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Effect of Delayed Inoculation After Wounding on the Development of Anthracnose Disease Caused by *Colletotrichum acutatum* on Chili Pepper Fruit

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Detached chili pepper fruits were inoculated with the conidial suspension of *Colletotrichum acutatum* JC-24 simultaneously (simultaneous inoculation, SI) and at delayed time (delayed inoculation, DI) after wounding with (delayed wound inoculation, DWI) or without additional wounding (delayed non-wound inoculation, DNI) at the inoculation time. Disease severity was significantly lowered by DNI, compared to SI. By DNI, the disease reduction rates were proportional with the length of delayed time, and greater at the high temperature range (18, 23 and 28°C) than at the low temperature (13°C) tested. DWI was also effective in reducing the disease severity especially at 18°C; however, its effectiveness was lower than for DNI. In light microscopy, parenchyma cells at the wounding sites were modified structurally, initially forming new cell walls crossing cytoplasm, enlarged with multiple periclinal cell divisions, and finally layered like wound periderms. In DWI, the above structural modifications occurred, showing the restriction of the fungal invasion by the cell walls in enlarged modified cells, while no definite cellular modifications were found with proliferation of fungal hyphae in SI. Sclerenchyma-like cells with thickened cell walls were proliferated around the wounding sites, which were partially dissolved by DWI, probably leading to some disease development. All of these results suggest that the decline of the anthracnose disease in pepper fruit by the delayed inoculations may be derived from the structural modifications related to the healing processes of the previous wound inflicted on the tissues.

Keywords **:** *Capsicum annuum*, *Colletotrichum acutatum*, delayed inoculation, structural modifications, wound periderm

Chili pepper is one of the most important vegetable crops in Korea. During the pepper cultivation, the plants suffer from various diseases, among which most frequently encountered and serious ones as top priority for control are anthracnose and Phytophthora blight caused by *Colletotrichum* spp. and *Phytophthora capsici*, respectively (Kim, 2004; Myung et al., 2005). Especially most anthracnose pathogens found in Korea are *C. gloeosporioides* and *C. acutatum* that damage pepper fruit, affecting directly fruit yield and quality loss (Park and Kim, 1992; Yoon, 2003). Use of chemical fungicides is one of the primary means of controlling this disease; however, it may provide the potential negative impact on the environment and human and animal health. In addition the occurrence of *Colletotrichum* isolates resistant to fungicides nullifies or reduces the control effects (Kim et al., 2005). One of the promising control methods against the pepper anthracnose is use of resistant pepper cultivars; however, no resistant cultivars have been developed until now for the control of the disease. Even little information is available on resistance and defense-related mechanisms of chili pepper to the disease.

Kim et al. (2004) reported that wound periderm (WP) formation is related to one of the resistant responses against *C. gloeosporioides* infection on chili pepper fruit. Wound periderms are formed in other plants such as peach responding to wounding and parasite invasion, which function as histological defense (Agrios, 2005; Biggs and Britton, 1988; Mullick, 1977; Rittinger et al., 1987). The wound periderm formation is a common phenomenon detectable in the wound healing processes in dicotyledons and certain monocotyledons after wounding (Esau, 1977), indicating wounding itself may induce a certain level of resistance in plants. Thus, this study is to know the relations of the induced resistance and wounding by comparing disease severities between simultaneous and delayed pathogen inoculations after wounding. Environmental factors, especially incubation temperature, were also examined in relation to the wound-induced resistance to provide basic information useful for the management of the anthracnose disease.

Materials and Methods

Plant, pathogen and inoculation. Fully grown green fruits of a commercial chili pepper (*Capsicum annuum* cv. Nokkwang) and *C. acutatum* JC-24, a strong pathogenic

