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Features of the rapid cell death induced in cowpea by the monokaryon of the cowpea rust fungus or the monokaryon-derived cultivar-specific elicitor of necrosis

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Rapid cell death induced in the resistant cowpea cultivar, Dixie Cream, by the monokaryotic stage of race 1 of the cowpea rust fungus was delayed by treatments with superoxide dismutase, mannitol, catalase and the lipoxygenase inhibitor salicyl hydroxamic acid. These treatments also delayed cell death caused by a cultivar-specific elicitor of necrosis present in intercellular washing fluids from monokaryon-infected susceptible plants. The data suggest that active oxygen species, hydrogen peroxide and lipid peroxidation may be involved in both elicitor- and fungal-induced cell death, and in both cases, treatments were effective when applied close to the onset of the first cytological signs of necrosis. Pre-inoculation treatment of cv. Dixie Cream with heat, or pre-necrosis treatments with the protein synthesis inhibitor blasticidin S or abscisic acid, also delayed fungal-induced cell death, but heat was the only treatment of all those tested that resulted in a slight increase in fungal growth. Cell death was also delayed by damaging or killing the fungus at various stages before the normal onset of necrosis suggesting the necessity for sustained fungal metabolism. All of these data, and the time-course of elicitor-induced necrosis, are consistent with the hypothesis that the elicitor is the primary cause of cell death in this incompatible plant–fungal interaction. However, the data also suggest that cell death may not be the primary cause of the cessation of fungal growth.

INTRODUCTION

Cultivar-specific resistance towards incompatible races of rust fungi is commonly expressed as rapid cell death (RCD), also known as the hypersensitive reaction (HR), at the site of infection and penetration. Since rust fungi, as obligate parasites, require living host cells for their growth and development, RCD *per se* may be sufficient to prevent them from further colonization.

Although RCD has been intensively studied since the HR was first described by Stakeman [*23*], the mechanisms leading to its occurrence in any host–pathogen system are not well understood. Many events have been associated with RCD, including the alteration of cell walls [*1*], depolarization of the cell membrane [*26*], accumulation of antimicrobial compounds such as phytoalexins [*19*, *22*], production and accumulation of free radicals such as active oxygen species [*7*, *9*, *20*, *24*, *27*, *28*], as well as changes in

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Abbreviations used in text: ABA, abscisic acid; BS, blasticidin S; CPR-1, cowpea rust fungus race 1; HR, hypersensitive reaction; IWFs, intercellular washing fluids; MES, 2-[N-Morpholino] ethanesulphonic acid; RCD, rapid cell death; SHAM, salicyl hydroxamic acid; SOD, superoxide dismutase.

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levels of abscisic acid (ABA) [2, 3] and the necessity for high levels of cytokinins [5]. However, whether these changes accompany RCD or are integral components of RCD is sometimes difficult to determine.

In terms of the genetic and molecular basis of RCD, the most extensively accepted hypothesis is that a specific elicitor (presumably the product of a gene for avirulence in the pathogen) is recognized by, and interacts with, a specific receptor (presumably the product of a gene for resistance in the host) on the cell membrane. This interaction causes the release of a secondary signal, which is then translocated to the host nucleus, and causes the activation of enzymes leading to the expression of resistance responses and RCD at the infection site [18]. If the above described model is correct, fungi that trigger RCD should release a specific elicitor of necrosis. To date, however, such elicitors have been isolated from only three systems: tomato–*Cladosporium fulvum* [8], bean–*Colletotrichum lindemuthianum* [25] and cowpea–*Uromyces vignae* [4].

In the latter system, a cultivar-specific elicitor of necrosis with the same cultivar-specificity as the pathogen has been isolated from both differentiated basidiospore germlings [4] and basidiospore-infected susceptible cowpea leaves [6]. Injection of this elicitor into cowpea leaves induces cell necrosis (RCD) in some resistant cowpea cultivars but not in susceptible cultivars [6]. However, the mechanisms that lead to the occurrence of RCD are not known.

To increase our knowledge of RCD in the cowpea–*Uromyces vignae* interaction, the present studies were initiated to investigate the factors that influence, or may be involved in, this necrosis. Where possible, pathogen-inoculated and elicitor-treated plants were compared to investigate further the role of the elicitor in the plant–fungus interaction.

MATERIALS AND METHODS

Plants and pathogen

Race 1 of the cowpea rust fungus, *Uromyces vignae* Barclay, (CPR-1) was used throughout these experiments. Teliospores of the pathogen were produced on the primary leaves of a susceptible cowpea cultivar and maintained on dried leaves at room temperature as described elsewhere [17].

Cowpea cultivars used in these experiments were cv. Dixie Cream (resistant, single-cell-death response phenotype) and cv. California Blackeye (susceptible). Seeds of plants were sown in sterile Pro-mix BX (Premier Brand Inc. N.Y.) and grown in a growth room at 21 °C with 16 h day^{-1} illumination providing 250 μE m^{-2} s^{-1}. Plants were inoculated with the pathogen, or treated with elicitor at 12 days after sowing.

Preparation of inocula and inoculation with pre-germinated teliospores

Leaf tissues bearing teliospores of CPR-1 were sterilized by soaking in 3% sodium hypochlorite solution for 35–45 s followed by several rinses in sterile double-distilled water. Teliospores were then scraped from leaves with a sterile knife and placed on 2% water agar plates, which were subsequently sealed with parafilm and incubated at 21 °C to allow for germination. After 50 h incubation, 1 cm^2 agar blocks bearing germinating teliospores were cut out and placed onto the upper surfaces of primary leaves of 12-day-old plants with the spore side facing the leaf. Inoculated plants were

kept in the dark in a humidity chamber for the first 24 h. In time-course studies, agar blocks bearing germinating teliospores were removed from leaves at 9 h after inoculation. In other types of experiments, the agar blocks were removed at 24 h after inoculation. If plant tissues were to be harvested at 2 or 4 days after inoculation, then the inoculated plants were returned to the growth room after the first 24 h in the humidity chamber.

