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Microbiological control of black leaf streak disease

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Abstract

Isolates of bacteria from the phyllosphere of tomato and banana were obtained from CATIE: *Bacillus cereus, Bacillus* sp., *Serratia marcescens, Serratia entomophila*, unidentified strains with glucanolytic and chitinolytic capacity (GS2-GS3-GC1-GBC2, SE/PO_2, White) and one isolate of *Mycosphaerella fijiensis* collected recently in Turrialba, Costa Rica. Crude culture filtrates of some microorganisms inhibited ascospore germination and the growth *in vitro* of *M. fijiensis* colonies. The two filtrates with the greatest effect resulted in changes to the ultrastructure of *M. fijiensis* hyphae, when examined under a scanning electronic microscope, in comparison with untreated tissue.

Resumen – Control microbiológico de la Sigatoka negra

Los aislados de la colección del CATIE obtenidos de la filosfera de tomate y hojas de banano: *Bacillus cereus, Bacillus* sp., *Serratia marcescens, Serratia entomophila* y cepas no identificadas con capacidad glucanolítica y quitinolítica (GS2-GS3-GC1-GBC2, SE/PO_2, White) y un aislado de *Mycosphaerella fijiensis* recolectado recientemente en Turrialba, Costa Rica, fueron utilizados para preparar filtrados de los cultivos líquidos. Los filtrados crudos de estos microorganismos se evaluaron bajo condiciones *in vitro* con el fin de determinar la germinación de las ascosporas y el crecimiento de las colonias de *M. fijiensis* (agente causal de la Sigatoka negra en banano y plátano). Los resultados muestran un efecto inhibitorio importante de algunos de estos filtrados en diferentes etapas de desarrollo de *Mycosphaerella*. La observación, bajo un microscopio electrónico de barrido, de las estructuras del hongo tratado con dos filtrados prometedores, muestra claras alteraciones de ultraestructura en el tejido tratado en comparación con el testigo sin tratamiento.

Résumé – Lutte microbiologique contre la maladie des raies noires

Des isolats de bactéries de la phyllosphère de tomates et de bananiers ont été obtenus du CATIE : *Bacillus cereus, Bacillus* sp., *Serratia marcescens, Serratia entomophila*, des souches non identifiées ayant une capacité glucanolytique et chitinolytique (GS2-GS3-GC1-GBC2, SE/PO_2, White) et un isolat de *Mycosphaerella fijiensis* collecté récemment à Turrialba, au Costa Rica. Les filtrats bruts de certains micro-organismes ont inhibé la germination des ascospores et la germination *in vitro* de colonies de *M. fijiensis*. Les deux filtrats qui ont eu le plus d'effet ont induit des changements de l'ultrastructure des hyphes de *M. fijiensis*, en microscopie électronique à balayage, par rapport aux tissus non traités.

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Introduction

During the seventies, agriculture was characterized by an undiscriminated use of agrochemicals. The situation has changed little since then, but international and national regulations have imposed changes aimed at reducing pollution and making agriculture sustainable. There are two types of agriculture: 1) high input agriculture characterized by high productivity that is limited by environmental factors and 2) low input agriculture with production that, in addition to environmental factors, is limited by pests, diseases and weeds.

Changes in the market and the influence of globalization are forcing a reconsideration of research in agriculture. The preferences of consumers play an increasingly important role that affects the work of multidisciplinary teams made up of researchers, ecologists, producers and industry.

The Tropical Agricultural Research and Higher Education Center (CATIE) has defined one of its research objectives as the implementation of methodologies focused on the biological control of the most common diseases and pests affecting economically important tropical crops, such as those belonging to the Musaceae family.

CATIE started studies on *Musa* and *M. fijiensis* with a project financed by AID/ ROCA (USA) the first phase of which started in July 1984. Other projects which followed were financed by RENARM (USA), CIRAD (France), INIBAP, INCO-*Musa*, Natural Resource Institute (NRI; UK), CINVESTAV (Mexico) and FONTAGRO.

