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Metrafenone: studies on the mode of action of a novel cereal powdery mildew fungicide†

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Abstract: Powdery mildew fungi are among the major pathogens causing diseases of cereals in the world. The mode of action of a novel systemic benzophenone fungicide, metrafenone, which is based on a precursor that is discussed in the preceding paper, has been analysed on the powdery mildew fungi of barley (*Blumeria graminis* Speer f. sp. *hordei* Marchal) and wheat (*Blumeria graminis* Speer f. sp. *tritici* Marchal). Preventive treatments reduced germination and blocked development beyond formation of appressoria, which penetrated less often. Moreover, metrafenone turned out to be an efficient curative fungicide, which rapidly affected fungal survival at low concentrations. The fungicide induced swelling, bursting and collapse of hyphal tips, resulting in the release of globules of cytoplasm. Bifurcation of hyphal tips, secondary appressoria and hyperbranching were also frequently observed. A histochemical analysis showed that metrafenone caused disruption of the apical actin cap and apical vesicle transport as well as weakening of the cell wall at hyphal tips. Finally, metrafenone strongly reduced sporulation. Reduced sporulation was associated with malformation of conidiophores that showed irregular septation, multinucleate cells and delocalisation of actin. Microtubules appeared to be only secondarily affected in metrafenone-treated *B. graminis*. The results suggest that the mode of action of metrafenone interferes with hyphal morphogenesis, polarised hyphal growth and the establishment and maintenance of cell polarity. Metrafenone likely disturbs a pathway regulating organisation of the actin cytoskeleton.

Keywords: metrafenone; *Blumeria graminis* f. sp. *hordei*; *Blumeria graminis* f. sp. *tritici*; cell polarity; actin cytoskeleton

1 INTRODUCTION

The ascomycete grass powdery mildew fungi, *Blumeria graminis* Speer ff. spp., continue to rank among the most harmful pathogens on cereals in the world. This is reflected by the size of the cereal mildew fungicide market, which is estimated to exceed $300 million per year.[1] In temperate regions, powdery mildew causes approximately 10% yield losses and occasionally as much as 40% when chemical control is neglected.[2] Along with cultural measures, the main measures of powdery mildew control in integrated crop protection systems are the use of less susceptible cultivars and the use of effective fungicides. A great challenge is the remarkable capacity of mildew populations to evolve virulent and fungicide-resistant genotypes that overcome control measures.[3,4] Indeed, powdery mildew has a number of characteristics, which support rapid adaptation, such as its relatively short generation time, with potential sexual recombination throughout the year, and the nature of its airborne spread. Consequently, finding novel and efficient fungicides against powdery mildew fungi represents an important challenge to crop protection research.

Powdery mildew is caused by the ectoparasitic obligate biotrophic fungus *B. graminis*: on barley by *B. graminis* f. sp. *hordei* Marchal (Bgh) and on wheat by *B. graminis* f. sp. *tritici* Marchal (Bgt). Following contact with the host surfaces, the conidia form a primary germ tube and an appressorial germ tube approximately 0.5–2 and 4–8 h after inoculation respectively. The appressorial germ tube begins to elongate and after 9–12 h differentiates a lobed appressorium. A peg forms under this appressorium, which penetrates the host cell wall and establishes a digitate haustorium within an epidermal cell. Papilla formation occurs in the leaf epidermal cell subjacent to the germ tubes. This local response in the outer epidermal cell wall excludes or delays a significant proportion of the attempted penetrations by the fungus.[5] Successful establishment of a haustorium, the only fungal organ that invades the host, is

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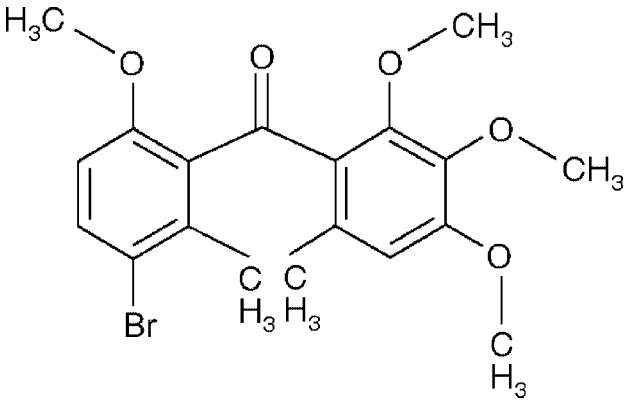

Figure 1. Structure of metrafenone.

followed by the formation of secondary hyphae from the appressorium. An elongating secondary hypha is the starting point for the development of a fungal colony. From the epicuticular hyphae, secondary appressoria are formed and, from these, secondary haustoria are established in epidermal cells. About 3–4 days after the primary infection, conidiophores are formed on the hyphae, sporulation starts and spores can be wind spread to initiate new infection cycles.[6]

This paper describes the mode of action of a novel systemic fungicide, metrafenone (3-bromo-2′,3′,4′,6-tetramethoxy-2,6′-dimethylbenzophenone, Flexity®, CAS registry number 220 899-03-6; Fig. 1), on the powdery mildew fungi of barley (Bgh) and wheat (Bgt). Metrafenone is a novel benzophenone-derived fungicide and represents an active ingredient that had not previously been used in chemical plant protection.[7] It was developed after earlier work had shown that a benzophenone compound exhibited promising effects.[8] Metrafenone differs from other commercial fungicides in that its action differs from currently known mechanisms. The research on its mode of action included evaluation of the preventive and curative activities of metrafenone, analysis of morphological anomalies of metrafenone-treated *B. graminis* and visualisation of different components of polar growth to see if the fungicide affects these. The results demonstrate that the potential target of metrafenone is involved in hyphal morphogenesis and the establishment and maintenance of cell polarity.

