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THE ACTIVITY OF ASPERGILLUS OCHRACEUS (FUNGI) ON REPLETE FEMALES OF RHIPICEPHALUS SANGUINEUS (ACARI: IXODIDAE) IN NATURAL AND EXPERIMENTAL CONDITIONS

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Abstract. *Aspergillus ochraceus* Wilhelm was isolated from *Rhipicephalus sanguineus* (Latreille) ticks infected under natural conditions, and developing an illness characterized by absence of oviposition, mummification and death. From the histological sections, it is deduced that *A. ochraceus* colonized the ticks via the anus, as posterior gut and Malpighian tubules are invaded. The experimental inoculation was carried out on replete female ticks; a disease alike to that observed in naturally infected ticks developed only when specimens are placed under a relative humidity near saturation.

Frequently, we found in the literature the notice of high mortality of ticks from "in vitro" culture (see e.g. Hendry and Rechav 1981) caused by some microorganism whose pathogenic effects are not at present well known. Sometimes, some workers (Ali et al. 1986) have tried to use the entomogenous material of some fungal and bacterial species as agents for biological control of ixodid ticks. Although some natural populations of insects and mites of agricultural and medical importance can be reduced by the action of some fungi (i.e. *Beauveria bassiana*, *Lagenidium giganteum*) or bacteria (i.e. *Bacillus thuringiensis*) it has not been possible yet to find the most indicated agent for biological control of ticks.

This paper describes the isolation of one fungal species from several *Rhipicephalus sanguineus* ticks, and also reports the results obtained from the experimental inoculation of this fungus into replete female ticks of this species.

MATERIALS AND METHODS

The *Rhipicephalus sanguineus* ticks were obtained from *Oryctolagus cuniculus*, captured in natural conditions at Pina de Ebro (Zaragoza province, Spain). Sixteen replete females were collected, placed separately in sterile flasks and carried to the laboratory in appropriate containers. These females were kept in an incubation oven, at 26 °C and 90 % ±2 relative humidity (RH) and observed every day. Fifteen days after their entry, the oviposition started only in 7 females; from the 9 remaining females, 4 were fixed and dehydrated in an ethanol series, embedded in paraffin and sectioned for histological studies. The sections were stained with haematoxylin-eosine (H–E) and the PAS methods. Ten days after, a fungal growth was observed on the body surface of the 5 remaining females; from these, two were processed for scanning electron microscopy. The material was fixed in 5 % glutaraldehyde in 0.1 M phosphate buffer for 1 hour, and dehydrated in a graded series of ethanols in order to remove particles of dirt from cuticular surface, the ticks were kept in a mixture of 100% ethanol and chloroform at 60 °C for 12 h. Prepared specimens were studied under a Jeol scanning electron microscope at 25 kV.

With the 3 remaining females, cultures in Agar-Saboureaud-Chloramphenicol were carried out, by touching their body surface on the Petri dish; besides this, these 3 remaining females were also processed for histological sections.

The experimental infection of the isolated fungal species from these ticks was carried out on twenty *R. sanguineus* engorged females, collected on *Canis familiaris*. The fungus was cultured on

Agar-Saboureaud, and, after 8 days, the spores and mycelium were collected and suspended in distilled water at pH 7; the concetration of this suspension was 6×10^7 colonies forming units ml^{-1}. Every female was plunged in this solution for 5 minutes with continuous stirring. After this time, the ticks were stored at 26 °C and 60% RH (experiment one) or at 26 °C and 60% RH (experiment two).

The fungal activity on the engorged females was measured with the following parameters: time of preoviposition (from the experimental infection to the onset of oviposition), time of oviposition (from the start to the end of egg laying), and time of incubation (from the day of laying to the egg hatching); egg mortality during the incubation; percent of the female weight transformed in egg weight (so called fecundity); "lost female weight" or difference between the initial and final female weights plus the egg weight. A sample of 100 eggs were obtained from each female to calculate egg mortality. With the above data, differences were calculated by means of an ANOVA test. When the oviposition (if done) was finished, cultures on Agar-Saboureaud-Chloramphenicol were carried out again by touching the medium with the tick body surface.

