Use of scanning and transmission electron microscopy to identify morphological and cellular damage on phytopathogenic fungi due to natural products application

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Due to their importance and the increasing concern over the use of synthetic fungicides to control phytopathogenic fungi, other methods for controlling their development have been evaluated. The antimicrobial properties of chitosan and essential oils from various plant species applied individually or in combination have been shown to affect and arrest fungal development on various horticultural commodities. Fungi were grown on chitosan alone or in combination with lime essential oil. Treated samples were processed for observations by scanning (SEM) and transmission (TEM) electron microscopy. Observations of F. oxysporum f. sp. gladioli and R. stolonifer by SEM showed diverse alterations in the hyphae such as swelling, depression and distortion along with a lack of spore development due to the effect of chitosan and essential oils, while observations of the mycelium and conidia of chitosan-treated A. alternata by TEM showed cell wall disintegration, cellular distortion, intense vacuolization and lyses of fungal cells.

Keywords chitosan; essential lime oil; Alternaria alternata; Fusarium oxysporum; Rhizopus stolonifer

1. Introduction

Fungi such as Alternaria alternata and Rhizopus stolonifer are important pathogens of fruits, vegetables and ornamentals. Fusarium oxysporum f. sp. gladioli is one of the usual agents of rot during gladiolus corm storage. Generally, it has been controlled by synthetic fungicide application due to its proven efficiency. Currently, however, this control method is strictly regulated due to increasing concern about environmental contamination, ‘side effects’ on human health and development of resistance in the treated pathogen [1]. Natural compounds that do not have any significant medical or environmental impact could potentially serve as effective alternatives to conventional antibacterial or antifungal agents. In this context, the activity of chitosan and several essential oils against phytopathogenic microorganisms and microorganisms that cause food spoilage have been studied. Chitosan, a chitin derivative that is a natural biodegradable compound derived from crustaceous shells such as crabs and shrimps, has been promoted as a natural fungicide. In numerous in vitro and in situ investigations the inhibitory effect of chitosan on these important phytopathogenic fungi has been demonstrated [2,3]. In previous studies, the degree of inhibition of mycelial growth and sporulation of A. alternata isolated from tomato fruit relied on the molecular weight of chitosan and its concentration [4], while in studies with a strain obtained from mango fruit, no conidial germination occurred at concentrations up to 1% [5]. For F. oxysporum f. sp. gladioli, a similar inhibitory response was obtained with chitosan at concentrations from 1% to 3% and germination did not take place at concentrations above 0.5% [6]. Rhizopus stolonifer was also seriously affected by chitosan application. Fungal isolates obtained from tomato and papaya fruit were inhibited at all development stages [6,7]. A number of investigations about the mechanism of action of chitosan highlight the alterations at the morphological and cellular level of the treated fungi; membrane and cell walls are reported to be the most affected organelles [8, 9]. The essential oils produced by plants have long been known to provide effective control over fungal phytopathogens. They are a mixture of volatile compounds resulting from secondary plant metabolism. With respect to their fungicidal potential, it has been reported that essential lime oil alone or combined with other natural compounds reduces rot caused by the fungus Colletotrichum gloeosporioides by up to 50% and it also blocks development of food-borne pathogens such as Escherichia coli on fresh tomato by 100% [10,11]. The reported effects of these compounds include, among others, toxicity at the cellular level, as they damage the cell wall and membrane, thereby inducing cytoplasm leakage [12]. Thus, the objective of this research was to observe, using scanning and electron microscopy, morphological and cellular alterations on A. alternata, F. oxysporum f. sp. gladioli and R. stolonifer after incubation with chitosan and essential lime oil.
2. Materials and experimental procedure

2.1. Microorganisms and treatments.

Fungi were isolated from infected mango (*A. alternata*), tomato (*R. stolonifer*) and gladiolus corms (*F. oxysporum* f. sp. *gladioli*). Strains were purified and maintained in potato dextrose agar (PDA) until further use. The chitosan concentration was 1.0 % for *A. alternata* and 1.0 mg ml⁻¹ for *F. oxysporum* f. sp. *gladioli*, while *R. stolonifer* was grown on a 1.0 % chitosan-based coating mixed with 0.1% beeswax and 0.1% lime essential oil. The control treatment consisted of growing fungi only on PDA. Chitosan preparation, inoculation methodology and sampling procedure were carried out following the methodologies reported by Sánchez-Domíngez et al [13], and Ramos-García et al [11].