2 MATERIALS AND METHODS

2.1 Plant material, pathogen and inoculation

Barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) were grown in a growth chamber at 18°C with 60% relative humidity and 16 h photoperiod (240 µmol photons $m^{-2}\,s^{-1}$). For microscopy, first leaves were inoculated with 50–100 conidia mm^{-2} from *B. graminis* f. sp. *hordei* or *B. graminis* f. sp. *tritici* at 7 days after germination. The fungus was cultivated on barley and wheat under the same conditions.

2.2 Antifungal agents

Metrafenone 300 g $litre^{-1}$ EC (BASF AG, Ludwigshafen, Germany) was used at concentrations from 0.004 to 250 mg AI $litre^{-1}$.

To quantify the preventive activity of metrafenone on *B. graminis* in wheat and barley, the fungicide was applied to first leaves placed in petri dishes 1 day before inoculation. To further determine whether metrafenone had a direct effect on *B. graminis* development, the chemical was applied to inoculated wheat leaves placed in petri dishes at 2 days post-inoculation (dpi). Subsequently we assessed mycelium formation and sporulation frequencies at 7 and 14 dpi. Observations were made on four pieces of primary leaf segment, counting interaction sites on each. These experiments were repeated four times. Observations were made by fluorescence microscopy after Uvitex 2B (Ciba Geigy, Basel, Switzerland) staining.

A comparison with other compounds with preventive activity, such as quinoxyfen (150 g $litre^{-1}$ SC, Fortress, Dow AgroScience, Indianapolis, IN, USA), and curative activity, such as spiroxamine (700 g $litre^{-1}$ EC, Impulse, Bayer, Leverkusen, Germany), was performed.

The effect of metrafenone was compared with that of specific inhibitors. For the inhibition of actin polymerisation, cytochalasin D and A (Molecular Probes, Leiden, The Netherlands) at a concentration of 0.5 mg $litre^{-1}$ were used. Benomyl at a concentration of 10 mg $litre^{-1}$ was included as an anti-microtubular drug. The inhibition of chitin synthesis was achieved by application of polyoxin D (Calbiochem, Darmstadt, Germany) at a concentration of 3 mg $litre^{-1}$. Chemical solutions were prepared in distilled water and applied to wheat and barley leaves by spraying with an atomiser until run-off.

2.3 Actin polymerisation assay

Actin polymerisation assays were carried out using a kit from Cytoskeleton Inc. (Cat. No. BK003, BMG Labtechnologies, Offenburg, Germany). Pyrene-conjugated actin was polymerised at room temperature in the presence of ATP and either the compound in question or DMSO. The increase in polymerisation was measured by detecting the increase in fluorescence signal emitted from the pyrene-conjugated F-actin using a Fluostar Galaxy plate reader (BMG Labtechnologies).

2.4 Cytological techniques

Uvitex 2B (Ciba Geigy) was used to stain the β-linked cell wall polymers.[9] Uvitex 2B dissolved in ethyl alcohol was sprayed directly onto the leaves prior to fluorescence microscopy and confocal laser scanning microscopy (CLSM, excitation 365 nm, emission 420–460 nm). CLSM images were recorded on a multichannel TCS SP2 confocal system (Leica Microsystems, Bensheim, Germany).

Staining of the fungal cytoplasm was performed with acridine orange (Sigma, Steinheim, Germany).

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Acridine orange was prepared as a 7 g litre^{-1} solution in sodium acetate buffer (0.2 M acetic acid + 0.2 M sodium acetate, 82 + 18 by volume; pH 4.0). Leaf segments were submerged for 10 min in acridine orange solution (100 ml) and then rinsed with acetate buffer. The probes were examined immediately by fluorescence microscopy (excitation 490 nm, emission 520–550 nm).

In order to determine the effect of metrafenone on fungal survival, phloxine B (ROTH, Karlsruhe, Germany) was applied to stain dead hyphal cells. A 1 g litre^{-1} solution in distilled water was prepared and the samples were plunged in this solution for 20 min, followed by rinsing with water. The evaluation was performed by bright field microscopy.

Fungal cell walls were stained with congo red (Merck, Darmstadt, Germany), which typically stains crystallised cellulose fibres. Leaf segments were immersed in a 10 μg ml^{-1} solution of congo red in distilled water for 20 min. After being rinsed with water, the specimens were directly examined by CLSM (excitation 585 nm, emission 600–615 nm).

To visualise cytoplasmatic vesicles, we used FM4-64 (Molecular Probes). Leaf segments were placed in 20 mM HEPES buffer (pH 7.2) containing 10 μM FM4-64 for 30 min. The samples were washed with HEPES and directly observed by CLSM (excitation 514 nm, emission 630–750 nm).

