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Self-inhibition of spore germination via reactive oxygen in the fungus *Cladosporium cucumerinum*, causal agent of cucurbit scab

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Abstract *Cladosporium cucumerinum* spore germination in vitro depended on spore suspension density. Different fungal isolates displayed germination maxima at different spore concentrations. For one isolate, the maximum was observed at the same spore density at both 18 and 25°C, although germination percentage increased slightly at the higher temperature. Diffusates originating in other spore suspensions of the same isolate reduced germination percentage of spores taken at optimal concentration. The least effect occurred in diffusate taken from spores kept at their optimal concentration. Self-suppression of spore germination at unfavourable concentrations was diminished more or less by antioxidants (superoxide dismutase, catalase, mannitol or formate). The same compounds, added to spore diffusates, reduced their fungitoxicities. All diffusates generated superoxide radical (assayed by adrenalin oxidation sensitive to superoxide dismutase). This activity correlated positively with diffusate toxicity. Leaf inoculation of the susceptible cucumber cultivar at 18°C with spore suspensions at extreme densities, at which they germinated poorly in vitro, led to less disease severity then that at optimal density. In contrast, no disease symptoms appeared at 25°C. It is suggested that spores germinating at their extreme concentrations produced reactive oxygen species, suppressing the pathogen; this effect could reduce disease development at low temperatures. At high temperatures, however, this mechanism seems not to work, suggesting that plant infection may be reduced by other disease inhibiting factors.

Keywords Cucumber · Fungitoxicity · Spore germination · Superoxide radical

Abbreviations

CAT	catalase
CAT inactiv.	catalase heat-inactivated
O_2^-	superoxide anion radical
ROS	reactive oxygen species
SOD	superoxide dismutase

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Introduction

Fungal spores germinate poorly in too dense and sometimes in too dilute spore suspensions. In general, various cells in dense populations may be self-inhibited

by their waste products. Some exo-metabolites of spores (for instance, *cis*-3,4-dimethoxycinnamate of rust fungi) are recognized as specialized inhibitors of their germination. In too dilute suspensions, spores may be inhibited due to deficiency in their diffusing self-stimulators; nonanal of rusts is an example (Gäumann 1951; Macko 1981; Tsurushima et al. 1995).

Apparently, the inhibition of early developmental stages of pathogenic fungi may hinder them from causing disease. For rice blast fungus *Magnaporthe grisea*, we reported the suppression of spore germination and appressorium formation on a neutral surface both in dilute and dense spore suspensions. If the suspension was used for leaf inoculation of the susceptible rice cultivar, its low concentration resulted in fewer compatible-type lesions than did the concentration optimal for spore germination in vitro. More incompatible-type lesions appeared at high concentration than at optimal concentration (Lapikova and Dzhavakhiya 1987). Consequently, self-inhibition of germination of pathogen spores at their extreme concentrations was associated with the reduction in the disease severity.

Self-suppression of the blast fungus is presumably caused to large extent by substances that it secretes into the external environment. In fact, spore diffusates taken from both dense and dilute suspensions suppressed germination of spores present at optimal concentration (Aver'yanov and Lapikova 1990). Fungal exo-metabolites potentially responsible for self-suppression are diverse (Macko 1981). Our data suppose reactive oxygen species (ROS) as additional candidates. Thus, exogenous antioxidants eliminating hydrogen peroxide, superoxide (O_2^-), or hydroxyl radicals restored *M. grisea* spore germination in suspensions at extreme densities or in diffusates prepared from these suspensions (Aver'yanov and Lapikova 1990). Generation of superoxide radical and hydrogen peroxide in suspensions and diffusates of germinating blast spores was confirmed by chemical assays (Aver'yanov et al. 2007b).

The aim of the present work was to elucidate whether other phytopathogenic fungi follow the same pattern as described above. To this end, the fungus *Cladosporium cucumerinum*, causing scab of cucumber, was studied. No evidence of its self-suppression as a mechanism involved in spore density-dependant regulation of spore germination has been found in the literature. In the present study, this question is addressed; furthermore, the relation between the level of aggressiveness and the suggested self-suppressing effect of ROS production by spores is investigated.

Materials and methods

Plant material

Cucumber plants (*Cucumis sativus* L.) of cv. Phoenix, highly susceptible to this disease, and cv. Edinstvo showing a certain level of resistance were used. They were grown in 200-ml plastic beakers with a natural soil without fertilization, in the growth chamber illuminated 12 h daily with luminescent lamps LB 40–2 at 80–90 μmol m^{-2} s^{-1} at 28°C (light), 18°C (dark) and 60% RH.

Fungus material

Causal agent of cucumber scab, *Cladosporium cucumerinum* Ell. et Arth. was initially isolated from cucumber plants in the Moscow region and obtained from the All-Russian Research Institute for Vegetable Breeding and Seed Production. The fungus was grown on potato glucose agar at 25°C. Two single-spore isolates indicated as C3 and C5 were derived from the initial culture. Annually, to refresh the culture, plants of the susceptible cultivar were inoculated. Leaf pieces with resulting distinct lesions were placed onto nutrient agar with 80 μg ml^{-1} streptomycin. The cultures were maintained on the same medium but without the antibiotic.

