Tick pathogenicity, thermal tolerance and virus infection in *Tolypocladium cylindrosporum*

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Abstract

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Tolypocladium cylindrosporum is a fungus which has been isolated from soil, from asymptomatic plants as an endophyte, and has been shown to be pathogenic to several species of arthropods. The objective of the present work was to study a collection of *T. cylindrosporum* strains in order to evaluate the characteristics of this fungus as a bioacaricide. The pathogenicity of five different strains of *T. cylindrosporum* was tested against two tick species, *Ornithodoros erraticus* and *Ornithodoros moubata*. Both tick species were susceptible to all the fungal strains. Mortality was greater for *O. erraticus*, and differed among the five developmental stages of the ticks tested, and among the fungal treatments. Mean mortality rates were close to 60% for *O. erraticus*, similar to those reported for other entomopathogenic fungi used for this purpose. The responses of eleven different strains of the fungus to 22ºC and 30ºC were also studied. Significant differences in temperature tolerance occurred among the strains, and growth inhibition was observed at 30ºC. Several mycoviruses were found infecting five of the eleven strains. However, no clear relationship was found between the presence of viruses and fungal growth or pathogenicity.

Keywords: Biological control, entomopathogens, mycovirus, endophyte, Argasidae, *Ornithodoros*

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Introduction

The fungal genus *Tolypocladium* is well known because of its biotechnological importance due to the production of cyclosporine (Aarnio & Agathos, 1989). *Tolypocladium cylindrosporum* produces cyclosporine A, a cyclic peptide that exhibits a strong and selective immunosuppressive activity, and became a crucial agent for the development of transplant surgery across histocompatibility barriers (Aarnio & Agathos, 1989). This compound is also used for the treatment of autoimmune and parasitic diseases, as well as in cancer chemotherapy (Reed & Thomas, 2008).

T. cylindrosporum was first reported as a soil-borne species, isolated from a variety of soils and turfs (Gams, 1971), and later it was found causing epizootics in mosquito populations of *Aedes sierrensis*, *Aedes australis*, and *Culex tarsalis* (Weiser & Pillai, 1981; Soares, 1982). Although *T. cylindrosporum* was originally thought to have a life cycle spent in soil and insects, this and other entomopathogenic species have been isolated as fungal endophytes capable of asymptomatically infecting plant tissues (Quesada-Moraga *et al*., 2006a; Vega *et al.,* 2008; Sánchez Márquez *et al.,* 2010).

In addition to cyclosporine A, *T. cylindrosporum* is known to produce high amounts of tolypin and efrapeptins, substances which are toxic to insects (Weiser & Matha, 1988; Bandani *et al.,* 2000). After these discoveries were made, this entomopathogenic fungus has been considered as a potential control agent for several mosquito genera, including *Anopheles* and *Aedes*, that are vectors of pathogens causing important human diseases such as malaria, dengue, and yellow fever (Scholte *et al.,* 2004). *T. cylindrosporum* has a wide host range, in addition to dipterans is pathogenic to insects of other orders such as *Lepidoptera* and *Ephemeroptera*, as well as to crustaceans like *Daphnia carinata* and *Tigriopus* spp. (Lam *et al.,* 1988). Recently a strain of this fungus has been reported as a pathogen of the argasid ticks *Ornithodoros erraticus* and *Ornithodoros moubata*, which have great medical and veterinary importance as vectors of African swine fever virus and several species of human relapsing fever borreliae (Zabalgogeazcoa *et al.,* 2008). *O. erraticus* is distributed in the Mediterranean basin. In southern Europe lives in close association with swine on free range pig farms, hidden in holes and fissures inside and around pig-pens (Oleaga-Pérez *et al*., 1990; Manzano-Román *et al*., 2007). *O. moubata* is distributed throughout South and East Africa and Madagascar, where it colonizes wild and domestic habitats and feeds on warthogs, domestic swine, and humans (Vial, 2009). To date, the primary means of tick control are based on the use of chemical acaricides. These compounds are very effective but have disadvantages such as higher costs, development of resistance, or environmental and food contamination. In order to overcome these problems researchers have evaluated the use of entomopathogenic fungi as bioacaricides. Many strains of *Beauveria bassiana* and *Metarhizium anisopliae* have been tested for this purpose (Kaaya & Hassan, 2000).

Viruses have been identified in many fungal species, including entomopathogens like *T. cylindrosporum, Beauveria bassiana, Metarhizium* spp., and *Paecilomyces* spp. (Inglis & Valadares-Inglis, 1997; Melzer & Bidochka, 1998; Martins *et al.,* 1999; Herrero *et al.,* 2009). A few mycoviruses are known to affect their hosts, causing hypovirulence in plant and insect pathogens, or growth distortion in mushrooms (Melzer & Bidochka, 1998; Ghabrial & Suzuki, 2009). However, unlike animal or plant viruses, most known fungal viruses rarely cause obvious symptoms in their hosts. For example, virus-infected strains of the

entomopathogen *M. anisopliae* do not show a distinct culture phenotype, reduced conidiospore production *in vitro*, or virulence against insects or ticks (Melzer & Bidochka, 1998; Frazzon *et al.,* 2000). Nevertheless, the fact that fungal viruses are common among fungi, that their vertical transmission to spores is often very efficient, and that their infections are very persistent, suggests that under some conditions some viruses could be beneficial to their hosts (Romo *et al*., 2007; Ghabrial & Suzuki, 2008; Herrero *et al*., 2009). For instance, a mutualistic virus which improves the thermal tolerance of a plant-endophyte association has been recently reported (Marquez *et al.,* 2007).

The main purpose of this research was to characterize a collection of *T. cylindrosporum* strains in order to evaluate the potential of this fungus as a bioacaricide. We studied how temperatures higher than optimal affected the growth of eleven *T. cylindrosporum* strains, and tested the pathogenicity of five different isolates against two species of *Ornithodoros* ticks. In addition, we analyzed the presence of mycoviruses in these strains, and studied if the presence of viruses had an effect on fungal pathogenicity or growth at higher temperatures.

