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RESEARCH PAPER

Volcanic materials as carriers for the formulation of mycoinsecticides using the fungus *Beauveria bassiana*

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Abstract

V.E. Sy, S. Schalamuk, A.C. Scorsetti, and I.L. Botto. 2016. Volcanic materials as carriers for the formulation of mycoinsecticides using the fungus *Beauveria bassiana*. Cien. Inv. Agr. 43(2):273-282. Improvements in the formulation of biological insecticides are crucial to increase their stability and competitiveness in the market. The aim of this work was to evaluate the use of volcanic materials with different hydric retention capacities to increase the thermotolerance of *B. bassiana* conidia. Two pyroclastic rocks and a zeolite clinoptilolite were selected for this study. In addition, a commercial silica gel was used due to its reported capacity to increase conidial thermotolerance. Dry conidia were mixed with the materials to obtain a 2% w/w granular formulation, while conidial powder alone served as the control. Mixtures were stored at room temperature for 30 days and then exposed to 50 ºC for two hours, and the viability was measured before and after the thermal exposure. No significant decrease in viability was observed for conidia stored with any of the pyroclastic materials, while the germination was reduced by 10, 17 and 23% for unformulated conidia, conidia stored with silica gel and conidia stored with zeolite, respectively. From these results, a pyroclastic rock was selected to test its capacity to maintain high viability under different temperatures (4, 25 and 35 ºC) and humidities (~0 and 20%). A decrease in viability was observed with an increase of temperature, and lower viability was also recorded in humid treatments, but only at 25 and 35 ºC.

Key words: Biological insecticide, entomopathogenic fungus, thermotolerance, volcanic ash.

Introduction

The massive use of chemical insecticides is increasingly controversial due to the side effects on human health and environmental pollution. Additionally, the extensive use of these products has led to the development of resistance by pest insects, with the concomitant loss of efficiency. Moreover, the low specificity typical of these insecticides elicits undesirable effects on the beneficial fauna that usually regulate the populations of phytophagous and pest arthropods (El-Wakeil

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et al., 2013). A promising alternative that might replace or at least decrease the use of chemical insecticides is the use of biological control agents such as viruses, fungi, nematodes and bacteria (Lacey *et al*., 2001).

The use of fungi as insecticides has been widely studied, and many commercial products have been developed, most of them based on the funguses *Beauveria bassiana* and *Metarhizium anisopliae* (Faria and Write, 2007). However, these new products have to compete with chemical insecticides, which in general, have a faster effect, are more stable, cheaper, easier to apply, and can be stored for longer periods under variable environmental conditions without the loss of effectiveness. A key factor for a biological formulation to be commercially successful is to maintain the viability and virulence of the infective units during storage and application. In general, before application, it is required for the product to keep its properties for at least a year under varied environmental conditions (Jackson *et al.,* 2010). Exposure to high temperatures during transport and storage is a critical issue. In addition, the conidia humidity content and moisture conditions of the atmosphere during storage are also key factors to maintain viability (Hong *et al*., 1997; Blanford *et al*., 2012). Some authors have observed that the addition of silica gel to oil formulations of *M. anisopliae* favors conidial viability due to the capacity of this material to adsorb humidity (McClatchie *et al*., 1994; Moore *et al*., 1996). Other studies have shown that the use of different desiccant materials enhances the conidial thermotolerance of the entomopathogenic fungi *Isaria fumorosea* and correlate this with water potential (Kim *et al*., 2014a). Thus, it is important for the ingredients in the formulation to have properties that favor the high temperature resistance of infective units and that can maintain suitable humidity conditions.

Several types of mycoinsecticide formulations have been developed, and the most common are the technical concentrates in the form of fungus-colonized substrates, followed by wettable powders and oil dispersions (Faria and Write, 2007). Although organic compounds are commonly used, inorganic materials also take part in formulations. Geomaterials such as zeolites, talc, bentonite and kaolin have been used as main components or as additives in fungal formulations (Daoust *et al*., 1983; Kucuk and Kivanc, 2005; Ezzati-Tabrizi *et al*., 2009; Ritu *et al*., 2012), providing stability and low cost and easy accessibility, mainly in countries where vast amounts of such materials are available and underexploited.