RESULTS

Natural infection

The natural pathological picture was observed after fifteen days of permanence in the incubation oven. This syndrome lies in an absolute absence of oviposition as well as the darkness and mummification of the body tick surface. The histological sections make up from these engorged ticks revealed the invasion of the idiosoma by a fungal organism: the Malpighian tubules and also the posterior gut were full of a septate fungus (Pl. 1, Fig. 1). No other body organ was affected by this organism; the absolute absence of the microorganism in the anterior gut, and the localization of the fungus, let us suppose that, in natural conditions, the fungus may penetrate via the anus.

Ten days after (25 days after the collection of the ticks) the presence of a whitish mycelium was observed on the tick body dorsal surface, and, in a lesser degree, on the ventral surface (Pl. 1, Fig. 2). The ticks, already dead at this time, showed a very dark surface under the fungal mycelium. In some areas the mycelium was manually removed, and the cuticle displayed the presence of widely extended necrotic zones.

The histological sections carried out at this time demonstrated the penetration of the fungus across the cuticle (Pl. 2, Fig. 1). The observations performed with SEM let the possibility of an active penetration; this features will be discussed later, as it suggests the postulated penetration mechanism of this fungus. The cultures accomplished from the body tick surface, revealed the presence of *Aspergillus ochraceus*, following the keys of Raper and Fennel (1965).

Experimental infection

The data relative to the oviposition and incubation in the experimental infection with a solution of *A. ochraceus* into engorged *R.sanguineus* ticks, as well as those from the control ticks are included in Table 1.

In the experiment one (26 °C and RH near saturation) the oviposition was observed only in two females. The parameters for the oviposition in these two females really resembled those of control ticks. The remaining 8 ticks never oviposited, developing a pathological picture as explained. Three days after the experimental inoculation, the presence of a whitish mycelium was noted, but the histological sections made from two specimens revealed no fungal invasion in the inner cavity of the tick body. However, two days after, the development of small "teats" on the dorsal tick surface, associated with intensive fungal growth areas was observed in the remaining six

female ticks. In some ticks, also, a small portion of the mycelium was manually removed, and again the necrotic cuticular areas were noted. One day after (six days from the experimental inoculation) the ticks were dead; at this time, new cultures and new histological sections were carried out.

In this experimental inoculation and subsequently starvation at near saturation, the invasion of Malpighian tubules or posterior gut was not observed, but the cuticle and the dermis were plenty of fungal mycelium. The microbiological cultures revealed the presence of only *A. ochraceus;* no other fungal organisms were isolated. The experimental inoculation showed also the sequence of the pathogenic events of the

Table 1. Data from natural and experimentally infected ticks, and control ticks

Female	WFB	PREOV-T	OV-T	WFA	WE	INC-T	MORT	FEC	LW
Experiment one									
1	292	dead						—	—
2	315	dead						—	—
3	488	4	21	123	279	22	0	57.17	86
4	152	dead						—	—
5	208	2	15	46	138	22	6	66.35	24
6	258	dead							
7	304	dead							
8	301	dead							
9	321	dead							
10	298	dead							
Experiment two									
1	207	4	21	45	132	21	2	63.77	30
2	258	3	20	55	178	22	5	68.99	25
3	271	4	21	66	186	21	4	68.63	19
4	258	5	20	58	166	21	5	64.34	34
5	266	4	19	57	169	22	1	63.53	40
6	304	3	21	80	201	23	4	66.12	23
7	248	4	21	66	165	24	2	66.53	17
8	244	3	21	63	142	21	1	58.20	39
9	199	4	20	55	124	20	4	62.31	20
10	260	5	20	66	168	22	6	64.62	26
Control									
1	179	4	13	46	110	21	1	61.45	23
2	204	4	14	48	125	21	4	61.27	31
3	250	4	13	49	150	24	2	60.00	51
4	210	4	15	50	139	21	1	66.19	21
5	205	5	14	55	130	22	2	63.41	20
6	199	4	15	58	124	23	5	62.31	17
7	201	5	15	40	120	25	1	59.70	41
8	209	4	14	41	144	21	2	68.90	24
9	210	5	15	52	128	21	1	60.95	30
10	215	4	14	41	122	20	2	56.74	52

