Regenerating Protoplasts from *Cercospora* and *Neurospora* Differ in Their Response to Cercosporin

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Supported in part by United States Department of Agriculture Competitive Research Grant 84-CRCR-1-1432 and by the North Carolina Biotechnology Center.

Paper 10975 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh 27695.

Accepted for publication 2 October 1987 (submitted for electronic processing).

ABSTRACT

Gwinn, K. D., and Daub, M. E. 1988. Regenerating protoplasts from *Cercospora* and *Neurospora* differ in their response to cercosporin. Phytopathology 78:414-418.

Cercospora species accumulate high quantities of the photosensitizing toxin, cercosporin, with no apparent harm. Growth of *Neurospora crassa*, however, is reduced in the presence of the toxin. Freshly isolated protoplasts from both *C. nicotianae* and *N. crassa* were killed by 10 μM cercosporin, but at 1 μM (a concentration lethal to plant cells), 50% of the fungal protoplasts remained viable. After an 8-hr incubation, all protoplasts of *C. nicotianae* were resistant to 1 μM cercosporin, and 35% were resistant to 10 μM cercosporin. Sensitivity of regenerating protoplasts of *N. crassa* did not differ significantly from that of freshly isolated protoplasts. Protoplasts of both species regenerated cell walls and started to divide during the time when differential resistance to cercosporin was expressed, but regeneration began earlier with protoplasts of *N. crassa*. Protoplasts of *N. crassa* became osmotically stable sooner than did protoplasts of *Cercospora*. Also, by 2 hr, some cells in the preparation of protoplasts of *N. crassa* had begun to divide, whereas no cell division was seen in preparations of *C. nicotianae* until 6–8 hr. The correlation between cell wall regeneration and acquisition of resistance to cercosporin suggests that cell wall components play an important role in resistance to cercosporin of regenerating *C. nicotianae* protoplasts.

Additional key word: nonspecific toxin.

Members of the genus *Cercospora* cause diseases of agronomically important crops and often create severe economic losses. Because *Cercospora* is a necrotrophic fungus, it must possess a means of killing the cells of its host. One mechanism by which the fungus may kill the host cells and thus advance through the tissue is through the action of the toxin, cercosporin. Although definitive proof is lacking, several lines of evidence strongly suggest that cercosporin is involved in disease development. First, most species of *Cercospora* that have been tested produce the toxin; in fact, Fajola (14) has suggested production of cercosporin as a taxonomic criterion for the genus. Second, *Cercospora* spp. have a broad host range, which suggests a generalized virulence mechanism; cercosporin is a nonspecific toxin, toxic to every plant that has been tested (3,9,14,21). Third, cercosporin has been isolated from lesions in plant tissues infected with *Cercospora* (14). Finally, cercosporin is toxic only in the light, and in some crops, light has been demonstrated to be critical for disease development (6,7,13,27).

Cercosporin is a photosensitizing compound that disrupts plant cell plasma membranes (8,9,11). In the presence of light, cercosporin is converted to an excited state (the triplet state), which reacts with molecular oxygen to form two toxic species, singlet oxygen and superoxide (12). These compounds are detrimental to many cellular components such as fatty acids, proteins, and nucleic acids (26). Cercosporin causes peroxidation of the fatty acids in plant cells, which leads to major alterations in membrane fluidity and composition; these changes eventually lead to rupture of the plasma membrane (9,11).

Given this generalized method of toxicity, cercosporin would be expected to be lethal to many organisms. Mice, bacteria, and plants (3,9,14,21,29) are all very sensitive to low concentrations of the toxin. By contrast, *Cercospora* and some other members of the divisions Ascomycotina and Deuteromycotina are not affected by cercosporin (10). Some members of these divisions such as *Neurospora crassa* Shear & Dodge have reduced growth in the presence of the toxin.

The purpose of this research was to identify the components involved in the resistance of these fungi to the toxin by comparing *Cercospora nicotianae* Ell. & Ev. to *N. crassa*. This paper focuses specifically on the cell wall and its function as a protective mechanism for the fungal plasma membrane. We chose to use protoplasts for these studies because the plasma membrane could be examined for toxin resistance. The effects of cell walls on toxin resistance could also be examined using regenerating protoplasts. Because protoplasts have not previously been isolated from *Cercospora*, a protocol for their efficient isolation was developed. Preliminary findings from these studies have been reported (16,17).