Staining of nuclei was performed with Hoechst 33 342 (Sigma). Leaf segments were immersed in a 1 μg ml^{-1} solution of Hoechst 33 342 in 25 mM phosphate buffer (PB; NaCl 8.0, KCl 0.2, $Na_2HPO_4 \cdot 2H_2O$ 1.4, KH_2PO_4 0.2 g litre^{-1} water; pH 6.8) for 10 min. After washing in PB, fluorescence was observed by CLSM (excitation 346 nm, emission 400–460 nm).

Visualisation of actin and microtubules was achieved by indirect immunofluorescence microscopy with anti-actin and anti-tubulin antibodies.[10,11] Leaf segments of size 5 mm × 5 mm were fixed in 4% formaldehyde in PIPES buffer (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) 25 mM, EGTA 2 mM, $MgCl_2$ 2 mM, Tween 20 0.5 g litre^{-1}; pH 6.8) at room temperature for 40 min. After being washed in 25 mM PIPES and in 25 mM PB, the leaf segments were rinsed in 25 mM PB (pH 6.5). They were then transferred to a solution containing 10 mg ml^{-1} driselase, 10 mg ml^{-1} chitinase, 16 mg ml^{-1} β-D-glucanase (enzymes provided by InterSpex Products, Inc., San Mateo, CA, USA) and 1 mg ml^{-1} bovine serum albumin (Sigma) dissolved in 25 mM PB (pH 6.5) at room temperature for 15 min. After being rinsed in 25 mM PB (pH 6.5), they were further treated with 5 g litre^{-1} Triton X-100 in 25 mM PB (pH 6.8) for 10 min. The specimens were washed with 25 mM PB (pH 6.8) then with TB buffer (Tris-HCl 50, NaCl 150 mM; pH 7.6). Following a rinse cycle, leaf segments were incubated with a monoclonal mouse anti-actin antibody (Clone C4, ICN BioMed, Inc., Aurora, OH, USA) for actin localisation or an anti-tubulin antibody (NeoMarkers, Fremont, CA, USA) for tubulin localisation, diluted 1:100 (w/v) in antibody solution (DAKO, Carpinteria, CA, USA), at room temperature for 30 min. Vacuum infiltration was repeated three times for 20 s at 25 mmHg to promote infiltration. The specimens were washed twice in TB before incubation in goat anti-mouse IgG Alexa Fluor 488® (Molecular Probes), diluted 1:100 (w/v) in antibody diluent, at room temperature for 1 h. Vacuum infiltration was repeated as described above. Finally the leaves were rinsed with TB (pH 7.6). The specimens were mounted in TB (pH 7.6) on glass slides and observed by CLSM (excitation 488 nm, emission 505–540 nm).

2.5 Cryofixation and low-temperature scanning electron microscope (LTSEM)

For observation by LTSEM, 6 mm × 5 mm pieces of leaf tissue were cut. Prior to cryofixation, samples were mounted on SEM stubs using a low-temperature mounting medium and rapidly frozen by plunging them into liquid nitrogen. After cryofixation the samples were placed on a Balzers specimen table (Balzers, Lichtenstein) under liquid nitrogen. Using a manipulator equipped with an anti-contamination cup, the table was transferred under nitrogen gas flow conditions to a Balzers SCU 020 cryopreparation unit attached to a Jeol JSM 6300 (Balzers, Lichtenstein). The samples were carefully warmed to −80°C and kept under high-vacuum conditions. Prior to planar magnetron (PM) sputter coating with 20 nm of gold, the total pressure was raised to 2 Pa with pure argon gas. After re-establishing high-vacuum conditions, the coated samples were transferred on-line into the SEM, observed and photographed at −150°C using an accelerating voltage of 10 kV.

3 RESULTS

3.1 Quantification of metrafenone effect on *Blumeria graminis*

3.1.1 Evaluation of preventive activity

Wheat and barley were sprayed with metrafenone, and leaves were inoculated 24 h later. Evaluation of the preventive activity of metrafenone on *B. graminis* in wheat and barley was carried out at 24 and 36 h post-inoculation (hpi) and 7 days post-inoculation (dpi). At 24 hpi the frequencies of germination of Bgt and Bgh were investigated. Metrafenone strongly reduced fungal germination compared with the untreated leaves. At the low concentration of 4 mg litre^{-1} the frequency of germination decreased by around 30%. At 36 hpi the frequencies of appressoria with one lobe or more lobes, the latter being indicative of unsuccessful penetration, and elongated secondary hyphae of Bgh were evaluated. At 4 mg litre^{-1} the preventive metrafenone treatment increased the proportion of conidia forming multilobed appressoria by 30% and lowered the frequency of elongated secondary hyphae by 25%. Moreover, metrafenone enhanced the percentage of appressoria giving rise to non-penetrated papillae while reducing the proportion

