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A 96-Well Epifluorescence Assay for Rapid Assessment of Compounds Inhibitory to *Candida* spp.

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A rapid method for the screening of potential antifungal compounds was developed. A variety of compounds were tested against regenerating protoplasts of *Candida* spp. in a microtiter format. The degree of cell wall formation was assessed by staining with Cellufluor (Polysciences, Inc., Warrington, Pa.), a fluorochrome with known affinity for chitin, followed by determination of fluorescence by using a Dynatech Microfluor reader (Dynatech Laboratories, Inc., Alexandria, Va.). Compounds with known activity against the cell wall or cytoplasmic membrane of fungi inhibited wall synthesis in a concentration-dependent fashion. Treatment with 5-fluorocytosine, however, resulted in no inhibition. In general, protoplasts of *C. albicans* regenerated more quickly and were more sensitive to the compounds tested than protoplasts of *C. tropicalis* and *C. parapsilosis*. While the described method is not specific for a given class of antifungal agents, it may prove useful for testing large numbers of compounds quickly.

Traditional methods of antifungal susceptibility testing, i.e., MIC testing with agar or broth dilution techniques, play an indispensable role in the screening of compounds for antifungal activity. These methods suffer several drawbacks, however, and have not yet achieved the level of standardization of antibacterial testing. In addition to these considerations, MIC testing can take from 48 h to 7 days, depending upon the fungi tested, and requires several milligrams of compound if done on a macro scale.

Because it has been suggested that compounds which inhibit the synthesis of fungal cell walls may have potential as chemotherapeutic agents against mycotic agents (1), the possibility of screening existing agricultural fungicides and pesticides has been reported (3, 14, 15). The practicality of screening such compounds by using present susceptibility methodologies would preclude all but a random sampling of the large numbers in existence. We report a novel method for the screening of compounds active directly or indirectly against chitin biosynthesis which has an extremely short incubation time and minimal raw material requirements.

MATERIALS AND METHODS

Organisms and conditions of culture. The following *Candida* strains were utilized in this study. *C. albicans* C-6, C-15, C-194, C-215, and C-278 were clinical isolates from M. D. Anderson Medical Center, Houston, Tex., Tulane University Medical Center, New Orleans, La., and Hartford Hospital, Hartford, Conn.; strain C-DCH was isolated from a case of diaper dermatitis by one of us (R.F.H.); strain B311 was originally obtained from H. Hasenclever, National Institutes of Health, Bethesda, Md. *C. tropicalis* C-8 and C-13 and *C. parapsilosis* C-14, C-20, and C-38 were clinical isolates obtained from M. D. Anderson Medical Center. The species of all strains were reconfirmed by germ tube tests, chlamydospore production on cornmeal agar, and appropriate carbon assimilation testing. Isolates were maintained on slants of modified Sabouraud dextrose agar by periodic transfer at least twice annually and were stored at 4°C. Prior to use, strains were grown overnight in 50 ml of glucose yeast-extract broth at 30°C with shaking (180 rpm) and then transferred to 50 ml of fresh broth for 2 h of additional incubation. The cells were centrifuged at $1,500 \times g$ for 5 min and washed with phosphate-buffered saline twice before the formation of protoplasts.

Formation of protoplasts. Approximately 1 ml of packed cell volume of each strain to be converted to protoplasts was transferred to a 250-ml flask containing 50 ml of 0.5 M sodium thioglycolate (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M Tris (Eastman Kodak Co., Rochester, N.Y.), pH 8.7. Flasks were incubated at 37°C with shaking at 100 rpm for 30 min. After centrifugation, the cells were washed once with 0.2 M phosphate–0.1 M citrate buffer containing 0.6 M KCl as an osmotic stabilizer (PCB-KCl). The cells were resuspended in 4.5 ml of PCB-KCl in a 50-ml flask, and 0.5 ml of β-glucuronidase type H-2 (Sigma) was added. The flasks were incubated at 37°C with shaking at 100 rpm for 1 h. The degree of formation of protoplasts was assessed by light-microscope observation of lysis in hypotonic solution. Preparations yielding <99% lysis were not used. Protoplasts were washed three times in PCB-KCl and were stored in 5 ml of the same buffer at 4°C until required, up to a maximum of 3 days.

