

Pyroclastic material from the Puyehue-Cordon-Caulle Volcanic Complex, Chile, as carrier of *Beauveria bassiana* conidia: Potential utilization in mycoinsecticide formulations

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ABSTRACT

The last volcanic eruption of the Puyehue-Cordon-Caulle Volcanic Complex in the Andes cordillera of western South America, occurring on 4 June 2011, ejected pyroclastic materials that were accumulated in a wide region of the northern Patagonia (Argentina), affecting the environment and health of residents within the area. The aim of this work was to evaluate the practicability of using this waste material as a low-cost carrier for mycopesticide formulations. *Beauveria bassiana* is a recognized fungal agent for arthropod biologic control. Lengthy storage is critical for the development of mycoinsecticide formulations. Accordingly, the search for adequate materials to improve the shelf life of biocontrol products becomes desirable. First, several analytical techniques were employed to characterize the pyroclast physicochemically; then the viability of the fungal conidia was evaluated after an 18-month storage in the volcanic material. Finally, the pathogenicity of the conidia after that prolonged maintenance in the vehicle was assessed on the beetle *Alphitobius diaperinus*, an insect pest in poultry houses that causes major economic losses. The results from those bioassays proved auspicious for the eventual utilization of the pyroclast as a bioinsecticide carrier especially since the formulation had proven to be stable for at least 18 months under

a wide range of environmental conditions. The constant moisture in a closed environment within a 5°C - 40°C temperature range insures a viable state during storage. The results indicate that what would otherwise be volcanic waste may be utilized as an efficient, abundant, inexpensive, and environmentally innocuous carrier of entomopathogenic fungi.

KEYWORDS

Volcanic Material; Bioinsecticide; Entomopathogenic Fungi; Viability; Vehicle

1. INTRODUCTION

The Andes cordillera in western South America is one of the most active tectonic and volcanic regions worldwide. The stratovolcano—part of the Puyehue-Cordon-Caulle Volcanic Complex (PCCVC), Chile (40°34'57"S, 72°06'53"W; 2236 meters above sea level)—has been active since the Miocene Epoch. The last volcanic eruption, occurring on 4 June 2011, ejected pyroclastic materials that were dispersed by the wind to accumulate in a wide region of the northern Patagonia (Argentina) [1]. The volcanic event not only affected the environment and the health of residents within the area, but also had negative effects on local and regional economies (related to agriculture, livestock, and fishing, among other natural commodities). Though volcanic ash always constitutes a significant environmental hazard [2]; nevertheless, upon

consideration of that pyroclast's high abundance and low cost of extraction, several investigations have been directed at analyzing the potential use of the ash as raw material for the development of different technological processes (e.g., in adsorbents and binders and as substrates). Different geomaterials (mainly stable mineral species) as well as organic and inorganic compounds are employed as vehicles for agrochemicals and biopesticides, with silicates being the most commonly used because of their properties, stability, abundance, and low cost [3]. In recent years, the use of microbial biocontrol agents such as bacteria, viruses, and fungi has raised interest as alternatives for reducing the use of chemical pesticides in integrated pest-management programs [4,5]. The development of fungal biocontrol formulations with clay minerals as carriers has highlighted the low environmental impact relative to other types of vehicles [6]. To the best of our knowledge, however, no information is as yet available on the utilization of pyroclastic material (ashes and pumices) for that purpose.

Among biocontrol agents, entomopathogenic fungi have attracted considerable attention as potential regulators of insect populations [7,8]. More than 100 insect biocontrol products based on those types of fungi are commercially available worldwide [9]. The fungus *Beauveria bassiana* is one of the most promising agents for biologic control of arthropods because of its persistence in the host population, the high mortality rates it causes in larvae and adults, and its facile dispersion [10]. For an effective control to be achieved, however, the asexual spores or conidia—the infective units—must retain a high viability (*i.e.*, the ability to germinate) and virulence (pathogenicity) against the target pest during sometimes prolonged storage before being dispersed in the field [11]. Within this context, research on potential inorganic carriers will be required before effective pest management can be achieved through the use of biocontrol agents. Moreover, a knowledge of the physicochemical properties of the carriers of entomopathogenic agents is essential for understanding the conditions affecting the shelf life and persistence of biocontrol products as well as the effect of their application to the environment.