MATERIALS AND METHODS

Fungal isolates. *Cercospora nicotianae* (ATCC 18366) was maintained on malt extract agar (8). *N. crassa* (strain ORS-6a) was obtained from Fungal Genetics Stock Center, University of Kansas Medical School, Kansas City, and maintained on potato-dextrose agar (Difco Laboratories, Detroit, MI).

Sensitivy of mycelium to cercosporin. Cercosporin was isolated and purified from *C. nicotianae* and *C. beticola* Sacc. (ATCC 24080) as previously described (8). Because cercosporin is not soluble in water, stock solutions of the toxin were prepared by dissolving crystals in acetone, and these solutions were stored at −15 C in foil-wrapped tubes. (Concentrations were confirmed by absorbance of the solutions at 473 nm.) Malt agar was amended after autoclaving with either 0.1% acetone or an equal volume of stock solution. Final cercosporin concentrations were 1 μM and 10 μM.

To test for senstivity to the toxin, an agar plug from the margin of a fungal culture was placed in a plate containing cercosporin-amended malt agar or liquid malt medium. Percentage growth was calculated either by comparison of the diameters of the colonies grown on agar medium or by dry weights of colonies formed on liquid medium. Growth diameter was measured after 16 hr for *N. crassa* and after 3 days for *C. nicotianae*.

Protoplast production. To produce stock cultures, one plate of a 1–2 mo-old culture of *C. nicotianae* or a 1-wk-old culture of

N. crassa was homogenized in 200 ml of glucose-yeast extract liquid medium (GYE) (15 g/L of glucose and 3 g/L of yeast extract) for 5 min at the low speed of a Waring blender. The homogenate was divided equally between two 500-ml Erlenmeyer flasks, and these were incubated on a rotary shaker (150 rpm) at 25 C. After 1 day, the cultures were diluted with equal amounts of GYE and incubated at 25 C as described above. These liquid cultures were termed stock cultures and were maintained for up to 1.5 mo with periodic transfers.

To produce cultures amenable to protoplast production in *Cercospora* spp., one liquid stock culture was homogenized in the blender for 1–2 min; 10 ml of this homogenate was added to 100 ml of GYE and incubated on a rotary shaker at 25 C. After 1 day, 10 ml of this culture was centrifuged at 800 *g* for 10 min in graduated centrifuge tubes. The pellet (1 ml) was suspended in 100 ml of GYE and incubated for 22–26 hr on the rotary shaker.

Four cultures of *Cercospora* produced from the procedure described above were centrifuged at 800 *g* for 10 min in 100-ml centrifuge tubes. The combined pellets were resuspended in 200 ml of buffer (5) and the resulting suspension centrifuged as before. The combined pellets were suspended in 200 ml of buffer containing 10 mg/ml of cellulase (Carolina Biological Supply, Burlington NC) and 10 μl/ml of β-glucuronidase (Sigma Chemical Co., St. Louis, MO). This mixture was incubated for 1.5 or 3.0 hr on a rotary shaker (80 rpm) at 27 C to allow protoplast release.

For the production of protoplasts from *N. crassa* one stock culture was blended for 10 min at the high speed of a Waring blender and the macerate divided equally among four flasks of GYE. These cultures were then allowed to incubate at room temperature for 1 day. Each culture was blended as before. After the second incubation, protoplasts were produced by treatment of the mycelium as described for *Cercospora* spp. with the exception that chitinase (50 units/ml) and amylase (500 units/ ml) (24) were added to the enzyme mixture. Amylase and chitinase were purchased from Sigma Chemical Co. (St. Louis, MO).

