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A mycoviral infection drives virulence and ecological fitness of the entomopathogenic fungus *Beauveria bassiana*

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ABSTRACT

Entomopathogenic ascomycetes are important natural regulators of insect pest populations and an increasingly adopted microbial control option. Fungal virulence in entomopathogenic ascomycetes can be modified by mycoviruses, viruses that infect fungi, whereas the possible role of these viruses on the physical and biochemical properties of the virus-containing fungal strains and on their ecological fitness has remained largely unexplored. Here, utilizing a *Beauveria bassiana* strain naturally infected with two mycoviruses, Beauveria bassiana partitivirus 2 (BbPV-2) and Beauveria bassiana polymycovirus 1 (BbPmV-1), we found that the mycovirus-containing strain is hypervirulent towards the experimental insect *Galleria mellonella* and shows major physical and biochemical changes in spore size, isoelectric point, and Pr1 activity, but even more impactful, the mycoviral infection confers a significant environmental- abiotic and biotic stress tolerance to the fungus. Hence, mycovirus infection expanded the temperature range for fungal growth and germination, and improved tolerance to osmotic stress, water stress, and UV-B radiation. Similarly, the antagonistic activity of the mycovirus-containing strain against *Trichoderma harzianum* was increased as compared to the mycovirus-free one. Taken together, these data suggest for the first time a mycovirus related adaptation of key traits indicators of environmental competence of a beneficial fungus, rendering these mycoviruses as potent tools for entomopathogenic fungal strain selection and development as mycoinsecticides.

1. Introduction

Mycoviruses, viruses that infect fungi, are present in almost all fungal groups including two major genera of entomopathogenic ascomycetes (EA), *Beauveria* spp. and *Metarhizium* spp. (Melzer and Bidochka, 1998; Giménez-Pecci et al., 2002; Dalzoto et al., 2006; Herrero et al., 2012; Perinoto et al., 2014; Koloniuk, et al., 2015; Kotta-Loizou et al., 2015; Kotta-Loizou et al., 2017; Santos et al., 2017; Filippou et al., 2018; Gilbert et al., 2019; Wang et al., 2002), but also in *Tolypocladium cylindrosporum* (Herrero and Zabalgogeazcoa, 2011) and *Isaria fumosorosea* (Azevedo et al., 2000). More importantly, several isolates of *Beauveria bassiana* (Balsamo) Vuill., probably the most widespread, widely distributed and commercially available entomopathogenic fugus, have been shown to be infected by double-stranded RNA (dsRNA) elements (Dalzoto et al., 2006; Herrero et al., 2012; Koloniuk, et al., 2015; Kotta-Loizou et al., 2015; Kotta-Loizou et al., 2017; Filippou et al., 2018).

The general evolutionary significance of dsRNA infections in fungi that threaten human, animal, plant, and ecosystem health is not yet fully understood. Mycovirus infections may be symptomless, not influencing host phenotype, or may lead from moderate to dramatic phenotypic changes in their hosts, including decrease or increase of *in vitro* fungal growth and hypo or hyper virulence (Nuss, 2005; Ghabrial et al., 2015; Hillman and Turina, 2024). An enhancement of *in vitro* growth, sporulation and/or virulence by mycoviruses has been reported in EA (Kotta-Loizou and Coutts, 2017; Kotta-Loizou et al., 2021), but the molecular mechanisms underpinning such phenomena remains poorly understood. It is hypothesized that mycovirus infection may modulate enzymes involved in cuticle degradation, particularly Pr1, which has received considerable attention in recent decades due to its crucial role in determining the pathogenicity of fungal strains (Gao et al., 2020; Ferreira et al., 2024).

In addition to the gaps in understanding these mechanisms, there is also a paucity of information on the ecological and environmental

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significance of mycovirus in EA, with no reports concerning possible differential responses of mycovirus-infected *B. bassiana* strains to abiotic and biotic environmental stresses. However, the success of EA in microbial pest control is highly affected by the environmental fitness of the selected fungal strains: temperature is a key climatic factor limiting fungal virulence, while relative humidity and ultraviolet (UV) radiation together with possible antagonistic relationships with other microbes are known to deplete and inactivate EA conidia in epigeal and hypogeal habitats (Quesada-Moraga et al., 2023). The success of EA-based biocontrol relies on selecting fungal strains that are both environmentally fit and highly virulent. Understanding how mycovirus infections influence this process is crucial to determining if certain mycoviruses provide evolutionary advantages under specific conditions (Son et al., 2015), and whether their role in environmental fitness can be genetically engineered (Kotta-Loizou, 2019).

In a previous survey of mycoviral population dynamics in Spanish EA, 18*B. bassiana* strains were found to harbour mycoviruses (Filippou et al., 2018; Rueda-Maillo et al., 2022). Among them, the already characterised Beauveria bassiana partitivirus 2 (BbPV-2) and Beauveria bassiana polymycovirus 1 (BbPmV-1) have shown promise for inducing hypervirulence in different strains against model insects (Filippou et al., 2018). Our aim is to establish the origin of this hypervirulence and unveil the effects of these mycoviral infections on the phenotype and ecological fitness of the host strains. In this regard, among the strains in our collection that contain mycoviruses (Filippou et al., 2018; Rueda-Maillo et al., 2022), the strain EABb 01/126-Su exhibited significant changes in virulence in preliminary studies, particularly in response to abiotic and biotic stresses, depending on the presence or absence of the mycovirus (Rueda-Maillo et al., 2022). Here, we report mycovirus-mediated physical and biochemical changes in *B. bassiana* spore size, isoelectric point, and Pr1 activity together with increased ecological fitness as illustrated by key traits of the fungal host, which overall promote the adaptation of EA to specialized niches for biocontrol.

2. Material and methods

2.1. Fungal strains and culture media

2.1.1. Beauveria bassiana strain

The *Beauveria bassiana* EABb 01/126-Su strain used in this study was obtained from the culture collection of the Department of Agronomy of the University of Cordoba (Spain) and was originally isolated from the soil of an olive crop at Bornos (Cadiz, Spain). The translation elongation factor 1-alpha (tef1a) of this strain was deposited in the GenBank database under accession number OQ944319. The presence of mycovirus was tested by dsRNA extraction from mycelium of the strain. The Real-Time qPCR was performed in the OneStepPlus Real-Time qPCR System (Applied Biosystems) to amplify the mycoviruses naturally present in this strain, Beauveria bassiana partitivirus 2 (BbPV-2) and Beauveria bassiana polymycovirus 1 (BbPmV-1).

2.1.2. Elimination of mycoviruses

To obtain a mycovirus-free strain (MFr) from the wild mycovirus-infected strain (WMI), treatment with the protein synthesis inhibitor cycloheximide was carried out as described Li et al. (2019). The strain was grown on potato dextrose agar (PDA) medium supplemented with cycloheximide at a concentration of 1 μg/ml. The culture was incubated at 32 °C for 7 days. This process was repeated five more times and subcultures were then grown on PDA alone at 27 °C.

2.1.3. Virus purification

Virus like particles (VLPs) process was carried out according to Howitt et al., 1995. Briefly, 10 g of frozen mycelium were ground into a fine powder using liquid nitrogen. The powder was mixed with cold 100 mM sodium phosphate buffer (pH 7.0), extracted with chloroform, and centrifuged at 11,000 g for 20 min at 4 °C. The aqueous upper phase was transferred into 3 ultracentrifuge tubes and centrifuged at 12,000 g for 80 min at 4 °C. The resulting pellet was resuspended in 1 ml of 20 mM sodium phosphate buffer (pH 7.0) for 2 h, stored overnight and clarified by centrifugation at 2,000 g for 10 min at 4 °C. The supernatant was adjusted to a volume of 10 ml by adding 20 mM sodium phosphate buffer and subjected to ultracentrifugation as described previously. The pellets obtained were dissolved in 0.5 ml of the buffer and left for at least 2 h before being transferred to a 1.5 ml Eppendorf tube and centrifuged at 1,700 g for 5 min at 4 °C. The supernatant was collected and either utilized immediately or stored at 4 °C until further processing.

2.1.4. Transmission electron microscopy (TEM)

Electron microscopy examination was recorded by use of JEOL JEM 1400 (Izasa Scientific). On a parafilm sheet, the grid (Formvar film, 200 mesh, Copper, UK) was sequentially moved through the following solutions: first, one drop of the sample (10 μL) for 5 min; then, two drops of distilled water (40 μL) for 1 min each; and finally, two drops of 2 % uranyl acetate (20 μL) for 1 min each. Subsequently, the excess liquid was carefully blotted from the grid using Whatman filter paper from the outer edge, and the grid was left to dry.