The aim of this work was therefore to evaluate the practicability of using volcanic material from the recent eruption within the PCCVC as a low-cost carrier for *Beauveria bassiana* conidia. Several analytical techniques—e.g., inductively coupled plasma atomic-emission spectroscopy (ICP-AES), differential thermal gravimetry, X-ray diffraction, scanning electron microscopy (SEM), Braunauer-Emmet-Teller (BET) adsorption, Raman spectroscopy, and particle-size-distribution measurement—were employed for physicochemical characterization of the pyroclast; the viability of the fungal conidia was evaluated after storage for 18 months in the

volcanic material; and the pathogenicity of the conidia after that prolonged maintenance in the vehicle was assessed on the beetle *Alphitobius diaperinus* (Coleoptera: Tenebrionidae), an insect pest in poultry houses that causes major economic losses [12,13].

2. MATERIALS AND METHODS

2.1. Characterization of the Carrier

The pyroclastic material (ashes and pumices) was collected in natural accumulations of Villa La Angostura (40°45'48"S, 71°38'46"W), situated at ~40 Km from the source, two months after the eruption (**Figure 1**). In this area, the depth of accumulated pyroclast was more than 30 cm. Bulk samples were dried and sieved (American Society for Testing and Materials mesh) for analyzing the grain-size distribution.

The pyroclast was examined under a Nikon polarizing microscope through the use of reflected light to visualize the opaque minerals. For this study, the samples were mounted in a resin that had been polished by means of different abrasives. Likewise, sheets of loose grain were prepared for analyzing the vitreous and crystalline particles constituting the volcanic mixture by transmitted light under a polarizing microscope.

Scanning-electron-microscopy measurements were performed in a Phillips 505 ESEM microscope with a tungsten filament and an Everhart-Thornley Detector (a high-vacuum secondary electron detector).

The BET surface area was measured by N₂ adsorption with a Micromeritics ASAP 2020 Automated Braunauer-Emmet-Teller Sorptometer.

X-ray-diffraction patterns for mineralogical analysis were carried out in a PHILIPS PW 1710 diffractometer, Cu Ka radiation Ni filtered.

A chemical analysis of the major elements was performed by the technique of inductively coupled plasma atomic-emission spectroscopy (expressed as % oxides; ALS Chemex Lab, Canada).

Raman spectra—obtained by a Via Renishaw micro-Raman spectrometer (785.0 nm laser line)—were analyzed by curve-fitting after background subtraction.

Thermal studies were done by means of a Shimadzu TG-50 for differential thermal gravimetric analysis. Additional thermal experiments were carried out in a furnace at controlled temperatures.

2.2. Fungal Isolate

The fungal strain used for testing the pyroclastic material as a carrier was *B. bassiana* (LPSC 1067) from the culture collection of the Spagazzini Institute (LPSC), La Plata, Argentina. The choice of this strain was based on its efficacy against pest grasshopper and locust species of Argentina in the laboratory [14,15]. Conidia of the fungal

