Cell Walls of *Phytophthora infestans* Contain an Elicitor of Terpene Accumulation in Potato Tubers

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ABSTRACT

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Homogenates of the mycelium of *Phytophthora infestans* were subjected to differential centrifugation and the fractions were applied to the upper surface of Kennebec potato slices. Lubimin and rishitin were determined in the upper 1 mm of slices 72–86 hr after application of the fractions. The fraction containing the 500-*g* pellet contained 70–90% of the terpene-eliciting activity, and most of the remaining activity was recovered in the fraction containing the 40,000-*g* supernatant. The fraction containing the 500-*g* pellet was also most active in eliciting browning. Living zoospores, cystospores, and sporangia of incompatible races of *P. infestans* caused the accumulation of high concentrations of lubimin and rishitin in potato slices. When the spores were killed by freezing followed by thawing, only cystospores and sporangia caused appreciable terpene accumulation. Empty sporangia had at least 75% of the terpene-eliciting activity of dead sporangia which had not released zoospores. Two methods were used to encyst zoospores. In one method, encystment was accomplished within 1 hr and in the other within 1.5 min. Dead cystospores produced by either method caused the accumulation of high concentrations of lubimin and rishitin in potato slices. It is concluded than an elicitor of terpene accumulation in potato tubers is associated with the cell wall of *P. infestans*.

Additional key words: phytoalexins, late blight, *Solanum tuberosum*.

The mycelium of *Phytophthora infestans,* but not culture filtrates or spore germination fluids, contains compounds (elicitors) which cause the accumulation of terpenes in potato (7,11,22–24,32). *Phytophthora megasperma* var. *sojae,* a pathogen of soybean, produces extracellular (1) and wall-associated (2,3) elicitors which cause the accumulation of isoflavonoids in soybean. Both elicitors produced by *P. megasperma* appear to be β-(1→3), β-(1→6) glucans containing 4% mannosyl residues (1,3). Lisker and Kuć (24) demonstrated that preparations from the Oomycetes, including *P. infestans,* elicited terpene accumulation in potato tubers. Similar preparations from other fungi did not.

Bartnicki-Garcia and his colleagues (4,5,17,20,27–31,34,35) have studied cell wall biosynthesis in Oomycetes and their data are pertinent to the work described in this publication. Bartnicki-Garcia and Hemmes (5) described the importance of β-glucans in the life cycle of Oomycetes: "The biochemical machinery of an Oomycete is largely committed to the production of different types of β-glucans used as pillars of the cell structure and as reservoirs of carbon and energy for differentiation (sporulation and spore germination)."

In all stages of the life cycle of *Phytophthora,* glucose polymers constitute 80–90% of the dry weight of cell walls (5,30). A major part of the glucose is present as insoluble highly branched β-(1→3), β-(1→6) glucans. The remaining glucose is present as a β-(1→4) glucan (36). The β-(1→3) glucan may be selectively removed from hyphal cell walls by exo- and endo-β-(1→3) glucanases (30), leaving an internal microfibrillar surface exposed.

In addition to cell wall glucans, the mycelium of *Phytophthora palmivora* also contains highly soluble β-(1→3) glucans as storage polysaccharides, mycolaminarans (5,35). These glucans are relatively small (DP 29–36), have up to two branches, and are neutral (35). Zoospores, sporangia, and cysts also contain mycolaminarans, but most are in the form of mycolaminaran phosphate (mycolaminaran-P). Mycolaminaran-P is absent from mycelia of *P. palmivora* (34).

Zoospores, which are natural protoplasts, apparently have the ability to synthesize a double layered cell wall de novo within 1–2 min, even in the presence of cycloheximide (4,5,14,17). Cyst germination, however, requires de novo protein synthesis and is inhibited by cycloheximide (17).

Upon encystment, the spores of *P. palmivora* excrete a sticky glycoprotein which contains mannose, glucose, and galactose (5,27). This glycoprotein, possibly secreted by peripheral vesicles, binds to concanavalin A (28). In the next (much longer) phase of encystment, zoospores lose the ability to adhere to solid surfaces or bind concanavalin A. They form a wall made of insoluble alkali-resistant β-(1→4) and β-(1→3) glucans. The glucans apparently arise from the complete hydrolysis of mycolaminarans and mycolaminarin phosphates to glucose and subsequent resynthesis. Glucans are then deposited in microfibrils formed from material excreted by peripheral vesicles (5). Zoospores and mycelium of *P. palmivora* contain an intracellular exo −β-(1→3) glucanase (20).