Preparation of elicitor

Leaves of cv. California Blackeye were inoculated with germinating teliospores and the intercellular washing fluids (IWFs) in which the elicitor of necrosis is present were extracted at 5–6 days after inoculation [*6*]. The isolated IWFs were then lyophilized and reconstituted with sterile double-distilled water to five times the original concentration just before use. IWFs from uninoculated, healthy leaves were treated in the same way as above and used as controls in those experiments involving elicitors.

Preparation of solutions

Metabolic inhibitors, enzymes, and free-radical scavengers used in these experiments and their functions are shown in Table 1; blasticidin S was prepared in sterile double-distilled water and all other inhibitors in 10 mM 2-[N-Morpholino] ethanesulphonic acid (MES) (Sigma) buffer at pH 6·5. Kinetin and abscisic acid (Sigma) were dissolved in a small amount of methanol and then diluted with sterile double-distilled water to 1 μg ml^{-1} to make stock solutions. Just before use, the stock solutions were further diluted to various concentrations employed in these experiments. The final concentration of methanol in the working solution was 0·1 %.

Disruption of elicitor production by killing the fungus

To investigate the role of the living fungus in eliciting RCD, invading basidiospores were removed from leaf surfaces by applying transparent adhesive tape to the inoculated areas several times followed by slightly rubbing these areas with a cotton Q-tip dipped in 95% ethanol. Alternatively, the fungus was killed by dipping inoculated leaves of intact plants in double-distilled water at 50 °C for 40 s [*15*]. The leaves were blotted dry immediately after the treatment.

Bioassay techniques

Solutions of phytohormones, metabolic inhibitors and free-radical scavengers were injected with a syringe into the intercellular spaces of cowpea leaf areas where germinating teliospores had been inoculated, or where IWFs had been infiltrated. These solutions were applied at various times either before, at the same time as, or after, inoculation with the pathogen or injection with the elicitor preparation. Treated leaf tissues were harvested at 24 h, 48 h or 4 days after inoculation as indicated in the text. For each set of treatments, at least three plants were used and one leaf piece was harvested from each plant.

Light microscopy

For observation under the light microscope, treated leaf areas were cut into 1 cm^2 pieces, decolorized in boiling 95% ethanol, and then cleared in saturated chloral hydrate solution for at least 7 days. Leaf pieces were mounted in modified Hoyer's

TABLE 1
Functions and concentrations of inhibitors used in experiments

Inhibitors	Functions and concentrations
Scavengers of active oxygen species	
Superoxide dismutase	A $O_2^{-\cdot}$ radical scavenger (900 units ml^{-1} in MES*) [27]
Mannitol	A $OH^{\cdot}$ radical scavenger (10 mM in MES) [27]
Catalase	A H_2O_2 scavenger (1100 units ml^{-1} in MES) [27]
Metabolic inhibitors	
Salicyl hydroxamic acid	An inhibitor of lipoxygenases, peroxidases, and other oxidases (10 mM in MES) [27]
Blasticidin S	An inhibitor of protein synthesis (1 μg ml^{-1} in water) [14]
Phytohormones	
Abscisic acid	Plant growth regulator (1 μg ml^{-1} in methanol†)
Kinetin	Plant growth regulator (1 μg ml^{-1} in methanol)

*MES buffer solution (pH 6·5, 10 mM).

†Phytohormones were first dissolved in a small amount of methanol, and its concentration in the final solution was adjusted with double distilled water to 0·1% before use.

medium as described elsewhere [17]. For the observation of cell death induced by the elicitor of necrosis, cell autofluorescence under epifluorescence and blue light irradiation was used as the sign of cell death [4], and the number of autofluorescent cells in a 1 cm^2 leaf area was counted. For each set of treatments, at least three 1 cm^2 leaf pieces each from a different plant were observed. For the observation of cell responses of cowpea to infection by the pathogen, leaf tissues were examined with differential interference microscopy and autofluorescence of infected epidermal cells was observed in the same way as described above.

RESULTS

The cowpea rust fungus is an autoecious fungus that can form both its dikaryotic and monokaryotic stages in susceptible cowpea cultivars. The dikaryotic urediospores produce germlings and infection structures that penetrate the leaf indirectly through stomata, while the monokaryotic basidiospores penetrate epidermal cells directly [17]. In cowpea cv. Dixie Cream, resistance to both stages of the fungus is expressed as RCD at the infection sites. Resistance to the dikaryon is activated only after the formation of the first haustorium in a mesophyll cell, whereas resistance to the monokaryon is exhibited in response to the primary hypha in the first infected epidermal cell [17]. All of the following experiments involving the fungus monitored RCD in epidermal cells invaded by monokaryotic primary hyphae formed from basidiospores.

Effects of removal of fungal basidiospores or post-inoculation heat treatment on RCD

The invading basidiospores of the fungus were removed using adhesive tapes followed by rubbing with a cotton Q-tip containing 95% ethanol at various times after inoculation to see whether removing surface spores, and presumably killing the

intracellular fungus, had any effect on the occurrence of RCD induced by the fungus. As seen in Fig. 1, these treatments had no effect on the susceptible cv. California Blackeye and no autofluorescence was induced in the tissue. On the other hand, the removal of invading basidiospores at 9, 12 or 14 h after inoculation significantly reduced RCD (viewed as autofluorescent cells under blue light) in resistant cv. Dixie Cream when examined at 24 h after inoculation.