Protoplast isolation and regeneration. Two methods were used to isolate protoplasts. In Method I, the amount of enzyme solution was reduced by centrifuging the mixture of mycelium and protoplasts at 800 *g* for 10 min. The pellet was suspended in approximately 40 ml of the supernatant and then filtered through Whatman No. 1 filter paper. The filtrate (17.5 ml) was layered on 2.5 ml of 1 M mannitol (28) and centrifuged as above. The pellet was suspended in regeneration medium (RM) (30 g of glucose, 15 g of malt extract, 3 g of peptone, and 24.4 g of NaCl per liter). In Method II, the mixture of mycelium and protoplasts was centrifuged at 250 *g* for 10 min. The pellet was suspended in 1 M $MgSO_4$ (1) and centrifuged at 500 *g* for 20 min. The top layer, which contained the protoplasts, was removed and filtered through Whatman No. 1 filter paper. The filtrate was diluted 1:50, 1:100, and 1:200 with regeneration medium. No differences were detected between protoplasts isolated by the two methods. Method I was used when a high concentration of protoplasts was required.

The total number of protoplasts obtained in each isolation was determined with a Fuchs Rosenthal Ultra Plane counting chamber. Percent regeneration was determined by plating a known number of freshly isolated protoplasts on solid regeneration medium (RM with 1.5% agar) and counting the number of colonies after a 4- to 5-day incubation. Protoplast regeneration was also recorded by light micrographs. All microscopy was performed on a Nikon inverted microscope (Nippon Kogaku K.K., Tokyo, Japan).

Osmotic stability. Protoplasts from *C. nicotianae* and *N. crassa* were produced and isolated as described above (Method II) and incubated in RM. At 4-hr intervals after isolation, a 0.3-ml aliquot of each dilution of the protoplast suspension was placed in 60- × 15-mm gridded petri plates. Each square of the grid measured 4 mm^2. Protoplasts were added to either 5 ml of RM or 5 ml of lysis medium (RM without NaCl). Two plates of each treatment were prepared from each dilution. The cultures were incubated at 25 C for 2 days (*C. nicotianae*) or for 24 hr (*N. crassa*) at which time the number of colonies/square was determined for each culture by counting the colonies in 10 squares and calculating an average value. The squares were selected by a systematic sampling scheme from a random starting point. Percent regeneration was determined from the ratio of the number of colonies/square in lysis medium to the number of colonies/square in RM. Values reported are the means of three experiments.

Cercosporin toxicity. Protoplasts were prepared and isolated as described above and were incubated in RM. Every 4 hr, 0.3 ml of each dilution of the protoplast suspension was mixed with either 5 ml of RM or 5 ml of RM that contained 1 or 10 μM cercosporin. (All cultures had a final concentration of 0.5% acetone). The cultures were exposed to light (15 $\mu E \cdot m^{-2} sec^{-1}$) for 8 hr and then stored in the dark for 2 days. The number of colonies/square was determined as in the osmotic stability studies. Percent survival was the ratio of the numbers of protoplasts in cercosporin-treated cultures that went on to regenerate as compared to the numbers of regenerating protoplasts in untreated cultures. Values reported are the means of five experiments.

RESULTS

Sensitivity of mycelium to cercosporin. The presence of exogenously added cercosporin did not affect the growth of mycelium of *Cercospora* spp. The growth of *N. crassa*, however, was inhibited by cercosporin. Percent inhibition of *N. crassa* by 1 and 10 μM cercosporin, respectively, was 62 ±3 and 78 ±4% when radial growth was measured, and 30 ±6 and 67 ±4% when cell mass was measured.

Protoplast isolation and regeneration. Because protoplasts have not been previously isolated from *Cercospora* spp., it was necessary to determine optimal procedures for protoplast isolation from these fungi. High yields of protoplasts (10^7–10^8 protoplasts per gram of mycelium dry weight) were obtained from *C. nicotianae* using the methods described. *C. beticola* and *C. zeae-maydis* also released protoplasts under these conditions. Protoplasts were variable in size but averaged 3–5 μm. Discernible regeneration began 8–10 hr after isolation, and visible colonies were formed on solid media after 4 or 5 days.

Yields of protoplasts from *C. nicotianae* depended on several factors. A 100-fold higher yield was obtained if the mycelium was incubated in the β-glucuronidase and cellulase mixture for 3 hr rather than 1.5 hr. Replacement of the phosphate buffer with the organic buffers, MES and BIS-TRIS, significantly lowered protoplast yield. The addition of chitinase to the digestion mixture did not increase yields of protoplasts from *C. nicotianae*. Acetone did not affect regeneration of the protoplasts in concentrations up to 1%, whereas ethanol and methanol inhibited regeneration at 0.1%.