Materials and methods

Fungal strains and taxonomy

The *T. cylindrosporum* isolates used in this study (Table 1) were obtained from the collections of the Centraalbureau voor Schimmelcultures (CBS), Colección Española de Cultivos Tipo (CECT), and the Merck, Sharp & Dohme collection (MSD). Strains 3398 and 11 were isolated as fungal endophytes from asymptomatic plants of the grasses *Holcus lanatus* and *Festuca rubra* in natural grasslands of western Spain (Sánchez Márquez *et al.,* 2010). Strain 3398M is a subculture obtained from strain 3398 which spontaneously lost one of the six dsRNA elements infecting the original 3398 strain. This characteristic was maintained in the strain after being stored in the laboratory and subcultured several times.

All isolates were identified according to morphological characteristics (Bissett, 1983), as well as with the nucleotide sequence of their ITS1-5.8SrRNA-ITS2 region. These sequences were obtained using the method described by Sánchez Márquez *et al.* (2007). Sequences of other taxa were retrieved from the EMBL nucleotide database (http://www.ebi.ac.uk/embl/). For sequence-based identification all sequences were aligned using the program ClustalX (Thompson *et al.,* 1997). A species dendrogram was constructed with MEGA 3.1 software using the neighbor-joining method and a Tajima-Nei model to calculate distances (Kumar *et al.,* 2004).

Growth response to temperature

To study isolate responses to a temperature higher than optimal, the eleven strains were cultured in potato dextrose agar (PDA) Petri plates at two different temperatures, 22ºC and 30ºC. Preliminary tests carried with isolates 11 and 3398M showed that in the conditions used for this study, the isolates did not grow at 32ºC. Bissett (1983) reported that 22ºC is the optimal temperature for growth on PDA for all the species of the genus.

A small block of mycelium, measuring about 3 x 3 mm was placed in the center of a 9 cm PDA plate which was sealed with Parafilm. Six plates of each strain were placed in two incubators set at 22º and 30ºC.

The position of the 66 culture plates incubated together at each temperature was randomized. After 21 days of incubation at each temperature, the diameter of the fungal cultures was measured (Zabalgogeazcoa *et al*., 1998). At each temperature, the statistical significance of the differences on the diameters of the fungal strains was analyzed using a one way ANOVA followed by an LSD procedure. Values of $p < 0.05$ were considered significant. To check if there was a relationship between the percentage of growth inhibition observed in each isolate at the higher temperature, and its diameter at 22ºC or 30ºC, simple linear correlation (Pearson correlation) was used. The statistical analyses were done using the Statistica 5.0 (StatSoft, USA) software package.

Pathogenicity on ticks

The *O. erraticus* and *O. moubata* ticks came from two colonies maintained in our laboratory. The colony of *O. erraticus* was established from specimens captured in Salamanca, western Spain, and the colony of *O. moubata* from specimens obtained from the Institute for Animal Health, Pirbright, Surrey, UK. These ticks are fed regularly on rabbits, and kept at 28ºC and 80 % relative humidity (RH).

Five strains of *T. cylindrosporum* were chosen for this assay, 11 and 3398M were obtained as grass endophytes, T4 was isolated from a mosquito host, and T1 and T6 were isolated from soil (Table 1). To obtain conidial suspensions, each strain was grown on PDA Petri plates at room temperature (22–25ºC). Conidia from 3-week old cultures were released from the mycelium with a glass rod, after adding 5 ml of sterile water containing 0.01% Tween 80 to each plate. The conidial suspensions from the plates were collected and centrifuged at 2000 x g for 5 min. The pellets were resuspended in sterile water and the concentration of conidia was estimated with a Bürker chamber (Zabalgogeazcoa *et al.*, 2008). To prepare these suspensions from *T. cylindrosporum*, on average we obtained 1.5×10^7 spores from each gram of fresh mycelium. A 20 day culture on a PDA Petri plate contained about 1.5 g of mycelium.

Five developmental stages from both *Ornithodoros* species were treated with the five strains of *T. cylindrosporum*. Namely, males, females, nymphs-4, nymphs-3 (20 individuals of each stage), and nymphs-2 (50 individuals). Three replicate groups were made from each tick species and developmental stage for testing each fungal strain. Each group of three replicates was inoculated in parallel at the same time. All the tick specimens used were newly moulted and unfed at the start of the pathogenicity experiments. For inoculation each treatment group was placed in a vial containing 2 ml of the corresponding conidial suspension (10^8 conidia/ml in water containing 0.01% Tween 80). After 5 minutes, the excess suspension was removed, and the ticks were incubated for 60 days at 28ºC and 80% RH. Also in parallel, three replicate groups of each tick species and developmental stage were treated with a 0.01% Tween 80 aqueous solution without conidia, and used as a negative control. Mortality was recorded for every group at 3, 7, 14, 28 and 60 days post-inoculation (d.p.i.), and the percentage of cumulative mortality was calculated.

For the statistical analyses, the mortality data was transformed as the arcsin of the square root of the proportion of dead ticks. The normality of the transformed data of each observation period (d.p.i.) was tested using a Kolmogorov-Smirnov test. Differences in mortality between tick species, developmental stages, and fungal treatments, were analysed at each observation period (3, 7, 14, 28, and 60 d.p.i.) using three way ANOVA. The control treatment was not included in the statistical analyses. Comparisons of the total

mortality caused by each fungal strain in each tick species at each observation period were made using LSD values obtained from one way ANOVAs for each observation period with fungal strain as main effect, and pooled values for all developmental stages of the ticks. Values of $p < 0.01$ were considered significant.