Before experiments, 10-day-old cultures were then refrigerated (4–8°C) for 2–30 days to stimulate the germinability of spores (conidia) isolated from it. Spores were washed off from the agar plates with distilled water and centrifuged 10 min at 8,000 × *g*. The pellet was rinsed with water and centrifuged again. The suspension was adjusted to the desirable concentration, which was counted with a haemocytometer.

Spore germination

Final concentrations of spores and reagents are indicated below. Conidia of C3 or C5 isolates were allowed to germinate in a Cellstar 96-well Suspension Culture Plate (Greiner Bio-One, Germany). Each well

contained 100 μl of the spore suspension at concentrations from 5×10^3 to 10^6 spores ml^{-1}. Spores were incubated for 20 h in the dark at either 18 or 25°C and were fixed with several droplets of ethanol per well. Those that developed germ tubes longer than a spore itself were taken as germinated. Percentage of germination was determined by examining 100 spores in each well with a Leitz-Diavert microscope (Wetzlar, Germany), with 4 replicates for each treatment. Staining or contrast-increasing optical techniques were not employed. Means ± SD (n=4 in each experiment) were calculated.

To reveal ROS participation, antioxidants were added to the spore suspensions. Those were superoxide dismutase (SOD, bovine erythrocuprein, 3.6 U μg^{-1}, Sigma-Aldrich Chemie Gmbh, Steinheim, Germany; E.C. 1.15.1.1) or catalase (CAT, from bovine liver, 11 U μg^{-1}, Sigma-Aldrich; E.C. 1.11.1.6.), 100 μg ml^{-1} each. To check the specificity of the enzymes, boiled catalase (CAT inactiv.) or bovine serum albumin (Sigma) were added at the same concentrations. Scavengers of hydroxyl radical (Halliwell and Gutteridge 2007), 10 mM mannitol (ICN, Eschwege, Germany) or 1 mM sodium formate (Merck, Darmstadt, Germany) were also used.

Preparation of spore diffusate and evaluation of its fungitoxicity

Spores of C5 isolate were incubated at different concentrations at either 18 or 25°C (see above). After 20 h, the suspensions were harvested with a drop collector described by Lapikova et al. (1995). It contained a paper filter, providing simultaneous removal of spores. The fluid prepared was referred to as spore diffusate.

In a 96-well plate, 80 μl diffusate was mixed with 10 μl water and 10 μl suspension of freshly isolated C5 spores (test-organism) at 10^5 spores ml^{-1}. In place of water, the same volume of one of the above-listed antioxidants at the same final concentration was added to probe ROS involvement in diffusate toxicity. After 24 h at 25°C, spores were fixed, and their germination was counted as above.

Assay for superoxide generation by spores

Superoxide radical in spore diffusates of the C5 isolate was detected by adrenaline oxidation (Bors et al. 1978). In Eppendorf 1.5-ml test tubes, 700 μl diffusate was incubated in a total volume 1,000 μl with 20 mM potassium phosphate buffer pH 7.8 and 1 mM adrenaline (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) dissolved initially in acid (pH 2) water. To validate the involvement of O_2^-, SOD was added at final concentration 100 μg ml^{-1}. The tubes were kept for 4 h in the dark at 25°C. In one experiment, six aliquots of 100 μl each were transferred from every sample to separate wells of 384-well plate (n=6). Optical densities at 480 nm were measured using a microplate spectrophotometer Bio-Rad Benchmark Plus, Japan. Means ± SD were calculated and normalized to the optical path 1 cm. Mean optical densities of six control wells with the same components, except water instead of diffusates were subtracted from values obtained with diffusates.

Plant inoculation and disease symptom estimation

Plants were inoculated with C5 spore suspensions at different concentrations at the stage of one true leaf. Ten droplets of inoculum, 10 μl each, were applied to the upper side of the leaf. Plants were kept for 24 h in the dark humid thermostat at either 18 or 25°C and were returned then to the illuminated growth chamber. Visual symptoms of disease were evaluated after 6–8 days.

Some infection droplets did not cause visual symptoms, while others brought about lesions after 3 days. Those were light-yellowish areas 0.5 mm in diameter or bigger, single or clusters of common diameter up to 5 mm. Later, some lesions did not change, and were described as chlorotic. In other lesions, their inner parts dried out and turned light-brown; they were described as necrotic. Sometimes, the damaged parts merged or disrupted completely to create a hole in a leaf blade.

On the highly susceptible cv. Phoenix, necrosis prevailed. On the more resistant cv. Edinstvo, this symptom was rare in favour of chlorosis or a symptomless state. It is important to note if the fungus is re-isolated from necrosis but not from chlorosis. Thus, necrosis, chlorosis, and absence of symptoms can be taken as markers of higher susceptibility, lower susceptibility and resistance, respectively. Percentages of necrosis-initiating inoculum droplets were calculated for individual, affected leaves.

Results

Effect of spore concentration on germination and secretion of toxicants

The percent of *C. cucumerinum* spores that germinated after 20 h under the defined conditions at 25°C was dependent on the spore concentration. Maximal germination of the isolate C3 was observed at 2.5×10^4 spores ml^{-1}; that of the isolate C5 was at 10^5 ml^{-1}, i.e. at four times higher concentration (Fig. 1). Isolate C5 was used for the rest of the study.

