effects of IWP on the fungal growth under *in vivo* and *in vitro* conditions.

## Materials and Methods

**Inoculum preparation.** An isolate (BD-3) of *B. dothidea* was obtained from a naturally infected apple fruit showing typical white rot symptoms. The isolate was grown on acidified (pH 4 to 5) potato dextrose agar (Difco Laboratories, Detroit, MI, USA) amended with streptomycin sulfate at 100 µg/ml to inhibit bacterial contamination. Barley grain media were prepared for inoculum production as previously reported (Kim and Park, 1998). The media were inoculated with two 5-mm-diameter mycelial plugs of *B. dothidea* and incubated at 25°C under continuous fluorescent light. The flasks were occasionally shaken to prevent caking of the grains. Pycnidia were produced on the barley grains after 3 days of inoculation. Conidia were harvested by flooding the inoculated barley grains with sterilized distilled water. The resulting suspension was filtered through two layers of cheesecloth to remove the mycelial fragments. The conidial suspension was adjusted to  $1.0 \times 10^6$  conidia/ml using a hemacytometer.

**Inoculation of apple fruit.** Mature apple (cv. Fuji) fruits were washed with mild detergent to remove dirt, insect honeydew and pesticide residues. Prior to inoculation, they were surface-sterilized with 0.1% sodium hypochlorite solution for 2 min, rinsed with tap water and allowed to air dry for 30 min. Ten inoculation sites were circled on each fruit using a marker pen for point inoculation, and two fruits were assigned to each treatment. A drop (200 µl) of conidial suspension from 4-week-old incubated barley grains was placed on each inoculation site on the fruits using an adjustable micropipette. Immediately after inoculation, the fruits were placed in a dew chamber at 25°C under the continuous darkness condition. Following an initial wetness period of 5 hr, the fruits were removed from the dew chamber and subsequently air-dried in an incubator at 25°C for 1, 2 or 4 hr. Visual observation of the droplet evaporation revealed that the suspension drops completely dried in the incubator at 25°C within 20 min. The relative humidity in the incubator at 25°C during the drying periods ranged from 31 to 34% throughout this experiment when measured using a psychrometer (CWKSIC-5425; Chungwon Science, Seoul, Korea). The fruits were then rewetted by dropping sterilized distilled water on the inoculation site, placed in the dew chamber and maintained at 25°C for another 5 hr. The fruits were removed and subjected to specimen preparation for light microscopy to examine conidial germination and germ tube elongation. Two checks were included; one in which conidia were allowed to germinate only for 5 hr and the other in which conidia germinated for 10 hr without interruption.

**Conidial germination and germ tube elongation on apple fruits.** Squares of epidermal strips (1 × 1 cm with approximately 1 mm of underlying tissues) were excised from each inoculation site of the fruits using a sterile razor blade. The strips were dipped in EtOH/chloroform (75:25, v/v) mixture amended with trichloroacetic acid at 0.15% (w/v) to prevent further conidial germination and decolorize the specimens (Wolf and Fric, 1981). The specimens were then immersed in 10 ml of distilled water amended with two drops (approximately 50 µl) of lactophenol-cotton blue (containing 100 ml of lactophenol, 3 ml of 1% aqueous solution of cotton blue and 10 ml of glacial acetic acid) and stained at room temperature for 10 min. The specimens were mounted on slide glasses and examined with a light microscope (Axiophot; Carl Zeiss, Oberkochen, Germany). A conidium was considered to have germinated if the germ tube was at least the length of the conidium. Percent conidial germination was determined by examining 10 conidia

selected randomly on each of 10 strips to give a total of 100 conidia per fruit. In addition, germ tube length was measured by examining five conidia on each of 10 strips to give a total of 50 conidia per fruit. Near real-time microscopic images were acquired and displayed onto a video monitor using a charge-coupled device camera (KP-C550; Hitachi, Tokyo, Japan) attached to the light microscope. Images of germ tubes on the video monitor were transcribed onto transparency films with a marker pen, and the transcripts were traced with a graphics digitizer (SummaSketch II; Summagraphics, Austin, TX, USA) connected to a personal computer (Seem et al., 1979). These length data were then converted and recorded for further statistical analysis. This experiment was conducted as a completely randomized design with two replications and repeated twice. Data from each experiment were pooled to calculate treatment means. Treatment means were compared by Fisher's protected least significant difference (FLSD) test using SAS PROC GLM (SAS Institute, Cary, NC, USA).