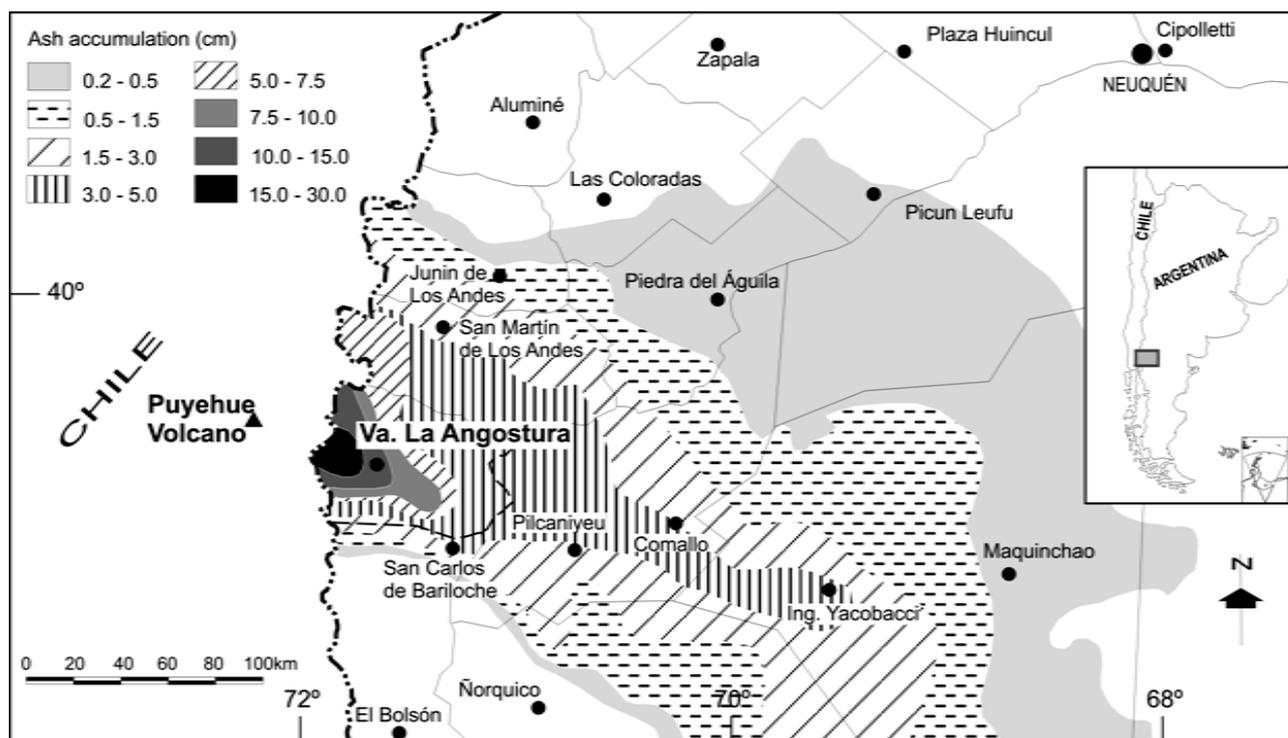


Figure 1. Distribution of the ash fall from the Puyehue-stratovolcano eruption (reported by the Instituto Nacional de Tecnología Agropecuaria, 2011).

strain were obtained from cultures on potato-dextrose-agar medium after incubation for 10 days at 25°C in the dark.

2.3. Preparation of the Conidial Suspension

Conidia were harvested with disposable cell scrapers (Fisherbrand®) from 10-day-old cultures and placed in test tubes containing 0.01% (v/v) Tween 80® (polyoxyethylene sorbitan monolaurate; Merck). Suspensions were vortexed for 2 min, filtered through four layers of sterile muslin, and adjusted to 1×10^8 conidia·ml⁻¹, according to Geden and Steinkraus [16] after cell counting in a Neubauer hemocytometer.

2.4. Impregnation in the Pyroclastic Material

In order to determine if the pyroclast constituted a potential carrier of *B. bassiana* conidia without loss of fungal viability and pathogenicity, the geomaterial was impregnated with the conidial suspension. The volcanic ash was first washed with sterile distilled water and left to dry at room temperature (in a laminar-flow chamber) for 24 h; then 1000 g were inoculated with a suspension of 1×10^8 conidia·ml⁻¹ of *B. bassiana* and placed in a sterile Erlenmeyer flask that was then capped tightly and stored at room temperature for 18 months. This entire process including the 18-month storage was carried out in triplicate.