In view of the claim that elicitors of phytoalexin accumulation are present in cell walls of *Phytophthora* spp. and related genera of Phycomycetes (3,6,18,22–24,32), the use of zoospores and cystospores provides an attractive model system for the localization and eventual identification of the elicitor.

MATERIALS AND METHODS

A semimicro method, based on ethyl acetate extraction and gas chromatography, was used to determine the ability of fractions to elicit terpene accumulation (19). A spectrophotometric method was used to estimate browning. Tissue residues from the semimicro extraction were heated for 1 hr on a steam bath in 15 ml of 0.5 N sodium hydroxide. The solution was cooled and transferred to 3 ml disposable cuvettes (Markson Co., Del Mar, CA 92014). The optical density of this solution at 400 nm was used as a relative measure of the intensity of browning. Experiments were performed three times or as indicated. Tuber slices of the cultivars Kennebec (R1) or PI 20396 (R1.4) were used in all experiments. *P.* infestans (Mont.) de By, races 0, 4, 1, and 1.4 were maintained on lima bean agar and transferred every 7–10 days. Races 0 and 4 are

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incompatible on Kennebec and races 0, 4, and 1 are incompatible on PI 20396. All other interactions are compatible.

Differential centrifugation of mycelial homogenates. *P. infestans* was grown on lima bean broth for 10–15 days (24). The cultures were filtered through two layers of cheesecloth and approximately 5 g of the mycelium were washed thoroughly in tap water followed by distilled water, and then transferred to a beaker containing 30 ml of cold (0–2 C) extraction medium. This medium contained mannitol (0.6 M), EDTA (Na_2) (1 mM), $CaCl_2$ (5 mM), cysteine (4 mM), ascorbic acid (2 mM), phosphate buffer pH 6.5 (0.1 M). The mycelium was homogenized first with a Polytron homogenizer (Brinkman Instruments, Westbury, NY 11590) at full power in five bursts of 1 min each. The beaker was kept in an icebath during homogenization. The suspension was then transferred to a glass homogenizer in 5-ml portions, and each was further homogenized using 12 strokes of the homogenizer. The final homogenate was free of living fungus. The homogenate was then subjected to differential centrifugation (Fig. 1). Pellets were washed twice in 5 ml of the extraction medium and the washings were discarded. All fractions were suspended in 10 ml of distilled water, dialyzed against three changes of 1 L of distilled water at 4 C, and adjusted to a final volume of 50 ml. The original homogenate, the pellets, and the 40,000 *g* supernatant were applied to the surface of potato slices (0.1 ml per slice) and terpene accumulation in the slices was determined 72–86 hr later by the semimicro method (19). Protein and carbohydrate content of the fractions were determined by the Lowry (25) and the phenol sulfuric acid (12) methods, respectively.

Eliciting activity of sporangia, zoospores, and cystospores. A modification of a method described by Doke and Tomiyama (10) was used to obtain sporangia and zoospores. Sterile distilled water was added to the surface of 3–4 petri plates containing 7–10 day cultures of *P. infestans* growing on lima bean agar. The plates were gently rubbed with a glass rod and the sporangial suspension was filtered with slightly reduced pressure through two layers of cheesecloth suspended over a Whatman #2 filter paper on a 4.5-cm-diameter Büchner funnel. The filter paper on the funnel was washed twice with sterile distilled water and transferred to a 4.5-cm-diameter culture jar to which 25–35 ml of sterile distilled water was added. After incubation at 12 C for 2.0–2.5 hr, zoospores were collected from the upper 0.5 cm of the water ($1–7 \times 10^5$ spores per milliliter). The cleaning procedure for glassware described by Harris and Dennis (16) was followed to obtain consistently good yields of viable spores.

In another experiment, the sporangial suspension was filtered through two layers of cheesecloth and the filtrate was incubated at 12 C for 2.5 hr. Released zoospores and sporangia which had not released zoospores were pelleted by centrifugation at 2,000 *g* for 10 min, whereas empty sporangia floated on the surface of the supernatant. The concentration of empty sporangia was adjusted to 5×10^5/ml and their terpene-eliciting activity was compared to that of dead sporangia which had not released zoospores.