**Germ tube elongation on slide glasses.** To further verify the conidial germlings' capacity to elongate, longer hours of rewetting and several exogenous substrates were applied to conidial germlings under *in vitro* conditions. New microscope slide glasses (20 × 60 mm) were washed with mild detergent, rinsed with tap water and autoclaved. Three sites were circled on each slide glass using a marker pen for point dropping of conidial suspension, and three slide glasses were assigned to each treatment. Four types of conidial suspensions were made as follows: Conidia were harvested by flooding 5-day-old inoculated barley grains with i) sterilized distilled water, ii) 0.3% yeast extract broth, iii) 0.3% potato dextrose broth and iv) 0.3% tryptic soy broth. Drops (200 µl/drop) of each conidial suspension were placed on slide glasses, respectively, using an adjustable micropipette. The slide glasses were placed in a polystyrene box (32 × 43 × 8 cm) lined with moist laboratory paper towels, and the box was placed in an incubator at 25°C under the continuous darkness condition.

After 5 hr of wetting, the slide glasses were removed from the box, and subsequently air-dried in the incubator at 25°C for 1 hr. The slide glasses were then rewetted by dropping sterilized distilled water or each broth on the inoculation sites, placed in the box and maintained at 25°C for up to 40 hr. At each rewetting period of 5, 10, 20 and 40 hr, the same 10 germ tubes per each of the three slide glasses, to give a total of 30 conidia per treatment, were observed under the light microscope and photographed using a video copy processor (P90U; Mitsubishi, Tokyo,

Japan) connected to the microscope. The germ tube length at each rewetting period was measured by tracing germ tubes on the photographs using the graphics digitizer as described earlier. Two checks were also included; one in which conidia were allowed to germinate only for 5 hr and the other in which conidia germinated for 10 hr without interruption. This experiment was conducted as a completely randomized design with three replications and was repeated twice. Treatment means were calculated and compared as described earlier.

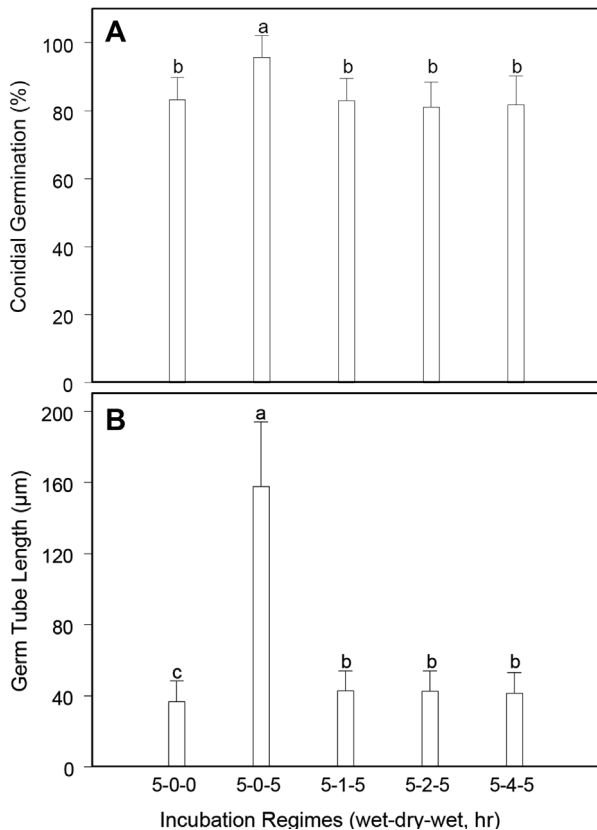
**Model application.** The infection model of apple white rot by Kim et al. (2005) was applied to evaluate the effect of IWP on infection periods of *B. dothidea*. Briefly, the infection model was derived from the effects of temperature and wetness period on conidial germination on apple fruits, and the quantitative relationship between conidial germination (G) and appressorium formation (A) as follows (Kim et al., 2005):