2.5. Viability of the Fungal Conidia

Fungal-conidial viability was determined as described by Lane *et al.* [17] both before and after 18 months [14,15]. For this purpose, 1 g of the impregnated volcanic material was removed from the original stored sample and placed in test tubes containing 10 ml of 0.01% (v/v) Tween 80 (Merck). The suspension was vortexed for 2 min and filtered through four layers of sterile muslin. Artificial culture medium (800 µl) was placed in the form of a thin layer of approximately 2-mm thickness onto a microscope slide (previously sterilized by autoclaving) located in a Petri dish containing filter paper of 100-mm diameter moistened with sterile distilled water. Of the fungal suspension, 400 µl were inoculated into the culture medium wetting each slide. The Petri dish containing this culture was incubated at 24°C in the dark to facilitate the germination of the spores. After 24 h the germinated and nongerminated conidia were counted. A conidium was considered germinated when the germ tube reached half its length. Three replicates were performed, and 300 conidia were counted in each one.

2.6. Pathogenicity Bioassays

To determine whether the conidia of *B. bassiana* within the volcanic material had conserved their pathogenicity, both before and after the 18-month storage period, 100 g of the impregnated ash was placed in 90 × 15 mm

sterile Petri dishes together with 12 adults of the pest of poultry houses, the darkling beetle *Alphitobius diaperinus* (Coleoptera: Tenebrionidae), a coleopteron that causes great economic losses to those producers. The beetles were kept in the Petri dishes with the impregnated pyroclast for 10 days. During the course of the trial all insects were fed *ad libitum*. At the end of the trial, the dead individuals were counted and immediately transferred to high-humidity chambers (sterile Petri dishes with filter paper dampened with sterile distilled water). Mycosis was confirmed by microscopical examination of the dead beetles. Three replicates of the impregnated ash before storage and five at the conclusion were performed with the treated ashes along with five control replicates undergoing the same treatment but without the fungal inoculum. The ash-treated and control samples were placed at 25°C with a 12:12 h light:dark photoperiod. The controls were included to exclude the possibility that exposure to the vehicle *per se* might produce significant mortality.

2.7. Statistical Analyses

The Student t-test was used for evaluation of significant differences between the mortality caused by volcanic ash inoculated with conidia of *B. bassiana* and the ash-minus-conidia controls, with the data having been previously square-root transformed.

3. RESULTS AND DISCUSSION

3.1. Characterization of the Carrier

Figure 2 shows the particle-size-distribution curve of the pyroclastic material, showing that the major proportion corresponds to the size of sand (0.105 to 1.68 mm). Likewise, polarizing-microscope studies indicated the predominance (~80% - 90%) of vitreous fragments; where crystalline phases such as quartz, orthoclase, plagioclase, titanite, iron oxides (magnetite and hematite), and pyrite were observed in minor proportions (not

shown). Figure 3(a) shows the more frequent morphology of the individual particles of the pumices, featuring those with subrounded, elongated, and angular shapes (these last in lower proportion). Figure 3(b) provides a detail of a sector containing the pumice fragments at higher magnification where the porous surface becomes clearly distinguishable.

The typical SEM morphology of the pumiceous volcanic material (Figure 4) was dominated by vesiculated particles with few connections among the channels and pores. The average BET-calculated surface area was $2.23 \text{ m}^2\text{g}^{-1}$, indicating pores of $\sim 114 \text{ \AA}$ within the mesopore size range. On the other hand, the material was found to contain a high degree (72.2% - 73.4% of the total volume) of macroporosity by the Hg-sortometer technique, thus indicating a low overall density.

The X-ray-diffraction pattern of the pyroclast indicated a major proportion of an amorphous vitreous phase. The low proportion of crystalline phases, when examined, showed the presence of substituted calcium plagioclase ($\text{CaAl}_2\text{Si}_2\text{O}_8$) and pyroxene ($\text{Mg}_2\text{Si}_2\text{O}_6$; Powder-Diffraction File [PDF] 89-1463, 85-1740, 88-2377). Likewise, iron oxides, observed as weak reflections, can be assigned to binary and/or mixed oxides, e.g. magnetite-type structures (PDF 80-0390) as well as to hematite or related phases (PDF 89-2810).