Analysis of the presence of double-stranded RNA

The presence of double stranded RNA (dsRNA) molecules of sizes ranging from 1 to 12 kbp was used as a method to diagnose virus infection in isolates. This type of nucleic acid can represent the genome of dsRNA mycoviruses, as well as replicative intermediates of viruses with single-stranded RNA genomes (Morris & Dodds, 1979). However, not all RNA viruses can be detected by dsRNA isolation (de Blas *et al*., 1996), and DNA viruses, recently discovered in fungi (Yu *et al*., 2010), would not be detected with this technique.

To determine if dsRNA might be present in the *T. cylindrosporum* strains, fungal isolates were cultured for three weeks over cellophane disks layered on top of PDA in Petri plates. Approximately 1.5 grams of fresh mycelium were harvested, ground with liquid nitrogen, and dsRNA was extracted by CF-11 cellulose chromatography (Morris & Dodds, 1979). The purified dsRNA samples were treated with DNase I (Ambion TURBO DNA-free), subjected to gel electrophoresis, and visualized after staining with ethidium bromide.

Results

Isolate identification

All eleven strains listed in Table 1 had the characteristic cylindrical conidia of *T. cylindrosporum* (Bissett, 1983). In addition, their ITS1-5.8SrRNA-ITS2 nucleotide sequences, including that of the type strain of *T. cylindrosporum* (strain T2, CBS718.70), were identical. In a dendrogram based on nucleotide sequences, all *T. cylindrosporum* strains grouped together in a clade separated from those of other species of the genus included in this analysis (Figure 1). The *T. inflatum* sequence differed only by four single nucleotide gaps from the *T. cylindrosporum* sequence. Therefore, morphological and molecular characters indicated that all our strains belonged to the same species: *T. cylindrosporum*.

Effect of temperature on growth of fungal strains

The radial growth of all strains was greater at 22ºC than at 30ºC (Table 2). The higher temperature was not favorable for fungal growth, one strain did not grow, and on average, culture diameters at 30ºC were about 59% smaller than those observed at 22ºC (Table 2). ANOVA indicated significant differences in growth among strains at 22ºC ($F_{10,55} = 340.97$; $P<0.01$), as well as at 30ºC ($F_{10,55} = 98.92$; $P<0.01$). At 22ºC strain diameters ranged from 47.3 to 13.9 mm, strains T5 and T7 had culture diameters significantly greater ($p < 0.05$) than the other strains, and strain T1 had the smallest cultures ($p < 0.05$) (Table 2). At 30ºC, strain T1 did not grow, the cultures of strains 3398 and T5 were significantly greater ($p < 0.05$) than those of the remaining strains, and strain T2 had the smallest cultures ($p < 0.05$) (Table 2).

When the percentage of change in diameter at 30ºC was estimated for each strain, a wide range of values, ranging from 100 to 32%, was observed (Table 2). There was a strong correlation between the percentage of change in diameter and the average diameter of each strain at 30ºC ($r = -0.96$, $p < 0.01$), and a

weak correlation at 22ºC, (r = -0.54, not significant). Therefore, the diameter of a strain at 30ºC could be considered as a good indicator of its heat tolerance.

Pathogenicity of fungi to *O. erraticus and O. moubata*

The five strains of *T. cylindrosporum* were pathogenic to both tick species (Figures 2-4). In both *Ornithodoros* species, dead ticks appeared swollen and with red coloration in their cuticle and legs. About seven days after death, fungal mycelium showing morphological characteristics of *T. cylindrosporum* were observed on the surface of dead ticks.

At the end of the experiment (60 d.p.i.), the mean mortality caused by the *Tolypocladium* strains in both tick species was 48.09%. Strain T6 was the least virulent, causing a mortality of 41.36%, significantly lower (LSD; $p<0.05$) than those of strains 3398 (51.73%) and 11 (52.83%). The mortalities caused by strains T1 (47.46 %) and T4 (47.10 %) were not significantly different from those caused by any other strain.

Mortality was significantly greater for *O. erraticus* than for *O. moubata* (Table 3; Figures 2-4). All developmental stages of *O. erraticus* were susceptible to all the strains of *T. cylindrosporum* tested (Figures 2, 4A). In most cases, the mortality of ticks began as early as 3 d.p.i., and after that increased rapidly, slowing down after 7 d.p.i. Little or no additional mortality took place between 28 and 60 d.p.i. The exception to this rule were the males treated with fungal strains T1 and T4, whose mortalities increased constantly throughout the whole period observed. At 60 d.p.i. all fungal strains except T6 induced mean mortality rates between 65% and 77%. In *O. moubata* mortality started at 3 d.p.i. and increased slowly until 60 d.p.i. (Figure 3). Mortality rates at 60 d.p.i. varied considerably among developmental stages and fungal strains, hardly surpassing 50%.

Significant differences in susceptibility to the fungi were observed among developmental stages of both tick species (Figures 2, 3, 4A). Although mortality was greater for *O. erraticus* than for *O. moubata*, the same trend in mortality according to the developmental stage was observed in both tick species (Figure 4A), females and nymphs-2 had the greatest mortality rates. As a result, a significant interaction between tick species and developmental stage was indicated by the ANOVA (Table 3). Similarly, the mortality caused by each fungal strain was greater for *O. erraticus* than for *O. moubata* (Figure 4B), resulting in a significant interaction between tick species and fungal strain. In addition, there was an interaction between developmental stage and fungal strains (Table 3). In *O. erraticus*, at all observation dates there were significant differences among some fungal treatments in the mortality of nymphs-3, and at four dates among nymphs-4 (Figure 2). In this tick species there were significant differences in mortality among some developmental stages at four dates for the treatments with strains T1 and T6, and at 3 dates for strain T4. In *O. moubata*, at all observation dates there were significant differences among some fungal treatments in the mortality of males, at three dates among nymphs-4, and at one date among nymphs-3 and nymphs-2 (Figure 3). There were significant differences among some developmental stages at four dates for treatment T6, at 3 dates for T4, and at one date for T1.