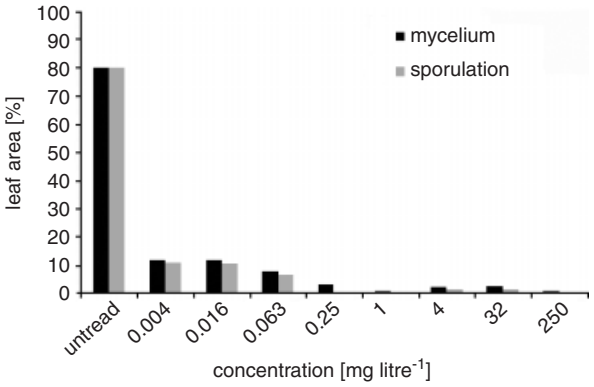

Figure 2. Effect of metrafenone on development of *Blumeria graminis* f. sp. *tritici* on wheat at 7 days after inoculation, following treatment 1 day before inoculation. The proportion of leaf area covered by mycelium and sporulating colonies was evaluated by fluorescence microscopy after Uvitex 2B staining.

that succeeded in forming a haustorium. Treatment of the fungal inoculum instead of the leaves resulted in the same situation that was observed after preventive treatment of the leaves, with the difference that the reduction in germination was stronger. At 7 dpi, preventive metrafenone activity on mycelium formation and sporulation of Bgt was examined in wheat (Fig. 2). The average reduction in mycelium compared with the untreated control corresponded to 98% at 7 dpi at the concentration of 1 mg litre^{-1}. A comparison of the preventive effect of metrafenone with that of quinoxyfen was carried out. Quinoxyfen at 1 mg litre^{-1} reduced mycelium development by 67% at 7 dpi. Thus metrafenone effectively prevented wheat from powdery mildew fungus invasion by reducing fungal germination and by inhibition of fungal development after appressoria formation.

3.1.2 Evaluation of curative activity

Metrafenone was directly applied to inoculated wheat leaves at 2 dpi when haustoria were fully developed. Subsequently, mycelium formation and sporulation frequencies were assessed at 7 and 14 dpi. At 4 mg litre^{-1} metrafenone, mycelium formation was reduced by 55 and 62% at 7 and 14 dpi respectively (Figs 3(a) and 3(b)). Uvitex 2B staining could not clearly distinguish between live and dead hyphae. Hence evaluation of hyphal cell death after metrafenone application was performed by phloxine B staining of dead hyphae at 4 dpi. Phloxine B staining revealed hyphal cell death as early as 1 h post-application of 4 mg litre^{-1} metrafenone. About 60% of the mycelium showed cell death at 12 h post-application. Hence, although mycelium was observed after treatment, most fungal hyphae did not survive metrafenone treatment. Metrafenone treatment at 2 dpi also reduced sporulation. At 4 mg litre^{-1}, sporulation decreased by 71 and 98% at 7 and 14 dpi respectively (Figs 3(a) and 3(b)). There were slight discrepancies between the proportions of leaf surface covered

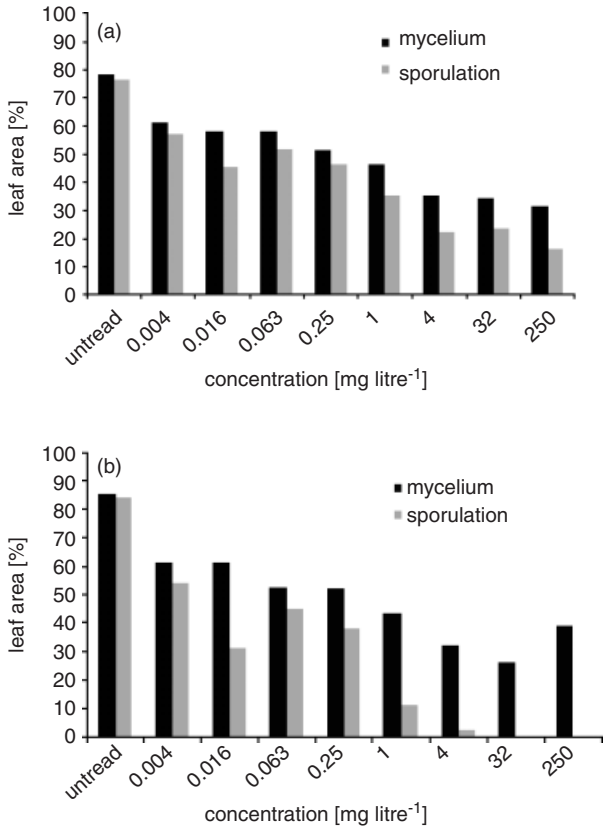

Figure 3. Effect of metrafenone on development of *Blumeria graminis* f. sp. *tritici* on wheat at (a) 7 and (b) 14 days after inoculation. Metrafenone was applied 2 days after inoculation. The proportion of leaf area covered by mycelium and sporulating colonies was evaluated by fluorescence microscopy after Uvitex 2B staining.

with mycelium and with spores in untreated leaves. However, sporulation was more strongly reduced than mycelium formation in metrafenone-treated leaves at 7 and 14 dpi. This indicated that, even if mycelium partially developed, sporulation was affected at low concentrations of metrafenone (Figs 3(a) and 3(b)). Consequently, conidiophore formation was often not observed even though mycelium was present after treatment with metrafenone. Notably, there was a significant reduction in sporulation between 7 and 14 dpi. A comparison of the curative effect of metrafenone with that of spiroxamine was performed. When compared with the control, sporulation was reduced by 87% after treatment with metrafenone and by 96% after treatment with spiroxamine at 14 dpi using concentrations of 1 mg litre^{-1} (data not shown). These data provided evidence that metrafenone inhibited sporulation and, in addition to its preventive activity, showed a substantial curative potential. It appeared that the conidiophores also showed rapid cell death after metrafenone treatment.

