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Experimental Study

Microscopic study of the morphology and metabolic activity of *Fusarium oxysporum* f. sp. *gladioli* treated with *Jatropha curcas* oil and derivatives

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ABSTRACT

The fungus *Fusarium oxysporum* f. sp. *gladioli* is one of the main pathogenic microorganisms of the ornamental genus *Gladiolus*. The attack of this microorganism includes corms and different plant phenological stages. In this study, different microscopic techniques and fluorochromes were used to evaluate the effect of *J. curcas* oil and acylglycerides, namely trilinolein, triolein, monomyristin and dimyristin, on the morphology, membrane integrity (%), viability (%) and germination (%) of *F. oxsporum* f sp. *gladioli*. Phase-contrast optical photomicrographs and scanning microscopy showed that *J. curcas* oil and the triglycerides triolein and trilinolein caused the formation of numerous vacuoles, alterations in the morphology of the outer covering of the mycelium and conidia, and inhibition of membrane activity in the fungus during 24 h of incubation. The fluorochromes used detected no permanent damage to the viability of the conidia. The high germination percentage of the conidia of *Fusarium oxysporum* f. sp. *gladioli* indicates that the damage caused by the application of the treatments was fungistatic rather than fungicidal and did not cause cell death.

1. Introduction

The fungus *Fusarium oxysporum* f. sp. *gladioli* is one of the main pathogenic microorganisms of the ornamental genus *Gladiolus*. The attack of this microorganism includes corms and different plant phenological stages [1]. The compounds generally used to control this pathogen are systemic fungicides, including Prochloraz, Thiophanate-methyl and Benomyl, among others, [2,3] however, their excessive use has generated environmental pollution problems. Because of this, research is being conducted to develop alternative methods to control this pathogen, including the application of plant oils and extracts, including from the genus *Jatropha* [4–6].

The botanical genus *Jatropha* is made up of about 186 species, highlighted by the species *J. curcas* [7,8]. The seeds of *Jatropha curcas* have a remarkable potential to control different fungal species including the genus *Fusarium* [9,10]. Regarding *F. oxysporum* f. sp. *gladioli*, Córdova-Albores *et al.* [11] reported the sensitivity of this fungal species to the application of *J. curcas* oil. The growth rate and germination of this fungal species were significantly inhibited with a concentration of 5 mg mL^{-1}. Subsequent

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studies indicated that the triglycerides triolein and trilinolein, reported as majority compounds in fractions of *J. curcas* oil, as well as the acylglycerides monomyristin and dimyristin, also had an inhibitory effect on *Fusarium oxysporum* f. sp. *gladioli* [12].

To date there is no published research concerning the effect of *J. curcas* oil on the morphology of pathogenic fungi; however, there are studies in relation to the essential oils of plant origin, in which it is reported that, in general, they induce morphological and structural changes in the affected cells. For example, Tripathi *et al.* [13] evaluated the activity of the essential oil of chia (*Hyptis suaveolens*) in the development and morphology of *F. oxysporum* f sp. *gladioli*. These authors reported that the main changes observed by optical microscopy after treatments were a decrease in the diameter of the hyphae, sometimes without cytoplasm and granule formation. In other studies associated with the effect of essential oils of oregano (*Origanum vulgare*) and thyme (*Zataria multiflora*) in the fungus *Aspergillus flavus*, abnormal structures, loss of pigmentation, intense vacuolization and detachment of the cell membrane were observed under the same microscopy [14–16]. In the case of the fungus *Botrytis cinerea*, alterations observed under phase contrast microscopy caused by the effect of oregano and bergamot (*Monarda dydyma*) oils were highlighted by cell damage associated with coagulation and cytoplasm leakage and therefore hyphal shrinkage [17].

Also, in the fungi *Alternaria alternata*, *Colletotrichum destructivum* and *Phytophthora parasitica*, considerable morphological degeneration associated with shrinkage, lysis and cytoplasm coagulation, resulting from the effect of the essential oil of different native plants, were observed with the use of electron scanning microscopy [18].

On the other hand, by optical epifluorescence microscopy and fluorocromatic techniques, several authors have reported the metabolic activity of pathogenic fungi treated with essential oils. For example, Carson et al. [19] reported that the application of tea tree (*Melaleuca alternifolia*) oil resulted in the uptake of the fluorochrome propidium iodide (PI) after 30 min, seriously altering the properties of the cell membrane of *Candida albicans*. Svircev et al. [20] observed by applying different fluorochromes (PI, fluorescein diacetate and 3,3' dihexyloxacarbocyanine iodide) a survival range of 17-23% of the *Monilinia fructicola* conidia treated with vapors of the essential oil of thyme (*Thymus vulgaris*).