The use of *B. bassiana* as a mycoinsecticide is promising. From a biological point of view, this fungus can infect many pest insect species, inducing high mortality rates (Uma Devi *et al*., 2008). From the commercial perspective, spore mass production has been successfully achieved, and there are many companies offering products based on *B. bassiana* (Seema *et al*., 2013). Improvements in the formulation that prolong the shelf life of the product using low cost materials will make mycoinsecticides more competitive, promoting eco-friendly solutions for pest control.

This study proposes the use of highly available and low cost volcanic materials as carriers for the formulation of mycoinsecticides using the fungus *B. bassiana*. Two volcanic ashes and one zeolite mineral were selected, which are amorphous and crystalline aluminosilicates, respectively, and have a common volcanic origin. First, the capacity to enhance the conidial thermotolerance of these materials was evaluated and correlated with their hydric retention properties. From the results obtained, one of these materials was selected to test its capacity to maintain spore viability under different temperature and humidity conditions.

Material and methods

Fungal culture

We used the fungal strain *B. bassiana* LPSc-1067 from the culture collection of the Spegazzini

Institute, La Plata, Argentina. The choice of this strain was based on its efficacy against pest grasshopper and locust species of Argentina in the laboratory (Pelizza *et al.*, 2012). The fungus was propagated in petri dishes containing potato-dextrose agar (APG) at 25 ºC in darkness for 30 days, and conidia were harvested by scraping the surface of the cultures onto a glass petri dish with a sterile metal spatula. The dish was placed in a desiccator containing silica gel for 4 days at 20 ºC to reduce the moisture content to < 5%.

Carriers characteristics

Three different volcanic materials were selected for this study: 1) pyroclastic material from the recent (2011) eruption of the Puyehue-Cordón Caulle volcano (Chile) (Schalamuk *et al.*, 2014a); 2) pyroclastic material from Palo Blanco (Catamarca, Argentina); and 3) zeolite clinoptilolite (La Rioja, Argentina). All the materials share a common volcanic origin and have a SiO_2:Al_2O_3 ratio close to 5, but differ in structure and porosity, which gives them different hydric retention capacities. The volcanic ash from the Puyehue volcano is a pumiceous material with a rhyolitic composition and was collected near Cardenal Samoré International Pass (Neuquén, Argentina). It has a predominance of vitreous fragments (~88%) and a lower proportion of crystalline phases (~12%). The volcanic ash from Palo Blanco is a redeposited material that originated during the eruption of the Cerro Blanco Volcanic complex beginning in the Quaternary. It is a pumiceous rock with a dacitic composition and ~90% vitreous phase, with the rest composed of crystalline particles. The zeolite is the variety clinoptilolite and was collected from Pagancillo village (La Rioja, Argentina). Unlike volcanic ashes, most of its structure consists of crystalline particles, being low in the vitreous phase (~10%). Zeolite structures have a uniform network of crystalline cavities and channels (meso and microporosity), which provide them a huge surface area. These channels hold cations that counteract the negative charge of the aluminosilicates and confer a high water adsorption capacity. Although volcanic ashes also have the capacity to retain water, they have only macro and mesoporosity due to their predominant vitreous nature, being smaller than the zeolites in terms of surface area and hydric retention capacity. These materials were selected based on their stability, abundance and low cost, as well as for their hydric retention capacity, which may enhance conidial thermotolerance. In addition, non-indicating silica gel (SiO_2) was used because of its known desiccant properties and reported capacity to enhance conidia viability (Moore *et al.*, 1996; Kim *et al.*, 2014a). Silica gel has an amorphous microporous structure with a distribution of pore opening sizes of approximately 3-60 angstroms. These interconnected pores form a vast surface area (~ 800 m^2 g^{-1}) that attracts and holds water by adsorption and capillary condensation, allowing the silica gel to adsorb up to 40% of its weight in water.