3.2 Morphological analysis of metrafenone effect on *Blumeria graminis*

3.2.1 Metrafenone effect on germling morphogenesis

To examine whether metrafenone affected the early development of conidia of Bgt and Bgh, 4 mg litre^{-1} metrafenone was applied on the first leaf segments

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1 day before inoculation. After 48 h, at 1 dpi, fungal germlings were observed by low-temperature scanning microscopy.

On untreated leaves the appressoria formed a typical apical hook-shaped lobe (Fig. 4a). When attempted penetration from the first lobe failed, a second was formed behind the first. On the metrafenone-treated leaves the fungicide caused a reduction in conidia germination, and, where conidia germinated, subsequent appressoria frequently presented two or three lobes, and sometimes lobes were malformed (Fig. 4b). The appressoria were functional in the sense that they could form infection pegs, but penetration was mostly blocked in host cell wall papillae. Hence the fungus rarely developed a haustorium and elongated secondary hyphae. If the germling established a haustorium, it was frequently malformed and encapsulated by host cell wall-like material, and consequently the pathogen stopped growing.

3.2.2 Direct effect of metrafenone on fungal morphogenesis

To investigate the direct effect on Bgt and Bgh, the fungicide was applied on the inoculated leaves at 8 hpi and 2 and 4 dpi. Fungal development was observed by low-temperature scanning microscopy.

In untreated controls, 3 days after inoculation the colonies consisted of a conidium with the primary and the secondary germ tube, primary appressorium and numerous hyphae (Fig. 4c). The tip-growing cells comprised a cylindrical shank of constant diameter and an apical dome (Fig. 4d). From the superficial hyphae, secondary appressoria were formed with small globular structures disposed alone or in pairs on opposite sides of the hyphae (Fig. 4e). At 4–5 days after inoculation, leaves showed spreading colonies with a dense surface of mycelium and numerous conidiophores consisting of a mother cell producing chains of conidia separated by regularly spaced septa (Fig. 4f).

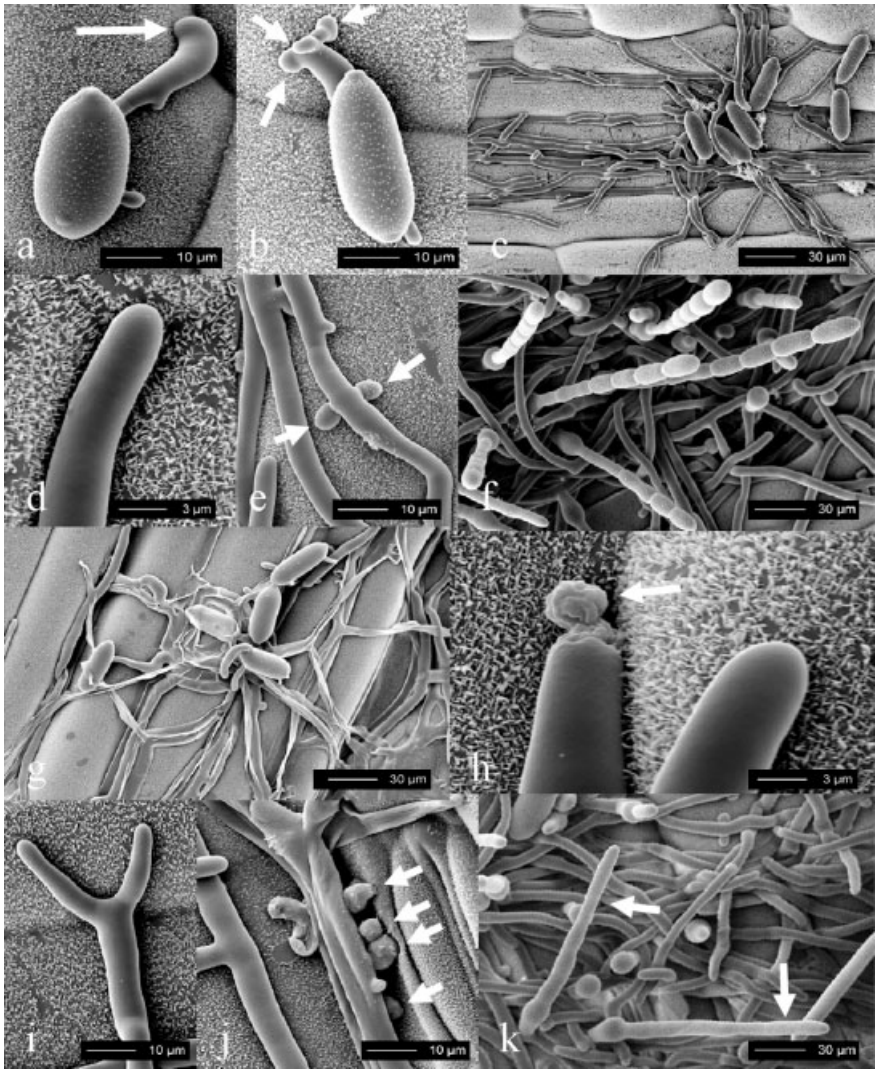

Figure 4. LTSEM of *Blumeria graminis* f. sp. *hordei* on barley leaves after treatment with metrafenone (4 mg litre^{-1}). Untreated leaves were included as a control. Conidia formed one lobe (arrow) in the control (a) and were malformed and multilobed (arrows) after preventive treatment (b). In the control a colony consisted of a conidium with numerous hyphae (c) that showed normal tips (d) and secondary appressoria (arrows) (e), and conidiophores that produced a chain of conidia separated by regularly spaced septa (f). After curative treatment the mycelium collapsed (g), the hyphal tips were swollen and extruded globules of fungal cytoplasm (arrow) (h) or bifurcated (i), the secondary appressoria were abundant (arrow), closely spaced and frequently bifurcated (j) and the conidiophores were often tubular (arrow) (k).