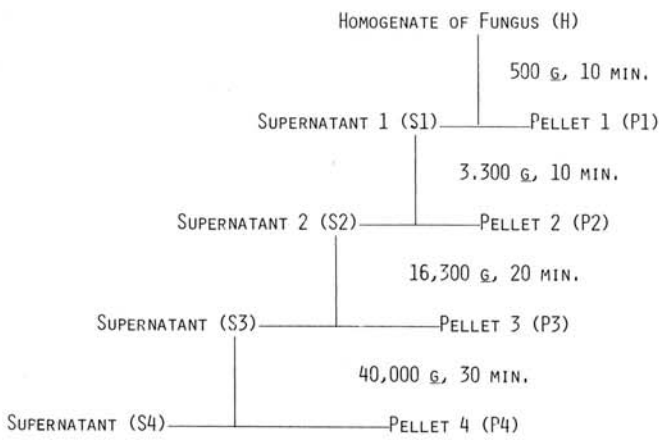

Fig. 1. Flow chart for differential centrifugation of a mycelial homogenate of *Phytophthora infestans*.

Zoospores were induced to encyst either by the "slow method" described by Doke and Tomiyama (11) or the "rapid method" described by Tokunaga and Bartnicki-Garcia (31) and applied to *P. infestans* by Harris and Dennis (16). In the slow method, zoospores encysted when placed on a shaker (20 C, 80–100 rpm) for 1 hr. Most cystospores formed a germ tube when left on the shaker for an additional hour. In the rapid method, a suspension of zoospores was shaken with a Vortex mixer for 1.5 min at moderate speed.

Immediately after preparation, samples of sporangia, zoospores, cystospores, or germinated cystospores were checked for homogeneity, and adjusted to a concentration of 5×10^4 spores per milliliter. Zoospore suspensions which did not contain sporangia and 5% or less contamination by cysts were used in tests. Suspensions of sporangia were free of mycelial fragments and other spore types. Suspensions of cystospores contained no sporangia and less than 5% of zoospores. These fractions were either directly applied to tuber slices (0.1 ml/slice) or frozen once in liquid nitrogen and applied after thawing. Freezing and thawing killed all spore types as evidenced by the granulation and coagulation of cytoplasm and the absence of spore germination or growth on lima bean agar.

RESULTS

Differential centrifugation. Most of the terpene-eliciting activity of the fungal homogenate (H) was recovered from the 500-*g* pellet (P1) (Figs. 1 and 2). Only the 40,000-*g* supernatant (S4) consistently had a residual activity. Based on specific activity with respect to either carbohydrate or protein, all fractions, except P4, were approximately equally active. Fraction P4 was extremely low in carbohydrate and protein, and though elicitation was low, specific activity was high. Tissue browning generally paralleled terpene accumulation; however, all fractions tested elicited some browning

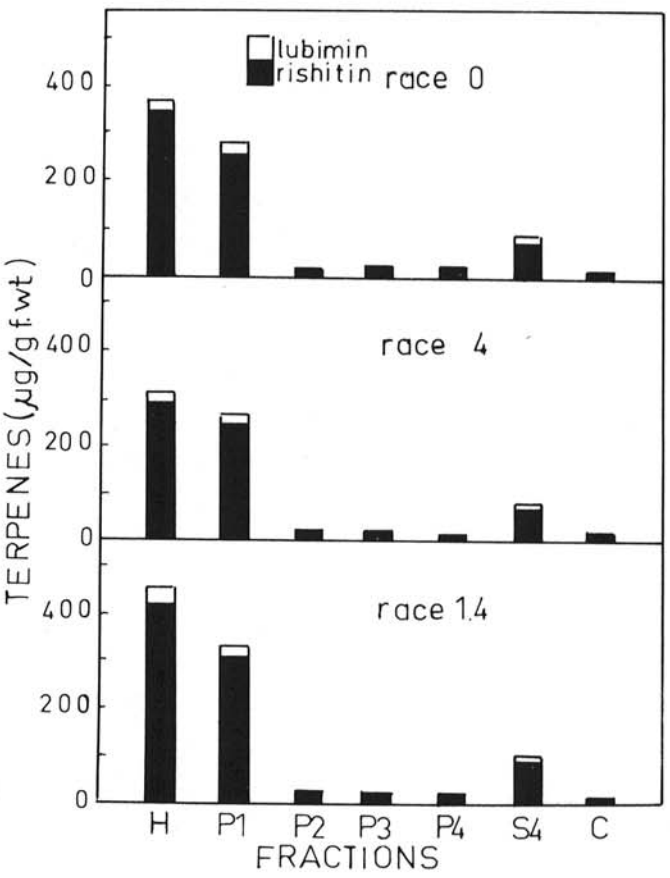

Fig. 2. Differential centrifugation of homogenates of three races of *Phytophthora infestans* and their ability to elicit the accumulation of terpenes in Kennebec potato tuber slices. The fractions are as described in Fig. 1.

when compared to water controls (author's, *unpublished*). Correlation between the spectrophotometric method for browning and a visual rating of tissue on a scale of 0–5 varied between 0.8–1.0.