Considering the total mortality caused for all developmental stages, some fungal strains appeared more virulent than others. In *O. erraticus*, strain T6 was significantly less pathogenic than the others at

several dates. In contrast, in *O. moubata* strain T1 was significantly less virulent than the other strains at several d.p.i. (Table 4).

Presence of viruses in *T. cylindrosporum* strains

Mycoviral dsRNA elements were detected in five of the eleven strains analyzed (Figure 5). The electrophoretic patterns observed revealed the existence of molecules of 11 different sizes, ranging from 1.2 to 5.1 kbp (Table 5). The size of these dsRNA molecules are within the range of sizes observed in mycovirus genomes (Ghabrial & Suzuki, 2008). All infected isolates contained at least two different dsRNA molecules. Isolates 3398 and 11, both obtained as endophytes from different grass species (Table 1), had an identical dsRNA electrophoretic pattern.

Strain 3398M had the same pattern as 3398 and 11, except for a 5.1 kbp band, which was not present. Strain 3398M was derived from a culture of strain 3398 which lost the 5.1 kbp dsRNA element. Therefore, this might indicate that strains 11 and 3398 are infected by more than one virus, and one of them was lost in isolate 3398M. Totiviruses have genomes consisting of a single dsRNA molecule of 4.5 to 7.0 kbp (Wickner *et al.*, 2005), the 5.1 kbp dsRNA band lost from isolate 3398 could have been a totivirus genome. The sets of dsRNA molecules detected in T5 and T7 are completely different from those detected in the strains obtained from grass endophytes. Both strains were obtained from mosquito hosts (Table 1).

The presence of mycoviruses did not seem to affect the average diameter of cultures of the isolates at 22 and 30ºC. No statistical significance was found when a Student's t-test ($p < 0.05$) was used to test the difference between the mean diameters of virus-infected and virus-free isolates. However, we observed a significant difference in the growth of strains 3398 and 3398M. These strains are isogenic, but 3398M is not infected by the 5.1 kbp dsRNA. At 22ºC the two strains did not differ significantly in their growth, but at 30ºC, the diameter of strain 3398 was significantly greater ($p < 0.05$) than that of 3398M.

At each developmental stage, the mean tick mortality caused by all virus-infected strains was compared to that caused by virus-free strains using a Student's t-test. No statistically significant differences between means were observed for any developmental stage. These results suggest that mycoviruses do not seem to affect the pathogenic processes of these fungi against *O. erraticus* and *O. moubata.*

Discussion

Fungi have been the main agents used in the early work in biological control developed between the 19th and 20th centuries, this might be because they are the most visible insect pathogens, and most are readily culturable (Lord, 2005). Nevertheless, research on fungi for control of ticks is rather new, because chemical acaricides are generally used for this purpose. However, biological control is becoming an attractive approach for tick management (Kaaya & Hassan, 2000; Fernandes & Bittencourt, 2008).

T. cylindrosporum is an interesting fungus that has not received as much attention as other entomopathogens traditionally used in biological control. Several characteristics of this fungus make it a very good candidate as a potential biological controller. The species has a very good sporulation capability, the strains we analyzed produced about $1.5x10^7$ spores per gram of fresh mycelium. Its spores are very resistant to temperatures under 0ºC; in our laboratory, a suspension of spores kept at -20ºC for one year germinated

efficiently. This characteristic might be useful for long term storage of spores. In addition, spores from *T. cylindrosporum* are capable to persist in the soil for long periods of time (Bissett, 1983). All the *T. cylindrosporum* isolates we included in the study had the same ITS1-5.8SrRNA-ITS2 nucleotide sequence as the type strain of the fungus, and could be distinguished from other related species (Figure 1). Therefore this molecular character seems useful for the identification of individuals of the species.

Another good characteristic of this species for its use in biological control is that several strains showed a relatively low growth inhibition at the higher temperature tested, and these strains might perform better than others in warm environments. To match the thermal tolerance of a prospective fungal strain to the climatic conditions expected at the target environment where it is going to be applied is desirable (Quesada-Moraga *et al.,* 2006b). We found that there is a good correlation between the percentage of growth inhibition observed at 30ºC, and the average diameter of a strain at 30ºC. Therefore, a screening for heat tolerant strains could be done in a collection by means of selecting those strains having the largest diameters at 30ºC.

The results of pathogenicity tests showed that *T. cylindrosporum* has a good potential for the biological control of ticks. The five different strains examined (3398M, 11, T1, T4 and T6) were pathogenic to both argasid species, *O. erraticus* and *O. moubata*, but their virulence varied notably among tick species and developmental stages (Figure 4). With the exception of T6, about one month after inoculation all strains induced mean mortality rates higher than 60% against *O. erraticus*. These rates are similar to those reported in the literature for *B. bassiana* and *M. anisopliae* in ixodid ticks, which range from 20% to 100% (Fernandes *et al.,* 2003; Samish *et al.,* 2004). On the other hand, all the strains of *T. cylindrosporum* were less effective against *O. moubata*. Strain T1 caused the lowest mean mortality rate (18%) in this species. This is curious because strain T1 caused the highest mortality rate in *O. erraticus*, showed the smallest diameter at 22ºC, and was not viable at 30ºC. This fact suggests that there might be no relation between growth rate and pathogenicity of *T. cylindrosporum*, at least in the experimental conditions used.

The presence of dsRNA molecules was observed in 5 of the 11 isolates analysed. These dsRNAs were detected in strains of different geographical origin (Ireland, Spain, and USA), obtained from different substrates (grass leaves and dead mosquitoes). All dsRNA elements observed (Table 5) presented sizes similar to those of mycovirus genomes, which range from 13 kbp in the replicative forms of some members of the Hypoviridae family (Nuss *et al.,* 2005) to 1.4 kbp in the bipartite genomes of the Partitiviridae family (Ghabrial *et al.,* 2005). This incidence of mycoviruses is comparable to those detected in other entomopathogenic fungi such as *Beauveria bassiana, Torrubiella confragosa*, and *Metarhizium anisopliae* (Melzer & Bidochka, 1998; Herrero *et al.,* 2009), and indicates that as in other insect pathogens, the presence of viruses seems to be common in *T. cylindrosporum*.

