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Efecto de la temperatura, la actividad del agua y la radiación UV-B sobre la germinación y crecimiento de aislados de *Beauveria bassiana* (Balsamo) Vuill. procedentes del suelo y filoplano de dos ecosistemas forestales

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Effect of temperature, water activity and UV-B radiation on conidia germination and colony growth of *Beauveria bassiana* (Balsamo) Vuill. isolates from soil and phylloplane of two forestry ecosystems

by

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A Jose, que sin su incansable ayuda y apoyo

no podría haberlo conseguido

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RESUMEN

Factores abióticos ambientales como la temperatura, la humedad y la radiación ultravioleta (UV-B) tienen una gran influencia sobre la presencia, distribución y persistencia de los hongos entomopatógenos (HE) en sus hábitats naturales (suelo o filoplano de las plantas). Sin embargo, la protección contra estos factores es mayor en el primer hábitat que en el segundo, por lo que uno de los objetivos del presente trabajo ha sido determinar si el filoplano podría proporcionar aislados fúngicos mejor adaptados a las condiciones extremas de temperatura, humedad y exposición a rayos UV-B que los del suelo.

Para ello, 20 aislados del HE *Beauveria bassiana* obtenidos de suelo y filoplano de dos ecosistemas del sur de España (dehesa y reforestación de encinas) fueron seleccionados para: (1) Ser caracterizados molecularmente con el factor de elongación EF1-α y la región intergénica nuclear Bloc, (2) Estudiar su diversidad utilizando 4 microsatélites (ISSR), (3) Evaluar sus requerimientos térmicos, hídricos y de UV-B.

El análisis genético reveló diferencias entre los aislados, y mostró relación entre los microsatelites y EF1-α/Bloc. De los 20 aislados estudiados, se obtuvieron dos haplotipos, uno que contenía los aislados de la secuencia tipo del grupo ST1 y un segundo haplotipo que contenía el resto de aislados agrupados en tres secuencias tipo (ST2, 3 y 4). Las secuencias tipo ST2, 3 y 4 son aparentemente polifiléticas, lo que sugiere heterogeneidad temporal aparecida entre estos tres grupos. Este análisis sugiere que no hay una relación directa entre el origen de los aislados y su diversidad genética.

Una vez caracterizados molecularmente, se procedió al estudio de la respuesta de los aislados frente a los principales factores climáticos (temperatura, humedad y radiación UV-B) para dilucidar una posible relación entre la ecología de estos aislados y su origen, secuencia tipo o haplotipo.

El efecto de la temperatura sobre la germinación y crecimiento diametral se evaluó sometiendo a los distintos aislados a temperaturas de entre 15 a 35 ºC. Estos presentaron un óptimo de temperatura en el crecimiento micelial que osciló entre de 23.8 y 28.7ºC y un porcentaje de germinación, máximo para todos los aislados a 25ºC, entre 64.6 y 94.3% tras 18 horas de incubación.

La actividad del agua (a_w) se evaluó en la germinación de los conidios enfrentándolos a distintos valores de a_w (1-0.862) obtenidos mediante la adición de glicerol al medio de cultivo. Todos los aislados presentaron valores máximos de germinación entre 1 y 0.996a_w, a partir del cual la germinación comenzó a decrecer en la mayoría de ellos. Tan sólo 4 aislados germinaron a 0.928a_w. Por debajo del mismo no germinó ningún aislado tras 24 horas de incubación.

Finalmente, los conidios de los aislados se expusieron a dos irradiancias (920 y 1200mW m^{-2}) durante 2, 4 y 6 horas. En este caso, se evaluó la germinación, las unidades formadoras de colonias (o culturability) y el crecimiento micelial. En el caso de la germinación, se observó que los conidios perdían capacidad germinativa cuanto mayor era la irradiancia y tiempo de exposición, un hecho que no fue tan evidente en la formación de colonias o el crecimiento micelial. Parece ser que los propágulos se "recuperan" tras la exposición, una recuperación que es menor cuanto mayor es el tiempo de exposición (6h) y la irradiancia (1200 mW m^{-1}).