Conditions of assay. The synthetic amino acid medium of Lee et al. (13) supplemented with 0.5 M $MgSO_4$ was used for all experiments. Except where noted, the following compounds were dissolved directly in the growth medium for assay. Nikkomycin X, Z, CZ, and D were kindly supplied by Klaus Schaller, Bayer AG, Wuppertal, Federal Republic of Germany. Polyoxin D was obtained from Calbiochem-Behring, La Jolla, Calif. 2-Deoxy-D-glucose was obtained from Eastman Kodak. Plumbagin was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., and was first dissolved in a minimal volume of 95% ethanol. Caffeine was obtained from Sigma. Amphotericin B, provided as Fungizone (dissolved as directed), and 5-fluorocytosine were kindly supplied by Ed Bernard, Memorial Sloan-Kettering Cancer Center, New York, N.Y. Ketoconazole (Janssen

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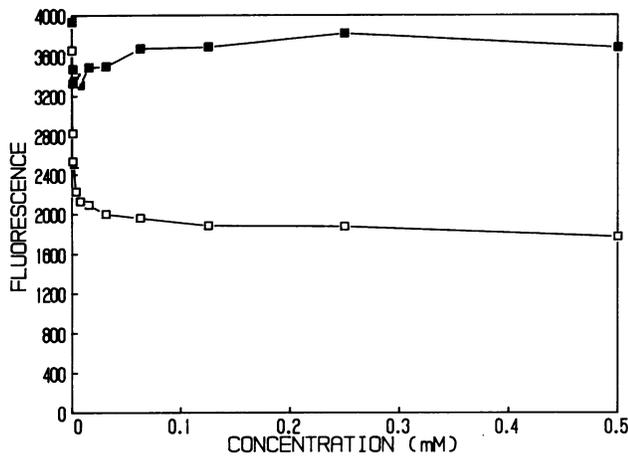
FIG. 1. Protoplasts of *C. albicans* C-215 treated with nikkomycin X (□) and polyoxin D (■). While there is demonstrable inhibition by nikkomycin X, the effects of polyoxin are negligible.

Pharmaceutica Inc., Piscataway, N.J.) was first dissolved in 0.2 N HCl. Bifonazole, clotrimazole, and BAY n 7133 are Miles Pharmaceuticals products and were dissolved in 95% ethanol.

U-bottomed, black plastic 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were used for all assays. Compounds to be tested were diluted in quadruplicate by using volumes of 100 μl in a twofold serial fashion. Four wells were reserved for untreated controls. Inocula were prepared by adjusting the concentration of the protoplasts to an optical density of 0.9 at 530 nm in PCB-KCl, which was then diluted 1:10 in the growth medium. Plates were inoculated immediately with 100 μl of the suspension per well and incubated at 30°C for 2 h.

Staining and reading of plates. At the end of the incubation period, 50 μl of Cellufluor (Polysciences, Inc., Warrington, Pa.), 300 μg/ml in phosphate-buffered saline, a fluorochrome with known affinity for chitin, was added to each well. The microtiter plates were incubated an additional 5 min followed by centrifugation at 800 × g for 6 min. The supernatants were removed by inverting the plates and snapping the

wrist in a downward motion. The cell pellets were washed with 200 μl of phosphate-buffered saline and centrifuged for 4 min at 800 × g, and the wash was repeated. Finally, the washed pellets were resuspended in 100 μl of phosphate-buffered saline, and the plates were read with a Dynatech Microfluor reader. Fluorescence was expressed as methylumbelliferone units per well, and values for each four-well set were averaged.

