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Effect of propagule size on the in vitro production of polygalacturonase and cellulase by *Rhizoctonia solani*[1]

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The physiological behavior of large (250–500 microns (μ)) infective and small (50–150 μ) non-infective mycelial fragments (propagules) of *Rhizoctonia solani* was compared. Liquid Czapek's medium containing sodium polypectate (NaPP) as the sole carbon source was inoculated with equal amounts (on a dry weight basis) of each kind of propagule. At NaPP concentrations of 0.005%–0.25% large propagules produced more polygalacturonase (PG) after a shorter period of incubation than small ones, whereas no differences in PG activity were observed at 1% NaPP. Differences in PG activity between large and small propagules were also observed in a medium consisting of 1:50 dilution of potato extract, but not in richer media such as potato dextrose broth and bean hypocotyl medium. These differences did not depend on pH of the medium or dry weight of the mycelium. Germination percentage of the large propagules and length of their emerging hyphae were greater than those of the small ones in media containing 0.005%–0.25% NaPP. Extracellular cellulase activity in Czapek's medium containing 0.25% carboxymethyl cellulose was also higher and appeared earlier in large propagules as compared with small ones. Viscometric and colorimetric methods for detecting PG and cellulase activities produced similar results. It is suggested that the capacity of the large propagules to produce hydrolytic enzymes at low nutrient levels may be important for their parasitic activity.

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Chez *Rhizoctonia solani* nous avons comparé le comportement physiologique de gros fragments mycéliens (propagules) infectifs (250–500 microns (μ)) et des petits fragments non infectifs (50–150 μ). Le milieu liquide Czapek contenant du polypectate de sodium (NaPP) comme seule source de carbone, fut inoculé avec des quantités égales sur une base de poids sec de chaque sorte de propagules. A des concentrations de NaPP de 0.005%–0.25%, les grosses propagules produisent plus de polygalacturonase (PG) après une courte période d'incubation, tandis que les petites propagules en produisent moins; il n'y a pas de différence dans l'activité de la PG à des concentrations de 1% de NaPP. Les différences dans l'activité de la PG entre les grosses et les petites propagules sont aussi observées dans un milieu composé de 1:50 d'extrait de patates, mais non pas dans des milieux plus riches tels que un bouillon au dextrose et patate et un milieu à l'hypocotyl de fève. Ces différences ne dépendent pas du pH du milieu ou du poids sec du mycélium. Le pourcentage de germination des grosses propagules et la longueur de leurs hyphes germinatifs sont plus grands que celles des petites dans des milieux contenant de 0.005%–0.25% de NaPP. L'activité extracellulaire de la cellulase dans un milieu Czapek contenant 0.25% de carboxyméthyl cellulose est aussi plus élevée et apparaît plus tôt chez les grosses propagules que chez les petites. Des méthodes viscométriques et colorimétriques pour détecter des activités de la PG et de la cellulase produisent des résultats semblables. Nous suggérons que la capacité des grosses propagules à produire des enzymes hydrolytiques à de faibles niveaux de nutrients peut être important pour leur activité parasitique.

[Traduit par le journal]

Introduction

The effects of inoculum density of soil-borne pathogens on plant diseases has been studied extensively and is well covered in the literature (8). Less attention has been paid to the relation between disease and inoculum quality. Inoculum quality may be defined as the specific pathogenic capacity of different types of propagules under similar conditions. Henis and Ben-Yephet (10) showed that propagule size has a pronounced effect on the pathogenicity of *Rhizoctonia solani*, large propagules (250–500 microns (μ)) being much more infective than small ones (150 μ). They attributed this phenomenon to the nutritional reserves in the propagule. The relationships between nutrition and the virulence of *R. solani* has been demonstrated by Weinhold *et al.* (19, 20). A positive correlation between inoculum size and infection has been found by Altson (1) and Whitney (21), who worked with *Fomes lignosus* and *Rhizoctonia crocorum*, respectively.

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The production of pectolytic enzymes, e.g. polygalacturonase (PG), is considered to be of great importance in the pathogenesis of *R. solani* (7, 12). However, the correlation between production of PG by this fungus in vitro and virulence of the pathogen is still being questioned (7, 9). *Rhizoctonia solani* also produces cellulase which assists the penetration of the fungus into host cells (6).

The purpose of this study was to compare the production of PG and cellulase by infective and noninfective propagules of *R. solani* under various physiological conditions.

