Title: Inside Pandora's box: development of the lethal myrmecopathogenic fungus *Pandora formicae* within its ant host

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Abstract

Parasites modify their host's appearance, behaviour and physiology to a certain extent. Many of these modifications are seen as purposefully manipulative, serving the parasite's interest. Endgames are particularly intriguing, where the parasite's development requires the host's death. 'Summit' or 'treetop' disease is one of such spectacular examples of parasite-induced death: the host dies attached to an elevated part of the vegetation. Many organisms induce summit disease including *Ophiocordyceps*, *Entomophthora* and *Pandora* species. We analyzed the development of *Pandora formicae* inside its ant host's body after the host's death. Our findings suggest that the fungus consumes the host's muscles and central nervous system quite fast, similarly to the related *Entomophthora muscae*. Unlike in *Ophiocordyceps*, no conidial anastomosis tubes were observed. Differences and similarities between the three fungi are discussed with regard to the extent of parasitic manipulation since *Pandora*'s manipulative traits seem less pronounced than in *Ophiocordyceps*.

Keywords: behavioural manipulation; extended phenotype; *Entomophthora muscae*; fungi; histological sections; *Ophiocordyceps*; summit disease; ants

1. Introduction

Parasites influence their hosts' behaviour, morphology and physiology to a different extent (Schmid-Hempel, 1998; Lefèvre et al., 2009; Molnár et al., 2010; Thomas et al., 2010; Lafferty and Shaw, 2013; Elya et al. 2018; Herbison et al., 2018). In many cases changes identified in the host are considered to be the consequence of parasitic manipulation, that is they come to serve the interest of the parasite, e.g. to facilitate its transmission to the next host, to assist its reproduction (Moore, 2002; Hughes et al., 2008; Poulin, 2010; Thomas et al., 2010). Such modifications that increase the parasite's fitness constitute the 'extended phenotype' of the parasite (Dawkins, 1982). Amongst all these parasitic effects, 'endgames' are the most intriguing, when the parasite effectively kills its host in an unusual way, just to aid its transmission (*see* Roy et al., 2006). Aquatic hairworms drive their terrestrial insect hosts to jump into water (Ponton et al., 2011), snail-infecting parasitic trematodes swell the host's tentacles to resemble caterpillars (Sorensen and Minchella, 2001), while the protozoan *Toxoplasma gondii* makes its rodent host attracted to cats, its definitive host (Berdoy et al., 2000; Webster et al., 2013).

Social animals such as ants are also frequently targeted by parasitic organisms. Bacteria, fungi, mites, fluke worms, nematodes, beetles, butterflies and even other ants can infiltrate the 'social fortress' (Schmid-Hempel, 1998; Csősz and Majoros, 2009; Espadaler and Santamaria, 2012; Csata et al., 2013; Otti et al., 2014; Witek et al., 2014; Csata et al., 2017a,b; Małagocka et al., 2017; de Bekker et al., 2018; Loreto et al., 2018). Among myrmecoparasitic organisms, fungi are one of the most diverse groups, with respect to both systematics, lifestyle and behavioural manipulations (*see* Weir and Blackwell, 2005; Roy et al., 2006; Hughes et al., 2016; de Bekker et al., 2018; Małagocka et al., 2019). Within parasitic fungi mostly the tropical species of the genus *Ophiocordyceps* (Ascomycota, Hypocreales) have received attention from the scientific community (e.g. Andersen and Hughes, 2012; de Bekker et al., 2014; de Bekker et al., 2015; Hughes et al., 2016; de Bekker et al., 2017; Fredericksen et al., 2017; Araújo et al., 2018; de Bekker et al., 2018; Loreto et al., 2018; Sakolrak et al., 2018; Loreto and Hughes, 2019; Mangold et al., 2019; Will et al., 2020) due to the bizarre endgame they cause. These fungi elicit the so-called 'summit disease' syndrome: moribund individuals climb on elevated parts of the vegetation (leaves, grass blades, twigs), fix themselves to it by their legs and/or mandibles, while after a certain latency the fungus breaks out of the ant cadaver spreading efficiently the airborne spores to the potential host population below. This behaviour is preceded by conspicuous changes in host activity that are caused by the fungal development inside the host (*see* Hughes et al., 2016; de Bekker et al., 2018 for reviews).