These collaborative projects not only spurred research on biological control but also on the control of black leaf streak disease, a disease that was already threatening banana and plantain production. Other outcomes were the development of systems for somatic embryogenesis, cell suspensions, plant pathology, cryopreservation, genetic transformation and the genetics of *M. fijiensis* populations.

Since then, the Plant Protection Unit at CATIE has developed integrated pest management (IPM) practices for black leaf streak disease based on the preservation of the environment, reduced risks to farmers, the rural population and consumers, and the sustainability of traditional agriculture. Countries included within the CATIE mandate have a rich biodiversity which may contain materials or products, e.g. genes of wild plants or biopesticides, that might be useful in IPM programmes.

Research into the biological control of black leaf streak disease at CATIE encouraged researchers involved in the AID/ROCAP-USA project, e.g. Dr Elkin Bustamante and his team who were the first to work on the project. After a careful study of the different aspects of the parasitic relationship between *Musa* and *M. fijiensis*: the biology and morphology of the pathogen, the phenology and physiology of the plant, the phyllosphere, soil (importance of rhizobacteria, endophytic fungi and organic amendments), and methods of internal and external inoculation, a research programme was constructed to better study these aspects (Figure 1).

Step 1. Identification of antagonistic microorganisms

The purpose was to isolate microorganisms antagonistic to *M. fijiensis* and to evaluate their effectiveness under greenhouse and field conditions. One hundred and twenty isolates with chitinolytic activity were obtained from plants of cv. 'Grande naine' coming

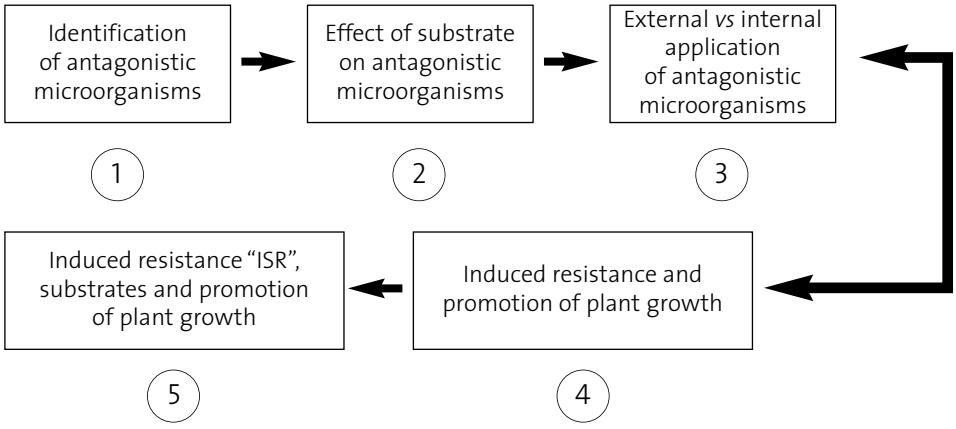

Figure 1. Steps involved in research on biological control at CATIE (1986-2001).

from two different locations: an area of high incidence of black leaf streak disease and an area of low incidence of the disease. The 'low incidence site' provided the highest population of microorganisms, which was evaluated on chitin agar. Thirteen bacterial strains were selected on the basis of their chitinolytic activity (*Serratia marcescens*, *Serratia entomophyla* and *Bacillus* spp.). Under greenhouse and field conditions the level of control of *M. fijiensis* was 40% in comparison with a level of 60% using fungicides (González, 1994; González *et al.* 1996).

Regarding glucanolytic activity, 196 strains of bacteria were collected from plants of cv. 'Grande naine' of which 37 belonged to the genus *Bacillus*. The microorganisms were purified and evaluated on glucan-agar and glucan nutrient-agar media. Seven strains with glucanolytic activity were selected. GS2, GBC2, BS3 and BC1 showed antagonistic effects, inhibiting germination of *M. fijiensis* ascospores in 25% of the cases and reducing germination tube length in 47%. Four strains were tested in the presence and absence of glucan. Commercial glucan being expensive, a common source of glucan from agricultural waste was used (Talavera-Sevilla, 1996; Talavera *et al.*, 1998a, b).