Metrafenone caused a fungal growth delay, a number of morphological anomalies and a rapid collapse of mycelium (Fig. 4g). The diameter of treated hyphae was larger than that of untreated hyphae. The form of the apices changed from cylindrical to spherical and the tip of the apical cells became swollen, often followed by a burst and release of globular structures (Fig. 4h). These globules were stainable with acridine orange but not with Uvitex 2B. Therefore they represented most likely fungal cytoplasm and not cell wall material. Hyphae with ruptured hyphal tips were often collapsed. Swelling and bursting were occasionally seen in subapical regions or near secondary appressoria. Furthermore, several hyphal tips were bifurcated (Fig. 4i). Secondary appressoria were more abundant than normal and grouped closely together (Fig. 4j). They were also more round or bifurcated.

Additionally, when inoculated leaves were treated with metrafenone at 4 dpi, sporulation was delayed or inhibited by metrafenone. Concurrently, metrafenone caused formation of aberrant conidiophores. The conidiophores formed elongated tubes of uniform diameter (Fig. 4k) or a chain of conidiospores with irregular or no septation. Irregular septation was also observed in surface hyphae.

3.3 Histochemical analysis of metrafenone effect on *Blumeria graminis*

Conventional fluorescence microscopy and confocal laser microscopy were used to analyse the distribution of different components involved in polar growth in *B. graminis*, to determine whether metrafenone could affect them.

The cell walls were stained with congo red. In untreated fungi, congo red-stained hyphal tips were brighter than subapical cell wall regions, indicating areas of polarised cell growth (Fig. 5a). In metrafenone-treated hyphae, staining was weaker in the hyphal tips and, instead, irregularly distributed along the hyphal walls (Fig. 5b). This indicated a defect in polarised cell growth and a loss of cell polarity, particularly in those hyphal tips that had become spherical. In parallel, we compared the metrafenone effect with that of polyoxin D, which caused a very strong swelling of the hyphal tip followed by bursting (data not shown). However, other typical effects of metrafenone were not observed after polyoxin D treatment of Bgh.

In living hyphae, apical vesicles were stained with FM4-64. In the control, FM4-64 labelled an accumulation of vesicles at the extreme hyphal tip (Fig. 5c), whereas such vesicles were delocalised from the apex after metrafenone application (Fig. 5d). As described already by Dijksterhuis,[12] FM4-64 also stains vesicles within the 'Spitzenkörper'. Hence the loss of cell polarity could be caused by a disturbance of the Spitzenkörper by metrafenone.

Furthermore, cellular patterns of actin and tubulin were visualised by indirect immunofluorescence microscopy. In control fungi, anti-actin antibodies revealed typical actin patches at tips of appressoria, hyphae (Fig. 5e), branches and immature conidiophores (Fig. 5k). Actin formed a peripheral cap in the hyphal apex. Filamentous actin, as usually stained with phalloidin-coupled dyes, is not highlighted by the anti-actin antibodies. In conidiophores, actin fluorescence occurred also at the site of septum formation. In metrafenone-treated fungi we discovered a change in actin organisation compared with the control. Indirect immunofluorescence microscopy showed a delocalisation of actin away from the apex to the subapical region of hyphae (Fig. 5f), branches and immature conidiophores (Fig. 5l). Actin fluorescence was undetectable in swollen hyphal tips and sites of septum formation. Simultaneously, the effect of metrafenone on fungal morphogenesis was compared with that of the actin polymerisation inhibitor cytochalasin D. Cytochalasin D led to very similar morphological anomalies and actin delocalisation to those observed after metrafenone treatment (data not shown). To test if metrafenone directly inhibits actin polymerisation, an actin polymerisation assay for metrafenone and cytochalasin A was carried out. This indicated that metrafenone does not inhibit actin polymerisation *in vitro* and thus the actin cytoskeleton was likely indirectly affected by metrafenone (data not shown).

In controls, anti-tubulin antibodies marked a longitudinal pattern of microtubules in appressoria, hyphae (Fig. 5g) and conidiophores (Fig. 5i). After metrafenone treatment, microtubules were mostly observed in the subapical region of hyphae (Fig. 5h) and conidiophores (Figs 5j and 5o). However, microtubules were often replaced by a 'star-bursting-like pattern'[13] in hyphae and conidiophores. The effect of metrafenone on the microtubules was compared with that of benomyl. Benomyl induced disruption of microtubules and looping of the hyphal tips (data not shown). Together, microtubules seemed not to be directly affected by metrafenone.

