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Potential of nanostructured alumina for leaf-cutting ants *Acromyrmex lobicornis* (Hymenoptera: Formicidae) management

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Abstract The present study assessed the contact toxicity of nanostructured alumina (NSA) and compared it to that of diatomaceous earth (DE) on workers of *Acromyrmex lobicornis.* Laboratory and field bioassays were also conducted to assess whether ants avoid NSA particles. Nanostructured alumina was more toxic to ants than DE (LC_{50} for NSA = 0.14 mg/g; CI 0.12–0.17; LC_{50} for DE = 0.36 mg/g; CI 0.25–0.77). The laboratory bioassays results showed that ants were not repelled by NSA. The average repellence percentage observed in the Petri dish bioassay was 0.66 ± 3.1. No repellence was observed in field tests either, given that ants collected oat flakes treated with NSA (12.7 ± 1.2 oat flakes) in similar amounts to control oats (13.9 ± 1.6 oat flakes) after 2 h. Scanning electron microscope pictures showed that NSA was more effective than DE in attaching to the cuticle of exposed insects.

This study reports for the first time the toxicity of NSA on leaf-cutting ants and shows that it has greater efficacy than DE in killing *A. lobicornis* at all the concentrations tested. Thus, NSA shows potential to be used as a dust insecticide that could be applied directly at the nest to induce mortality of worker ants. Further studies should address the effect of NSA on the symbiotic fungus, as well as its potential as carrier for other insecticides or fungicides.

Key words inert dust, nanoinsecticide, pest management.

INTRODUCTION

Leaf-cutting ants *Acromyrmex* spp. and *Atta* spp. are amongst the most important agricultural and forest pests in South America, reducing wood production as well as affecting tree establishment (Folgarait *et al.* 1996; Della Lucia *et al.* 2014; Zanetti *et al.* 2014). These genera are grouped in the tribe Attini of Nearctic and Neotropical distribution, limited to the American continent between latitudes of 40° N and 44° S (Farji-Brener 1992), and are the only ants that show a forced dependence on fungal symbionts as a food source (Farji-Brener & Ruggiero 1994; Currie 2001). The association with a fungus enables these ants to exploit a wide range of plant species, causing direct losses to many agricultural crops, notably cacao, cassava, citrus, coffee, cotton and maize (Cherrett 1986; Hölldobler & Wilson 1990).

Acromyrmex lobicornis extends from the north of Argentina to 44° S in Patagonia (Franzel & Farji-Brener 2000), and it is the only species in the genus *Acromyrmex* that harvests both mono and dicotyledons (Pilati *et al.* 1997).This species is polyphagous, given the wide range of species that can be used to grow the symbiotic fungus and opportunistic given that it can exploit food resources when available and adapt its activity patterns to marked seasonal conditions (Franzel & Farji-Brener 2000). These characteristics confer the species great plasticity as shown by its wide geographical distribution.

Among control methods, the use of toxic baits is probably the most efficient method to manage *Acromyrmex* spp. ants (Zanetti *et al.* 2014). In fact, control strategies against these leaf-cutting ants have essentially focused on the use of broad spectrum conventional insecticides targeted at the major ant workers (Antunes *et al.* 2000). Sustainable agriculture demands new environmentally friendly pesticides that adhere to increasingly stricter international regulations and product certification (Lemes *et al.* 2017; Zanuncio *et al.* 2016). Part of the research on new biorational pesticides focuses on natural products such as plant extracts, oils and inorganic insecticides. Plant extracts show potential as management tools for *Acromyrmex* spp. ants due to their toxic and repellent properties (Boulogne *et al.* 2012). Biocontrol agents of leaf-cutting ants, including Phorid flies, may contribute as mortality agents although are not effective in suppressing the colony by themselves (Elizalde & Folgarait 2010; Della Lucia *et al.* 2014). The use of pathogens such as the microfungi *Escovopsis* spp. (anamorphic Hypocreales) showed promising results under laboratory conditions (Folgarait *et al.* 2011), but results have not been translated into field use (Della Lucia *et al.* 2014). Among other possible alternatives are insecticidal inert dusts, with low toxicity to vertebrates and non-target organisms, and low impact on the environment (Athanassiou *et al.* 2016).