Validity of assay. The use of Cellufluor and the determination of fluorescence as a quantitative tool to assess chitin synthesis in regenerating protoplasts was tested in experiments to be described elsewhere (R. F. Hector, P. C. Braun, M. Kamark and J. Hart, submitted for publication). Briefly, in parallel experiments protoplasts of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* were introduced into duplicate sets of regeneration medium. In one group, *N*-[³H]acetylglucosamine was also introduced. Samples were removed at intervals and processed for staining with Cellufluor or for scintillation counting after acid-alkali digestion. Samples stained with Cellufluor were analyzed by flow cytometry with excitation in the UV range, and histograms were collected. Data were compared for peak and mean fluorescence versus incorporation of label over time and judged to be similar.

RESULTS

Chitin synthetase inhibitors. Initial experiments were conducted with compounds known to be competitive inhibitors of fungal chitin synthetase. Figure 1 shows representative data from an isolate of *C. albicans* treated with various concentrations of nikkomycin X and polyoxin D (note that although several isolates were tested against each compound, only representative figures are presented). While chitin and cell wall synthesis, as measured by the staining intensity of Cellufluor, was depressed in an exponential fashion with increasing concentrations of nikkomycin X, polyoxin D appeared to have little effect with the conditions employed. The isolates of *C. tropicalis* appeared less affected by nikkomycin X, while polyoxin D again appeared ineffectual (Fig. 2). Two isolates of *C. parapsilosis* showed minimal responses to both compounds (Fig. 3). Nikkomycin X and nikkomycin Z were compared by using eight isolates
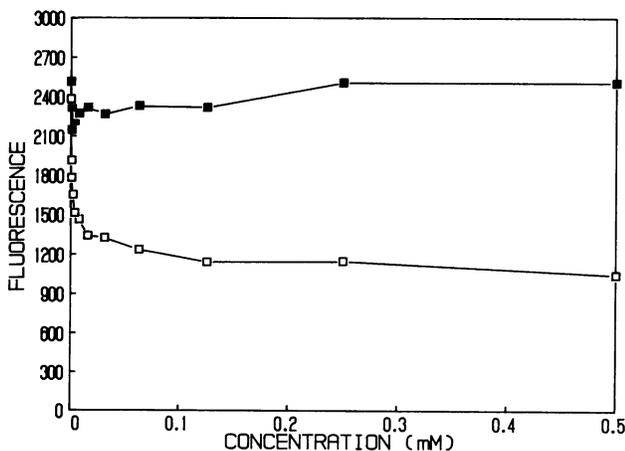

FIG. 2. Protoplasts of *C. tropicalis* C-13 treated with nikkomycin X (□) and polyoxin D (■). In general, the effects are similar to those shown in Fig. 1, except that the levels of fluorescence at all concentrations are not as high.
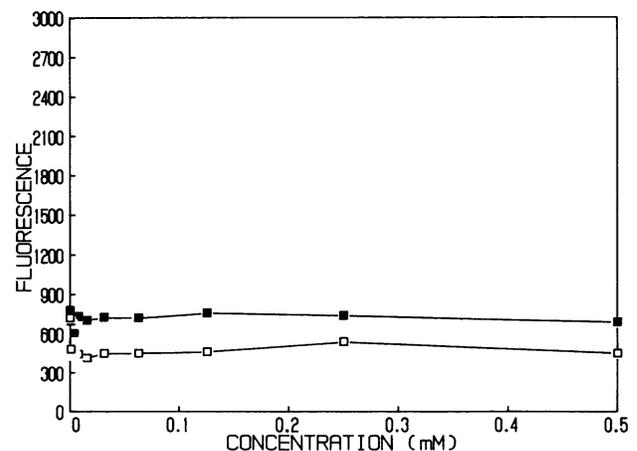