Materials and Methods

Inoculum

The isolate of *Rhizoctonia solani* Kuehn used in this experiment was highly pathogenic to beans, onion, and watermelon. It was grown in liquid yeast extract medium (10) at 27C for 7 days in standing cultures. The resulting mycelial mat was removed and homogenized in a Waring Blendor for 1 min with sterile 0.02 M citrate buffer containing 0.1 M KCl at pH 4.6. The homogenized mycelium was separated on plastic sieves into "large" (250–500 μ) and "small" (50–150 μ) propagules which were separately washed with the same buffer solution. Buffer was used for washing the propagules since preliminary experiments indicated that if sterile water was used instead, a considerable amount of PG was released upon transferring the homogenized mycelium to a fresh medium. This is probably the result of release of cell-bound enzyme which is thought to be due to the ionic effect of the solution (3). The propagules on each sieve were further washed with a solution containing the mineral salts of Czapek's medium; they were then transferred separately to flasks and washed twice by decantation. The whole process of propagule preparation was carried out aseptically at 4C.

Samples of large and small propagules equivalent in weight to 1 mg dry weight (80C) were used for media inoculation unless otherwise stated. Pathogenicity tests with bean seedlings confirmed a previous report (10) that only large propagules are pathogenic.

Enzyme Production

The rate of enzyme production as well as propagules outgrowth were determined on the following media: (i) basal medium consisting of Czapek's salt solution in which the sole carbon source was either sodium polypectate (NaPP, Sigma Chemical Co.) as PG inducer or carboxymethyl cellulose (CMC, British Drug Houses (BDH)) as cellulase (C_x) inducer. (ii) Wiese *et al.* medium (22) containing dextrose, citrus pectin grade II (Sigma Chemical Co.), and potato extract (PDP). (iii) One to 50 diluted extract of 200 g potatoes boiled in 1 liter distilled water (P/50). For suppression of bacteria, 250 μg/ml chloramphenicol (Abic, Israel) was added to these media. (iv) Autoclaved hypocotyls of 9-day-old bean seedlings cut into segments of 0.5 cm length. Fifteen grams of the segments were mixed with 2 ml distilled water in 100-ml flasks before they were autoclaved. Unless otherwise stated, equivalents of 1 mg dry weight of either large or small propagules were inoculated into 100-ml flasks containing 17 ml liquid medium. The flasks were incubated at 27C in the dark in a shaker bath (90 strokes/min). Preliminary experiments showed that less enzyme was produced at a higher shaking speed. After various periods of incubation the contents of each flask were centrifuged at 12 500 rpm for 20 min at 4C. Enzymatic activity was assayed in the supernatant liquid whereas the dry weight of the mycelium was estimated in the sediment after drying at 80C for 48 h. Cultures containing autoclaved bean segments were incubated without being shaken. The enzyme was extracted at 4C by homogenizing the flask content for 3 min with 15 ml distilled water, using a Sorvall omni-mixer. The homogenate was centrifuged at 12 500 rpm for 20 min at 4C. Enzymatic activity was assayed in the supernatant liquid. Experiments were conducted in duplicate and were repeated at least once.

Assay of Enzymatic Activity

The substrate solutions were prepared 1 day before use and stored at 4C. Aliquots of 2.5 ml of the supernatant to be tested were mixed with 6 ml of either 1.2% NaPP or 1% CMC both in 0.05 M citrate buffer at pH 4.8 for the determination of PG and cellulase activities, respectively. Viscosity of the reaction mixture was determined with an Ostwald viscometer at 30C, after an incubation period at 30C.

Unless otherwise stated, relative enzymatic activity (RA) was defined as 1000 × the reciprocal of time in minutes required for a 50% reduction in the viscosity of the substrate (5). Reduction in the viscosity of the reaction mixture containing NaPP was considered to be 100% if it reached the viscosity of a reaction mixture without NaPP under identical conditions. Zero reduction in viscosity was taken as the viscosity of a reaction mixture containing a supernatant which had been collected immediately after inoculating the medium and which was mixed at once with the substrate.