Time expressed in days, weight in mg, mortality and fecundity in %

Abbreviations: WFB, weight of female before the oviposition; PREOV-T, preoviposition timing; OV-T, oviposition timing; WFA, weight of females after the oviposition; WE, weight of eggs; INC-T, incubation timing; MORT, eggs mortality; FEC, fecundity, LW, „lost weight".

fungus on the cuticle. In the first step (Pl. 2, Fig. 2), and after the colonization of near the entire cuticle, some fungal filaments were introduced in the epicuticle; later on, the mycelium grew between the cuticle layers (Pl. 3, Fig. 1), extending also in the dermis-cuticle interleave. At that time, the cuticle was separated from the dermis, and the fungus colonized extensive areas of the dermis; in that point concentrations of mycelium were accumulated; the final step is an absolute cuticular degradation (Pl. 3, Fig. 2). The "teats" mentioned above, well observed in macroscopic and SEM observations (Pl. 4, Fig. 1), belong to these areas of intense fungal penetration and cuticular degradation (Pl. 4, Fig. 2).

In the experiment two (at the same temperature, but with 60% RH) the infected female ticks performed a normal oviposition (see Table 1) without fungal growth on their body surface. The parameters for the oviposition and incubation were alike to those of the control ticks; no significant differences were found with the data of control ticks. The histological sections as well as the SEM observations showed no fungal invasion but the cultures from these ticks revealed the presence of *A. ochraceus* in all the experimentally infected specimens.

DISCUSSION

From these results, two facts of the activity of fungi on *R. sanguineus* can be deduced. First, in natural conditions there are fungal species with pathogenic action on the ticks, whose effects may reduce or control the natural populations of ticks; second, in experimental conditions the fungi may be easily inoculated and the pathological pictures on ticks may be reproduced.

The studies on the activity of fungi on ticks are scarce and, in general lines, have been oriented toward the isolation of the species of microorganism carried by the ticks. Some authors, like Lipa (1971) and Samšiňáková et al. (1974) have mentioned the existence of fungi carried on tick cuticle, but no studies on the pathogenic experimental effect seem to have been performed.

The great pathogenic activity of *A. ochraceus* seems evident on *R. sanguineus* engorged females. Two kinds of penetration can be deduced from the reports above: one by an "anal route" and another one by means of a "cuticular route". Both classes seems to be temperature and RH dependent. In natural conditions, with a low temperature and RH (the last ranging from 40 to 70 %), *A. ochraceus* may parasitize the tick inner body by means of an anal route, since the low climatic parameters make unable the sporulation of the fungus on the tick cuticle; the low temperatures have influence on the female oviposition, delaying the onset of egg laying and allowing the fungus to penetrate and invade the female body. These suppositions were made bearing in mind the data from the invasion pattern in the natural infection by *A. ochraceus*.

However, when the ticks are placed in an environment with near saturation RH (similar to that existing in the incubation oven, in the experiment one), the fungus may sporulate on the body surface. It seems possible that, under these conditions, the expression of some enzymes, breaking the tick cuticle, take place allowing the fungus to penetrate the cuticle. In other words, when environmental climatic factors prevent the fungal sporulation, the fungus must colonize the natural tick apertures in order to invade their body. In this paper, the anal route has been checked, even though other routes seem to be plausible. The possibility of lytic enzyme expression also in the anal invasion route was not confirmed, as in the natural infection, the histological damage can not be attributed to a determined pathogenic mechanism.

Hall and Papierok (1982) have mentioned the possible importance of enzymatic components in the potential entomopathogenic fungi, whose action would be the active penetration across the arthropod cuticle; anyway, these authors do not exclude the possibility of an anal penetration in situations of low RH. The results obtained in our paper, with the comparison of data from the two experiments, seem to confirm this hypothesis.

In terrestrial arthropods, the fungal invasion usually takes place directly across the insect cuticle (Broome et al. 1976) and several papers suggested the inexistence of preferences to the penetration site (Fargues and Vey 1974), Pekinl and Grula 1979) although the head is less frequently affected than the rest of the body (Brobyn and Wilding 1977). However, in *Beauveria bassiana*, the body areas with a major sensitivity are the anus and the head appendages (Delmas 1973). In our results, there is not a determined affinity to any cuticular area, since in both natural and experimental infections, all the tick cuticle was fast and progressively affected by the fungal growth.

