



# Regulation of hyphal growth and sporulation of the insect pathogenic fungus *Entomophthora thripidum* in vitro

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## Abstract

*Entomophthora thripidum* is an obligate biotrophic insect pathogenic fungus that grows as protoplasts within the hemocoel of thrips. Prior to penetration through the insect cuticle and spore formation at the insect surface the protoplasts switch to hyphal growth. In vitro, the differentiation to hyphal growth was a prerequisite for the subsequent formation of infectious spores and was detected 10–20 days after inoculation. *E. thripidum* secreted a factor that autoinduced the differentiation to hyphal growth. The discovery of this activity inducing hyphal growth made possible the reliable production of spores, the infection of host insects and the consecutive re-isolation of the fungus from the infected insects.

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**Keywords:** Entomophthorales; Differentiation; Hyphal growth; Nitrogen limitation; Sporulation; Zygomycetes

## 1. Introduction

The life cycle of obligate biotrophic insect pathogenic fungi can be separated into three distinct steps: the infection of the host, vegetative growth within the host insect and the breaking through the insect cuticle in order to sporulate and to re-infect. In contrast to infection and vegetative growth, the last step in the life cycle, the penetration through the insect cuticle by outward growth and subsequent spore formation, has received less attention. The induction of the sporulating stage brings about fundamental changes in the morphology of the fungus. Insect pathogenic fungi belonging to the order of the Entomophthorales often grow in the form of discrete cells with some species multiplying as protoplasts [1]. For sporulation fungal cells grow coordinately outward, break through the insect's cuticle and spores are formed at the tip of hyphae that are surrounded by a cell wall [2]. Sporulation is timed

to assure that all cells form spores simultaneously before the insect cadaver is overgrown by saprophytic and opportunistic bacteria or fungi. Regulated sporulation has to occur in those species of Entomophthorales that sporulate from insects that are still alive or that only break through the cuticle at defined places (summarised in [3,4]).

*Entomophthora thripidum* (Zygomycetes: Entomophthorales) is a pathogen of *Thrips tabaci* (Thysanoptera: Thripidae; onion or potato thrips) [5], a common pest insect in vegetables and ornamentals [6]. This fungus is especially noteworthy for its localised sporulation from the abdomen of still living thrips [5]. In vitro *E. thripidum* grows and multiplies in the form of irregularly shaped protoplasts that aggregate to form pellets (Fig. 1) [7]. After prolonged cultivation in the same growth medium the protoplasts of *E. thripidum* will differentiate to hyphae that are surrounded by a cell wall (Fig. 1). The aggregates and pellets formed by these hyphae are referred to as mycelium [7].

In vitro hyphal growth was a prerequisite for the formation of infectious spores in *E. thripidum*. It was therefore attempted to characterise the factors influencing this differentiation in order to optimise sporulation. Thereby it was observed that *E. thripidum* autoinduced hyphal growth and mycelium formation by a secreted factor. This finding enabled the reliable production of infectious

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**Abbreviations:** CMM, conditioned medium from a culture that had formed mycelium; FBS, foetal bovine serum

spores and the completion of the life cycle of *E. thripidum* under laboratory conditions. From these results it was hypothesised that autoinduction of hyphal growth and sporulation is important in fungi that sporulate from insects that are still alive or break through the cuticle at defined places.

