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Morphological and biochemical modifications induced by a static magnetic field on *Fusarium culmorum*

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Abstract

The effects of the exposure to a static magnetic field (sMF) of 0.3 ± 0.03 T on the *Fusarium culmorum* were investigated in vitro. sMF inhibition of mycelia growth was accompanied by morphological and biochemical changes. Fungal conidia germination and cell viability were also reduced. We provide evidence of the influence of sMF on Ca^{2+}-dependent signal transduction pathways involved in conidia germination. Perturbation of these pathways by adding different compounds (i.e. $CaCl_2$, phorbol 12-myristate 13-acetate, neomycin, EGTA, LiCl) to the medium, suggested that exposed conidia are unable to mobilise calcium from intracellular stores and that the hindered mechanism may be IP_3-dependent.

Keywords: Static magnetic field; *Fusarium culmorum*; Germination; Ca^{2+}-dependent signal transduction pathways; Antifungal agents

1. Introduction

Fusarium head blight (FHB) and Fusarium ear rot (FER) are worldwide damaging cereal diseases and the common causal organisms include *Fusarium graminearum, Fusarium culmorum, Fusarium avenaceum, Fusarium poae* and *Microdochium nivale. F. culmorum* and *M. nivale* are the agents causing foot rot of wheat [1,2]. *F. graminearum* is responsible of head blight of wheat [3]. FHB and FER cause severe yield and quality losses [4], but the most serious concern is the possible contamination of mycotoxins, some of which have relevant impact on human and animal health [5–7]. At present there are two major approaches to reduce mycotoxin contamination of grain: (i) selective breeding of cultivars resistant to *Fusarium* pathogens; (ii) application of fungicides to reduce FHB and FER. The use of resistant cultivars is a must economical and sustainable option [8] but the different climatic conditions of growing areas seem to play a determinant role on the growth of toxigenic fungi and the subsequent accumulation of mycotoxin in cereal kernels [3]. Application of fungicides could, therefore, be an alternative measure to reduce FHB and FER and mycotoxin contamination, but conflicting evidence exists regarding their use. For example, applications of propiconazole significantly reduce the incidence of FHB caused by *F. graminearum* but did not affect the mycotoxin concentration in harvested grain [9]. In contrast, it has been shown that propiconazole [10], triadimefon [11], thiophanate-methyl [12], and tebuconazole [13] all could reduce the severity of diseases and the accumulation of mycotoxins. However, a formulation of tebuconazole plus triadimenol applied to ears of winter wheat artificially inoculated with *F. culmorum* reduce symptoms of FHB but increase up to 16-fold mycotoxin content in harvested grain [10,14]. Furthermore, it is emerging that the use of tebuconazole to inhibit Fusaria growth is accompanied by marked morphological and cytological alterations, most likely associated with biochemical changes [13].

We proposed static magnetic fields (sMF) as a safe preservative agent substitute for chemical fungicides. Since a very large number of cellular components, cellular processes, and cellular systems can conceivably be affected by sMF, mecha-

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nistic studies are essential to guide and interpret the experimental work. Most studies have been performed on cultured cells exposed in vitro to investigate sMF effects on cellular membranes, general and specific gene expression and signal transduction pathways [15–18]. The great advantage of in vitro exposure is its precision, since the geometry and physical properties of the system can be well-controlled.

It is well-known that calcium ions are regulators of cell proliferation and morphogenesis in many cell systems [19–21] and recently numerous experimental investigations have interested the interaction between Ca^{2+} fluxes and sMF [18]. Effects of calcium ions on conidia germination have already been described indicating a stimulatory role, since the removal of calcium with Ca^{2+}-chelators inhibits germination [22]. Any change in the intracellular free Ca^{2+} content can then have various effects, via cytoskeletal interaction or other Ca^{2+}-binding proteins [23]. All the substances that interfere with Ca^{2+}-mediated processes can disrupt germination (e.g. calcium channel-blockers, calcium ionophores, calcium chelators, calmodulin inhibitors, caffeine, TMB-8, etc.).