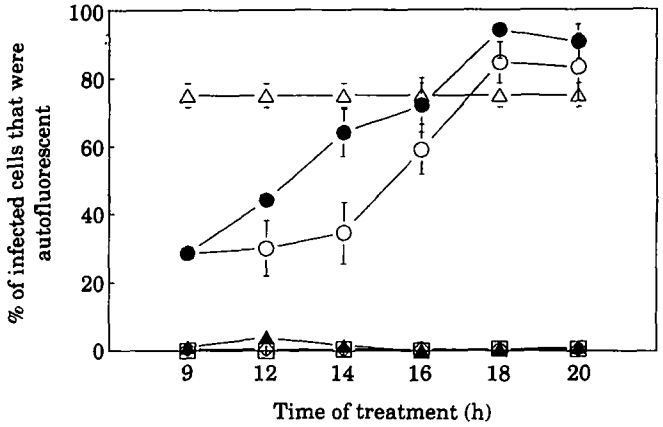

FIG. 1. Effects of removal or killing of the fungus on rapid cell death induced by cowpea root fungus race 1 in cv. Dixie Cream (DC, resistant) or cv. California Blackeye (CB, susceptible). Treatments were carried out at 9, 12, 14, 16, 18 or 20 h after inoculation. The invading pathogen was removed from leaf surfaces using an adhesive tape, or killed by dipping inoculated leaves in 50 °C distilled water for 30 s. All data were collected at 24 h after inoculation. CB-control (◇——◇) and DC-control (△——△) indicate that cv. California Blackeye and cv. Dixie Cream were inoculated with the pathogen as usual but no further treatments were made. (○——○), DC-removal; (●——●), DC-killing; (▲——▲), CB-removal; (□——□), CB-killing.

The effect of killing the basidiospores and invading fungal structures by heating the inoculated cowpea leaves at various times after inoculation was also investigated. This treatment had been shown to be effective in killing rust fungi without visible influence on the plant [*14*]. As shown in Fig. 1, cell death was reduced in cv. Dixie Cream as long as the pathogen was killed before 14 h after inoculation.

When treated tissues were observed at 48 h after inoculation, all infected cells in cv. Dixie Cream had died in situations in which basidiospores and infection structures were either removed or heat-killed at 12 h after inoculation (Fig. 2). Similar treatments at 9 h after inoculation caused slightly lower, but statistically non-significant, percentages of dead infected cells (72 and 80%, respectively) as compared to that of the control (Fig. 2).

Time course of RCD elicited by intercellular washing fluids (IWFs)

IWFs were injected into the intercellular spaces of cowpea leaves and examined at various times after application to see when cell death occurred. As shown in Fig. 3, the number of autofluorescent cells per 1 cm^2 of leaf tissue began to increase at 4 h after injection, and reached a significantly high level at 8 h in cv. Dixie Cream but not cv. California Blackeye.

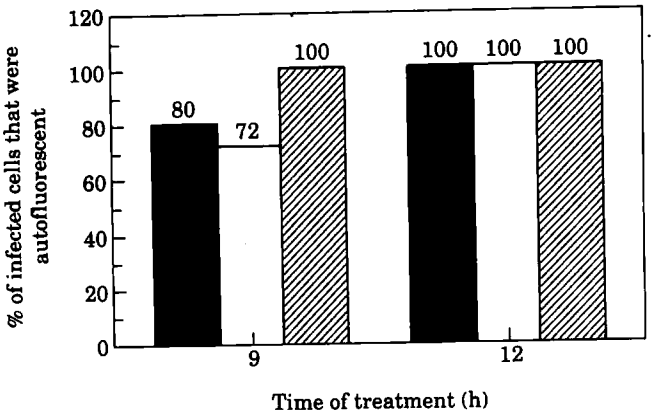

FIG. 2. Effects of removal or killing of the fungus on rapid cell death induced in cv. Dixie Cream by CPR-1 at 48 h after inoculation. (■), Removal; (□), killing; (▨), control.

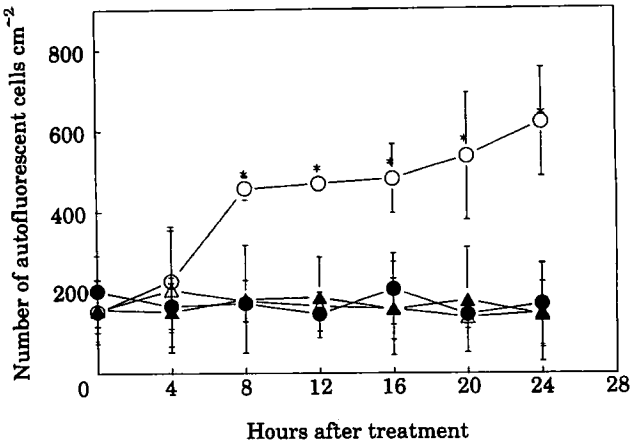

FIG. 3. Time course of rapid cell death elicited by intercellular washing fluids from monokaryon (cowpea rust fungus race 1) infected susceptible cowpea plants. Numbers of autofluorescent cells were recorded from a 1 cm^2 leaf piece. IWFi+DC (○——○) and IWFi+CB (●——●) represent the elicitor-containing IWFs-treated cv. Dixie Cream and cv. California Blackeye, respectively. IWFc+DC (△——△) and IWFc+CB (▲——▲) indicate that cv. Dixie Cream and cv. California Blackeye, respectively, were treated with IWFs from uninoculated cowpea plants. * Significantly different from control.

