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SHORT NOTE

Antifungal Effect of Silver Nanoparticles on *Rickia wasmannii* Cavara (Ascomycota: Laboulbeniales) Infecting *Myrmica scabrinodis* Nylander (Formicidae) Ants

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Abstract

Rickia wasmannii Cavara (Ascomycota: Laboulbeniales) is an ectoparasitic fungus infecting *Myrmica* ants. Ant-parasitic Laboulbeniales and their interactions with the hosts have been in the focus of several studies. To assess the effects of these fungi, comparison of infected and uninfected or completely treated ants are needed. So far, treating Laboulbeniales infection was only achieved with cockroaches, but not with ants. We present a simple, yet relatively long, AgNP topical treatment that reduces or eliminates *Rickia* infection from *Myrmica scabrinodis* ants without affecting their lifespan. We discuss the possibilities of the proposed treatment in the light of the biology of *Rickia*.

Laboulbeniales are obligate ectoparasites of Arthropods with a peculiar biology (Weir & Beakes, 1995; Haelewaters et al., 2015). Insect-Laboulbeniales relationships have several interesting but rather understudied aspects. Recent results showed that ant parasitic Laboulbeniales fungi can influence the physiology and behavior of their hosts (Csata et al., 2014; Báthori et al., 2015a; Konrad et al., 2015), although penetration of the host cuticle was not observed (Tragust et al., 2016).

Ants are optimal model organisms for studying the effects of insect parasitic microorganisms, as there is usually a high number of infected (often heavily infected) hosts available (and easily maintained) and methods for studying survival, overall health, behavioral changes, etc. have been established. Furthermore, ant-associated Laboulbeniales are also relatively easy to count using a binocular microscope (Csata et al., 2014; Báthori et al., 2015a; Markó et al., 2016).

The importance of uninfected controls in such survival/behavior oriented studies is beyond question. This may constitute a problem as infected and uninfected specimens are not necessarily available from the same colony or even the same location (Báthori et al., 2015a). Thus, an alternative approach may be treating the hosts with an antifungal compound and thereby making comparisons between infected and uninfected insects possible. In the case of ants, it could give the possibility to have infected and uninfected individuals from the same colonies (e.g. sister or closely related workers).

Control of a Laboulbenialean genus, *Herpomyces* Thaxt. on cockroaches was achieved using the fungicide benomyl {methyl N-[1-(butylcarbamoyl)benzimidazol-2-yl]carbamate} (Gemeno et al., 2004). Recently, this compound was found to be ineffective in treating *Myrmica rubra* L. ant queens infected with *R. wasmannii* and the antifungal treatment also negatively affected the host (Pech & Heneberg, 2015). The main target of benomyl is the polymerization of tubulin, resulting in the inhibition of cell proliferation and division (Wride et al., 2014). *Rickia* thalli are attached firmly to the

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host, presumably even if the cells of the thallus are dead, *e.g.* we observed *Rickia* and other Laboulbeniales thalli on decades-old museum specimens (Báthori et al., 2014, 2015b) and the fungus does not produce hyphae at all. Thus, antimycotics inhibiting cell proliferation may not be suitable to decrease the number of mature *Rickia* thalli on infected hosts at all. Additionally, the lack of structures penetrating the host in the case of ant-infecting Laboulbeniales fungi (Tragust et al., 2016) also probably accounts for the ineffectiveness of oral treatments administered to the host insect. On the other hand, antimicrobial compounds with cytotoxic effects may be more effective against *Rickia*. Silver nanoparticles (AgNPs) can interact with the cell surface causing ruptures in the cell membrane of eukaryotic cells, they are known to generate oxidative stress and disturb metabolic pathways and are also promising anti-biofilm agents (You et al., 2012; Cavalieri et al., 2014).

Our aim was therefore to test whether AgNPs are able to exterminate *R. wasmannii* efficiently from its host ants.