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fungus, isolated from Jincheon, Korea in 2002 (Kim et al., 2008), were used in this study. The fungus was grown on potato-dextrose agar (PDA) at 25°C for 7 days. Ten ml of sterile distilled water was added to the culture to obtain conidial suspension. The conidial suspension was filtered through four layers of cheesecloth to remove mycelia and cultural debris, and centrifuged at 10,000 × g for 1 min, washing three times in sterile distilled water by decanting the supernatant after centrifugation. The conidial suspension was adjusted to 1.0×10^6 conidia per ml with a hemacytometer. Pepper fruits were surface sterilized in 1% sodium hypochlorite for 3 min, washed in sterile distilled water three times. The conidial suspension was dropped on four or five sites on each fruit (6 μl per site) after pin-prick wounding (ca. 0.6 mm diameter × 1.2 mm depth) using a syringe needle (wound inoculation) or without wounding (non-wound inoculation). As the other inoculation test, the conidial suspension was sprayed on the fruit surface to run-off, for which wounding was made using 0.01% 400-mesh carborundum suspension sprayed on pepper fruit at pressure of 0.8 MPa with the aid of an air compressor. For both inoculation methods, sterile distilled water instead of the conidial suspension was used as control (or treatment of wounding alone). After inoculation, the pepper fruits were placed in a plastic container ($30 \times 20 \times 7$ cm^3) lined with four layers of paper towel moistened with sterile distilled water to produce a humid environment. Symptoms on the pepper fruit were examined daily, and longitudinal lesion diameter was measured with a ruler.

Effect of delayed inoculation after wounding on the disease development. Disease severities were examined on pepper fruit inoculated with *C. acutatum* JC-24 simultaneously (simultaneous inoculation, SI) and at delayed time (delayed inoculation, DI) after primary wounding as mentioned above with or without additional wounding for DI. For the delayed non-wound inoculation (DNI), the conidial suspension of *C. acutatum* JC-24 was dropped on wounds or sprayed to run-off evenly on fruit surface without additional wounding 0 h (for SI), 1 h , 6 h, 12 h, 24 h, 72 h, and 120 h (for DI) after primary wounding. Inoculated pepper fruits were placed in the plastic containers and incubated at 23°C. Symptom development on pepper fruit was examined 7 days after inoculation. For the delayed wound inoculation (DWI), additional pin-prick wounds were made on pepper fruit when pepper fruits were inoculated with *C. acutatum* JC-24 24 h after primary wounding. Simultaneous wound inoculation at the time of wounding served as control. The inoculated pepper fruits were incubated at two temperature conditions (18°C and 23°C). Symptom development (longitudinal lesion size) on pepper fruit was examined 6 days after inoculation.

Effect of incubation temperature on wound-induced resistance by DI. The delayed non-wound inoculation (DNI) with spraying the conidial suspension on pepper fruits was only applied for examining the effect of wounding and delayed pathogen inoculation on the disease development of the pepper anthracnose. For SI as control, *C. acutatum* JC-24 was inoculated at the same time of (0 h after), and for DI, 20 min, 40 min, 1 h, 6 h, 24 h, and 72 h after the carborundum spray wounding. After inoculation, the pepper fruits were incubated in different temperature conditions of 13°C, 18°C, 23°C and 28°C. Symptom development on pepper fruit was examined 7 days after inoculation. Disease severity was expressed as the sum of longitudinal lesion diameters relative to the total length of the fruit inoculated.

Histological responses of pepper fruit tissues to wounding and pathogen inoculation. Specimens for light microscopy of pepper fruit tissues to examine the effects of delayed inoculation after wounding were obtained from the experiments mentioned above. Hand-cut sections of fresh fruit and thin sections of resin-embedded specimens were made and observed under a compound light microscope. For the hand-cut sections, wounding and inoculation sites of pepper fruit were sliced out and hand-sectioned with a razor blade at given periods of days after inoculation. The sections were observed under a light microscope (Axiophot, Zeiss, Germany) after staining with 0.1% toluidine blue O (O'Brien and McCully, 1981). For the resin-embedded thin sections, wounding and inoculation sites of pepper fruit tissues were cut off at different time after wounding and inoculation, and fruit tissue segments were fixed in Karnovsky's fixative in 0.1 M sodium cacodylate buffer (pH 7.0) for 4 h (Karnovsky, 1965). The segments were rinsed with the same buffer three times for 20 min each, and post-fixed in 1% osmium tetroxide in the same buffer for 2 h. The specimens were washed briefly in distilled water, *en bloc* stained in 0.5% uranyl acetate at 4°C overnight, and dehydrated in a ethanol series of 30, 50, 70, 80, 90, and 100% for 10 min each with the final exposure to 100% ethanol repeating three times. The specimens were further treated with propylene oxide as a transition fluid two times for 15 min each and embedded in Spurr's epoxy resin (Spurr, 1969). The embedded specimens were sectioned 1-2 μm in thickness with a glass knife on an MT-X ultramicrotome (RMC, Inc., Tucson, AZ). The sections were stained with 1% toluidine blue O, and observed under the light microscope.