Thermotolerance of conidia formulated with different materials

Prior to formulation, volcanic materials were sieved to obtain particles between 1.2 and 1.7 mm, washed three times with abundant distilled water, dried at 100 ºC to constant weight and sterilized. A sample of 4.9 g of each material was mixed with 0.1 g of conidial powder in a Falcon tube to obtain a 2% w/w granular formulation. Each mixture was then separated into three samples and placed in 4 x 4 cm aluminum bags. Three bags containing 0.1 g of dry conidial powder alone served as the controls. Twenty-four hours after formulation, the initial viability was measured. The samples were stored at room temperature (21 ± 4 ºC) for 30 days and then exposed to 50 ºC for two hours. The viability was measured before and after the thermal exposure to investigate the capacity of the carrier to enhance the conidial thermotolerance.

Measurement of conidial viability

From each bag, 0.1 g of formulated conidia were added to a tube containing 5 mL of 0.05% Tween 80 and vortexed for three minutes. From the control bags, a pinch of unformulated conidia was suspended using the same procedure. The concentration was adjusted to $10^5 - 10^6$ spores mL^{-1}, and 0.3 mL of this suspension was dropped by duplicate onto a glass slide provided with a thin layer of APG. Slides were incubated at 25 ºC in darkness for 24 h in a moist chamber, and then the spores were examined for germination under a compound microscope (Goettel and Inglis, 1997). Conidia were counted as germinated if the germ tube had at least the diameter of the spore. A total of at least 300 conidia were counted per slide, and the viability was calculated as a percentage of the total.

Medium term viability of conidia formulated with Puyehue volcanic ash and stored under different temperature and humidity conditions

Conidia were formulated as described above using the pyroclastic material from the Puyehue volcano. To generate different humidity conditions, distilled water was added to part of the carrier prior to formulation, obtaining a material with 20% humidity content (moist treatments), while no water was added to the rest of the material (dry treatments). For each humidity condition, 9 replicates consisting of 5 g of formulated product were prepared into 100 mL plastic containers, covered with a lid and sealed with Parafilm®. Containers provided with 0.3 g of dry conidial powder served as control treatments. Samples (three of each treatment) were stored under one of three different temperatures: 4, 25 and 35 ºC. The viability was measured as described above 24 h after formulation and then at 7, 14, 30, 60 and 120 days.

Data analysis

Data on the percentage of germination was analyzed using a multiple factor analysis of variance (ANOVA). Data were previously transformed using a Probit transformation. The differences between treatments were detected using Tukey's honestly significant difference (HSD) test. The analyses were conducted using Statistica v. 7.0.

Results

Conidial viability using volcanic materials as carriers

Conidial viability varied with the material used as the carrier ($F_{4,30} = 75.0$, $P \leq 0.001$), and it was evident soon after formulation. Although the initial viability was high in all treatments (> 90%), unformulated conidia and formulations with Puyehue and Palo Blanco pumice rock showed a significantly higher viability compared with the zeolite and silica gel formulations (Table 1). The same tendency was observed after 30 days of storage and after exposure to 50 ºC for two hours (Table 1). On the other hand, the viability significantly decreased after 30 days of storage and after thermal exposure ($F_{2,30} = 608.4$, $P \leq 0.001$). All the formulations and the control treatment showed a lower viability after storage, but only the zeolite, silica gel and unformulated conidia were affected by high temperature exposure (Figure 1). The reduction in germination was more pronounced in the zeolite and silica gel treatments, where the final viability was lower than 30% (Figure 1).

Viability of conidia formulated with Puyehue volcanic ash under different storage conditions

Conidial viability was affected by temperature ($F_{2,108} = 282.2$, $P \leq 0.001$), humidity ($F_{2,108} = 55.9$, $P \leq 0.001$) and storage time ($F_{5,108} = 608.4$, $P \leq 0.001$). With low temperature (4 ºC), the viability was high in all treatments (> 91%) and more or less

Table 1. Germination (mean percent) of conidia of *Beauveria bassiana* mixed with different materials 24 h after formulation, after storage at room temperature for 30 days and after exposure to 50 ºC for 2 h.

	Germination of conidia ± SE (%)		
Carrier	After formulation	After 30 days storage	After 50 ºC exposure
Puyehue pumice	96.4 ± 0.4 a	70.8 ± 4.0 ab	65.9 ± 3.3 a
Palo Blanco pumice	97.0 ± 0.7 ab	70.2 ± 2.5 b	69.5 ± 1.7 a
Zeolite	92.7 ± 0.7 c	52.9 ± 3.1 c	29.5 ± 1.5 b
Silica gel	93.8 ± 0.8 bc	41.0 ± 3.9 c	24.0 ± 5.1 b
Control (unformulated conidia)	97.4 ± 0.3 a	82.7 ± 1.8 a	72.8 ± 1.3 a

Means followed by different letters within the same column are significantly different according to Tukey's HSD (P≤0.05).