2.1.5. Trichoderma spp. strains

Trichoderma was selected as a model microorganism to evaluate the impact of the mycovirus infection on the antagonistic capacity of EABb 01/126-Su strain. The two strains of the genus *Trichoderma* were provided by Prof. Enrique Monte from the culture collection of the Spanish-Portuguese Center for Agricultural Research (CIALE), in the Department of Microbiology and Genetics from University of Salamanca (Spain). *Trichoderma harzianum* Rifai T115 strain and *Trichoderma reesei* Simmons T112 strain were selected because their chitinolytic and cellulolytic enzymes, respectively, and cultivated in PDA medium.

2.2. Physical and biochemical properties of conidia

2.2.1. Size and shape

To measure the spore size, 50 conidia from each of the WMI and MFr strains were randomly selected from photos taken using light and electron microscopy. ImageJ software (https://imagej.nih.gov/ij/index.html) was used to measure the major and minor axis, the area and the perimeter of the conidia. Conidia diameter (Ø), aspect ratio (AR), circularity (C) and roundness (R) were calculated using the formulae of van den Brule et al., 2020:

$$Ø = 2 \times \sqrt[3]{\frac{major}{2} \times \left(\frac{minor}{2}\right)^2},\ AR = \frac{major}{minor},\ C = \frac{4\pi \times area}{perimeter^2} \text{ and } R = \frac{4 \times area}{\pi \times major^2}$$

The full experiment was performed twice with fresh fungal inoculum.

2.2.2. Electrostatic properties

Conidia of the WMI and MFr strain at a concentration of 10^7 ml^{-1} were suspended in 10 ml of distilled water containing Tween 80 (0.01 %). Isoelectric point was obtained determining the pH value where the zeta potential was zero and zeta potential was determined at pH = 7. Measurements were made at room temperature with a Dynamic light scattering (DLS) using Malvern Zetasizer Nano, ZSP with 633 nm laser, equipped with a MPT-2 Autotitrator. The measured data were processed using the software provided by the manufacturer. The full experiment was performed twice with fresh fungal inoculum.

2.2.3. Total Pr1 enzymatic activities

To assay the total levels of extracellular Pr1 activity (EPA) an intracellular Pr1 activity (IPA), 50 ml of suspensions adjusted to 10^7 conidia/ml were prepared from CBD-BSA [3 % sucrose, 0.3 % BSA, 0.1

% K_2HPO_4, 0.05 % KCl, 0.05 % $MgSO_4$, 0.001 % $FeSO_4$] according to Gao et al., 2020. Following a 3 h of incubation on a shaking bed at 150 rpm and 25 °C, each culture was separated into supernatant and hyphal cells by centrifugation at 4 °C and the latter was immediately dried for 3 h at 70 °C. For EPA, 100 µl of supernatant were mixed 50 µl of 1 mM substrate [*N*-succinil-Ala-Ala-Pro-Phe p-nitroanilida], 850 µl 15 mM Tris-HCl buffer pH 8.5. After 1 h incubation at 28 °C, 250 µl of 30 % acetic acid were added. Subsequently, the reaction was kept for 15 min in ice and centrifuged at 1,250 g for 5 min at 4 °C. For IPA, dried hyphal cells were grinded with liquid nitrogen, suspended in 50 mM phosphate buffer pH 7.4 and centrifuged at 12,000 g at 4 °C. Total EPA and IPA were quantified by a spectrophotometer with a reading optical density at 410 nm (OD_{410}). One unit of enzyme activity was defined as the enzyme amount required for a 0.01 increase in optical density after 1 h reaction of each treatment versus control, according to Gao et al., 2020. The full experiment was performed twice with fresh fungal inoculum.

2.2.4. Salt aggregation test for conidial hydrophobicity

The salt aggregation test involves inducing cellular flocculation by increasing the salt concentration. The order in which cells precipitate is a measure of their hydrophobicity, with the most hydrophobic cells precipitating at the lowest salt concentration (Lindahl et al., 1981; Mozes and Rouxhet, 1987). Serial dilutions of $(NH_4)_2SO_4$ were prepared over a concentration range from 4 M to 0.2 M, differing by 0.2 M per dilution. Sodium phosphate, pH 6.8, was used to dilute $(NH_4)_2SO_4$ over a range from 0.2 to 0.02 M, differing by 0.02 M. The pH was adjusted using NH_4OH. Suspensions were adjusted to 5×10^9 conidia ml^{-1} in 0.002 M pH 6.8 sodium phosphate, and 25 µl of each suspension were mixed with an equal volume of salt solution. The mixture was gently shaken for 2 min at 20 °C and visualized against a black background according to Lindahl et al., 1981. Conidial suspension mixed with 0.002 sodium phosphate (pH 6.8) without addition of salt was used as a control. A reaction causing white aggregates in a clear solution was regarded as a positive result. The full experiment was performed twice with fresh fungal inoculum.

2.3. Virulence bioassay

Five suspensions were prepared for WMI and MFr, including 1×10^6, 5×10^6, 1×10^7, 5×10^7, 1×10^8 conidia ml^{-1}. For control, sterile 0.1 % (v/v) Tween 80 aqueous solution was used. Fourth instar larvae of the greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae) were immersed in 10 ml of the different concentrations of conidia for 30 s. Three replicates were performed for WMI, MFr and control, with 10 larvae per replicate. The mortality was evaluated for 10 days. Dead larvae were immediately surface sterilized with 1 % (v/v) sodium hypochlorite, followed by two rinses with sterile distilled water. Subsequently, the cadavers were placed on sterile wet filter paper in Petri plates and kept at 25 °C. After five days, fungal outgrowth was observed on the surface of the insect cuticle. The full experiment was performed twice with fresh fungal inoculum.

2.4. Abiotic stresses

2.4.1. Temperature effects on conidial germination and radial growth

Suspensions adjusted to 1×10^5 conidia ml^{-1} were prepared from malt agar (MA) 12-day-old cultures of WMI and MFr strains, by flooding the plates with sterile, distilled water containing 0.01 % (v/v) Tween 80. The suspensions were mixed using a vortex, sonicated for 5 min, and filtered. Subsequently, 40 µl of each suspension were placed into the centre of water-agar Petri dishes (60 mm diameter). In parallel, circular plugs 8 mm diameter were cut from MA 4-day-old cultures of WMI and MFr strains and individually placed upside down in the centre of fresh MA Petri dishes (60 mm diameter) according to Fernandez-Bravo et al. (2016). The plates were incubated in the dark at 10, 15, 20, 25, 30, 35 and 38 ± 2°C. Five replicates were performed for each strain and temperature combination. The full experiment was performed twice with fresh fungal inoculum.

For plates inoculated with conidial suspensions, germination was stopped at 18 h according to Fernandez-Bravo et al. (2016), by transferring 0.5 ml of lactophenol cotton blue to each plate. Germination percentages were observed by counting 100 conidia on each plate at 400 × magnification.

For plates inoculated with mycelial plugs, two orthogonal diameters of each colony were measured every two days with a digital caliper. To obtain the current growth, the 8 mm diameter of the initial circular plugs was subtracted from the mean diameter.

Radial growth was calculated by regression analysis for each combination of strain and temperature. Radial measurements (from day 2 to 10) were fitted to a linear model according to Fernandez-Bravo et al. (2016):

$$Y_{(\text{mm diameter})} = v \times t_{(\text{incubation days})} + B$$

The linear regression slope (v) indicates the growth rate in mm per day at each temperature, evaluated using the generalized β function of a nonlinear model according to Bassanezi et al. (1998):

$$V(T) = T_{Yopt} \times \left(\frac{T - Tmin}{Topt - Tmin}\right)^{TB \times \left(\frac{Topt - Tmin}{Tmax - Topt}\right)} \times \left(\frac{Tmax - T}{Tmax - Topt}\right)^{TB}$$

The equation contains four parameters. T is the incubation temperature. T_B is the shape parameter that influences the temperature range around T_{opt} in which the curve remains close to TY_{opt}. TY_{opt} represents the fungal growth at the optimal temperature T_{opt}. T_{min} and T_{max} are the minimal and maximal temperature for the fungal growth, respectively. The former was set at 5 °C because it is known that this strain does not grow at that temperature and Tmax was given by the model. These four parameters were estimated by the method of Newton and pairwise comparisons were made by performing Student's *t*-tests (P = 0.05) on the estimated values (p1 and p2) and their standard errors according to Fernandez-Bravo et al. (2016):

$$t = \frac{p1 - p2}{\sqrt{SE_1^2 + SE_2^2}}$$

2.4.2. Water activity related effects on conidial germination

Different levels of osmotic potential (ψ) were established in water-agar amended with eight different glycerol concentrations according to Fernandez-Bravo et al. (2016), while glycerol-free water-agar was used as control. Water activity (a_w) values ranged from 0.999 to 0.862. For WMI and MFr, 40 µl of conidial suspension adjusted to 1×10^5 conidia ml^{-1} were spread on Petri dishes (60 mm diameter) with different glycerol concentrations. The plates were incubated in the dark at 25 °C for 24 h. To stop germination, 0.5 ml of lactophenol cotton blue were transferred to each plate and germination percentages were observed by counting 100 conidia on each plate at 400 × magnification. Three replicates were performed for each strain and glycerol concentration combination. The full experiment was performed twice with fresh fungal inoculum.