At the lower temperature, 18°C, which is generally more favorable for cucumber infection, germination efficiency of isolate C5 was lower at all but the highest concentrations and also displayed the maximum at 10^5 spores ml^{-1} (Table 1). At both temperatures, germ tubes were mainly shorter at high spore concentrations than at optimum and low ones (Fig. 2).

To test whether spore exo-metabolites were associated with decreased germination rates, the diffusates of spores allowed to germinate at optimal and at two extreme concentrations were tested. The diffusates were prepared to be used as media for germination at optimal concentration. It was observed that spore diffusates from the suspension of optimal concentration (10^5 spores ml^{-1}) did not affect germination of the test organism, but diffusates from both dilute (10^4 spores ml^{-1}) and dense (5×10^5 spores ml^{-1}) suspensions suppressed the germination rate significantly (Table 2). Therefore, self-suppression of spore germination may be partially driven by exo-metabolites.

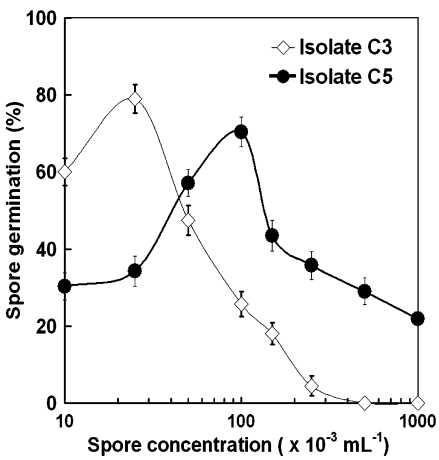

Fig. 1 Effect of spore concentration of two *C. cucumerinum* isolates on their germination in water at 25°C. Means ± SD (n=4) are reported for one typical experiment of 2 with similar results. For each isolate, the germination value of the maximum differs significantly from all other values at $p\leq0.01$ (Student's t-test)

Table 1 Germination of *C. cucumerinum* spores in water at different concentrations of spore suspensions and different temperatures

Spore [a] concentration (ml^{-1})	Spore germination (%) [b]	
	at 18°C	at 25°C
5×10^3	$11^{**}\pm3$	$20^{**}\pm4$
10^4	$22^{**}\pm3$	$41^{**}\pm8$
10^5	68±5	81±4
5×10^5	$35^{**}\pm8$	$22^{**}\pm4$

[a] Spore suspensions of C5 isolate were incubated for 20 h

[b] Means ± SD (n=12) of three independent experiments are reported. For each temperature, differences from the levels at 10^5 spores ml^{-1} were significant at $^{**}P\leq0.01$ according to Student's t-test

Effects of antioxidants on spore germination in suspensions at different concentrations and on fungitoxicity of spore diffusates

To probe the possibility of ROS involvement in the self-suppression, antioxidants SOD, CAT, mannitol or formate were added to spore suspensions of different densities in attempts to restore the germination (Fig. 3). The additions had little effect on germination rate at 10^5 spores ml^{-1}, the optimum concentration for germination (Fig. 3b). In the dilute suspension, 10^4 spores ml^{-1}, CAT or mannitol (but not SOD or formate) restored the germination level close to that observed in the optimal spore concentration (Fig. 3a). In the dense suspension, 5×10^5 spores ml^{-1}, self-suppression was diminished by both antioxidant enzymes and both hydroxyl radical scavengers (Fig. 3c). At both extreme spore concentrations, albumin or inactivated catalase did not affect germination. Consequently, the protective action of native CAT was accounted for by its specific activity.

Diffusate toxicities of spores germinated in dilute (Fig. 4a) or dense (Fig. 4c) suspensions to spores kept at optimal concentration were reduced by some