$$A = 0.381 - 0.227G + 0.005G^2 \quad (1)$$

From Eq. (1), appressorium formation was hardly initiated on apple fruits until conidial germination reached approximately 43.7% at a given temperature and wetness period (Kim et al., 2005). The daily infection period estimated by the model was the accumulated hours of appressorium formation after 43.7% of conidial germination in a day based on hourly weather data. Two sets of weather data collected from apple orchards in Suwon and Naju, Korea in 1994 and 2003, respectively, were used to calculate season-long profiles of infection periods. To ascertain the effects of dry period interrupting wetness period on infection period estimation under field conditions, season-long profiles of wetness periods were calculated in two ways: First, if two wetness periods were discontinued by a dry period of less than or equal to 1 hr long, the two wetness periods were regarded as one continuous wetness period (CWP); second, if the interrupting dry period was longer than 1 hr, the two wetness periods were calculated separately as IWP. The weather data were collected using automated weather stations installed at apple orchards in Suwon and Naju, Korea in 1994 and 2003, respectively. The automated weather stations consisted of a datalogger (CR10; Campbell Scientific, Logan, UT, USA) and various sensors including temperature (Model 107; Campbell Scientific) and leaf wetness sensors (Model 237; Campbell Scientific). Each weather data set consisted of hourly temperature and wetness period from June 1 to October 31.

## Results

**Conidial germination and germ tube elongation on apple fruits.** Most conidia produced germ tubes from one end or both ends of conidia, whereas in other instances germination took place from the lateral part of conidia. Following an initial wetness period of 5 hr,  $83.3 \pm 6.6\%$  of the conidia germinated, and conidial germination increased to  $95.8 \pm 6.4\%$  after 10 hr of continuous wetness period without interruption by a dry period (5–0–5) (Fig. 1A). However, conidial germination was no longer increased after interruption of wetting by a dry period of 1, 2 and 4 hr, resulting in  $83.0 \pm 6.5\%$ ,  $81.0 \pm 7.4\%$  and  $81.8 \pm 8.4\%$ , respectively. Percent conidial germination was significantly higher in the 10 hr check than in any other treatments ( $P = 0.01$ ). No significant differences in percent conidial germination were observed either between the 5 hr check and interrupted wetting treatments or among the three interrupted wetting treatments. In addition, the mean lengths

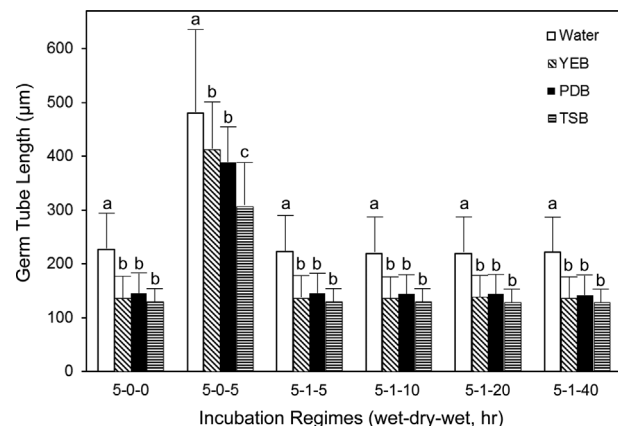


**Fig. 1.** Effects of interrupted wetness periods on development of *B. dothidea* on apple fruits. (A) Conidial germination. (B) Germ tube elongation. Bars in the columns indicate the standard deviations. The same letter denotes no significant difference at  $P = 0.01$  according to FLSD test.

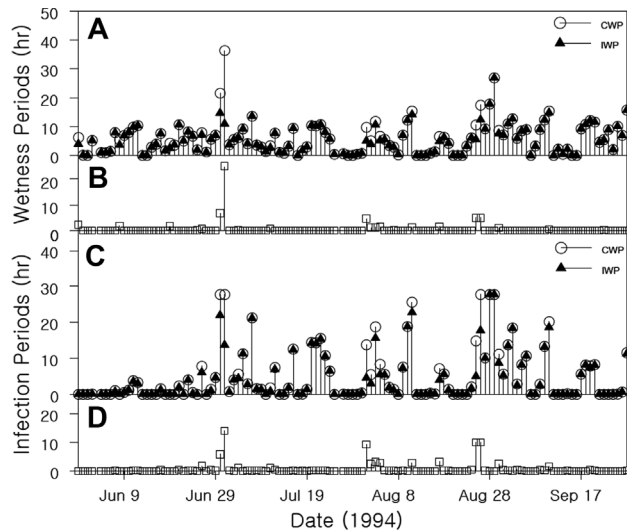
of the germ tubes were  $36.5 \pm 12.0 \mu\text{m}$  and  $157.1 \pm 37.3 \mu\text{m}$  after 5 and 10 hr of continuous wetting, respectively (Fig. 1B). On the other hand, interruption of wetting by a dry period of 1, 2 or 4 hr was effective in arresting germ tube elongation at approximately  $42 \mu\text{m}$  long. There was a significant difference in germ tube length between the 5 hr check and interrupted wetting treatments. However, no significant differences in germ tube length were found among the three interrupted wetting treatments.