Se podría decir que los hongos entomopatógenos procedentes del filoplano, aparentemente más expuestos a valores extremos de los factores ambientales evaluados, podrían haber evolucionado para resistir las condiciones más desfavorables. Los resultados de este trabajo muestran claramente que el hábitat no siempre origina genotipos con mejor competencia ambiental.

Palabras clave: habitat, factor de elongación (EF1-α), Bloc, ISSR, ecología

ABSTRACT

Environmental abiotic factors such temperature, moisture and ultraviolet radiation (UV-B) highly influence presence, distribution and persistence of entomopathogenic fungi (EF) in their natural habitats (soil or plants phylloplane). However, protection against these factors is greater in the soil than in the plant phylloplane. For that, one of the objectives of the present work has been to determine whether the phylloplane could harbour fungal isolates better adapted to extreme conditions of temperature, humidity and UV-B exposure than those from the soil. For that, twenty isolates of the EF *Beauveria bassiana* obtained from soil and phylloplane of two ecosystems from the south of Spain (holm oak dehesa and reforestation) were selected to: (1) be molecularly characterized with elongation factor 1-α(EF1-α) and the intergenic nuclear region Bloc, (2) Study their diversity using with 4 microsatellites primers (ISSR); (3) Evaluate their thermal, humidity and UV-B requirements.

From the 20 *B. bassiana* isolates, two haplotypes were obtained, one contained the type sequence of TS1 group and the second one contained the rest of isolates grouped in three type sequences (TS2, 3 and 4), which were apparently polyphyletic, and could represent temporal heterogeneity between these three groups. Besides, similar patterns of isolate grouping were observed by using microsatellites (ISSR) and TEF/Bloc. The analysis revealed that these TS were not linked to isolate habitat (soil or phylloplane) or origin, holm oak dehesa or reforestation.

The temperature effect on germination and colony growth was evaluated in the range 15-35 ºC. The optimal temperature for mycelia growth ranged between 23.8 and 28.7 ºC, and the germination percentage (maximum at 25ºC for all isolates) ranged between 64.6 and 94.3 % after 18 hours of incubation.

The water activity (a_w) effect on conidia germination was evaluated against different values of a_w (1 – 0.862), which were obtained adding glycerol to the medium. All isolates showed maximum germination values between 1 and 0996 a_w. Only 4 isolates germinated at 0.928 a_w. Germination at a_w values lower than 0.928 was not observed for any isolate.

Finally, conidia were exposed to different irradiances (920 and 1200 mWm^{-2}) during 2, 4 and 6 hours, and germination, culturability and mycelia growth were evaluated. All isolates exhibit an initial delay on germination for higher irradiance and longer exposition time, whereas a recovery seemed to occur as revealed by the higher culturability rates and colony growth values. It could be concluded that a "recovery" of the fungal propagules could occur after exposition to UV-B, even if such recovery is lower for longer exposure times (6h) and irradiance (1200 mW m^{-1}).

It could be argued that isolates of entomopathogenic fungi from the phylloplane that are most exposed to abiotic environmental factors could have evolved to resist more unfavorable environmental conditions, the present work results clearly shows that such habitat not always provide the more environmentally competent genotypes.

Keywords: habitat, elongation factor 1-alpha (EF1-α), Bloc, ISSR, ecology

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1.- Introduction

Despite the vital role that entomopathogenic fungi (EF) play in the natural pest control, they have been overlooked in studies performed to determine their diversity in different ecosystems. A better understanding of their ecology can improve our ability to use them as a pest control measure and predict their response to agricultural practices (Quesada-Moraga y Santiago-Alvarez, 2008).