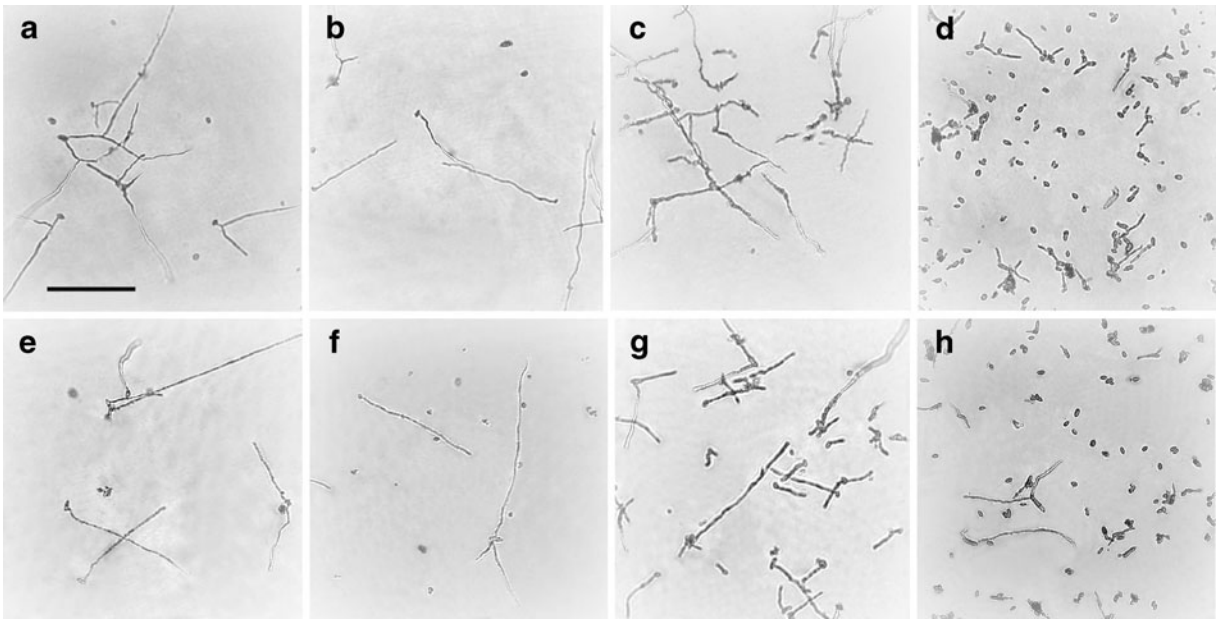

Fig. 2 Spores of *C. cucumerinum* (isolate C5) after their 20-h incubation in water at 18°C (**a**–**d**) or 25°C (**e**–**h**) at concentrations 5×10^3 (**a**, **e**), 10^4 (**b**, **f**), 10^5 (**c**, **g**) or 5×10^5 spores ml^{-1}(**d**, **h**). All images are on one scale, bar=100 μm

exogenous antioxidants. CAT, SOD or mannitol detoxified diffusates prepared at low concentration of spores. CAT and formate were active against the dense suspension, respectively. None of the antioxidants stimulated spore germination in diffusate of the suspension at optimal (10^5 spores ml^{-1}) concentration; SOD even reduced it slightly (Fig. 4b).

The self-inhibition of spore germination in either water or diffusate was reduced by antioxidants (Figs. 3 and 4). Thus, both spore inhibiting effects appeared to depend on hydrogen peroxide along with superoxide and hydroxyl radicals produced apparently by germinating spores. It is difficult to interpret the roles of particular ROS, inasmuch as antioxidants may not show full activity in a heterogeneous biological system, due to their hampered access to targets and metabolization (Czapski 1984).

Table 2 Germination of *C. cucumerinum* spores in water or in diffusates prepared from suspensions of other spores germinated at different concentrations

Medium [a] for test-organism spores [b]	Test-organism spore germination (%) [c]
Water	67±4
Diffusate from suspension 10^4 spores ml^{-1}	40^{**}±9
The same, 10^5 spores ml^{-1}	60±8
The same, 5×10^5 spores ml^{-1}	32^{**}±9

[a] Spore suspensions of C5 isolate at the indicated concentrations were incubated for 20 h in water at 25°C. The liquid phase of suspensions was harvested to give spore diffusates

[b] Another suspension (10^5 spores ml^{-1}) of the same isolate (test-organism) was incubated in water or in spore diffusates aforementioned for 20 h at 25°C followed by spore germination count

[c] Means ± SD (n=12) of three independent experiments are reported. Differences from the mean of water counterpart were significant at $^{**}P\leq0.01$ according to Student's t-test

Superoxide radical generation in spore diffusates from suspensions of different densities

To verify ROS formation, one of them, O_2^-, was chemically assayed in spore suspensions as related to their densities.

Spore diffusates were found to oxidize exogenous adrenaline (Table 3). The reaction was prevented by addition of SOD, suggesting it is dependent on superoxide radicals. Diffusates prepared at 18°C from two dilute suspensions (5×10^3 and 10^4 ml^{-1}) generated O_2^- more readily than those from suspensions of