We already described the antibacterial effect of sMF on *Serratia marcescens* [24] and in this paper we extend our experimental approach to *F. culmorum* colonies to investigate the effects of sMF on the hyphal growth and the conidial germination. Ultrastructure hyphae modifications are correlated with the changes in the glyoxylate cycle, a metabolic pathway involved in fungal virulence [25]. The use of germinating conidia gave us the possibility both to determine biochemical alterations during sMF exposure and to speculate on the role of calcium ions in the process of conidia germination and in the mechanism of action of magnetic field.

The observed sMF effects are cytostatic (as reported for the reversible antiapoptotic effects observed on U937 by [18]), but our proposal as preservative agents may be intended for an alternative to cold.

2. Materials and methods

2.1. Fungal cultures preparation

Discs (5-mm diameter) from *F. culmorum* (strain No. 1 isolated from wheat ears) culture were inoculated in the centre of Petri dishes containing potato dextrose agar (PDA; Oxoid) and grown for 1 week in a control chamber at 24 °C and 4000 lux with 12-h photoperiod. Petri dishes were used either for sMF exposure experiments or for conidia production. In the latter case, Petri dishes were exposed again for one more week in a control chamber at 24 °C and N-UV lux with 12-h photoperiod to stimulate conidia germination.

2.2. Cell extraction

Cultures of *F. culmorum* were used for mycelium collection by gently removing hyphae from Petri dishes. Mycelia were suspended in 2 ml (final volume) of 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 50 mM NaCl, pH 6.5 and lysed by sonic disruption (30 × 3 s, in a Heat Systems Model 350 sonicator, Plainview, NY). The lysate was centrifuged at 15 000 × *g* for 30 min at 4 °C and the supernatant fluid was used for metabolic studies. The pellet was utilised for fresh and dry weight determination: briefly, the pellet was immediately weighted (FW) and left for 24 h in an oven at 95 °C and then weighted again (DW). To calculate the "adjusted dry weight" on a total volume of 1 ml the following formula was applied: aDW = (FW – DW)/FW/total volume.

2.3. Protein determination

Protein was determined by the method of Bradford [26] using the Bio-Rad protein assay and bovine serum albumin as standard. Protein concentration was calculated as mg/g of aDW.

2.4. Enzymatic and metabolic assays

Isocitrate lyase (ICL) activity was measured as previously described [27]. Lipase was assayed according to Mauck [28]. Glucose was determined enzymatically as described by Bergmeyer et al. [29]. Triglyceride concentrations were assayed by routine enzymatic method [30]. Enzyme activities were calculated as Units per gram of proteins (U/g Prot). Lipid and glucose concentrations were expressed as mg/g aDW.

2.5. Conidia isolation

Conidia suspensions were prepared by washing mycelia of *F. culmorum* with sterile Czapek liquid medium (Oxoid) and by filtering through glass-wool filters to free the conidia from mycelial contaminations. Conidial suspension aliquots were placed in different Petri dishes on glass coverslips treated with 0.01% poly-L-lysine solution. The coverslips were gently overlaid with Czapek liquid medium and kept at 24 °C for 6 h in the absence or in the presence of sMF. The coverslips with tightly adhering conidia were used for optical and fluorescence microscope observations. To calculate the percentage of germination, conidia counting was performed by using a Thoma chamber and the formula: germinating conidia/total conidia × 100, was applied.

2.6. Static MF exposure of mycelia and conidia

sMF was produced by 28.5 × 10 mm Neodymium magnetic disks of known intensities supplied by Calamit Ltd (Milan, Italy) which were placed under the Petri dishes. The field was axial, magnitude and homogeneity of 0.3 ± 0.03 T were checked by means of a gaussmeter (Hall-effect gaussmeter, GM04 Hirst Magnetic Instruments Ltd, UK). Mycelia from *F. culmorum* were exposed to sMF immediately after their inoculation on Petri dishes. Simultaneous experiments by omitting the magnets were performed as controls (sham).

