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Effectiveness of several nutritional sources on the virulence of *Beauveria bassiana* s.l. CEP147 against the planthopper *Delphacodes kuscheli*

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Abstract

Delphacodes kuscheli Fennah (Hemiptera: Delphacidae) is the main planthopper vector of *Mal de Río Cuarto virus* (MRCV), a *Fijivirus* that severely affects maize production in Argentina. The effect of several nutritional sources on the virulence of *Beauveria bassiana* s.l. CEP147 (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) against this planthopper was evaluated with the aim to select some of them to be incorporated in future mass production studies. Ten agar-agar 2% media were used. Media were supplemented with 10 g of sucrose and 4% flour of either amaranth, chia, flax, oat bran, parboiled rice, poppy, quinoa, or wheat germ. Sabouraud dextrose agar with 1% yeast extract (SDAY) was used as control, and sucrose agar medium supplemented with 2% chitin was used as a possible inductor of enzymatic activity. We evaluated the effect of each medium on colony growth, conidial yield, viability, size, adherence, hydrophobicity, protease activity, cumulative mortality, and median survival time (MST). Principal component analysis indicated that conidial viability and adherence explained the most of the total variance, indicating that these variables can be used as indicators of fungal virulence. Medium supplemented with chia flour not only increased conidial viability (100%) and adherence (0.09 A_{550}) with respect to the control medium, but also produced high *D. kuscheli* mortality (91.7%), with the lowest MST (5.2 days). We suggest that chia flour, which is rich in carbohydrates, proteins, vitamins, and minerals, might be incorporated as a nutritional supplement to solid substrates in order to increase the virulence of *B. bassiana* s.l. CEP147 against this and other pests. However, future studies are necessary to evaluate the effectiveness of this nutritional source on solid-state fermentation.

Introduction

The planthopper *Delphacodes kuscheli* Fennah (Hemiptera: Delphacidae) is the most important vector of *Mal de Río Cuarto virus* (MRCV), a *Fijivirus* (Reoviridae) that is transmitted in a persistent propagative manner and severely affects maize, *Zea mays* L. (Poaceae) production in the main growing areas of Argentina (Giménez Pecci et al., 2012). MRCV also infects wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.) (all Poaceae), and several grass weeds, which are potential reservoirs of the virus and natural hosts for its vector during fall and winter (Laguna et al., 2000). Since more than 2 decades, the most effective strategies to manage MRCV have been early or late seeding, according to the prediction system of the vector population level, the application of systemic insecticides to the seeds, and mainly, the use of tolerant cultivars (Ornaghi et al., 2011; Giménez Pecci et al., 2012). However, when the viral load is too high,

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tolerance might be overcome, thereby increasing the prevalence, incidence and severity of the disease (Lenardón et al., 1999), a situation that also is favored when outbreak populations of *D. kuscheli* occur as was reported in the year 2019 (Rossi, 2019).

Considering the resistance to systemic insecticides developed in pest insect populations and their negative environmental impact, pathogenic fungi have been proposed as a more eco-friendly tactic to control insects, especially those with piercing-sucking mouth parts (Li & Lee, 2014; Bugti et al., 2018; Parys & Portilla, 2020). In this context, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) has been one of the most commonly used species, mainly due to its easy mass production, wide host range, and compatibility with some pesticides and fungicides (Rehner, 2005; de Faria & Wraight, 2007; Wraight et al., 2007). Previous studies by Toledo et al. (2007) showed that *B. bassiana* s.l. CEP147 was highly virulent against *D. kuscheli* and other planthopper vectors of corn pathogens in Argentina, indicating its potential as biocontrol agent of these pests. The use of fungi as biocontrol agents of insect pests depends on numerous biological constraints, including the ability to produce high concentrations of stable propagules at a reasonable cost (Jaronski, 1986; Latgé, et al., 1986). Although most research has focused on increasing conidial yield, currently there is an increasing interest in improving also conidial quality, considering that conidial features such as size, viability, surface characteristics, and enzymatic activity are key indicators of fungal virulence in the pathogenesis process (Altre et al., 1999; Safavi et al., 2007; Shan et al., 2010; Mascarin et al., 2013; Zulfiana et al., 2020). Several studies have shown that all these parameters fluctuate according to the composition of the medium in which the fungus is cultivated (Ibrahim et al., 2002; Shah et al., 2005; Safavi et al., 2007; Ali et al., 2009; Shan et al., 2010; Pedrini et al., 2011; Mascarin et al., 2013; Butt et al., 2016; Zulfiana et al., 2020). Nutrients, minerals, and vitamins supplied by the substrate are key elements to support fungal growth, providing the energy source and co-factors for biochemical reactions as well as affecting fungal morphogenesis, growth rate, and propagule quality and fitness for use in biological control (Jackson, 1997; Jaronski & Mascarin, 2016). For this reason, it is important to define the appropriate nutritional conditions for the production of large quantity of high-quality conidia. Taking this into account, we evaluated the effect of various nutritional sources added to sucrose agar medium on virulence factors of *B. bassiana* s.l. CEP147 in order to select the best source to use in future mass production studies using solid-state fermentation (SSF).