FIG. 3. Protoplasts of *C. parapsilosis* C-20 treated with nikkomycin X (□) and polyoxin D (■). The effects of both compounds are minimal, and the levels of fluorescence indicate that little regeneration has occurred.
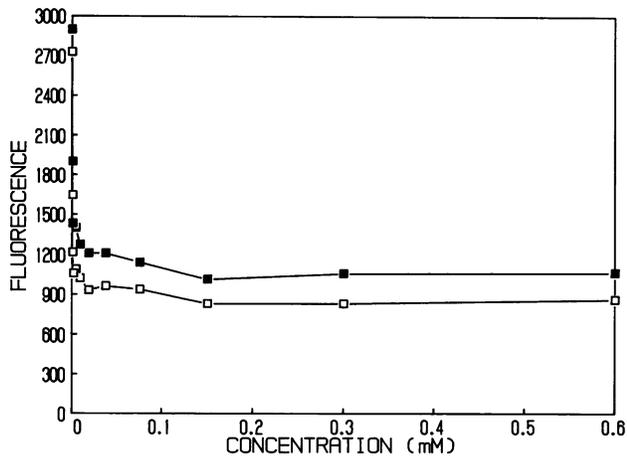
FIG. 4. Protoplasts of *C. albicans* C-278 treated with nikkomycin X (□) and Z (■). While the curves are similar, all strains tested showed slightly greater inhibition with nikkomycin X.

FIG. 6. Protoplasts of four strains of *C. albicans* treated with 5-fluorocytosine. Overall, changes in fluorescence indicate minimal effects by the compound over the interval tested.

of *C. albicans*, and a representative graph is presented (Fig. 4). Nikkomycin X showed greater inhibition of the isolates than did nikkomycin Z. Nikkomycin CZ and D showed no inhibition at any of the concentrations tested (data not shown).

The effects of temperature on the assay system with nikkomycin X were investigated. Duplicate plates inoculated with *C. albicans* C-6 and C-15 were incubated at 30 or 37°C for the 2-h incubation period. Although the curves were similar in shape (not shown), the plates incubated at 37°C had a higher base line.

Other antifungal compounds. Three classes of antifungal compounds available for human use were tested in the assay system to determine their effects on the regenerating protoplasts. Figure 5 shows the rather pronounced effects of amphotericin B on four isolates of *C. albicans*. Use of 5-fluorocytosine in the assay (Fig. 6) resulted in little decrease in fluorescence in treated *C. albicans* protoplasts versus untreated controls. Use of four azoles against isolates of *C. albicans* (Fig. 7) showed the two topical agents bifonazole and clotrimazole to be more active than ketoconazole and BAY n 7133. Ketoconazole was tested against *C.*

tropicalis and *C. parapsilosis* (Fig. 8), and it caused a net reduction in fluorescence in *C. tropicalis* somewhat greater than that seen against *C. albicans*, but it had no activity against *C. parapsilosis*.

Less "traditional" compounds were also tested for their ability to inhibit cell wall regeneration. Plumbagin, a reputed inhibitor of chitin synthase (12), was found to be inhibitory at submicrogram concentrations (Fig. 9). 2-Deoxy-D-glucose was tested against six isolates of *C. albicans* and was found to be moderately inhibitory (Fig. 10). Caffeine, a naturally occurring xanthine (18), a class of compounds with known antifungal properties, demonstrated an ability to inhibit the cell wall regeneration process at millimolar concentrations (Fig. 11).