Aliquots of conidia suspensions (450–500 × 10^3 per ml) were induced to germinate in the presence or absence of sMF. In basal condition of germination, the conidia were grown in the absence of added calcium, with intracellular stores as sole source of Ca^{2+}. With the aim to influence Ca^{2+}-dependent signal transduction pathways during conidia germination, different concentrations of $CaCl_2$ (to increase intracellular calcium), EGTA (to deplete free intracellular calcium), neomycin (to inhibit phospholipase C), phorbol 12-myristate 13-acetate (PMA; to stimulate protein kinase C) and LiCl (to block IP_3 recycling) were separately added to the medium.

2.7. Optical and fluorescence microscopy

Coverslips with adherent conidia were fixed with 37% formaldehyde and 0.2% Triton X-100 in phosphate buffer (50 mM, pH 7.0) for 30–45 min at room temperature. For optical microscope investigation, the coverslips were rinsed with distilled water, stained with hematoxylin–eosin for 45 min at room temperature and observed with a ZEISS axyoplane light microscope. For acridine orange fluorescence observation, the coverslips were rinsed with distilled water and stained with 0.01% acridine orange for 5 min at room temperature. Subsequently, the coverslips were mounted on clear glass slides and observed with a ZEISS 40× objective (filterset 14 excitation BP 546/12, emission LP 590). The conidia suspensions used for different treatment and exposure conditions, were fixed with 2% paraformaldehyde [31] in phosphate buffer (10 mM, pH 7.2) and incubated with the calcium fluorescent indicator chlortetracycline (CTC, 10 μM) for 15 min in the dark. Conidia were washed twice with the same buffer and resuspended in 100 μl (final volume) before transferring the sample on a gelatinised slide for fluorescence emission estimation (filterset 0.9 excitation BP 450/490, emission LP 520). As already reported [32], after CTC staining two fluorescent patterns are generated: the first one referred to as "diffuse" with the fluorescence uniformly distributed in the cytoplasm and the second one designated "punctate" with the fluorescence mainly concentrated in the organelles. All the fluorescence evaluations were performed by using a ZEISS 40× objective.

2.8. Scanning (SEM) and transmission (TEM) electron microscopy

For SEM, samples were fixed with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.3) for 1 h and quickly washed with the same buffer. Thereafter, the samples were post-fixed in 1% OsO_4 in phosphate buffer (0.1 M, pH 7.3) for 1.5 h, progressively alcohol dehydrated and finally point-dried with a Emitech K 850 device. After mounting with adhesive ribbon on conventional SEM stubs and outlining with silver glue, slides were gold-coated by a Emitech K 550 sputtering device. Observations were carried out with a Philips 515 at 15 kV. For TEM, the agar pieces were cut to 1 mm, washed with phosphate buffer (0.1 M, pH 7.3) and immediately fixed with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.3) for 1.5 h. Subsequently, the samples were post-fixed with 1% OsO_4 in phosphate buffer (0.1 M, pH 7.3) for 1.5 h, alcohol dehydrated and embedded in araldite [33]. Semithin sections were stained with 1% toluidine blue in distilled water at 60 °C. Thin sections were collected on nickel grids, stained with uranyl acetate and lead citrate, and analysed with a Philips 300.

2.9. Statistical analysis

In order to test the significance ($\alpha = 0.05$) of the differences between regression lines, the 95% confidence interval of the difference between the slopes has been computed. If the 95% confidence interval includes 0, then the difference is not statistically significant with respect to the slopes. The same procedure has been performed with respect to the intercepts.

2.10. Chemical

Analytical grade chemicals were from Sigma (USA).