The aim of this study was to thus assess, by different microscopic and fluorocromatic techniques, the effect of *J. curcas* oil and its derivatives triolein, trilinolein, monomyristin and dimyristin on the morphology and metabolic activity of *F. oxysporum* f. sp. *gladioli*.

2. Materials and methods

2.1. Microorganism

The strain of *Fusarium oxysporum* f. sp. *gladioli* was isolated from infected gladiolus corms. *Fusarium oxysporum* species had been previously corroborated morphologically and molecularly [11].

2.2. Compounds and treatments

Jatropha curcas seed oil was extracted on Soxhlet using petroleum ether [21]. Sigma-Chemical Co. (St. Louis, MI, USA) provided commercial triacylglicerides trilinolein (glyceryl trilinoleate) and triolein (glyceryl trioleate). Synthetic compounds monomyristin (glyceryl monomyristate) and dimyristin (glyceryl dimyristate) were obtained as follows: *Monomyristin*. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, 155 mg, 1.0 mmol), 4-dimethylaminopyridine (DMAP, 134 mg, 1.1 mmol) and N,N-diisopropylethylamine (DIPEA, 0.10 mL, 1.1 mmol) were added dropwise to a well-stirred solution of glycerin (92 mg, 1.0 mmol) in CH_2Cl_2 (15 mL). The reaction was stirred at room temperature for 30 min and then myristic acid (228 mg, 1.0 mmol) was added dropwise. The reaction was stirred at room temperature for 12 h. Brine solution (25 mL) and CH_2Cl_2 (25 mL) were added to the reaction mixture. The organic phase was separated. The aqueous phase was extracted with CH_2Cl_2 (2×25 mL). Combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo* to give 188 mg (62.5% yield) of crude reaction. The residue was purified by FCC (silica gel, 100-230 mesh; 3.6 cm i.d. x 22 cm) performed with petroleum ether-CH_2Cl_2 (100:0 to 40:60), then CH_2Cl_2 (100:0) and then acetone (100:0) collecting 120 fractions (12 mL each). 42 mg of pure monomyristin (14% yield) were obtained as waxy white solid from 82-88 fractions. *Dimyristin*. The same procedure was used for monomyristin by adjusting the mmol of each reactive as follows: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, 310 mg, 2.0 mmol), 4-dimethylaminopyridine (DMAP, 244 mg, 2.0 mmol) and N,N-diisopropylethylamine (DIPEA, 0.19 mL, 2.1 mmol), glycerin (92 mg, 1.0 mmol) and myristic acid (456 mg, 2.0 mmol), yielding 326 mg (63.8% yield) of crude reaction. The residue was purified by FCC (silica gel, 100-230 mesh; 3.6 cm i.d. x 22 cm) performed with petroleum ether-CH_2Cl_2 (100:0 to 40:60), then CH_2Cl_2 (100:0) and then acetone (100:0) collecting 108 fractions (12 mL each). 107 mg of pure dimyristin (21% yield) was obtained as waxy white solid from 66-70 fractions. Both compounds were identified by analysis of their ^{1}H NMR spectra parameters. *Monomyristin* δ 4.27-4.14 (5H, m, H_1-H_3), 2.34 (2H, t, J = 7.2 Hz, $H_{2'}$), 1.60 (4H, m, $H_{3'}$), 1.26 (20H, br s, $H_{4'}$-H-$_{13'}$), 0.88 (3H, t, J = 7.0 Hz, H-$_{14'}$). *Dimyristin* δ 4.27-4.14 (5H, m, H_1-H_3), 2.34 (4H, t, J = 7.2 Hz, $H_{2'}$), 1.60 (4H, m, $H_{3'}$), 1.26 (40H, br s, $H_{4'}$-H-$_{13'}$), 0.88 (6H, t, J = 7.0 Hz, H-$_{14'}$).

The concentrations applied in this study of *J. curcas* seed oil (2.5 mg mL^{-1}), trilinolein, triolein, monomyristin and dimyristin (200 mgL^{-1} each compound) correspond with those showing outstanding antifungal activity on *F. oxysporum* f. sp. *gladioli* in previous studies reported by Córdova-Albores et al. [12].