Environmental factors such as temperature, moisture and UV radiation are conditioning host insects, soil and plants, which are habitat for EF (Vega et al., 2009; Quesada Moraga et al., 2006a; Meyling and Eilenberg, 2006). Indeed, these environmental factors and their complex interactions may influence EF presence, distribution, persistence and infection process (Jaronski, 2010).

Ambient temperature may influence germination rate, micelial growth and infection progress of EF (Roberts and Campbell, 1977), with responses being either lineal or showing bell shaped curves (Yeo et al., 2003; Quesada-Moraga et al., 2006b; Garrido-Jurado et al., 2011). Although the optimal temperatures for germination, growth rate, and infection often range between 20-30ºC, these can occur within in a wide range of temperatures (5.0–37.0ºC) (McCammon and Rath, 1994). Usually, the vegetative growth decreases close to 30ºC, and normally ceases at 34-37ºC, thus they cannot involve a risk for mammals.

Some studies have revealed that the ability of entomopathogenic fungi to germinate and infect the host under conditions of low ambient humidity is attributed to sufficient moisture within microhabitats (Fargues et al., 2003; Inglis et al., 2001; Wraight et al., 2000). Besides, moisture is not only determining spore germination, but also conidiogenesis after the host death (Inglis et al., 2001). In the habitat , the water availability is a critical abiotic parameter, being higher in the limit layer of the host than

in the habitat environment (Willmer, 1986). In contrast, ambient temperature influences the rate of infection and time to death of insects treated with entomopathogenic fungi and is a key factor influencing efficacy of these biocontrol agents (Inglis et al., 2001). Therefore, it is important to match the thermal tolerance of a prospective fungal isolate to the climatic conditions expected at the targeted environment (Faria and Wraight, 2001).

Solar radiation is a key factor determining inoculum persistence in epigeous habitats, with a dramatic fall in the inoculum viability detected after short-term exposure to sunlight (Roberts and Campbell, 1977; Braga et al., 2001). Both conidia and hyphae of EF are highly susceptible to the damage caused particularly by the ultraviolet radiation (UV) (Inglis et al., 2001). UV radiation is a part of the electromagnetic spectrum emitted by the sun that can be divided into three types of radiation depending on the wavelength (λ): UV-A; UV-B and UV-C. The most important and harmful for the biological processes is UV-B (λ between 285-315 nm), which is practically retained by O_3 from the stratosphere, together with all the UV-C of λ short (lethal for life). Only the 10% of the UV-B radiation reaches the Earth surface, as well as 95% of UV-A radiation (λ between 315-400 nm), which also may damage to some extend most of the living beings.

The photodegradation of the conidia depend on the surface in where they are placed, since differences can be found if they are deposited on glass, on the plant surface, or in culture growth media (Inglis et al., 1997). As aforementioned, UV radiation is one of the greatest challenges for the commercial use of EF, in view of the fact that this radiation reduces the probability of epizooties with the consequent increase in the cost of new applications. Therefore, it is essential to find and incorporate solar

protectors (e.g. oils or pigments) to the commercial formulations which improve UV radiation tolerance, or select high-tolerant isolates to UV radiation for field applications.

Generally, the thermal, water activity and UV features of the isolates are adapted to the microhabitats in which they grow, e.g. phylloplane, providing environmental (Meyling and Eilenberg, 2006). That suggests there are differences between genetic materials which result in some of them could be selected for high tolerance to environmental stresses. The new fungal phylogeny studies have supplied new insights that should allow to better understand the environmental features of EF (Vega et al., 2009).These phylogenetic studies have been used extensively in the field of ecology, by the proposal of a variety of methods to examine to which extent the ecological traits are conserved or not (Webb et al., 2002). In recent years, many molecular techniques have been developed to investigate genetic diversity or population genetics of EF (Pu et al., 2010).