3. Results

3.1. Morphological and biochemical changes in F. culmorum mycelia exposed to sMF

One week after inoculation, the diameters of *F. culmorum* colonies grown in the absence and in the presence of 0.3 ± 0.03 T sMF, were 8.50 and 4.87 cm (Table 1), respectively, indicative of a strong inhibitory effect of sMF on fungal growth. Optical microscopy observation (not shown) also revealed that *F. culmorum* cultures exposed to sMF did not produce any conidia and the hyphae from the colony edges showed pronounced alterations in comparison with control. SEM observation revealed that following exposure to sMF, the cell walls of the hyphae became considerably shrivelled (Fig. 1a vs. b). TEM also revealed that sMF exposure induced an increase of vacuoles and lipid bodies and organelle disorganisation in the cytoplasm of the hyphae (Fig. 1c vs. d).

Table 1
Morphometric and biochemical parameters evaluated in *F. culmorum* colonies exposed to sMF

	Control samples	Exposed samples
Colony diameter (cm)	8.5 ± 0.5 *	4.87 ± 0.91
Protein concentration (mg/g aDW)	4.95 ± 0.67 **	3.08 ± 0.72
Isocytrate lyase (U/g Prot)	0.31 ± 0.013 *	0.23 ± 0.033
Lipase (U/g Prot)	0.42 ± 0.058 **	1.38 ± 0.14
Lipid concentration (mg/g aDW)	2.65 ± 0.60 *	3.46 ± 0.45
Glucose concentration (mg/g aDW)	1.85 ± 0.34 *	1.30 ± 0.17

Results are the mean ± S.D. of six different experiments.
aDW stands for adjusted dry weight. Details of these experiments are given under Section 2.
*, $P < 0.05$; **, $P < 0.005$: significance vs. the value for control cells.

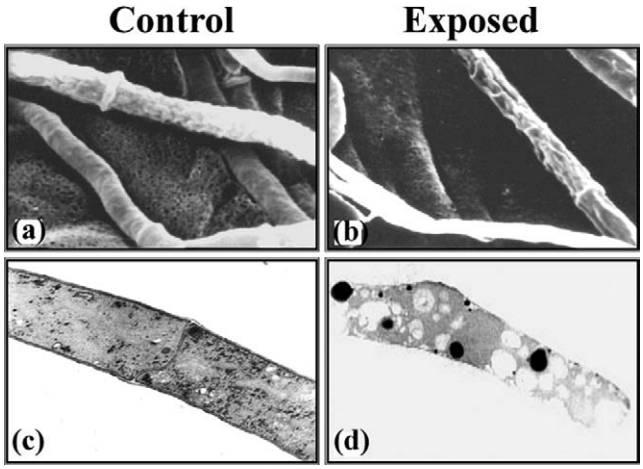

Fig. 1. sMF effects on *F. culmorum* growing mycelia. Morphology was observed by (a and b), SEM; and (c and d) TEM. SEM evidenced the presence of shrivelled hyphae in exposed samples (b) in comparison with controls (a). TEM revealed hyphae with cytoplasmic vacuolisation (lipid and electron dense bodies) in exposed samples (d) in contrast to homogenous cytoplasm and normally distributed organelles of controls (c). Cells were photographed at the same magnification, SEM (×4780) and TEM (×7000).

In addition to the observed decrease of colony diameter of *F. culmorum* caused by sMF exposure, we also detect a reduction of the protein concentration, supporting that sMF could exert an inhibitory effect on the mycelial growth rate (Table 1). From Table 1, it can be seen that the exposure of fungal cells to sMF induced a decrease in isocytrate lyase enzyme activity. This decrease was accompanied by a reduction of glucose concentration (1.30 vs. 1.85 mg/g aDW), by an increase of lipase activity (1.38 vs. 0.42 U/g Prot) and by an elevation of lipid concentration (3.46 vs. 2.63 mg/g aDW). The latter confirms TEM images showing the formation of vacuoles and lipid bodies in the cytoplasm of the exposed hyphae.