The optimum age of cultures of *C. nicotianae* for protoplast release was 22–26 hr. Cultures produced at times outside this frame released fewer protoplasts. The age of the stock cultures also influenced protoplast yield. Low yields of protoplasts were obtained from cultures derived from stock cultures more than 1.5 mo old.

The number of colonies of *C. nicotianae* formed after 4 or 5 days represented only 35–55% of the original number of protoplasts. The total number of protoplasts present in the RM decreased with time. At 4 hr, the number of protoplasts was reduced by 24 ±5% and by 8 hr, this number was decreased by an additional 20 ±5%. At 24 hr, 80–100% of the remaining protoplasts were regenerating.

Published protocols for protoplast isolation from *N. crassa* utilize spores of the fungus (4). In this study, protoplasts were isolated from young hyphae to provide greater consistency between physiological states of the cells of the two fungi. When mycelium of *N. crassa* was incubated in the enzyme preparation used in the preparation of protoplasts of *C. nicotianae*, few protoplasts were obtained. Addition of chitinase to the digestion mixture doubled the number of protoplasts. Amylase increased yields from *N. crassa* by 10-fold. Culture age was not critical.

Regeneration of protoplasts was not synchronous with either fungus. However, as a rule protoplasts of *N. crassa* regenerated more quickly than those of *C. nicotianae* (Fig. 1). Some cells in the preparation of protoplasts of *N. crassa* had begun to divide by 2 hr, whereas all protoplasts of *C. nicotianae* remained as single cells

until 6–8 hr. By 24 hr, *N. crassa* had formed colonies that were large enough to settle to the bottom of the petri plate and thus be counted reproducibly. Colonies of *C. nicotianae* could not be counted for 2 days.

Osmotic stability. In both fungi, freshly isolated protoplasts were sensitive to osmotic shock (Fig. 2). Cell wall regeneration (as measured by resistance of the protoplasts to osmotic shock) was not synchronous in either fungus. However, in *N. crassa* the number of protoplasts resistant to osmotic shock started to increase rapidly after 4 hr, whereas protoplasts of *C. nicotianae* did not start this rapid increase until 12 hr. With both fungi, the rate of increase in osmotic resistance was linear after the 4- or 12-hr lag period. The linear rate of increase did not differ significantly between the two fungi ($P < 0.05$).

Cercosporin toxicity. Protoplasts of both species were killed when they were exposed to 10 μM cercosporin immediately after isolation (Fig. 3). When protoplasts of *C. nicotianae* were preincubated in regeneration medium before exposure to cercosporin, numbers of protoplasts resistant to cercosporin increased with time; almost 40% of the protoplasts were resistant when treated with cercosporin 4 hr after isolation, and 50% were resistant 12 hr after isolation. Protoplasts of *N. crassa*, by contrast, remained sensitive up to 12 hr.

Approximately 50% of the protoplasts of both species survived treatment with 1 μM cercosporin immediately after isolation (Fig. 3). With *C. nicotianae*, survival increased rapidly with increased preincubation time in RM. After 8 hr, survival reached maximum levels. Preincubation time had no effect on the sensitivity of regenerating protoplasts of *N. crassa* exposed to 1 μM cercosporin.

Treatment of freshly isolated protoplasts with 10 μM cercosporin in the dark had no effect on protoplasts of either fungus.

DISCUSSION

Production of protoplasts through enzymatic degradation of cell walls of filamentous fungi depends on the presence of young actively growing hyphae (1,5,19,20). The culturing procedures used in our study were designed to provide actively growing hyphae and were similiar to strategies employed by Rawn and Van Etten (25) to ensure metabolically active hyphae in *Phytophthora* and *Pythium*. Although these methods were designed for *C. nicotianae*, other *Cercospora* species released protoplasts readily from cultures that had been grown under these conditions. The procedure described may prove valuable for other fungi that do not readily produce the numbers of conidia necessary for high protoplast yields from conidia. Although *N. crassa* readily produces high numbers of conidia, protoplasts were obtained from mycelium in order to provide greater consistency in the comparison between the two fungi.