The most critical environmental factor influencing fungi is relative humidity. Saturated or near-saturated air or a waterfilm is necessary for spore germination in the vast majority of fungi. Some earlier reports, reviewed by Madelin (1963), have suggested that insects may support their own microclimate and some recent studies have shown that isolated insects can be infected at low humidity (Ferron 1981, Dobersky 1981). In our results, it seems reasonably evident that the RH plays a fundamental role in the colonization rate of *A. ochraceus*, even with only a variation of 30 %. Similar differences were detected by Ramoska (1984) and Simandl (1988) for *Beauveria bassiana* and by Nnakumusana (1985) for *A. parasiticus*.

The high pathogenic activity observed in the laboratory, greater than observed in the ticks collected with the natural infection, may be due to the higher spore concentration in the inoculation vehicle. In general terms, the amount of spores used in experimental inoculations ranges between 10^8 and 10^{12} colonies forming units ml^{-1} of dilution (Ferron 1981, Zimmermann 1981). In this way, *A. ochraceus* may have a greater pathogenic activity in orders of several tens. It is noticeable the absence of symptoms in two from ten inoculated females, whereas the remaining ticks developed an intense parasitation picture. The tick defenses against several parasitoids, fungi and/or bacterial organism are a little studied field, although Whitcomb et al. (1974) have mentioned the encapsulation as a mechanism of evasion against the presence of determined microbial organism, and particularly against the fungi. Anyway, the individual resistance against certain entomopathogenic fungi seems to be a very common feature in the infection pattern.

Truly, more studies are needed to understand the intimate mechanism of infection of *A. ochraceus*, although this species may be considered as a candidate in the integrated pest management against the ticks.

ВЛИЯНИЕ *ASPERGILLUS OCHRACEUS* (FUNGI) НА УПИТАННЫХ САМОК *RHIPICEPHALUS SANGUINEUS* (ACARI: IXODIDAE) В ЕСТЕСТВЕННЫХ И ЭКСПЕРИМЕНТАЛЬНЫХ УСЛОВИЯХ

А. Эстрада-Пеня, Х. Гонзалез и А. Касасолас

Резюме. Грибок *Aspergillus ochraceus* Wilhelm изолированный из клещей *Rhipicephalus sanguineus* (Latreille) зараженных в естественных условиях вызывает заболевание характеризующееся отсутствием отложения яиц, мумификацией и гибелью; из гистологических срезов сделано заключение, что *A. ochraceus* заселяет клещей с анального отверстия, так

как обнаружено поражение дистальной части кишечника и трубочек Мальпиги. Проведено экспериментальное заражение самок клещей; заболевание похоже на то, которое обнаружено в естественных условиях, развивается только тогда, когда экземпляры помещали в относительную влажность близкую насыщению.