2.3. Evaluation of morphological changes

2.3.1. Phase Contrast Microscopy

Conidia of *Fusarium oxysporum* f. sp. *gladioli* at 2×10^6 mL^{-1} concentration were cultured in microtubes

containing potato dextrose broth (PDB) nutrient added with the treatments: *J. curcas* oil, triolein, trilinolein, monomyristin and dimyristin. After 24 h incubation at room temperature, observations were made with a Zeiss Axioskop 40 microscope at 100X magnification with immersion oil.

2.3.2. Scanning Electron Microscopy (SEM)

Conidia and mycelia of a 6-day culture of *F. oxysporum* f. sp. *gladioli* from the above mentioned treatments were fixed in solutions of 2.0% glutaraldehyde for 24 h at 5 °C. Samples were rinsed three times with 0.02 M phosphate buffer for 2 h and postfixed with 2% osmium tetraoxide for 2 h at room temperature (26 °C). Dehydration was made with ethanol at gradual concentrations (30-100%) for 15 min each one. They were dried in the presence of CO_2 for 40 min (SAMDRI-780B Tousimis), and mounted on stubs with carbon tape and covered with gold in a metal ionizer (Baltec SDC 50) for 15 min. Samples were observed in an electronic scan microscope (Jeol model JEM 7600F) with a resolution of 4 nm at 15 Kv.

2.4. Evaluation of the metabolic activity

2.4.1. Membrane integrity (MI) and viability of conidia by Fluorescence Microscopy

For evaluation of MI and viability, a conidial concentration of *F. oxysporum* f. sp. *gladioli* sampled from the above treatments was adjusted to 2×10^5 conidia mL^{-1}, cultured on potato broth (PB) and added with the fluorescent dyes. Prior to staining with the fluorochromes, conidia were centrifuged and rinsed three times with 0.02M-phosphate saline buffer. Then they were stained with the following fluorescent dyes: propidium iodide (PI, Sigma Chemical, St. Louis, MO, USA), 3, 3′-dihexyloxacarbocyanine iodide ($DiO6_{(3)}$, Sigma Chemical), and the combination of PI and fluorescein diacetate (FDA, Sigma Chemical). The fluorescent dyes were prepared according to the protocol described by Svircev et al. [20]. The PI stain was prepared by using a 3 mg mL^{-1} stock solution adjusted to a working solution of 18 μg mL^{-1} in 50 mL sterile distilled water. The FDA stain stock solution consisted of dissolving FDA 1 mg mL^{-1} in acetone and storing at −20 °C, whereas the $DiO6_{(3)}$ was dissolved in ethanol at a concentration of 5 mg mL^{-1}, and the working aqueous solutions were prepared at a concentration of 50 mg mL^{-1}. PI alone or combined with FDA stain (each 5 μL), and a drop of 10 μL of the above- mentioned spore suspensions were mixed and examined after 0, 4, 8, 12, 16 and 24 h for membrane integrity evaluation and, within 10 min for spore viability under a fluorescent microscope. Viable conidia fluoresced bright green-yellow with FDA, while dead cells fluoresced red with PI. The final concentration of the stain $DiO6_{(3)}$ used with the conidia was 10 μg mL^{-1}. The viable cells stained green while the non-viable were orange in color. Examinations were carried out under a Zeiss Axioskop 40, Leitz Dialux 20 microscope (Carl Zeiss, Oberkochen, Alemania) fitted with epifluorescence optics using a blue excitation (450–490 nm) and 520-590 nm barrier filters.

Percentage MI and conidial viability were calculated as follows:

MI (%) = No. spores–No. stained spores/Total No. of spores;

Viability (%) = No. stained spores–Total No. of spores/ Total No. of spores.

2.4.2. Germination of conidia

10 ml of sterile distilled water were added to Petri dishes containing the growth of *F. oxysporum* f. sp. *gladioli*, of the above treatments. The surface was scraped with a bent metal rod and the filtrate was passed through cotton gauze. Of this suspension, 20 μl of 5×10^5 conidia mL^{-1} were placed on PDA discs of 10 mm in diameter and incubated for 8 h at 26 °C. Later a few drops of lactophenol methylene blue were added and the number of germinated spores was determined. Germination was evaluated on nine PDA discs.

2.5. Statistical analyses

A completely randomized design was used for statistical analysis of percentages of MI, viability and germination parameters. One-way analysis of variance with a significance level of $p < 0.05$ was applied. Similarly, when significant differences were found, a comparison of means was performed using Tukey's multiple comparison tests ($p < 0.05$). Means and standard deviation were also calculated.

