Plant Pathol. J. 15(6) . 330-334 (1999)

The Plant Pathology Journal

Pycnidiospore Production and Dispersal from the Warts Produced by Infection of *Botryosphaeria dothidea* on Apple Stems

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(Received on October 27, 1999)

Applying the method of quantitative analysis of pycnidiospore from the detached warts produced by the infection of *Botryosphaeria dothidea* on apple stems, repeated productivity of spores within the detached warts, variations in the amount of spores by the length of induction time for sporulation, and the effects of temperature and moisture on the sporulation were investigated. In addition to these experiment, the changes in the state of spores within the pycnidia contained in the warts accompanied by the induction of sporulation and dispersal of spores were also investigated. When detached warts were kept in moist conditions, the sporulation and discharge of spores could be repeated several times, and the amount of spores were almost constant after each repeat of sporulation induction and dispersal of spores in a given period. The fact that the pycnidia filled with spores were observed at considerable rates within the warts which were subjected to the shaking in the water to release spores indicated that the spores might never be released until the pycnidia were fully matured. From the high rate of empty pycnidia even in the warts which were kept in moist conditions for induction of sporulation, the pycnidiospores might be produced through the development of new pycnidia. A considerable amount of pycnidiospores were produced at 5°C, and the sporulation was accelerated with the rise of temperature until 35°C. When the warts were supplied with sufficient moisture, sporulation was further accelerated. The results obtained in these experiment will be applied in developing the method for assessing the inhibitory efficacies of fungicides on the sporulation of this fungus, with which a new control measure would be developed.

Keywords : apple white rot, pycnidiospore formation and dispersal.

Apple white rot caused by *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not. is one of the most serious apple diseases in Korea. The pathogenic fungus infects both stem and fruit (Brown and Hendrix 1981; Hayashi, 1984), the latter infection is much more serious than the former (Hayashi, 1984). The inoculum of the fruit infection is the ascospores or pycnidiospores produced in the respective fruiting bodies formed within the warts or raised blister developed by the infection of the pathogene to the stems of apple tree (Hayashi, 1984). The pycnidiospores play a much more important role than ascospores as an inoculum source of fruit decay (Ogata, 1997), and are dispersed during the whole growing season of the apple (Kim et al., 1995; Ogata, 1997; Sutton, 1981). The infection of the fruits begins to occur at around 7 weeks after the petal fall and continues until late August (Hiraragi and Sekizawa, 1981; Ogata, 1997; Parker and Sutton, 1993).

The control of this disease is largely dependent on the periodical spraying of fungicides, usually at a 10-day interval, during the possible infection period (Uhm et al., 1995). In spite of frequent spraying, many farmers fail to control the disease (Uhm 1998), since the spores are always dispersed whenever it rains during almost all of the growing season (Kim et al., 1995; Ogata, 1997; Sutton and Arauz, 1981), and the fruits are also susceptible until late August (Hiraragi and Sekizawa, 1981; Ogata, 1997).

From the ecology of this disease, it can be conceivable that the disease can be controlled or at least can increase the control efficacy of the fungicides by reduction of inoculum density through inhibition of sporulation at the lesions on the stem. Actually the fungicides sprayed during the growing season of apple might have influence on the sporulation of this fungus, and might play some role in controlling the disease, since the chemicals would usually be applied to the stems where the warts are produced. However, the *in vitro* assessment of inhibitory efficacy of the chemicals on the sporulation is usually very difficult, since the vegetative growth of the fungus would be inhibited if the fungus are grown on the media amended with chemicals. This fungus, however, produces spores within the warts which can be detached from the stems, and the sporulation can be amenable to quantitative analysis by counting the number of spores contained in a given volume of water in which the detached warts were shaken (Yang et al., 1998). This property of the sporulation of this fungus might be favorable to assess the inhibitory efficacy of the chemicals on the sporu-

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lation. In order to obtain the basic date required to develop a method for assessing the efficacy of the chemicals which would be used in developing a new control measure, a series of experiments were conducted. Applying the method for the quantification of sporulation from the detached warts, the repeated productivity of spores within the detached warts, variations in the amount of spores by the length of induction time for sporulation, and the effects of temperature and moisture on the sporulation were investigated. In addition to these experiment, the changes in the state of spores within the pycnidia contained in the warts accompanied by the induction of sporulation and dispersal of spores were also investigated.