Insecticidal dusts represent the oldest group of substances used by men for pest management, and their efficacy is based on physical phenomena. Diatomaceous earth (DE), inert dust made up of fossilised diatoms, has been reported to be very effective in combination with pathogens against fire ant *Solenopsis invicta* (Brinkman & Gardner 2001). However, when used alone, it causes very low ant mortality and has been shown ineffective against *Atta sexdens rubropilosa* Forel colonies (Ferreira-Filho

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et al. 2015). Moreover, these tools would not work in nests with complex architecture and multiple fungus chambers such as those found in Atta spp. Recently, the discovery of nanoinsecticides provides new alternatives to expand the spectrum of applications of inorganic dusts (Stadler *et al.* 2010a). The current use of nanotechnology in a wide array of fields and products as well as their potential in crop protection (Parisi *et al.* 2015) suggests that nanomaterials have a great potential to impact agriculture, although its development is in its initial stages (Kah & Hofmann 2014). Further studies have shown the potential of nanosilica (Debnath *et al.* 2011; Barik *et al.* 2012) and nanostructured alumina (NSA) for insect pest control (Stadler *et al.* 2010a, 2012; Buteler *et al.* 2015). Nanoparticles may provide an alternative strategy to traditional broad spectrum insecticides, to manage pests which have become resistant to conventional pesticides or to enhance the efficacy of traditional pesticides, by improving delivery methods and increasing potency of nanoformulations (Sekhon 2014). One of the key effects of insecticide dusts on insects is to disturb the external protective wax layer of the cuticle, turning it vulnerable to water loss and subsequent insect dehydration. After contact with the insect outermost layer by electrostatically induced binding, adsorption on the epicuticle and the absorption of cuticular waxes (paraffins, polyphenols, esters) occur, resulting in a reduction of the outer epicuticular layer (Mewis & Ulrichs 1999; Stadler *et al.* 2017 in press). Through the disturbed cuticle, in accordance with Fick's law, there is higher diffusion of body water along the concentration gradient into the surrounding air. On the other hand the water permeability of the insect integument increases with the number of particles attached and their surface area. The result of the action of NSA is death of the insect by dehydration.

Stadler *et al.* (2012 and 2017, in press) demonstrated the efficacy and the mechanism of insecticide action of NSA in insect pest species of stored grain. Nanostructured alumina efficacy was comparable and, in some cases, greater than DE (Stadler *et al.* 2012). Further, Stadler *et al.* (2017, in press) confirmed that the main mechanism of insecticide action of NSA is through physical phenomena rather than biochemical mechanisms. Hence, it has been definitively demonstrated that electrically charged NSA particles attach to the insects cuticle due to tribo-electric forces, sorbing its wax layer by surface area phenomena, resulting in insect dehydration. However, potential adverse effects of the NSA particles that could add to its insecticidal effect as a negative impact on ant bacterial symbionts (Andersen *et al.* 2015) should not be discarded.

The present study assessed the contact toxicity and repellent effect of NSA on workers of *A. lobicornis* to investigate the potential of these dusts as a management tool for leaf-cutting ants.

MATERIALS AND METHODS

Insect material

Experiments were carried out with *A. lobicornis* workers. Five colonies of about 1000 ants were collected around Villa La Angostura in Neuquen province-Argentina and placed in plastic containers. Fungal isolates were collected as basidiocarps states from the nests and placed in the plastic containers used as rearing nests in the laboratory. Nests were fed with fresh leaves of roses, oats and rice and also supplied with a 0.4 M sucrose solution '*ad libitum*', which was applied to floral foam (OASIS®). Colonies were kept at 20 ± 5°C and 65 ± 5% RH, and light–dark cycle of 12:12 h.

Chemicals

Nanostructured alumina (NSA) was obtained by glycine–nitrate combustion synthesis technique (Toniolo *et al.* 2005). The bulk density of the powder was measured at 0.108 g/cm^3. Nanostructured alumina tends to cluster and may behave as larger particles (350 ηm up to 150 μm), depending on particle surface reactivity (Karasev *et al.* 2004; Stadler *et al.* 2012). Diatomaceous earth was obtained from diatom fossilised sedimentary deposits from San Juan-Argentina, which contains over 85% amorphous SiO_2, commercially available as DiatomiD®. Specific gravity of DE is 0.22 g × cm^{-3} (Bilbao *et al.* 2007). Particles range from 1 to 45 μm, and the median particle size is 10 μm.