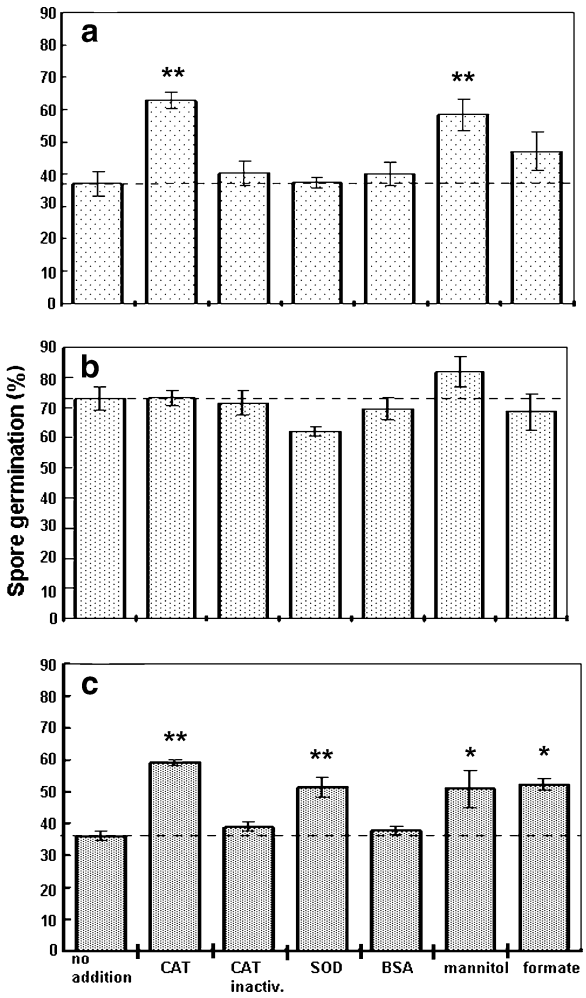

Fig. 3 Effect of antioxidants on *C. cucumerinum* (isolate C5) spore germination for 20 h in water at 10^4 (**a**), 10^5 (**b**) or 5×10^5 spores ml^{-1} (**c**) at 25°C. The compounds were added at zero time at these final concentrations as follows: 50 μg ml^{-1} catalase, boiled catalase or superoxide dismutase, 10 mM mannitol and 1 mM sodium formate. Means ± SD (n=8) of two independent experiments are reported. The dashed lines show the levels of spore germination without additions. Differences from these levels were significant at * $P\leq0.05$ and ** $P\leq0.01$ according to Student's t-test

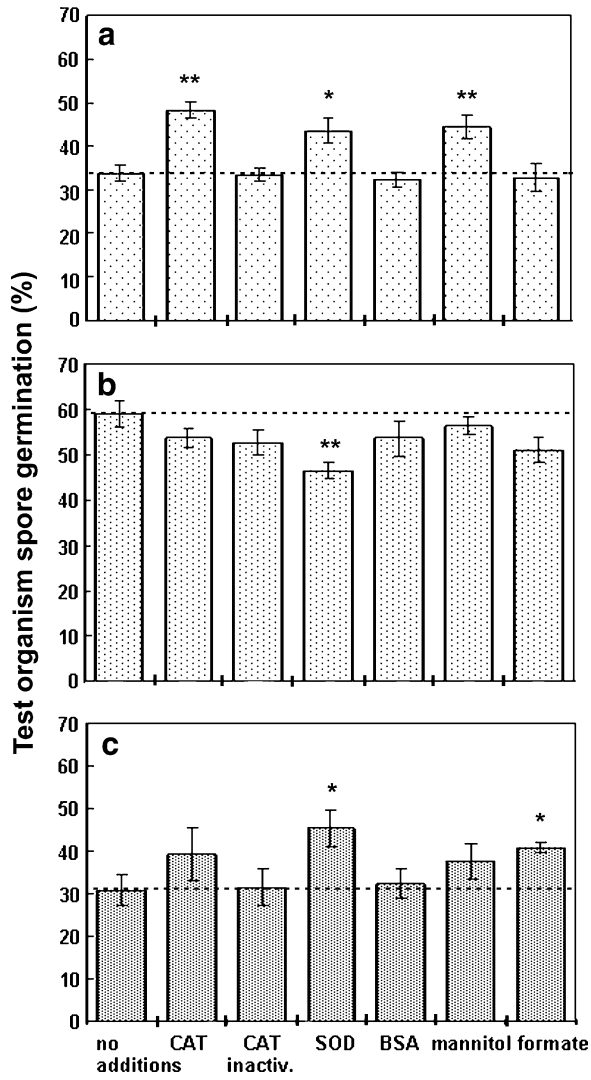

Fig. 4 Effect of antioxidants on *C. cucumerinum* spore germination in diffusates of other spores of the same isolate C5. Suspensions at 10^4 (**a**), 10^5 (**b**) or 5×10^5 spores ml^{-1} (**c**) were incubated for 20 h in water at 25°C to give a liquid phase referred to as a diffusate. Other spores were suspended (10^5 ml^{-1}) in water or some diffusate with or without antioxidants and kept for 20 h at 25°C followed by spore germination count. The compounds added were: 50 μg ml^{-1} catalase, boiled catalase or superoxide dismutase, 10 mM mannitol and 1 mM sodium formate. Means ± SD (n=8) of two independent experiments are reported. The dashed lines show the level of spore germination without additions. Differences from these levels were significant at * $P\leq0.05$ and ** $P\leq0.01$ according to Student's t-test

optimal or high concentrations. Diffusates prepared at 25°C from either dilute or dense suspensions produced more superoxide than that from the suspension at optimum concentration.

Comparison between amount of adrenaline oxidation and spore germination in spore diffusates revealed the inverse correlation of these indices at all suspension concentrations and temperatures tested (Fig. 5). In other words, the more O_2^- accumulated in spore suspensions, the more germination was inhibited.

Table 3 Production of superoxide radical in *C. cucumerinum* spore diffusates

Spore [a] concentration, (ml^{-1})	Adrenaline oxidation ($A_{480}\times10^4$) in diffusates of spores germinated [b]			
	at 18°C		at 25°C	
	without SOD	+ SOD	without SOD	+ SOD
5×10^3	1,172**±88	−84±43	481**±42	41±17
10^4	1,399**±130	−6±37	487**±73	−3±17
10^5	641±67	30±27	208±30	68±14
5×10^5	538±122	−3±30	980**±182	42±30

[a] Spore suspensions of C5 isolate at the indicated concentrations were incubated in water for 22–24 h at temperatures specified followed by a harvest of spore diffusates. The assay system of 1 mM adrenaline in 20 mM potassium phosphate buffer pH 7.8 with or without 50 μg ml^{-1} superoxide dismutase were added to diffusates (the concentrations and pH are final). The samples were kept for 4 h at 25°C, then their optical densities were read

[b] Means±SD (n=18) of three independent experiments are reported. For SOD-free diffusates prepared at each temperature, differences from the means derived for 10^5 spores ml^{-1} were significant at $^{**}P\leq0.01$ according to Student's t-test

Visual disease symptoms after leaf inoculation with spore suspensions of different densities

Symptoms caused by inocula at different concentrations were compared, to help elucidate how self-suppression of spore germination could influence spore aggressiveness.