Results

Effect of DI on the anthracnose disease development. For DNI, disease severities were lowered significantly on

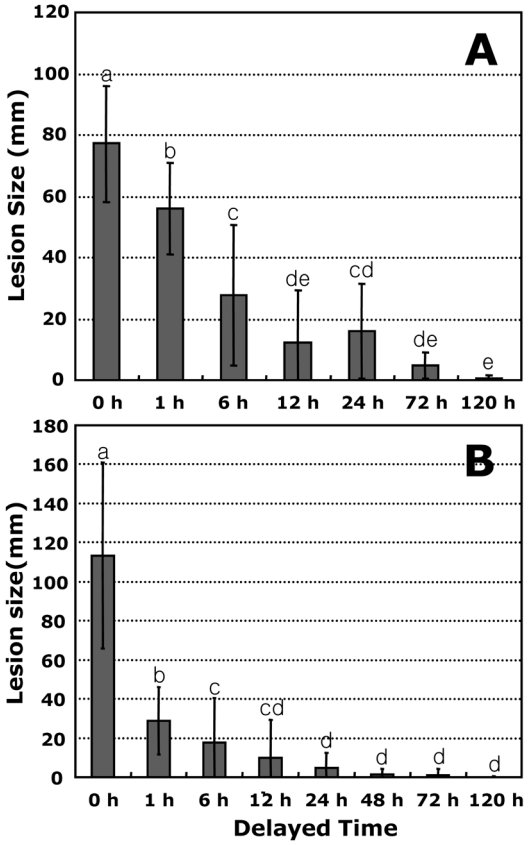

Fig. 1. Effect of delayed non-wound inoculation (DNI) after primary wounding (A: pin-prick wounding, B: carborundum spray wounding) on disease severity of chili pepper anthracnose caused by *Colletotrichum acutatum* JC-24. Bars and vertical lines are averages and standard deviations of 10 replications (A) and 30 replications (B). Lesion size (length) was examined at 7 days after inoculation. 0 h: simultaneous pathogen inoculation at the time of wounding. The same letters above the bars denote no significant difference at P=0.01 by least significant difference (LSD) test.

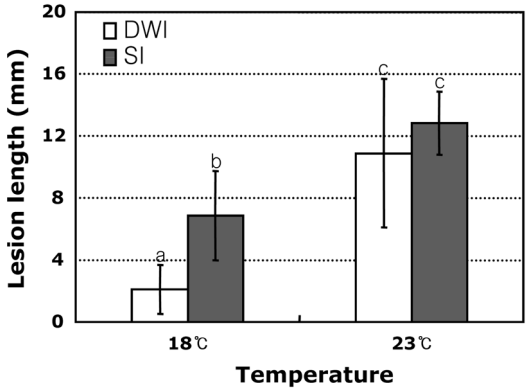

Fig. 2. Effect of delayed wound inoculation (DWI) after primary wounding by pin pricking on disease development of chili pepper anthracnose caused by *Colletotrichum acutatum* JC-24 at different incubation temperatures. DWI: 24 h delayed wound inoculation after wounding, SI: simultaneous inoculation at the time of wounding. Lesion size is longitudinal lesion diameter that was examined at 6 days after inoculation. Bars and vertical lines are averages and standard deviations of 10 replications. The same letters above the bars denote no significant difference at P=0.01 by LSD test.

pepper fruit inoculated with *C. acutatum* JC-24, regardless of the wounding methods (pin-prick and carborundum spray wounding), when the time of inoculation after primary wounding was delayed for 1 h to 120 h (Fig. 1). The disease severity was proportionally decreased to the time lengths of DNI, which was remarkably decreased from 6 h and almost none at 72 and 120 h after primary wounding. For DWI, disease severities were also reduced by DI; however, the disease reduction rates were not so high as in DNI (Fig. 2). Significant disease reduction was noted in DI at 18°C, but not at 23°C compared to the simultaneous inoculation.

Effect of incubation temperature on DNI for the disease development. When pepper fruits were wounded by carborundum spray and then inoculated with the conidial suspension of *C. acutatum* JC-24, the amount of the disease was the highest at the incubation temperature of 28°C and by simultaneous inoculation (0 h DNI) at each temperature (Fig. 3). For all the temperatures examined, the severity of the anthracnose disease was decreased proportionally with delayed time after wounding. For all the temperatures tested (even at 13°C), significant disease decline was noted from 1 h DNI after wounding compared to SI. However, the disease severities were still high with the sum of longitudinal lesion diameters over 70% of the total fruit length in 20 min and 40 min DI at 23°C and 28°C, while almost no disease occurred in simultaneous inoculation as well as in all DI at 13°C.