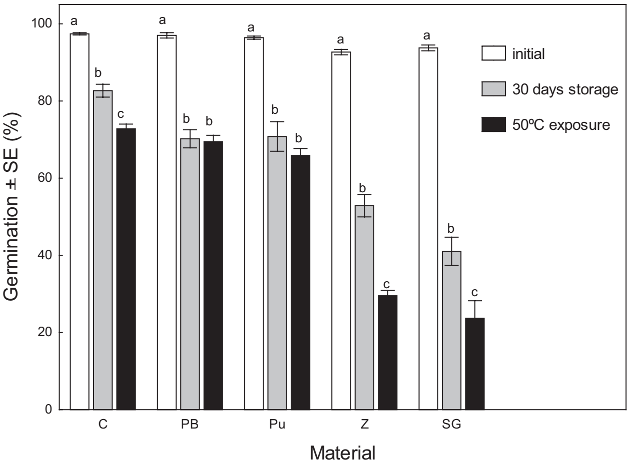

Figure 1. Germination (percent) of conidia of *Beauveria bassiana* mixed with different materials 24 h after formulation (initial), after storage at room temperature for 30 days and after exposure to 50 ºC for 2 h. For the same material, different letters indicate significant differences according to Tukey's HSD (P≤0.05). C: control (unformulated conidial powder), PB: Palo Blanco pumice rock, Pu: Puyehue volcano pumice rock, Z: zeolite clinoptilolite, SG: silica gel.

constant throughout the four-month period. Only the control and dry treatments at day 7 showed significant differences with all the treatments stored more than 7 days (Figure 2A). Conidia stored at 25 ºC showed high viability during the first two months (> 83%), and except for the unformulated conidia stored for 7 and 60 days, which presented the highest and the lowest viability, respectively, there were no significant differences among treatments. After four months of storage there was a decline in the viability, which was more evident in the moist treatment where no germination was observed. Dry formulation and unformulated conidia were similar at this time (52 and 48%, respectively), but differed from the moist formulation and from all the treatments stored for 30 days or less (Figure 2B). Storage at high temperature (35 ºC) produced a decrease in viability after 14 days of storage, which was again more pronounced under moist conditions, with no germination registered from this time forward. Dry treatments and unformulated conidia showed a gradual decrease in viability and almost zero germinated conidia were observed after 60 days or more in storage. Overall, the control and dry treatments were similar and differed from the moist treatments at the intermediate storage time (14 and 30 days) (Figure 2C).

Discussion

The use of entomopathogenic fungi as biological control agents represents a promising alternative to the widespread use of chemical insecticides (Lacey *et al.*, 2001). Among the benefits offered by these microorganisms are their safety to human beings and animals, their relatively high specificity and the absence of the development of resistance in target organisms. Moreover, the efficiency of these microorganisms to control important pests has been proven in multiple studies (Li *et al.*, 2010; Lacey *et al.*, 2011; Pelizza *et al.*, 2012; Reddy *et al.*, 2014). A crucial step prior to its use as an insecticide is the formulation, which should assure the viability and pathogenicity of infective units during storage and after application and should improve the product form for ease of application (Jackson *et al.*, 2010). The temperature and humidity during storage are key factors that may affect conidia viability, and different materials can be added to the formulation to enhance stability of the product.