Myrmica scabrinodis Nylander ants infected with *R. wasmannii* were obtained from Rakaca (48°27'N, 20°47'E, NE Hungary) and maintained as a colony in laboratory. Workers of age groups 3 and 4 (see: Cammaerts-Tricot, 1974) were selected to avoid variation in expected lifespan. Treated (19 specimens) and water-treated control workers (18 specimens) were kept in individual plastic containers. Conditions are described in Báthori et al. (2015a). Thalli of *R. wasmannii* were counted using a Leica binocular microscope, using 20x to 100x magnification with hosts handled with a delicate pincer. Only thalli on the dorsal part of the heads of specimens were counted (antennae and mouthparts excluded; the mean number of thalli counted on a single ant was 26.6, SD= 12.98, for 37 specimens). Thalli were counted before the experiment and every 7 days during the four-week-long treatment. Ants were monitored and thalli counted for 2 more weeks after treatment. Thalli on dead specimens were not counted.

Treatment involved submerging ants into 20, 10 or 5 ppm AgNP (Pérez et al., 2008) solution (Bay Nano, Miskolc) for 5 seconds using a pincer on a daily basis. Specimens of the control group were treated the same way with distilled water.

Survival of AgNP-treated and water-treated hosts was compared by Kaplan-Meier survival analysis and its log-rank test using the MedCalc Software. As *Rickia* is not a mycelial fungus (Weir & Beakes, 1995), we could also assess the survival of the thalli (with a weekly resolution) using the same method (considering thalli on dead hosts as censored). Student's 2-sample T-test (or Welch's test depending on the equality of variance) was applied to test the significances of the differences in the proportional decrease of *Rickia* thalli on the two groups of ants.

During our initial trial experiments, we determined the optimal concentration of AgNPs to treat *M. scabrinodis* workers with *R. wasmannii* infection. 20 to 10 ppm AgNPs proved to be unsuitable for the treatment of ants as these concentrations caused argyria and death of the host. The 5 ppm concentration showed no such effects: compared to the control (water-treated) group, the mortality of the ants was not different (log-rank test for Kaplan-Meier survival analysis, $p<0.6$), with only 4-4 hosts surviving the whole 6-week-long period of observations. Thus, we concluded that the AgNP treatment did not affect the lifespan of workers during the period of experiments. The low survival rate in both groups is probably associated with the negative effects of social isolation (Koto et al., 2015). It is noted that isolation was only necessary for tracking the changes on the number of thalli on each specimens. Treatment to ants may be applied without isolation in further experiments.

The effect of the 4-week-long daily AgNP treatment resulted in the decrease in the number of thalli on the ants significantly more effectively than water treatment (number of thalli on ants in the group treated only with water also decreased in most cases). Significance was $p<0.05$ for the first week of treatment and $p<0.01$ for the second to fourth week. After 4 weeks of AgNP treatment, the mean decrease of thalli number was 82.99 % (SD=18.13) and all thalli disappeared from 4 of the surviving 10 AgNP treated ants (40 %) (Fig 1c). None of the control water-treated ants lost more than 75 % of thalli during this period and the mean decrease was 39.68 % (SD=26.62) for the surviving 9 control ants. Post treatment observation after 2 weeks also confirmed the significant ($p<0.05$ and $p<0.1$) difference between the two groups' loss of thalli, although the number of surviving ants was low. Fig 1a summarizes the observations on the decrease in thalli number on the two ant groups. The results of the analysis and the comparison of the two survival curves using Kaplan-Meier survival analysis for the thalli of *Rickia* confirms that the treatment was effective with high statistical support ($p<0.0001$), backed by a high number (n=983) of tracked thalli (Fig 1b).

Our results offer a possible treatment of Laboulbeniales infection of ants that could be exploited in the study their host-parasite interactions. Infected and post-treatment uninfected ants from the same colony could be used for experiments, allowing better comparisons to assess the effect of *Rickia* on behavior (especially social behavior between infected/uninfected sister ants), mortality, etc. As far as we know, our method is the first topical treatment of an insect for a fungal parasite and also the first effective treatment for Laboulbeniales on ants. We also tried to apply the treatment to *Blatta lateralis* L. cockroaches infected with *Herpomyces stylopygae* Speg., but the number of thalli quickly increased in both the treated and control groups (results not shown). This may be related to the fact that *Hesperomyces* spp. penetrate the host's cuticle (Richards & Smith, 1956), while *R. wasmannii* was recently shown to be only attached to the surface of the ant cuticle (Tragust et al., 2016), but this question requires further study. Studying other host-Laboulbeniales pairs was not possible due to the rarity of these fungi and the usually small number of thalli on other insects (e.g. beetles). Thus, topical treatment is so far only effective for ants.