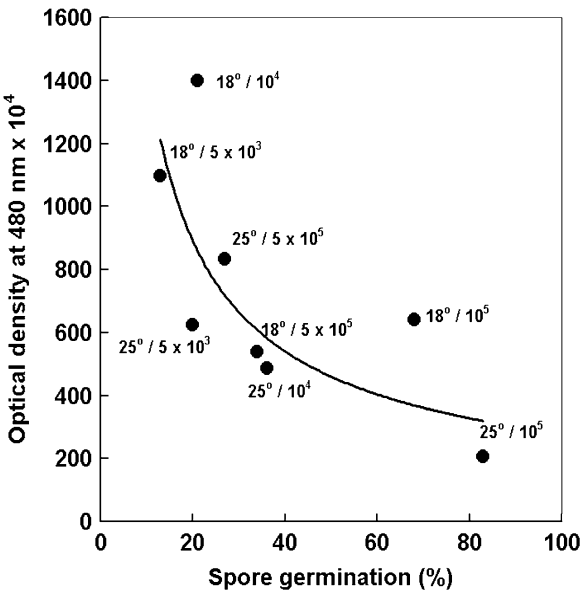

Fig. 5 The relationship between *C. cucumerinum* (isolate C5) spore germination in water and superoxide formation in spore diffusates. Temperature (°C)/spore concentration (ml^{-1}) is indicated near experimental dots. Values of germination and those of optical densities are taken from Tables 1 and 3, respectively

Plants of the susceptible cv. Phoenix kept at 25°C for 24 h after inoculation did not exhibit symptoms. Lesions appeared on leaves only if the temperature was 18°C.

Disease occurrence as a whole (Fig. 6), as well as frequencies of necrotic lesions (Table 4) depended on the inoculum density. At spore concentration 10^5 ml^{-1}, optimal for their germination in vitro, the disease was the most severe, with necrosis predominating. At both extreme inoculum concentrations, the total number of lesions was lower, and necrosis was absent.

Consequently, different factors may hinder infection at different temperatures. At 18°C, concentrations of *C. cucumerinum* spores unfavourable for their germination in vitro were also disadvantageous for the disease. This mechanism, however, does not seem to underlie the resistance observed at 25°C.

Discussion

The disease outcome is determined not only by genes of the hosts and pathogens but also by the environmental conditions and the physiological state of the partners. The last depends specifically on the inoculum density. Some causal agents (for example, wheat rust fungi, TMV or potato X-virus) can cause disease at very low doses, for example, one fungal spore or several virus molecules. In other diseases (canker of potato, snow mold and bunt of wheat), the threshold of the successful infection is much higher. For several

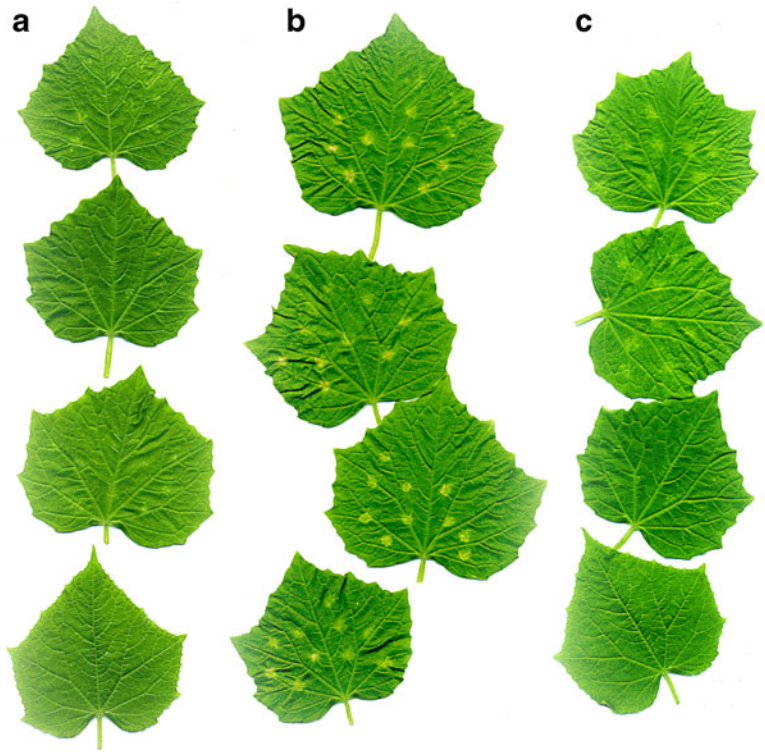

Fig. 6 Symptoms of cucurbit scab on cucumber leaves challenged at different inoculum concentrations. The first true cucumber leaf of the highly susceptible cv. Phoenix was inoculated with 10 µl droplets (ten per leaf) of *C. cucumerinum* (isolate C5) spore suspension at 10^4 (**a**), 10^5 (**b**) or 5×10^5 spores ml^{-1} (**c**). The plants were then kept for 24 h at 18°C under high humidity and returned to the growth chamber. The photographs were taken 8 days post inoculation

diseases, an upper limit also exists, such that inoculum densities above it are less infective. For instance, the occurrence of successful wheat infection by *Erysiphe graminis tritici* is 4, 100 and 2%, respectively, with inocula at 1.5×10^3, 1.5×10^6 and 1.5×10^7 spores ml^{-1}. Consequently, for a successful challenge, some intermediate inoculum concentrations are needed (Gäumann 1951).