Materials and Methods

Collection of the warts. Warts of S-2 stage which was charaterized by the development of cracks on the apex of the wart and high productivity of spores (Yang et al., 1998), were cut off at the bottom with a small knife from the stems of apple trees infected by *B. dothidea* at an commercial orchard near Taegu.

Induction of spore discharge and spore counting. The detached warts were vortexed for 30 seconds in distilled water to eliminate the dust and plant debris attached on them. The warts, usually 40 to 50, were put in a 100 ml Erlenmeyer flask with sterilized distilled water in the ratio of one ml per one wart. Spore discharge was induced by shaking the flask containing the warts for 4 hours with a shaking incubator (K.M.C-8480SF, Vision Scientific, Korea) at 150 rpm under 20°C, which was the optimum temperature for spore discharge (Yang et al., 1998). After the shaking, a household detergent Trio (Aekyung, Korea) was added to the suspension at the concentratiom of 0.1% (V/V), and shook for an additional 10 minutes to release the spores attached to the glassware (Yang et al., 1998). One milliliter of spore suspension, thus prepared, was passed through a membrane filter (Nucleopore, USA) of which the pore size and diameter was 2.0 μm and 11 mm, respectively. The number of spores attached to the filter was counted under a microscope (250 X) after staining them with lactophenol amended with aniline blue. In every experiment the counting of the number of spores was conducted with 3 replications, and 40 to 50 warts were used in every experiment to minimize the sampling error based on the results of a previous experiment that at least 30 warts should be used simultaneously in a single shaking to obtain a statistically consistent result (Yang et al., 1998). The number of spores contained in 1 ml of suspension was considered to be as the number of spores discharged from one wart

Repeated productivity of spores within the detached warts and variations in the amount of spores by the length of induction time for sporulation. In order to synchronize the sporulation in each wart, the preexisting spores in the detached warts were discharged by shaking them in water for 4 hours. The water on the warts were eliminated with paper towels, and kept in a moist chamber at 25°C. The chambers were prepared with a Petri dish (9 cm) containing 1.5% water agar as source of moisture. The dishes were placed upside down so as to the warts were placed on the lid, and were sealed with parafilm to avoid evaporation of the moisture. The moist chamber of this sort was devised to avoid the development of free water on the surface of the warts which might wash out the spores produced or make them germinate. The quantitative analysis of sporulation was conducted by the shaking methods described above after respective incubation period of 1, 3, 5 and 7 days, and the warts which had discharged spores were kept in the moist chamber repeatedly. The discharge of the spores and induction of sporulation by the preservation in the humidity chamber were repeated 5 times, and amount of sporulation after each repetition were examined.

State of spores in the pycnidia contained in the detached warts before and after shaking in water and those kept in the moist chamber. Warts were harvested 10 days after rainfall to permit sufficient sporulation. Some of the warts were directly fixed with FAA solution (EtOH : Acetic acid : Formalin=10 : 1 : 2) for 24 hours, and others were shaken in water for 4 hours in order to make the spores be discharged. The shaken warts were subdivided into two parts, one was fixed directly after shaking and the other was fixed after incubation in the moist chamber at 30°C for 5 days. The fixed warts were embedded in paraplast (Fisher Sci., USA) after dehydration with *n*-butanol series (Sass, 1958), and they were sectioned (20 μm) with a sliding microtome (TU-213, Yamato Koki, Japan). The sections were deparaffined by the xylene-alcohol process, and the state of spores in the pycnidia were examined with a microscope (100X) after staining the sections with aniline blue. In each treatment, about 100 pycnidia from 5 warts were examined.

Influence of incubation temperature on the sporulation. The warts which were shaken in the water for 4 hours to discharge the preexisting spores were placed in the moist chamber of a Petri dish, with 40 warts per each chamber. The dishes containing warts were kept in incubators adjusted to 10, 15, 20, 25, 30, and 35°C for 5 days, and the number of spores discharged from a wart was examined by the methods described above.

Influence of moisture on the sporulation. The detached warts which were shaken in the water to eliminate the preexisting spores were dried up with a hair dryer. The warts were divided into 3 parts, 50 in each part, and were kept in 3 different moist conditions The warts were 1) kept in a dried Petri dish (dry-dry), 2) kept in the moist chamber of Petri dishes which was previously referred to (dry-humid), and 3) was vortexed in the water for 5 minutes to be thoroughly wetted, and then kept in the moist chamber after elimination of the free water on the surface of the warts with paper towels (immersion-humid). Those Petri dishes containing the warts under different moisture conditions were kept in an incubator at 25°C for 3 days.

