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Effect of Heat Shock on Red Light-Induced Resistance of Broad Bean against *Botrytis cinerea*

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Abstract Heat shock treatment at 55 ℃ for 5 to 10 sec before inoculation induced the disease susceptibility of broad bean to *B. cinerea*. Such heat-induced susceptibility was manifested by lesion development and hyphal growth of pathogenicity-lost strains of *B. cinerea* in heat-shocked broad bean leaflets. On the other hand, when virulent strain of *B. cinerea* was inoculated to the broad bean, appressorium formation and infection hyphal growth were also promoted in the dark. Under red light, however, infection behaviors were inhibited even in heat treated broad bean. Higher anifungal activity was observed in inoculation droplet recovered from heat-shocked broad bean leaflet kept under red light compared with that in the dark. These studies suggested that red light suppressed the heat-induced susceptibility in broad bean leaves inoculated with *B. cinerea* by accumulation of antifungal substance(s)in inoculation droplet.

Key words: *B. cinerea*, broad bean, red light, heat-shock, antifungal substance(s)

Introduction

It was reported that plants have some disease resistance mechanisms for their survival in nature against the wide variety of plant pathogenic agents. Understanding the mechanism of host-plant resistance to pathogens has attracted much attention, especially for the control of plant disease using new biotechnology to improve host resistance (Gasser and Fraley, 1989; Staskawicz *et al*., 1995). A new aspect for plant disease management, red light induced resistance has been reported in some plants (Islam *et al*. 1998, 2002 b; Rahman *et al*. 2003; Umezu *et al*. 1999). However, the specific mechanism of red light induced resistance in broad has not yet been elucidated clearly.

Heat shock can alter or negate the disease resistance even to non-pathogenic agents (Cruickshank and Perrin 1965; Heath 1979). Heat shock treatment is an established method which is commonly used to prevent or delay resistance response (Elliston *et al*. 1977; Jerome and Müller 1958; Oku *et al*. 1975 a & b; Yarwood 1956). Lesion development and sporulation are useful criteria for measuring heat-induced susceptibility of rice plant against blast fungi (Arase *et al*. 1982 a & b). Yoshida *et al*. (1999) reported that pre-inoculated heat treatment of cruciferous plant cotyledons suppress defense reactions against *Peronospora parasitica*. The effect of heat shock on red light-induced disease resistance in plant is yet to be revealed, though, the response of plants to heat-shock has recently received much attention. Thus, thermo-biological approaches on red light induced resistance in broad bean against *B. cinerea* seem to provide some valuable information on the mechanism of red light-induced disease resistance in plant. Therefore, the effect of heat treatment on the lesion development and infection behaviors in red light irradiated broad bean leaflet inoculated with *B. cinerea* was investigated.

Materials and Methods

Plant

Broad bean plants (*Vicia faba* L., cv. Taito) were grown in a glasshouse throughout the experiment. Seeds were sown in 16-cm-diameter pots containing 3 kg of commercial garden soil (Kureha Chemicals, Osaka, Japan), 1 seed/pot (Islam *et al*., 1998).

Pathogen

A virulent strain BC 304 of *B. cinerea* was isolated by single spore culture from infected strawberry fruit, and stocked on potato-strawberry agar (100-100-20g/l) in slants for maintaining its pathogenicity. An avirulent strain BC 1 which had lost the pathogenicity during the continuous subculture was also used in this study. Both strains of *B. cinerea* were cultured on potato sucrose agar medium at 22.5℃ under continuous near ultraviolet irradiation provided by fluorescent lamp (FL 10BLB, 360 μW/cm^2, Toshiba, Japan). A lawn of uniform

sporulation was formed within 6 to 8 days. A spore suspension was prepared by flooding plates of 6-8-day-old cultures with sterilized, distilled water and dislodging spores with a glass rod. Spore densities were estimated with a hemacytometer and adjusted to 2-3×10^5 spores/ml.