## 2. Materials and methods

### 2.1. Isolates and cultivation

For all experiments *E. thripidum* (isolates ARSEF 5868, 6517, 6518, 6519, 6520, 6525 and 6521 and 6522, two re-isolates of ARSEF 6518; all deposited in the ARSEF Collection of Entomopathogenic Fungal Cultures, USDA-ARS Plant Protection Research Unit, US Plant, Soil, and Nutrition Laboratory, Tower Road, Ithaca, NY 14853-2901, USA) was grown in 48-well or 24-well microtitre plates in 500  $\mu$ l or 1 ml of culture medium, respectively. The standard growth medium was Grace's insect cell culture medium supplemented with lactalbumin hydrolysate and yeastolate (Gibco) and 10% foetal bovine serum (FBS, Gibco). Alternatively, supplemented GLEN medium, containing 0.4% glucose, 0.5% yeast extract, 0.65% lactalbumin hydrolysate, 0.77% NaCl (all w/v) and 10% FBS (v/v) [8] was used. This medium made it possible to omit the carbon (glucose) and/or the nitrogen sources (yeast extract and lactalbumin hydrolysate) in order to determine their influence on the formation of mycelium. The cultures were inoculated with approximately  $10^4$  cells  $\text{ml}^{-1}$ , kept in the dark at 20°C and shaken at 160 rpm. Re-isolation was performed as described by Grund-schober et al. [9] from *T. tabaci* that had been infected by the method developed for *Neozygites parvispora* [10].

### 2.2. Morphological observations and sporulation

*E. thripidum* was examined daily under an inverse microscope for 20 days to follow the process of mycelium formation. The presence or absence of a cell wall was determined by addition of 1  $\mu$ l of Calcofluor (Sigma, 0.1 mg  $\text{ml}^{-1}$  final concentration) to 10  $\mu$ l of fungal culture followed by detection with fluorescence microscopy using a Zeiss Axiophot with a 365 nm excitation filter, 395 nm beam splitter and 420 nm barrier filter. All cultures were grown in duplicate.

To induce spore formation fungal cultures were transferred to 1.5% water agar and incubated in a humid, translucent box overnight at 20°C in light. The ability to form spores was assessed qualitatively.

### 2.3. Analytical measurements

The density of living cells was determined by the MTT assay as described elsewhere [11]. The glucose concentra-

tion in the supernatant growth medium was measured enzymatically (Sigma Diagnostic kit, No. 16-20), while the concentration of total  $\alpha$ -amino acids was determined colorimetrically by the OPA method using leucine as a standard [12]. The pH was determined by using a pH electrode (Mettler Toledo, InLab<sup>®</sup> 423, Electrolyte 9811) and a pH meter (Knick pH-meter 761, Calimatic) prior to freezing supernatants for further analysis.

All measurements were performed in four independent replicates and experiments were performed twice with different fungal isolates.

### 2.4. Mycelium formation in different media

To test the influence of medium compositions on the formation of mycelium the standard cultivation medium was removed after 7 days and replaced by the medium to be tested. Protoplast pellets of *E. thripidum* were left to sediment in the microtitre plates, the supernatant was aspirated with a pipette and the new medium added to the cultures.

To determine the influence of carbon and nitrogen sources on mycelium formation GLEN medium where glucose and/or lactalbumin hydrolysate and yeastolate had been omitted was tested. To assess the activity of conditioned medium, it was filter-sterilised and used to replace half of the culture medium of 7-day old *E. thripidum* cultures. Medium from cultures that had formed mycelium (CMM) was tested at different concentrations (100%, 50%, 25%, 12.5%) by removing only part of the old culture medium and replacing it with CMM. In one experiment the old medium was not changed, instead different volumes were removed in order to decrease the culture volume (and thus increasing the cell density) after 7 days by 20%, 40%, 60% or 80%.

In addition, the medium of a 7-day old test culture was replaced by a mixture of equal amounts of CMM and GLEN medium (either complete or without the C- and/or N-source). As a control only half of the medium of a 7-day old culture of *E. thripidum* was replaced with either CMM or one of the variations of the GLEN medium.

For all experiments two or four replicates were used and each experiment was performed at least twice with different isolates. In all cases a control was used where the culture medium was not changed.

### 2.5. Characterisation of factors from conditioned medium

To characterise the factor(s) that induced the switch to hyphal growth and mycelium formation conditioned medium was subjected to different treatments. CMM was either heated to 100°C for 10 min, extracted with equal volumes of chloroform, fractionated by centrifugation through membranes with 30, 10 and 3 kDa cutoff (Centricon concentrators, Amicon), concentrated 20-fold with

the 30 kDa cutoff filter or digested with 8000 U of trypsin (Sigma; 37°C, 1.5 h). Trypsin was inactivated by the addition of trypsin inhibitor (4 mg, ~20 kDa, Fluka).