Histological responses of pepper fruit tissues to wounding and pathogen inoculation

Effect of DNI. Wound-induced resistant responses were examined by DNI because the pathogen development is to be influenced only by the healing processes on the primary wounding sites without the secondary wounding that may hamper the processes. Initial cell divisions were noticed one day after wounding beneath the wounding site of pepper fruit tissues, forming new cell walls adjacent to the pin-prick wounding (Fig. 4A). Then cell divisions with parallel cell walls were evident at 4 days after wounding beneath the wounding sites, which were composed of modified isometric rectangular parenchyma cells (MC) with thickened cell walls differentiated from the surrounding cells (Fig. 4B). At 10 days after wounding, cell divisions occurred in a radial file and began to form a layer with thickened dark green (maybe suberized) outmost cell walls beneath the wounding sites, which is similar to wound periderm (Fig. 4C).

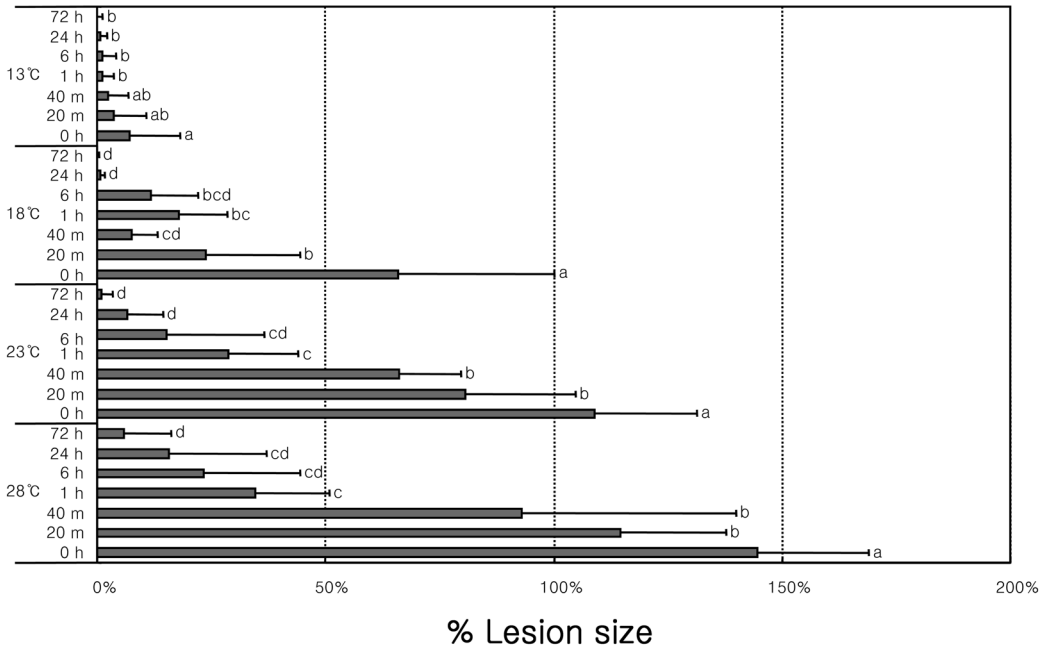

Fig. 3. Effect of delayed non-wound inoculation after primary wounding with high pressure spraying with carborundum on disease development of chili pepper anthracnose caused by *Colletotrichum acutatum* JC-24 at different incubation temperatures. Lesion size is longitudinal lesion diameter (% of the fruit length) that was examined at 7 days after inoculation. 0 h: simultaneous inoculation at the time of wounding. Bars and vertical lines are averages and standard deviations of 10 replications. The same letters above the bars denote no significant difference at P=0.01 by LSD test for each temperature.