Results and Discussion

Repeated productivity of spores within the detached warts and variations in the amount of spores by the length of induction time for sporulation. The mean number of discharged spores increased steadily as the incubation period extended up to 5 days, and almost no difference was found

thereafter (Table 1). From the data, it was determined that the number of spores contained in a wart might reach its maximum state at around 5 days of incubation in the moist chamber. It was also revealed that the spores could be produced repeatedly, but the number of repeats with which the initial productivity could be maintained was different with the incubation period. When the cycles of the induction of sporulation and discharge were repeated within 1 or 3 days, the number of discharged spore did not vary throughout the 5 repetitions, except for the 4th repeat in the 3-day cycle. The drastic reduction during the 4th repeat in the 3-day cycle (Table 1) was thought to be due to the development of free water on the surface of warts, since the number of spores was recovered at the 5th repeat. However, the reduction of spores at the 5th repeat in the 5 and 7-day cycle was thought to be due to the depletion of nutrition, since the warts were kept in nutrient-poor condition for 25 or 35 days, respectively.

In this experiment, it was elucidated that the pycnidiospores could be produced repeatedly within the detached warts kept in an artificially controlled environment, and that the amount of spores produced would not vary when environmental conditions were kept constant. This fact can be applicable to the study of elucidating the ecological and physiological aspects of sporulation of this fungus, and can also be used in the screening of sporulation inhibitors and evaluation of their efficiency.

State of spores in the pycnidia contained in the detached warts before and after shaking in water and those kept in the moist chamber. The warts which were just harvested 10 days after rain, 85.2% of the pycnidia were filled with spores (Fig. 1A), 12.0% was completely empty (Fig. 1B), and 2.8% contained a small number of spores as can be seen in Fig. 1C (Table 2). After the warts were shaken in the water for 4 hours, the pycnidia filled with spores were reduced to 12.2%, but those containing a small number of spores or none increased to 69.3% and 18.4%, respectively (Table 2). The warts which were kept in the moist chamber for 5 days after primary discharge by the shaking contained 60.2% of pycnidia which were full of spores, 6.2% of pycnidia with a small number of spores, and the proportion of the empty pycnidia increased greatly to 33.6% (Table 2).

Table 1. Effect of incubation periods and repeat of spore discharge on the spore formation in pycnidia of *Botryosphaeria dothidea*

Incubation Period (day)[a]	No. of spores discharged from the wart at each cycle of incubation-shaking of respective period[b]					
	1st	2nd	3rd	4th	5th	mean
1	688	657	660	580	625	642
3	2493	2214	2281	1424	2452	2173
5	3518	3704	2805	3233	1122	2876
7	3884	3206	3416	3070	779	2871

[a] Period during which the discharged warts were kept in the moist chamber.
[b] Repeat of sporulation induction and discharge of spores