3.2. Effects of sMF on F. culmorum conidia germination

Fig. 2 shows the percentage of germination of conidia incubated in the presence of different concentrations of $CaCl_2$, EGTA, neomycin, LiCl and PMA, separately, compared with the percentage of cells grown without added calcium. No significant differences were found in the conidia germinating in the presence of all concentrations tested for $CaCl_2$, LiCl and PMA after 2, 4 and 6 h incubation. On the contrary, a significant reduction of germination occurred at increasing concentrations of neomycin (from 29% to 0%) and of EGTA (from 43% to 37%) after 6-h incubation. As shown in Fig. 3, conidia germinating in the absence of calcium exhibited free cytoplasmic calcium ions as diffuse fluorescence after CTC staining. Perturbation of the cellular Ca^{2+} distribution with EGTA or neomycin resulted in a transition from diffuse to punctate fluorescence. There was no modification of diffuse fluorescence by adding $CaCl_2$, LiCl and PMA to the medium (not shown).

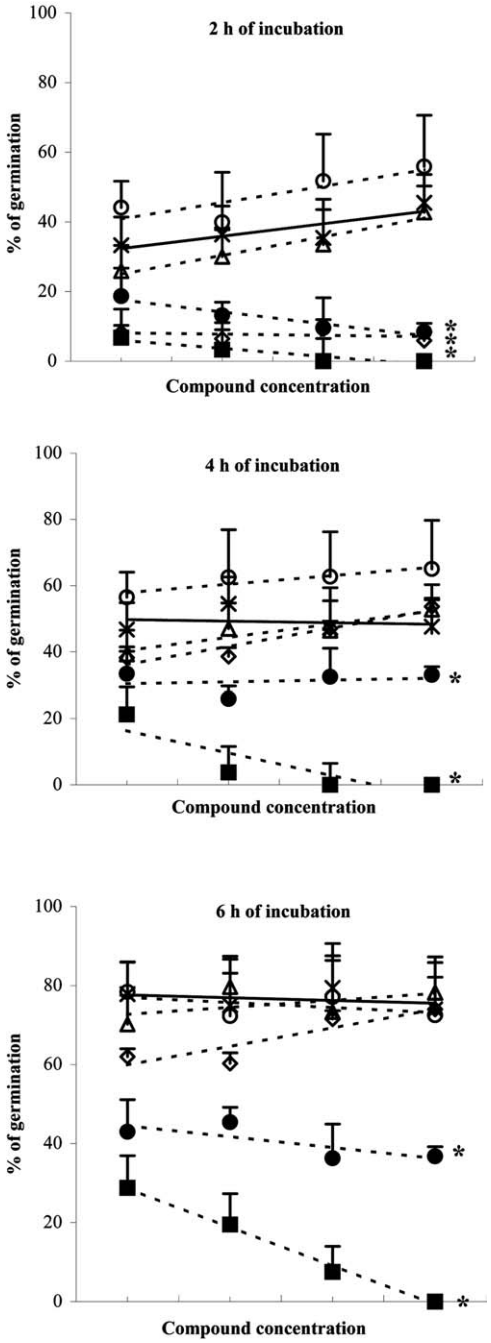

Fig. 2. Influence on germination percentage of *F. culmorum* conidia observed after 2, 4 and 6 h of incubation in the absence (-×-, control) and in the presence of the following compounds: -●-, EGTA (0.5, 1, 1.5, 2 mM); -■-, neomycin (0.5, 1, 1.5, 2 mM); -◇-, LiCl (1.25, 2.5, 5, 10 mM); -△-, PMA (0.25, 0.5, 1, 1.5 mM); -○-, $CaCl_2$ (1.25, 2.5, 5, 10 mM) (results are the mean ± S.D. of 20 different experiments; * significantly different from the control).