**Germ tube elongation on slide glasses.** All of the conidia germinated on slide glasses under all treatments after 5 hr of wetting. The mean germ tube length was  $227.1 \pm 55.4 \mu\text{m}$  in sterilized distilled water after 5 hr, and increased to  $481.1 \pm 134.9 \mu\text{m}$  after 10 hr (Fig. 2). Prolonged rewetting for 10, 20 or 40 hr after 1 hr of drying did not increase germ tube length. There was no significant difference in germ tube length either between the 5 hr check and interrupted wetting treatments or among the rewetting treatments in the sterilized distilled water ( $P = 0.01$ ). Among the substrate treatments, the maximum germ tube elongation occurred in yeast extract broth and potato dextrose broth, followed by tryptic soy broth ( $P = 0.01$ ). There was no increase in germ tube length after 1 hr of drying. Prolonged rewetting up to 40 hr did not restore germ tube elongation under substrate treatments. There was a significant difference in germ tube length between sterilized distilled water and substrate treatments ( $P = 0.01$ ).

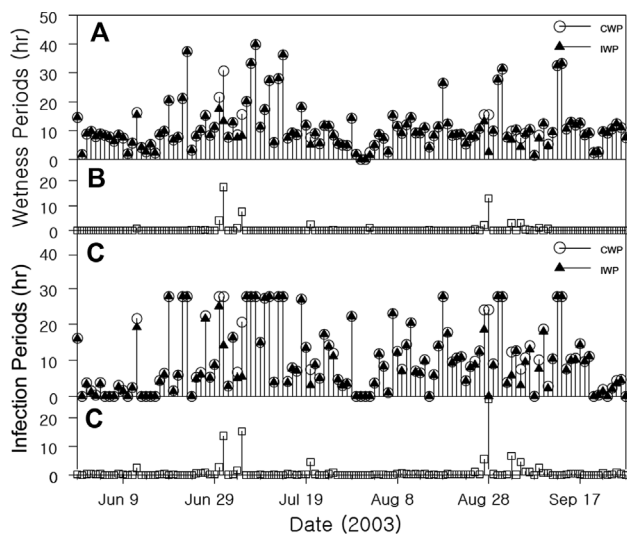
**Model application.** Differences were found in estimated



**Fig. 2.** Effects of interrupted wetness periods on germ tube elongation of *B. dothidea* on slide glasses. Water = conidial suspension in sterilized distilled water, YEB = 0.3% yeast extract broth, PDB = 0.3% potato dextrose broth and TSB = 0.3% tryptic soy broth. Bars in the columns indicate the standard deviations. The same letter denotes no significant difference at  $P = 0.01$  according to FLSD test.



**Fig. 3.** Effects of interrupted wetness periods on wetness periods and infection periods of *B. dothidea* at Suwon in 1994. (A) Continuous wetness periods (CWP) and interrupted wetness periods (IWP) from June 1 to October 31. CWP was calculated by adding wetness periods interrupted by a dry period of less than or equal to 1 hr long. (B) Subtraction of wetness periods from CWP to IWP. (C) Infection period calculated from CWP and IWP from June 1 to October 31. (D) Subtraction of infection periods from CWP to IWP.



**Fig. 4.** Effects of interrupted wetness periods on wetness periods and infection periods of *B. dothidea* at Naju in 2003. (A) Continuous wetness periods (CWP) and interrupted wetness periods (IWP) from June 1 to October 31. CWP was calculated by adding wetness periods interrupted by a dry period of less than or equal to 1 hr long. (B) Subtraction of wetness periods from CWP to IWP. (C) Infection periods calculated from CWP and IWP from June 1 to October 31. (D) Subtraction of infection periods from CWP to IWP.

wetness periods and infection periods depending on the calculation logics of wetness periods used in the infection model. The estimation based on the CWP generated higher levels of estimated wetness periods and infection periods. The wetness periods and infection periods from the two sets of wetness periods were similar overall, but differed in days when the interruption of wetness periods occurred in the two years. Season-long profiles revealed frequent occurrences of interruption of wetness periods under orchard conditions from June 1 to October 31 in 1994 (18 cases of over 1 hr difference) (Figs. 3A and 3B). The overestimation of infection periods in 1994 was severe mainly in July and August (20 cases of over 1 hr difference) (Figs. 3C and 3D). The frequencies of overestimation of wetness periods were relatively low during the two months (4 cases of over 1 hr difference).