### 3. Results

#### 3.1. Hyphal growth was a prerequisite for vegetative spore formation in vitro

In vitro, *E. thripidum* grows and multiplies as protoplasts (Fig. 1) [7]. Hyphal growth and mycelium formation (Fig. 1), concomitant with cell wall formation, was observed after prolonged cultivation without subculturing and usually occurred between 10 and 20 days after inoculation [7]. The switch from protoplasts to hyphal growth was an irreversible process and affected all cells in the respective well (not shown). This differentiation to mycelium was observed in the isolates AJ6518, AJ6519, AJ6521, AJ6522 and AJ6525.

Following transfer to water agar mycelium of *E. thripidum* readily formed actively ejected spores after overnight incubation. In contrast, protoplasts treated the same way did not form spores (data not shown). It was possible to infect *T. tabaci* with spores formed on water agar and to re-isolate the fungus from the artificially infected thrips (data not shown).

#### 3.2. Mycelium formation occurred in stationary phase

The *E. thripidum* isolates tested (AJ6518, AJ6519) grew exponentially and 10 days after inoculation the cultures had formed mycelium (indicated by arrow in Fig. 2a). At this time point the cultures had reached the stationary phase because the measure for the density of living cells was decreasing (Fig. 2a). The glucose concentration in the culture medium dropped to zero on the 4th day, but increased again between days 6 and 16 (Fig. 2b). The concentration of  $\alpha$ -amino acids started to decrease after the 6th day (Fig. 2b), 4 days before the formation of mycelium (at day 10). However,  $\alpha$ -amino acids present in the broth were only partially utilised by the fungus (Fig. 2b). The pH of the culture medium behaved similarly to the time course of cell density (Fig. 2c). Starting at 6.5 the pH rose by almost one unit to pH 7.4, before dropping to between 6.8 and 6.9 (Fig. 2c).

#### 3.3. CMM-induced differentiation to hyphal growth and formation of mycelium

If conditioned medium from a 10-day old culture (CMM; Fig. 3, x-axis) was given to a culture of *E. thripidum*, mycelium formation was induced within 2 days (Fig. 3, y-axis). Medium from younger cultures prevented mycelium formation (0–4-day old cultures) or induced dif-