These crystalline compounds were corroborated by micro-Raman spectroscopy, a technique that reveals the individual components within a mixture of complex composition. Figure 5(a) with lines at 325, 380, 530, 665, 888 and 999 cm^{-1} corresponds to clinopyroxene (characterized by signals at 322, 667 and 997 cm^{-1} [18]) and plagioclase (typical strong signal at 510 cm^{-1} and weak signals in the 1000 cm^{-1} region [18]). Spectrum of Figure 5(b) shows bands at 221, 290, 406, 605 and 1308 cm^{-1} indicates the presence of hematite (identified by lines at ~ 225 , 291, 411, 611 and 1321 cm^{-1} [19,20]) whereas a shoulder at 662 cm^{-1} could be assigned to magnetite (signal at $\sim 665 \text{ cm}^{-1}$ [19,20]).

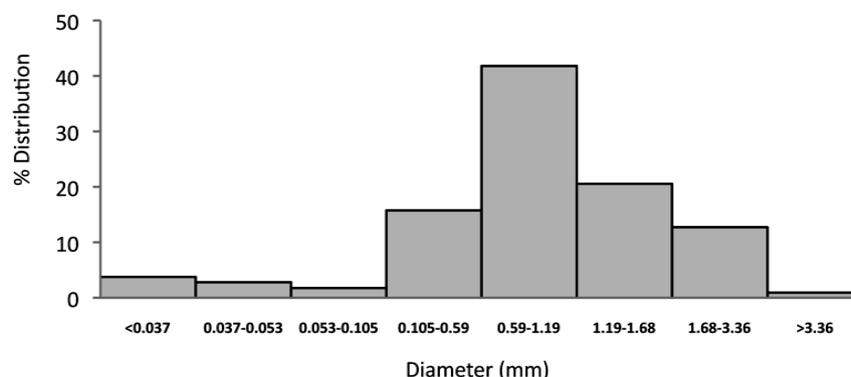


Figure 2. Grain-size-distribution curve. In the figure the percent size distribution is plotted versus the grain diameter in mm.

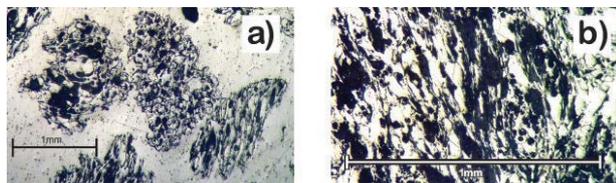


Figure 3. Reflection polarized light microscope images of the pyroclast pumice fragments showing (a) The shapes of the pumices; (b) Details of their porous surface.

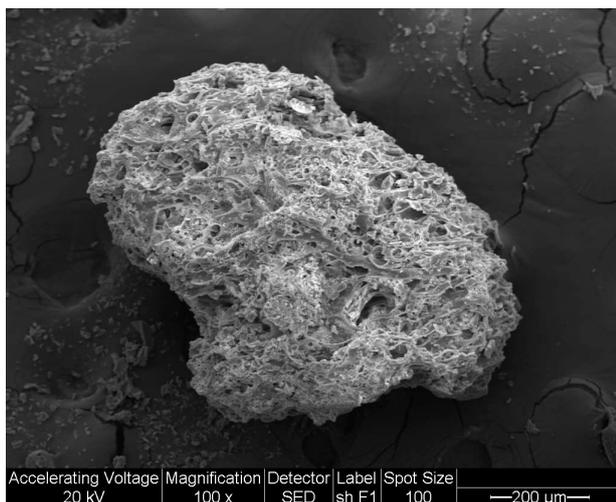


Figure 4. SEM appearance of a typical pumicite grain.

