Notas Científicas

Susceptibility of *Atta sexdens* worker ants treated with the immunosuppressant Sandimmun Neoral to *Metarhizium anisopliae*

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Abstract – The objective of this work was to evaluate if the immunosuppressant Sandimmun Neoral enhances the activity of *Metarhizium anisopliae* against the leaf-cutting ant *Atta sexdens*. The vulnerability to the pathogen was measured by comparing the mortality rate of worker ants subjected to the following treatments: immunosuppressant+control, immunosuppressant+*M. anisopliae*, excipient+control, excipient+*M. anisopliae*, control+*M. anisopliae*, and control. Worker ants treated with immunosuppressant+*M. anisopliae* showed the highest mortality rate in comparison with those subjected to all other treatments. The use of the immunosuppressant together with entomopathogenic fungus controlled leaf-cutting ants in laboratory conditions.

Index terms: biological control, Formicidae, immunity, immunosuppression.

Suscetibilidade de operárias de *Atta sexdens* tratadas com o imunossupressor Sandimmun Neoral a *Metarhizium anisopliae*

Resumo – O objetivo deste trabalho foi avaliar se o imunossupressor Sandimmun Neoral favorece a atividade de *Metarhizium anisopliae* contra a formiga cortadeira *Atta sexdens*. A vulnerabilidade ao patógeno foi medida ao se comparar a mortalidade de operárias expostas aos seguintes tratamentos: imunossupressor+controle, imunossupressor+*M. anisopliae*, excipiente+controle, excipiente+*M. anisopliae*, controle+*M. anisopliae* e controle. Operárias tratadas com imunossupressor+*M. anisopliae* apresentaram a maior taxa de mortalidade em comparação às submetidas a todos os outros tratamentos. O uso do imunossupressor juntamente com fungo entomopatogênico controlou as formigas cortadeiras em condições de laboratório.

Termos para indexação: controle biológico, Formicidae, imunidade, imunossupressão.

Large quantities of entomopathogenic fungi, such as *Metarhizium anisopliae* (Metchnikoff) Sorokin and *Beauveria bassiana* (Bals.-Criv.) Vuill., have been found in close proximity to nests of leaf-cutting ants in tropical forests. However, worker ants are usually uninfected by these fungi in the field (Loreto & Hughes, 2016), which can be explained by behavioral and physiological mechanisms and by the complex association of these ants with actinomycetes (Mattoso et al., 2012; Souza et al., 2013).

In this scenario, research on the immunity of leaf-cutting ants could help elucidate their resistance mechanisms against pathogens and, consequently, in the development of new control strategies. This is especially interesting since the use of the main active ingredients for leaf-cutting ant control (sulfluramid and fipronil) is not recommended, being only possible due to the derogation approved by the Forest Stewardship Council, an important voluntary certification system of sustainable forest management (Zanuncio et al., 2016).

One strategy that could be adopted to improve the efficacy of entomopathogenic fungi in the control of leaf-cutting ants is the concomitant use of immunosuppressant agents, i.e., substances that allow reducing the activation or efficacy of the immune system. Among these, stands out cyclosporine A, a cyclic, hydrophobic peptide produced by several fungal species of the genera *Beauveria*, *Verticillium*, and *Tolypocladium*. Previous studies have shown that cyclosporine A is able to decrease the resistance of *Galleria mellonella* larvae to pathogenic bacteria, for example (Fiolka, 2008). Sandimmun Neoral, an immunosuppressive drug used in organ transplant patients, is one of the main formulations of cyclosporine A (Colombo & Egan, 2010).

The objective of this work was to evaluate if the immunosuppressant Sandimmun Neoral enhances the activity of *M. anisopliae* against the leaf-cutting ant *Atta sexdens*.

Worker ants from a three-year-old *A. sexdens* colony were collected in April 2011 in the municipality of Gurupi, in the state of Tocantins, Brazil (11º43'45"S, 49º04'07"W), and were kept in a climate room (25±2°C, 12:12 light-dark cycle, and 75±5% relative humidity). The colony was fed on yellow bell [*Tecoma stans* (L.) Griseb.] (Bignoniaceae) leaves and flowers, mango (*Mangifera indica* L.) (Anacardiaceae) leaves, *Citrus* spp. (Rutaceae) fruits, and oat (*Avena sativa* L.) flakes. At the time of the experiments, the colony had a population of approximately 10,000 worker ants.

Metarhizium anisopliae was isolated from *A. sexdens* forager. The fungus was identified by Dr. André Rodrigues from Universidade Estadual Paulista Júlio de Mesquita Filho and was kept at the center for microbial resources (CRM) of the university, where it received the identification code LESF 206 – CRM 530. On previous bioassays, it was found that the inoculation of 1 μL suspension containing 1×10^7 conidia mL^{-1} caused the death of approximately 30% worker ants within ten days. This conidial suspension was prepared in a solution of 0.05% Tween 80 as a surfactant, using colonies that had sporulated in < three days and that had a spore viability of >90%. Conidia were quantified by counting in a Neubauer chamber, and the suspension was diluted until the desired concentration. The pharmaceutical formulation Sandimmun Neoral, containing 100 mg mL^{-1} cyclosporine A, was used. The excipient was prepared in 12.5% ethanol dissolved in corn (*Zea mays* L.) oil, one of the substances found in the microemulsion excipient (Strickley, 2004). All *A. sexdens* medium worker ants were removed from the interior of the colony with a forceps to reduce age-related variations. First, 1 μL of the immunosuppressant was applied between the mandibles of each worker ant; then, 1 hour later, 1 μL of a conidial suspension of *M. anisopliae* (1×10^7 conidia mL^{-1}) was applied on the abdomen surface. For the immunosuppressant treatment, two controls were used, consisting of ants treated with: an excipient composed of a mixture of corn oil and ethanol; and sterile distilled water. For the fungal treatment, the control consisted of ants that received the application of autoclaved 0.05% Tween 80 in distilled water, in order to verify the effects of Tween solution.