The 'summit disease' or 'tree top disease' syndrome can be linked to a number of parasitic organisms infecting various insect groups (Roy et al., 2006; Boer, 2008; van Houte et al., 2012; Hughes et al., 2016; Steinkraus et al., 2017; de Bekker et al., 2018; Elya et al., 2018; Lovett et al., 2020). Particularly in ants, besides *Ophiocordyceps* species, *Dicrocoelium* fluke worms, and also infection by another fungal group, members of the genus *Pandora* (Entomophthoromycota, Entomophthorales) cause summit disease. Entomophthoralean fungi infect insects from every major insect order from Orthoptera to Diptera and Hymenoptera (Gryganskyi et al., 2013). The genus *Pandora* is the only known Entomophthoralean fungal group that attacks eusocial insects; *P. formicae* parasitizes species of the genus *Formica* (Hymenoptera: Formicidae) (Hughes et al., 2016). The fungus produces infective spores that attach to, germinate on, and penetrate the cuticle of its hosts. It proliferates then in the form of single- or multicellular infective structures in the haemocoel, ultimately killing the insect host while producing infective conidia and/or resting spores on the surface of the cadaver. Overwintering resting spores are mostly produced in late autumn, these can probably survive in the soil or in the nest material (Małagocka et al., 2017).

The similarities between *Ophiocordyceps* and *Pandora* let us hypothesize that the fungal colonisation process could also be similar, even though Entomophthoromycota fungi are phylogenetically quite unrelated to Ascomycota (James et al., 2006; Gryganskyi et al., 2013). A recent comprehensive study on *Entomophthora muscae* (Elya et al., 2018), a close relative of *Pandora* that induces summit disease in solitary insects, namely flies, could also help us identify the specificity of *Pandora*-ant relationship. Contrary to *Ophiocordyceps*, where parasitic interferences are known from the molecular to the population level (*see* de Bekker et al., 2018 for a review), only a few studies dealt with the invasion process in *Pandora* (Boer, 2008; Małagocka et al., 2015; Małagocka et al., 2017; Małagocka et al., 2019). In the frame of our study, we investigated for the first time the dynamics of *P. formicae* development and outbreak after the death of its host, with the use of histological sections to identify convergences, but also differences between these fungal groups, which seem to manipulate their hosts in a strikingly similar manner.

2. Materials and methods

2.1 Study species and collection methods

Workers of *Formica* (*Coptoformica*) *exsecta* Nylander, 1846, infected with the fungus *Pandora formicae*, were collected from a large polydomous system in the southern part of the Giurgeului depression (46°36'N, 25°36'E, 780 m a.s.l.) in the Eastern Carpathians, Romania, in the summer of 2014 and 2016. This system is the largest known European polydomous system of *F. exsecta* and contains 3,347 permanent nests over an area of ca. 22 ha with a density of ca. 153.25 nests/ha (Markó et al., 2012). *Formica exsecta*, the narrow-headed ant, is a relatively common mound-building pan-Palaearctic ant species that inhabits areas of mixed and deciduous forests, from England to eastern Siberia and from Sweden to southern Europe (Schultz and Seifert, 2007).

We collected cadavers of ant workers that were already fixed to the grass blades in the typical summit disease manner. In all cases, we looked for the existence of fungal rhizoids that grew out from the intersegmental parts of the basisternum and laterocervical plates, and bind the corpse to grass, to confirm *P. formicae* infection prior to dissection. The collected corpses belonged to the early, middle and late necrotic stages. These categories were established on the basis of the level of external fungal growth (*see* also Andersen et al., 2009 for *Ophiocordyceps*): cadavers attached to grass blades, with no visible fungal conidiophores were considered early necrotic stage corpses (Fig. 1A), the middle necrotic stage was established based on the thin strip-like appearance of fungal emergence at intersegmental parts of the abdomen (Fig. 1B), and the late necrotic stage was considered when the fur-like conidiophores with conidia densely covered major parts of the abdomen (Fig. 1C).

Six individuals were collected for scanning electron microscopy on 18 August 2014: 1 individual belonging to the early, 2 to the middle and 3 to the late necrotic stage. For histological sections altogether 23 individuals were collected on 24 July and 28–30 August 2016, out of which 7 were placed in 96% ethanol solution as reference material, while 6 individuals belonging to the early necrotic stage, 4 to the middle and 6 to the late necrotic stages were used for histological analysis further on. Altogether 784 *F. exsecta* nests were checked for infected individuals, and each infected worker ant was found at a different nest (< 3% prevalence). The cadavers were all located at a height of 21.17 ± 12.33 cm (N = 23) above ground at the border of the nest mound. In *Formica exsecta*, unlike in other *Formica* species (*see* Małagocka et al., 2017) infected ants have never been observed along trails further from the nest.

2.2 Preparation of SEM images and histological sections

Samples for scanning electron microscopy were fixed in 2.7% glutaraldehyde, dehydrated in acetone, and sputter-coated with 7 nm gold. Images were taken with a Jeol JSM5510LV (Japan) at 30kV.