Heat treatment and inoculation

Fully expanded unblemished leaflets were collected and dipped into hot water at 50 and 55 ℃ for 5 and 10 sec. Each leaflet was divided into two-half leaflets by its midrib, and placed on a frame of glass rod in a Petri dish lined with sterilized moist filter paper. Heated and unheated half leaflets were inoculated with 20μl drops of a spore suspension of *B. cinerea* (2-3×10^5 spores/ml) and then kept under red light (FL 20S・R-F, National: 600-700 nm, max. 650 nm, 258 μW/cm^2) or in the dark for 48 h at 25℃.

Light microscopic observation

Leaf tissue under inoculation droplets were cut into a small pieces and boiled in a lactophenol-alcohol solution (1:2, v/v) until the chlorophyll was removed. The leaf pieces were immersed into saturated chloral hydrate solution for 1 day to make the leaf tissue transparent. The tissue was stained with 1% cotton blue in lactophenol on a glass slide and the fungal infection behavior was observed under a microscope. Three hundred appressoria from six pieces were observed for each replication. Each experiment was repeated at least twice with three replications.

Bioassay of anti-fungal activity of infection droplets

The heated and unheated broad bean leaflets were washed thoroughly with distilled water. Each leaflet was divided into two-half leaflets by its midrib and placed in plastic case lined with moist paper. The leaflets (upper surface) were inoculated with three to four drops (50μl) of *B. cinerea* (2-3×10^5 spores/ml) and then incubated under red light from a fluorescence lamp (FL 20S・R-F, National: 600-700 nm, max. 650 nm, 258 μW/cm^2). For dark control, one half was inoculated with a spore suspension and incubated in plastic cases covered with aluminum foil. After 48 hr, inoculation droplets were recovered from the leaflets by micropipette. The droplets were centrifuged (2500 rpm for 10 min) to remove the spores. New spores of *B. cinerea* from 5-6 days old culture were suspended in supernatants from inoculation droplets giving the concentration of 2×10^5 spores/ml. The drops (50μl each) of spore suspension were incubated on glass slide at 25℃. After 24 h, spore germination was investigated. Approximately 400 spores were evaluated for each replication.

Results

Effect of red light on heat-induced susceptibility in broad bean against *B. cinerea*

1) **Avirulent strain of *B. cinerea***

When an avirulent strain BC1 of *B. cinerea* was inoculated to the unheated broad bean, small necrotic lesions appeared slightly in some inoculated sites of broad bean leaflets in the dark (Fig 1, A). In the heated leaflets, however, small necrotic lesions appeared at some inoculated sites 12 h after inoculation in the dark and then after 24 and 48 h, large necrotic lesions appeared at all inoculated sites irrespective of heat doses (Table 1) and developed around the healthy tissues (Fig. 1, B). Under red light, however, lesion formation was suppressed even in heated broad bean, as compared to that in the dark (Fig. 1, B).

Table 1 Effect of heat shock before inoculation on lesion formation in broad bean inoculated with an avirulent strain of *B. cinerea* in the dark

Strain	Heat dose	Lesion formation		
		12h	24h	48h
BC1	50 ℃— 5 sec	±[a]	+[b]	+
	50 ℃—10 sec	±	+	+
	55 ℃— 5 sec	±	+	+
	55 ℃—10 sec	±	+	+
	Control	—[c]	—	—

a) Small necrotic lesions were formed in some inoculated sites.
b) Large necrotic lesions were formed in all inoculated sites.
c) No lesion formation was observed.

2) **Virulent strain of *B. cinerea***

When heated and unheated broad bean leaflets were inoculated with a virulent strain of *B. cinerea* and kept in the dark, respectively, large necrotic lesions were formed in the inoculated sites of both heated and unheated leaflets (Fig. 1, C and D). There was no difference in lesion formation between the heated and unheated broad bean leaflets. Under red light, however, lesion formation was significantly inhibited even in heated broad bean, as compared to that in the dark.