Dry dust exposure bioassays

Laboratory bioassays were conducted to test the toxicity of NSA and DE using dry dust applications and evaluated at four different concentrations of the product: 500, 250, 125 and 80 mg/kg. The bioassay methodology was the same as that reported in Stadler *et al.* (2010b). Briefly, wheat seeds were used as a natural substrate for the dust. Nanostructured alumina was mixed thoroughly to allow an even distribution of the powder through the entire grain mass, and dilutions were conducted to achieve the desired concentrations in 10 g of wheat, and distributed uniformly over the bottom of 9.5 cm Petri dishes. These experimental arenas were supplied with 1 M sucrose solution in distilled water, soaked in floral foam (OASIS®) contained in uncapped 1.5 mL capacity Microcentrifuge tubes. A Petri dish containing 10 g of untreated wheat and 1 M sucrose solution was used as control. Ten worker ants collected from laboratory nests were placed in each arena and dishes properly capped. Thus, ants could move freely, having access to an *ad libitum* water source. Dishes were placed in a growth chamber, and temperature and humidity inside the chamber were monitored with dataloggers (HOBO® Onset Computer, Bourne, MA, USA). The experiments were conducted at 25 ± 1°C and constant photoperiod of 12:12 (L:D). Ant mortality was assessed 2, 4, 6, 8 and 10 days after continuous exposure to the treated wheat. The data were analysed using the Mixed Procedure (PROC MIXED) of the Statistical Analysis System (SAS Institute 2011) with mortality as the response variable, and dust concentration and treatment (NSA or DE) and their interaction as the fixed factors. Date of observation was the repeated measure. A separate analysis of the mortality at the end of the bioassay was conducted where overall mortality was the response variable, and dust concentration, replicate and their interactions were the main effects. The variance–covariance structure was modelled as compound symmetry. Control mortality was corrected using Abbott's (1925) formula. LSMEANS comparisons were conducted with the Tukey option in SAS.

Contact toxicity was analysed using the Probit procedure in SAS PROC PROBIT (SAS Institute 2011). Lethal concentration to cause 50% mortality of ants (LC_{50}) on the 10th day after treatment was calculated and compared among treatments. To test the equality of the LC_{50}s, we examined the ratio of LC_{50}s and their confidence interval according to Robertson & Preisler (1992). If the CI for the LC_{50} ratio contained 1, the population LC_{50}s were considered to be the same.

Preference and avoidance laboratory and field bioassays

Laboratory and field bioassays were conducted to assess whether ants avoid NSA. The laboratory bioassay was similar to that used by Appel *et al.* (2004). The choice test consisted of placing 10 worker ants in 95 mm diameter, 17 mm high, glass petri dishes. The Petri dishes were divided in two equal parts. Half of the dish contained NSA, and the other half contained soil. The amounts of soil and NSA used were sufficient to cover the surface of the dish completely. The ants were acclimatised for 1 h, after which the number of ants on each side was recorded every 30 min for 4 h. No food or drink was added to avoid confounding effects. The experiment was replicated 15 times. The laboratory data were analysed with a Wilcoxon Signed rank test for paired samples comparing the number of ants in the control vs. the treated areas.

A field bioassay was conducted to test whether ants collected baits treated with NSA. Two foraging trails with similar traffic were identified in each of 10 nests. Thirty oat flakes were used as bait and placed on a filter paper next to a foraging trail. One foraging trail had treated oats that had been mixed with 500 mg/kg of NSA as described above, and the other foraging trail had untreated oats. The number of flakes removed by the ants was recorded after 30 min, 1 h and 24 h. The experiment was replicated 10 times in 10 different nests. The field data were analysed with a Wilcoxon Signed rank test for paired samples, comparing the number of flakes removed in the control vs. the treated foraging trail. A repellence percentage (RP) was calculated according to Talukder and House (1994): RP = 2 × (C-50).