The interruption of wetness periods also occurred in 2003 (10 cases of over 1 hr difference) (Figs. 4A and 4B). The resulting overestimation of infection periods in 2003 (14 cases of over 1 hr difference) was noted mostly from July to early September (Figs. 4C and 4D). The difference up to 24 hr in infection periods was estimated in late August, 2003.

## Discussion

This study demonstrated that interruption of a wetness period by a dry period as short as 1 hr ceased conidial germination and germ tube elongation of *B. dothidea*, suggesting that non-germinated conidia and germ tubes of *B. dothidea* are sensitive to drying. The fungal responses to IWPs in the present study were in good agreement with those obtained from *B. obtusa* (Arauz and Sutton, 1990; Sutton and Arauz, 1991). Water availability was apparently a critical factor in the fungal growth, such as conidial germination and germ tube elongation after being initiated by wetting conditions. Thus these fungi can be considered as xero-sensitive fungi as opposed to xero-resistant fungi such as *Alternaria porri* f. sp. *solani* (Bashi and Rotem, 1974) and *Cercospora musae* (Good et al., 1967).

The results revealed that non-germinated conidia of *B. dothidea* were vulnerable to drying regardless of the surface on which conidia germinated, such as cover glass and host surface. Fungal tolerance to low water availability has been reported to be greatest at its optimum growth temperature (Griffin, 1994). The optimum temperatures for the germination of ascospores and conidia of *B. dothidea* range from 24.6 to 29.5°C depending on fungal isolates. The germination can occur in as quickly as 90 min at 28°C (Sutton, 1990; Sutton and Arauz, 1991). Based on the complete ab-

sence of any resumption of the conidial germination when rewetted at 25°C for an additional 5 hr, it is assumed that a short dry period not only inhibited the germination process, but also degraded the conidial viability as proposed in the case of spore germination of *B. obtusa* and *B. dothidea* (Arauz and Sutton, 1989; Arauz and Sutton, 1990; Sutton and Arauz, 1991). A dry period following discharge from pycnidia and deposition on fruits in the outer canopy of apple trees could reduce the probability of host infection by conidia of *B. dothidea* (Aylor and Sanogo, 1997).

Short exposure to desiccation also resulted in a rapid decline in the viability of germ tubes of *B. dothidea*. In the present study, conidial germ tubes of *B. dothidea* were found to be vulnerable to drying for less than 1 hr. Prolonged rewetting up to 40 hr did not restore the germ tube elongation, and the viability of the germ tubes was irreversibly destroyed by IWP. These results were consistent regardless of the exogenous substrate conditions. Once the germ tubes of *B. dothidea* are exposed to drying, fungal utilization of the exogenously applied substrates is likely to have no stimulatory effects on subsequent germ tube elongation after drying. Under natural conditions in apple orchards, germ tube growth toward natural openings and following appressorium formation are arrested on the fruit surface by intermittent drying. The significant difference in germ tube length between the 5 hr check and the interrupted wetting treatments may be attributed to the germ tube elongation on the fruit surface during which the suspension drops were subjected to drying (within 20 min) in the incubator at 25°C before rewetting. Preliminary experiments showed that the time necessary for drying, as measured by visual observation of the droplet evaporation, was in close accordance with that measured by the same type of wetness sensors used in the collection of weather data sets (data not shown).

In comparison with the daily infection periods calculated from the two sets of wetness periods, it was apparent that IWP often occurred in apple orchards throughout the growing seasons. In addition, the season-long infection period profiles of both 1994 and 2003 revealed that adding wetness periods interrupted by a dry period of less than or equal to 1 hr long may have led to overestimation of the daily infection periods in calculating the infection periods. The wetness periods and infection periods were dependent on climate factors including temperature, rainfall events and others. The shorter wetness periods and the resulting lower levels of the infection periods in 1994 compared to those in 2003 were attributed to less frequent rainfall events over the period (data not shown). As shown in 1994 and 2003, the overestimation would probably increase under

favorable weather conditions prevalent from July to early September, resulting in more fungicide sprays per season. These results suggest that ending infection periods by IWP of at least 1 hr could save possible fungicide sprays to control white rot of apple in practice.

From this study we provided a rationale for integration of the effects of IWP on the fungal growth into disease forecast models. Considering the drastic effects of IWP on the fungal growth, effects of intervening dry periods on the infection period of *B. dothidea* should be included in disease forecast models to improve white rot control. Further field evaluation of disease forecast models with consideration of IWP is needed before wide applications in apple disease management.

### Acknowledgments

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