Inhibition of lesion development in heated broad bean un-

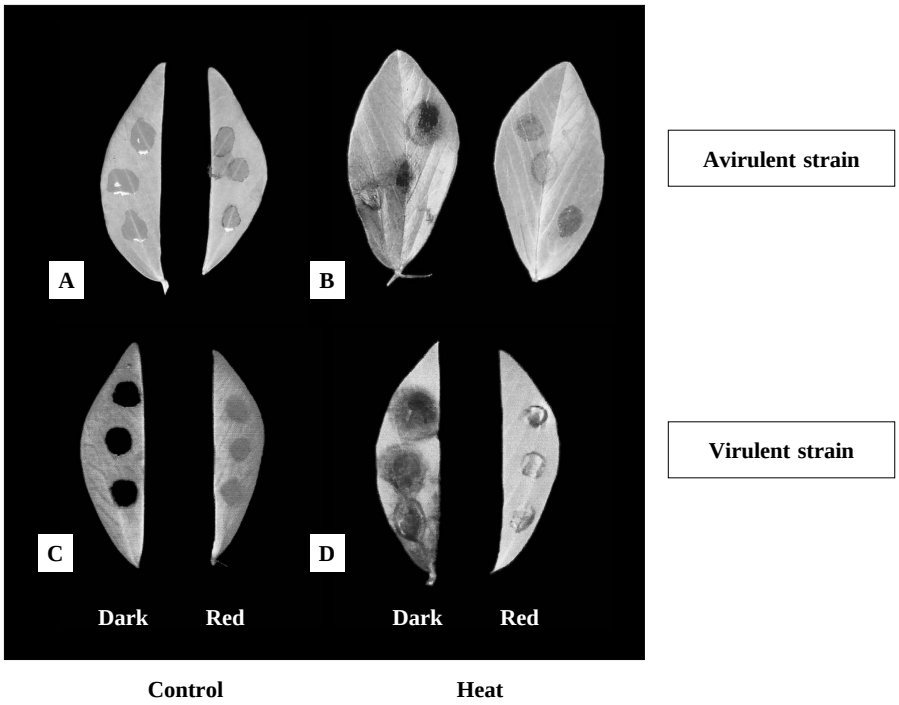

Fig. 1. Effect of red light on lesion formation by *B. cinerea* in heated and unheated broad bean leaflets. Broad bean leaflets were immersed in hot water at 50 ℃ for 5 sec. Pre-heated (Heat shock) and unheated (Control) leaflets were inoculated with avirulent (A and B) or virulent (C and D) strain of *B. cinerea* and kept in the dark (A and C) or under red light (B and D) for 48 h.

der red light was also recognized at the hyphal growth level by the microscopical observation. There was no difference in appressorium formation of *B. cinerea* between light treatments or heat treatments. Under red light, however, the infection hypha formation and also hyphal growth of *B. cinerea* were significantly inhibited even in heated broad bean tissues. More than 98% of appressoria produced infection hyphae in heated broad bean leaflets in the dark, whereas 75% of appressoria produced under red light. Number of infected cells per infection hypha in heated broad bean was 2 to 7 in the dark, whereas that under red light was 1 to 3 (Fig. 2).

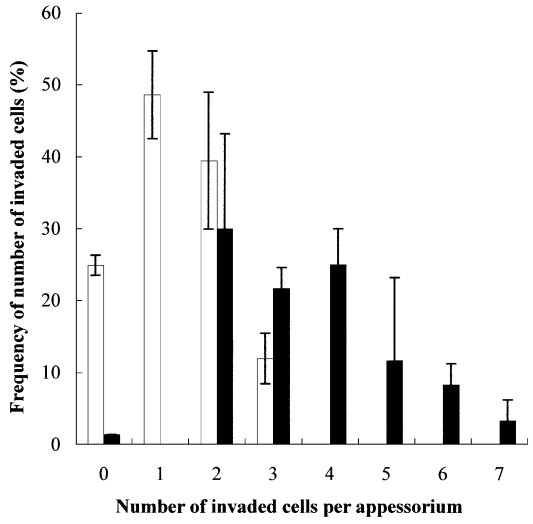

Fig. 2. Effect of red light on development of infection hyphae of *B. cinerea* in heated broad bean leaflets kept under red light (□) or in the dark (■) for 12 h. Data are the means of three experiments with three replications. Bars represent ± SD.