Materials and methods

Insects rearing

The planthopper *D. kuscheli* was collected in the endemic area of MRCV (Río Cuarto, Córdoba province, Argentina) from oat plants. A colony from this natural population was maintained for over 5 years on oat plants at 22 ± 3 °C, 50–55% r.h., and L16:D8 photoperiod in the bioterium of Division Entomology, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Buenos Aires, Argentina.

Fungal strain

A highly virulent isolate of *B. bassiana* s.l. previously evaluated against planthopper pests of maize crops by Toledo et al. (2007) was used. Isolate namely CEP147 was obtained from infected adults of *Cycloneda sanguinea* L. (Coleoptera: Coccinellidae) collected on maize in Tucumán province, Argentina (26°49′S–65°16′W), and was preserved at the mycological collection of the USDA-Agricultural Research Service Collection of Entomopathogenic Fungal Cultures-ARSEF (Ithaca, NY, USA) under the accession number ARSEF 8372. In addition to its morphological identification, its identity was confirmed by its internal transcribed spacer (ITS) sequence available at the GenBank database (www.ncbi.nlm.nih.gov) under the accession number KF308683.1 (Toledo et al., 2019).

Culture media

Ten culture media were used in the assays. Nutrient-rich medium Sabouraud dextrose agar with yeast extract (SDAY: 20 g dextrose, 20 g peptone, and 10 g yeast extract per liter of distilled water) was used as control. The other media were made up of 10 g of sucrose (commercial sugar; Ledesma, Buenos Aires, Argentina) and 20 g agar per liter of distilled water, and were supplemented with 4% flour of either amaranth (*Amaranthus quitensis* H.B.K.), chia (*Salvia hispanica* L.), flax (*Linum usitatissimum* L.), oat bran, parboiled rice (*Oryza sativa* L.), poppy (*Papaver rhoeas* L.), quinoa (*Chenopodium quinoa* Willd.), or wheat germ. Nutritional composition of seeds used is shown in Table 1. Sucrose agar medium supplemented with 2% chitin (Sigma-Aldrich, Burlington, MA, USA) was used as possible inductor of enzymatic activity. Chitin was directly incorporated into the media and dissolved together with the sucrose before being autoclaved. Media were sterilized in an autoclave for 20 min at 121 °C and 1 atm and their pH was measured (culture media with quinoa or chitin: pH = 5.5; media with wheat germ, oat bran, parboiled rice, flax, or amaranth: pH = 6; SDAY and

Table 1 Nutritional composition of seeds (values per 100 g) added as flour to the various culture media tested

	Amaranth	Chia	Flax	Oat bran	Parboiled rice	Poppy	Quinoa	Wheat germ
Protein (g)	13.56	16.54	18.29	17.3	7.51	17.99	14.12	23.15
Total lipid (g)	7.02	30.74	42.16	7.03	1.03	41.56	6.07	9.72
Carbohydrate (g)	65.25	42.12	28.88	66.22	80.89	28.13	64.16	51.8
Calcium (mg)	159	631	255	58	71	1438	47	39
Iron (mg)	7.61	7.72	5.73	5.41	0.74	9.76	4.57	6.26
Magnesium (mg)	248	335	392	235	27	347	197	239
Phosphorus (mg)	557	860	642	734	153	870	457	842
Potassium (mg)	508	407	813	566	174	719	563	892
Sodium (mg)	4	16	30	4	2	26	5	12
Zinc (mg)	2.87	4.58	4.32	3.11	1.02	7.9	3.1	12.29
Thiamin (mg)	0.12	0.62	1.64	1.17	0.22	0.85	0.36	1.88
Riboflavin (mg)	0.2	0.17	0.16	0.22	0.05	0.1	0.32	0.49
Niacin (mg)	0.9	8.83	3.08	0.93	5.05	0.89	1.52	6.81
Vitamin B-6 (mg)	0.59	0	0.47	0.17	0.45	0.25	0.49	1.3
Folic acid (μg)	82	0	87	52	8	82	184	281
Vitamin A (UI)	2	54	0	0	0	0	14	0
Vitamin E (mg)	0.19	0.5	0.31	1.01	0.03	1.77	2	0

Source: USDA National Nutrient Database for Standard Reference (https://fdc.nal.usda.gov/).

medium with chia: pH = 6.5; medium with poppy: pH = 7).

Colony growth and conidial yield

To evaluate the effect of culture medium composition on colony growth and conidial yield, each medium was inoculated with a 7-mm-diameter mycelial plug taken from the edge of 3-day-old cultures grown on SDAY at 26 ± 1 °C in darkness. Inoculated Petri dishes were incubated for 10 days at 26 ± 1 °C in darkness. Mycelial growth was estimated based on colony radial increase, which was measured between two drawn orthogonal diameters. To determine the conidial yield, a 7-mm-diameter plug was taken near to the center of each colony (n = 6), suspended in 5 ml of 0.01% (vol/vol) Tween 20 (Biopack, Buenos Aires, Argentina), and conidia were counted using a Neubauer chamber.