For histological examination, the ants were fixed for 12 h in cold 2% glutaraldehyde buffered at pH 7.3 with 50 mM Na-cacodylate and 150 mM saccharose. 1 h after post-fixation in 2% osmium tetroxide in the same buffer, tissues were dehydrated in a graded acetone series, embedded in Araldite, and sectioned with a Leica EM UC6 ultramicrotome. Serial semithin 1 µm sections were stained with methylene blue and thionin and viewed in an Olympus BX-51 microscope. Comparative histological sections were also prepared of uninfected individuals collected from the nest surface.

3. Results

Before the fungal outbreak the moribund workers typically become agitated, climb up and down on the nearby vegetation and in the end fix themselves with their mandibles (Fig. 1A), and finally die in this 'death grip'. While in most cases infected ants die with their head upwards, it is also common to find cadavers with their head oriented downward (Fig. 1A). In 1 or 2 d the fluffy, fur-like conidiophores appear at the intersegmental parts of the thorax and abdomen (Figs. 1B, C). The infective conidia are then actively discharged from the cadaver and are showered on the potential host population below.

The fungus develops rapidly inside the host's body after killing the ant worker (Małagocka et al., 2015). In the death grip phase fungal rhizoids grow out from the intersegmental parts of the basisternum and laterocervical plates, and bind the corpse to grass (Figs. 1D, E). During this **early necrotic stage**, hyphal bodies already filled up considerable portions of the host ant's body (Fig. 1F; a healthy head is shown for comparison in Fig. 1G). The fungus at this stage was restricted to the 'interior tissues' of the host, since it was not found inside the infrabuccal cavity (Fig. 1F), the oesophagus (Fig. 1H), and the tracheal system (Fig. 1I), as these structures all represent the anatomical 'exterior'. Muscle fibres were already invaded in this early necrotic stage (Fig. 1I), while the ganglia were still untouched. However, the brain was already invaded and considerably reduced by the fungus (Figs. 1F, H). The thorax and abdomen were also invaded, but the reservoir of glands (e.g. venom and Dufour gland), that also form part of the anatomical 'exterior' were still intact (Fig. 1J).

In the **middle necrotic stage,** the head was almost completely devoid of host tissue, while the brain and the muscles were entirely consumed by the fungus (Fig. 2A). Hyphal bodies were invading all other parts of the ant host, including the compound eyes (Fig. 2B), mandibles (Fig. 2C) and other mouthparts (Fig. 2D). At this stage the mycelium could be observed in the soft intersegmental membranes, as is shown for the membrane that connects the mandibles to the head capsule (Fig. 2C) and the intersegmental membrane between the metathorax and the petiole (Fig. 2E). The hard and sclerotized cuticle, however, was not affected by the fungus (Figs. 2A-E).

The **late necrotic stage** was the most conspicuous, as fungal tissue started covering much of the exterior of the host's body (Figs. 3A-D). The host body was completely emptied by the fungus (Fig. 3E). Fungus broke through the intersegmental membranes, through the antennal sockets (Fig. 3F) and the mandibular articulation (Figs. 3G, H). The fluffy external layer of fungal tissue consisted of conidia bearing conidiophores (Figs. 3G, H). The fungal growth inside the host were generally filamentous with a diameter around 10-15 μm, although bifurcations and constrictions are often observed (Figs. 1H-I, 2B-C, 3I). We did not observe CATs (Conidial Anastomosis Tubes) as described in *Ophiocordyceps* by Fredericksen et al. (2017). High magnification observation of hyphal bodies for eventual CATs only showed bifurcations within a hyphal body, rather than a connection between two hyphal bodies (Fig. 3I).

4. Discussion

Parasitic organisms can modify their host's behaviour even in a relatively simple manner by capitalizing on the behavioural changes resulting from the interplay between the nervous system and the immune system of the host, or could directly interfere with the host's physiology (*see* Cézilly et al., 2010; Lafferty and Shaw, 2013; Hughes et al., 2016; Elya et al. 2018; Will et al., 2020). While taken for granted in many cases, the actual demonstration of true parasitic manipulation is not very straightforward in most situations. One could truly suspect parasitic manipulation when the infected host manifests a certain behaviour and/or morphology that is not part of its normal repertoire or its appearance, its frequency is unusual, while it also clearly benefits the parasite and not the host (Moore, 2002; Hughes et al., 2008; Thomas et al., 2010; Cézilly et al., 2010; Poulin, 2010; van Houte et al., 2012; Hughes et al., 2016; Herbison et al., 2018). The host–pathogen relationship among ascomycete fungi, *Ophiocordyceps* spp. and their carpenter ant hosts or fungi from the Entomophthoralean genus *Pandora* and its *Formica* ant hosts most certainly lead us to think about elaborate mechanisms of manipulation. At first glance both fungal organisms, although distantly related, manipulate the behaviour of their host in a remarkably similar manner almost serving as a textbook example for convergent evolution. Yet beyond striking similarities quite important differences occur between the two fungal organisms (*see* also Małagocka et al., 2019), which we address in the frame of this study from a histological perspective. When interpreting the results of this study caution should be applied, however, since data obtained from host necrotic stages have limited potential with regards to the characterization of the invasion process in live hosts.