The 500-*g* pellet contained the cell wall debris. The other pellets contained numerous vesicles and optically dense granules. Fraction S4 was free of microscopically visible structures.

Elicitation by sporangia, cystospores, and zoospores. Terpene elicitation by living spores of incompatible races is a function of the numbers of spores rather than the type of spore (18). This observation was confirmed using four races and two cultivars of potato (Figs. 3 and 5). Compatible races elicited accumulation of low amounts of terpenes.

When spores killed by freezing and thawing were added to potato slices, the relative terpene-eliciting activity of the spore types was sporangia > cystospores > zoospores (Figs. 4 and 6). Zoospores had very low activity. These results were consistent in all experiments. Upon germination, the activity of cystospores markedly increased (Fig. 7). There was little or no difference in activity of cystospores formed by the slow (1 hr for encystment) and fast (1.5 min for encystment) methods (Fig. 7). In one

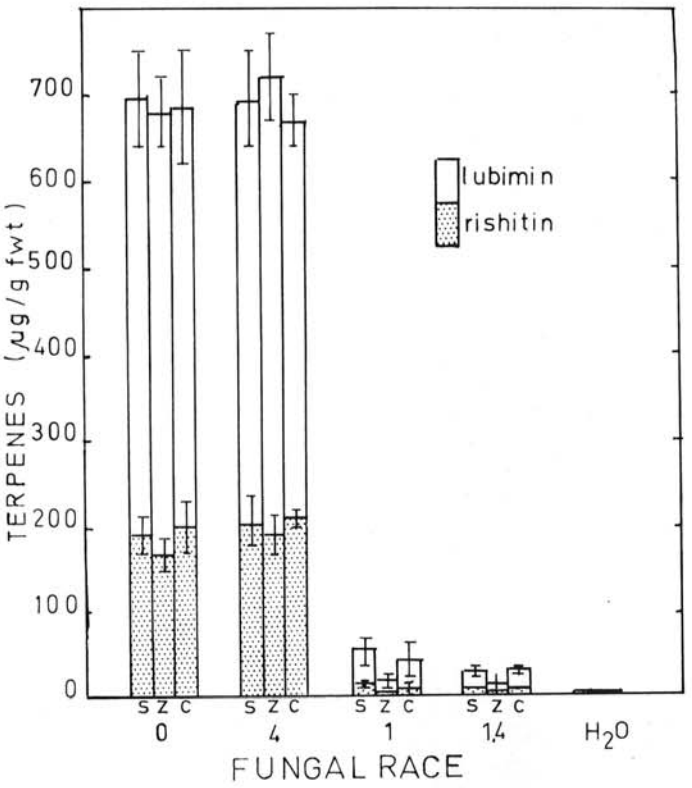

Fig. 3. The ability of different spore types (living) of *Phytophthora infestans* to elicit the accumulation of rishitin and lubimin in Kennebec (R1) potato tuber slices. The means and standard errors are indicated.