Table 4 Occurrence of disease symptoms caused by *C. cucumerinum* on cucumber leaves as depended on inoculum concentration

Inoculum concentration [a] (spores ml^{-1})	Necrotic lesions (%) [b]
10^4	$1^{**}\pm1$
10^5	56±6
5×10^5	$0^{**}\pm0$

[a] The first true leaf of the highly susceptible cv. Phoenix was inoculated with ten droplets of isolate C5 spore suspensions at concentrations indicated. The plants were then kept for 24 h at 18°C under high humidity

[b] The symptoms were estimated after 6–8 days post inoculation. The percentage of inoculum droplets (of their total number on one leaf) that gave necrosis is represented. Means ± SE (n=14) of 3 independent experiments are reported. The differences from the means derived for 10^5 spores ml^{-1} were significant at $^{**}P\leq0.01$ according to Student's t-test

At extreme inoculum densities (in comparison to the optimal one), low disease severity may be a result of unfavourable condition of the pathogen, irrespective of the host's responses. Presumably, at extremely low inoculum concentrations, the percentage of successful fungal development is inadequate for successful colonization of the host. The spores may also secrete insufficient amounts of toxins, lytic enzymes and self-stimulators of germination necessary for ingress. At extremely high inoculum concentrations, pathogen cells may suffer from competition for nutrients in the infection droplet. Also, higher amounts of pathogen elicitors produced in dense inoculum may evoke stronger defence responses from the plant; crowded spores might be more vulnerable to the responses. Therefore, inocula at extreme concentrations may cause less severe disease, for several reasons. In addition to these mechanisms, we showed earlier that the reduction of rice blast severity at both extremely low and high concentrations may be associated with the self-suppression of fungal development (Lapikova and Dzhavakhiya 1987; Aver'yanov and Lapikova 1990). The present work revealed that

similar relationships may be operating in cucurbit scab of cucumber. The self-suppression of *C. cucumerinum*, like that of *M. grisea*, was related to its exo-metabolites because diffusates from either dilute or dense spore suspensions suppressed development of other spores kept at optimal concentration.

The chemical mechanism of the self-suppression is interesting. Self-inhibitors of fungal development, including those secreted by germinating spores, are diverse (Macko 1981; Tsurushima et al. 1995). Some, such as mycosporine-alanine of *Colletotrichum graminicola,* are water-soluble and are present in spore mucilage (Leite and Nicholson 1992). Volatile self-inhibitors, for example, 1-octen-3-ol of *Penicillium paneum* are also known (Chitarra et al. 2004). In addition, we showed for *M. grisea* that the role of self-inhibitors might be performed by ROS in both dilute and dense suspensions (Aver'yanov and Lapikova 1990). The similar dependence was revealed for *C. cucumerinum* here. It is known that exogenous H_2O_2 suppresses germination of its spores (Peng and Kuc 1992; Aver'yanov et al. 2007a), whereas self-suppression at extreme suspension concentrations was diminished by antioxidants (Figs. 3 and 4), suggesting the involvement of fungal endogenous ROS. This assumption agrees with the chemically assayed superoxide production by spores and the inverse relation between this activity and germination (Fig. 5).

In the self-suppression of fungi, ROS may act independently of or in association with other exo-metabolites. In the latter case, ROS may mediate, as signals, reactions of organic compounds known as fungal self-inhibitors. Just trace amounts of ROS appear to be sufficient for signalling, since hydrogen peroxide even at picomolar concentration suppressed germination of *M. grisea* and *C. cucumerinum* spores (Aver'yanov et al. 2007a).

In general, the biological role of ROS is not only suppressive; small amounts of these compounds produced by fungi are required for their development under normal conditions (Egan et al. 2007; Scott and Eaton 2008). This may explain the reduction of spore germination by SOD in the diffusate of other spores germinated at optimal density (Fig. 4b).

We found that cucumber plants, susceptible to the isolate tested at lower temperature, exhibited apparent resistance at higher temperature. Such phenomena are known (Ramakrishnan 1967) and may involve host-originated ROS, as shown for rice blast disease (Aver'yanov et al. 1993). Presumably, it holds true for cucurbit scab, and the high-temperature resistance is due to strengthening defence of the host rather than self-suppression of the pathogen. But this suppression seems to diminish disease severity during compatible interactions at lower temperature. In addition, fungal exo-metabolites (including ROS) secreted at extreme spore densities might elicit plant defence responses, which again may involve ROS. Presumably, cucumber is more resistant at 25°C because this temperature is generally preferable for this plant.