Before killing the infected ant, *Ophiocordyceps* fungi manipulate the behaviour of the ant hosts by driving them away from the vicinity of the nest, and making them fix themselves with their mandibles and legs to vegetation parts (Andersen et al., 2009; Hughes et al., 2011). The death grip occurs as a consequence of hypercontraction of mandibular muscles caused by the fungus (Mangold et al., 2019), as the fungus invades the muscle fibers throughout the host's body (head, thorax, abdomen, legs). However, the brain is spared until the death grip (Fredericksen et al., 2017). Uniquely, within the host body *Ophiocordyceps* fungal cells establish a network through conidial anastomosis tubes (CATs) (Fredericksen et al., 2017) that makes possible faster germination than in the case of a single conidium (Roca et al. 2005). It is known already that bacterial-like enterotoxins are involved in host manipulation (de Bekker et al., 2017; Will et al., 2020), but the putative protein-tyrosine phosphatase (*ptp*) gene is also up-regulated both in the ant and in the fungus during manipulation (de Bekker et al., 2015), which could induce hyperactivity in the host as already documented in other insects (van Houte et al., 2012). The increase in the production of ergot-like alkaloids by the fungus, could also affect the central nervous system of ants (de Bekker et al., 2015; Will et al., 2020). Similarly to *Pandora*, within 24 h after death, sparse hyphae grow out from the host's body at the thin non-sclerotized parts of the cuticle, as intersegmental membrane (Andersen et al., 2009). Nevertheless, the spore-producing fruit body appears only several weeks after the host ant's death (Chung et al., 2017), while the cadaver can last for even 1 y (Loreto et al., 2018).

Entomophthora muscae applies a similar strategy to *Ophiocordyceps*, while its host group is quite different both with regards to systematic position (Diptera) and lifestyle (solitary). The comprehensive study of Elya et al. (2018) offers a detailed view on how this particular fungus invades the host's body after infection, and how it develops after its death, yielding an accurate basis for comparison with *Pandora* and *Ophiocordyceps*. Here as well the whole body is invaded, while the fungus reaches the central nervous system of the host already in the first 48 h while it is still alive. After the host's death there is an explosive developmental phase, and within 1 d the first infective spores could appear, but no fruit body is produced.

In stark contrast to *Ophiocordyceps*, in the case of *Pandora* infection, the host dies in the immediate vicinity of the ant nest, in most cases attached to grasses surrounding the nest mound, or on grass blades bordering ant trails (Marikovsky, 1962; Boer, 2008; Małagocka et al., 2017; pers. obs.). It is known here as well that a wide array of different chitinase to lipase enzymes are employed (Małagocka et al., 2017), similarly to *Ophiocordyceps* and *E. muscae* (de Bekker et al., 2017; Elya et al. 2018; Will et al., 2020), that help subdue the host's resistance. Based on our results in the necrotic stages both muscles and the central nervous system were invaded by the fungus, which is consistent with both *Ophiocordyceps* and *E. muscae*. *Pandora* was omnipresent inside the host, not just in the capsules of the head, thorax, and abdomen, but also in more specialized parts such as the compound eyes, the mandibles, mouthparts, and legs. Unlike in *Ophiocordyceps*, and similarly to *E. muscae*, conidial anastomosis tubes were not observed. Also, conidiospores produced by *P. formicae* develop on the ant's body within almost 24 h after death (Boer, 2008; Małagocka et al., 2017; pers. obs.), similarly to *E. muscae*. Thus, *Pandora* 'strikes' closer to the nest, and also reaches full maturity much faster.