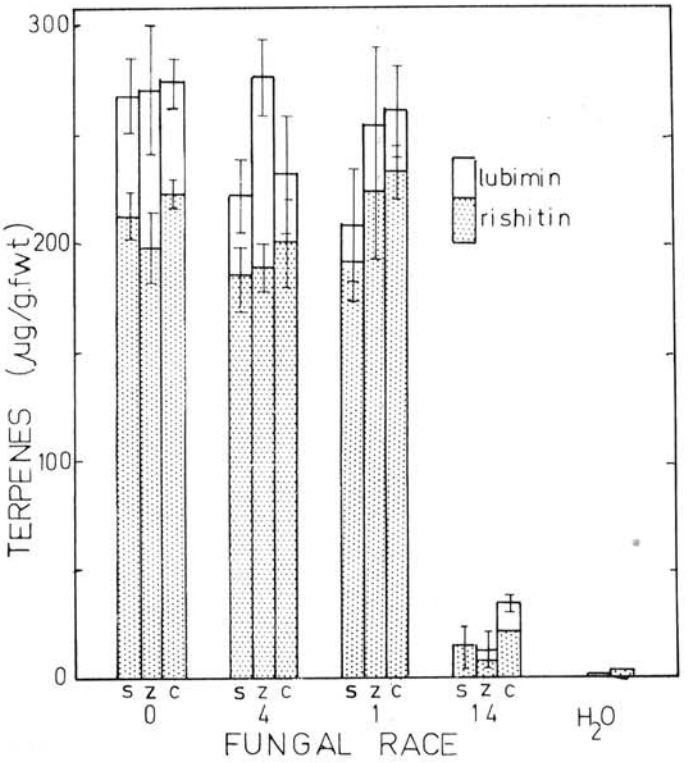

Fig. 5. The ability of different spore types (living) of *Phytophthora infestans* to elicit the accumulation of rishitin and lubimin in tuber slices of PI 203906 (R1.4). The means and standard errors are indicated.

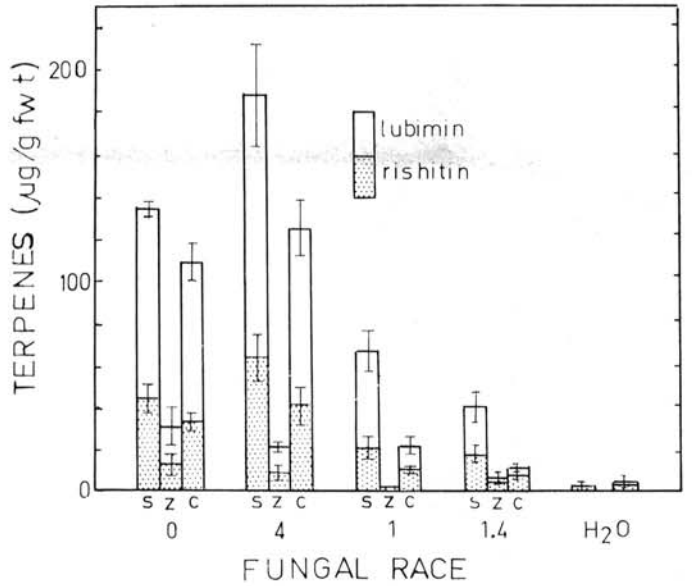

Fig. 4. The ability of different spore types (killed) of *Phytophthora infestans* to elicit the accumulation of rishitin and lubimin in Kennebec (R1) tuber slices. The means and standard errors are indicated.

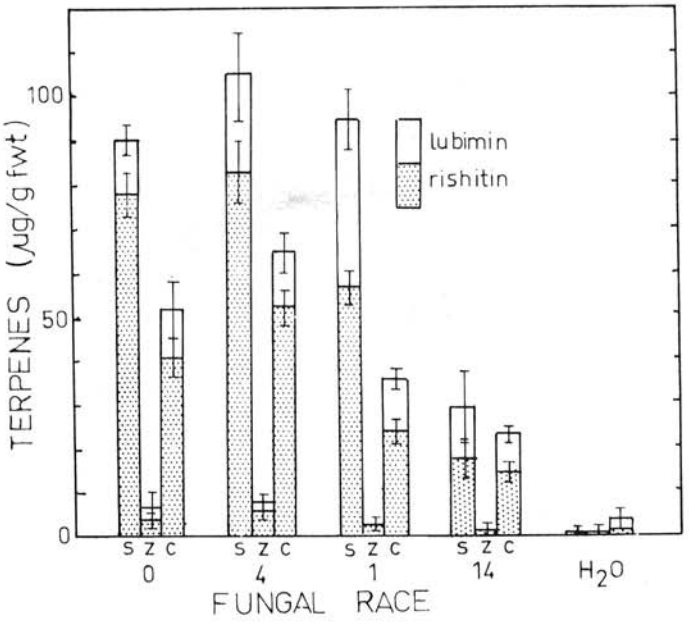

Fig. 6. The ability of different spore types (killed) of *Phytophthora infestans* to elicit the accumulation of rishitin and lubimin in tuber slices of PI 203906 (R1.4). The means and standard errors are indicated.

experiment, empty sporangia had at least 75% of the terpene-eliciting activity of dead sporangia which had not released zoospores.