2.4.3. Osmotic stress related effect on radial growth

Conidial suspensions were prepared from MA 12-day-old cultures of WMI and MFr strains, mixed by vortexing, sonicated for 5 min and filtered. For each suspension, 5 µl containing 1000 conidia were placed into the centre of Petri dishes (90 mm diameter) containing MA supplemented with 0.8 M NaCl according to Stevens et al., (2023), while NaCl-free MA was used as control. The plates were incubated in the dark at 25 °C until the strains reached the edges of the plate. The diameter of each colony was measured every day, and the growth area was calculated using the formula πr^2. Four replicates were performed for each strain and their respective controls. The full experiment was performed twice with fresh fungal inoculum.

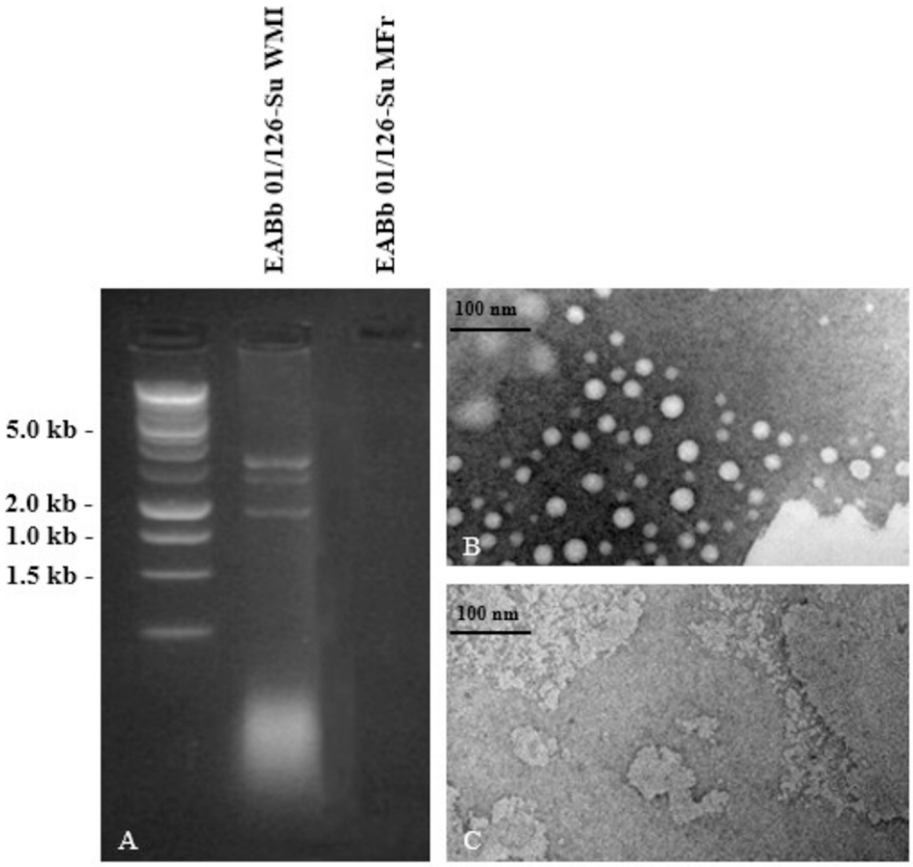

Fig. 1. (A) **Agarose gel electrophoresis of viral dsRNA elements** isolated from the wild mycovirus-infected (WMI) EABb 01/126-Su *Beauveria bassiana* strain. No dsRNA elements were founded in the mycovirus-free strain (MFr). (B) and (C) **Visualisation of virus-like particles (VLPs) on formvar film grid using TEM.** (B) Isometric VLPs 30–45 nm in diameter were purified from WMI. (C) No VLPs were visible in preparations from Mfr.

2.4.4. UV-B radiation effects on conidial germination, culturability and radial growth

For WMI and MFr, 40 µl of suspension adjusted to 1×10^5 conidia ml^{-1} were placed into the centre of water-agar Petri dishes (60 mm diameter). Each treatment Petri dish was covered with cellulose-diacetate film, while aluminium foil was used as control. The plates were exposed at 1200 mW m^{-2} UV-B radiation for 2, 4 and 6 h and incubated in the dark at 25 °C for 24 h. Three replicates were performed for each strain and exposure time combination and their respective controls. The full experiment was performed twice with fresh fungal inoculum.

To stop germination, 0.5 ml of lactophenol cotton blue were transferred to each plate and germination percentages were observed by counting 100 conidia on each plate at 400 × magnification. The relative germination percentage for each exposure time was calculated by the following formula:

$$Relative\ germination(\%) = \frac{Wt}{Wc} \times 100$$

where Wt is the number of conidia germinated at different exposure times (2, 4 or 6) for each treatment and replicate and Wc is the mean number of germinated conidia obtained in each replicate for each exposure time on the control plates.

Relative culturability was calculated using the following formula:

$$Relative\ culturability\ (\%) = \frac{Tt}{Mc} \times 100$$

where Tt represents the number of colonies forming units (CFUs) evaluated by counting them from the replicate of each treatment plate at different exposure times (2, 4 or 6) at 40 × magnification and Mc is the mean number of CFUs in the replicate of the control plates.

Radial growth was calculated by measuring two orthogonal diameters of each colony and using the formula:

$$Growth\ index\ (\%) = \frac{Cc - Ct}{Cc + Ct} \times 100$$

where Ct represents the mean diameter at different exposure times (2, 4 or 6) of each treatment replicate and *Cc* is the mean diameter of the control plates. For each treatment, 10 colonies were measured. Values close to 100 indicate that the strain is highly susceptible to UV radiation, while values close to 0 indicate that the strain is not susceptible to UV radiation.

2.5. Biotic stresses: Antagonistic activity against Trichoderma spp

The antagonistic activity of WMI and MFr was evaluated *in vitro* against *Trichoderma* spp. by the dual culture test method (Morton and Stroube et al., 1955). Four strains, WMI and MFr together with two *Trichoderma* spp., were grown separately on PDA. After 5 days of growth, circular plugs 5 mm diameter were cut and pairs of plugs from *B. bassiana* and *Trichoderma* spp. were placed upside down on the same plate equidistantly, with both having the same opportunity for growth, 10 mm from the edge according to Cherkupally et al. (2017). Controls were placed individually upside down in the centre of a PDA Petri dish (90 mm diameter). The plates were incubated at 25 °C for 5 days. Three replicates were performed for each *B. bassiana* and *Trichoderma* strain combination and their respective controls. The full experiment was performed twice with fresh fungal inoculum.