The results from this study show that the use of pyroclastic material either from the recent eruption of the Puyehue volcano or from the ancient activity of the Cerro Blanco volcanic complex may enhance the conidial thermotolerance of *B. bassiana*. Conidia stored with these materials and exposed to 50 ºC for two hours showed no significant decrease in viability, while the germination was reduced by 10, 17 and 23% for unformulated conidia and conidia stored with silica gel and or with zeolite, respectively. It is surprising that those materials with higher hydric retention capacity, i.e., silica gel and zeolite, did not enhance the thermotolerance, because other studies have shown that this property may protect spores when exposed to high temperatures (McClatchie *et al.*, 1994; Moore *et al.*, 1996; Kim *et al.*, 2014a). It is possible that the higher proportion of material used in this study has influenced conidial viability. The authors cited tested the use of silica gel and other desiccant materials as adjuvants in the formulation, thus a small proportion (~10%) was added to the conidia. On the other hand, 98% of our formulation consisted of silica gel or the volcanic material, because it was intended to serve as carrier. Daoust *et al.* (1983) also found that the addition of a high proportion (80%) of silica gel or anhydrous $CaCl_2$ reduced the

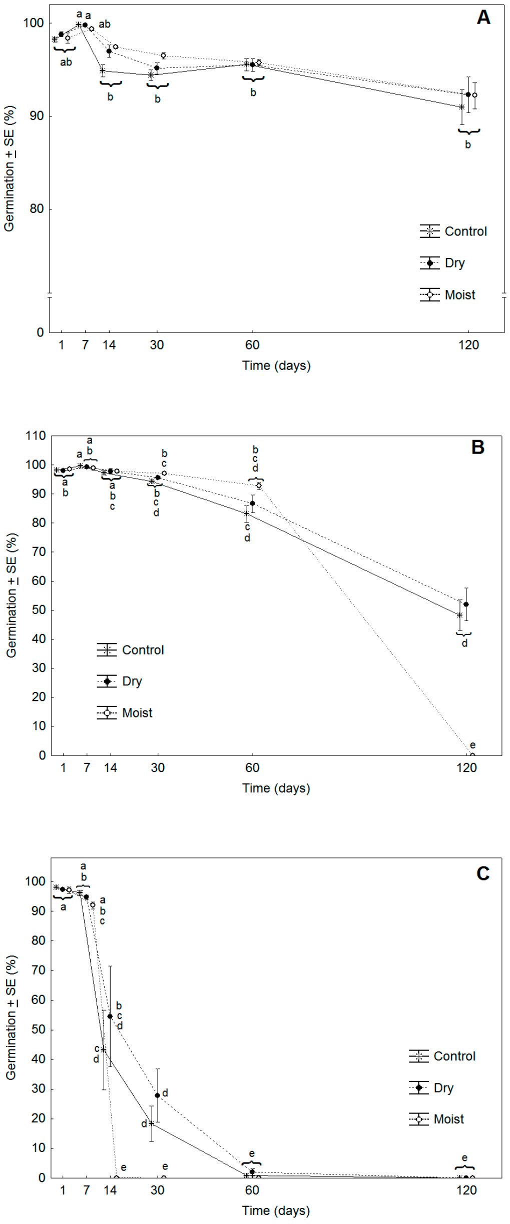

Figure 2. Germination (percent) of conidia of *Beauveria bassiana* mixed with dry (0%) and moist (20%) pumice rock from the Puyehue volcano after different storage times at 4 ºC (A), 25 ºC (B) and 35 ºC (C). The control corresponds to dry conidial powder. Different letters indicate significant differences between treatments according to Tukey's HSD ($P \leq 0.05$).

viability of conidia, compared with formulations containing 20% desiccant, 60% kaolinite clay and 20% conidia. Thus, although the use of desiccants may enhance the viability of conidia, its use as diluents could be detrimental, being preferable the use of materials with a lower hydric retention capacity, such as pyroclastic rock.

When *B. bassiana* conidia was formulated with volcanic ash from the Puyehue volcano, it was possible to maintain a high viability of the infective units for long periods provided that low temperatures could be set, without observing a negative effect from humidity. However, with intermediate and high temperatures, a decrease in viability with storage time was observed, which was more pronounced in moist treatments. Under these conditions some condensation was observed on the walls of the recipients. This free water might trigger spore germination followed by death as the requirements to complete germination were not fulfilled in the storage environment, as was proposed by Jackson *et al.* (2010). Thus, if this material is to be used as carrier, it would be necessary for a previous treatment to remove the humidity from the material.