DISCUSSION

Zoospores are natural protoplasts that are readily lysed by detergents, terpenes (15,16), and steroid glycoalkaloids (26). Cysts and germtubes of *P. infestans,* however, are resistant to lysis by detergents and phytuberin, a sesquiterpene. A major difference between zoospores and cystospores is the absence of a cell wall in the former (5,14). The spore types of *P. palmivora* have been studied extensively with regard to their development and glucan transformation. Sporangia, zoospores, cysts, and mycelium of *P. palmivora* are rich in soluble mycolaminarans, whereas only the spores contain mycolaminaran P. During encystment, a large proportion of mycolaminaran and mycolaminaran P are hydrolyzed, and the insoluble glucans (absent in zoospores) are deposited in the wall (5,30).

P. infestans contains mycolaminaran and mycolaminaran phosphates in zoospores, germinated cystopores, and, unlike *P. palmivora,* also in the mycelium (8,9,13). Since cystospores are much more effective elicitors of terpene accumulation than are zoospores, irrespective of the manner of encystment (slow or rapid), it appears that mycolaminarans and mycolaminaran P are not active elicitors of terpene accumulation. Data from experiments with differential centrifugation and studies of the activity of killed spore types are consistent with the conclusion that an elicitor of terpene accumulation in potato is localized in the cell wall of *P. infestans.* The relatively low level of activity in killed spores of compatible as compared to incompatible races of the fungus (Figs. 4 and 6) may be explained by the specificity of the elicitor or by the presence of suppressors which determine specificity (8,9,13,33). It remains to be ascertained whether the elicitor is on the surface of the living fungus and whether metabolic processes in host or fungus modify the activity, availability, or stability of the elicitor (21).

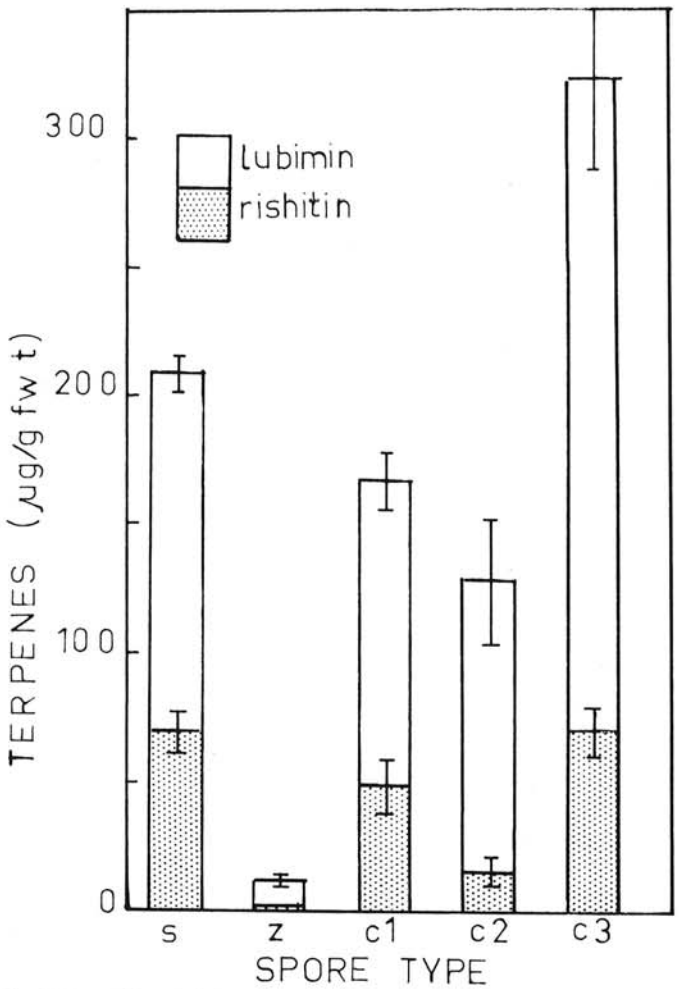

Fig. 7. The ability of different spore types (killed) of race 4 of *Phytophthora infestans* to elicit the accumulation of terpenes in Kennebec tuber slices. Sporetypes assayed: S = sporangia, Z = zoospores, C1 = rapid cysts (encystment in 1.5 min), C2 = slow cysts (encystment in 1 hr), C3 = cysts aged for 8 hr at 12 C (more than 90% have germinated).

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