Attachment of dust particles on *A. lobicornis* workers body surface

The interaction between the insect cuticle and the dusts (NSA and DE) was analysed with electron microscopy, to determine the affinity of particles of each dust in attaching to the insect cuticle. The coverage of the insect body after exposure to NSA or DE particles was qualitatively and quantitatively studied by means of a scanning electron microscope (SEM) and the X-ray analyser (energy dispersive spectrometer (EDS) JEOL mod 6610 LV – Thermo Scientific). The EDS system software Noran System 7 version 2.1 analyses the energy spectrum in order to create element composition maps over the raster area and to determine the abundance of an element (Al). Specimens were C coated. Al was examined by the interaction of Al_2O_3 with a high energy (20 kV) electron beam focused to a diameter of a few microns.

RESULTS

Dry dust exposure bioassays

Adult mortality increased with time (F = 89.93, d.f. = 4, 20, $P < 0.0001$) and concentration (F = 1016.9, d.f. = 3, 12, $P < 0.0001$) (data not tabulated). There was also a difference in mortality between treatments (F = 2939, d.f. = 1, 4, $P < 0.0001$). The greatest differences were observed at the highest concentrations of 500 mg/kg, in which all the test organisms were dead in the NSA treatment in comparison to only around 50% mortality in the DE treatment (Table 1). Tukey comparisons revealed that NSA was more effective than DE at every concentration tested ($P < 0.05$).

Cumulative mortality at the end of the bioassay was different between treatments (d.f. = 1, 4, F = 1416, $P < 0.0001$) and concentrations (d.f. = 3, 12, F = 2979, $P < 0.0001$) (Table 1). Probit analysis revealed that NSA (LC_{50} for NSA = 0.14 mg/g; CI 0.12–0.17) was more toxic to ants than DE (LC_{50} for DE = 0.36 mg/g; CI 0.25–0.77) (Table 2).

Preference–avoidance

Ants were not repelled by NSA (Wilcoxon Signed rank test S = −48.5, P = 0.7) in laboratory bioassays (data not tabulated). The average RP observed in the Petri dishes was 0.66 ± 3.1. No repellence was observed in field tests either, given that ants collected oat flakes treated with NSA (12.7 ± 1.2 oat flakes) in similar amounts to control oat flakes (13.9 ± 1.6) after 2 h exposure (Wilcoxon Signed rank test S = −9.5, P = 0.36).

Attachment of dust particles on *A. lobicornis* workers body surface

The EDS analysis of the backscattered electron image of a control insect shows a minimal amount of silica (0.45 ± 0.11%wt), detached in principle from wheat kernels which are originally rich in silica, and no aluminium (Table 3). Silica accounts for 5.05 ± 0.35%wt, in the insect treated with DE, representing the amount of DE attached to the insect cuticle. An insect treated with NSA contains an 11.84 ± 0.74%wt aluminium. The ant body surface is uniformly covered by small particles of NSA with some larger agglomerates distributed randomly throughout the cuticle. The Al was the most abundant metal on the scattered

Table 1 Cumulative mortality at the end of the toxicity bioassay

Concentration (mg/g)	Treatment	Mean mortality
0.5	NSA	100 ± 0.0a
0.25	NSA	85.83 ± 0.83b
0.25	DE	53.3 ± 0.83c
0.5	DE	52.5 ± 1.44c
0.125	NSA	25 ± 0.0d
0.08	NSA	24.17 ± 0.83d
0.08	DE	15.83 ± 0.83e
0	NSA	15.0 ± 1.44e
0	DE	15.0 ± 1.44e
0.125	DE	14.16 ± 0.83e

Rows followed by the same letter are not statistically different at $P < 0.05$.

Table 2 Median lethal concentration values for DE and NSA, tested on *Acromyrmex lobicornis*

Product	Time (days)	LC_{50}mg/g (CI)	LC_{95}mg/g (CI)	Slope (SE)	Intercept (SE)	Goodness of fit Chi square/*P* value
DE	10	0.36a(0.25, 0.77)	4.04b(1.4, 9.02)	0.68(0.18)	0.69(0.30)	4.36/0.92
NSA	10	0.14b(0.12,0.17)	0.37b(0.28,0.60)	1.68(0.26)	3.29(0.53)	6.67/0.75

LC_{50} = median lethal concentration; LC_{95} = lethal concentration 95%; CI = confidence interval; SE = standard error. Values followed by the same letter in a given column are not statistically different according to Robertson and Preisler (1992).