Viability and conidial size

Viability of conidia was assessed by harvesting them from colonies grown on each medium for 7 days at 26 °C in darkness. Conidial suspensions were made in 0.01% (vol/vol) Tween 20 and were adjusted to 10^6 conidia ml^{-1} using a Neubauer chamber. Aliquots of 100 μl of each suspension were distributed over the surface of a translucent SDAY medium in 9-cm-diameter Petri dishes that were incubated 17 h at 26 °C in darkness. In total, 1200 conidia per culture medium were counted as described by Inglis et al. (2012) and were considered viable if germ-tube lengths were 2× their diameter.

Conidia produced by the isolate grown for 10 days at 26 °C in darkness were harvested, stained with lactophenol cotton blue, and their length and width were measured under a WILD M-20 microscope at 1000× magnification. Conidia (n = 30 per medium) were considered as ellipsoidal, and their surface area (S_a, μm^2) was calculated using the formula $S_a = 2(2b)^{1/2}(a^2 + b^2)^{1/2}$, where a is the length and b is the width of a conidium (Shan et al., 2010).

Conidial adherence and hydrophobicity

To evaluate the effect of culture medium composition on conidial adherence, we modified the methodologies described by Pierce et al. (2008), Melo et al. (2011), and Melloul et al. (2016). Conidia were harvested from 10-day-old cultures grown at 26 °C in darkness and suspended in phosphate-buffered saline (PBS) pH 7.4 containing 0.025% (vol/vol) Tween 20. Conidial suspension was adjusted to 10^8 conidia ml^{-1}. One hundred μl of each conidial suspension was transferred to 96-well plates as well as 100 μl of the control (only PBS), and incubated for 4 h at 25 °C. The remaining conidial suspension was preserved as negative control at 4 °C. After 4 h, 100 μl of negative control suspension was added to the plates, the supernatant was carefully removed, and the wells were gently washed twice with 200 μl of PBS and dried for 20 min at 30 °C. A 250-μl volume of 0.1% crystal violet (CV) aqueous solution was added to each well and plates were incubated for 30 min at 30 °C. Then, wells were gently washed twice with 300 μl of PBS and dried. Finally, each well was loaded with 200 μl of methanol and plates

were incubated for 20 min in agitation (80 rpm). The quantity of CV dissolved in methanol, corresponding to the conidia that remained attached to the wells, was estimated by measuring absorbance at 550 nm using a 96-well plate reader (BioTek, Winooski, VT, USA).

Conidial hydrophobicity was assessed using a modification of the aqueous–solvent partitioning method (Shan et al., 2010). Conidia were harvested from cultures incubated for 10 days at 26 °C in darkness and suspended in PM buffer (6.97 g K_2HPO_4, 2.99 g KH_2PO_4, and 0.2 g $MgSO_4\,7H_2O\,l^{-1}$ of distilled water; final pH 7.12) containing 0.02% (vol/vol) Tween 20. Conidial suspensions were standardized to a concentration of 10^7 conidia ml^{-1}. Mineral oil (USBTM, Cleveland, OH, USA) was used as organic phase and was added to the conidial suspension at the ratio of 40 µl over 4 ml. Three replicates were made for each treatment. The mixture was placed in 15-ml disposable tubes, vortexed for 2 min, and then transferred to three 10-ml glass burettes. Burettes were preserved at 4 °C for 1 h and when the aqueous/organic phase was separated, one aliquot of 200 µl of each burette was collected in Eppendorf tubes. Conidia present in each aqueous phase were counted 2× using a Neubauer chamber. Finally, a total of six counts per treatment were made. The hydrophobicity rate (H_r) was assessed as $H_r = (1–C/C_0) \times 100$, where C_0 is the conidial concentration of the aqueous suspension before organic phase was added and C corresponds to the conidial concentration in the aqueous phase after phase partitioning.