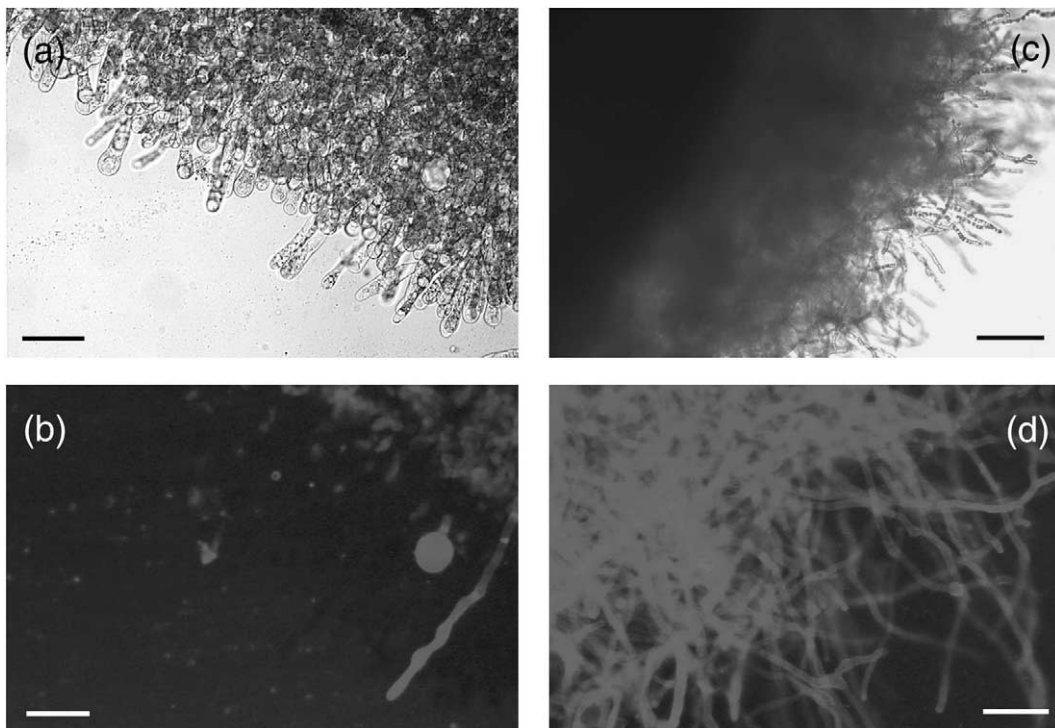


Fig. 1. Light microscopy pictures of *E. thripidum* before (a) and after (c) formation of mycelium. To assess for the presence of a cell wall, cultures were stained with calcofluor and observed under fluorescent light (b,d). A 7-day old culture of *E. thripidum* (panel a) consisted mainly of protoplasts with only a few cells having a cell wall (panel b). Formation of a cell wall and mycelium occurred within 2 days (panel d). Bars in panels a, b, d, 50  $\mu$ m; in panel c, 100  $\mu$ m.

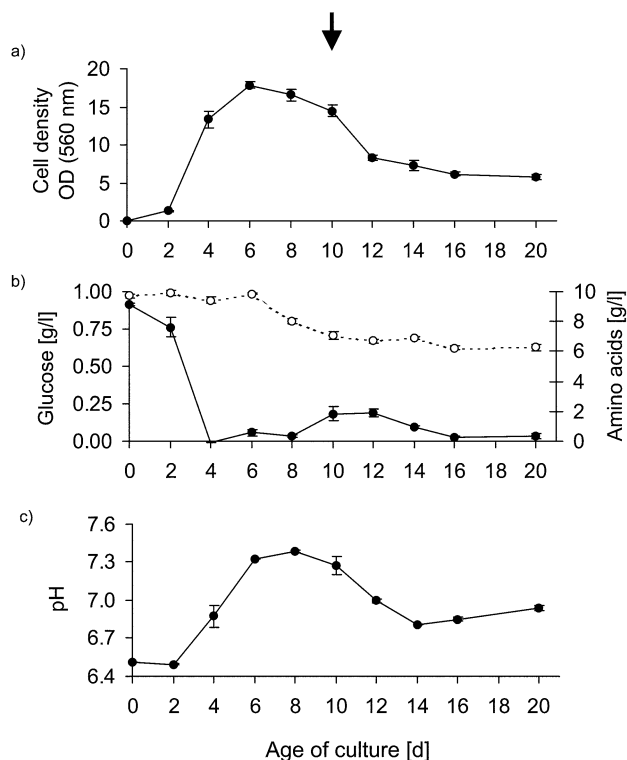


Fig. 2. Time course of an *E. thripidium* culture that formed mycelium spontaneously after 10 days (arrow). (a) Change of cell density as measured by the MTT assay over time. (b) Glucose (●) and total  $\alpha$ -amino acid (○) measurements in the growth medium. Amino acid concentrations were determined with reference to leucine as a standard. (c) Change of the pH in the medium during growth of *E. thripidium*.

fermentation slower (6- and 8-day old cultures; Fig. 3). Medium from cultures older than 10 days (CMM) always induced the switch to mycelium within 2 days (Fig. 3). In addition, CMM induced mycelium in a concentration-dependent manner and increasing the cell density after 7 days by removing culture medium led to a faster mycelium formation (data not shown).