While in the case of *Ophiocordyceps* manipulation seems obvious even at the death grip phase since infected ants do something which is not in their normal behavioural repertoire, this is not entirely so in *Pandora*. The hosts of *P. formicae*, members of the genus *Formica*, mostly mound-building species of subgenera *Formica* s. str. and *Coptoformica* (Marikovsky, 1962; Boer, 2008; Erős et al., 2009; Csata et al., 2012; Małagocka et al., 2017) quite frequently climb on grasses surrounding their nest mounds or bordering their trails, in *Coptoformica* they even cut the tip of the grasses usually. Therefore, *Pandora* could use an already well-established set of behaviour rather than pushing the ant workers to do something utterly unusual. In *Pandora*'s case moving the infected ant workers farther from the nest would dramatically decrease transmission chances and lower the fungus' fitness. Recently, Lovett et al. (2020) proposed a comprehensive framework for explaining summiting behaviour elicited by different pathogens. They also suggest that already existing ancestral behavioural patterns are used by the pathogens, arguing that these are mostly related to period of quiescence and circadian rhythms.

It is easy to hypothesize that the central nervous system is the primary candidate for manipulative interferences, and in many cases, indeed, it is the most targeted site by parasites (Lafferty and Shaw, 2013). Parasites could even be able to use immune defenses on the level of the central nervous system to their benefit since the inflammation of the brain can be linked to the production of various neuromodulators, which, in turn, could result in behavioural alterations (*see* Lafferty and Shaw, 2013). *Ophiocordyceps* fungi and *E. muscae* apply different strategies seemingly. While the former invades the brain only after the death grip of the host (Fredericksen et al., 2017), *E. muscae* appears in the central nervous system already in live hosts (Elya et al., 2018). Interference with the brain could exist in both cases though. While our approach, the analyses of necrotic stages of *Pandora* infected hosts, certainly does not tell us what actually goes on in a live host, an analogy with *E. muscae* would suggest that the brain could already be invaded before the death grip. Elya et al. (2018) proposes different scenarios for why this could happen in *E. muscae*, suggesting that it is most plausible that invader fungal cells could start interacting with the central nervous system quite early, thus manipulating the host.

The answer to why *Pandora* develops at a faster rate might, at first glance, also lie in the efficiency of social prophylaxis performed by ants. As suggested by Małagocka et al. (2019) workers could be highly successful in discovering infected ants based on chemical cues and might dispose of them earlier than the fungus could fully develop. This also implies that the fungus' behaviour is subject to heavy constraints: while driving the host to perform the death grip near the ant nest mound is relatively easy and very advantageous with respect to increased transmission probability, it is also highly risky since infected cadavers might be easily discovered and then disposed of potentially zeroing the fitness of the fungus. Nevertheless, the fact that the phylogenetically related *E. muscae* also displays a similarly fast development (Elya et al., 2018), while parasitizing solitary insects as e.g. fruitflies, instead of social ones, offers a more parsimonious scenario: the developmental rapidity of *Pandora* could be an ancestral trait that, luckily, fits well with the need to minimize the negative effect of social prophylaxis in ants. In *Ophiocordyceps* slower development might be related to its reproductive strategy which implies the growth of a fruit body that takes time and requires a relatively safe environment with minimal intrusion from e.g. ants.

As a general rule, we must bear in mind also that in most cases of parasitic interferences with host behaviour it is rather hard to tell where the actual parasitic manipulation begins and host response ends, but also whether what we observe is actual adaptation or just a perfect coincidence (*see* de Bekker et al., 2018). In our specific case, we suggest that although phenotypically convergent the case of *Pandora* is rather different from that of *Ophiocordyceps*, where evidence for parasitic manipulations seems to be much more obvious, occurring on different levels. In *Pandora* it is a faster process, where well established behavioural features of the host are used. and ancestral traits might function as seemingly specific adaptations.

Acknowledgements

We are indebted to An Vandoren for her considerable help with the preparation of the histological sections and to Katalin Erős, Kriszta Kincső Keresztes, and Ágota Szabó during field collections. The suggestions of two anonymous reviewers helped us improve considerably the quality of the manuscript. B.M. was supported by a grant of the Romanian National Authority for Scientific Research and Innovation, CNCS–UEFISCDI, project number PN-II-RU-TE-2014-4-1930. B.M. was also supported by the Bolyai János scholarship of the Hungarian Academy of Sciences. E.C. was supported by a grant of the Ministry of Research and Innovation, CNCS – UEFISCDI, project number PN-III-P1-1.1-PD-2016-1272, within PNCDI III.