Effects of scavengers of active oxygen species on RCD induced by the pathogen

To investigate whether active oxygen species are involved in the process of RCD, some specific scavengers of free radicals ($O_2^{-\cdot}$, $OH^{\cdot}$), and H_2O_2 were applied to cowpea leaves at the same time as, or at various times after, inoculation with the pathogen. Results in Fig. 4 show that when applied at the same time as the inoculation of the pathogen, mannitol and superoxide dismutase (SOD) partially inhibited RCD (as observed 24 h after inoculation), but catalase did not have any obvious effect. When applied at 9 h after inoculation, all the above three treatments significantly decreased cell death. SOD had a lesser effect when applied later after inoculation, while the other treatments had maximal effect when applied at 12 h. When applied at 16 h after

inoculation, all tested scavengers of active oxygen species exhibited no effects on RCD. All treatments delayed rather than completely inhibited RCD, since the incidence of the latter was similar to control leaves by 48 h after inoculation. No effect on fungal growth of any treatment was observed.

Effects of scavengers of active oxygen species on RCD induced by the necrosis elicitor

To investigate whether the application of scavengers of active oxygen species affect the RCD induced by elicitor treatment, solutions were injected into cowpea leaf tissues at 8 h after injection with IWFs from monokaryon-infected plants. As seen in Table 2, the $OH^{\cdot}$ scavenger mannitol, and the H_2O_2 scavenger catalase, significantly inhibited cell death elicited by the IWFs in cv. Dixie Cream when observed at 24 h after IWFs injection. Similar to its effect on RCD induced by CPR-1, $O_2^{-\cdot}$ scavenger SOD was less active than the other scavengers in delaying cell death. However, the number of fluorescent cells was still significantly lower than observed with IWF alone. IWF elicited little necrosis in the compatible cv. California Blackeye, and the used concentrations of scavengers had no visible effect on the viability of cells in either cultivar.

Effects of inhibitor of lipoxygenases and peroxidases on RCD induced by the pathogen

Salicyl hydroxamic acid (SHAM), an inhibitor of lipoxygenases, peroxidases and other oxidases, when applied to cv. Dixie Cream at the same time as, or between 9 and 14 h after inoculation, significantly inhibited RCD; maximum effect was exhibited when applied at 12 h after inoculation (Fig. 4). However, RCD was not inhibited when SHAM was applied at 16 h after inoculation (Fig. 4). When observed at 48 h after inoculation, the incidence of RCD in SHAM-treated leaf tissues was similar to the control leaves, and no increase in fungal growth was observed.

Effects of inhibitor of lipoxygenases and peroxidases on RCD induced by the elicitor of necrosis

As seen in Table 2, application of SHAM at 8 h after the treatment of cv. Dixie Cream with IWFs dramatically decreased the frequency of dead cells observed at 24 h after elicitor injection.

Effects of kinetin and abscisic acid (ABA) on RCD induced by the pathogen

Kinetin solutions at concentrations of 0·1 or 1 $\mu g\ ml^{-1}$ were injected into the leaves of cowpea cv. Dixie Cream at various times before, or after, inoculation with the pathogen. When observed at 24 h after inoculation, the percentage of infected cells that were autofluorescent under blue light were similar to those of the untreated controls (i.e. leaf tissues that were inoculated with CPR-1, but not treated with phytohormones nor with the control solution) in leaves treated at the same time as, or at 5 or 10 h after, inoculation (Fig. 5). When the kinetin solutions were applied at 2 h before inoculation, RCD was reduced, but the same effect was observed with the solvent control solution (i.e. the solution used to prepare phytohormones).

In contrast to the effect of kinetin, ABA significantly reduced the RCD detectable at 24 h after inoculation regardless of when it was applied (Fig. 5). However, when tissues were harvested and examined 4 days after inoculation, the percentage of infected cells that were autofluorescent was not significantly different from that of the control, and the mean length of primary hyphae had not increased (Table 3).