Enzymatic activity was also determined colorimetrically with a Coleman Junior II spectrophotometer, using dinitrosalicylic acid (DNS) reagent (13) in which the Rochelle salts were removed (18). The reaction mixture was the same as that used for the viscometric method, but with acetate buffer pH 4.8, 0.02 M instead of citrate buffer in the PG activity assay (13). The incubated reaction mixture was diluted 10-fold and 3 ml was mixed with an equal amount of the DNS reagent for the colorimetric determination. The developing color was read in 10 × 75 nm photometric tubes at 575 nm. Standard curves of α-D-monogalacturonic acid (Sigma Chemical Co.) and glucose were used for calculation of PG and cellulase activity, respectively.

Characterization of the Pectolytic Enzyme

Characterization of polygalacturonase was conducted by chromatography. Thin-layer plates were coated with microcrystalline cellulose (Sigmacell type 19, Sigma Chemical Co.). Chromatograms were loaded with 15-μl aliquots of enzyme preparation. They were run ascendingly to 17 cm using ethyl-acetate : acetic acid : water (2:1:2 v/v) as the solvent system (2) and dried under a fan at room temperature, and compounds were located by spraying with 0.5% $AgNO_3$ (16). α-D-Monogalacturonic

acid (Sigma Chemical Co.) was used as a standard ($R_f = 0.57$). Chromatography of the incubated reaction mixture revealed the appearance of five reducing products formed from the NaPP substrate in addition to monogalacturonic acid. Since these products had R_f values at constant intervals (0.05–0.07), which were lower than those of monogalacturonic acid, it was assumed that they were oligomers of galacturonic acid, ranging from the dimer through the hexamer (2, 15).

Results

Effects of R. solani Propagule Size and NaPP Concentration on PG Activity

Large and small propagules of *R. solani* were incubated in a medium containing various concentrations of NaPP. PG activity, pH of the growth medium, and dry weight of mycelium were recorded periodically during the incubation period, and these parameters were affected by the concentration of NaPP in the medium (Fig. 1, A–C). In all cases, PG activity followed a typical pattern of an initial increase up to a peak, followed by a decrease. At a concentration of 0.005% NaPP, the PG activity of large propagules developed faster and reached a higher peak than the activity of small ones (Fig. 1A). The peaks reached 290 and 150 RA and were obtained after 28 and 40 h, by the large and the small propagules, respectively. After 28 h the PG activity of the large propagules was six times that of the small ones. Slight but detectable PG activity was observed 8 h after medium inoculation only in the case of the large propagules. No enzymatic activity could be detected in the case of small propagules even after extending the incubation period of the reaction mixture. Similar results were obtained with media either containing or lacking chloramphenicol. No increase in dry weight of mycelium could be detected. Apparently the amount of carbon in the medium was too low to allow such an increase. However, the propagule did germinate, giving rise to protruding hyphae.

In another experiment, the PG activity of 1 mg of large propagules was compared to that of 1–4 mg of small propagules in a medium containing 0.005% NaPP. Polygalacturonase activity of both small and large propagules reached its peak (300 RA) when the inoculum concentrations were 2 mg and 1 mg per flask, respectively.

When NaPP concentration was increased 50-fold to 0.25%, PG activity of large propagules again developed earlier, reached its peak faster, and was higher than that of small ones (Fig. 1B). However, the ratio between peaks of PG activity of the two fractions decreased to 4:3, from the 2:1 ratio obtained with 0.005% NaPP. Increasing NaPP concentration resulted in a delay in the appearance of the peak of enzymatic activity, and in an increase in dry weight of mycelium and pH. However, PG activity was unexpectedly lower than that obtained when the inducer was at a concentration of 0.005%.

At the highest concentration of NaPP tested (1%), the pattern of development and level of PG activity was very similar in both fractions (Fig. 1C); about 2000 RA units were detected after 78 h. At this concentration of NaPP, the highest values of PG activity and pH as well as greatest dry weights of mycelium were observed. The increased pH might result from the release of Na^+ ions from NaPP. The small propagules produced detectable amounts of PG 8 h after inoculation only with this medium. In the two media where increases in dry weight of mycelium were observed, the total dry weight of large propagules was higher than that of small ones and the peak in enzymatic activity preceded maximum growth.

In the absence of NaPP or at a concentration of 5×10^{-4}%, PG activity of both fractions was lowest. Large propagules produced more enzyme than the small ones; peaks of the PG activity being 6.0 and 2.5 RA in the absence of NaPP, and 26.0 and 8.0 RA at 5×10^{-4}% NaPP, for the large and the small propagules, respectively. No changes in dry weight of mycelium or in pH values were observed throughout the experiment. When the medium consisted of only sterile tap water, no enzymatic activity could be detected even with an inoculum of 10 mg/flask and after the incubation period with the substrate was extended to 2 h. In this medium the propagules appeared as nonpigmented aggregates in contrast to the normally pigmented germinating propagules.