Although the self-suppression of the fungus hinders the disease, this phenomenon may be somewhat beneficial for the pathogen. The reversibility of the action of some self-inhibitors suggests their relation with spore dormancy (Tsurushima et al. 1995). Thus, under conditions unfavourable for infection, pathogen development would be retarded until the spore concentration becomes optimal due to the droplet drying up or, on the contrary, dilution by dew or rain.

Conclusions

Spores of *C. cucumerinum* germinated poorly in too dilute or too dense spore suspensions. Diffusates from suspensions of extreme densities suppressed germination of other spores kept at optimal concentration. Therefore, diffusible inhibitors may account for the density-dependent self-suppression. The spore-inhibiting effects reported were sensitive to antioxidants. Fungitoxicity of diffusates correlated with their production of superoxide radicals. Therefore, mechanisms of spore self-inhibition may involve reactive oxygen species. Temperature did not markedly affect spore germination in different suspensions but dramatically changed their interaction with the susceptible host-plant. At 18°C, typical compatible symptoms appeared on cucumber leaves but were less pronounced with inocula at extreme densities. At 25°C, none of the inocula caused symptoms. It is suggested that low temperatures favour disease development, which may be diminished by self-suppression of the causative agent. At higher temperatures the plant infection may be reduced by other inhibiting factors, regardless of spore germination.

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References

Aver'yanov, A. A., & Lapikova, V. P. (1990). Activated oxygen as a possible factor in the autoinhibition of spore germination of the fungus *Pyricularia oryzae*. *Biokhimiya (Moscow), 55*, 1397–1402.

Aver'yanov, A. A., Lapikova, V. P., & Dzhavakhiya, V. G. (1993). Active oxygen mediates heat-induced resistance of rice plant to blast disease. *Plant Science, 92*, 27–34.

Aver'yanov, A. A., Lapikova, V. P., Pasechnik, T. D., Kuznetsov, V. V., & Baker, C. J. (2007a). Suppression of early stages of fungus development by hydrogen peroxide at low concentrations. *Plant Pathology Journal, 6*, 242–247.

Aver'yanov, A. A., Pasechnik, T. D., Lapikova, V. P., Gaivoronskaya, L. M., Kuznetsov, V. V., & Baker, C. J. (2007b). Possible contribution of blast spores to the oxidative burst in the infection droplet on rice leaf. *Acta Phytopathologica et Entomologica Hungarica, 42*, 305–319.

Bors, W., Michel, C., Saran, M., & Lengfelder, E. (1978). The involvement of oxygen radicals during the autoxidation of adrenalin. *Biochimica et Biophysica Acta, 540*, 162–172.

Chitarra, G. S., Abee, T., Rombouts, F. M., Posthums, M. A., & Dijksterhuis, J. (2004). Germination of *Penicillium paneum* conidia is regulated by 1-octen-3-ol, a volatile self-inhibitor. *Applied and Environmental Microbiology, 70*, 2823–2829.

Czapski, G. (1984). On the use of ·OH scavengers in biological systems. *Israel Journal of Chemistry, 24*, 29–32.

Egan, M. J., Zheng-Yi, W., Jones, M. A., Smirnoff, N., & Talbot, N. J. (2007). Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. *Proceedings of the National Academy of Sciences, USA, 104*, 11772–11777.

Gäumann, E. (1951). *Pflanzliche infectionslehre*. Basel: Birkhaeuser.

Halliwell, B., & Gutteridge, J. M. C. (2007). *Free Radicals in Biology and Medicine*. Oxford: Oxford University Press.

Lapikova, V. P., Aver'yanov, A. A., & Pasechnik, T. D. (1995). Devices for the preparation of drop diffusates of cereal leaves. *Mikologia i Fitopatologia (Mycology and Phytopathology, in Russian), 29*, 44–47.

Lapikova, V. P., & Dzhavakhiya, V. G. (1987). Early development stages of *Pyricularia oryzae* Cav. on rice leaves. *Mikologia i Fitopatologia (Mycology and Phytopathology, in Russian), 21*, 358–365.

Leite, B., & Nicholson, R. L. (1992). Mycosporine-alanine: a self-inhibitor of germination from the conidial mucilage of *Colletotrichum graminicola*. *Experimental Mycology, 16*, 76–86.

Macko, V. (1981). Inhibitors and stimulators of spore germination and infection structure formation in fungi. In G. Turian & H. R. Hohl (Eds.), *The fungal spore: Morphogenetic Controls* (pp. 565–584). London-New York: Academic.

Peng, M., & Kuc, J. (1992). Peroxidase-generated hydrogen peroxide as a source of antifungal activity in vitro and on tobacco leaf discs. *Phytopathology, 82*, 696–699.

Ramakrishnan, L. (1967). Studies in the host-parasite relations of blast disease of rice. III. The effect of night temperature on the infection phase of blast disease. *Phytopatologishe Zeitschrift, 57*, 17–23.

Scott, B., & Eaton, C. J. (2008). Role of reactive oxygen species in fungal cellular differentiations. *Current Opinion in Microbiology, 11*, 488–493.

Tsurushima, T., Ueno, T., Fukami, H., Irie, H., & Inoue, M. (1995). Germination self-inhibitors from *Colletotrichum gloesporioides* f. sp. *jussiaea*. *Molecular Plant-Microbe Interactions, 8*, 652–657.