The most common method used in the past to differentiate entomopathogenic fungal isolates was randomly amplified polymorphic DNA (RAPD) but now was replaced due to low reproducibility among laboratories. This technique has also been widely employed to study intraspecific variation within species or associations of fungal genotypes with specific hosts (Enkerli and Widmer, 2010). For EF, the SSU rRNA gene and the internal transcribed spacer region (ITS) region have been the main target loci for previous analyses of fungal community structure; nevertheless the region of elongation factor 1-alpha (EF1-α) and other nuclear intergenic regions (e.g. Bloc, EFutr,...) have proven to be more suited for one deep analysis (Kepler and Rehner, 2013). Moreover, inter simple sequence repeat (ISSR) or microsatellites markers have revealed to have a great potential for population structure and genetic variation analysis, showing considerable intraspecific variability among the isolates (Wang et al., 2005), that owing

to the abundant in the genome, high level of variation, statistical independence, and codominant pattern of the inheritance. The evaluation of the sources of genetic variation for local adaptation can provide different patterns of ecological requirements for the EF isolates.

It is important to bear in mind that the EF show complex relationships of their environmental features in the natural habitat. As environmental fluctuations produced in normal conditions are not as the same as that those reproduced in laboratory, so it should check whether EF which showed desirable environmental features *in vitro* can be successfully applied in the field experiments (Inglis et al., 2001). In this regard, the introduction of predictable models will allow the proper use of EF in an ecosystem with certain environmental characteristics. Only using predictive modelling, adapted fungal strains to the main conditions of the ecosystem could be select for a successfully field application (Jackson and O'Callaham, 1997).

Therefore, the objective of this study was to determine the effect of temperature, water activity, and UV-B radiation on germination and colony growth of *B. bassiana* isolates by using predictable models. In addition to that it sought to determine the adaptive advantages according to their genotypes for future field applications.

2. Materials and methods

2.1 Fungal isolates

The 20 fungal used belong to the Research Group "Agricultural Entomology" (PAIDI AGR 163 group) from the Department of Agricultural and Forestry Sciences of the University of Córdoba (Table 1). Eight isolates were obtained from the soil (five from a Holm oak dehesa and three from a Holm oak reforestation) and twelve from the phylloplane (seven from a Holm oak dehesa and five from a Holm oak reforestation).

Table 1.- Fungal isolates used in the present study

Isolate	Origin	Ecology	Habitat
EABb 10/126-Su	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	Soil
EABb 10/147-Su	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	Soil
EABb 10/275-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	*Quercus ilex*
EABb 10/150-Su	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	Soil
EABb 10/156-Su	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	Soil
EABb 10/225-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	*Quercus ilex*
EABb 10/282-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	*Quercus ilex*
EABb 10/129-Su	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	Soil
EABb 10/223-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	*Quercus ilex*
EABb 10/261-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	*Quercus ilex*
EABb 9/28-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	*Quercus ilex*
EABb 10/329-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak dehesa	*Quercus ilex*
EABb 10/143-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak reforestation	*Quercus ilex*
EABb 10/235-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak reforestation	*Quercus ilex*
EABb 10/121-Su	Castilblanco de los Arroyos (Sevilla)	Holm oak reforestation	Soil
EABb 9/20-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak reforestation	*Quercus ilex*
EABb 10/133-Su	Castilblanco de los Arroyos (Sevilla)	Holm oak reforestation	Soil
EABb 10/169-Su	Castilblanco de los Arroyos (Sevilla)	Holm oak reforestation	Soil
EABb 9/29-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak reforestation	*Quercus ilex*
EABb 09/16-Fil	Castilblanco de los Arroyos (Sevilla)	Holm oak reforestation	*Quercus ilex*

2.2.- Molecular characterization

2.2.1.- DNA extraction, PCR and sequencing

Mycelia for DNA extraction were grown on 90 mm Petri plates of malt agar medium (MA) (Oxoid, Basingstoke, Hants., England) under sterile conditions. The plates were incubated at 25ºC for 15 days in dark.