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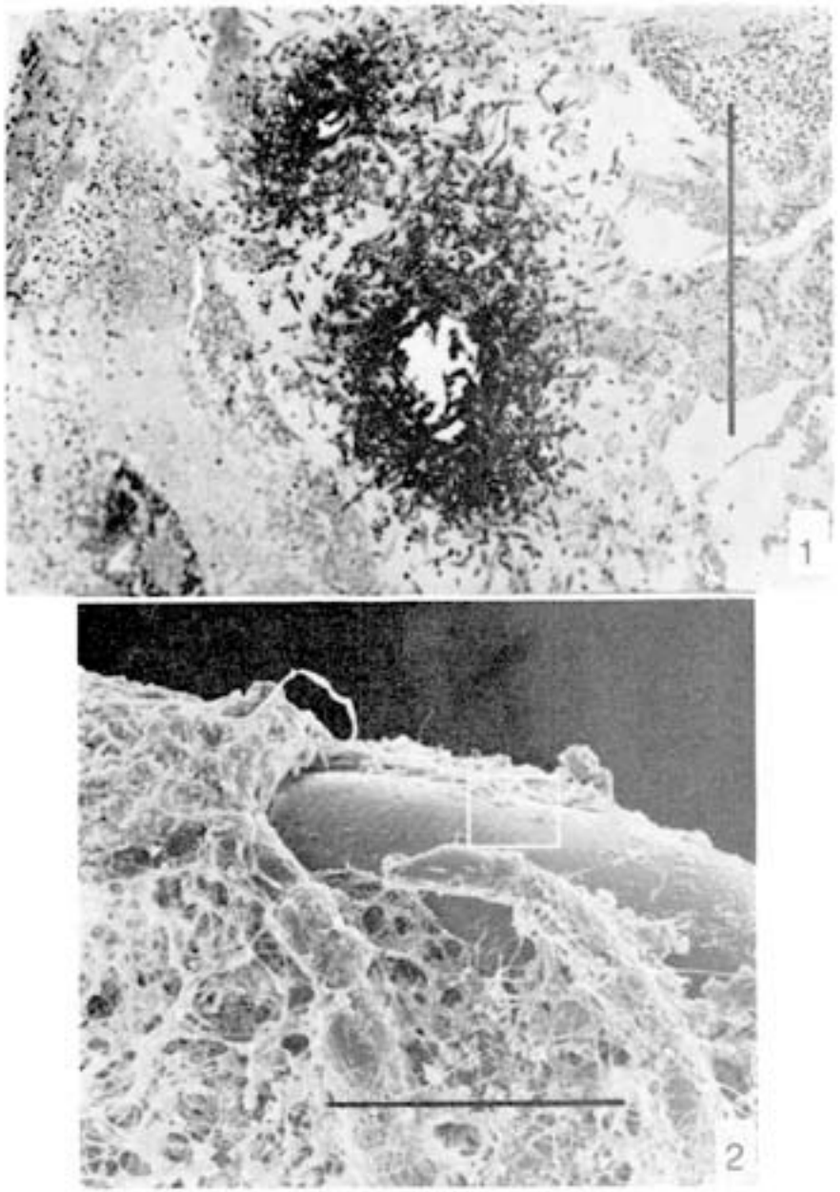

Fig. 1. Histological section from naturally infected *R. sanguineus* females showing the invasion of septate fungus in the tick body cavity. Stain: HE; bar: 250 μm. **Fig. 2.** SEM image of a ventro-lateral view of *R. sanguineus* female naturally infected with *Aspergillus ochraceus*. Bar: 1 mm.