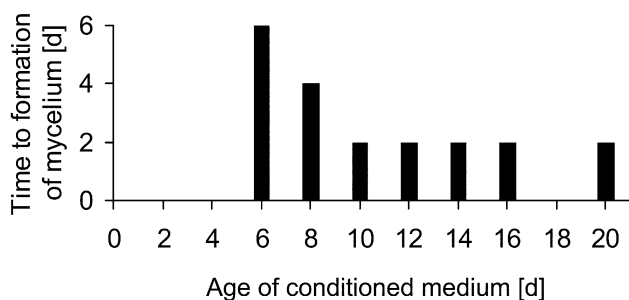


Fig. 3. Conditioned medium from cultures of different age was used to replace half of the culture volume of a 7-day old culture of *E. thripidium* (AJ6518). The age of the culture from which the conditioned medium originated is indicated on the x-axis. In medium from a culture 10 or more days old mycelium had been formed (CMM). The time required to form mycelium after the change of the medium was recorded (y-axis).

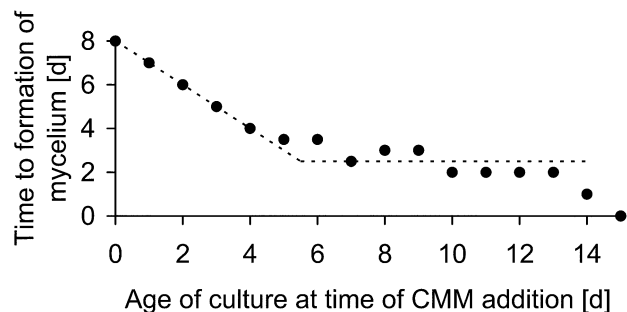


Fig. 4. Competence of mycelium formation at different ages of an *E. thripidium* culture (AJ6521). A culture was grown for 15 days. Every day half of the culture medium was replaced with CMM (x-axis) and the time from the change of the medium to the formation of mycelium was recorded (y-axis). The curve that is drawn is eye-fitting.

### 3.4. Induction of mycelium by CMM required a responsive stage of *E. thripidium*

A culture (AJ6521, AJ6522) that was inoculated into medium containing CMM (addition at culture age 0 days) formed mycelium after 8 days (Fig. 4). The time required for the formation of mycelium after the addition of CMM decreased with culture age to only 3 days when a 7-day old culture was induced by CMM addition (Fig. 4). *E. thripidium* cultures older than 7 days consistently formed mycelium within 2–3 days upon CMM addition (Fig. 4). After 15 days the cultures had formed mycelium without the addition of conditioned medium and the experiment was terminated (Fig. 4). These results suggested that the fungus had to grow for 5–7 days (the time point when the curve in Fig. 4 bends) and to reach a certain cell density in order to become responsive to CMM. This experiment also showed that the CMM-induced transformation of protoplasts to the mycelium stage required at least 2–3 days (height of the horizontal part of the curve in Fig. 4).

### 3.5. Mycelium formation was inhibited by complex nitrogen sources

*E. thripidium* (AJ6521, AJ6522, AJ6525) grown in complete GLEN medium formed mycelium after 14 days (7 days after the medium was changed in other cultures; Fig. 5a). However, replacement of the medium after 7 days by complete medium or by medium lacking the C-source suppressed mycelium formation (Fig. 5b,c). Replacement of the growth medium by a medium lacking the N-source, irrespective of the presence or absence of the C-source, induced mycelium formation within 5 days after the addition (Fig. 5d,e). This was considerably faster than the 7–10 days observed without an exchange of medium (Fig. 5d,e,a, respectively). The effect of CMM in the presence or absence of C- and N-sources was also studied (Fig. 5f–i). Interestingly, CMM was able to induce mycelium formation in the presence of a C- as well as an N-source. In all cases where CMM was used mycelium was formed