The evaluated treatments were: immunosuppressant +*M. anisopliae*; immunosuppressant+0.05% Tween 80; corn oil/ethanol+*M. anisopliae*; corn oil/ethanol+0.05% Tween 80; water+*M. anisopliae*; and water only. For each treatment, 15 worker ants were used, totaling 90 for the entire experiment.

After treatments, worker ants were individually transferred to 9-cm diameter Petri dishes and supplied with honey and water under controlled conditions (26±1.70°C, 10% relative humidity, and a 12-hour photoperiod). Surviving ants were examined ten days after the beginning of the experiment.

To confirm the mortality caused by the fungus, dead worker ants were disinfected in a series of solutions – 70% ethanol, 4% sodium hypochlorite, and sterile distilled water –, for 10 s in each one, then dried on filter paper in a laminar flow hood, placed in centrifuge tubes containing a moist sterile cotton, and kept in an incubator at 28±1°C until fungal growth was observed (Ribeiro et al., 2012).

The survival curves were compared between multiple groups using the Kaplan-Meier method and between each group with the nonparametric log-rank test, at 5% probability, to verify the differences between two treatments. All analyses were performed using the Statistica software, version 7.0 (Dell Statistica, Tulsa, OK, USA).

The treatment with the immunosuppressant enhanced the pathogenic activity of *M. anisopliae* on the worker ants (Figure 1), leading to a mortality rate of 80% within six days; this was observed when using a spore load that would otherwise result in low mortality in the absence of the immunosuppressant. According to Fiolka (2008), cyclosporine A is effective in the initial phase of the insect immune response but loses its immunosuppressive effect after a certain period.

The mortality rate of ants injected with water and the fungus reached 30% by the tenth day of the experiment. The survival curves of the groups treated with immunosuppressant+*M. anisopliae* and control+*M. anisopliae* were significantly different by the log-rank test (p<0.0001) (Table 1).

These results show that it is essential to understand the potential of these substances to suppress the immune responses of leaf-cutting ants, in order to devise strategies to make ants more susceptible to fungi that normally do not cause epizootic events. *Aspergillus ochraceus* is an example of a fungus with widespread distribution that is found in 40% of *Atta bisphaerica* forager worker ants. Although *A. ochraceus* has been reported to be highly virulent under laboratory conditions, there is still no evidence that it can control ant populations in the field (Ribeiro et al., 2012). In combination with an immunosuppressant agent, several of these opportunistic species could become natural control agents of leaf-cutting ants.

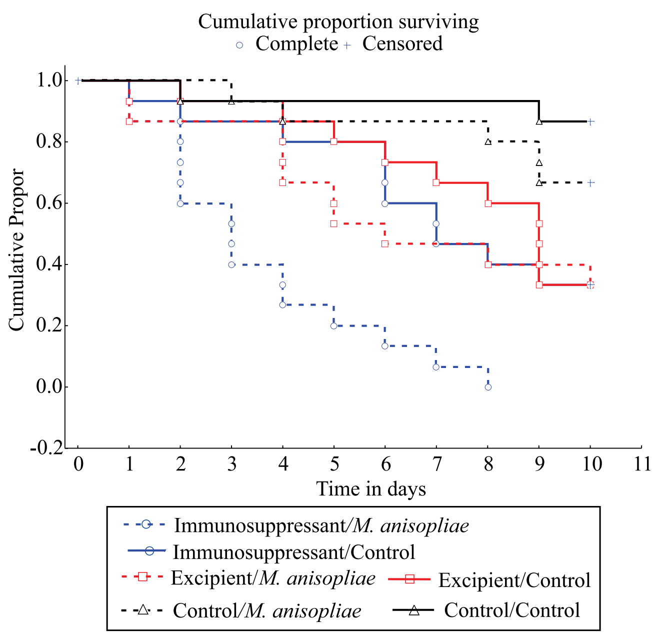

Figure 1. Kaplan-Meier survival curves of *Atta sexdens* worker ants subjected to different treatments

Table 1. P-values for pairwise comparisons with the log-rank test between the survival curves of *Atta sexdens* worker ants subjected to different treatments.

	CT	CT+Ma	EX+CT	EX+Ma	IM+CT	IM+Ma
CT	-	0.21	0.004	0.004	0.003	0.00000
CT+Ma	0.21	-	0.08	0.05	0.05	0.00002
EX+CT	0.004	0.08	-	0.58	0.66	0.0003
EX+Ma	0.004	0.05	0.58	-	0.78	0.006
IM+CT	0.003	0.05	0.66	0.78	-	0.002
IM+Ma	0.00000	0.00002	0.0003	0.006	0.002	-

(1)CT, control; Ma, *Metarhizium anisopliae*; EX, excipient; IM, immunosuppressant.

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