The results obtained with the viscometric method were also verified using a colorimetric method (Fig. 1A).

PG Activity in Media Containing Plant Material

When *R. solani* was grown in a relatively poor medium (P/50), the ratio between the activity level of PG of large and small propagules was highest. Medium pH values and dry weight of

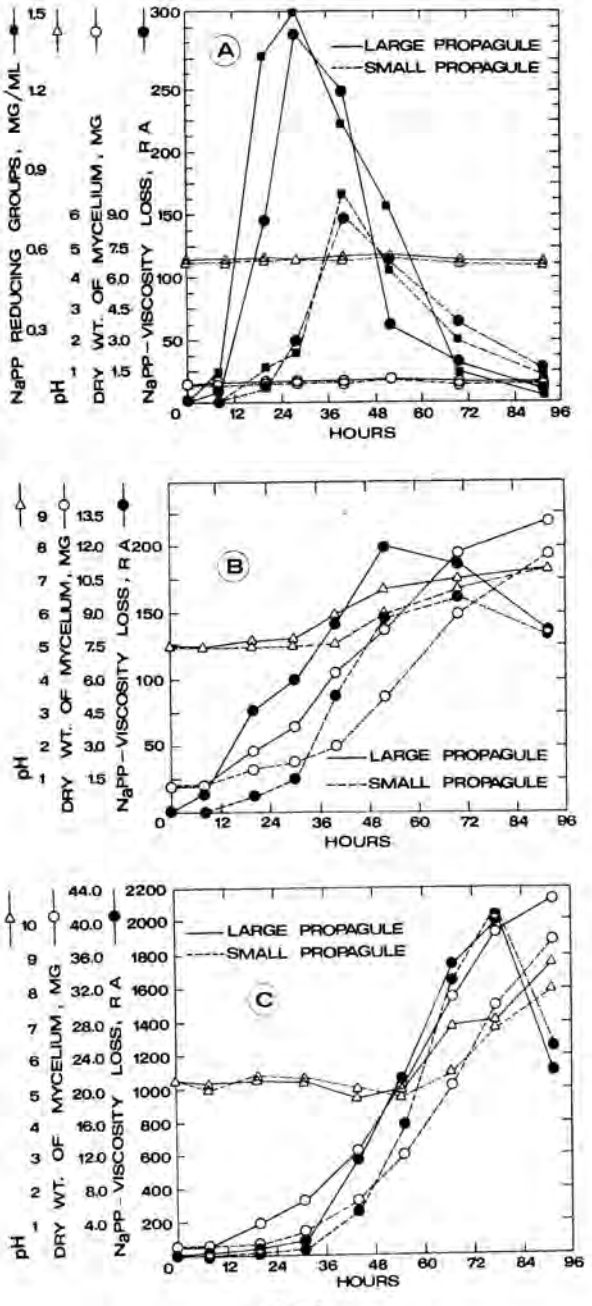

FIG. 1

FIG. 1. Effect of sodium polypectate (NaPP) concentration and *Rhizoctonia solani* propagule size on changes in time of polygalacturonase activity (viscometric (●) and colorimetric (■) methods), weight of mycelium, and pH of the growth medium. Colorimetric tests expressed as mg/ml of monogalacturonic acid. NaPP concentration: (A) 0.005%; (B) 0.25%; (C) 1%.

FIG. 2. Effect of media containing plant material and of *Rhizoctonia solani* propagule size on development of polygalacturonase activity, weight of mycelium, and pH of the medium. (A) 1:50 potato broth; (B) potato–dextrose–pectin medium; (C) autoclaved bean hypocotyls.