Total DNA was extracted from mycelia following the Raeder and Broda (1985) method. One nuclear intergenic region developed specifically for *Beauveria* (Bloc) and one nuclear protein-encoding genes (translation elongation factor 1- α (EF1-α)) were amplified, sequenced and analyzed. A 1100 bp fragment spanning the 3' 2/3 of the EF1-α gene was amplified with primers tef1fw (5'-GTGAGCGTGGTATCACCA-3') (O'Donnell et al., 1998) and 1750-rw (5'-GACGCATGTCACGGACGGC-3') (Garrido-Jurado, et al., 2011). A 1500 bp fragment spanning the 3' 2/3 of the Bloc gene was amplified with primers B5.1fw (5'-CGACCCGGCCAACTACTTTGA-3') and B3.1rw (5'-GRCTTCCAGTACCACTACGCC-3') (Rehner et al., 2006). The total reaction volume was 50 µl and contained 1.5 µl of genomic DNA, 10 µl of PCR reaction buffer (5x), 1 µl of each primers (0.20 mM), and 0.5 µl of *Taq* polymerase (MyTaq^{TM} Red DNA Polymerase, Bioline Ltd, UK). Finally, add ultrapure water up to 50 µl. The amplification program included an initial denaturing cycle of 3 min at 95ºC, followed by 35 cycles of 15 s at 95ºC, 15 s at 50ºC for EF1-α primers and 65ºC for Bloc primers,10 s at 72ºC, and a final extension step of 7 min at 72ºC. Negative (no DNA) and positive controls (fungal DNA from pure culture) were included in each set of reactions.

The PCR products were electrophoresed on 1% agarose gels buffered with 1 X TAE and stained with SYBR® Green (Invitrogen, Paisley, UK). A 100-bp ladder molecular weight standard (Solis Biodyne, Tartu, Estonia) was also used. The PCR

products were purified from agarose gels using the Geneclean II kit® system (QBiogene, Inc., Carlsbad, CA), following the manufacturer's protocol. All PCR products of EF-1α amplification were sequenced in both directions.

Microsatellite amplification reactions were carried out in 25 µl reaction mixture containing 5 µl of PCR reaction buffer (5x), 1 µl of each primers (Table 2), 0.25 µl of *Taq* polymerase, 1 µl of genomic DNA, and ultrapure water up to 25 µl. The amplification program included an initial denaturing cycle of 3 min at 95ºC, followed by 35 cycles of 15 s at 95ºC, annealing at the specific annealing temperature for 15 s, 10 s at 72ºC, and a final extension step of 7 min at 72ºC. The annealing temperatures were 61, 49, 43 and 47 for primer 6, 7, I and D, respectively. The PCR products were electrophoresed on 2% agarose gels buffered with 1 X TAE and stained with SYBR® Green (Invitrogen, Paisley, UK). A 100-bp ladder molecular weight standard (Solis Biodyne, Tartu, Estonia) was also used. Gels were photographed in UV light using a GelDoc^{TM} EZ Imager (Bio-Rad, Hércules, CA, USA).

Table 2.- ISSR primer sequences with details of the number and the size of amplified fragments (Ormond et al., 2010)

Primer	Sequence	Total number of polymorphic bands	Amplicon size range (bp)
6	5'-GATATCCGTCCGACGACGACGA-3'	43	180-1500
7	5'-CTATCCTGTGTGTGTGTG-3'	41	180-1600
I	5'-GCCTCCTCCTCCTC-3'	34	220-1500
D	5'-GTTGTGTGTGTGTGTG-3'	40	130-1600

2.2.2.- Data analysis

Sequences were alienated with DNASTAR 5.0. Maximum Parsimony (MP) analyses were implemented in MEGA 4.0, using the heuristic search option close-neighbor-interchange. A heuristic MP bootstrap analysis consisted of 500 pseudoreplicates and with gapped and parsimony-uninformative characters excluded. Clades with bootstrap values >50% were considered supported by the data.