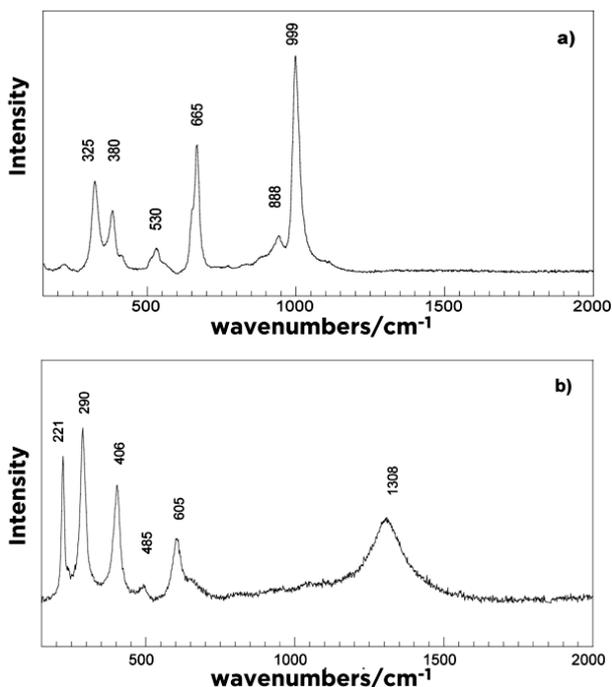


Figure 5. Micro-Raman spectra registered in different point locations within the pyroclast.

The water loss proved variable, depending on the atmospheric moisture. Differential-thermal-gravimetry stu-

dies indicated two consecutive weight-loss signals in general: room temperature (RT)—80°C and 80°C - 200°C, suggesting the presence of adsorbed water to different degrees. The pumiceous particles lost 7% of the adsorbed moisture at 50°C, 14% at 100°C and 18% at 200°C. The hydric retention under adequate conditions can reach about 30%. A thermal stability between RT and 800°C was indicated by X-ray diffraction, while the dehydration process (between the RT and ~100°C) was reversible—a behavior similar to that observed in zeolite minerals.

Furthermore, the chemical analysis of a dried sample (**Table 1**) indicated the rhyolite-like composition characteristic of pumiceous material, with an iron content (expressed as % Fe₂O₃) of 5.58. Notably, Ca, Mg, Na, K, Mn, Ba, Sr, and P were present as minor components.