A clear relationship between the presence of viruses and pathogenicity was not found. This result is in concordance with those obtained in similar experiments performed with strains of *M. anisopliae*, the presence of dsRNA did not affect the virulence of *M. anisopliae* strains against ticks or insects (Frazzon *et al.,* 2000; Giménez-Pecci *et al.,* 2002). In the same way, the presence of viruses did not seem to affect the radial growth of fungal strains grown at 22 or 30ºC. However, it is interesting that strains 3398 and 3398M, which differ in the presence of a 5.1 kbp dsRNA molecule, but presumably not in the fungal genotype,

showed different behaviors at 30ºC (Table 3). It is possible that the presence of the 5.1 kbp dsRNA, which could represent a totivirus genome, might confer some advantages at the higher temperature to the fungus. However, strain 11 has a dsRNA pattern similar to 3398 (Figure 5), and its behavior at 30ºC was like that of strain 3398M.

In conclusion, *T. cylindrosporum* has good characteristics to be considered as a tick control agent. It is known the fungus sporulates abundantly, and its spores are persistent in storage. The present work shows that the species can be easily separated molecularly from other members of the *Tolypocladium* genus, and strains are pathogenic against all stages of *O. erraticus*, and to a less extent *O. moubata*. Although mycoviruses are present, these did not appear to affect pathogenicity.

Given that some strains are functional at higher than optimal temperatures (30ºC) and the host range is known to be wide, this fungus may have the potential to be applied topically as a mycoinsecticide to eliminate at the same time ticks and other arthropod pests in livestock (Polar *et al.*, 2008). However, additional studies are necessary in order to better understand the effects of dsRNA on conidiogenesis, persistence, production of secondary metabolites, and other important traits that could interfere with the performance of virus infected strains as biological control agents.

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Table 1. *Tolypocladium cylindrosporum* strains used in the study. Strain T2 is the type strain of the species (Gams, 1971).

Strain	Source	Country of origin	Original substrate
3398	IRNASA	Spain	*Holcus lanatus*[a]
3398M[b]	IRNASA	Spain	*Holcus lanatus*
11	IRNASA	Spain	*Festuca rubra*[a]
T1	CBS 719.70	Czech Republic	Soil
T2	CBS 718.70	UK	Peat soil
T4	CBS 276.82	New Zealand	Mosquito
T5	CBS 612.80	USA	Mosquito
T6	CBS 550.75	Argentina	Soil
T7	CECT 20414	Ireland	Mosquito
T8	MSD	Unknown	Unknown
T9	MSD	Unknown	Unknown

[a]*Holcus* and *Festuca* are two genera of grasses. [b] Strain 3398M was derived from a subculture of strain 3398 which spontaneously lost a 5.1 kbp dsRNA element.

Table 2. Average diameter of cultures and percentage of change in diameter observed at 30ºC in eleven strains of *Tolypocladium cylindrosporum* after 21 days of growth at 22ºC and 30ºC.

Strain	Diameter 22ºC (mm)	Diameter 30ºC (mm)	Diameter reduction at 30ºC (%)
3398	36.5	24.8	32.1
T5	46.5	23	50.5
T8	45.6	20.8	54.4
T6	39.1	17.4	55.5
T9	44.9	19.5	56.6
11	33.3	14.1	57.7
3398M	37.1	14.9	59.8
T4	43.3	14.1	67.4
T7	47.3	12.6	73.4
T2	39.6	8.7	78.0
T1	13.9	0	100
[a]LSD; d.f.	1.45; 55	1.99; 55	

[a]Least significant difference and degrees of freedom; $p<0.05$

Table 3. F values produced by the analyses of variance of mortality data at each of five periods post inoculation.

Effect	df	Days post inoculation				
		3	7	14	28	60
Tick species (T)	1	550.79**	469.91**	460.25**	297.21**	249.33**
Stage (S)	4	11.50**	13.70**	15.83**	17.58**	20.14**
Fungus (F)	4	8.71**	5.60**	6.51**	3.38*	2.70*
TxS	4	4.28**	10.86**	9.03**	2.95*	1.48 [ns]
TxF	4	5.58**	6.65**	11.01**	9.67**	10.48**
SxF	16	2.19**	2.67**	2.60**	2.41**	2.28**
TxSxF	16	1.16 [ns]	1.78*	2.50**	1.41 [ns]	1.39 [ns]

** $p<0.01$; * $p<0.05$; ns: not significant

Table 4. Transformed values (arcsin[proportion of dead ticks]^{½}) of the mean mortality across all developmental stages observed on each tick species after each observation period. The significance of the differences between the transformed means can be determined using the LSD ($p<0.01$).

Days post inoculation	**Fungal strain**					
	T1	**T4**	**T6**	**3398M**	**11**	**[a]LSD; d.f.**
O. erraticus						
3	0.78	0.62	0.62	0.84	0.72	0.19; 70
7	0.85	0.67	0.68	0.87	0.77	0.20; 70
14	0.98	0.81	0.68	0.92	0.85	0.21; 70
28	1.05	0.89	0.73	0.98	0.91	0.20; 70
60	1.11	0.94	0.79	1.06	0.99	0.21; 70
O. moubata						
3	0.19	0.09	0.04	0.07	0.31	0.17; 70
7	0.22	0.174	0.266	0.174	0.406	0.20; 70
14	0.28	0.266	0.358	0.276	0.52	0.19; 70
28	0.35	0.45	0.49	0.47	0.58	0.20; 70
60	0.41	0.55	0.60	0.55	0.65	0.21; 70

[a]Least significant difference and degrees of freedom; $p<0.01$

Table 5. *T. cylindrosporum* isolates where mycovirus-like dsRNA elements where detected. Size estimates were determined using agarose gel electrophoresis and dsDNA size markers.