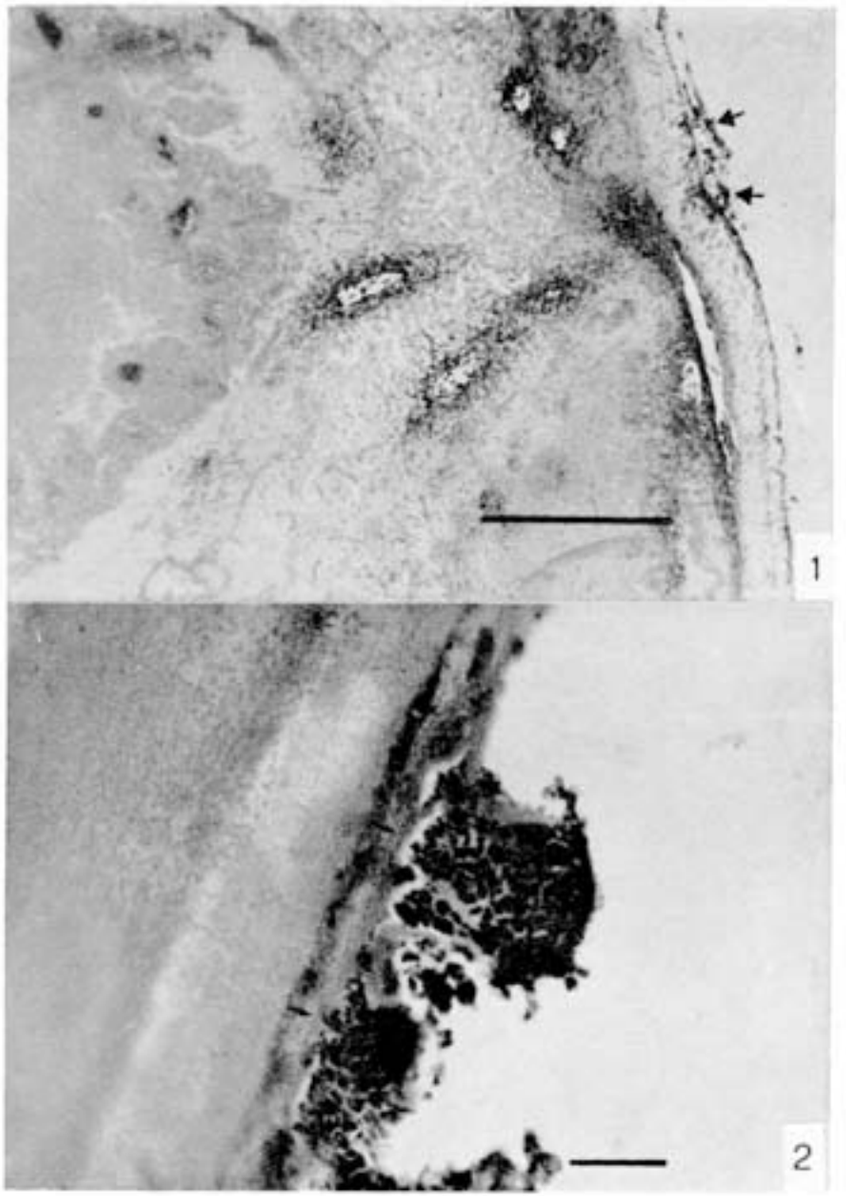

Fig. 1. Histological section (stain: PAS) showing the infiltration of *A. ochraceus* through the tick cuticle (arows). Bar: 500 μm. **Fig. 2.** Histological section of the cuticle of an experimentally infected *R. sanguineus* female. Several portions of fungus are slightly introduced into the tick cuticle (arrows). Stain: PAS, bar: 5 μm.