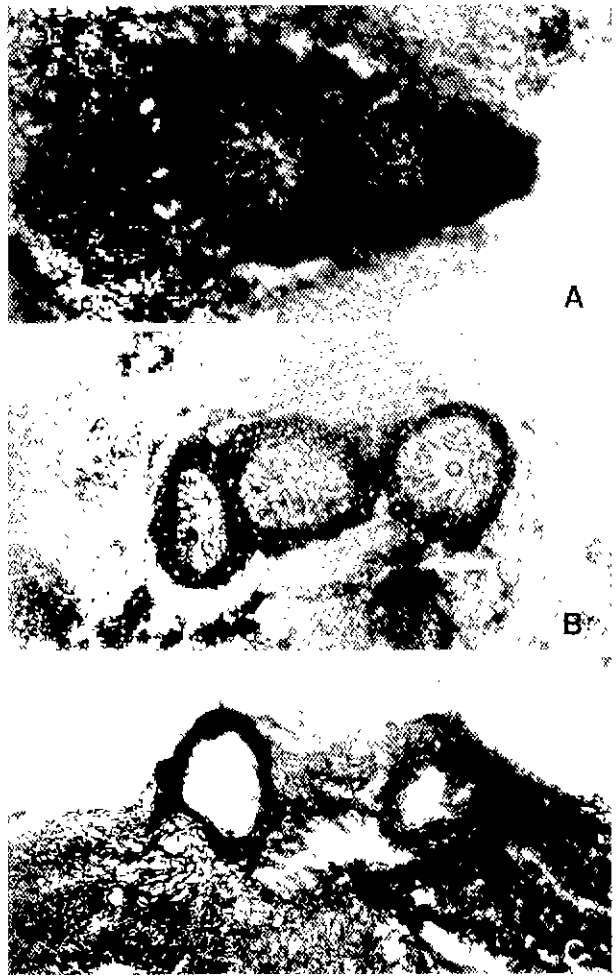

Fig. 1. Transverse sections of detached warts produced by infection of *Botryosphaeria dothidea* on apple stems (100 X).
A: Pycnidia filled with conidia just before spore dispersal, B: Pycnidia with small number of spores found after dispersal, C: Empty pycnidia found after spore dispersal.

Table 2. State of spores in the pycnidia contained in the warts which were sampled before and after release of spores, and those sampled after 5 days of keeping in humidity chamber at 30°C

Time of sampling	No. of pycnidia examined[a]	% of pycnidia containing spores		
		Full	Small number	Without
Before discharge	108	85.2	2.8	12.0
After discharge	98	12.2	69.4	18.4
After 5 days of incubation	113	60.2	6.2	33.6

[a] Pycnidia contained in 5 warts

With these results, the detailed aspects of spore production and dispersal within the warts can be explained.

In an experiment under the assumption of continuous rain Ogata (1997) reported that more than 80% of spores were discharged within 2 hours when the warts were soaked in water. The present authors had also conducted an experiment to investigate the time course of spore dispersal with detached warts, and found that 86.9% of spores were discharged within 3 hours, 94.4% within 4 hours, and only a small number of spores were continuously discharged without variation in numbers after 5 hours and thereafter (Yang et al., 1998). From this fact the spore production and discharge seemed to take place simultaneously. However, the results obtained in this experiment suggest somewhat different aspects in spore production and dispersal. From the fact that a considerable proportion of pycnidia filled with spores were found even in the warts which had been shaken 4 hours in the water, it was considered that the spores might be produced during the shaking, but not be discharged unless the pycnidia were fully matured. If the spores were released from the pycnidia as soon as they were formed, the pycnidia filled with spores would not be appeared in the warts after shaking. Additionally, it was also possible to suppose that the pycnidia filled with spores might not be those of discharged spores, but of newly formed ones. The rationale behind this supposition is that 4 hours is not a long enough time for a pycnidia to discharge their spores and reproduce them within it, since it took 5 days for the detached warts to reach the platitude in the number of spores as was elucidated in the experiment to investigate the repeatability of sporulation within the detached warts (Table 1). Another circumstantial evidence for this supposition is that the proportion of the empty pycnidia in the warts incubated for 5 days was much higher (33.6%) than those found in the warts before and after shaking in which 12.0% and 18.4% of the empty pycnidia were found, respectively (Table 2). If the spores were repeatedly formed in the pycnidia, the warts which were kept in such a good condition for sporulation would not contain such a high proportion of empty pycnidia. And the empty pycnidia was supposed to disappear by the lapse of the time, since the proportion was not so high in the warts just harvested from the tree (Table 2) in spite of their relative high ages.

Influence of temperature on the sporulation. A considerable number of spores were produced at a temperature as low as 5°C, and the number of spores increased as the temperature rises until it reach 20°C (Fig. 2). The increasing rate became greater above than 20°C, and continued to increase steadily until 35°C (Fig. 2). The range of temperature for sporulation of this fungus is different from that for mycelial growth at the upper limit. It was reported that the mycelia of this fungus grew at 10 to 35°C, with the optimum at 30°C, and the mycelial growth decreased rapidly as the temperature reach 30°C, and completely ceased at 40°C (Sutton and Arauz, 1991). The optimum temperature for sporulation was not elucidated in this experiment, since the sporulation was steadily promoted even at 35°C which was the highest temperature tested in this experiment. Therefore, it can be supposed that the optimum temperature might be higher than 35°C or the sporulation can, at least, be possible at a temperature higher than it. It could be also supposed that the lower limit for sporulation might still be lower than 5°C, since a considerable number of spores were produced at that temperature. Actually, Kim et al. (1995) reported that spore dispersal was confirmed during late November around which the mean temperature was 3.6°C.