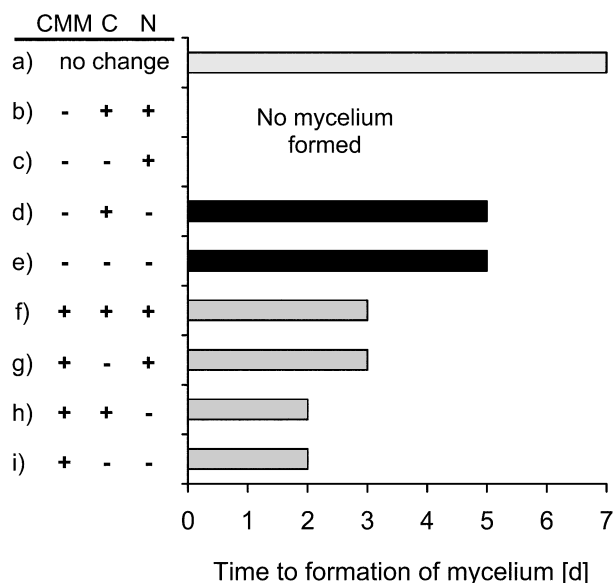


Fig. 5. Mycelium formation in different media. 7 days after inoculation the medium of a culture of *E. thripidum* (AJ6522) was left unchanged (a), exchanged with complete fresh medium containing the C- and N-source (b), or with medium lacking the N- and/or the C-source (c,d,e as indicated). In addition, these four medium compositions (complete or depleted fresh media) were used in combination with CMM (f–i as indicated). The x-axis indicates the time elapsed from the moment of medium change to mycelium formation.

after 2–3 days, even if complete GLEN medium was added together with the conditioned medium (Fig. 5f–i). This was substantially faster than the 5 days required for the formation of mycelium in media lacking the nitrogen source (Fig. 5d,e).

### 3.6. Characterisation of mycelium-inducing factor

CMM activity was destroyed by boiling, was inactivated by extraction with chloroform and did not pass through filters with 30 kDa cutoff size. Thereby it was possible to concentrate CMM activity. Finally, the mycelium-inducing activity was abolished by digestion with trypsin.

## 4. Discussion

This report describes the differentiation from protoplast to hyphal growth that occurs during the life cycle of the entomopathogenic fungus *E. thripidum* and that is an absolute requirement for sporulation. Five of the eight isolates used during this study regularly showed hyphal growth. However, similar to the loss of pathogenicity [13,14] or the sporulation capability [10] in other entomopathorean fungi, some cultures lost the ability to grow as hyphae and to form mycelium during continuous subculturing.

At first glance the synthesis of the cell wall and the switch from the protoplast to the hyphal stage is similar to the cell wall regeneration in protoplasts of *Entomophaga*

*ga aulicae* [16,17] and the dimorphism between yeast-like and hyphal stages observed in many fungi (reviewed, for example, in [18–20]). However, the differentiation of the protoplasts of *E. thripidum* is particular in many aspects. In *E. thripidum* and also in *Entomophthora muscae*, a closely related species pathogenic to house flies, the switch from the protoplasts to the hyphal form is regulated differently than in *E. aulicae* [7,8,21]. In addition, the distinct stages observed during cell wall regeneration of protoplasts in *E. aulicae* neither occur in *E. thripidum* nor in *E. muscae* [7,17,21,22]. In *E. thripidum* the synthesis of the cell wall is also accompanied by extensive hyphal growth, another difference in comparison to *E. aulicae* [7]. The hyphal stage in *E. thripidum* is also not an alternative growth form of the fungus. Vegetative growth and cell multiplication occur as protoplasts. The hyphal stage, in contrast, is an essential step in the process of sporulation enabling *E. thripidum* to break out of the insect and to produce and eject spores. The switch to hyphal growth is thus a chronologically distinct step in a sequence of events that lead to the formation of infectious spores.