Finally, the organisation of nuclei in metrafenone-treated fungi was also different from that in untreated controls. Hoechst staining showed that, in the control, conidiophores were normally composed of a uninucleate mother cell (Fig. 5k) producing chains of uninucleate conidiospores (Fig. 5m). After metrafenone treatment, some mother cells were multinucleated (Fig. 5o) and the condiospores, which showed irregularly spaced septa, contained several nuclei or nuclear fragments (Fig. 5n). Likewise, Hoechst staining revealed proliferation or fragmentation of nuclei in the regions of hyphal swelling, bifurcation and at abundant secondary appressoria (not shown).

4 DISCUSSION

Treatment of barley and wheat with metrafenone resulted in effective protection against *B. graminis*. The preventive treatments caused a significant reduction in germination (around 30%) of both Bgh and Bgt and

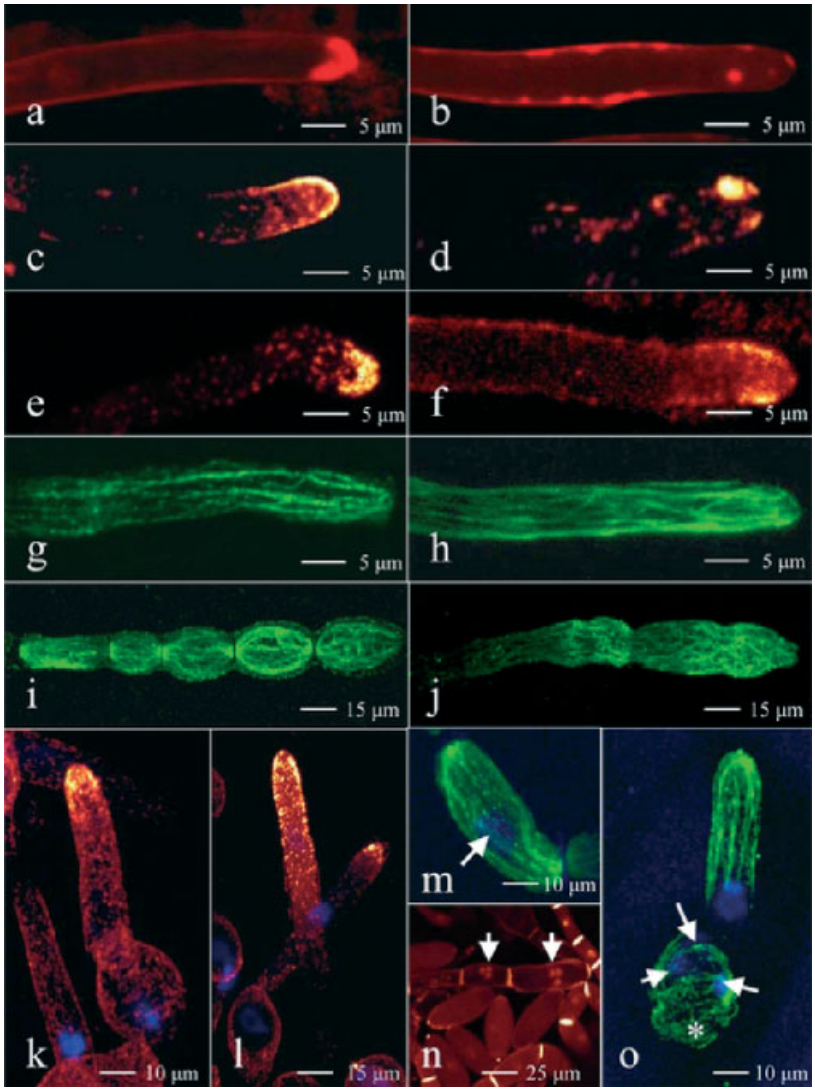

Figure 5. Micrographs showing cytological features of *Blumeria graminis* f. sp. *hordei* on barley leaves, (a, c, e, g, i, k, m) untreated and (b, d, f, h, j, l, n, o) after treatment with metrafenone (4 mg litre^{-1}). The hyphal tip (a–h) and the conidiophore (i–o) were observed by confocal laser scanning microscopy (CLSM). The distribution of glucan was stained by congo red (a, b), cytoplasmic vesicles by FM4-64 (c, d). Actin was localised after staining with anti-actin antibodies (e, f, k, l). Tubulin was visualised after staining with anti-tubulin antibodies (g, h, i, j, m, o). The distribution of nuclei (arrows) was visualised together with actin after staining with Hoechst and anti-actin antibodies (k, l), or together with tubulin after staining with Hoechst and anti-tubulin antibodies (m, o), or together with septa after staining with Uvitex 2B and Hoechst (n). The basal cell is indicated by an asterisk.

blocked development beyond the primary appressoria. On metrafenone-treated leaves, appressoria typically formed two or three lobes. Similar multilobed appressoria appear on resistant host cultivars in the case of unsuccessful penetration by the apical lobe.[6] Metrafenone enhanced the number of appressoria giving rise to papillae whilst reducing the proportion that succeeded in forming a haustorium. Additionally, treatment of the conidiospores used for the inoculation also caused an increase in multilobed appressoria and a reduction in germination. Since inoculum treatment and leaf treatment had similar effects, we conclude that metrafenone exerts a direct effect on the fungus and likely does not induce host resistance. The fungicide might rather delay fungal development, giving the host more time to prevent penetration.