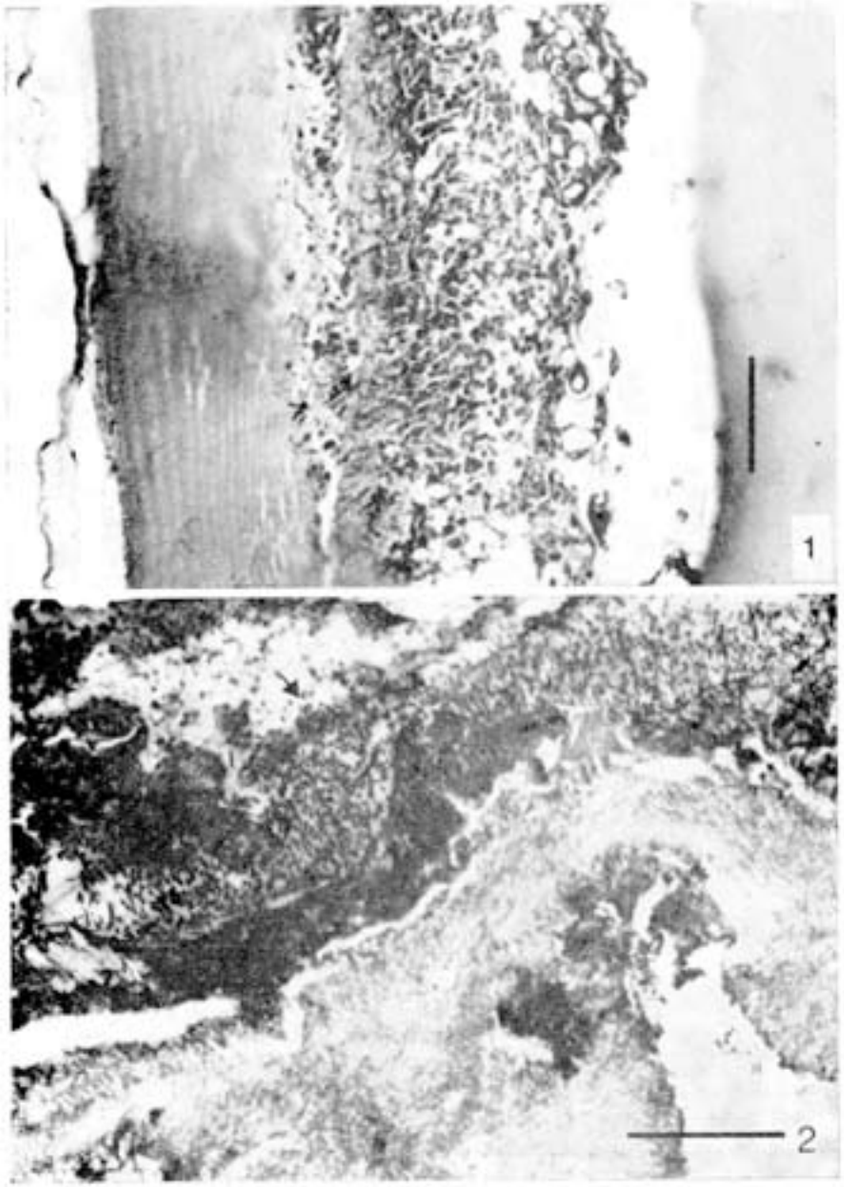

Fig. 1. The fungal mycelium extends into the epicuticle, which is dislodged. Stain: PAS; bar: 10 µm.
Fig. 2. The fungus is growing on the tick surface; there are no traces of cuticle and the fungus invades the tick body (arrow) Stain: PAS; bar: 400 µm.

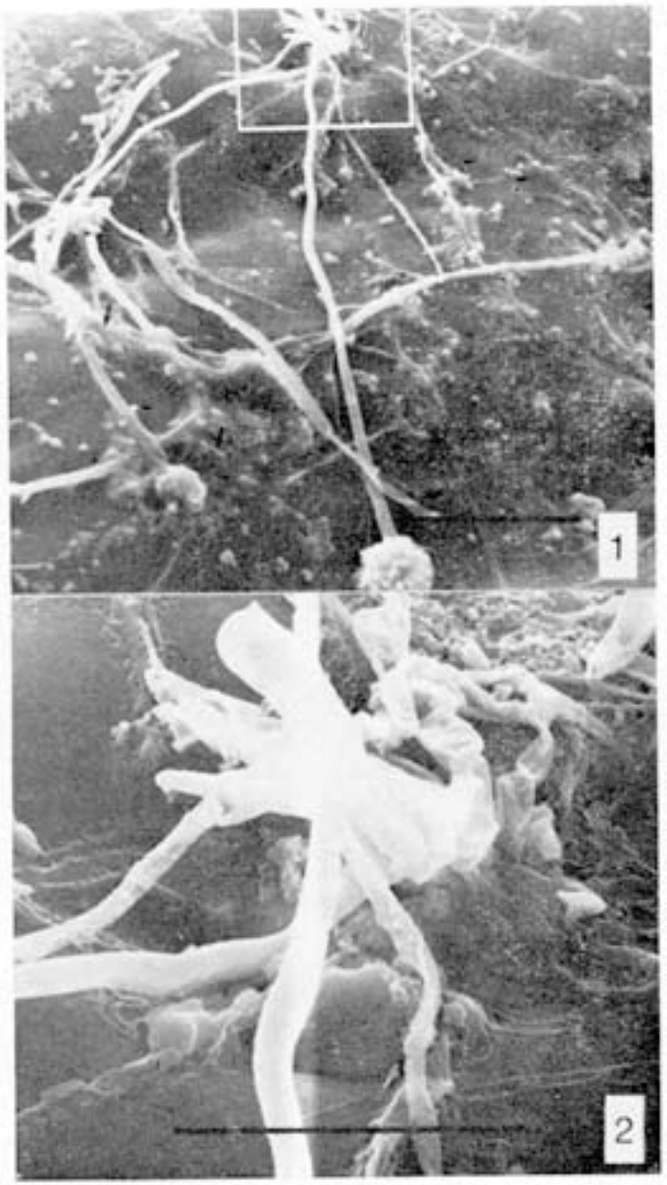

Fig. 1. SEM image of the dorsal tick body in an experimentally infected female. Several areas in the cuticle (arrows) are associated with fungal growth zones and degeneratives. Bar: 100 µm.
Fig. 2. SEM magnification of the little square of the Fig. 1. Cuticular degradation associated to fungal growth in an experimentally infected tick. Bar: 50 µm.