Hamlyn et al (18) found that crude commercial enzyme preparations that contained additional enzyme activities more efficiently released protoplasts from fungal cells than did enzyme preparations that were essentially pure. We did not test the side activities of the enzymes used in our study, but the β-glucuronidase was a crude extract with high sulfatase actvity (as determined by the manufacturers analysis). Yabuki et al (28) showed that the cooperative action of β-glucuronidase and chitinase is necessary for release of protoplasts from *Aspergillus* when highly purified preparations of these enzymes are used. Because the addition of

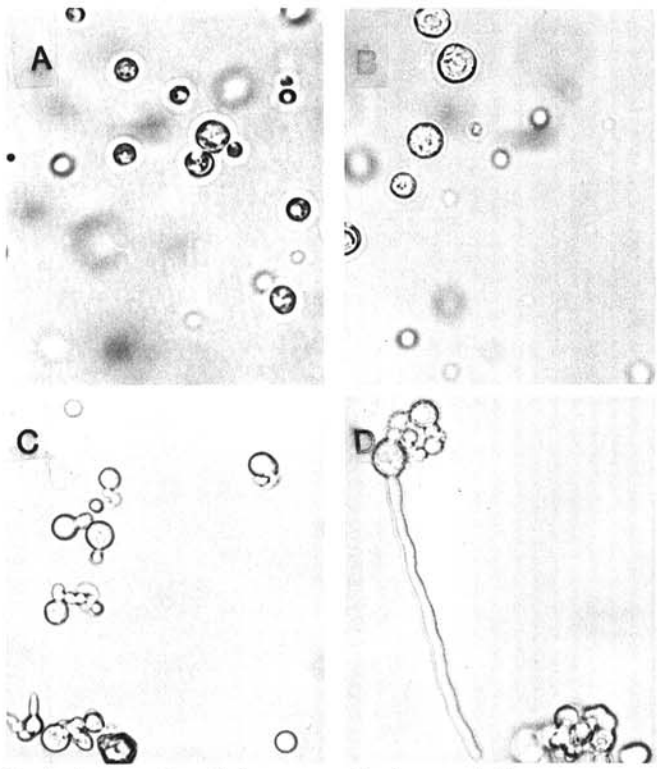

Fig. 1. Regeneration of *Cercospora nicotianae* and *Neurospora crassa* protoplasts. **A and C,** Protoplasts from *C. nicotianae*. **A,** Freshly isolated (850×). **C,** 8 hr after isolation (400×). **B and D,** Protoplasts from *N. crassa*. **B,** Freshly isolated (400×). **D,** 8 hr after isolation (300×).