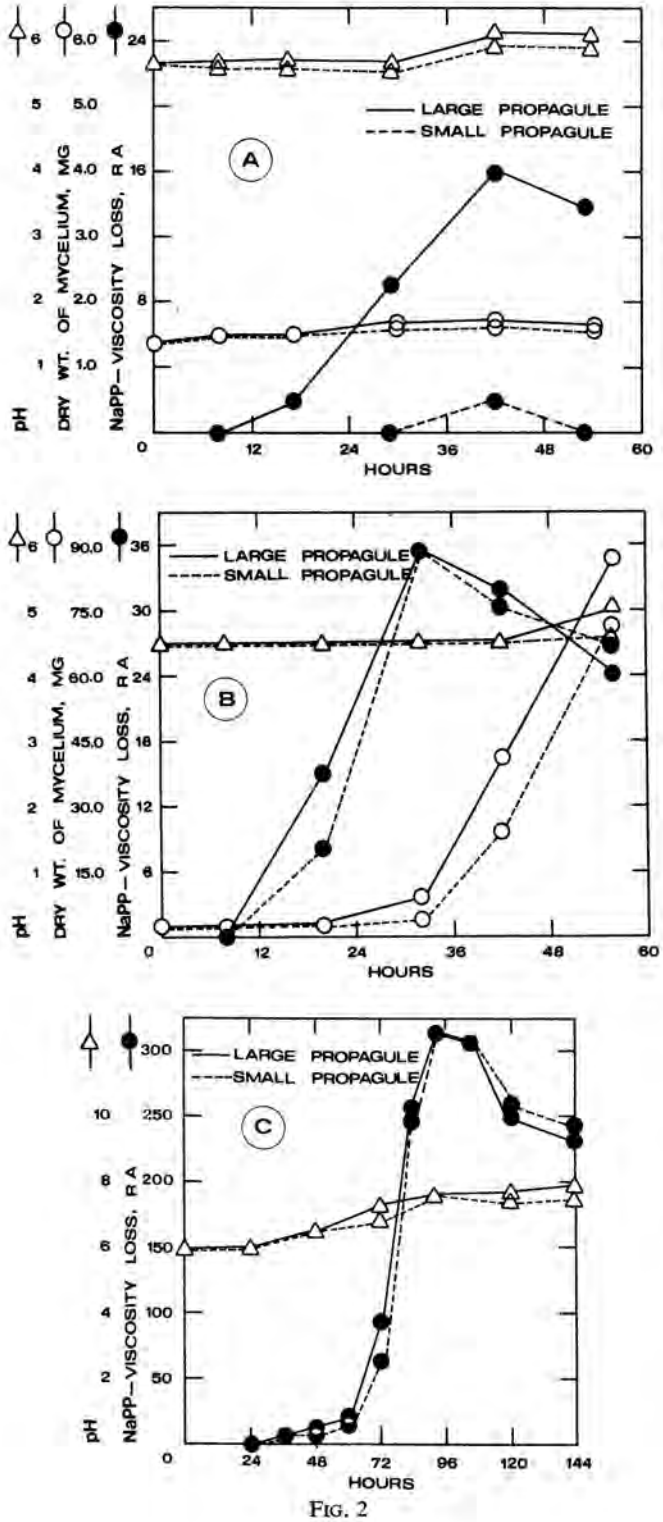
A
LARGE PROPAGULE
SMALL PROPAGULE
pH
DRY WT. OF MYCELIUM, MG
NaPP–VISCOSITY LOSS, R A
HOURS
B
LARGE PROPAGULE
SMALL PROPAGULE
pH
DRY WT. OF MYCELIUM, MG
NaPP–VISCOSITY LOSS, R A
HOURS
C
LARGE PROPAGULE
SMALL PROPAGULE
pH
NaPP–VISCOSITY LOSS, R A
HOURS

FIG. 2

mycelium remained constant (Fig. 2A). These results are similar to those obtained with media containing low concentrations of NaPP (Fig. 1A). When grown on a relatively rich medium such as PDP or bean hypocotyl medium, PG production by the two types of propagule was similar (Fig. 2, B–C). Large and small propagules have therefore a similar saprophytic capacity on autoclaved bean hypocotyls. Enzyme production in PDP medium was poor, but fungal growth was greatest. No changes in the pH of the medium were observed (Fig. 2B). High activity of PG was observed on autoclaved bean hypocotyls (Fig. 2C). Since separation of the fungus from the medium is not possible, fungal growth was not measured. In this medium the pH value increased from 6.0 to 7.5.

The differences in enzymatic activity between the two propagule fractions inoculated in relatively poor media could not be correlated with changes in pH, dry weight of mycelium, or to their total enzymatic activity.

PG Activity of R. solani as Related to Propagule Germination

Germination of propagules, growth of the extruding hyphae, and PG activity in media with low (0.005%) and high (0.25%) concentrations of NaPP were followed during 10 h of incubation. Results illustrated in Fig. 3 indicated that the very low production of PG by small propagules at the beginning of the experiment was correlated with a lag in germination. However, the lag in germination and PG production was shorter in a richer medium containing 0.25% NaPP. In no case did enzyme production precede propagule germination.