2.2. Samples processing for studies by Scanning and Transmission Electron Microscopy (SEM, TEM).

For SEM observations, both mycelia and conidia of *A. alternata* and *F. oxysporum* f. sp. *gladioli* were fixed with 6% glutaraldehyde for 24 h at room temperature, rinsed three times with 0.02 M phosphate buffers and subsequently fixed with 2% osmium tetraoxide for 24 at 20 °C, dehydrated in a graded ethanol series for five minutes each, CO₂ dried (SAMDRI 780-B Tousimis) and sputter coated with gold palladium in a Nanotech sputter coater (BAL-TEC SDC 050). Samples were kept in a desiccator until examination with a Carl Zeiss DMS 940 scanning electron microscope operated at 30kV. The Rhizopus samples containing the formulations of chitosan + beeswax + lime essential oil and PDA were mounted on aluminum stubs with double-sided carbon adhesive tape and directly observed under a XL-30 Environmental Scanning Electron Microscope (Philips) at 25 kV accelerating voltage, working in a pressure range in the sample of 2.0 – 3.3 torr using a gaseous secondary electron detector (GSE) for image formation. *Alternaria alternata* was the only fungus subjected to TEM observations. Previously fixed samples of treated mycelium and conidia of this fungus were dehydrated in a graded ethanol series and embedded in London white resin (EMS cat. 14380). Ultra-thin sections (60 nm thick) were cut with a diamond knife (Ultracut R, Leica) and collected in Formvar-coated copper grids (EMS cat. FCF200). They were contrasted in uranyl acetate and lead citrate, washed and then examined in a Zeiss EM900 transmission electron microscope at 60 kV.

3. Results and discussion

Except for *A. alternata*, chitosan and chitosan combined with beeswax and lime essential oil caused morphological alterations in treated mycelium of *F. oxysporum* f. sp. *gladioli* and *R. stolonifer*. The control mycelium of *F. oxysporum* f. sp. *gladioli* showed the typical structures belonging to this fungus such as monophialides bearing micro and macroconidia (Fig. 1a). Well-defined chlamydospores were also observed (Fig. 1b). The absence of these structures was common in the chitosan-treated mycelium (Fig. 1c). The mycelium was severely damaged in the presence of chitosan. In regard to *F. oxysporum* f. sp. *gladioli* conidia, no damage was observed in the untreated specimens compared with the intense dehydration shown with chitosan (Figs. 2a, b). With respect to the fungus *R. stolonifer*, it was observed that the control treatment grown on PDA developed well-formed sporangiophores and mycelia (Fig. 3a), while *R. stolonifer* subjected to the formulation with chitosan + beeswax + lime essential oil showed no development of the typical reproductive structures of *R. stolonifer* called sporangia, and the mycelia was distorted and swollen (Fig. 3b). Apparently no damage was observed in the morphology of the sporangiophores.

![Fig. 1](image-url) Transmission scanning micrographs of nontreated mycelium and chitosan-treated mycelium of *Fusarium oxysporum* f. sp. *gladioli*. Well-formed mycelium containing phyalides bearing microconidia (a) and well defined chlamydospores (b). Dehydrated and distorted chitosan-treated mycelium (c)
As reported by other authors, the treated fungi showed serious morphological alteration. Previous SEM observations have demonstrated that chitosan may alter the morphology of hyphae of various fungi. El Ghaouth et al [4] reported that \textit{R. stolonifer} hyphae in contact with chitosan were smaller and also bifurcated, distorted and swollen. The same observations were reported for \textit{F. oxysporum} f. sp. \textit{radicis-lycopersici}, isolated from tomato plants [8]. Ruptures along the chitosan-treated mycelium were observed at concentrations of 1 mg ml⁻¹. Contrary to other reports, in our observations, mycelia of \textit{A. alternata} and conidia of both \textit{A. alternata} and \textit{R. stolonifer} did not present morphological alterations. In the first study [4], \textit{A. alternata} was isolated from infected tomato whilst our strain was obtained from mango fruit. The sensitivity to chitosan might also be associated with a specific strain. As for \textit{R. stolonifer}, the absence of chitosan-induced alterations on the sporangiospores as previously reported [7] might be due to the concentration used, which was lower (0.1 %) than ours (1.5 %).

In TEM observations, there were clear cellular alterations in chitosan-treated \textit{A. alternata} conidia and mycelium. Examinations of ultra-thin sections of chitosan-treated \textit{A. alternata} hyphae and conidia revealed marked alterations in the cell wall and intense vacuolization along the cell (Figs. 4a,b). The chitosan-treated mycelium showed membrane rupture resulting in the loss of cellular content. Surrounding the mycelium and conidia, a mucilaginous material was observed. The nontreated mycelium showed well-defined organelles, and an even cell wall and membrane (Figs. 4c, d). Similar cellular alterations due to chitosan were reported by Sánchez-Domínguez et al. [13]. For \textit{A. alternata}, isolated
from tomato fruit, they reported disintegration of the cell wall, shrinkage and lysis at 1.5% concentration. In other phytopathogens such as *R. stolonifer*, *F. oxysporum* f. sp. *radicis-lycopercisi* and *Phythium aphanidermatum* cell wall and membrane alterations and cellular disorganization were observed in both mycelium and conidia when incubated on different chitosan concentrations [8,9,14].

![Fig. 4](image)

*Fig. 4* Transmission electron micrographs of *A. Alternata*. Chitosan treated mycelium (a) and conidia (c), showing intense vacuolization and nontreated mycelium (b) and conidia (d) showing well defined cell wall and organelles. cw = cell wall, m = membrane, n = nucleous, o = organelles, v = vacuoles

### 4. Conclusions

Morphological and cellular alterations in *A. alternata*, *F. oxysporum* f. sp. *gladioli* and *R. stolonifer* due to chitosan application were observed in detail and defined by the use of MEB and TEM. This technology is a valuable tool that enables visualizing internal and external changes occurring in fungi treated with these two natural compounds.

### References