Step 2. Effect of substrate on antagonistic bacteria

The effect of different substrates on the growth and survival of antagonistic bacteria were investigated. The aim was to modify the physical and nutritional conditions in order to inhibit germination and establishment of the pathogen and favour antagonistic organisms.

The bacterial strains used were: *Serratia marcescens* R1, *Serratia entomophila* A100 and *Bacillus cereus* A30. The substrates tested, singly or in combination, were leaf extract, milk, foliar fertilizers, molasses, cassava starch, glucan and chitin. The highest recovery level of bacteria was observed in molasses which had positive effects on antagonistic microorganisms. A combination of milk and molasses increased multiplication and survival of R1 and A30 (Ruiz-Silvera *et al.*, 1997a, b).

Plants treated with a combination of chitin, yeast and calcium nitrate alternated with commonly used fungicides, reduced the number of fungicide treatments by 40% in comparison with fungicides alone (Arango-Ospina, 2000).

Step 3. Internal vs external application of antagonistic microorganisms

The objectives of the study were to evaluate the effects of R1 and A30 applied externally in combination with Silwet L-77, Nu-Film 17 and mineral oil, and to evaluate an endophytic inoculation method via the roots. R1 was compatible with stickers, mainly mineral oil. Mineral oil in combination with antagonistic microorganisms reduced disease severity in comparison with the controls. The best colonization of internal tissues was when A30 was applied directly inside the plant (Miranda, 1996).

Step 4. The phenomenom of induced resistance and promotion of plant growth

Stimulation by pathogens, non-pathogenic microorganisms, and by substances of biological or non-biological origin can induce resistance in susceptible plants Induced resistance to disease and growth promotion have potential for controlling disease (Figure 2).

Four bacterial and one fungal suspensions were applied to the rhizosphere; KH_2PO_4 and K_2HPO_4 solutions were applied to the leaves as abiotic exogenous inducers. In a second experiment, microorganisms were evaluated with the addition of sugarcane pulp, sugarcane filter press and coffee husks to the rhizosphere. *Pseudomonas fluorescens* (PRA25), *P. cepacia* (AMMD) and *Trichoderma harzianum* (Th) plus substrates increased growth the most and reduced disease in comparison with the controls (water and substrates). However, the lowest percentage was obtained with propiconazole (Tilt®). There was a significant and negative correlation between them (Gutiérrez, 1996).

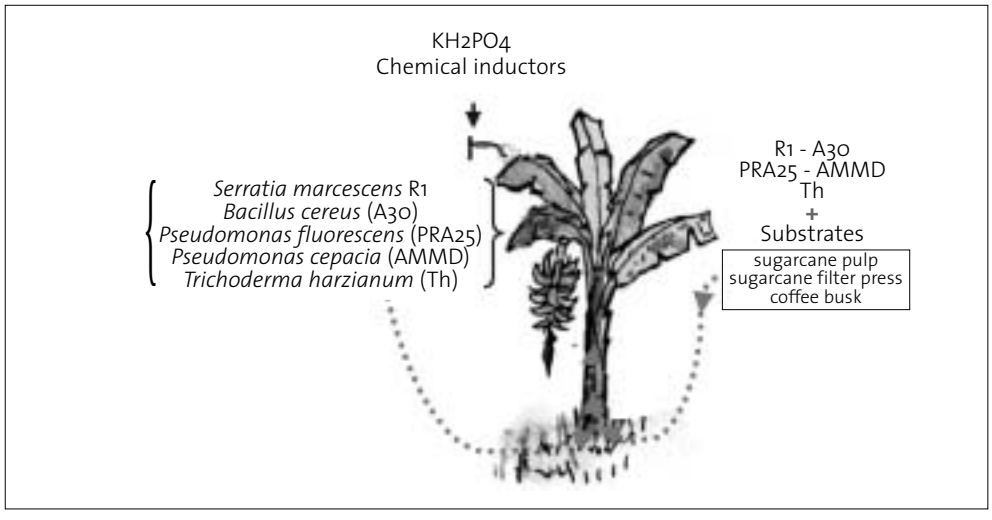

Figure 2. Illustration of the concept of induced resistance to control black leaf streak disease (© S. Belalcázar, 2002).