Cellulase Activity of R. solani

In a medium containing 0.25% CMC, cellulase activity appeared earlier, progressed faster, and reached a higher peak in cultures containing large propagules as compared with cultures containing small ones (Fig. 4). This phenomenon is similar to that observed with PG activity. However, the

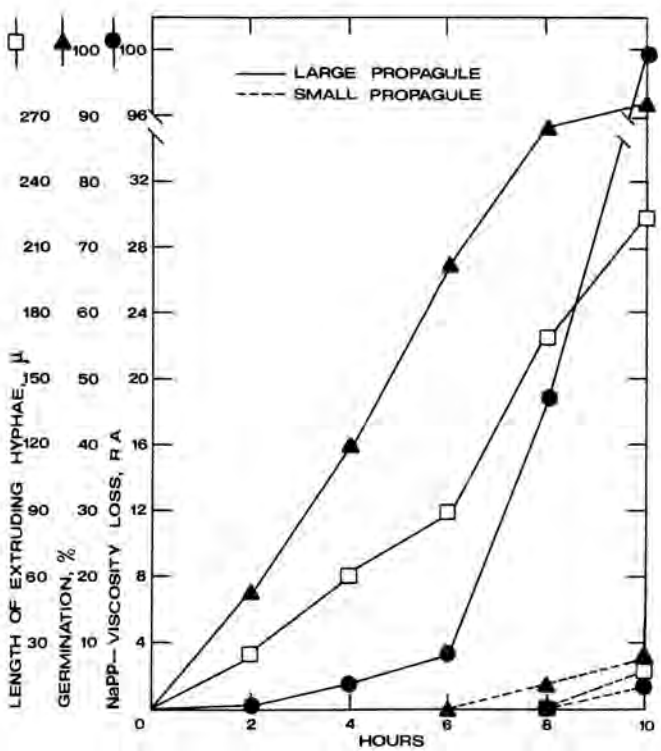

FIG. 3. Development of polygalacturonase activity, growth of extruding hyphae, and germination percentage of large (250–500 μ) and small (50–150 μ) propagules of *Rhizoctonia solani* in a 0.005% sodium polypectate basal medium. Inoculum concentration: 3 mg per flask.

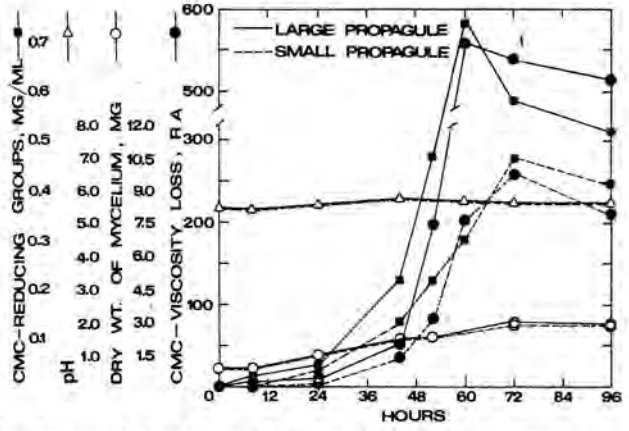

FIG. 4. Effect of carboxymethyl cellulose (CMC) at 0.25% and *Rhizoctonia solani* propagule size on cellulase activity (viscometric (●) and colorimetric (■) methods), weight of mycelium, and pH level of the growth medium. Colorimetric test expressed as mg/ml glucose.

decrease in enzyme activity once having reached the peaks is more gradual than was the case with PG activity. The higher ability of the large propagules to produce cellulase was also demonstrated by the colorimetric method (Fig. 4). Cellulase activity was much lower in media where the CMC concentration was reduced 50-fold to 0.005%. The large propagules, however, still produced more enzyme than the small ones; peaks of activity being 14 and 5 RA, respectively. In this medium no change in pH and dry weight was observed. Germination percentage, length of the extruding hyphae, and cellulase activity were followed in a medium containing 0.25% CMC (Fig. 5). Small propagules did not germinate during the first 12 h of incubation whereas