Strain	**dsRNA elements observed**	
	Number	**Size (kbp)**
3398M	3	3.1 - 3.2 - 3.4 - 3.7 - 4.2
3398	4	3.1 - 3.2 - 3.4 - 3.7 - 4.2 - 5.1
11	4	3.1 - 3.2 - 3.4 - 3.7 - 4.2- 5.1
T5	2	1.8 - 2.2
T7	6	1.2 - 1.3 - 1.5 - 2.2 - 2.3 - 2.6

Figure 1. Dendrogram built using sequences from the ITS1-5.8SrRNA-ITS2 region of *T. cylindrosporum* isolates included in the study. Other reference sequences were obtained from the EMBL nucleotide database; numbers between parentheses indicate their accession numbers. The dendrogram was constructed using the neighbor-joining method and a Tajima-Nei model to calculate distances. The type strain of *T. cylindrosporum* (T2) is indicated by an asterisk.

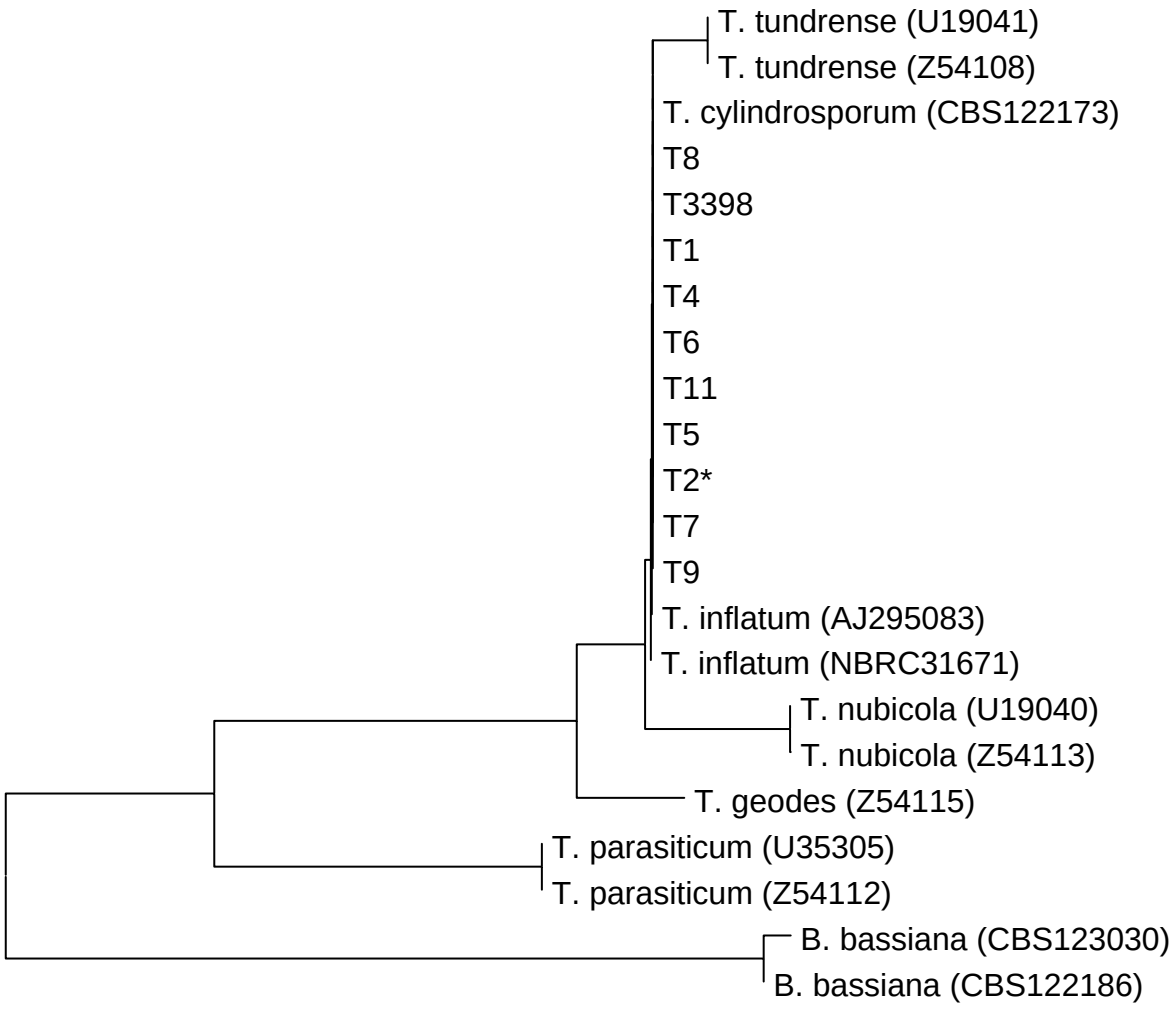


Figure 2. Cumulative mortality (%) in males, females, nymphs 4 (N4), nymphs 3 (N3), and nymphs 2 (N2) of *O. erraticus* at 3, 7, 14, 28, and 60 days post inoculation with *T. cylindrosporum* strains T1, T4, T6, 3398M and 11. Control: tick specimens treated with 0.01% Tween 80 without fungal conidia.