When *F. culmorum* conidia were induced to germinate in the absence and in the presence of sMF, an inhibition of the germination was observed. As represented in Fig. 4, a 6 h exposure produced about 45% germination in comparison with 76% of the control. The decreased germination was confirmed by hematoxylin–eosin and acridine orange stainings. The former giving an assessment of the conidial cell

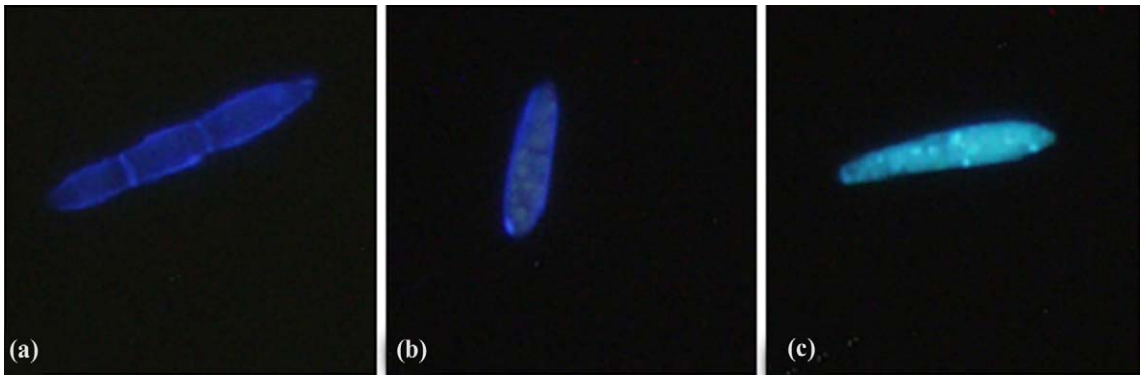

Fig. 3. Fluorescence microscopy of germinating conidia. Cells incubated in the absence and in the presence of different compounds were observed after CTC staining. Images are representative of the fluorescence observed in: (a) control and conidia treated with $CaCl_2$, LiCl and PMA; (b) conidia treated with EGTA; (c) conidia treated with neomycin. Fluorescence emitted in (a) is referred as "diffuse" in contrast to "punctate" emitted by (c). Both types of fluorescence are emitted in (b). Cells were photographed at the same magnification (×60).

number (Fig. 5a vs. b), the latter assessing conidial cell viability (Fig. 5c vs. d). Moreover, the inhibitory effect of sMF caused the appearance of punctate fluorescence after CTC staining (Fig. 3). The interference of the different compounds that alter Ca^{2+}-dependent signal transduction pathways in conidia germination during sMF exposure, was also evaluated. As far as $CaCl_2$, PMA and LiCl are concerned, we observed a removal of germination inhibitory effect of sMF at all incubation times tested (Fig. 4). This result is consistent with the observation of the restoration of diffuse from punctate fluorescence (not shown). As expected, any influence of EGTA and neomycin on sMF effect was observed (Fig. 4).

4. Discussion

Many species of *Fusarium* are recognised as cereal pathogens causing diseases like FHB in wheat and barley and FER in maize. They are also sources of the important mycotoxins of concern in animal and human health [5–7] and in this context many studies have centred on the use of fungicides to control fungal growth [13].

In the present study we proposed sMF as physical antifungal agent by examining the effect of 0.3 ± 0.03 T sMF on mycelia growth and conidia germination of *F. culmorum.* Possible interaction of the sMF with fungal metabolism has also been studied. The results of our experiments revealed that growth and germination inhibition of *F. culmorum* as a response to sMF exposure was accompanied by either morphological or biochemical changes. The marked morphological alterations, including shrivelled hyphae and increased vacuolisation, were similar to those occurring in plant pathogenic fungi treated with chemical fungicides [13]. Herein we show that such sMF induced structural modifications are associated with biochemical changes. Since it is already demonstrated [25] that fungal virulence depends on the glyoxylate cycle activity, that permits the use of acetyl-CoA to synthesise carbohydrates, we evaluated either the activity of one of the principal enzymes of the cycle, namely ICL, and the intracellular concentrations of glucose and lipids. The