It is noteworthy that the actin was delocalised from the apex in appressorial lobes, indicating that the fungicide might affect polar growth of the penetration organ. In *Magnaporthe grisea* (Hebert) Barr, polarisation of the actin cytoskeleton to the site of penetration hypha formation was suggested to be involved in localised wall modification, which is essential for penetration.[14–16] Together, the fungicide might cause disturbance of the peg cell polarity and prevent successful fungal penetration of the host cell. Bgh, when treated with quinoxyfen, another fungicide with preventive activity, also shows less effective appressorium formation. Gene expression and mutant analysis revealed that a Ras-type GTPase activating protein is most likely involved in quinoxyfen resistance of Bgh, and hence this fungicide might disrupt Ras signalling in appressorium formation.[17]

When metrafenone was applied directly to *B. graminis*, the mycelium proportion was greatly reduced compared with controls in the treatment

at 2 dpi. Metrafenone is an efficient curative fungicide at very low concentrations if it is applied early enough after infection. Moreover, metrafenone also has a significant impact on fungal survival. Mycelium cell death occurred as early as 1–3 h after metrafenone application. The reduction in mycelium formation was associated with a rapid collapse of hyphae. Hyphal collapse was associated with swelling and bursting of hyphal tips. Burst hyphal tips released globules of cytoplasm. Our cytological analysis indicated that swelling of hyphal tips might be caused by weakening of the cell wall at the apex, disturbance of apical vesicle delivery and disruption of the F-actin cap at the apex. It has been reported that cytochalasin A or latrunculin B treatment results in swelling of hyphal tips, with disruption of actin, disturbed vesicle delivery and irregular wall deposition at the hyphal tips.[13,18,19] However, although the effect of cytochalasin D on *B. graminis* was very similar to that of metrafenone (data not shown), an *in vitro* actin polymerisation assay indicated that metrafenone did not directly inhibit actin polymerisation, suggesting that the chemical rather induced changes in the actin pattern. Proteins that play an important role in actin organisation include the Ras and Rho GTPases Rho, Cdc42 and Rac.[20–22] In *Ashbya gossypii* Guill the *Agrho3* mutant showed swelling with delocalised actin at the hyphal tips.[23] In *Colletotricum trifolii* Bain & Ess the mutational activation of *Ras* gene exhibited hyphal tips tending to burst.[24]

Moreover, metrafenone caused bifurcation of hyphal tips and secondary appressoria, likely indicating the loss of cell polarity. A mutation in the *Neurospora crassa* Shear & Dodge actin gene results in branching of hyphal tips and alteration of actin at the tip.[25] In a temperature-sensitive mutant of *Aspergillus niger* van Teighem, apical branching involves dislocation and disappearance of the Spitzenkörper.[26] The process of apical branching was suggested to be caused by a shift of vesicle deposition from the tip to the side, consequently initialising the formation of new hyphal outgrowths.[27] Similarly, metrafenone might cause bifurcation by delocalisation of actin, vesicles and the Spitzenkörper at hyphal tips. Thus in metrafenone-treated hyphae the defects in cell polarity could be a consequence of the failure in maintaining polarity of the actin cytoskeleton. Additionally, metrafenone treatment resulted in abundant secondary appressoria grouped together at abnormally close distance. A high number of lateral branches were also observed in hyphae of *Saprolegnia ferax* (Gruithuisen) Thuret after application of latrunculin B. The phenomenon was preceded by formation of radial arrays of actin in regions without detectable surface protrusion. These sites were consistent with future branches.[13] Accordingly, in the absence of focused actin at the apex, hyphal growth might be not directed and thus cells grew in multiple directions. Hyperbranching possibly revealed multiple randomly distributed initiation sites of polar growth after metrafenone treatment.

Microtubules have been reported to be implicated in polarised growth and vesicle transport.[21,28] However, indirect immunofluorescence microscopy did not indicate disruption of microtubules by metrafenone. However, microtubules were often replaced by a 'star-bursting-like pattern' in hyphae. This reorganisation of microtubules was also observed in *S. ferax* after disruption of actin and therefore might be a secondary effect of actin defects.[13]

Finally, metrafenone inhibited and delayed sporulation. Metrafenone treatment at 4 mg litre^{-1} at 2 dpi reduced sporulation by 98% at 14 dpi. Hence metrafenone has curative activity. The inhibition of sporulation was associated with the malformation of conidiophores that showed multinucleate cells and irregular septation. This suggests that mitosis proceeded without septum formation, indicating that cytokinesis was impaired. Actin is known to play a central role in septum formation and to be involved in cytokinesis.[29] In turn, actin was delocalised in the tip of the young conidiophores, and actin associated with septa was hardly detectable after metrafenone treatment. The effect of metrafenone on *B. graminis* shows interesting similarities to the deletion of *cflB*, the *Penicillium marneffei* RAC homologue. Δ*cflB* results in cell division and growth defects in conidiophores so that cells become multinucleate and exhibit inappropriate septation.[30]

These results, taken together, suggest that the mode of action of metrafenone and its potential target are involved in hyphal morphogenesis, polarised hyphal growth and the establishment and maintenance of cell polarity in *B. graminis*. Metrafenone might interfere with processes that are essential to establish and maintain polar actin organisation.

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