Step 5. Induced systemic resistance (ISR)

Investigations improved the understanding of the use of organic amendments, antagonistic microorganisms, substrates as energy sources in combination with mycorrhizal fungi and organic extracts known as efficient microorganisms, EMs (Okumoto *et al*., 2001; Ayuso, 2000; Sanchez Garita *et al*., 1998; Okumoto, 1992).

Induced systemic resistance (ISR) to disease results from the inoculation of lower leaves, or roots, with restrictive pathogens, non-pathogenic races of pathogens, non-pathogens, products of pathogen or non-pathogens, and organic or inorganic chemicals. ISR is also referred to in the scientific literature as SIR or SAR (Ku,? 2001).

The objective of the research was to evaluate, under greenhouse conditions, the response of cv. 'Grande naine', as an example of a banana cultivar susceptible to black leaf streak disease, and of 'FHIA-23' as an example of a clone resistance to the disease, in the presence of 3 resistance inducers and one foliar substrate as an energy source. The inducers were PRA25 bacteria and culture filtrate from germinating spores of *M. fijiensis* strains according to Riveros and Lepoivre (1998), and Acilbenzolar-S-metil (BION®,), a synthetic inducer provided by Syngenta. ISR was higher in 'FHIA-23' than in cv. 'Grande naine'.

Vermicompost increased ISR. BION® resulted in high ISR in both cultivars. Rhizobacteria and *M. fijiensis* filtrate induced resistance only in 'FHIA-23' when in the presence of an energy source. In the field, BION® reduced disease incidence in cv. 'Grande naine' in comparison with conventional control measures (Patiño, 2001).

Massive applications of antagonistic bacteria or fungi on crops could have unforeseen effects on the environment. The objectives of the study were to evaluate the *in vitro* biological activity of microbiological filtrates on a *M. fijiensis* ascospore preparation, the growth of *M. fijiensis* colonies, and the effects of two filtrates on the cell structure of *M. fijiensis*. Emphasis was put on the isolation of strains with glucanolytic and chitinolytic activity.

Materials and methods

Bacterial strains used in this study were obtained from CATIE's Plant Protection Unit Collection:

- *Bacillus cereus* (A30), isolated from tomato leaves (*Lycopersicon pimpinelli*), Turrialba, Costa Rica (Okumoto, 1992).
- *Serratia marcescens* (R1), isolated from banana leaves (*Musa* sp.), Limon province, Costa Rica (González, 1994).
- *Serratia entomophila* (SE), isolated from Canterbury Valley (New Zealand) from the digestive tract of the scarabid *Costelytra zealandica* (donated by Trevor Jackson from the AgResearch Lincoln Laboratory in 1994).
- GS2, GS3, GC1, GBC2, bacteria with glucanolytic activity isolated from banana leaves, Indiana farm, Siquirres, Costa Rica (Talavera-Sevilla, 1996).
- SE/PO2, isolated from deep well water, Carmen de Siquirres, Costa Rica (Gamboa, personal communication).

• White, chitinolytic bacteria, isolated from plantain leaves, La Montaña farm, CATIE, Costa Rica (Arango-Ospina, 2000).

• Extracts of the fluid obtained from suspensions of the conidia of *M. fijiensis* isolated from La Montaña farm, Turrialba, Costa Rica.