For this reason the regulation of this differentiation from protoplasts to mycelium was studied further with the goal to make sporulation of *E. thripidum* more reliable and efficient. Protoplasts of *E. thripidum* grew exponentially and formed mycelium when the stationary phase was reached and the cell density started to decrease. This decline in the measure for the density of living cells was ascribed to lysis of cells within the pellets [7]. Mycelium formation was observed after the amino acid content in the medium had started to decrease. At this time the glucose concentration had dropped to zero. The subsequent increase in the glucose concentration between days 6 and 16 was considered an artifact. It was possibly also due to the lysis of cells within the protoplast pellets that led to the release of glucose or production of substances that interfered with the enzymatic assay used for the determination of the glucose. Similar to the time course for cell density the pH rose by almost one unit and decreased again after 8 days (Fig. 2c). This rise in pH may be ascribed to the utilisation of amino acids as carbon sources, thereby releasing ammonium. A possible relation of the pH change with the utilisation of amino acids as C-sources was also supported by the coincidence of glucose depletion and pH rise at day 4 (Fig. 2b,c). The subsequent drop of the pH could have been caused by the production of organic acids after prolonged cultivation. In contrast to considering the change in pH as a secondary effect it is also possible that the fungus actively changes the pH, which has been shown for the entomopathogenic fungus *Metarhizium anisopliae* [15].

It was then found that CMM induced the formation of mycelium within 2–3 days. However, *E. thripidum* had to be in a competent state in order to become responsive to CMM; the fungus had to grow for 5–7 days before CMM-induced transformation of protoplasts could occur. Com-

paring mycelium formation in media lacking either the C- or N-source it was found that omitting the N-source induced mycelium within 5 days. This was considerably faster than the 7–10 days observed in complete medium and indicated that N-limitation was essential to initiate mycelium formation. However, CMM activity was able to bypass this requirement and it was possible to extract, inactivate (by boiling or digestion with trypsin) and concentrate CMM activity. These results are compatible with the hypothesis that an additional proteinaceous factor, produced and secreted by the fungus under nitrogen limitation, was present in the conditioned medium and induced mycelium formation.

Based on these results we propose a model for the induction of the mycelium stage in *E. thripidum* where complex nitrogen sources repress the production of a ‘mycelium factor’ that stimulates the differentiation processes by autoinduction. *E. thripidum* grows within the body of its host insects, which is an environment rich in complex nitrogen sources as well as in free amino acids. Nevertheless, it could be imagined that the dense and aggregated growth of *E. thripidum* in the abdomen of its hosts locally uses up all insect components. This localised N-limitation inside the host would then cause the secretion of the ‘mycelium factor’ that results in coordinated mycelium formation of all fungal cells present in the insect. This could provide the mechanism that enables insect pathogenic fungi such as *E. thripidum* to sporulate coordinately and localised from only a selected spot of the host insect and at a moment when the host is still alive. The time window that allows sporulation from the living and moving insect is very narrow and autoinduction of mycelium formation and subsequent sporulation could provide a mechanism to optimise this process. The selective advantage of sporulation from living and moving hosts might be the spreading of the infectious spores over a larger area, thereby increasing the probability of reaching a new host. Within the framework of this hypothesis it is predicted that entomophthoralean fungi sporulating from cadavers do not require such an autoinduction mechanism, because there is no selective pressure favouring highly organised sporulation. In Entomophthorales that sporulate from dead hosts N-limitation as the primary signal might be sufficient for the direct induction of the sporulation process. Indeed, *E. muscae*, which sporulates only from dead insects, forms mycelium more readily and does not rely on a secreted factor for this differentiation (data not shown). In contrast, the regulation of mycelium formation and sporulation via a ‘mycelium factor’ could represent a general mechanism for fungi sporulating while the host is still alive. Besides for the *E. thripidum*–*T. tabaci* interaction [5], sporulation from living hosts is also known for *Entomophthora erupta* [23] and species of the genera *Strongwellsea* and *Massospora* [24,25]. It will be interesting to learn if these species also rely on a self-produced factor

for the initiation of hyphal growth and subsequent sporulation.

In conclusion, the discovery of this regulation mechanism in *E. thripidum* made possible the reliable and controlled production of mycelium and infectious spores, which embodies a first and essential step for the utilisation of this fungus as a mycoinsecticide. Reliable sporulation also made it possible to infect healthy onion thrips and to complete the asexual life cycle of *E. thripidum* under laboratory conditions.

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