3. Results

3.1. Evaluation of morphological changes

3.1.1. Phase Contrast Microscopy

Along the hyphae of *F. oxysporum* f. sp. *gladioli*, in the control and triolein treatments, homogeneous structures including cytoplasm, regular and defined septa, and various organelles were observed (Fig. 1a, d). Germinated hyphae of the conidia incubated with *J. curcas* oil and trilinolein showed irregular shapes, without septa, swollen and little or no presence of organelles. (Fig. 1b, c). In the case of the monomyristin and dimyristin, it was observed that after germination there was little hyphal growth and intense vacuolization (Fig. 1e, f).

3.1.2. SEM

The application of *J. curcas* oil and the triglycerides trilinolein and triolein substantially altered the morphology of the mycelium and conidia of *F. oxysporum* f. sp. *gladioli*. In all treatments, except for the control (Figs. 2a, 3a), there was widespread thinning along the hyphae of the fungus (Fig. 3b-f). In hyphae treated with trilinolein and triolein, the mycelium appeared distorted with discontinuous areas due to the effect of the hypha breaking (Fig. 3c, d). In the treated conidia, the formation of pore-type collapsation stood out, being most notable with the *J curcas* oil and trilinoein (Fig. 3b, c).

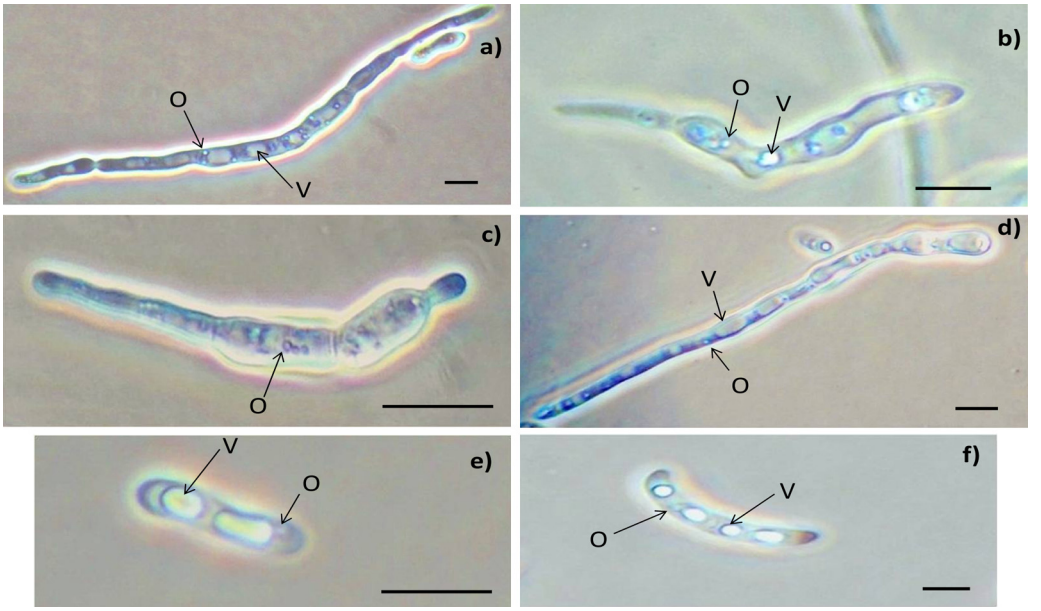

Fig. 1. Phase contrast micrographies of *Fusarium oxysporum* f. sp. *gladioli* conidia treated with: a) PDB, b) *J. curcas* oil, c) trilinolein, d) triolein, e) monomyristin and f) dimyristin. Bar 10 μm. v = vacuole, o = organelle.