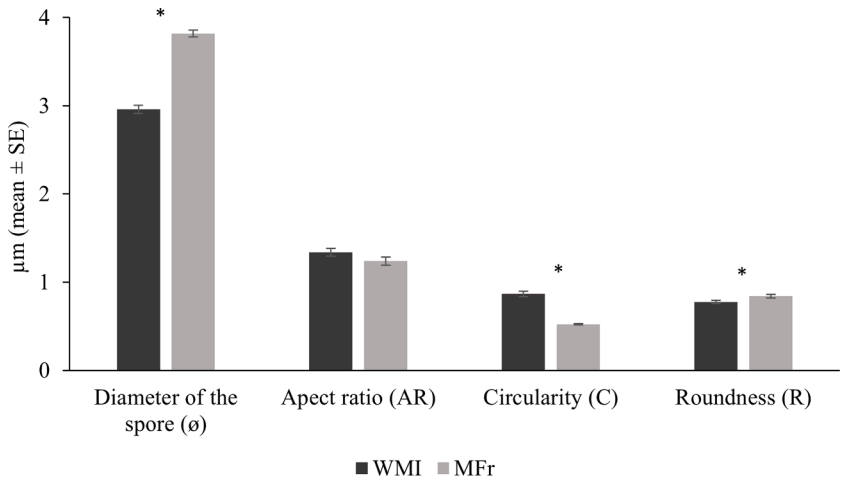

Fig. 2. Effect of mycovirus infection on conidia size and shape. Conidia diameter (Ø), aspect ratio (AR), circularity (C) and roundness (R) were calculated for WMI and MFr. Each column represents the mean value of 50 randomly selected conidia, and the error bars represent standard error of the mean. Asterisks indicate statistically significant differences between WMI and MFr ($P < 0.05$ LSD test).

The percentage of inhibition of the mycelia growth (IMG) was evaluated using the following formula:

$$IMG\ (\%) = \frac{C - T}{C} \times 100$$

where C is the radial growth of the control (mm) measured with a digital calliper and T is the radial growth of the treatment.

The ability of *Trichoderma* spp. to overgrow and inhibit the growth of the *B. bassiana* strains was given by Bell scale according to Cherkupally et al. (2017), being: R1) 100 % *B. bassiana* overgrowth; R2) two-thirds of the medium surface *B. bassiana* overgrowth; *R*3) one-half of the medium surface *B. bassiana* overgrowth; R4) locked at the point of contact.

The spherical index (α/β) measures the antagonism exhibited by two opposing organisms. The α parameter measures the antagonistic effect on each other and the β parameter measures the orthogonal progression of the antagonists according to Rigerte et al. (2019). An organism growing radially uninhibited has a spherical index equal to 1, since α = β. A spherical index < 1 for *B. bassiana* indicates that it is being antagonized by *Trichoderma*, whereas a spherical index > 1 indicates that *Trichoderma* has no effect on the *B. bassiana* growth.

2.6. Statistical analysis

Analysis of variance (ANOVA) was used to assess statistically significant differences in spore size, total Pr1 activity, effects of temperature and water activity on germination, effects of UV-B radiation on relative germination, relative tillering ability and growth rate and least significant difference (LSD) test was performed to compare means. The Tukey HSD test was used for antagonistic activity on the percentage inhibition, both tests using Statistix 9.0 (free version) (Statistix 9.0® Analytical Software, 2008). Student's *t*-tests (P = 0.05) was performed to assess statistically significant differences in isoelectric point, zeta potential and osmotic stress using SPSS 19.0 for Windows (IBM Company, 2010).

In virulence assays, the lethal concentration (LC_{50}) values were calculated through probit analysis, following the method described by Finney 1952. To assess the parallelism of probit regression lines for all tested isolates, χ^2 goodness-of-fit tests were employed. Relative median potencies and their corresponding 95 % confidence intervals were computed for different treatments, provided that their slopes exhibited no significant differences, as outlined by Finney 1952. The time required to achieve 50 % insect mortality (LT_{50}) was determined utilizing the probit analysis method for correlated data, following the approach

Table 1
Effect of mycovirus infection on virulence and biochemical characteristics.

Strain	Regression analysis of probit mortality for *Galleria mellonella* virulence bioassays						Total Pr[1] activity (x 100 U/ml)[3]				
	Regression equation	Se[2]	χ2 (2 d. f.)	LC_{50} (Confidence interval 95 %, conidia ml^{-1})	Relative potency (Confidence interval 95 %)	LT_{50}[2] (Confidence interval 95 %, conidia ml^{-1})	Extracellular Pr1 activity	Intracellular Pr1 activity	Hydrophobicity[4]	Zeta potential (mV)[5]	Isoelectric point (IP)[5]
WMI	y = 0.88x + 4.83	0.17	1.84	1.97×10^7 (1.11×10^7-3.84×10^7)	3.40	6 (5.61–6.53)	1.95 ± 0.07a	0.55 ± 0.07a	0.04 M	3.95 ± 0.05a	4.31 ± 0.06a
MFr	y = 0.59x + 4.84	0.16	0.30	1.03×10^8 (3.89×10^7-1.29×10^8)	1	6.80 (6.29–7.51)	1.25 ± 0.21b	0.15 ± 0.07b	0.03 M	8.50 ± 1.50b	2.94 ± 0.01b

[1] Slope error.
[2] LT_{50} measures in days.
[3] The means of total Pr1 activity of WMI and MFr conidial suspensions were compared using the least significant difference (LSD) test and different letters indicate statistically significant differences.
[4] The hydrophobicity of WMI and MFr conidial suspensions was the same for both repetitions of the experiments.
[5] The means of zeta potential and isoelectric point of WMI and MFr conidial suspensions were compared using Student's *t* test and different letters indicate statistically significant differences.

proposed by Throne et al., 1995. Statistical analyses were performed using SPSS 19.0 for Windows (IBM Company, 2010). The analysis of the two real repetitions of each experiment have been pooled into one.

3. Results

3.1. Confirmation of virus absence in the cured EABb 01/126-Su strain

Following treatment of the EABb 01/126-Su strain with cycloheximide, the absence of mycovirus was tested by electrophoresis in a 1 % (w/v) agarose gel from the total nucleic acid extract of the sample (Fig. 1A). The absence of mycovirus was also tested by partial purification of VLPs. Whilst VLPs of different sizes (30–45 nm) were observed from the WMI strain under, no viral structures were observed in the MFr strain, confirming that the MFr stain is indeed virus-free (Fig. 1B and C).

3.2. Mycovirus-related alterations in the physical and biochemical properties of EABb 01/126-Su conidia

3.2.1. Conidia size and shape

The conidia diameter, circularity and roundness were compared for the WMI and MFr strains (Fig. 2). A statistically significant mycovirus-related 23.7 % decrease in spore diameter was noted, together with a decrease in roundness and an increase in circularity ($F_{1,\ 97} = 209.66$; $F_{1,\ 97} = 107.42$; $F_{1,\ 97} = 6.35$; $P < 0.001$). No statistically significant differences were observed between the aspect ratio of WMI and MFr conidia ($F_{1,\ 97} = 2.54$; $P > 0.05$).

3.2.2. Electrostatic properties of conidia

The isoelectric point of conidial solutions was observed at zero value of the zeta potential and compared for the WMI and MFr strains. There was a statistically significant effect on the isoelectric point ($p = 0.001$), with pH values of 4.31 and 2.94 for WMI and MFr, respectively (Table 1). Additionally, there was a statistically significant effect on the zeta potential at pH 7 ($p < 0.05$), with values of –3.9 mV and –8.5 mV for WMI and MFr, respectively (Table 1).

3.2.3. Total Pr1 activities

The total EPA and IPA of conidial suspensions were assessed and compared for the WMI and MFr strains. There were statistically significant effects on the total EPA ($F_{1,3} = 19.60$; $P < 0.05$) and the total IPA ($F_{1,3} = 32.00$; $P < 0.05$), with EPA values of 1.95 × 100 U/ml and 1.25 × 100 U/ml and IPA values of 0.55 × 100 U/ml and 0.15 × U/ml for WMI and MFr, respectively (Table 1).

3.2.4. Hydrophobicity

The hydrophobicity of conidial suspensions was assessed using the salt aggregation test and compared for the WMI and MFr strains. MFr exhibited a more hydrophobic cell surface, as its conidia precipitated at a lower molarity of 0.03 M, as compared to WMI, whose conidia precipitated at a molarity of 0.04 M (Table 1).