Microbiological extracts were prepared on Petri dishes containing nutrient agar medium (DIFCO). Two boxes per bacterial strain were inoculated with 20 ml bacterial solution previously maintained at 4°C. The bacterial suspension was uniformly distributed over the medium using a glass handle and the boxes were incubated at 30°C for 2 days. Once the bacteria started growing, sterile distilled water was added to the medium and the bacterial suspension removed with a scalpel; approximately 20 ml of each bacterial suspension was transfered to sterile vials. The absorbency at 600 nm of a 3-ml solution was measured with a spectrophotometer. One ml of each suspension with an optical density of 1.2 was transferred to 200 ml of sterile nutrient medium (DIFCO) and incubated for 12 hours at 30°C and 150 rpm. Absorbency was measured again at 600 nm and gave values of 1.2 ±0.02 after 12 hours

Suspensions were adjusted to an absorbency of 1.1 by adding sterile nutrient medium. Cell-free extracts were obtained by centrifugation at 5000 rpm for 40 minutes followed by vacuum filtration on 0.22 mm membranes. Extracts were kept at 4°C in sterile flasks and protected from the light.

A suspension of $2x10^5$ conidia/ml of *M. fijiensis* in 700 ml of sterile distilled water was agitated at 100 cycles/min for 48 hours in darkness and then filtered using ethamine and Whatman paper. The residue was lyophilized to obtain 0.341 mg of powder, which was diluted in 70 ml sterile distilled water to obtain a final 20x concentration and filtered through 0.22 µm Nalgene Disposable Filterware filters. The filtrate was protected from light and kept at 4°C until its utilization.

Samples of plantain leaves with black leaf streak disease were transferred to La Montaña farm, which belongs to CATIE. Using a magnifying glass, fragments of viable perithecia were removed and transferred to the laboratory in paper bags. The samples were checked using a stereomicroscope, and sections with abundant sporulating lesions were cut into 2x2 cm pieces. Two to four of these pieces were stapled to pieces of paper and incubated in a humid chamber for 24 hours at room temperature. They were then transferred to sterile distilled water for 5 minutes to hydrate the perithecia. Ascospores discharged on water agar (4% w/v). Treatments were 0.5, 0.1 and 0.01 ppm dilutions of microbiological filtrates and the controls were without microbiological filtrate or with fungicide.

Diluted culture filtrates were mixed with 15 ml of V8 medium, with constant agitation and then transferred to Petri dishes; there were 3 replicates per treatment. Seven-day-old sub-cultures of a strain of *M. fijiensis* isolated from La Montaña were used. Twenty colonies of 1–1.5 cm in diameter were excised with a scalpel and transferred to an assay tube with 3 ml of 0.05% (v/v) Tween 20 and agitated in a vortex. The assay tubes were left to rest for 10 minutes and 10 drops of liquid were transferred to each Petri dish and spread with a glass handle. Dishes were sealed, and incubated in darkness for 5 days at 26°C.

After 5 days of incubation, the diameters of *M. fijiensis* colonies were measured using a micrometer in the 4x field of a microscope. Thirty readings were taken,

frequency intervals of amplitude 10 were done and only the 10 data from the interval with higher frequency were registered to conduct the analysis of the inhibiting effect of the microbiological filtrates.

The longest germination tubes were measured using a micrometer in the 10x or 40x field of a microscope. One hundred readings were taken per treatment. Since bacteria were cultured in nutrient medium, bioassays included a treatment with this medium.

Initial data were multiplied by a correction factor to transform the values to microns, 10 and 2.5 for 10x and 40x, respectively. The difference in germ tube length between the treatment and the control was used to calculate the inhibiting effect of the microbiological filtrates.

Results and discussion

Figure 3A shows preliminary results obtained for growth inhibition of *M. fijiensis* ascospores discharged onto different concentrations of microorganism filtrates. The percentage of inhibition was almost 50% and in some cases higher when the medium included filtrates of bacteria with glucanolytic activity (GBC2) and chitinolytic activity (SE/PO2 and White).

Regarding colony diameter (Figure 3B) the general tendency remained similar except that another bacterium with glucanolytic activity (GC1) showed a higher inhibition which was not fully revealed during the sexual phase of ascospore development.