Several reports on the effect of temperature on spore dispersal of this fungus can be available (Hayashi, 1984; Kim et al., 1995; Ogata, 1997; Sutton and Arauz, 1981), but those on the spore production cannot be available. Spore production and dispersal may certainly be a different facet, however, most of the research has been focused on the relation between spore dispersal and climatic conditions (Hayashi, 1984; Kim et al., 1995; Sutton and Arauz, 1981). Ogata (1997) reported that spores began to disperse when the minimum temperature of a day was higher than 16°C, and Kim et al. (1995) also reported that the number of spores trapped showed a high correlation with the minimum temperature. The temperature condition in relation to the spore dispersal reported by researchers was thought to be that for spore production. More recently, Ogata (1997) also asserted that the temperature was of no importance for spore dispersal, if the pycnidia were mature enough and filled with spores. The present authors had conducted an experiment to determine the effect of temperature on the spore dispersal by using the detached warts. It was elucidated that a large number of spores were dispersed even at a

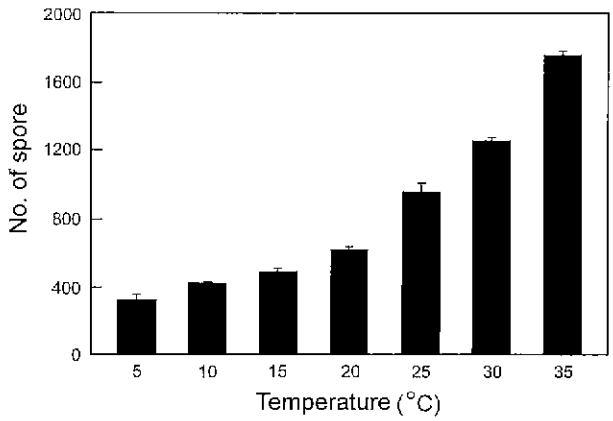

Fig. 2. Effect of temperature on the sporulation of *Botryosphaeria dothidea* determined by keeping the detached warts in the humidity chamber at different temperature for 4 days.

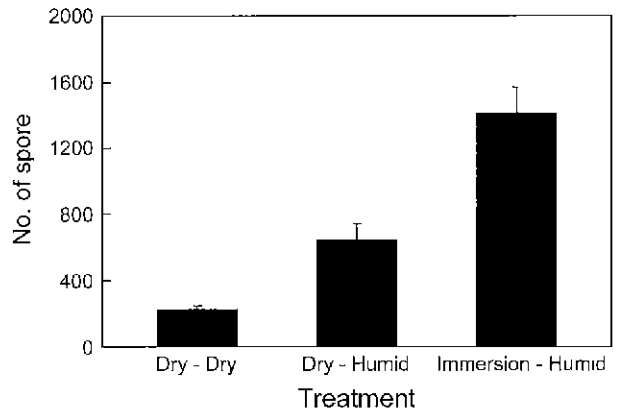

Fig. 3. Effect of moisture on the sporulation of *Botryosphaeria dothidea* determined by keeping the detached warts at different moisture levels. Dry-Dry. kept dried warts in dry condition; Dry-Humid: kept dried warts in the moist chamber: Immersion-Humid: kept thoroughly wet warts in moist chamber.

temperature as low as 10°C which is lower than that of mycelial growth, and the maximum dispersal was observed at 20°C, above which the amount of dispersal reduced drastically (Yang et al., 1998). Consequently, it can be said that the optimum temperature for sporulation and for spore dispersal was quite different.

Influence of humidity on sporulation. When the dried warts which had discharged spores were kept in the moist chamber for 3 days, the amount of spores produced were almost 3 times as much as those kept in dry condition (Fig. 3). Much more spores, more than 6 times of those kept in dry condition, were produced when the warts were kept in a moist chamber after wetting. This fact indicated that sporulation could occur even under high humid condition, and might be accelerated if the warts were soaked by rain, dew and even by sprinklers. Moreover, as the tissue of warts is hyperplasia and die when the pycnidia are formed within it (Lee et al., 1993), the tissue can reserve much water in the loose texture and is able to supply the pycnidia with water for a long period of time.

Acknowledgments

This study was supported by the academic research fund of the Ministry of Education, Republic of Korea.

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