DISCUSSION

The use of regenerating protoplasts of fungi as a tool for assessing certain classes of antifungal substances has several advantages over the more traditional methods of susceptibility testing or isolated enzyme assays. While MIC methods can be used in a microtiter format, the periods of incubation
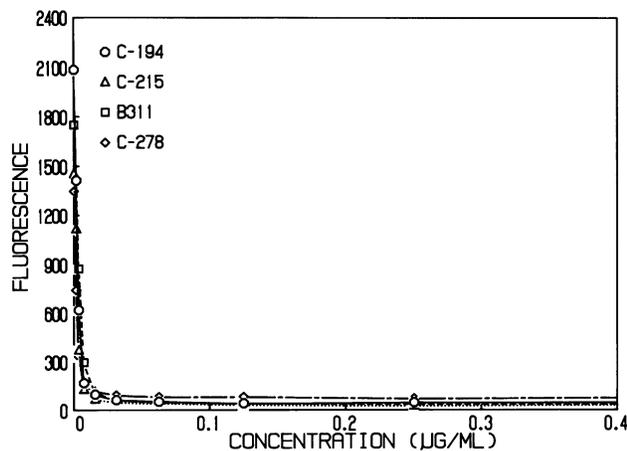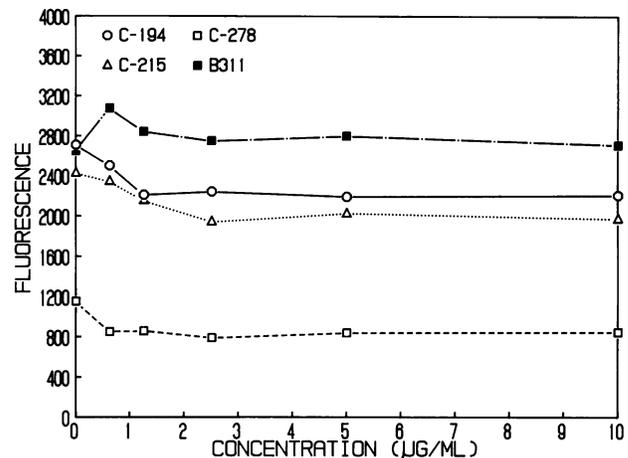

FIG. 5. Protoplasts from four strains of *C. albicans* treated with amphotericin B showing complete inhibition of growth at submicrogram concentrations.
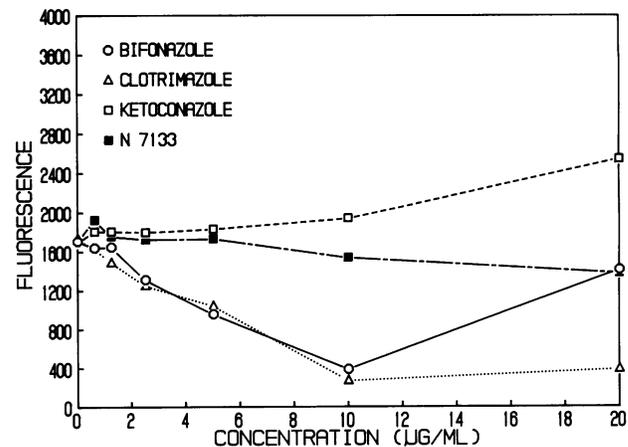

FIG. 7. Protoplasts of *C. albicans* C-215 treated with four azole compounds. Increases in fluorescence seen at concentrations greater than 10 μg/ml are most likely because of the insolubility of azoles at higher concentrations.
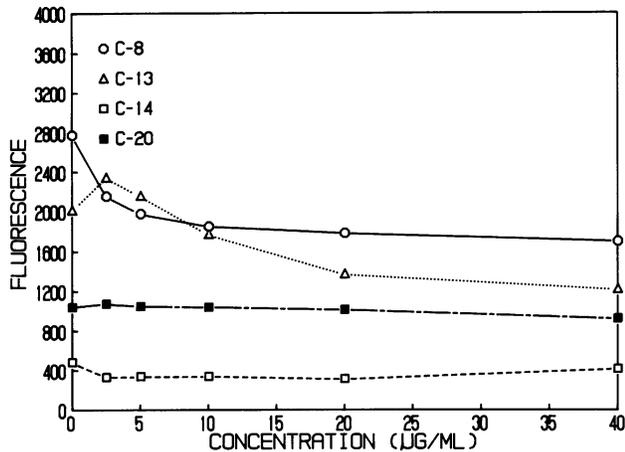
FIG. 8. Protoplasts of *C. tropicalis* (C-8 and C-13) and *C. parapsilosis* (C-14 and C-20) treated with ketoconazole. While inhibition is evident with the strains of *C. tropicalis*, no inhibition was seen with *C. parapsilosis*.