The crude *M. fijiensis* filtrate (FCMf) also revealed, a clear inhibiting effect on ascospore and colony growth at a concentration of 0.5 ppm but not at the other concentrations. This poses the question as to whether the toxin(s) produced by *M. fijiensis* spores during the germination process can inhibit the pathogen in a "suicidal" type of action.

After five days of incubation, physical growth had stopped in the cultures with the crude filtrates of GBC2 and SE/PO2 at the 0.1 ppm concentration in comparison to the absolute control (water). Electronic transmission microscopy revealed modifications at the level of cell organelles with a strong presence of electrodense osmophylic globules that were not found neither in the control nor in the transversal longitudinal cut (Figure 4).

The preliminary results suggest that liquid culture filtrates of four bacterial strains with glucanolytic or chitinolytic activity, and the liquid filtrate of germinating spores of a Costa Rican strain of *M. fijiensis* inhibited the growth of *M. fijiensis* germ tubes and colonies.

Crude liquid preparations diluted from antibiotic(s) or toxin(s) and without bacterial or fungal cells had similar effects as the fungicide Tilt®.

The promising microbiological filtrates need to be evaluated under greenhouse and field conditions with or without adjuvant applications.

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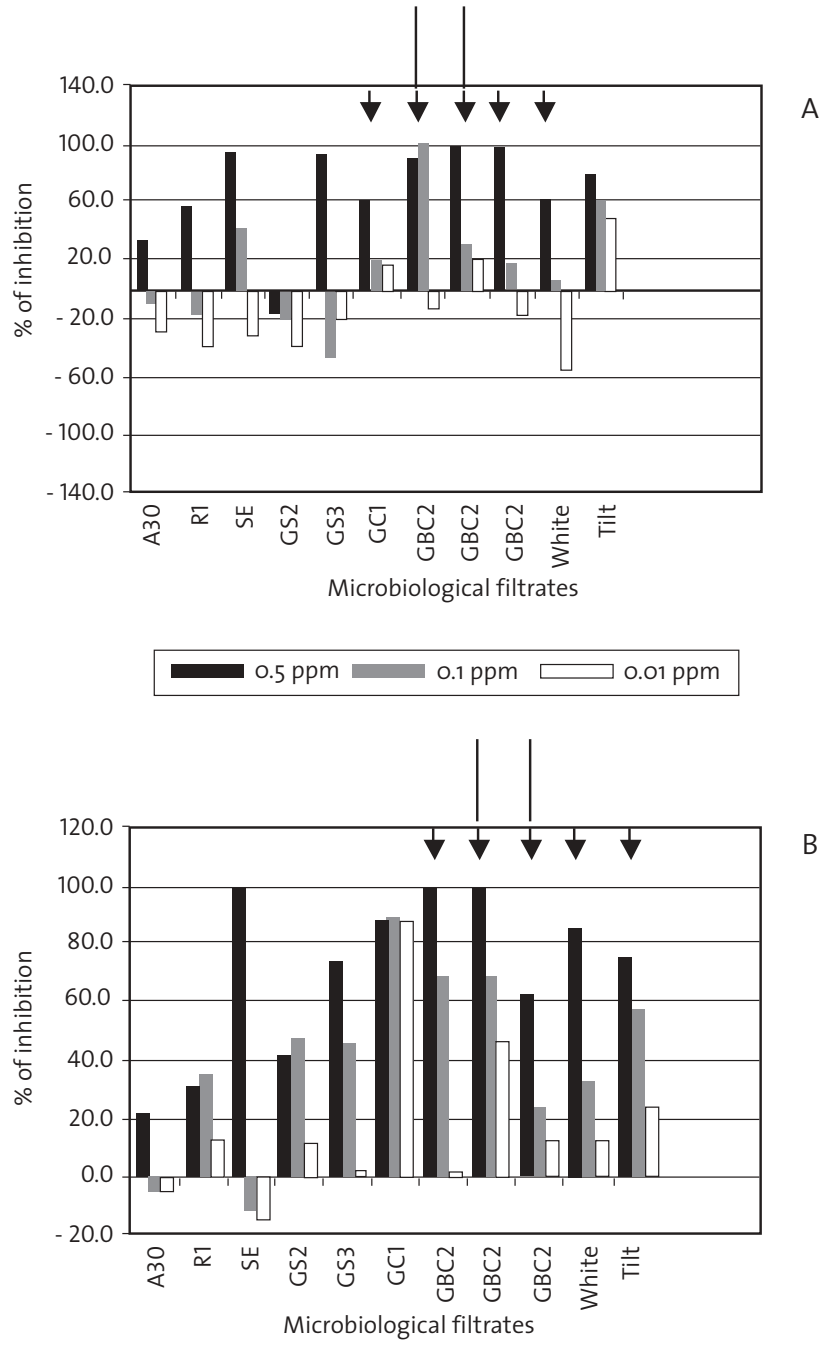