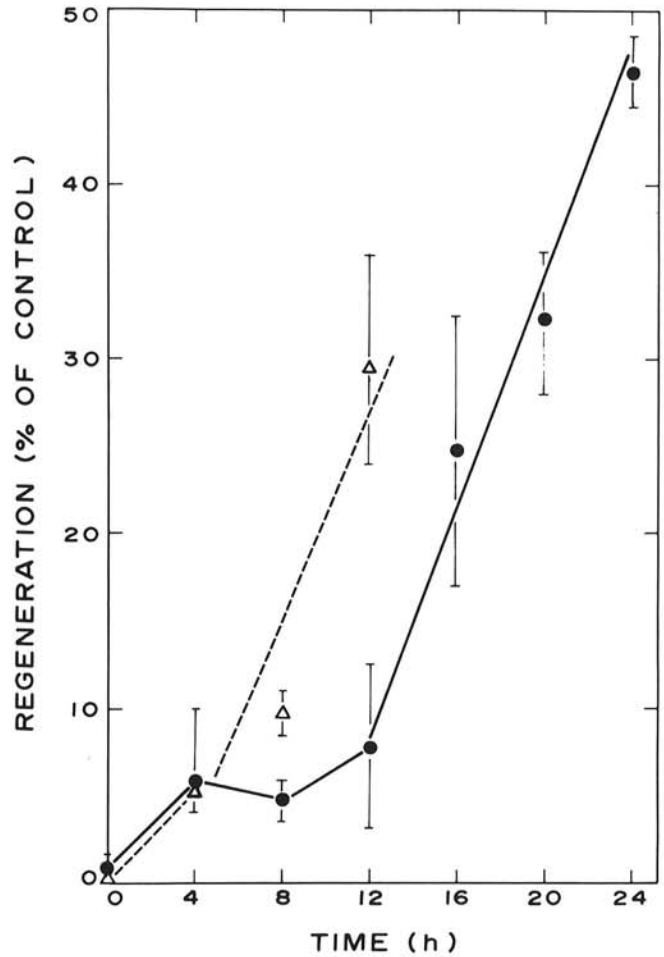

Fig. 2. Changes in the resistance of regenerating *Cercospora nicotianae* (•) and *Neurospora crassa* (Δ) protoplasts to osmotic shock. Protoplasts were incubated in regeneration medium and transferred to lysis medium at varying times after isolation (as indicated on the abscissa). Values are the ratio of the number of protoplasts regenerating in lysis medium to the number regenerating in regeneration medium. Regenerated protoplasts were counted at 1 (*N. crassa*) or 2 days (*C. nicotianae*) after isolation. After 4 hr the resistance to osmotic shock in *N. crassa* increased linearly with time. In *C. nicotianae* resistance to osmotic shock began to increase linearly after 12 hr. Slopes did not differ significantly ($P = 0.05$) between replications or treatments. Bars represent the standard error of the mean values given.

chitinase did not affect protoplast release from *C. nicotianae*, our enzyme mixture may have had chitinase activity. If so, the mixture did not have chitinase activity sufficient to efficiently release protoplasts from *N. crassa*.

Only 35–55% of the *C. nicotianae* protoplasts observed immediately after isolation formed colonies on solid agar. After an 8-hr incubation period, only 55% of this original population could be counted with a counting chamber. Those *C. nicotianae* protoplasts that died in the first 8 hr probably lacked a nucleus. Fifteen to 25% of *C. nicotianae* protoplasts viewed 8 hr after isolation lack a nucleus (Gwinn, *unpublished results*). Previous reports indicate that 50% of the protoplasts of *Aspergillus* released in the first hour of incubation had no nucleus (22). In our studies on osmotic sensitivity and cercosporin resistance, numbers of treated protoplasts that survived were compared to numbers of regenerating protoplasts in controls. Therefore, only regenerating protoplasts were counted.

Protoplasts from *C. nicotianae* and *N. crassa* were more resistant to the toxin, cercosporin, than tobacco suspension cultured cells. Under conditions used in our assay, 1 μM cercosporin is lethal to all cells in a tobacco suspension culture (approximately 10^6 cells) when exposed to light for 1 hr (Daub, *unpublished results*). In this study, approximately 50% of freshly isolated fungal protoplasts survived treatment with 1 μM cercosporin for 8 hr in the light, but exposure to 10 μM in the light for 8 hr was lethal for all cells (Fig. 3). This suggests that the fungal membranes are less sensitive than plant membranes to cercosporin.