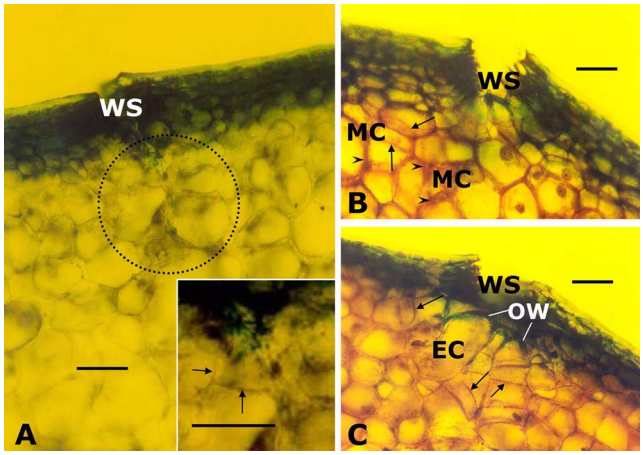

Fig. 4. Light micrographs of hand-cut sections of pepper fruit with pin-prick wounding in *Capsicum annuum* cv. Nokkwang (at A, 1; B, 4; and C, 10 days after wounding), showing new cell walls (arrow) formed beneath the wounding site (WS), an indication of cell division (A), proliferated isometric rectangular cells (modified cells, MC) with thickened wall (arrowhead) and new cell wall (arrow) beneath the wounding site (B), and enlarged cells (EC) layered beneath the wounding site, having periclinally divided cell walls (arrow) and thickly stained (probably suberized) outmost cell walls (OW) to form wound periderm (C). Inset of A: Magnification of the circled area in A. Bars=100 μm.

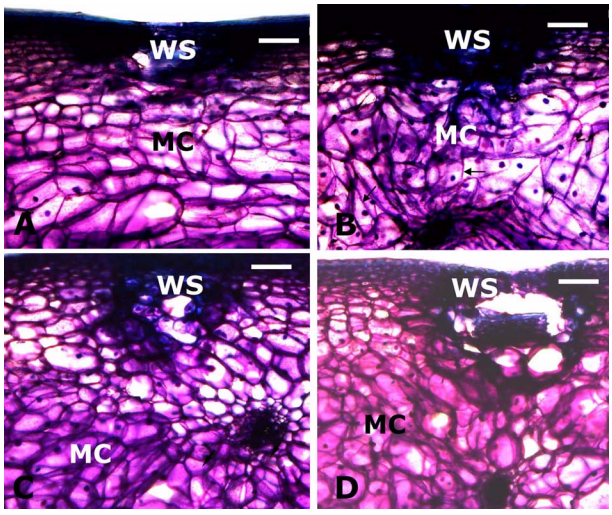

Fig. 5. Light micrographs of hand-cut sections of pepper fruit with pin-prick wounding in *Capsicum annuum* cv. Nokkwang at different temperatures (A, 13°C; B, 18°C; C, 23°C; and D, 28°C), showing the formation of sclelenchyma-like modified cells (MC) beneath the wounding sites (WS) at 12 days after wounding. Note somewhat more proliferated modified cells at 18°C (B) than other temperatures. Arrow: new cell wall. Bars = 200 μm.

Effect of incubation temperature on wound-induced resistance by DI. The pepper fruit tissues wounded by pin pricking and incubated under different temperature conditions were hand-cut sectioned and viewed by light microscopy. For all the temperature conditions at 12 days after wounding, the fruit tissues beneath the wounding sites

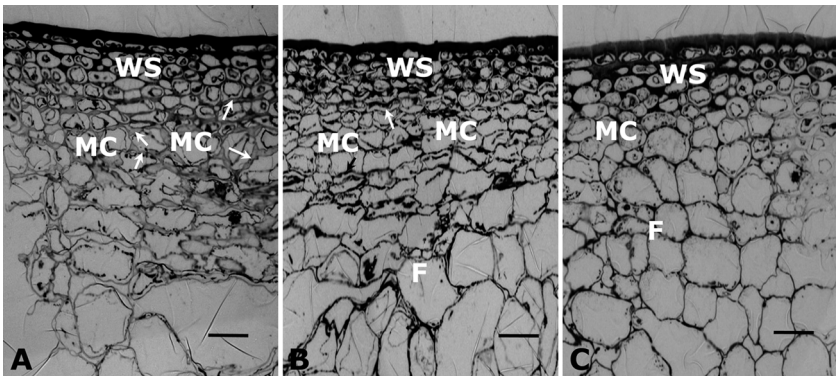

Fig. 6. Light micrographs of resin-embedding sections of pepper fruit tissues (*Capsicum annuum* cv. Nokkwang) with pin-prick wounding alone (A), 24 h delayed wound inoculation after primary wounding (B), and simultaneous inoculation by *Colletotrichum acutatum* JC-24 at the time of wounding (C), and incubated at 18°C. Specimens were examined 6 days after wounding (inoculation). Extensive formation of sclelenchyma-like modified cells (MC) in A and B, but no or poor formation in C. Note the thickened cell walls (arrows) of the modified cells in A are partly dissolved (arrows) in B by the infection of the fungus (F). Bars = 100 μm.

contained modified parenchyma cells (MC) differentiating from other surrounding intact cells (Fig. 5). The modified cells were somewhat more prominent at 18°C than other temperatures, in which new cell walls were sometimes found especially at 18°C.