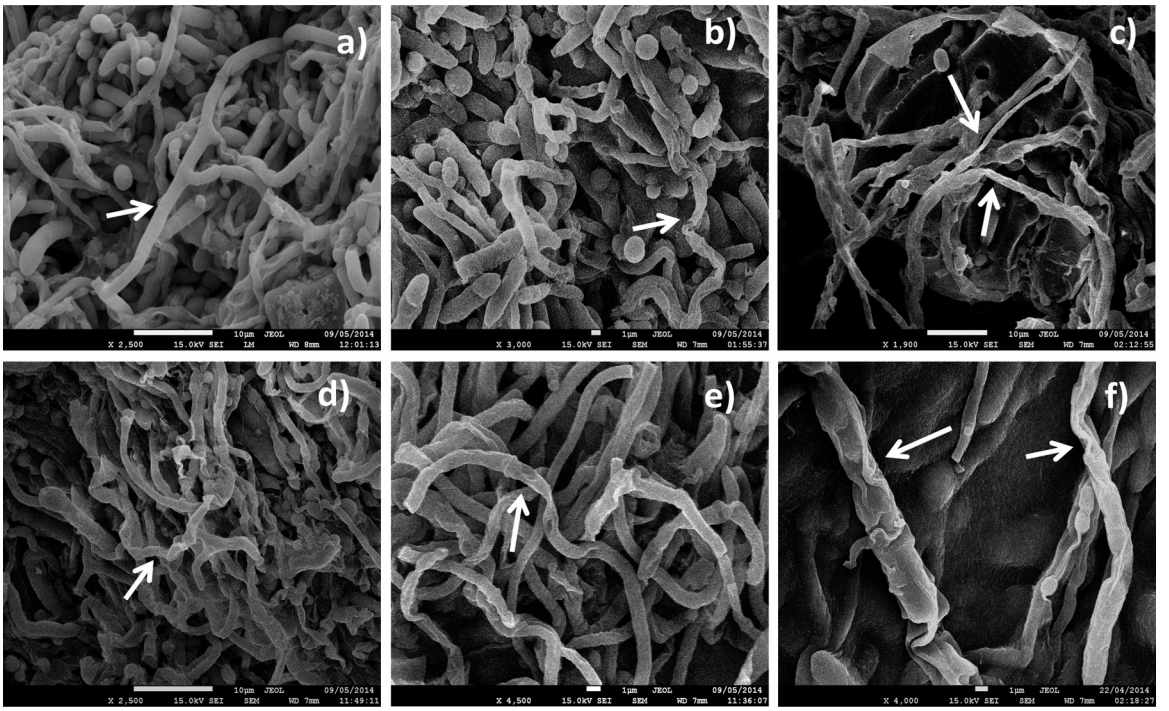

Fig. 2. Scanning Electron micrographies of *Fusarium oxysporum* f. sp. *gladioli* treated mycelia with: a) PDB, b) *J. curcas* oil, c) trilinolein, d) triolein, e) monomyristin and f) dimyristin. Arrows point out in a) well-formed and continuous mycelia and b-f) contorted, dehydrated and uneven mycelia.

3.2. Evaluation of the metabolic activity

3.2.1. Membrane integrity (MI) and viability of conidia by Fluorescence Microscopy

In relation to membrane integrity, statistical differences ($p > 0.05$) among treatments and the incubation period in each applied fluorochrome were observed. During the 24 h of incubation, it was noted that, in general, the most damage to the cell membrane of *F. oxysporum* f. sp. *gladioli* was with the *J. curcas* oil and trilinolein. Thus, with the PI fluorochrome, from 8 h the main damage to the membrane in conidia treated with trilinolein was observed, while at 12 h it was noted with the *J. curcas* oil. In this case, in general, damage increased during the incubation time in the