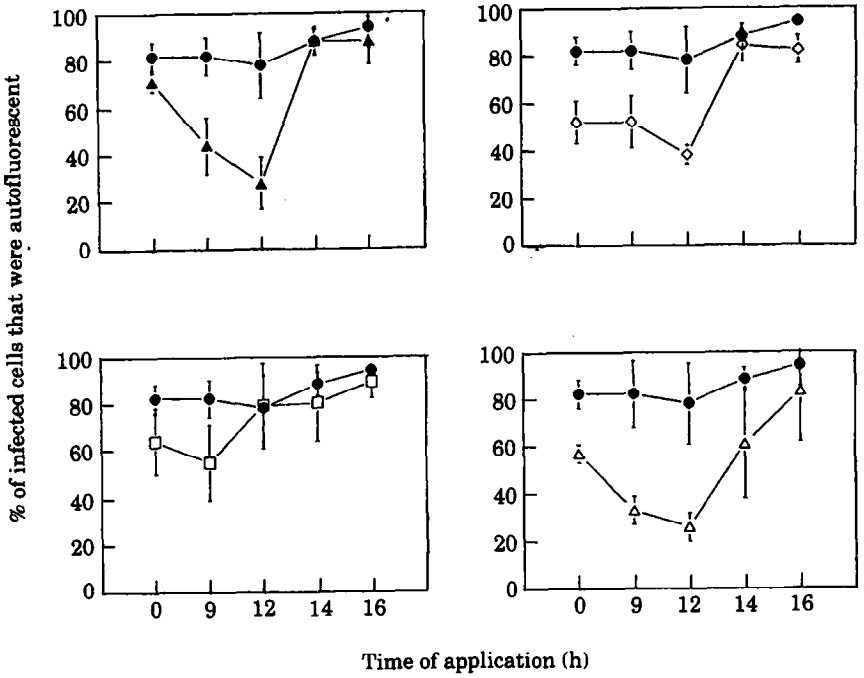

FIG. 4. Effects of injection of scavengers of active oxygen species and inhibitor of lipoxygenases at various times after inoculation with basidiospores of the cowpea rust fungus on rapid cell death in cv. Dixie Cream. All data were collected at 24 h after inoculation with the pathogen. Scavengers and inhibitors were prepared in a MES buffer solution (pH 6·5, 10 mM). (●——●) Control was injected with buffer solution only. (▲——▲), Catalase; (◇——◇), mannitol; (□——□), superoxide dismutase; (△——△), salicyl hydroxamic acid.

TABLE 2

*Effect of some scavengers of active oxygen species and an inhibitor of lipoxygenase and lipid peroxidation on rapid cell death in cowpea induced by intercellular washing fluids (IWFs) from cowpea rust fungus race 1-infected plants**

Treatments	No. of fluorescent cells cm^{-2} leaf tissue (mean ± SD)	
	cv. Dixie Cream	cv. California Blackeye
IWFs + mannitol	88 ± 3·06a †	NT
IWFs + SHAM	159 ± 73·1a	NT
IWFs + catalase	274 ± 47·3a	NT
IWFs + SOD	394 ± 288b	NT
IWFs + MES‡	542 ± 20·7c	121 ± 17·6a
IWFs	515 ± 94·3c	148 ± 25·2a
Control§	197 ± 138a	84 ± 16·5a

*Cowpea leaves were treated with inhibitors at 8 h after their treatment with the elicitor and observed at 24 h after elicitor treatment. NT, Not tested; SHAM, salicyl hydroxamic acid; SOD, superoxide dismutase.

†ANOVA test, values with the same letter are not significantly different at $P = 0·05$.

‡MES was the buffer used to prepare the inhibitor solutions.

§Plants treated with IWFs from the uninoculated cowpea leaves were employed as control.

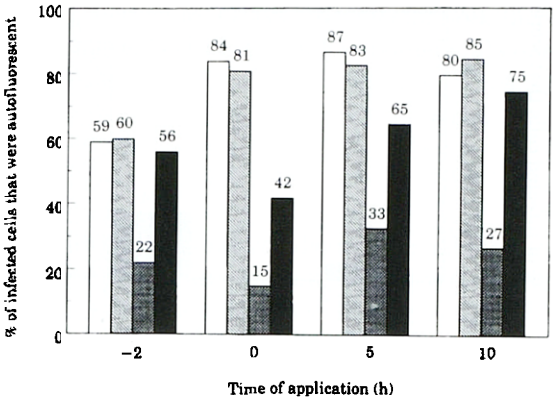

FIG. 5. Effects of kinetin and abscisic acid (ABA) on rapid cell death induced by cowpea rust fungus race 1 in cv. Dixie Cream. All data were collected at 24 h after inoculation. (□), Kinetin at concentration of 0·1 μg ml^{-1}; (▨), kinetin at concentration of 1 μg ml^{-1}; (▩), ABA at concentration of 1 μg ml^{-1}; (■), control, indicates that the inoculated tissues were treated with the solution used to dissolve kinetin and ABA. In this experiment, inoculated leaf tissue without any other treatment was also employed as a control, the percentage of infected cells that were autofluorescent in this control was 88 when observed at 24 h after inoculation.

TABLE 3

Duration of the effects of kinetin, abscisic acid (ABA), blasticidin S (BS) and heat treatment applied at the time of inoculation on the disease responses and on the development of basidiospore-derived primary hyphae in cowpea cvs Dixie Cream and California Blackeye

	% Autofluorescent infected cells				Length of primary hyphae (μm)			
	cv. Dixie Cream		cv. California Blackeye		cv. Dixie Cream		cv. California Blackeye	
Treatment	1 D*	4 D	1 D	4 D	1 D	4 D	1 D	4 D
Kinetin	84	100	0	4	12·9	15·6	18·5	66·1
ABA	21	100	0	4	15·2	16·4	32·5	249·6
BS	25	92	0	0	16·2	18·6	30·4	203·0
Heat	18	52	4	0	23·2	56·1	32·5	264·0
Control	84	95	0	0	14·4	14·2	27·7	209·0

*1 D, observed at 1 day after inoculation of the pathogen; 4 D, observed at 4 days after inoculation.

†Each value represents the mean of four experiments.

Effects of protein synthesis inhibitor blasticidin S and heat treatment on RCD induced by the pathogen

In cv. Dixie Cream, heating of the plant tissues at 2 h before, or at the same time as, inoculation with the pathogen, significantly decreased the RCD observed 24 h after inoculation (Fig. 6). Heating was the only treatment in these experiments that increased the growth of primary hyphae in this resistant cultivar (Table 3), and had an effect on RCD for at least 4 days after treatment (Table 3). Four days after heat treatment, the percentage of RCD was still significantly lower than the control (28 %

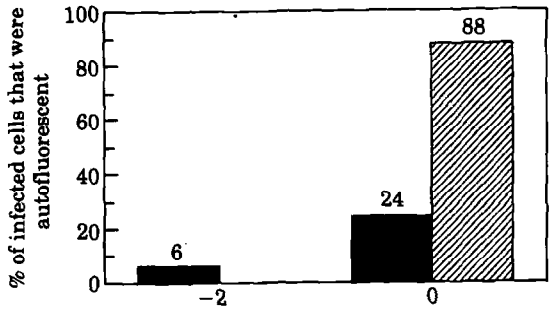

FIG. 6. Effects of heat treatment of cowpea leaves before or at the same time as inoculation on rapid cell death induced by basidiospore-derived infection by the cowpea rust fungus. Leaf tissues were harvested at 24 h after inoculation and observed as described in the Materials and Methods section. (■), Heat treated; (▨), control. −2 indicates that heat treatments were carried at 2 h before inoculation; 0 indicates that heat treatments were carried out at the time of inoculation. Value on the top of each column represents the percentage of infected cells that were autofluorescent under blue light.