FIG. 10. Protoplasts from six strains of *C. albicans* treated with 2-deoxy-D-glucose, a nonmetabolizable sugar which is a competitive inhibitor with glucose.

range from 48 h to 7 days, depending upon the fungi tested. Such methods give quantitative data on the effects on cell growth but no information on specific effects of a given compound. Isolated enzyme assays require tedious procedures to purify the desired enzyme system, require rather defined conditions and substrates, typically involve the use of expensive radiolabels, and are not practical for testing several compounds at several different concentrations.

The described assay system appears to be well suited for compounds known to be inhibitors of fungal chitin synthase, with most data compatible with previously known properties of this class of substances. As an example, nikkomycin X proved to be slightly more active than nikkomycin Z, which is consistent with previous data on the inhibition constants of both compounds (0.5 versus 3.5 μM, respectively) (17). Nikkomycins D and CZ are known to be biologically inactive (8) and showed no activity in the test system. Somewhat puzzling, however, was the apparent lack of activity of polyoxin D in the assay system since polyoxin D has a K_i value nearly identical to those of the nikkomycins (6).

Factors other than the inhibitory potential of the compound for chitin synthase would appear to play a role, however. In a study on germ tube emergence by *C. albicans*, Chiew et al. (4) found that polyoxin D did not affect germ tube emergence, concluding that the compound was unable to permeate the cell envelope. Supporting this hypothesis is the report of McCarthy et al. (16), who found polyoxin D to be transported intracellularly at a much slower rate in *C. albicans* than nikkomycin Z. Becker et al. (1), however, reported that polyoxin D did have an effect on cell viability at millimolar concentrations, but that this effect was strain dependent. Therefore, our findings seem to indicate the superiority of the nikkomycins over polyoxin D against *Candida* spp. in vitro.

The other compounds tested gave variable results, demonstrating that inhibition of regeneration by protoplasts could be affected by compounds other than chitin synthase inhibitors. The antifungal drug amphotericin B showed high levels of activity at submicrogram quantities. This probably was due to lysis of the protoplasts, which is consistent with the known mechanism of activity of the compound, rather than a direct inhibition of chitin synthase (9). It should be pointed
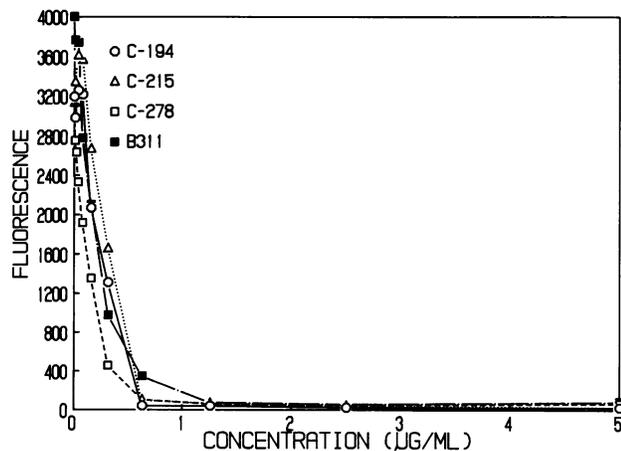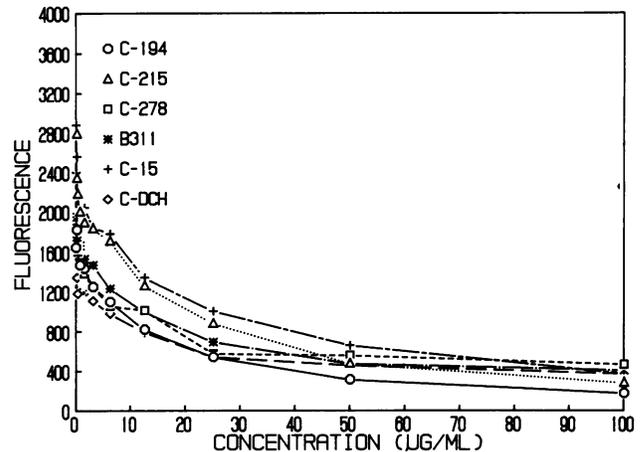