A30:*Bacillus cereus*; R1:*Serratia marcescens*; SE: *Serratia entomophila*; GS2, GS3, GC1 and GBC2: bacteria with glucanolytic activity; SEPO2 and White: bacteria with chitinolytic activity; FCMf: conidial fluid; Tilt: triazole fungicide.

Figure 3. Effect of three dilutions of nine antagonistic microbiological filtrates on A) germ tube growth and B) colony diameter of *M. fijiensis* . Arrows indicate where crude filtrates affected growth.

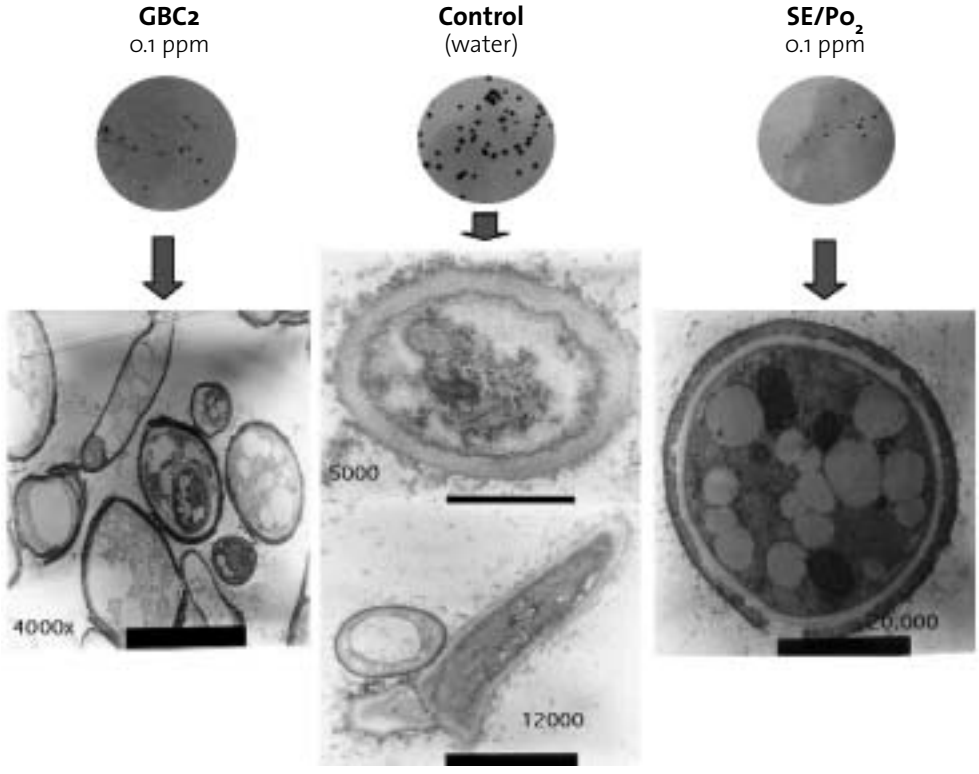

Figure 4. Cytological changes revealed by transmission electron microscopy of *M. fijiensis* hyphae tissues treated with crude filtrates of bacteria with glucanolytic (CBC2) and chitinolytic (SE/PO2) activity.

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