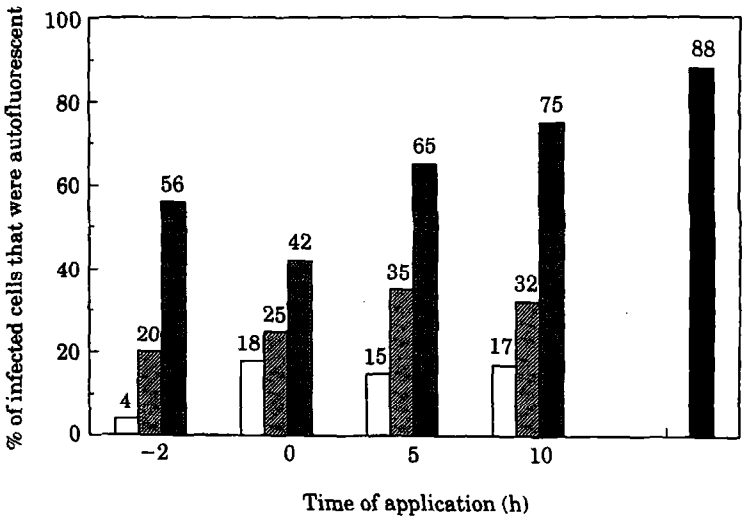

FIG. 7. Effects of blasticidin S (BS) on rapid cell death induced in cv. Dixie Cream. All data were collected at 24 h after inoculation. −2 indicates that BS treatment was carried out at 2 h before inoculation; 0 indicates that treatment with BS was carried at the time of inoculation; while +5 and +10 indicate that BS treatments were carried out at 5 or 10 h after inoculation, respectively. Value on the top of each column is the percentage of infected cells that were autofluorescent under blue light. (□), BS at concentration of 1 μg ml^{-1}; (▨), BS at concentration of 0·1 μg ml^{-1}; (⊠), water control; (■), untreated control.

v. 90%), while the length of primary hyphae was longer than the control (57·4 μm *v.* 16·3 μm) (Table 3) although the fungus usually was still restricted to the first-invaded epidermal cell.

Application of blasticidin S (BS), an inhibitor of protein synthesis, decreased the RCD observed at 24 h after inoculation at concentrations of both 1 and 0·5 μg ml^{-1} at all treatment times (Fig. 7). However, the effects of BS on RCD did not last long. As

shown in Table 3, when observed at 4 days after inoculation, there was no difference in the frequency of dead infected cells between BS-treated and control plants and little difference in fungal growth.

DISCUSSION

Previous cytological studies suggest that cell death induced by the monokaryon of the fungus usually begins at about 15 h after inoculation [*5*] whereas the fact that exudates from differentiated basidiospore have necrosis-eliciting activity [*4*] suggests that the elicitor may be produced as soon as the basidiospore forms an appressorium. In the present studies, the crude IWF elicitor did not elicit significant numbers of dead cells in cv. Dixie Cream until about 4–8 h after the treatment. Taking into account the fact that it takes at least 7–8 h for the pathogen to penetrate into epidermal cells (unpublished work) and another 4–8 h for the elicitor to induce cell death, the data are consistent with the necrosis elicitor being the factor that causes RCD *in planta*. If this interpretation is correct, then the fact that removal or killing the fungus between 9 and 14 h after inoculation delayed RCD may indicate that the plant was sensitive to the low concentration of elicitor released before fungal death but required a longer time to respond. The possibility that this cell death was caused by some toxic molecule released from the fungus as it died is less likely since fungal damage did not induce cell death in cv. California Blackeye. Overall, the data suggest that continued fungal metabolism is required for RCD.

As mentioned earlier, the mechanisms leading to the occurrence of RCD are not well understood in any pathogen–plant system. In the case of the cultivar-specific elicitor released by the tomato pathogen *C. fulvum*, it has been shown that active oxygen species are involved in the initiation of the process of RCD as early as 2 min after the application of the elicitor to cultured resistant tomato cell lines [*27*]. Therefore, we decided to investigate whether active oxygen species, particularly $O_2^{-\cdot}$, $OH^{\cdot}$, and H_2O_2 are involved in the process of RCD caused either by infection of CPR-1 or by treatment with the elicitor of necrosis of CPR-1 origin. The delay of both types of RCD upon treatment with scavengers of active oxygen species such as SOD, catalase, mannitol and with the lipoxygenase and peroxidase inhibitor SHAM, suggests that, in both cases, the process of RCD involves the formation and accumulation of $O_2^{-\cdot}$, $OH^{\cdot}$, H_2O_2, and membrane alteration such as lipid peroxidation. These data also suggest that the elicitor of necrosis may have a similar mode of action in inducing RCD to that of the pathogen itself.