Conidial protease activity

The protease activity of conidia was determined according to the method using by Coêlho et al. (2016), with some modifications. Azocasein (Sigma-Aldrich) was used as substrate. Conidia were collected from fungal cultures grown for 10 days at 26 °C in darkness and suspended in 10 ml of saline solution adjusting conidial concentration to 10^8 conidia ml^{-1} using a Neubauer chamber. A 500-µl aliquot of conidial suspension was mixed with 500 µl of azocasein solution (2% azocasein, 50 mM Tris-HCl, pH 7), vortexed and incubated for 4 h at 28 °C with gentle vortexing every 30 min. A 500-µl aliquot of a 10% trichloroacetic acid solution (TCA) was added to each sample, shaken vigorously and centrifuged for 10 min at 10000 *g*. A 0.6-ml aliquot of the supernatant was added to an Eppendorf tube containing 0.7 ml of 1 M NaOH and vortexed. Finally, 200 µl of the mixture was transferred to a 96-well plate avoiding the formation of air bubbles. Treatments included conidial suspension, azocasein and TCA, whereas controls were designed as shown below: control 1 (azocasein and TCA without conidial suspension), control 2 (conidial suspension and TCA without azocasein), control 3 (conidial suspension, azocasein, and TCA incubated at 95 °C), and control 4 (conidial suspension, azocasein and TCA + 10 mM phenylmethylsulfonyl fluoride, a serine protease inhibitor). The average control value was subtracted from each of the treatments to correct the results. Absorbance was determined at 450 nm, and the units of enzymatic activity were calculated by means of the following formula: $U = \Delta Abs\ g^{-1}$ substrate $ml^{-1}\ h^{-1}$, where ΔAbs = reaction absorbance−control absorbance.

Cumulative mortality and median survival time

The virulence of conidia of fungal isolate generated on each culture medium was evaluated on 30 adults (2–6 days old) of *D. kuscheli*. Adults were placed in groups of 10 into PET plastic bottles (500 ml capacity), whose bottoms were cut off and sealed with Parafilm that was punched to make small holes to allow air diffusion according to Toledo et al. (2007). Conidia were harvested from cultures grown at 26 °C in darkness for 10 days and were suspended in 5 ml of 0.01% (vol/vol) Tween 20. Conidial suspensions were standardized to 10^7 conidia ml^{-1} and 300 µl per group of 10 insects were inoculated using a professional airbrush (model 180, nozzle diameter 0.25–0.3 mm, fluid cup capacity 9 ml). The bottle assemblies were placed upside down on 200-ml glass flasks containing young oat plants (two expanded leaves) with their roots wrapped in cotton and immersed in distilled water. In total, 30 insects in groups of 10 were used as control and were sprayed with 300 µl of 0.01% (vol/vol) Tween 20. Treated and control insects were maintained at 25 ± 1 °C, 80% r.h., and L12:D12 photoperiod. Insects were checked every 24 h up to 7 days and young oat plants were changed every 3 days. Dead insects were removed daily and were superficially sterilized in 70% ethanol for a few seconds, washed in sterile distilled water, and placed in 0.5% sodium hypochlorite for 1 min. Finally, they were rinsed again in distilled water, placed in Petri dishes with filter paper moistened with sterile distilled water, and incubated at 25 °C for 3–5 days. Only those insects showing external mycelia growth were considered to be dead due to fungal infection. After 7 days, the cumulative mortality (%) was recorded and median survival time (MST; days) was calculated.

Data analysis

Experiments included 10 treatments, each with six replicates when radial growth, conidial yield, and viability were analyzed, and three replicates when adherence, hydrophobicity, protease activity, cumulative mortality, and MST were analyzed. Conidial adherence, hydrophobicity and virulence assays were performed twice, and conidial

protease activity assay was performed in triplicate. After appropriate transformation, data that reached the assumptions of normality and homoscedasticity (conidial yield, hydrophobicity, and cumulative mortality) were analyzed by parametric ANOVA followed by Tukey's honestly significant difference (HSD) procedure for all pairwise comparisons ($\alpha = 0.05$). Data that did not reach the assumptions of normality and homoscedasticity (radial growth, surface area, viability, adherence, and protease activity) were analyzed by non-parametric Kruskal–Wallis test, and medians were separated by the method described by Conover (1999). Survival analysis based on the Kaplan–Meier method was used to determine survival probability functions as well as to estimate the MST. The survival curves for different treatments were compared by the log rank χ^2 test ($\alpha = 0.05$). Correlations between cumulative mortality, MST, and conidial features such as surface area, adherence, hydrophobicity, and protease activity were calculated by Pearson's method. A principal component analysis (PCA) was used in order to show how nutritional sources can be grouped according to quality parameters of the conidia. Because the variables were measured in different scales, the PCA was performed with standardized data. In addition, a cluster analysis was performed with Ward's method (Ward, 1963) to calculate the Euclidean distances and to classify the different culture media on the basis of the meaningful observed variables derived from PCA. All analyses were performed using the InfoStat-Professional software, version 2020 (Di Rienzo et al., 2020).

Results

Colony growth and conidial yield

Growth of *B. bassiana* s.l. CEP147 was affected by the culture medium (H = 40.16, d.f. = 9, P<0.0001). Only on media supplemented with chitin or parboiled rice, colony growth was significantly less than on SDAY control medium. In the rest of the media, colony growth was similar to that on SDAY (Table 2). Conidial yield was also affected by culture medium composition ($F_{9,50} = 12.03$, P<0.0001). It was significantly less in all media in comparison to the SDAY control medium, but among them there were no statistically significant differences (Table 2).