3.3. Mycovirus-related alterations in the virulence of EABb 01/126-Su conidia against G. mellonella

The virulence of the WMI and MFr strains was evaluated against the greater wax moth *G. mellonella* larvae. The concentration-mortality response regression analysis for the WMI and MFr strain was calculated using five different concentrations of conidial suspensions. All χ^2 values were non-significant according to goodness-of-fit Pearson's test ($\alpha = 0.05$), with regression coefficients varying from 0.16 to 0.17. The WMI strain was more virulent with a LC_{50} value of 1.97×10^7 conidia/ml, while the LC_{50} value of the MFr strain was 1.03×10^8 conidia/ml, showing statistically significant differences between them (Table 1). Furthermore, the parallelism test showed that the relative potency of the WMI strain compared with the MFr strain was 3.6 with a 95 %

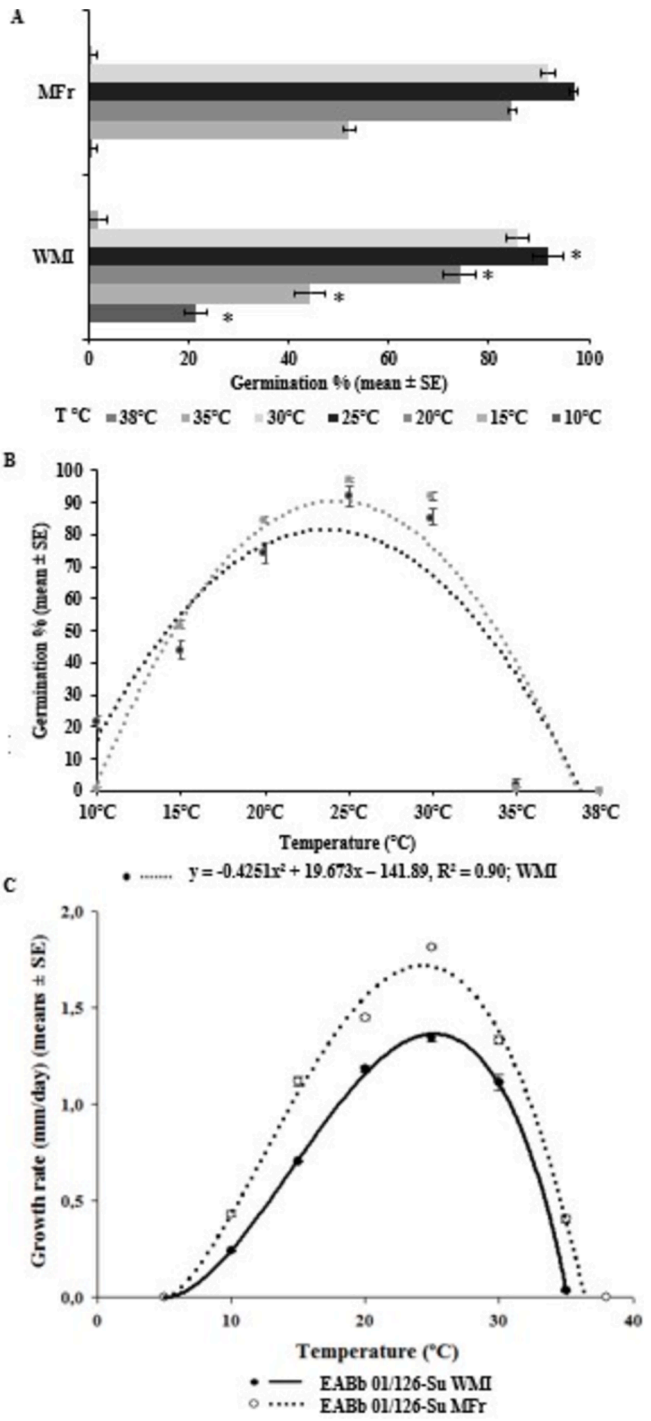

Fig. 3. Effect of mycovirus infection on conidial germination and radial growth under different temperatures. (A) Conidial germination (%) of WMI and MFr at different temperatures. Each column represents the mean value of five replicates and the error bars represent standard error of the mean. For each temperature, asterisks indicate statistically significant differences between WMI and MFr ($P < 0.05$ LSD test). (B) Polynomial regressions explaining the evolution of germination of the wild mycovirus-infected (WMI) and mycovirus-free (MFr) EABb 01/126-Su of B. bassiana strain at different temperatures. (C) Model predictions for the effect of temperature on the growth rate of colonies of WMI (solid line) and MFr (dotted line) B. bassiana EABb 01/126-Su of strain. Lines represent fitted curves obtained using the generalized β function modified according to Bassanezi et al. (1998).

Table 2
Estimated parameters (± SE) of the generalized β function of a nonlinear model for temperature radial growth of WMI and MFr.

Strain	Estimated parameters[1, 2]			
	T_{opt} (°C)	T_{max} (°C)	TY_{opt} (mm/day)	T_{B3}
WMI	25.14 ± 0.16 a	35.07 ± 0.05 a	1.36 ± 0.01 a	0.90 ± 0.05 a
MFr	24.30 ± 0.26b	36.34 ± 0.36b	1.69 ± 0.02b	0.96 ± 0.10b

[1] The generalized β function of a nonlinear model is given by the formula: $V(T) = ((T - T_{min})/(T_{opt} - T_{min}))^{\wedge}(Tb3 \times ((T_{opt} - T_{min})/(T_{max} - T_{opt}))) \times ((T_{max} - T)/(T_{max} - T_{opt}))^{\wedge}Tb3$ where V(T) indicates the fungal growth in mm per day; T is the incubation temperature; T_{B3} is the shape parameter that influences the temperature range around T_{opt}; TY_{opt} is the fungal growth at the optimal temperature T_{opt}; T_{min}, T_{max} and T_{opt} are the lowest, highest, and optimal temperature for the fungal growth, respectively.

[2] The means of radial growth of WMI and MFr were compared using the least significant difference Student's *t* test and different letters indicate statistically significant differences.

confidence limit of 1.29–11.84 (Table 1). Conversely, there were no significant differences in the LT_{50} virulence measurements for WMI and MFr, with values of 6.0 and 6.8 days, respectively (Table 1).

3.4. Abiotic stresses

3.4.1. Mycovirus-related response of Beauveria bassiana to temperature

The effects of temperature on conidial germination and radial growth were assessed and compared for the WMI and MFr strains. Conidial germination of both strains was observed in the entire temperature range tested from 10 °C to 35 °C but not at 38 °C. A statistically significant impact of temperature on germination was detected, with lower germination rates for WMI ($F_{6,69} = 1301.8$; $P < 0.001$) as compared to MFr ($F_{6,69} = 18636.6$; $P < 0.001$) after 18 h of incubation (Fig. 3A). The high F-values observed in this analysis suggest substantial differences between the treatment groups, indicating a strong effect of the treatments on the response variable. Statistically significant lower germination rates for WMI as compared to MFr was also observed for 15 °C ($F_{1,19} = 42.25$; $P < 0.05$), 20 °C ($F_{1,19} = 47.86$; $P < 0.05$), 25 °C ($F_{1,19} = 13.94$; $P < 0.05$) and 30 °C ($F_{1,19} = 22.09$; $P < 0.05$). Conversely, statistically significant higher germination rate for WMI as compared to MFr was noted for 10 °C ($F_{1,19} = 322.87$; $P < 0.01$). WMI conidia are capable of germinating across a more extensive range of temperatures as revealed by an increase of 21.4 and 2.0 % in their germination rates at the extreme temperatures of 10 and 35 °C as compared with MFr conidia (Fig. 3A and B). The relationship between temperature and germination was adequately described by second-order polynomials, with maximum germination rates of 85.7 % at 23.1 °C for the WMI strain and 95.6 % at 23.6 °C for the MFr strain (Fig. 3B).

Radial growth of both strains was observed in the entire temperature range tested from 10 °C to 35 °C but not at 38 °C (Table 2). Radial growth measurements from day 2 to 10 at all temperatures were used to fit linear models, and coefficients of determination of regression lines for the WMI and MFr strains varied from 0.928 to 0.989 and from 0.917 to 0.984, respectively. Colony extension rates for the WMI and MFr strain were 0.24 and 0.43 mm/day at 10 °C, 0.70 and 1.11 mm/day at 15 °C, 1.18 and 1.44 mm/day at 20 °C, 1.34 and 1.81 mm/day at 25 °C, 1.11 and 1.33 mm/day at 30 °C and 0.03 and 0.40 mm/day at 35 °C, respectively (Fig. 3C). By using these growth rates in mm/day at each temperature, optimum curves well described by the generalized β function were fitted (Table 2). Statistically significant differences ($P < 0.05$) were detected between WMI and MFr in their maximal growth temperatures (T_{max}), 35.1 and 36.3 °C, and optimal growth temperatures (T_{opt}), 25.1 °C and 24.3 °C, respectively (Table 2). There was also a statistically significant difference ($P < 0.05$) between WMI and MFr radial growth at optimal temperature (TY_{opt}), 1.36 and 1.69 mm/day, respectively (Table 2).