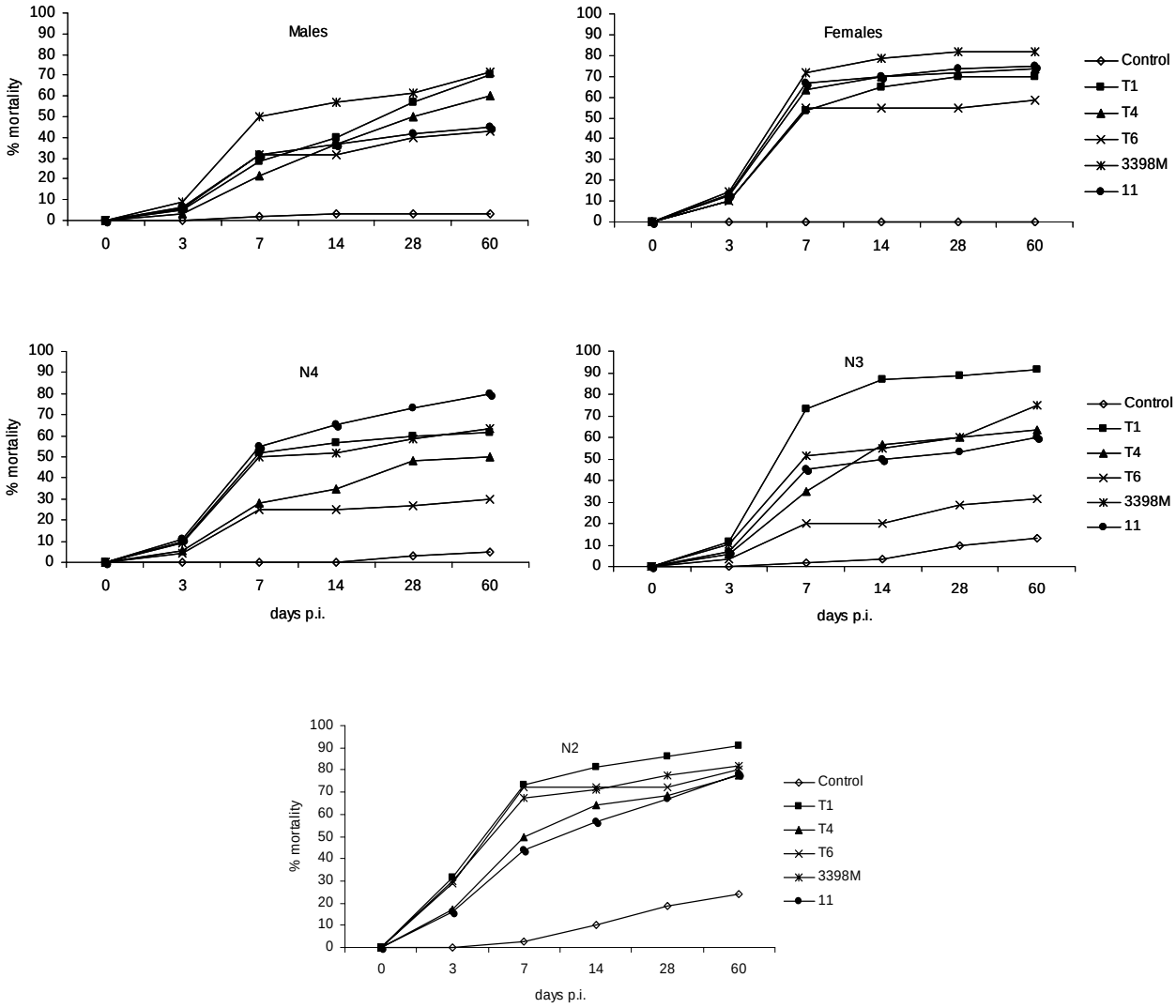


Figure 3. Cumulative mortality (%) in males, females, nymphs 4 (N4), nymphs 3 (N3), and nymphs 2 (N2) of *O. moubata* at 3, 7, 14, 28 and 60 days postinoculation with *T. cylindrosporum* strains T1, T4, T6, 3398M, and 11. Control: tick specimens treated with 0.01% Tween 80 without fungal conidia.