Viability and conidial size

Conidial viability was significantly affected by the culture medium (H = 45.03, d.f. = 9, P<0.0001). In media supplemented with chia, chitin, parboiled rice, poppy, or wheat germ it was similar to that produced on SDAY. In the other media, conidial viability was lower than that recorded on SDAY (Table 2). Conidial size also was modified by culture medium composition (H = 98.85, d.f. = 9,

Table 2 Influence of culture composition on growth and virulence of *Beauveria bassiana* s.l. CEP147

Medium	Radial growth[1] (cm)	Conidial yield[2] (mean no. conidia $ml^{-1} \pm$ SE)	Viability[1] (%)	Surface area[1] (μm^2 per conidium)	Adherence[1] (A_{550})	Hydrophobicity[2] (mean % ± SE)	Protease activity[1] (ΔAbs)	Cumulative mortality[2] (mean % ± SE)
Amaranth	4 (1.1)ab	$5.8 \times 10^7 \pm 6.3 \times 10^7$a	86.45 (5.2)a	13.62 (0)e	0.02 (0.01)a	87.1 ± 1.5cd	0.45 (0.02)fg	95 ± 3.4b
Chia	5.3 (0.5)c	$7.5 \times 10^7 \pm 5.3 \times 10^7$a	100 (0)d	13.62 (3.19)c	0.09 (0.07)c	86.3 ± 1.2cd	0.15 (0.01)ab	91.66 ± 6.5ab
Chitin	1.4 (0.3)a	$1.3 \times 10^8 \pm 1.3 \times 10^8$a	98.75 (2)bcd	13.62 (0)de	0.1 (0.01)c	89.9 ± 0.9de	0.006 (0.01)a	90 ± 3.6ab
Flax	5.45 (0.3)c	$1.05 \times 10^8 \pm 3.4 \times 10^7$a	96.65 (4.7)ab	10.43 (0)a	0.05 (0.03)ab	30.5 ± 3.3a	0.37 (0.01)efg	73.33 ± 12.2ab
Oat bran	5.45 (0.1)c	$7.2 \times 10^7 \pm 7.2 \times 10^7$a	83.45 (5.6)a	10.43 (3.19)bc	0.03 (0.02)ab	77.7 ± 2.2b	0.32 (0.02)cdef	60 ± 4.5a
Parboiled rice	3.05 (0.4)a	$9.4 \times 10^7 \pm 3.8 \times 10^8$a	99.25 (1.5)bcd	13.62 (3.19)cd	0.048 (0.003)ab	81.1 ± 0.6bc	0.22 (0.01)abc	71.66 ± 6.5ab
Poppy	5.2 (0.5)c	$5.2 \times 10^7 \pm 5 \times 10^7$a	98.8 (1.9)bcd	13.62 (0)de	0.1 (0.02)c	95.8 ± 0.5f	0.68 (0.04)g	76.66 ± 8.8ab
Quinoa	5.1 (0.8)c	$6.7 \times 10^7 \pm 6.8 \times 10^7$a	97.3 (1.4)ab	13.62 (3.19)c	0.07 (0.04)bc	95.1 ± 0.9f	0.35 (0.02)defg	85 ± 6.2ab
Wheat germ	5.35 (0.01)c	$6.7 \times 10^7 \pm 3.4 \times 10^7$a	97.95 (2.8)bc	10.43 (3.19)ab	0.14 (0.06)c	94.7 ± 1.1ef	0.28 (0.01)bcde	81.66 ± 5.4ab
SDAY[3]	5 (1)bc	$8.1 \times 10^8 \pm 1.3 \times 10^8$b	99.75 (1)cd	10.43 (3.19)ab	0.023 (0.04)ab	95.5 ± 0.5f	0.26 (0.02)abcd	85 ± 5. ab

[1]Radial growth, viability, surface area, adherence, and protease activity are presented as median and interquartile range (IQR). Values within a column followed by different letters denote significant differences among treatments (Kruskal–Wallis tests followed by comparison of medians according to Conover: P<0.05).
[2]Conidial yield, hydrophobicity, and cumulative mortality are presented as mean ± SE. Values within a column followed by different letters differ significantly (Tukey's HSD test: P<0.05).
[3]Sabouraud dextrose agar with 1% yeast extract.

P<0.0001). The surface area of conidia produced on media supplemented with amaranth, chia, chitin, parboiled rice, poppy, or quinoa was greater than that produced on SDAY (Table 2).

Conidial adherence and hydrophobicity

Adherence of conidia was affected by culture medium composition (H = 44.29, d.f. = 9, P<0.0001). Conidial adherence increased with respect to the control when the fungal isolate was grown on chia, chitin, poppy, or wheat germ (Table 2). Conidial hydrophobicity was also affected by culture medium ($F_{9,50}$ = 119.65, P<0.0001). Hydrophobicity recorded on media containing poppy, quinoa, or wheat germ was similar to that on SDAY. In cultures grown on the other media hydrophobicity of conidia was lower than that of those produced on control medium (Table 2).