3.4.2. Mycovirus-related response of EABb 01/126-Su to water activity

The effects of water activity on conidial germination were assessed and compared for the WMI and MFr strains. There was a mycovirus-related impact on the effect of water activity on conidial germination after 24 h of incubation for both WMI and MFr ($F_{1,19} = 1285.71$, $F_{1,19} = 10.24$, respectively) with statistically significant () higher germination rates for MWI as compared to MFr detected at 0.985 and 0.970 a_w (Fig. 4). In particular, no germination was observed for the MFr strain at 0.970 a_w while the WMI strain showed a 2.6 % germination rate. No germination was observed at 0.959, 0.928, 0.895 and 0.862 a_w in neither WMI nor MFr (Fig. 4).

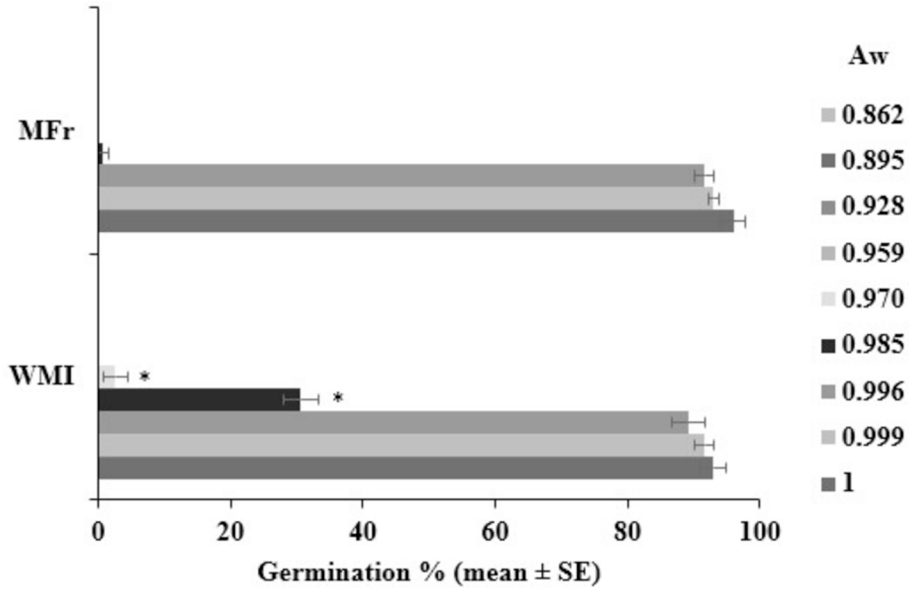

Fig. 4. Effect of mycovirus infection on conidial germination under different water activities. Conidial germination (%) of WMI and MFr at different water activity regimes (a_w). Each column represents the mean value of three replicates and the error bars represent standard error of the mean. For each water activity regime, asterisks indicate statistically significant differences between WMI and MFr ($P < 0.05$ LSD test).

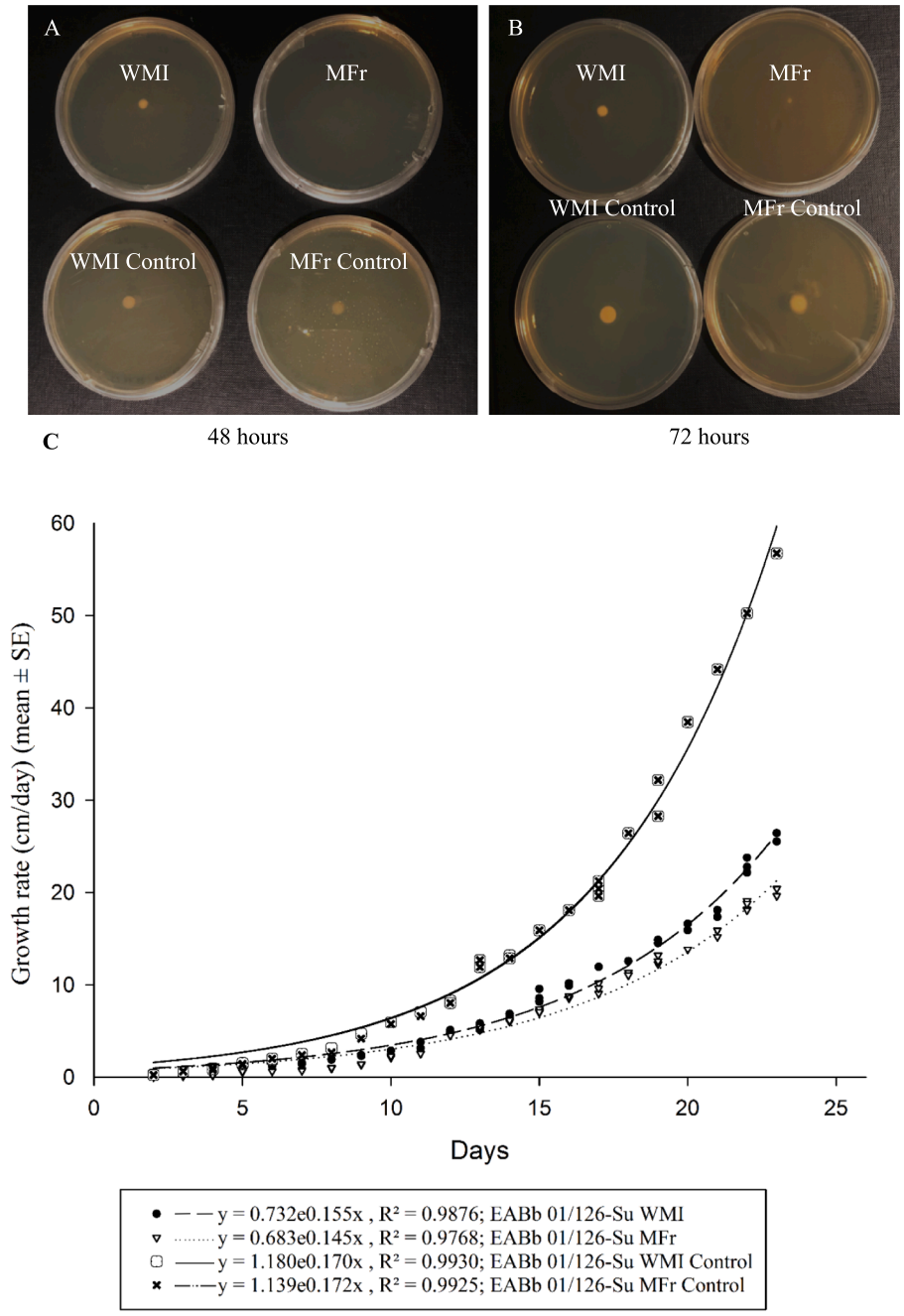

Fig. 5. Effect of mycovirus infection on radial growth under osmotic stress. Radial growth of WMI and MFr (A) 48 hpi and (B) 72 hpi on MA medium without NaCl (control) and supplemented with 0.8 M NaCl. (C) Exponential regressions of radial growth for WMI and MFr.

3.4.3. Mycovirus-related response of EABb 01/126-Su to osmotic stress

The effects of osmotic stress on radial growth were assessed and compared for the WMI and MFr strains. The WMI strain grew faster on 0.8 M NaCl than the MFr strain and this difference was statistically significant ($P < 0.01$) (Fig. 5A and B). Additionally, there was a statistically significant growth inhibition evident when comparing WMI and MFr with their respective controls ($P < 0.001$), while there were no significant differences between controls ($P > 0.05$) (Fig. 5A and B).

3.4.4. Mycovirus-related response of Beauveria bassiana to UV-B radiation

The effects of UV-B radiation on conidial germination, culturability and radial growth were assessed and compared for the WMI and MFr strains. No statistically significant differences were detected between WMI and MFr conidial germination and radial growth in response to UV-B radiation after 2 and 4 h of exposure, although a trend was noted for higher germination and increased radial growth in MFr. However, a statistically significant ($F_{1,11} = 60.41$; $P < 0.05$) higher germination rate and growth index was observed for MFr as compared to WMI after 6 h of exposure (Fig. 6A and C). Statistically significant ($F_{1,11} = 44.26$; $P < 0.05$) higher culturability was observed for MFr after 4 h of exposure, but the effect was reversed for 6 h of exposure with WMI showing statistically significant ($F_{1,11} = 21.81$; $P < 0.05$) higher culturability (Fig. 6B). Notably, MFr colonies showed a reduction in hyphal proliferation as compared to WMI colonies, after 6 h of exposure to 1200 mWm^{-2} (Fig. 6D and E).