Effect of red light on production of antifungal substances in heated broad bean

Antifungal activity in infection droplets on heated and unheated broad bean leaflets was investigated (Fig. 3). In the dark, spore germination in inoculation droplets was more than 92% in both heated and unheated broad bean leaflets. Under red light, however, antifungal activity was observed in droplets recovered from heated broad bean leaflets (41.1%), although its activity decreased, as compared to that in droplets from unheated broad bean leaflet (10.2%).

Discussion

Heat-induced susceptibility of beans to some viruses and fungi was demonstrated by Yarwood (1956). Temporary suppres-

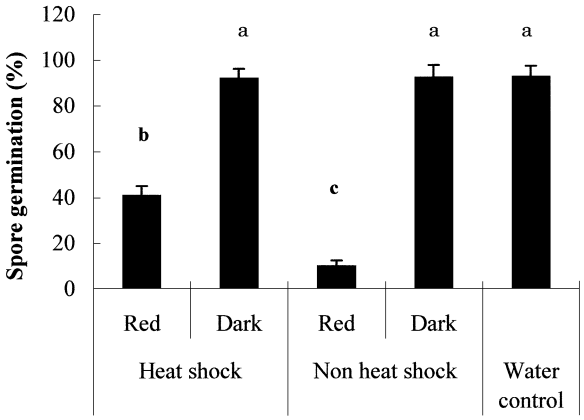

Fig. 3. Antifungal activity of inoculation droplets collected from heated and unheated broad bean leaflets inoculated with *Botrytis cinerea*. *B. cinerae*-inoculated broad bean leaflets were kept under red light or in the dark. After 48 h, inoculation droplets were used for antifungal activity test. Antifungal activity was checked by spore germination of *B. cinerea* on a glass slide 24 h after incubation. Data are the means of three experiments with three replications. Bars represent ± SD. Mean followed by the same letters are not significantly different at 5% level according to New Duncan's multiple range test.

sion of resistance by heat shock has been also demonstrated in cabbage and French bean infected with the non-pathogen cowpea rust fungi (Heath, 1979) and in cucumber inoculated with *Cladosporium cucumerinum* or *Helmonthosporium carbonum* (Stermer and Hammerschmidt, 1982). Avirulent strains used in this study formed lesion in heated broad bean leaflets within 24 h irrespective of heat doses. Moreover, in some cases, lesion was also formed 12 h after inoculation. No lesion or any visible symptom was observed in heat shocked broad bean leaflets treated with distilled water even 48 h after inoculation. This result showed that necrotic spots on heated broad bean leaves were lesions induced by an avirulent strain of *B. cinerea*, not physiological products brought by heat shock. Arase and Oka (1985) also observed that penetration and hyphal growth of avirulent isolates of *Pyricularia oryzae* increased remarkably into the sheath cells which had been preliminarily treated with hot water and cycloheximide. In this study, however, the percentages of infection hypha formation and hyphal growth of *B. cinerea* were much lower in heated leaflets under red light than that in the dark.

In previous reports (Rauscher *et al*., 1999; Deverall, 1967; Lecher *et al*., 1970; Islam *et al*., 2002 a), it was demonstrated that antifangal substances, such as phytoalexins (wyeronic acid and its derivaties) or proteins were produced in broad bean by the fungal infection. In order to elucidate the mechanism of resistance recovery in the heated broad bean leaflets under red light, antifungal activity in inoculation droplets recovered from heat shocked broad bean leaflets was checked by spore germination test. High antifungal activity was observed in inoculation droplet recovered from heated broad bean leaflets kept under red light. In the dark, however, antifungal activity was not observed in inoculation droplets. These studies suggested that the suppression of heat-induced susceptibility under red light was due to the accumulation of antifungal substance(s) in inoculation droplet.

In conclusion, present studies demonstrated that red light induced resistance was heat stable and red light-dependent production of antifungal substance(s) were involved in resistance of heated broad bean leaflets aganinst *B. cinerea*.

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