Table 3 Quantitative chemical analysis of body surface of *A. lobicornis* using dispersive X-ray spectroscopy (EDS) showing the main elements present on the insect body surface of control insect, or insect treated with nanostructured alumina or diatomaceous earth

Treatment	DE	NSA	Control
Element	Element wt%	Element wt%	Element wt%
CK	62.31 +/– 1.96	57.76 +/– 2.39	62.46 +/– 0.91
NK	–	–	11.04 +/– 4.08
OK	31.18 +/– 1.84	29.54 +/– 2.00	25.49 +/– 1.21
AlK	–	11.84 +/– 0.74	–
SiK	5.05 +/– 0.35	–	0.45 +/– 0.11
KK	–	0.85 +/– 0.52	0.56 +/– 0.19
CaK	1.46 +/– 0.54	–	–
CK	62.31 +/– 1.96	–	–
Total	100.00	100.00	100.00

insect surface, and it is directly associated with the nanoinsecticide (Al_2O_3) (Table 3). These figures demonstrate that NSA shows a higher affinity than DE in attaching to the insect cuticle.

DISCUSSION

This study reports for the first time the toxicity of NSA on leaf-cutting ants and shows that it has a greater efficacy than DE in killing *A. lobicornis* at all the concentrations tested. Inert dusts may have abrasive, sorptive or combined effects (Wigglesworth 1944; Ebeling & Wagner 1961; Ebeling 1971). Nanostructured alumina is an amorphous nanostructured material with greater sorptive properties than DE given the size and shape of particles (Stadler *et al.* 2012), which could explain its greater efficacy (Ebeling & Wagner 1961; Mewis & Ulrichs 1999). Moreover, there are wide differences in the insecticidal potency of different DE dusts (McLaughlin 1994; Subramanyam & Roesli 2000). The efficacy of DE is a function of the physical properties attributable to the morphological characteristics of the diatoms depending on the quarry from where they were obtained (Korunic 1998). Nanostructured alumina is expected to be more reliable given that it is synthetised in the laboratory under controlled conditions. Diatomaceous earth, on the other hand, shows a high variability in efficacy (Mvumi *et al.* 2006).

Our results are consistent with DE on fire ant *Solenopsis invicta* where DE (at 0.1 g per dish) induced around mortality of 30% in workers (Brinkman & Gardner 2001). Ferreira-Filho *et al.* (2015) found that DE caused low level mortality *Atta* spp. and did not achieve nest control when applying DE directly to the nests. Our results show a marked increased efficacy of NSA in comparison to DE as it resulted in total mortality of ants at concentrations of 500 mg/kg. Thus, NSA shows potential to be used as a granular insecticide that could be applied directly to *Acromyrmex* spp. nests given that they have typically smaller nests than *Atta* spp. However, such a method is of limited value for established nests containing hundreds of fungus chambers at a depth of several metres (Boaretto & Forti 1997). Mature nests of *Atta* spp. may contain thousands of subterranean chambers, while nests of *Acromyrmex* spp. are generally small with only one or a few chambers (Verza *et al.* 2007). Thus, inert dusts, and NSA, in particular may be more useful for the control of *Acromyrmex* spp.

One of the major control methods used for leaf-cutting ants is the use of attractive baits with an insecticide, so is it particularly interesting that NSA is not repellent to ants. The fact that ants did not recognise the NSA coupled with the strong adhesion to the insect body surface suggest that NSA could be added into a bait and used as carrier particles for synthetic insecticides, entomopathogens or pheromones in insect control systems. Nanostructured alumina could also aid in the use of insecticidal baits by reducing the amount of active ingredient used or by developing novel control systems or to improve formulations of plant based repellents and disruptive compounds such as eudesmol (Marsaro *et al.* 2004). Further studies should address the effect of NSA on the symbiotic fungus, ant bacterial symbionts as well as its potential as carrier for other insecticides or fungicides.

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