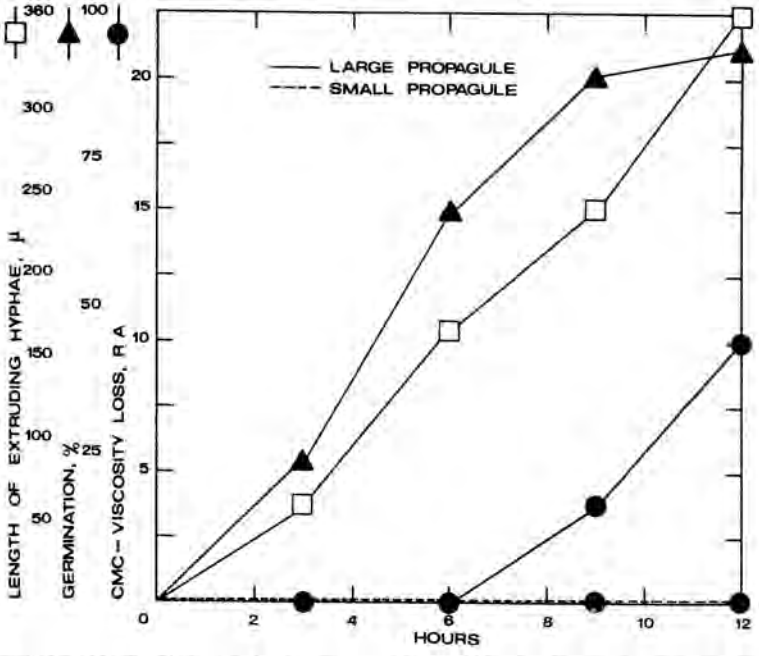

FIG. 5. Development of cellulase activity, growth of extruding hyphae, and germination percentage of large (250–500 μ) and small (50–150 μ) *Rhizoctonia solani* propagules in a basal medium supplemented with 0.25% carboxymethyl cellulose. Inoculum concentration: 3 mg per flask.

most of the large ones did so. In contrast to PG activity, no cellulase activity could be detected during the early stages of germination of large propagules.

When the cellulase activity of 1 mg of large propagules was compared with that of 1–4 mg of small ones in a medium containing 0.25% CMC, it was found that 4 mg of small propagules produced about the same peak of cellulase activity (550 RA) as that produced by 1 mg of the large ones.

Discussion

The size of the individual propagules is of importance in the study of PG and cellulase production by *R. solani*. The use of a fraction of homogenized mycelium of uniform size ascertains a high uniformity in pathogenicity (10) and rate of enzyme production, and shortens considerably the time period required for the appearance of the peak of PG production (14). Thus, pattern of enzyme production by *R. solani* propagules in our system resembles that occurring in infected tissues since lesion formation and high PG activity in tissues occur within 2 days (17).

Differences were found in vitro between the large infective, and the small noninfective propagules of *R. solani* regarding several properties which are considered of significance during pathogenesis. In relatively poor media containing a relatively low concentration of inducer and (or) nutrients, large propagules germinated and produced higher levels of PG and cellulase, faster and earlier than the small ones. These differences were greatest during the early stages of incubation. Increasing inoculum concentrations of the small propagules resulted in production of similar peaks of enzymatic activity. Since the small propagules were noninfective, the production of PG and cellulase activities by the two fractions could only be compared in vitro.

The higher PG activity produced by infective large propagules in all the poor media tested indicates a better ability to synthesize hydrolytic enzymes. The development of their PG activity supports its possible involvement in pathogenesis: it occurs concurrently with germination, in the presence of a very low amount of inducer or even in its absence, and its development is not accompanied by a mass increase of fungus mycelium nor by a rise in pH level which may have suppressed PG production (unpublished data). However, the correlation between the ability to produce PG in vitro and virulence is still an open question (7). Bateman (4) also showed that different types of PG are produced by this fungus under different growth conditions. Our results demonstrate that the correlation between PG production in vitro and virulence in *R. solani* depends on medium composition, as shown by Keen and Erwin (11) with *Verticillium*.

Weinhold *et al.* (19, 20) showed that virulence of *R. solani* is markedly influenced by inoculum nutrition and by the level of external nutrients available to the pathogen. Our results indicate that these factors also determine the capacity of the pathogen to produce pectolytic and cellulolytic enzymes. The capacity to produce these enzymes in response to small amounts of external nutrients depends on propagule size which determines the internal nutrients reserves of the pathogen. It may also be of great value to the pathogen in its competition with soil microflora for plant exudates at the infection site.

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