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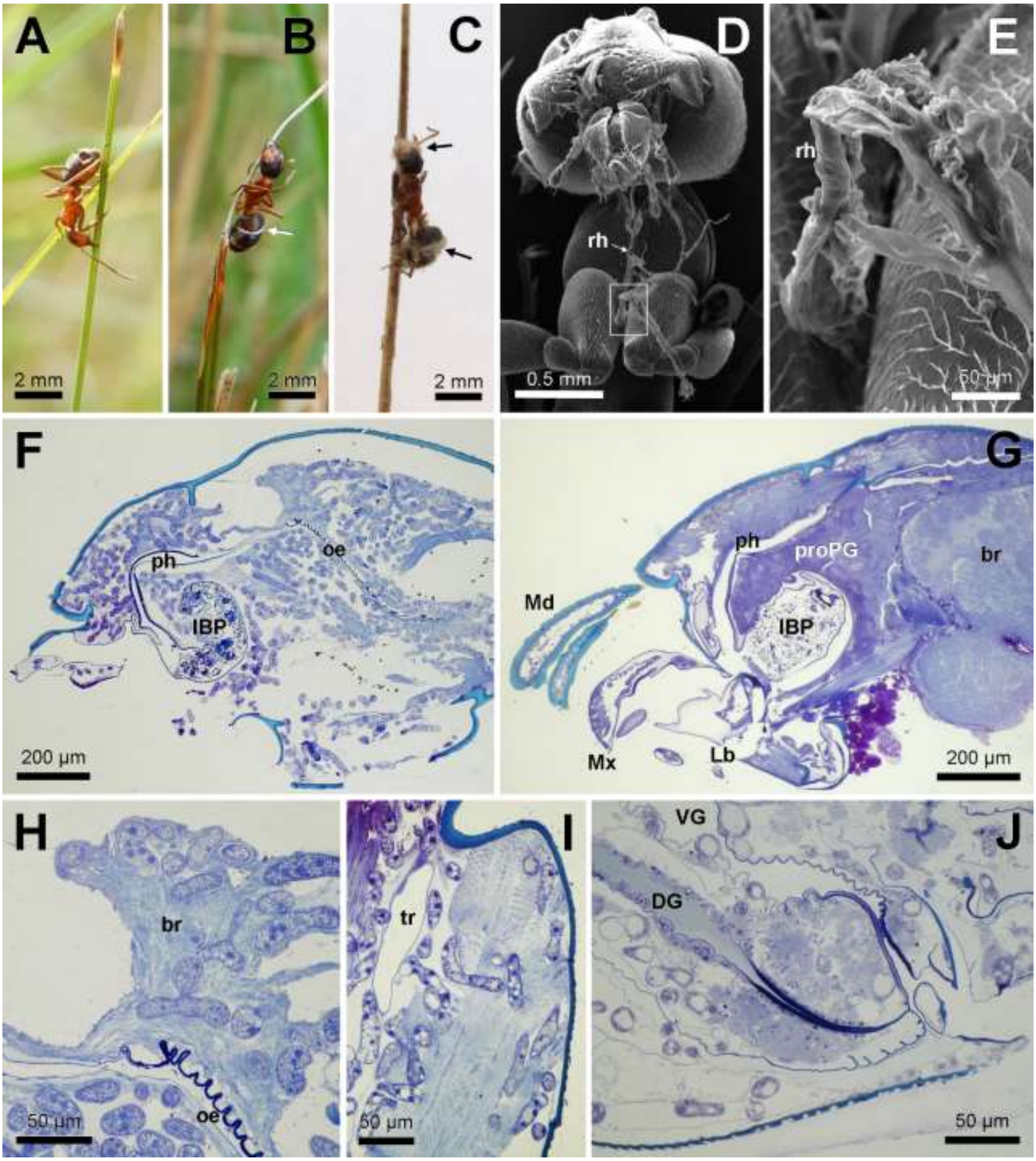

Fig. 1. (**A**) *Pandora* infected *Formica exsecta* worker attached to a grass blade in the field in the early necrotic stage. (**B**) Middle necrotic stage with fungus outbreak through abdominal intersegmental membranes (arrow). (**C**) Late necrotic stage with fungus covering greater parts of the host ant (arrows). (**D**) Ventral view of infected *F. exsecta* worker in the early necrotic stage, showing the fungal rhizoid (rh) through which the ant is anchored to the grass blade. (**E**) Detail of fungal rhizoid (framed part of 1D). (**F**) Longitudinal semi thin section through infected worker head in the early necrotic stage. Fungal tissue is entirely confined inside the host ant, anatomically external parts as infrabuccal pocket (IBP), pharynx (ph) and oesophagus (oe) do not contain fungus. (**G**) Head of non-infected worker for comparison (br: brain, Lb: labium, Md: mandible, Mx: maxilla, proPG: propharyngeal gland). (**H**) Detail of brain invaded by fungus. (**I**) Detail of foreleg coxa showing fungal cells attacking muscle fibres, whereas trachea (tr) does not contain fungus. (**J**) Longitudinal section of abdomen tip, the fungus is spread over internal tissues but not inside Dufour gland (DG) and venom gland (VG).