3.2. Use of Volcanic Material as Vehicle of *Beauveria bassiana* Conidia

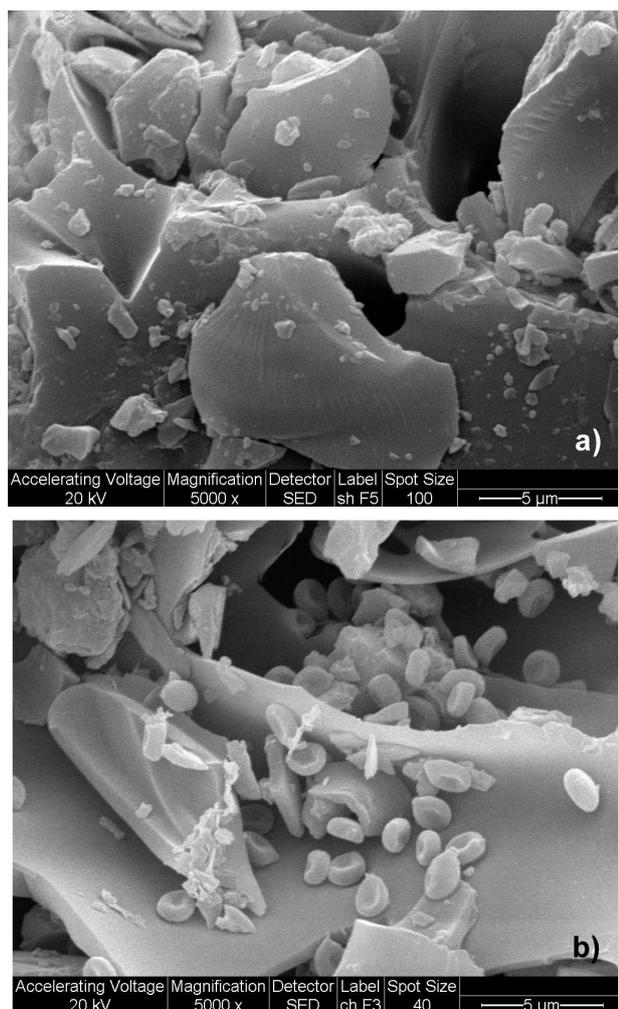
The use of dust carriers may improve the storage of the conidia as well as its distribution and application in the field [21]. Indeed, even under these conditions of nonrefrigeration for such a long period the viability of the *B. bassiana* (LPSC 1067) conidia was 97.4% ± 0.7% (where the average initial values were determined at 98.5 ± 0.8). An optimal shelf life of fungal spores under non-refrigerated storage conditions is of paramount practicality since mycopesticides may be exposed to high temperatures during transport, warehousing, or on-farm storage [22-24]. Therefore, the very high viability determined after this prolonged period of storage demonstrates that the pyroclast obtained after the recent PCCVC eruption is a promising geomaterial for use as a carrier for the biocontrol agent *B. bassiana* and possibly for other fungi.

The SEM micrograph of **Figure 6(b)** shows the distribution and relative size of the conidia among the particles of the inoculated volcanic ash after 18 months at room temperature. The conidia shown exhibit a certain morphologic deformation, probably an artifactual result of the preparation used for scanning electron microscopy since their viability was as high as 97% before the preparation of the samples for SEM.

Previous studies indicated that the spore longevity of entomopathogenic fungi was inversely proportional to temperature [25-27]. Blanford *et al.* [28] studied the interaction between temperature and humidity on the viability of nonformulated *B. bassiana* spores and found half-lives of decay of 31, 49, and 71 days at 32°C, 26°C, and 22°C, respectively, in environments with low relative humidities, but a slower decay in viability with half-lives of 67 and 117 days at 26°C and 22°C, respectively, in environments with high relative humidities. Therefore, compared to those data with nonformulated spores, the present results showed that the volcanic material tested

Table 1. Major and minor elements (expressed as % oxides) by ICP-AES.

Sample	PM
SiO ₂	67.04
TiO ₂	0.89
Al ₂ O ₃	14.17
Fe ₂ O ₃	5.58
MnO	0.14
MgO	1.05
CaO	2.95
Na ₂ O	5.04
K ₂ O	2.37
P ₂ O ₅	0.17
BaO	0.08
SrO	0.02
LOI	0.50

**Figure 6.** SEM micrograph of (a) The pyroclastic material without *B. bassiana*; (b) The same material impregnated with *B. bassiana*.

clearly favoured the prolongation of conidial viability. Since biocontrol products must remain stable for at least one year in order to be commercially acceptable [25]; the utilization of pyroclastic material could constitute a stable, simple, and low-cost vehicle for *B. bassiana* formulations.