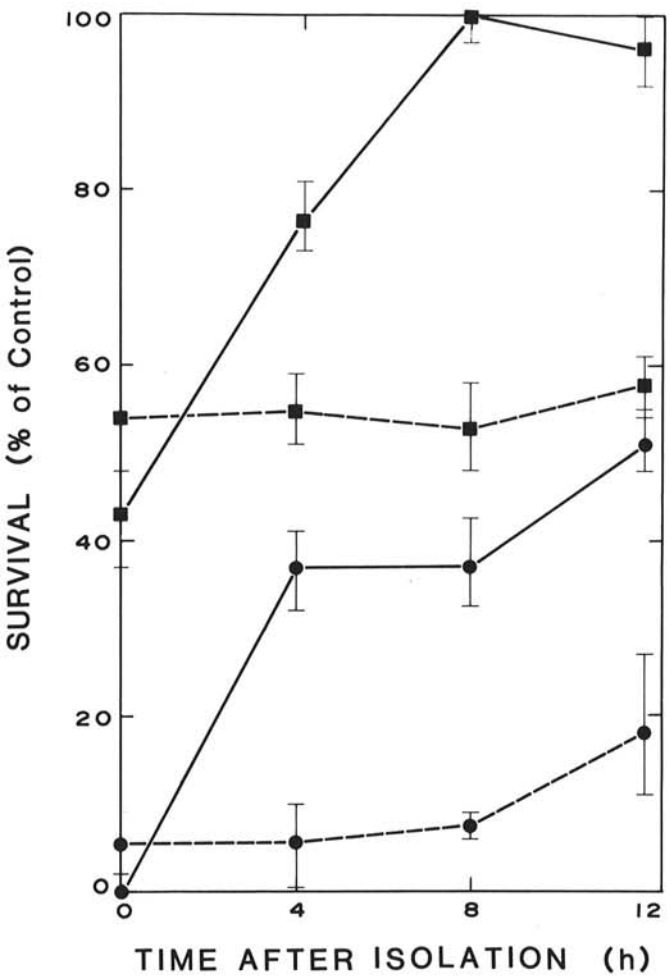

Fig. 3. Numbers of *Cercospora nicotianae* (—) and *Neurospora crassa* (- -) protoplasts surviving treatment with cercosporin. Protoplasts were incubated in regeneration medium and transferred to regeneration medium containing 1 μM (■) or 10 μM (●) cercosporin at varying times after isolation (as indicated on the abscissa). Percent survival was the number of protoplasts going on to regenerate in the cercosporin-containing medium as compared to the number of protoplasts that regenerated in medium lacking cercosporin. Bars represent the standard error of the mean values given.

Our data also support the hypothesis that fungal cell wall components are important in resistance of *Cercospora* species to cercosporin. Although freshly isolated protoplasts from both fungi are equally sensitive to cercosporin, regenerating protoplasts from *C. nicotianae* gain resistance to 1 μM cercosporin within 8 hr, whereas *N. crassa* protoplasts that were allowed to regenerate for up to 12 hr were as sensitive to cercosporin as freshly isolated protoplasts.

This study also indicates that an intact cell wall is not necessary for resistance of *C. nicotianae* to cercosporin. A high proportion of the *C. nicotianae* protoplasts were resistant to cercosporin before a significant proportion had become osmotically stable. At any given time the number of protoplasts of *C. nicotianae* resistant to cercosporin exceeded the number of protoplasts that tolerated osmotic shock.

Lack of resistance in regenerating protoplasts of *N. crassa* cannot be attributed to the inability of the protoplasts isolated from that fungus to produce a cell wall. Regenerating protoplasts of *N. crassa* became osmotically stable approximately 8 hr before regenerating protoplasts of *C. nicotianae*. Furthermore, microscopically detectable hyphal development from protoplasts begins approximately 4 hr earlier in *N. crassa* than in *C. nicotianae* even though protoplast populations of neither fungus demonstrated synchronicity in regeneration. Because the capacity for cellular division indicates at least partial presence of a cell wall (15), earlier hyphal development in *N. crassa* implies that production of the cell wall of *N. crassa* occurs before its production in *C. nicotianae*.

Osmotic sensitivity of regenerating protoplasts of *C. nicotianae* does not indicate that they completely lack cell wall components. Perberdy and Gibson (23) demonstrated that osmotic sensitivity continues after the production of cell wall glucans. They subjected protoplasts that had bound calcofluor (an optical brightener that binds to cell wall carbohydrates) to osmotic shock; the protoplasts lysed leaving behind a fluorescent 'ghost.' In preliminary studies, we have demonstated that both *C. nicotianae* and *N. crassa* protoplasts bind Tinopal 5BM (an optical brightener similiar to calcofluor) within 10 min after isolation (17) indicating that β-glucans are synthesized rapidly by the protoplasts. Studies to further characterize the surface of the regenerating protoplasts are currently being conducted in this laboratory.

The data presented in this paper demonstrate that differences in the sensitivity to cercosporin of *N. crassa* and *C. nicotianae* may be attributed to differences in their cell walls. Production of cell wall components in *C. nicotianae* correlates with the ability of this fungus to grow apparently unharmed in the presence of high concentrations of the toxin, whereas presence of a cell wall in *N. crassa* does not bestow increased resistance on regenerating protoplasts. If cell wall components are responsible for resistance in *C. nicotianae*, then these components must therefore either be missing or inactive in *N. crassa*.

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