Effect of DWI. Wound-induced resistant responses and their effect on disease development were examined by DWI, focusing on structural modifications of inoculated pepper fruit tissues and the fungal growth and invasion in the tissues. The histological responses of pepper fruit tissues were compared among wounding alone, simultaneous and one-day delayed wound inoculations. At 6 days after wounding, the fruit tissues beneath the wounding sites contained prominent modified cells (MC) with extensively thickened cell walls in the wounding alone, which were similar to sclerenchyma-like cells (Fig. 6A). The thickened cell walls appeared to be partly dissolved by the fungal infection in the delayed inoculation (Fig. 6B), and no or minimal cellular modifications were found in the simultaneous inoculation (Fig. 6C). At 9 days after wounding or inoculation, cell divisions occurred in the enlarged (modified) cells in the wounding alone (Fig. 7A) as in Fig. 4C. In DWI, the fungal hyphae were bounded by the cell walls of the enlarged modified cells, by which they appeared to be restricted and deviated in growth and invasion (Fig. 7B). Only a few hyphae were observed in the infected cells with DWI. In the simultaneous inoculation, however, no cellular modifications accompanying dividing cell walls were found, and the fungal hyphae were proliferated in the infected cells (Fig. 7C).

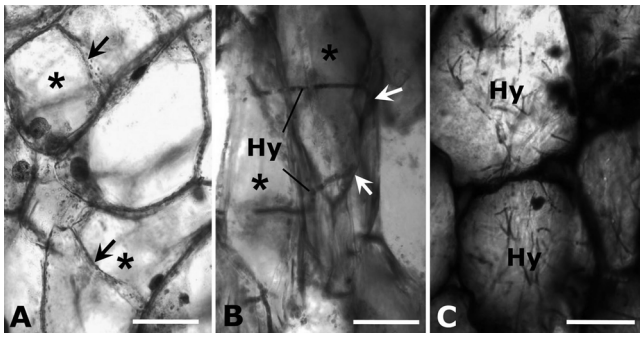

Fig. 7. Light micrographs of hand-cut sections of pepper fruit tissues (*Capsicum annuum* cv. Nokkwang) with pin-prick wounding alone (A), 24 h delayed wound inoculation after primary wounding (B), and simultaneous inoculation by *Colletotrichum acutatum* JC-24 at the time of wounding (C), and incubated at 18°C for 9 days, showing the formation of new cell walls (arrows) in enlarged cells (asterisk) in A and B, but none in C. Fungal hyphae (Hy) are restricted and deviated in their invasion and orientation by the cell walls (arrows) in B, but they colonized non-modified cells profusely in C. Bars=50 μm.

Discussion

Wound is an important infection court for many plant pathogens lacking direct and other means of indirect penetration (Agrios, 2005). Although the chili pepper anthracnose develops readily on pepper fruit of susceptible cultivars regardless of wounding (Kim et al., 2008), the disease severity is enhanced by the wound inoculation compared to non-wound inoculation (Kim et al., 2004). After wounding, however, many plant organs develop healing processes, resulting in the wound periderm formation (Esau, 1977), which is related to the structural defense to plant pathogens (Agrios, 2005). Thus, the disease is most severe when the pathogen arrives at the wounding site (infection court) immediately, and if the pathogen arrives later, the disease frequency and severity decline with time until the wounded tissues express resistance comparable to that of uninjured tissues (Biggs, 1986; 1989).

In our study, the anthracnose disease of chili pepper was significantly reduced in disease severity when the susceptible pepper fruit were inoculated at delayed periods with no secondary wounding after the primary wounding; i.e. delayed non-wound inoculation (DNI). The disease reduction rates were proportional with the longer delayed periods after wounding. These results suggest that the disease severity is declined and the tissues are strengthened to have resistance against the pathogen infection with delayed time (with age) of inoculation after wounding. It was also demonstrated in several host-pathogen interactions that wounds become increasingly less susceptible to infection with age (Bostock and Middleton, 1987; Cline and Neely, 1983; Riffle and Peterson, 1986; Russin and Shain, 1984).