Conidial protease activity

Culture medium also affected conidial protease activity (H = 55.97, d.f. = 9, P<0.0001). The enzymatic activity of conidia significantly increased compared to the control in media supplemented either with amaranth, flax, or poppy (Table 2).

Cumulative mortality and MST

Cumulative mortality of *D. kuscheli* was affected by the culture medium in which the fungus was grown ($F_{9,50}$ = 2.25, P = 0.034). Higher mortality (>85%) was caused by conidia produced on media supplemented with amaranth, chia, or chitin, whereas the lowest mortality was caused by conidia produced on medium supplemented with oat bran (Table 2). Values of MST were also affected by the composition of culture medium. Kaplan-Meier survival curves differed significantly (χ^2 = 26.77, P = 0.002). Values of MST ranged from 5.17 days (chia) to 5.98 days (parboiled rice) (Figure 1).

Correlation between variables

Some interesting direct correlations were observed between variables associated with fungal virulence. According to Pearson's method, we found some positive correlations between *D. kuscheli* cumulative mortality and *B. bassiana* s.l. CEP147 conidial surface area (r = 0.2, P = 0.0005) and adherence (r = 0.12, P = 0.05). Likewise, high values of conidial surface area were positively associated with high values of conidial hydrophobicity (r = 0.26, P = 0.000007) and protease activity (r = 0.14, P = 0.02). In addition, conidia with high values of adherence were positively associated with high values of conidial hydrophobicity (r = 0.25, P = 0.00004). On the other hand, an indirect correlation was observed between conidial adherence and *D. kuscheli* MST (r = −0.12, P = 0.00004).

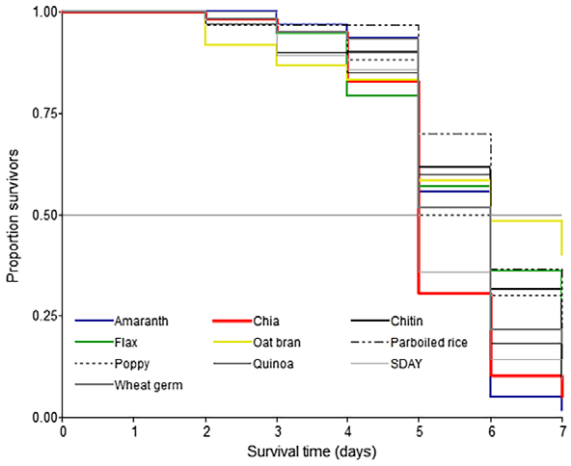

Figure 1 Survival curves of *Delphacodes kuscheli* inoculated with conidial suspensions of *Beauveria bassiana* s.l. CEP147 grown on various media. SDAY, Sabouraud dextrose agar with 1% yeast extract.

Principal component and cluster analysis

The PCA provided additional information about the variability between the performance and the virulence of isolate *B. bassiana* s.l. CEP147 grown on different culture media. Components 1, 2, and 3 explained 82% of the total variance and displayed eigenvalues >1 (Table 3). In general, all culture media contributed to the distribution shown in the biplot graph (Figure 2), where it was clearly observed that conidia produced on media supplemented with chia, chitin, or wheat germ showed high values of viability and adherence. Likewise, conidial surface area and hydrophobicity were higher when the fungus was cultivated on medium supplemented with poppy. On the other hand, conidia with the highest protease activity were differentiated when the fungus grew on media supplemented with poppy or amaranth. Conidial adherence and viability were the most important variables for the first component, which together explained the maximal amount of total variance (37%). The second component explained 30% of total variance and was associated with conidial surface area and hydrophobicity. Finally, the third component explained 16% of total variance and was related with the variable protease activity. The cluster analysis showed two distinct groups with similar profiles (Figure 3). The first group comprised media supplemented with poppy, SDAY, quinoa, parboiled rice, wheat germ, chitin, or chia, in which conidial viability was >97%. In this cluster, media supplemented with wheat germ, chitin, or chia were

Table 3 Principal component analysis with eigenvalues and variation explained for each principal component

Component[1]	Eigenvalue	Variation (%)	Cumulative variation (%)
1	1.83	37	37
2	1.49	30	66
3	0.78	16	82
4	0.49	10	92
5	0.41	8	100

	Component[1]		
Variable	1	2	3
Surface area	−0.16	0.67	−0.38
Viability	0.63	−0.05	0.08
Adherence	0.57	0.16	0.44
Hydrophobicity	0.27	0.66	−0.07
Protease activity	−0.42	0.29	0.81

[1]Eigenvectors are presented only for components 1–3. There were just three principal components retained which together account for 82% of the variability in the original data.