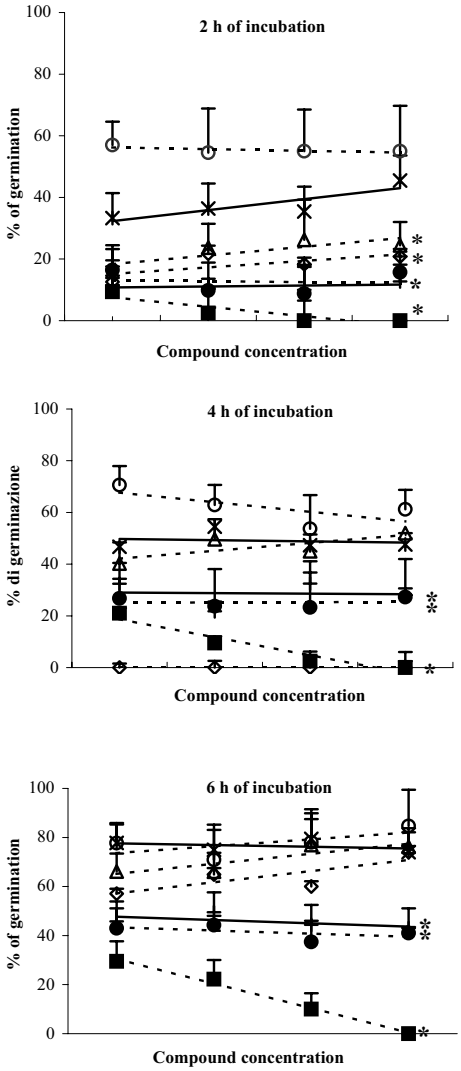

Fig. 4. Influence of sMF on germination percentage of *F. culmorum* conidia observed after 2, 4 and 6 h of incubation in the absence (-×-, control; -+-, control plus sMF) and in the presence of the following compounds: -●-, EGTA (0.5, 1, 1.5, 2 mM); -■-, neomycin (0.5, 1, 1.5, 2 mM); -◇-, LiCl (1.25, 2.5, 5, 10 mM); -△-, PMA (0.25, 0.5, 1, 1.5 mM); -○-, $CaCl_2$ (1.25, 2.5, 5, 10 mM) (results are the mean ± S.D. of 20 different experiments; * significantly different from the control).

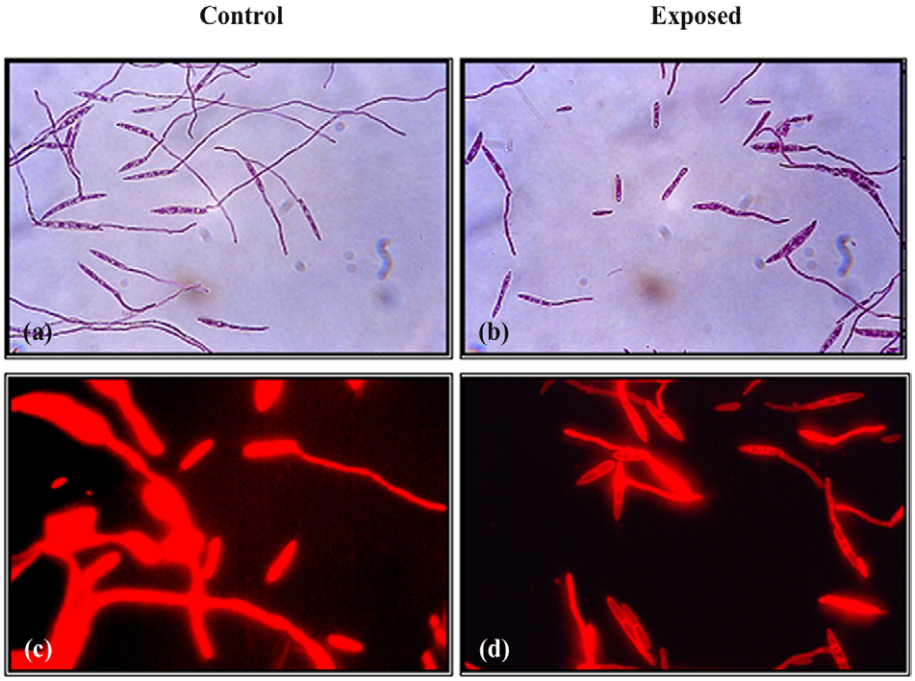

Fig. 5. Fluorescence microscopy of germinating conidia observed in the absence and in the presence of sMF. Cells were stained with hematoxylin–eosin (a, b) and acridine orange (c, d). Images are representative of the fluorescence observed in: control (a, c) and sMF exposed conidia (b, d). Cells were photographed at the same magnification (×60).