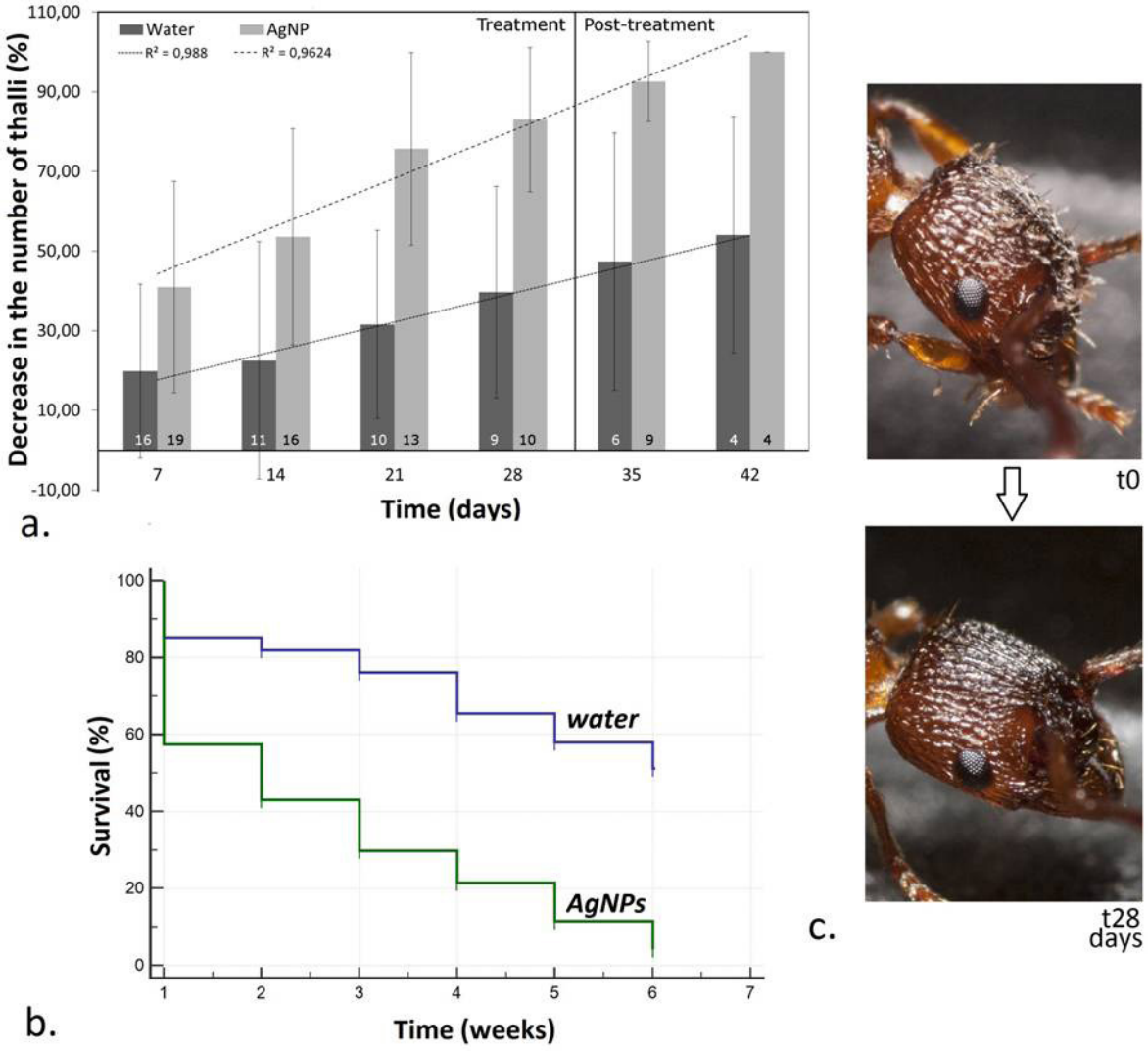

Fig 1a. Decrease in the number of thalli (in percentage) on AgNP-treated and control (water-treated) ants during the 4 weeks of treatment and the 2 weeks of post-treatment tracking. Number of hosts in each group on bars, error bars show SD. b. Survival of thalli in the two groups. c. Example of a completely treated host ant on 1st and 28th day of AgNP treatment.

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