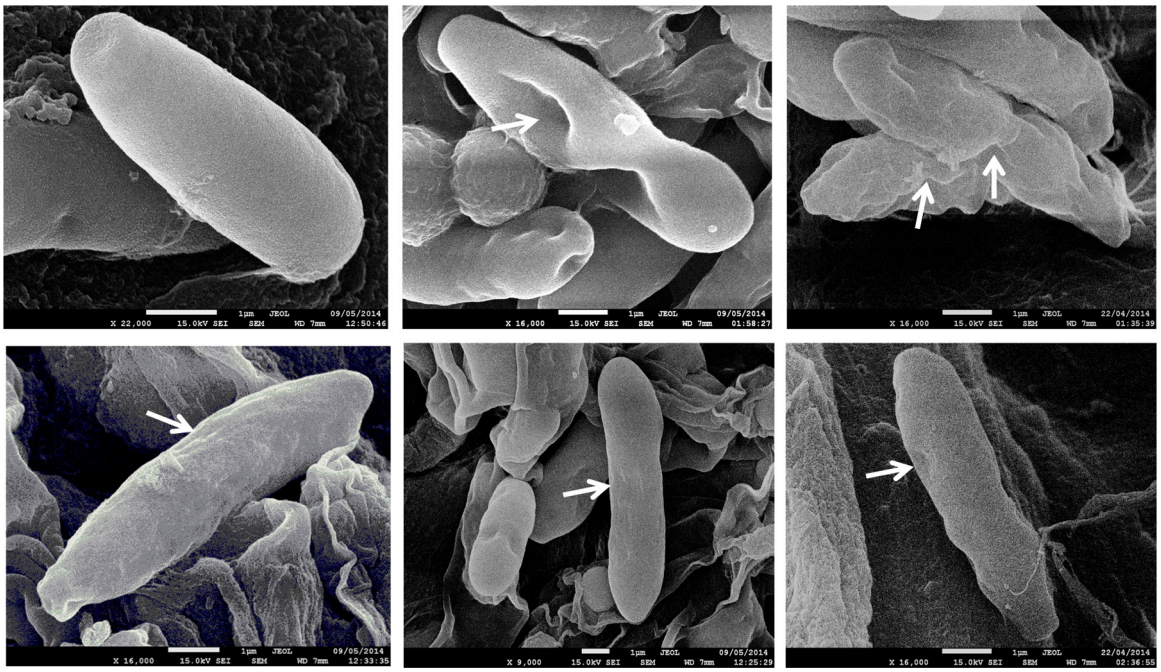

Fig. 3. Scanning Electron micrographies of *Fusarium oxysporum* f. sp. *gladioli* treated conidia with: a) PDB, b) *J. curcas* oil, c) trilinolein, d) triolein, e) monomyristin and f) dimyristin. Arrows point out in a) well-formed, and b-f) uneven, damaged and dehydrated conidia, showing sunken areas.

Table 1
Mean percentage viability of *F. oxysporum* f. sp. *gladioli* conidia stained with different fluorochromes.

	Fluorochrome		
Treatment	**PI**	**PI+FDA**	**$DiO_{6(3)}$**
PDA	97.92 ± 3.06	99.30 ± 1.20	100 ± 0.00
J. curcas oil	84.66 ± 13.53	87.33 ± 9.33	97.56 ± 4.22
Trilinolein	95.65 ± 3.77	94.51 ± 8.14	98.72 ± 0.69
Triolein	98.87 ± 1.95	90.83 ± 8.66	90.06 ± 2.03
Monomiristin	89.53 ± 10.19	87.96 ± 18.49	99.16 ± 0.79
Dimiristin	98.36 ± 1.59	74.32 ± 18.4	99.26 ± 0.99

treatments (Fig. 4a). With the PI and FDA fluorochromes, it was observed that the treatment with *J. curcas* oil affected membrane activity from 4 h, followed by trilinolein, triolein and dimyristin, after 12 h; however, recovery of the metabolic activity of the membrane was observed at the end of the incubation period in these treatments (Fig. 4b). With $DiO6_{(3)}$, in all treatments there was a decrease in the metabolic activity of the cytoplasm at 12 h, being greater with triolein, monomyristin and *J. curcas* oil. From 16 h of incubation, there was a notable recovery in all treatments (Fig. 4c).

Regarding the viability percentage of the conidia, there were no significant differences among treatments (Table 1). With the PI fluorochrome, the lowest viability percentage (85%) was observed in conidia treated with *J. curcas* oil; with the FDA and PI fluorochromes, the lowest percentage occurred with dimyristin with an approximate value of 75%, and with $DiO6_{(3)}$ the lowest viability (90%) was seen with triolein.

3.2.2. Germination of conidia

In relation to the germination percentage, there were statistical differences ($p > 0.05$) among the applied treatments relative to the control (Fig. 5). The lowest percentage was observed with trilinolein (38%), followed by triolein (50%) and dimyristin (60%). For the control, 100% germination occurred.

4. Discussion

Results showed that overall the application of *J. curcas* oil and its derivatives, including acylglycerides and synthetic products, caused morphological and cell damages in the mycelium and conidia of *F. oxysporum* f. sp. *gladioli*. Phase contrast microscopy mainly showed intense vacuolization in the treated hyphae of this fungus. In this regard, Henics and Wheatley [22] state that the phenomenon of 'vacuolization' may correspond to adaptive physiological responses to external agents, in this case the application of *J. curcas* oil and its derivatives. For their part, Calahorra et al. [23] emphasize that the functions of vacuoles in fungal cells include homeostatic intracellular mechanisms and regulation of various enzyme activities. On the other hand, and in line with other studies, the induction of visible changes in the morphology of *F. oxysporum* f. sp. *gladioli* was noteworthy in the present study. SEM micrographs showed serious alterations in the outer structures with *J. curcas* oil, trilinolein and triolein. In this study, the use of fluorochromes allowed quantifying the cell damage at membrane and cytoplasm level, and the survival of conidia of *F. oxysporum* f. sp. *gladioli*, exerted by *J. curcas* oil and its derivatives. With the applied fluorochromes,