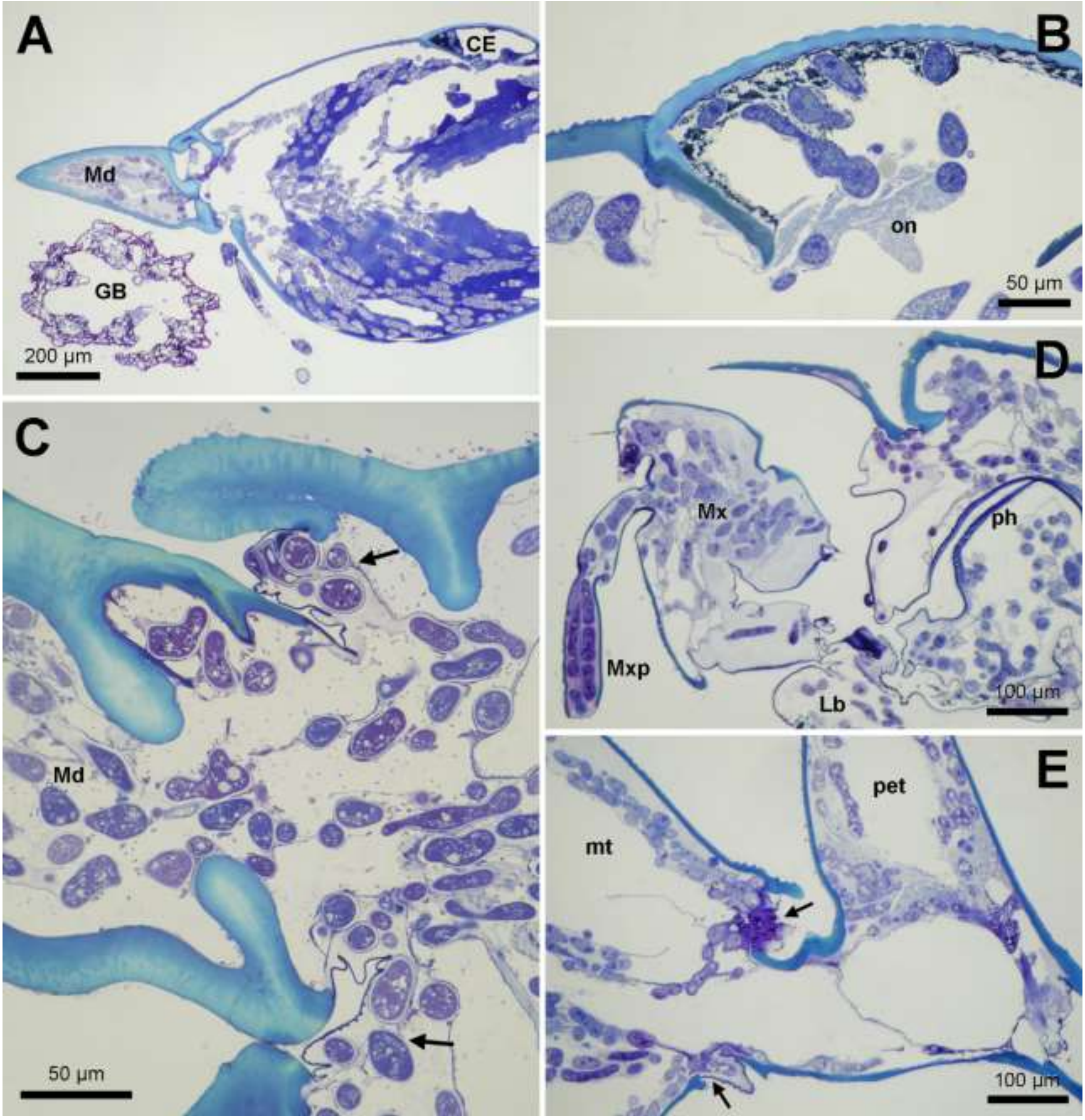

Fig. 2. Semi thin sections of an infected worker in middle necrotic stage. (**A**) Longitudinal view of the head in which host tissues have been replaced by the fungus (CE: compound eye, GB: grass blade, Md: mandible). (**B**) Detail of fungus in compound eye with remnant of optical nerve (on). (**C**) Junction of mandible with head capsule, showing fungal cells penetrating soft intersegmental membrane (arrows). (**D**) Presence of fungal tissue inside maxilla (Mx) and labium (Lb), but not inside pharynx (ph). (**E**) The junction between metathorax (mt) and petiole (pet), showing fungal cells in soft intersegmental membrane (arrows).