The time-course studies indicate that the relative importance of the different active oxygen species may differ at different stages of pathogenesis. All scavengers or inhibitors had an effect when applied at the same time as, or at 9 h after, inoculation, suggesting that active oxygen species are formed very early during the infection process. However, when applied at 12 h, SOD expressed significantly lower activity in inhibiting RCD than the other scavengers tested, while SHAM, catalase, and mannitol exhibited their maximum effects on RCD when applied at this time. These data indicate that $O_2^{-\cdot}$ may play a less important role, while $OH^{\cdot}$, H_2O_2 and lipid peroxidation may play a more important role, at this stage of pathogenesis. Similarly,

it may be concluded that $OH^{\cdot}$ and lipid peroxidation may be more prevalent than $O_2^{-\cdot}$ and H_2O_2 at 14 h after inoculation, since at this time only mannitol and SHAM reduced the frequency of dead infected cells. However, when applied at 16 h after inoculation, RCD was not inhibited by any treatment, suggesting that at this stage of pathogenesis, the process of RCD had become irreversible.

In this study, at all concentrations tested, kinetin did not decrease the incidence of RCD in cv. Dixie Cream in response to the monokaryon of CPR-1. In contrast, treatment with ABA significantly decreased the proportion of infected cells that underwent RCD when observed at 24 h after inoculation although the majority of fungus-containing cells had died within a further 24 h and the mean length of primary hyphae was not significantly different from that of the control by 4 days after inoculation. Although it is difficult to explain this effect of ABA, it is consistent with the effect of this phytohormone on other plant–fungal interactions. Application of ABA to resistant soybean hypocotyls made them susceptible to *Phytophthora megasperma* f.sp. *glycinea* and ABA concentrations decreased rapidly in localized infection sites in resistant soybean hypocotyls at 2–4 h after inoculation, while no significant decrease was detected in compatible plants [*2*]. The authors therefore concluded that low ABA levels are required for the activity of resistance genes in soybean. However, for dikaryotic infections of *U. vignae*, ABA levels drop rapidly in cowpea infected with either a compatible race of *U. vignae* or the incompatible *U. appendiculatus* [*21*], suggesting that low ABA levels are associated with rust susceptibility *and* resistance in cowpea. It should also be noted that application of ABA does not increase susceptibility in every plant–pathogen system. For example, Dunn *et al.* [*10*] reported that exogenous ABA decreased symptom severity of susceptible hypocotyls of *Phaseolus vulgaris* infected with *Colletotrichum lindemuthianum*, while application of an ABA biosynthesis inhibitor to resistant hypocotyls increased symptom severity. Similar results were also published by Fraser and Whenham [*13*] who reported that tomato genotypes resistant to tobacco mosaic virus contained higher concentrations of ABA than did susceptible plants.

In the present studies, pre-inoculation heat treatment of leaves of cv. Dixie Cream prevented RCD in response to the infection by CPR-1, as has been reported in many other systems [e.g. *14*, *26*, *29*]. More importantly, pre-inoculation heat treatment was the only one in which the effect on RCD lasted for at least 4 days and was associated with slightly increased fungal growth. Heat treatment similarly reduced the incidence of cell death but did not dramatically increase susceptibility in cv. Dixie Cream inoculated with urediospores of CPR-1 [*11*]. The effect of heat treatment on non-host–fungus interactions often mimics that of protein synthesis inhibitors [*12*]. Similarly, treatment with BS, a translation inhibitor of protein synthesis, delayed RCD in cv. Dixie Cream inoculated with the monokaryon of CPR-1, suggesting that *de novo* synthesis of proteins is required for the expression of RCD in this system.

In summary, the effects of BS, ABA or heat treatment on the cell death induced in the resistant cv. Dixie Cream by CPR-1 suggest that *de novo* synthesis of proteins and other metabolic activities of the plant are required for this cell death to occur. The delaying effect of fungal damage on this response suggests a similar need for fungal metabolism and support previous conclusions from studies with the dikaryotic stage of rust fungi that the release of toxins from the dying fungus is not the common cause of necrosis in incompatible host cultivars [*15*, *16*]. The similar sensitivity of both fungus-

and elicitor-induced cell death to the tested treatments, and the timing of elicitor-induced necrosis, all are consistent with the conclusion that the elicitor is the cause of necrosis in the CPR-1–cv. Dixie Cream interaction. However, the fact that a delay in cell death in this cultivar did not necessarily result in increased fungal growth suggests that this response, *per se*, may not be the primary cause of rust resistance.