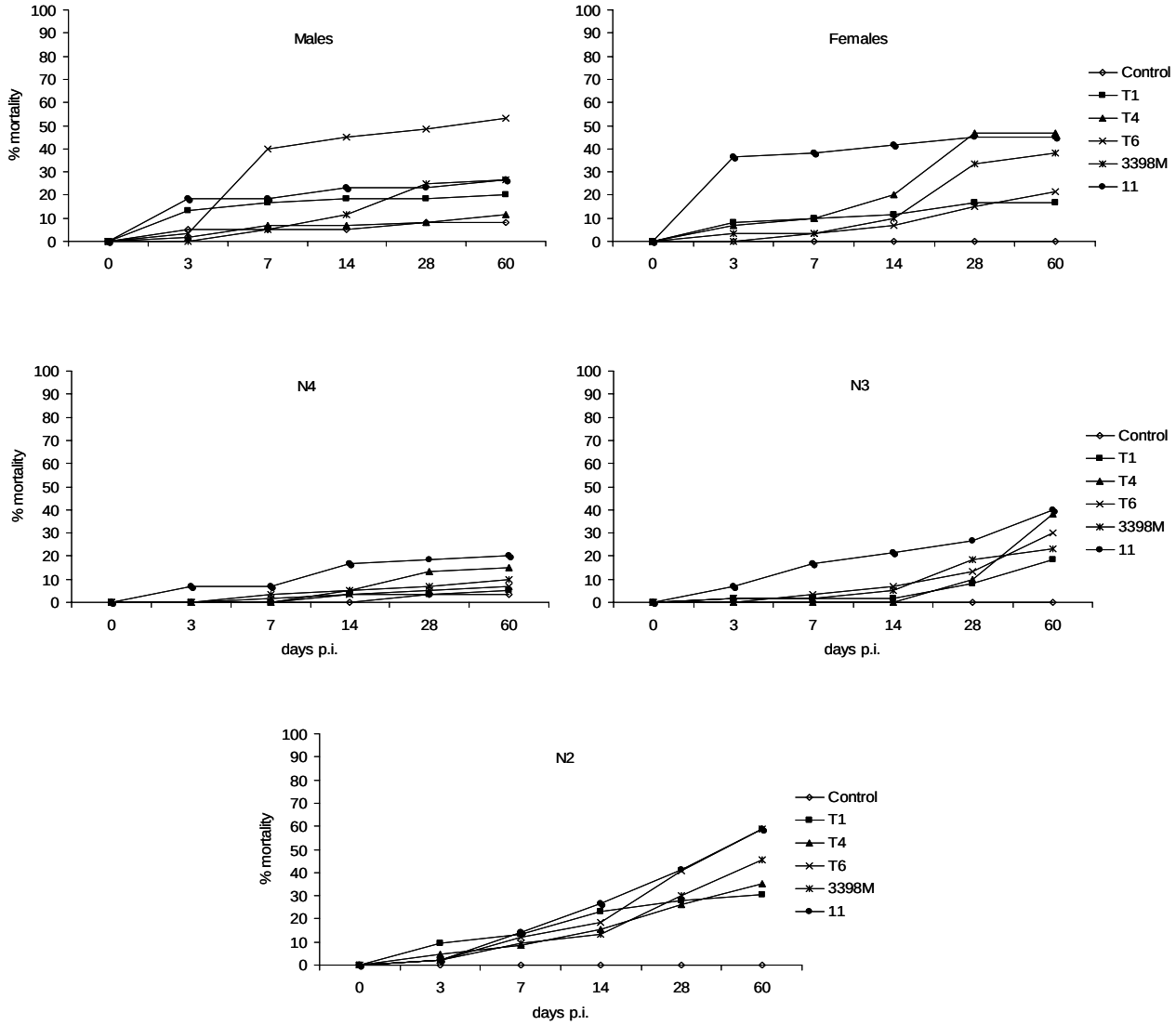
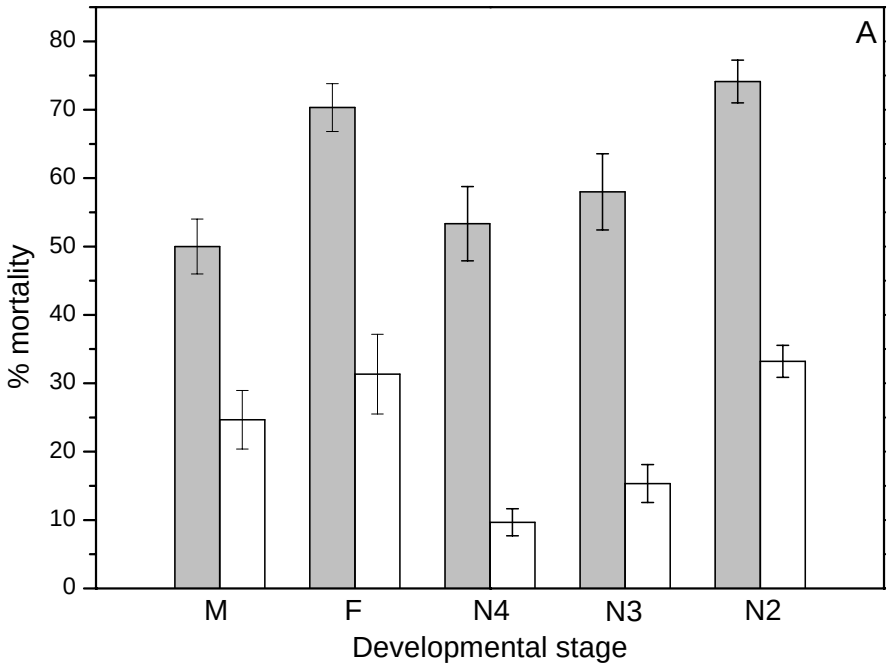


Figure 4. A. Mortality (mean ± standard error) observed on each developmental stage of *O. erraticus* (grey bars) and *O. moubata* (white) at 28 days post inoculation. B. Mortality (mean ± standard error) caused by each fungal strain in each tick species in the same time period. M, males; F, females; N4, nymphs-4; N3, nymphs-3; N2, nymphs-2.



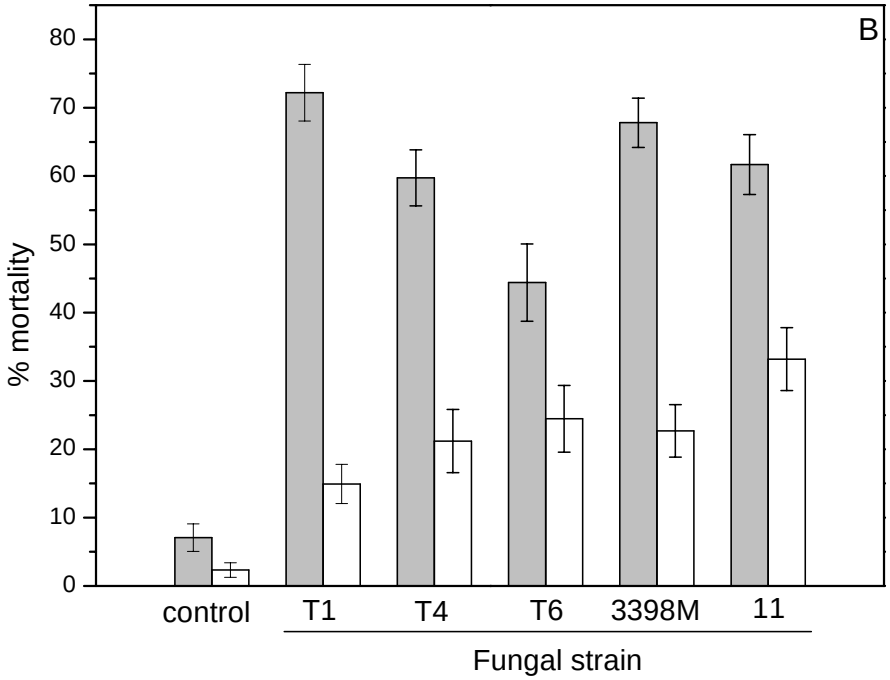


Figure 5. Electrophoretic banding patterns of dsRNA elements present in eleven *Tolypocladium cylindrosporum* isolates. Lanes M1 and M2 contain size markers and numbers on left and right indicate size in kbp.