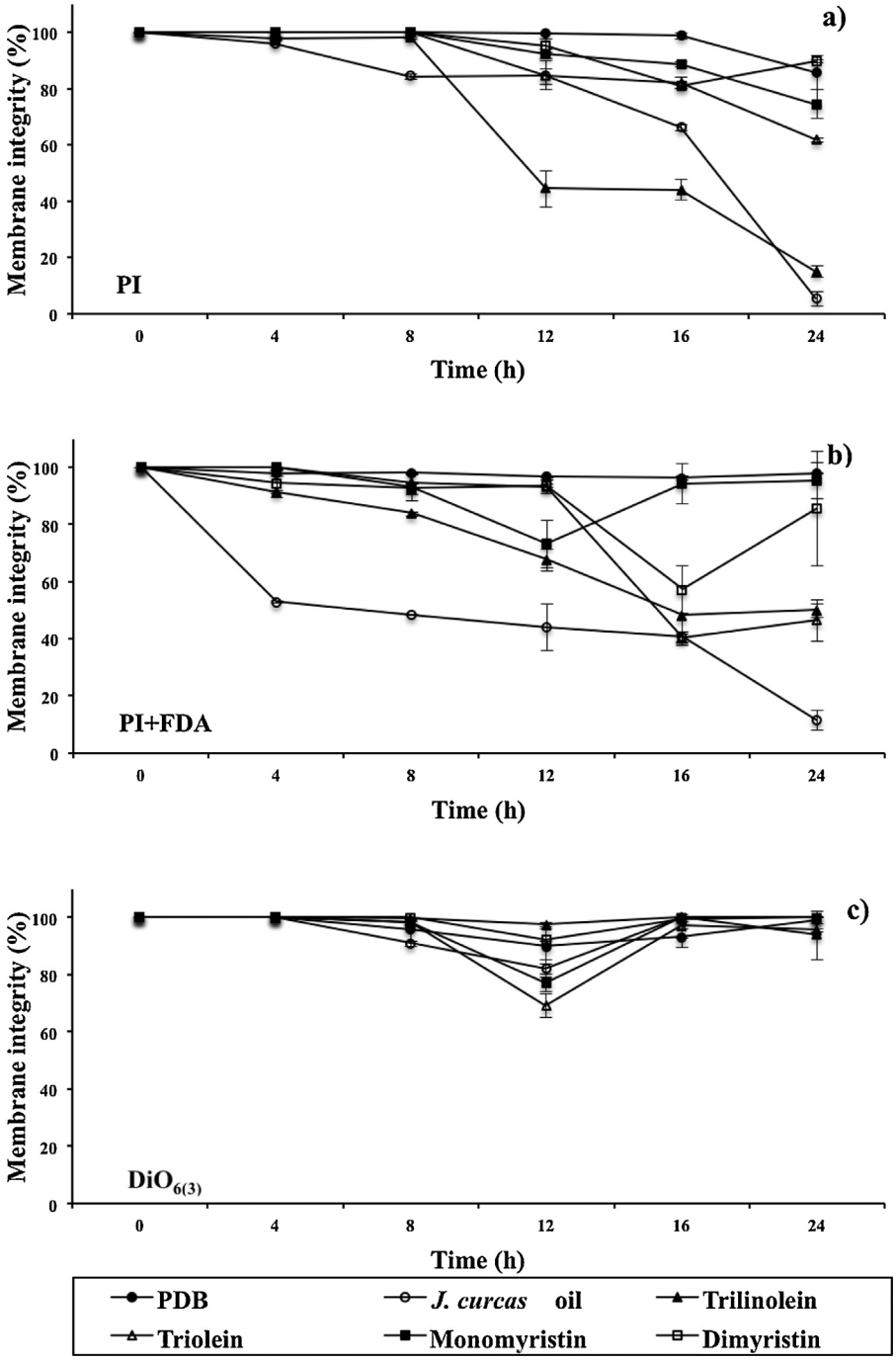

Fig. 4. Evaluation of membrane integrity by different fluorochromes of *Fusarium oxysporum* f. sp. *gladioli* during 24 h incubation in *J. curcas* oil and derivatives.