The disease severity was also reduced by the delayed wound inoculation (DWI) in which the secondary wounding was made for the pathogen inoculation. However, the reduction of disease severity was less prominent in DWI than in DNI, and was significant at the incubation temperature of 18°C but not 23°C, suggesting that the secondary wounding in DWI may interfere in the wound healing processes induced by the primary wounding.

Our study revealed that there were structural modifications in pepper fruit tissues beneath the wounding sites with age after wounding, which are identical to those (suggested to be wound periderm) formed in resistant pepper fruit tissues infected with *C. gloeosporioides* at the later stages of infection (Kim et al., 2004). Wound periderm itself may serve as a structural barrier to the pathogen infection, and moreover, the formation of primary ligno-suberized tissues (Biggs, 1986) and the accumulation of phenolics (Koga et al., 1988; Lummerzheim et al., 1993; Mayama and Shishiyama, 1978; Von Röpenack et al., 1998) during the structural modifications may play an important role in inhibiting the initial pathogen growth (Silva et al., 1998). In the delayed inoculation after wounding in our study, the histopathological aspects that few fruit tissues were colonized by the fungal hyphae before definite wound periderm formation at the later stages of infection may be related to these physico-chemical changes accompanied by the structural modifications. However, as indicated by Biggs (1986), the presence of wound periderm is more critical for inhibition of the pathogen than primary ligno-suberized tissues possibly including the accumulation of phenolic compounds. These aspects are also derived from the fact that anthracnose pathogens of some host plants including chili pepper are hemibiotrophs which develop secondary necrotrophic hyphae (Kim et al., 2005; Latunde-Dada et al., 1996; O'Connell et al., 1985), possibly overcoming the physico-chemical modifications prior to the wound periderm formation. It was also clearly shown in our study that a few remaining fungal hyphae appeared to be obstructed in their growth and deviated in their orientation in confrontation with the cell walls of such modified cells in the process of wound periderm formation.

The effects of delayed inoculation after wounding differed among different temperatures, and were somewhat more prominent at 18°C than other higher temperature conditions examined, especially when the delayed time was less than 1 h in DNI, showing high (susceptible) disease severities in 20 min and 40 min delayed inoculation. Furthermore in DWI, the disease reduction effect by DI was remarkably different between the incubation temperature of 18°C and 23°C; a significant disease reduction was only detected at 18°C. Accordingly, structural modifications leading to the wound periderm formation were also somewhat more prominent at 18°C than other temperature conditions. This suggests that the pepper fruit may be subjected to the most rapid wound periderm formation at 18°C among the temperature conditions examined. This is, however, contradictory to temperature effects for curing of underground plant organs such as storage roots and tubers before their long-term storage, which refers to the process of wound healing with development and suberization of new epidermal tissues called wound periderm. It generally requires relatively high temperature for the rapid wound periderm formation in these storage organs (Morris et al., 1989; Thomas, 1982; Wigginton, 1974). Contrary to our present study, wound periderm was formed in inoculation sites at 28°C, but not at 18°C in ginseng root tissues inoculated with a virulent isolate of *Cylindrocarpon destructans*, in which no black root rots developed at the higher temperature conditions (Kim et al., 2008). Besides the relatedness of wound periderm formation to the disease severity decline at different temperature conditions, the pathogen activity could not be ruled out from the temperature effects. This is because the disease severity was heightened proportionally with temperature increase in our study. The dissolution of thickened cell walls of the modified cells in the delayed inoculation (Fig. 6B) also supports the disease development may be governed by the pathogen activities to overcome the host defense reactions.

Jeon and Kim (2008) reported that wound periderm was induced in ginseng root tissues by the treatment of a low concentration of *Paenibacillus polymyxa* suspension, on which no rot was developed. As the wound periderm formation was stimulated by wounding, making artificial wounds on pepper fruit surface at an appropriate time during the chili pepper growth may reduce the anthracnose disease development, providing a way of the disease control. Especially the carborundum spray wounding can provide the whole fruit surface with the wounding stimulus evenly, so that this wounding method can be modified further for the improved disease control.

Acknowledgement

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