FIG. 9. Protoplasts of four strains of *C. albicans* treated with plumbagin, a reputed inhibitor of chitin synthetase. The dramatic drop in fluorescence suggests effects other than inhibition of chitin synthesis may also be contributory to cell death.
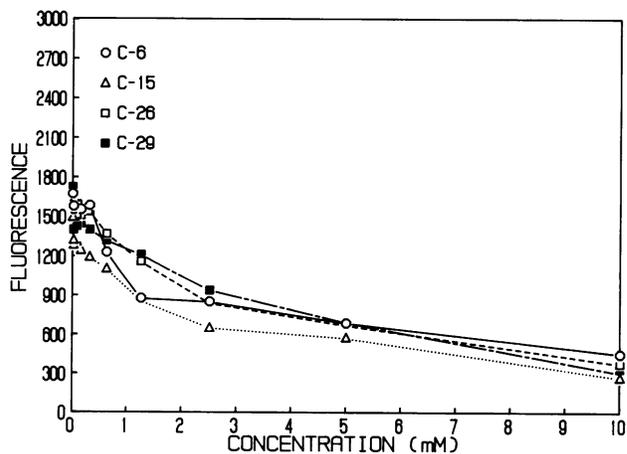

FIG. 11. Protoplasts from four strains of *C. albicans* treated with caffeine, a naturally occurring methylxanthine.

out, however, that amphotericin B does repress chitin synthesis in an isolated enzyme system, as was reported by Rast and Bartnicki-Garcia (20). Results from tests of azole compounds, agents active against the cytoplasmic membrane, indicate greater inhibition of regeneration in *C. albicans* by bifonazole and clotrimazole compared to the orally administered ketoconazole and BAY n 7133. The lack of activity by 5-fluorocytosine on regenerating protoplasts, however, was not unexpected as the early events in the regeneration process appear to be limited to synthesis of cell wall materials which are mostly polysaccharide in nature (10). In a study of germ tube emergence by stationary-phase cells of *C. albicans*, Shepherd et al. (22) found that while emergence required net synthesis of protein and RNA, there was no synthesis of DNA. Thus, a lack of inhibition of the regeneration of the protoplasts by 5-fluorocytosine suggests a similar situation.

2-Deoxy-D-glucose has previously been demonstrated to serve as a competitive inhibitor against various metabolic pathways in fungi (2, 7, 21, 23), and the data reflect this with a smooth, concentration-dependent decrease in fluorescence.

The use of Cellufluor as a quantitative tool has, with one exception (11), been exploited only recently (19). The use of regenerating protoplasts followed by staining is a more sensitive system than use of whole cells for two reasons. (i) Since protoplasts start with negligible staining material while whole cells have a substantial component of chitin, depression of new wall synthesis is easier to detect. (ii) Regenerating protoplasts of *C. albicans* have a higher content of chitin than whole cells (5), thus further increasing the sensitivity of those cells to inhibitors of cell wall synthesis. In practical terms, this translates into an ability to assay by using short incubation times. The availability of a commercial reader able to detect fluorescence at a suitable wavelength has allowed the adaption of these principles into a rapid procedure which should prove useful for both basic research and antifungal screening programs.

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