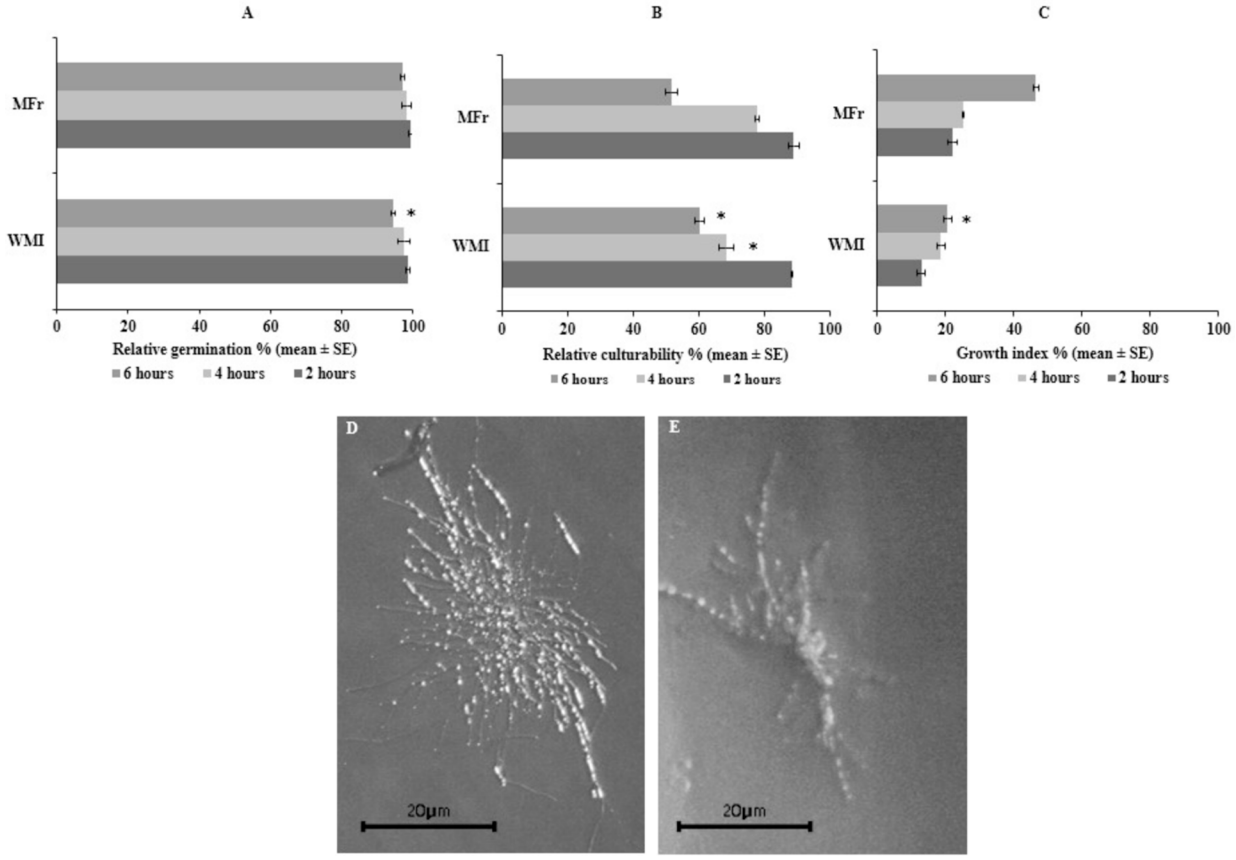

Fig. 6. Effect of mycovirus infection on conidial germination, culturability and radial growth following UV-B radiation. (A) Relative germination rate (%) 24 hpi; (B) relative culturability (5) 48 hpi; and (C) growth index (%) of WMI and MFr after 2, 4 and 6 h exposure to 1200 mW m^{-2}. Each column represents the mean value of three replicates and the error bars represent standard error of the mean. Asterisks indicate statistically significant differences between WMI and MFr ($P < 0.05$ LSD test). Hyphal proliferation of (D) WMI and (E) MFr after 6 h exposure to 1200 mW m^{-2}.

Table 3
Inhibitory activity of *Trichoderma* sp. on the radial growth of *B. bassiana* WMI and MFr.

Strain	*Trichoderma harzianum* T115		*Trichoderma reesei* T112
	Inhibition (%)[1]	Spherical index[2]	Bell scale[3]
WMI	45.69 a	1.00	R4
MFr	43.85 a	0.89	R4

[1] Inhibition percentage was calculated using the formula IMG=((C-T)/C) × 100, where C is radial growth in the control and T is radial growth in the treatment. Different letters indicate statistically significant differences (P = 0.05 Tukey HSD test).

[2] Spherical index was calculated using the formula α/β. If the spherical index is 1, *Trichoderma* spp. has no effect on the growth of *B. bassiana* and the latter grows radially without inhibition.

[3] Bell's scale. R1) 100 % *B. bassiana* overgrowth; R2) two-thirds of the medium surface of *B. bassiana* overgrowth; *R*3) one-half of the medium surface of *B. bassiana* overgrowth; R4) locked at the point of contact.

3.5. Biotic stress: Mycovirus-related effect on antagonistic activity of EABb 01/126-Su against Trichoderma spp

The antagonistic activity against *Trichoderma* spp. was assessed and compared for the WMI and MFr strains. There was not a statistically significant antagonistic effect against *T. harzianum*, with growth inhibition rates of 45.7 % and 43.9 % for WMI and MFr, respectively. Conversely, the spherical index values of WMI and MFr revealed a significant mycovirus-related effect on *T. harzianum* antagonism, with no change detected in the WMI growth a marked change in the one of the MFr strain revealing that it was antagonized by *T. harzianum* (Table 3, Fig. 7A). The Bell scale (R4, locked at the point of contact) revealed that growth of both WMI and MFr was blocked by *T. reesei* (Table 3, Fig. 7B).

4. Discussion

Whilst there have been numerous studies on optimizing the inoculum quantity in the mass production of EA, little attention has been paid to inoculum quality, which is a critical bottleneck limiting the consistency of biocontrol under field conditions and their widespread use (Magan, 2011; Quesada-Moraga et al., 2024). One of the main types of ecological fitness, the environmental competence, is the ability of the EA strain to realise its maximal potential for pest control under fluctuating abiotic and biotic factors, which it is a prerequisite for its successful development in the field (Quesada-Moraga et al., 2024). Therefore, research on improving the ecological fitness of EA inoculum is becoming increasingly important to improve the EA efficacy (Magan, 2011; Quesada-Moraga et al., 2024). In this context, mycovirus research in EA is not only meaningful towards a biotechnological exploitation of hypervirulence induction, but crucial as a driver of ecological fitness and quality of the fungal inoculum (Filippou et al. 2018; García-Pedrajas et al., 2019; Kotta-Loizou, 2021; Kondo et al., 2022).

The findings of this study are consistent with the emergence of a key mutualistic interaction between the EA and popular biocontrol agent *B. bassiana* and mycoviruses: specifically, BbPV-2 and BbPmV-1 provide both a virulence and ecological fitness advantage to their host, particularly under environmental stress conditions. Some morphological changes associated with mycovirus infection are surprising and reported for the first time in EA. For instance, we only have evidence of similar