REFERENCES

1. **Beardmore J, Ride JP, Granger JW.** 1983. Cellular lignification as a factor in the hypersensitive resistance of wheat to stem rust. *Physiological Plant Pathology* **22**: 209–220.
2. **Cahill DM, Ward EWB.** 1989. Rapid localized changes in abscisic acid concentrations in soybean in interactions with *Phytophthora megasperma* f.sp. *glycinea* or after treatment with elicitors. *Physiological and Molecular Plant Pathology* **35**: 483–493.
3. **Cahill DM, Morris PF, Ward EWB.** 1993. Influence of metalaxyl on abscisic acid levels in soybean hypocotyls infected with *Phytophthora sojae*. *Physiological and Molecular Plant Pathology* **42**: 109–121.
4. **Chen CY, Heath MC.** 1990. Cultivar-specific induction of necrosis by exudates from basidiospore germlings of the cowpea rust fungus. *Physiological and Molecular Plant Pathology* **37**: 169–177.
5. **Chen CY, Heath MC.** 1991. Cytological studies of the hypersensitive death of cowpea epidermal cells induced by basidiospore-derived infection by the cowpea rust fungus. *Canadian Journal of Botany* **69**, 1199–1206.
6. **Chen CY, Heath MC.** 1992. Effect of stage of development of the cowpea rust fungus on the release of a cultivar-specific elicitor of necrosis. *Physiological and Molecular Plant Pathology* **40**: 23–30.
7. **Devlin WS, Gustine DL.** 1992. Involvement of oxidative burst in phytoalexin accumulation and the hypersensitive reaction. *Plant Physiology* **100**: 1189–1195.
8. **De Wit PJGM, Spikman G.** 1982. Evidence for the occurrence of race and cultivar-specific elicitors of necrosis in intercellular fluids of compatible interactions of *Cladosporium fulvum* and tomato. *Physiological Plant Pathology* **21**: 1–11.
9. **Doke N.** 1983. Involvement of superoxide anion in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiological Plant Pathology* **23**: 345–357.
10. **Dunn RM, Hedden P, Bailey JA.** 1990. A physiologically-induced resistance of *Phaseolus vulgaris* to a compatible race of *Colletotrichum lindemuthianum* is associated with increases in ABA content. *Physiological and Molecular Plant Pathology* **36**: 339–349.
11. **Elmhirst JF, Heath MC.** 1989. Interactions of the bean rust and cowpea rust fungi with species of the *Phaseolus–Vigna* plant complex. II. Histological responses to infection in heat-treated and untreated leaves. *Canadian Journal of Botany* **67**: 58–72.
12. **Fernandez M, Heath MC.** 1991. Interactions of the nonhost French bean plant (*Phaseolus vulgaris*) with parasitic and saprophytic fungi. IV. Effect of preinoculation with the bean rust fungus on growth of parasitic fungi nonpathogenic on beans. *Canadian Journal of Botany* **69**: 1642–1646.
13. **Fraser RSS, Whenham RJ.** 1989. Abscisic acid metabolism in tomato plants infected with tobacco mosaic virus: relation with growth, symptoms and Tm-1 gene for TMV resistance. *Physiological and Molecular Plant Pathology* **34**: 215–226.
14. **Heath MC.** 1979. Effects of heat shock, actinomycin D, cycloheximide and blasticidin S on nonhost interactions with rust fungi. *Physiological Plant Pathology* **15**: 211–218.
15. **Heath MC.** 1984. Relationship between heat-induced fungal death and plant necrosis in compatible and incompatible interactions involving the bean and cowpea rust fungi. *Phytopathology* **74**: 1370–1376.
16. **Heath MC.** 1988. Effect of fungal death or inhibition induced by oxycarboxin or polyoxin D on the interaction between resistant or susceptible bean cultivars and the bean rust fungus. *Phytopathology* **78**: 1452–1462.
17. **Heath MC.** 1989. A comparison of fungal growth and plant responses in cowpea and bean cultivars inoculated with urediospores or basidiospores of the cowpea rust fungus. *Physiological and Molecular Plant Pathology* **34**: 415–426.
18. **Keen NT.** 1982. Specific recognition in gene-for-gene host–parasite systems. In: Ingram DS, Williams PH, eds. *Advances in Plant Pathology, Vol. I.* New York: Academic Press, pp. 35–82.
19. **Mansfield JW, Porter ACA, Smallman V.** 1980. Dihydrowycrone derivatives as components of the furanoacetylenic phytoalexin response of tissue of *Vicia faba*. *Phytochemistry* **19**: 1057–1061.
20. **Moreau RA, Osman SF.** 1989. The properties of agents released by treatment of *Solanum tuberosum* with elicitors from *Phytophthora infestans*. *Physiological and Molecular Plant Pathology* **35**: 1–10.

21. **Ryerson DE, Li A, Young JP, Heath MC.** 1993. Changes in abscisic acid levels in bean leaves during the initial stages of host and nonhost reactions to rust fungi. *Physiological and Molecular Plant Pathology* **43**: 265–273.
22. **Snyder BA, Nicholson RL.** 1990. Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. *Science* **248**: 1637–1639.
23. **Stakeman EC.** 1915. Relation between *Puccinia graminis* and plants highly resistant to its attack. *Journal of Agricultural Research* **4**: 193–200.
24. **Sutherland MW.** 1991. The generation of oxygen radicals during host plant responses to infection. *Physiological and Molecular Plant Pathology* **39**: 79–93.
25. **Tepper CS, Anderson AJ.** 1986. Two cultivars of bean display a differential response to extracellular components from *Colletotrichum lindemuthianum*. *Physiological and Molecular Plant Pathology* **29**: 411–420.
26. **Tomiyama K, Okamoto H.** 1989. Effect of pre-inoculational heat-treatment on the membrane potential change of potato cell induced by infection with *Phytophthora infestans*. *Physiological and Molecular Plant Pathology* **35**: 191–201.
27. **Vera-Estrella R, Blumwald E, Higgins VJ.** 1992. Effect of specific elicitor of *Cladosporium fulvum* on tomato suspension cells: evidence for the involvement of active oxygen species. *Plant Physiology* **99**: 1208–1215.
28. **Vera-Estrella R, Blumwald E, Higgins VJ.** 1993. Non-specific glycopeptide elicitor of *Cladosporium fulvum*: evidence for involvement of active oxygen species in elicitor-induced effects on tomato cell suspensions. *Physiological and Molecular Plant Pathology* **42**: 9–22.
29. **Woods AM, Fagg J, Mansfield JW.** 1989. Effects of heat-shock and inhibitors of protein synthesis on irreversible membrane damage occurring during the hypersensitive reaction of *Lactuca sativa* L. to *Bremia lactucae* Regel. *Physiological and Molecular Plant Pathology* **34**: 531–544.