It is well documented that a reduction in the spore moisture content increases the shelf life of the spores, and it is an important step in standard procedures for the mass production of *Beauveria* and *Metarhizium* spores (Seema *et al.*, 2013). It has also been stated that keeping the humidity low during storage is advantageous and may enhance thermotolerance. Our results show that a very humid atmosphere that allows water to remain as a molecular layer reduces the stability of spores. However, a very desiccant atmosphere can also be disadvantageous and might cause the death of spores from strong dehydration. Similar results were obtained by Blanford *et al.* (2012), who observed that unformulated spores stored in an open environment subjected to 80% humidity survived longer than those maintained in sealed packages protected from humidity.

From a practical point of view, a granular formulation using pyroclastic material as a carrier could be used for the control of soil pests through its incorporation in substrates used for greenhouse production. There are some studies in which granular formulations of *B. bassiana* have been successfully applied for the control of soil pests, highlighting the preferability of this control method compared with the application of more common products, such as wettable powders or suspension concentrates (Kim *et al.*, 2014b). Moreover, the Puyehue pyroclastic rock has been to replace perlite in the elaboration of substrates for greenhouse vegetable production because it is safe for plant growth (Schalamuk *et al.*, 2014b), an important aspect that should be tested before its use in a formulation (Leggett *et al.*, 2011). Thus, the product we propose would have a dual function, as an insecticide and as a constituent of the substrate used for plant growth, which represents a novel approach.

Another advantage of this material is its abrasiveness, which would lacerate the insect cuticle, enabling germinated fungal conidia to penetrate more easily into the insect hemocoel. Other abrasive materials, such as diatomaceous earth, have been used to enhance the insecticidal effect of entomopathogenic fungi, and there is an extensive bibliography on their synergic effect (Lord, 2001; Riasat *et al.*, 2011). Moreover, Fernandez-Arhe *et al.* (2013) found that the pyroclastic material from the Puyehue volcano has an insecticidal effect and attributed this capacity to its abrasiveness and adsorptive properties, which would remove the lipid monolayer from the cuticle, causing the insect to desiccate.

In summary, the results from our research demonstrate that the use of pyroclastic material as a carrier for the formulation of *B. bassiana* could be an effective alternative approach to pest control. As a novel application that has been proposed for the control of soil pests in containerized plants, it would be interesting to test its efficacy under simulated conditions. It would also be interesting to test the synergic effect of the use of a pyroclastic material in combination with an entomopathogenic fungus for pest control, which would enhance the competitiveness of the product.

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Resumen

V.E. Sy, S. Schalamuk, A.C. Scorsetti y I.L. Botto. 2016. Materiales volcánicos como vehículo para la formulación de micoinsecticidas usando el hongo *Beauveria bassiana*. Cien. Inv. Agr. 43(2):273-282. La optimización de las formulaciones de insecticidas biológicos resulta crucial para poder mejorar su estabilidad y competitividad en el mercado. El objetivo de este trabajo fue evaluar el uso de materiales volcánicos con diferente capacidad de retención hídrica para incrementar la termotolerancia de los conidios de *B. bassiana*. Para ello se seleccionaron dos tipos de rocas piroclásticas y una zeolita clinoptilolita. Además se usó sílica gel comercial debido a su conocida capacidad de incrementar la termotolerancia de los conidios. Los conidios secos fueron mezclados con los materiales para obtener una formulación en gránulos al 2% p/p, mientras que el polvo de conidios sin aditivos se usó como control. Las mezclas se almacenaron a temperatura ambiente por 30 días y luego se expusieron a 50 ºC durante dos horas. La viabilidad se midió antes y después de la exposición a alta temperatura. La viabilidad no se vio afectada por las altas temperaturas en los conidios almacenados con materiales piroclásticos, mientras que hubo una reducción del 10, 17 y 23% en la germinación en los conidios no formulados y en los conidios almacenados con sílica gel y zeolita respectivamente. Sobre la base de estos resultados se seleccionó una de las rocas piroclásticas para testear su capacidad de mantener una alta viabilidad bajo diferentes temperaturas (4, 25 y 35 ºC) y humedades (~0 and 20%). Se observó una disminución en la viabilidad al aumentar la temperatura y también fue menor la viabilidad en los tratamientos húmedos, pero solamente a 25 y 35 ºC.

Palabras clave: Ceniza volcánica, hongos entomopatógenos, insecticida biológico, termotolerancia.

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