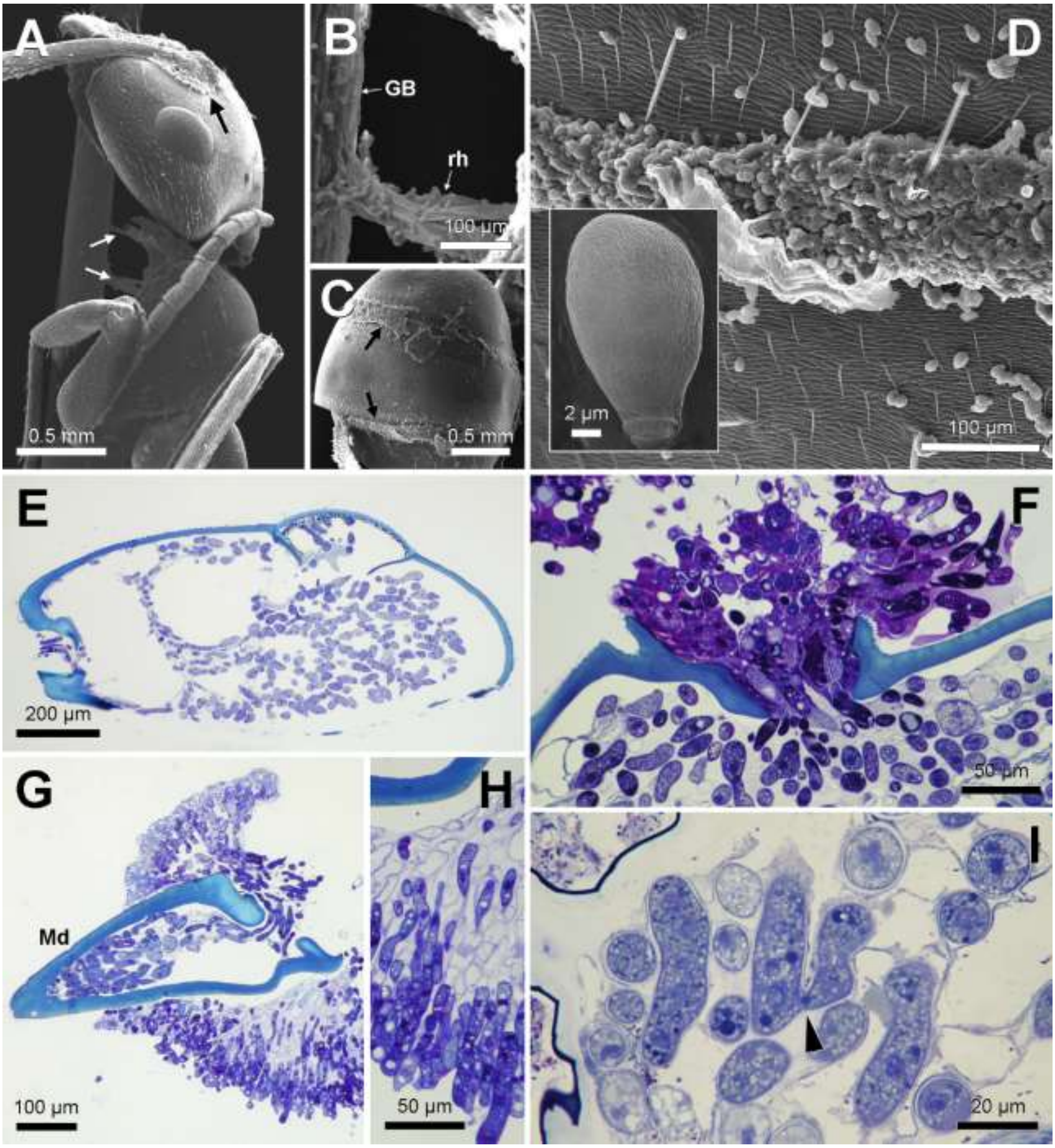

Fig. 3. Scanning micrographs (A-D) and semi thin sections (E-I) of infected worker in late necrotic stage. (**A**) Worker ant attached to grass blade through fungal rhizoid (white arrows), note fungal outbreak on anterior head portion (black arrow). (**B**) Detail of contact between rhizoid (rh) and grass blade (GB). (**C**) Abdomen with fungal outburst through intersegmental membranes (arrows). (**D**) Detail of fungal outbreak at abdominal intersegmental membrane with numerous conidia (inset shows detail of conidium). (**E**) Longitudinal view of head containing only fungal tissue and air sacs (as) belonging to the respiratory tract of the host. (**F**) Fungal outbreak through antennal socket. (**G**) Mandible covered with fluffy fungal tissue. (**H**) Detail of fungal tissue on host ant exterior. (**I**) Detail of fungal cells, showing bifurcation (arrowhead), but no CAT structures.