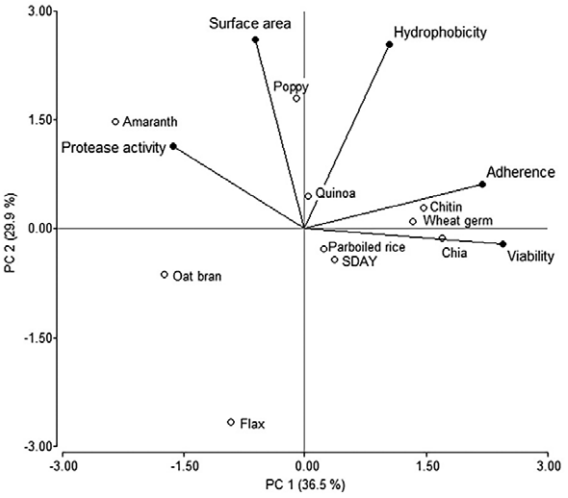

Figure 2 Principal component analysis biplot between components 1 and 2. It is clear that high values of conidial adherence and viability were obtained mainly on medium supplemented with chia.

grouped together showing the highest values of conidial adherence, whereas medium supplemented with poppy was separated from the other media due to the highest values of conidial hydrophobicity. In addition, on media supplemented with chitin or chia, fungal isolate did not show significant differences between any variable tested, and coincidently the shortest Euclidean distance between these

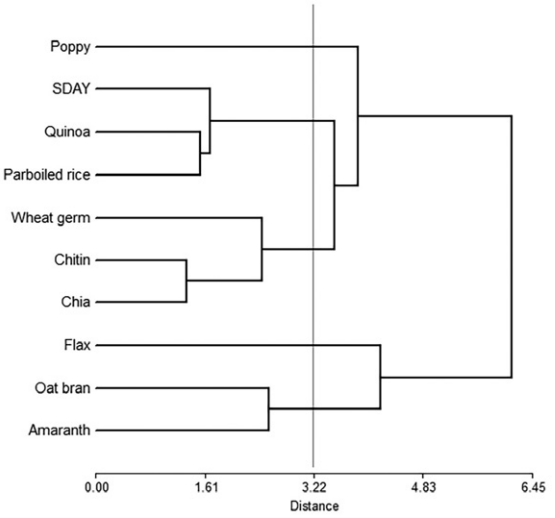

Figure 3 Cluster dendrogram showing 10 culture media distributed in two distinct groups. Dendrogram was constructed taking into account the meaningful response variables retained by principal component analysis and using Ward's method to calculate Euclidean distances.

media was observed. The second group clustered media supplemented with flax, oat bran, or amaranth, in which conidial viability was <97%. In these media, values of conidial adherence and protease activity were also similar. Nevertheless, medium supplemented with flax was separated from the rest due to the lowest values of conidial hydrophobicity.

Discussion

The ability of entomopathogenic fungi to parasitize and to kill insects is governed by many factors, which are dependent on the fungal isolate (Gao & Liu, 2010; Barra et al., 2018), the target insect, and the environment (Lu & St. Leger, 2016). Different mechanisms, such as those involving the participation of extracellular enzymes, the synthesis of low molecular weight toxins, and/or the ability to avoid the insect immune response, could explain the variation in fungal virulence (Xiao et al., 2012; Valero-Jiménez et al., 2014). Additionally, virulence is conditioned by other factors such as the amount of applied conidia, their size and viability, as well as functional properties related to their adherence, hydrophobicity, and level of enzyme activity, which are under the influence of the medium in which the fungus is grown (Ibrahim et al., 2002; Shah et al., 2005; Safavi et al., 2007; Ali et al., 2009; Shan et al., 2010; Pedrini et al., 2011; Mascarin et al., 2013; Butt et al., 2016; Zulfiana et al., 2020). In this study, the addition of certain

nutritional sources to sucrose agar medium significantly increased some virulence factors of *B. bassiana* s.l. CEP147. We showed that conidial viability and adherence explained the maximal amount of total variance, demonstrating that both variables can be used as indicators of fungal virulence against *D. kuscheli*. According to Butt & Goettel (2000), the amount of viable conidia adhered to the tegument of the insect is key in fungal entomopathogenic activity. Results of this study showed that all media produced higher percentages of viable conidia ranging from 84% on medium supplemented with oat bran to 100% on medium supplemented with chia. However, our previous studies (Toledo et al., 2007) indicated that viability should not be individually used as an indicator of fungal virulence, as the conidia that have greater viability do not always cause the highest mortality rate in host insects.