data obtained show that the exposure of *F. culmorum* to sMF induced significant decrease of both ICL activity and glucose concentration, suggesting that ICL is likely responsible for the impairment of acetate conversion to the sugars needed for germination. Concurrently, sMF increased the number of lipid bodies which may correlate with increased lipid concentration. This result apparently contrasted with the increased lipase activity. Actually, we have assayed general hydrolytic activity of lipases, but it is well known that these enzymes are reversible and can catalyse the synthesis of fatty acid esters [34]. Thus, the increased lipase activity we have observed may signify a synthetic rather than a hydrolytic action responsible for lipid bodies accumulation.

The use of conidia was a suitable system to point out Ca^{2+}-dependent signal transduction pathway involvement during germination and possible sMF interference with them. Fungal conidia are important asexual cells for colony dispersion and survival. They are the starting point of fungal infection, dependent on the infective mechanism by ongoing conidia germination [35]. In germinating conidia the metabolism increases greatly and RNA and protein synthesis occur [22]. When conidia germination process is inhibited by sMF exposure, microscope observation revealed, beside a reduced percentage of germination, a decreased cell viability. It has already been demonstrated that during conidia germination calcium plays an important role in the overall process of germination by exerting a stimulatory effect [35]. It is important to maintain a constant intracellular concentration of calcium and this equilibrium is well preserved by either freeing calcium from intracellular stores (endoplasmic reticulum, mitochondria, cytosolic Ca^{2+}-binding proteins) and activating calcium influx from extracellular medium. Since in our experimental model *F. culmorum* conidia naturally germinate in a calcium-free medium, they provide cytosol with calcium from the intracellular stores. Normally, mycelia conidiation in agar occur by taking up calcium from the culture medium itself [36]. Our results show that germination of *F. culmorum* conidia was inhibited by EGTA and neomycin: the former directly regulates cytosolic calcium [37], the latter hinders calcium release from intracellular stores by inhibiting phospholipase C. In both cases CTC staining showed a “punctate” fluorescence typical of discrete intracellular calcium stores (Fig. 3b and c vs. a). This is more evident for neomycin treatment, since calcium stores remain intact (Fig. 3c vs. b). On the contrary, when calcium ions are free in the cytosol, fluorescence is “diffuse” and its signal overcomes that of calcium stores (Fig. 3a). The presence of added $CaCl_2$, LiCl and PMA (Fig. 2) neither modify conidia germination nor CTC fluorescence (not shown). These results are expected since $CaCl_2$ (directly) and LiCl (indirectly) [38,39] increase cytosolic Ca^{2+}, while PMA has been reported to stimulate a conidia germination without added Ca^{2+} [22]. In this paper, we have shown that sMF inhibited conidia germination. To obtain hints on the site of action of sMF on the process of germination we have repeated the above discussed experiments also in the presence of sMF. The comparison of the data obtained in the absence and in the presence of sMF suggests that Ca^{2+} signalling pathways are likely influenced by sMF. In fact, the addition of $CaCl_2$ totally removed sMF inhibition as well as LiCl and PMA at the highest concentration used. On the contrary, EGTA and neomycin did not modify the inhibitory effect. Taken to-

gether these results provide evidence that sMF exposed cells are unable to mobilise calcium from intracellular stores and, more precisely, the hindered mechanism might be dependent on IP_3 second messenger. With this meaning we interpret the effect of LiCl and PMA, the former by increasing IP_3 level [38] and the latter by activating diacylglycerol-dependent protein kinase C.

Further studies are in progress to support the findings herein presented. Anyhow, as already proposed for bacteria [24] we indicate the use of sMF to reduce the incidence of infection and virulence of pathogenic fungi.

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