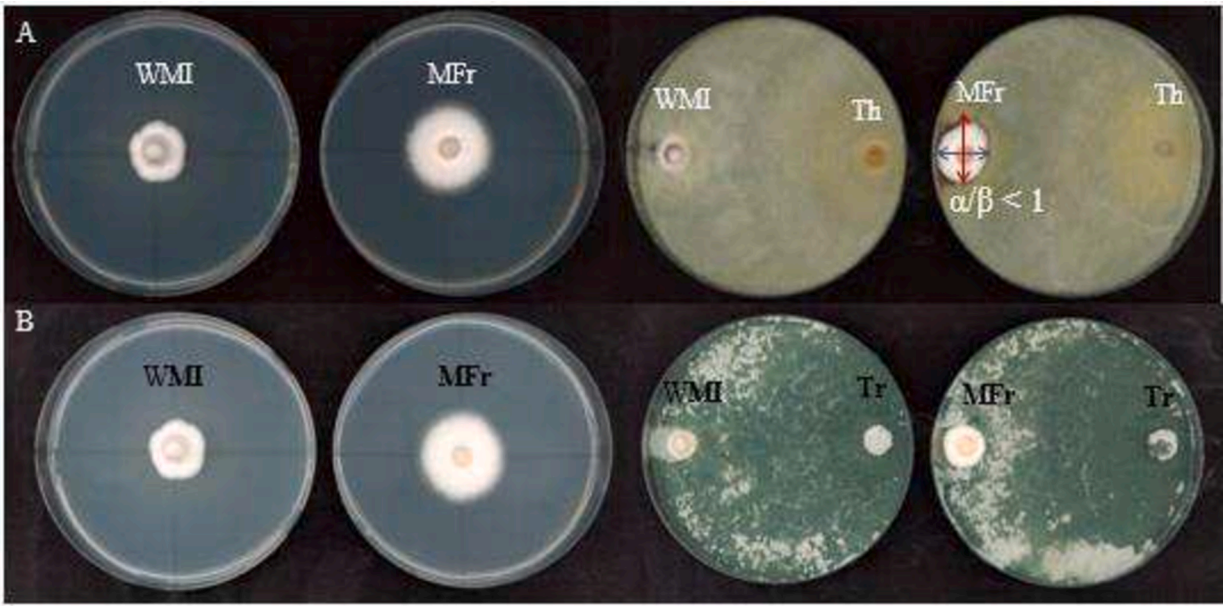

Fig. 7. Effect of mycovirus infection on antagonism between *B. bassiana* and *Trichoderma* spp. (A) Antagonistic activity of *T. harzianum* (Th) against *B. bassiana* WMI and MFr. The circular shape of the WMI colony and the ellipsoidal shape of the MFr colony are noted. (B) Antagonistic activity of *T. reesei* (Tr) against *B. bassiana* WMI and MFr.

changes in spore size, circularity and roundness in the saprophytic ascomycete and opportunistic human pathogen *Aspergillus fumigatus*, whereas the mycovirus-free strain exhibited a larger conidial diameter as compared to the wild-type, mycovirus-infected strain (Takahashi-Nakaguchi et al., 2020).

Pr1 is an important EA virulence factor for cuticle degradation during host penetration (Song & Feng, 2011; Gao et al., 2020; Wang et al., 2002), but has not been examined in association with mycovirus infection in the past. In our *B. bassiana* strain, mycovirus infection led to a 3.4-fold increase in virulence accompanied by a 35.9 % and 72.7 % increase in the levels of intracellular and extracellular Pr1 activity, respectively. Additionally, the increased virulence may be related to better adhesion of mycovirus-infected conidia to the insect cuticle as indicated by their higher isoelectric point (IP) and lower zeta potential (Holder & Keyhani, 2005; Cho et al., 2007; Song & Feng, 2011). Further, the impact of mycovirus-related changes in hydrophobicity and zeta potential on the interaction of the host strain with soil and plant surfaces remains to be elucidated, since such phenomena could influence the endophytic behaviour of the host strain and its use for biological control of soil dwelling pests.

We showed that mycovirus infection broadens the temperature range for growth and germination of the host strain, with 2.0 and 21.4 % increase in the germination rates of the wild mycovirus-infected strain compared to the mycovirus-free strain at the extremes of the evaluated range, 10 and 35 °C, respectively. Despite the paucity of data on the effect of mycoviruses in EA ecological adaptation, previous reports on other fungi point towards the same direction; for instance, a mycovirus provides the phytopathogenic fungus *Curvularia protuberata* with thermal protection as reveled by the host's increased growth at 30 °C and survival to 38 °C exposure (Morsy et al., 2010). Similarly, a mycovirus-infected strain of *Tolypocladium cylindrosporum* showed increased growth at 30 °C (Zabalgogeazcoa et al., 2018). Our results show that the virus-free strain shows higher germination and colony extension rates at intermediate thermal regimes (20–30 °C) as compared with the virus-infected strain, implying that the benefits of a wider temperature range come at an ecological cost of slower growth at intermediate temperatures. This was also noted by both Ejmal et al. (2018) in *Aspergillus* spp. and Zheng et al. (2014) in *Rhizoctonia solani*, with the mycovirus-free strain showing increased growth at 20 °C and 28 °C, respectively.

The present work reports for the first time mycovirus infection promoting drought tolerance for a beneficial fungus, with the virus-infected strain showing a 2.6 % germination rate at 0.970 a_w as opposed to no germination for the virus-free strain. Osmotic stress significantly impairs fungal growth, but the virus-infected strain showed a higher growth rate as compared to the virus-free strain. Tolerance to osmotic stress was previously observed in mycovirus-infected *Cryphonectria parasitica* (Nerva et al., 2017). In our study, mycovirus infection improves the UV-B stress tolerance of *B. bassiana*, increasing germination, culturability and growth index rates after 6-hour exposure to 1200 mWm^{-2}; similarly, mycovirus-infected *Cordyceps chanhua* (Zhu et al., 2022) and *Cryphonectria parasitica* (Santos et al., 2017) had higher tolerance to UV radiation, probably due to alterations in the pigment synthesis responsible for UV-B protection (Santos et al., 2017). In summary, we show that mycovirus infection promoted drought, salinity and UV-B tolerance in a beneficial EA, important findings highlighting the positive evolutionary effect of the mutualistic interaction between *B. bassiana* and two mycoviruses, BbPV-2 and BbPmV-1, on pest control (Quesada-Moraga et al., 2023).

The effects of soil and host microbiota, whether synergistic or antagonistic, have been reported as important for the development and survival of EA, which have evolved a spectrum of strategies for exploiting or suppressing these microbes (Jackson and O'Callaghan, 1997; Jackson et al., 2000; Boucias et al., 2018; Quesada-Moraga et al., 2023). The enhanced antagonistic potential provided by mycovirus infection may confer a selective advantage to *B. bassiana* in environments where it must compete with other soil microorganisms for survival (Meyling & Eilenberg, 2007; Bruck, 2010). Hence, the greater antagonistic potential of the strain EABb 01/126-Su associated with the presence of the mycovirus demonstrates the evolutionary selective pressure in favour of mycovirus infection, allowing the host strain to have a greater capacity to persist in complex microbial environments and therefore improving their ability to function as microbial control agents. In practical conditions, the presence of the mycovirus enhances the ability of the strain to express its higher virulence potential in both epigeous and hypogeous treatments, where it may encounter a suppressive microbiota (Meyling & Eilenberg, 2007; Bruck, 2010; Arjona-López & López-Herrera, 2021). Previous studies show that mycovirus

infection improves antagonistic activity, as reported for *Entoleuca* spp. against *Rosellinia necatrix* (Arjona-López & López-Herrera, 2021) and for *Trichoderma harzianum* against *Pleurotus ostreatus* and *Rhizoctonia solani* (Chun et al., 2018), whereas to date there was no information regarding beneficial EA. Therefore, the mycovirus related improvement of *B. bassiana* antagonistic potential against *T. harzianum* found in the present work is also relevant in terms of environmental competence and successful use in pest control.

Our present research is directed towards understanding the molecular basis of this impactful effect of the mycovirus infection on the biocontrol potential of EABb 01/126-Su strain by investigating the identify mycovirus genes responsible for the increased virulence and ecological fitness of the mycovirus infected strain as reported previously for the mycovirus-related hypovirulence of an *Aspergillus fumigatus* Fresenius strain. (Takahashi-Nakaguchi et al., 2020). The present work provides novel information on the selection pressure to the wild-type mycovirus-infected EA strains over the mycovirus-free ones. In the favourable interactions between *B. bassiana* and its mycoviruses reported in the present work, the mycoviruses appear to hijack host survival pathways and thereby modify host adaptation (Lerer & Shlezinger, 2022). These mycovirus-derived traits that enhance fungal fitness in specialized niches should be biotechnologically explored for improving the environmental competence of EA strains used in pest control.

5. Conclusions

In the favourable interaction of the strain EABb 01/126-Su with its mycovirus reported in the present work, the mycovirus seems to hijack fungal survival pathways and thereby modify fungal host adaptation (Lerer and Shlezinger, 2022). These mycovirus-derived traits that enhance fungal fitness in specialized niches for microbial control underlie fungal adaptation and should be biotechnologically explored for improving the environmental competence of the EA strains used in microbial pest control. Moreover, the present work provides novel information on the positive selection pressure to the wild mycovirus-infected EA strains over the mycovirus free ones.

CRediT authorship contribution statement

F. Rueda-Maíllo: Writing – original draft, Methodology, Investigation, Formal analysis. **I. Garrido-Jurado:** Writing – review & editing. **I. Kotta-Loizou:** Writing – review & editing. **E. Quesada-Moraga:** Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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