Correlation analysis showed that mortality of *D. kuscheli* was directly correlated with conidial surface area and adherence. Similarly, significant correlation between conidial size and virulence was also reported by Altre et al. (1999) during the interaction between *Cordyceps fumosorosea* (Wize) Kepler, B. Shrestha & Spatafora and *Plutella xylostella* L. larvae. Adhesion of conidia to the host surface is the first step of fungal infection and it is affected by several conidial surface properties like charge, texture, and hydrophobicity. The surface of aerial conidia of most hypocrealean fungi, including *B. bassiana*, is covered with a rodlet layer composed of hydrophobin proteins that confer a hydrophobic charge facilitating the passive attachment of conidia to the hydrophobic surfaces of insects (Holder & Keyhani, 2005). Hydrophobins in *B. bassiana* were related to conidial surface hydrophobicity, adhesion and virulence (measured in terms of MST) (Zhang et al., 2011). In this study, we found that sucrose agar medium supplemented with chia or chitin was associated with high conidial adherence. Furthermore, we observed that 25% of the variation in *B. bassiana* s.l. CEP147 conidial adherence was attributed to conidial hydrophobicity. In our experiments, conidia recorded higher adherence when the fungus grew on media supplemented with chia, chitin, poppy, or wheat germ, whereas conidial hydrophobicity was similar to that produced on SDAY only when the fungus was grown on media containing poppy, quinoa, or wheat germ. Additionally, a positive correlation between conidial hydrophobicity and surface area of *B. bassiana* s.l. CEP147 was observed, indicating that 26% of the variation in conidial hydrophobicity was attributed to conidial size. Our results are in agreement with those reported by Shan et al. (2010), who observed a positive correlation between hydrophobicity and conidial surface among several isolates of three species of Hypocrealean fungi including *B. bassiana*. Higher *D. kuscheli* cumulative mortality (>85%) was recorded on media supplemented with amaranth, chia, or chitin where the conidia showed a significantly higher surface area than those produced on control medium. However, only in media supplemented with chia or chitin, a higher adherence was also recorded.

Entomopathogenic fungi produce an extensive cocktail of extracellular hydrolytic enzymes including lipases, proteases, and chitinases (Santi et al., 2010; Schrank & Vainstein, 2010). Among them, proteases are the main enzymes produced during the infection process and their hydrolytic role is essential to the degradation of the integument; 55–80% of the insect cuticle consists of protein (Neville, 1975; St. Leger, 1995). The serine protease Pr1, identified in several entomopathogenic fungi including *B. bassiana* (Dias et al., 2008), plays a major role in insect penetration and subsequent pathogenicity (St. Leger et al., 1987; Goettel et al., 1989). In this study, proteases produced by *B. bassiana* s.l. CEP147 were not identified. However, when we evaluated the proteolytic activity of fungal conidia, we found that this variable was not correlated with *D. kuscheli* cumulative mortality or MST. On medium supplemented with poppy, conidial protease activity was the highest with low values of cumulative mortality of *D. kuscheli*. Conversely, conidia produced on medium supplemented with chitin recorded the lowest protease activity with high values of cumulative mortality. In addition, conidia produced on both media recorded similar values of MST. These results are in accordance with those reported by Safavi et al. (2007) who did not find any relationship between Pr1 activity and virulence of *B. bassiana* against larvae of *Tenebrio molitor* L. The absence of correlation could be related to the fact that the infection process has multiple components including multiple enzymes and is not a single factor, regardless of whether the factor is a protease, a lipase, or a chitinase.

SSF using sterilized moistened cereal grains has emerged as an appropriate technology for the mass production of aerial conidia (Jaronski, 2014; Mascarin & Jaronski, 2016). Cereal grains generally provide an adequate effective surface for mycelial growth and an adequate nutritional balance, aeration and humidity (Bhanu-Pakrasha et al., 2008). However, some studies demonstrated that *B. bassiana* conidial production increased with the addition into the substrate of sugars, nitrogen-containing compounds or minerals (Hallsworth & Magan, 1994; Balakrishnan et al., 2011; Mishra et al., 2016). Recently, we observed that aerial conidia of *B. bassiana* s.l. CEP147 produced on white rice grains without nutritional supplements, showed a high viability (98.6%) and caused 83.3% *D. kuscheli* mortality within 7 days (AV Toledo, unpubl.) suggesting that rice could be a promising substrate for mass

production of this fungal isolate. In this study, we demonstrated that chia flour not only increased conidial viability (100%) and adherence (0.09 A_{550}) with respect to the control medium, but also produced a high mortality of *D. kuscheli* (91.7%) recording the lowest MST (5.17 days). For this reason, we suggest that chia flour, which is rich in carbohydrates, proteins, vitamins, and minerals, might be incorporated as a nutritional supplement to solid substrates to increase the virulence of *B. bassiana* s.l. CEP147 against *D. kuscheli* and other pests. However, future studies are necessary to evaluate the effectiveness of this nutritional source on SSF.

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Author Contributions

Andrea Toledo: Conceptualization (equal); Formal analysis (lead); Funding acquisition (equal); Methodology (lead); Writing – original draft (lead); Writing – review & editing (equal). Eugenia Brentassi: Conceptualization (equal); Funding acquisition (equal); Writing – original draft (equal); Writing – review & editing (equal).

Conflict of Interest

The authors have declared that they have not conflict of interest.

Data Availability Statement

Data used for the analysis in this manuscript are openly available. Data citation: Andrea Toledo. (2021). Effect of nutrition on growth and virulence of *Beauveria bassiana* s.l. against the planthopper *Delphacodes kuscheli*. http://doi.org/10.5281/zenodo.4687857. More information can be requested from the corresponding author.

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