Because of the normal local seasonal variations occurring during the storage period, this formulation (*i.e.*, geomaterial with bioinsecticide) was necessarily subjected to a substantial temperature variability (*i.e.*, 5°C - 40°C). Nevertheless, this degree of variation did not affect the viability of *B. bassiana* conidia. A physico-chemical characterization of the matrix dust used under the conditions of experimental storage (*i.e.*, the mass of the pyroclastic material and the volume of the receptacle) revealed an average weight loss of only 3% at up to 40°C because the loss depended on the nature of the components of the pyroclastic mixture (0.5% for the crystalline phases and between 4% and 6% for the pumiceous particles). Considerable disagreement exists in literature about the effect of moisture content on the viability of entomopathogenic fungal spores, a question that is even more complicated considering the influence of temperature. Liquid water is cited as diminishing the viability of mycopesticide products by promoting spore germination [25]. Nevertheless, the decline of *B. bassiana* viability because of high temperatures can be significantly lower at high humidity environments [28]. The specific interactions with water that take place within the mass of this pyroclastic material may produce an environment with a suitable intermediate vapor pressure so as to protect the spores from desiccation at high temperatures but still minimize the accumulation of liquid water that would necessarily cause a decline in viability. Therefore, the type of water in the material—*i.e.*, that which is only adsorbed to the vesicles by a net physical process, thus leaving the material subject to a suitable vapor pressure (e.g., from water evaporation in a closed container dependent on the temperature, or by effect of the relative humidity of the environment)—may help prolong the longevity of spores.

Statistical analysis of the pathogenicity bioassays demonstrated that the high percentages of mortality among the insects that were placed in the inoculated volcanic material after 18 months of storage ($63.3\% \pm 3.5\%$) were significantly different ($t = 2.31$, $p = 0.001$) from the mortality of those exposed to the control substrate ($8.31\% + 0.7\%$; **Figure 7**). These results also indicated that contact of the insects with the ash alone produced only minimal mortality. In addition these same mortality determinations when performed on the conidia immediately after impregnation in the volcanic ash gave levels of $65.3\% \pm 2.5\%$, which values were statistically indistinguishable from the results represented in the figure (not shown).

That level of mortality is high, compared to other studies evaluating the effects of *B. bassiana* on darkling beetles [29].

All the dead beetles in contact with the impregnated volcanic material exhibited an external growth of the fungus after a 24-h incubation in a humid chamber, thus demonstrating that death had been caused by mycosis (Figure 8). Darkling beetles represent one of the most pernicious pests in poultry production since they are known to transmit the viruses causing Marek's disease, fowl pox, avian reovirus, infectious bursal disease, and Newcastle disease along with *Aspergillus* spp. and coccidia [12,13]. Because of the physical characteristics of the geomaterial tested, the ashes could be easily spread as a dust carrying *B. bassiana* for the biocontrol of *A. diaperinus* in poultry houses.

4. CONCLUSION

The results from bioassays on the killing efficiency of entomopathogenic fungi contained in volcanic materials from the PCCVC as a carrier proved auspicious for the

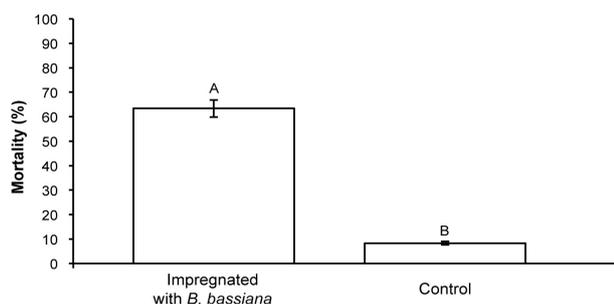


Figure 7. Percent mortality (means \pm SD) of *Alphitobius diaperinus* exposed to volcanic ash impregnated with *Beauveria bassiana* conidia and the volcanic-ash control. Values with different letter superscripts indicate significant differences according to the Student t-test.



Figure 8. Dead adults of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) infected by *Beauveria bassiana* conidia from impregnated volcanic ash after a 24-h incubation in a humid chamber.

eventual utilization of the pyroclast as a bioinsecticide vehicle. The biologic results in combination with the physicochemical properties of the carrier revealed that the formulation is stable for at least 18 months under a wide range of environmental conditions. We believe that the material tested can serve as an excellent substitute for other dust vehicles used to maintain the viability and pathogenicity of conidia of *B. bassiana*. In conclusion, this geomaterial would appear to be highly suitable for the preparation of formulations for pest control; thus utilizing what would otherwise be volcanic waste as an efficient, abundant, and inexpensive carrier of entomopathogenic fungi.

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