the ratio/relationship of living and dead cells relative to the metabolically-active of plasma membrane (PI and/or FDA) and cytoplasm ($DiO6_{(3)}$) was detected. Thus, it was observed with the PI and/or FDA fluorochromes that the treatments that primarily affected the metabolic activity of the membrane during the 24-h incubation time were the *J. curcas* oil and the triglycerides, trilinolein and triolein, while the damage at the level of metabolic activity of the cytoplasm was at 12 h of incubation. To date, there are no reports about the effect of *J. curcas* oil and these acylglycerides on the metabolic activity in a fungal cell; however, there are reports of antifungal activity of other monoacylglycerides and fatty acids such as lauric acid and its derivatives (monolauroylglycerol, D-laurate, T-laurate and 6-*O*-lauroysucrose) in *Aspergillus niger*, where a noticeable effect was reported on germination as a result of their application [24]. In subsequent studies, other compounds synthesized from coconut oil, namely lauroylglycerol and

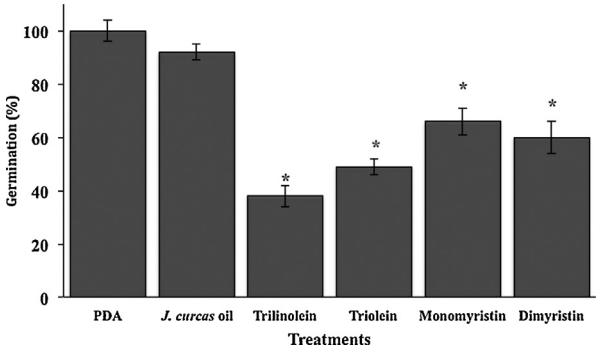

Fig. 5. Percentage germination of *Fusarium oxysporum* f. sp. *gladioli* after 8 h incubation on *J. curcas* oil and derivatives. *Significant differences Tukey's multiple comparison test ($p < 0.05$).

mixtures of monoacylglycerols, also influenced other *in vitro* development stages of this fungus [25]. Similarly, Liu et al. [26,27] demonstrated that fatty acids (butyric, caproic, lauric, etc.), alone or in combination, had strong antifungal activity against several phytopathogenic fungi including *F. oxysporum*.

In this research it was interesting to note that within 10 min of incubation with the applied fluorochromes none of them could detect any effect of the treatments on the viability percentage of the conidia; thus, the data indicate values of close to 100%, including the control. However, it was observed that, due to the application of the treatments, conidia germination was significantly lower with trilinolein and triloein, as the average values for these two treatments were 30% and 50% respectively. In this regard, Pohl et al. [28] in a study on the antifungal activity of fatty acids, highlighted the effect of various analogues of tetradecanoic (myristic) acid to inhibit N-myristoyltransferase (NMT), which is considered an essential enzyme for the growth and survival of several fungi and yeasts, including *F. oxysporum*. These authors, in agreement with previous studies among others, by Liu et al. [26] and Carballeira [29] in *A niger* and *Candida albicans*, respectively demonstrated, first of all, the ability of these pathogens to synthesize a variety of N-myristoyl proteins using NMT as a catalyst, and secondly, that the incorporation of these myristic acid analogues to the fungi competed for binding NMT, thereby producing myristoylation of the fungus proteins. Consequently, this event disrupted protein functions, such as folding, resulting in the inhibition of fungal growth.

Bakkali et al. [30] noted that most essential oils, particularly in bacterial and fungal cells, cause a series of physiological events and reactions in affected cells, which may even lead to death by apoptosis or necrosis. In this study despite the observed morphological and cellular (vacuolization) alterations, and the apparent damage to the metabolic activity of the membrane of the *F. oxysporum* f. sp. *gladioli* conidia due to the treatments, the germination process took place once the conidia were transferred to a suitable nutrient medium, indicating that the damage exerted by both the *J. curcas* oil and its derivatives was partial (fungistatic effect), allowing their recovery and survival.

5. Conclusions

This research showed, by means of different microscopic and fluorocromatic techniques, some of the possible mechanisms of action of *J. curcas* oil and its derivatives, including: changes in the morphology of the outer covering of the mycelium and conidia, the presence of numerous vacuoles and inhibition of the metabolic activity of the fungus membrane. However, the damage was not so substantial as to cause a complete fungicidal effect.

Conflict of interest

Authors declare that there is no conflict of interest.

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