For the microsatellites analysis, gels were scored manually for band size and the resulting binary (presence/absence of amplified bands) data matrix was analyzed using FreeTree (Hampl et al., 2001) where 1000 bootstrap replicates of trees generated via the Jaccard coefficient and neighbor joining algorithms were produced for each of the four ISSR primers. For visualization of the clusters TreeView package were used (Page, 1996)

2.3.- Ecological characterization

2.3.1.- Temperature effect on the fungal growth

For each selected isolate, circular plugs (8-mm diameter) were cut from non-sporulating mycelia of 4-day-old culture dishes using a cork-borer and a single plug was placed upside down in the center of a new Petri plate (60mm diameter) of MA medium (Oxoid, Basingstoke, Hants., England). Plates were sealed with Parafilm® (Pechiney Plastic Packaging Co., Chicago, IL) and incubated in the dark in separated incubator at 15, 20, 25, 30 and 35 ºC during 10 days. Five replicates plates were prepared for each isolate/temperature combination, with each replicate being submitted to a different model adjustment.

Surface radial growth was evaluated for 2, 4, 6, 8, and 10 days, with a digital calibrator, measuring two perpendicular diameters. After that, the average diameter was calculated and expressed in real growth, subtracting 8 mm because of the initial sown disc.

Radial growth data were fitted by regression analysis. The radial measurements (from 2nd to the 10th day) were fitted a linear model

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

The linear regression slopes (v) indicates the growth rate (velocity in mm per day) at a certain temperature. The regression analysis was carried out for each isolated/temperature repeats.

Temperature effect on the fungal growth rates (V) was evaluated by nonlinear model ß according to Bassanezi et al. (1998). The generalized β function is given by:

$$V(T) = TY_{opt} \times \left(\frac{T - T_{min}}{T_{opt} - T_{min}}\right)^{T_B \times \left(\frac{T_{opt} - T_{min}}{T_{max} - T_{opt}}\right)} \times \left(\frac{T_{max} - T}{T_{max} - T_{opt}}\right)^{T_B}$$

where V(T) is the fungal growth in mm per day (dependent variable) and T is the incubation temperature (independent variable). T_{min}, T_{max} and T_{opt} are, respectively, the lowest, the highest, and the optimal temperature for fungal growth. TY_{opt} is the fungal growth at the optimal temperature T_{opt}. Tb3 is the shape parameter that influences the temperature range around T_{opt} in which the curve stays near to TY_{opt}. T_{min} was fixed at 5 ºC (similar researches have proved that does not exist any growth at that temperature) for all the strains. So then, a better fit was achieved and the standard error estimation of the other parameters was also improved.

For each strain, non-linear model ß and the estimation of parameters and its standard error, were realized by using 5 values of V obtained (mm/day) from 5 repetition to each temperature. $T_{max,}$ $T_{opt,}$ TY_{opt} and Tb3 values was estimated by the method of Newton. The comparisons were made two by two with Student's t-test (P=0.05) from estimated values (p_1 and p_2) and their standard errors.

$$t = \frac{p_1 - p_2}{\sqrt{SE_1^2 + SE_2^2}},$$

2.3.2.- Temperature effect on germination

Conidial suspensions of twenty *B. bassiana* isolates were obtained by scraping the surface of 15-days old cultures maintained on MA medium. Conidia were then suspended in sterile distilled water. This suspension was shaken, sonicated for 5 minutes, and filtered. The number of conidia in the suspension was counted with Malassez chamber (Blau Brand, Germany) at 400X magnification and finally all suspensions were adjusted to $1x10^5$ conidia ml^{-1}.

Conidial suspensions were spread on Petri plate (60mm diameter) of water-agar medium. Each inoculated plate were sealed with Parafilm® and incubated at 15, 20, 25, 30 and 35 ºC in complete darkness. Germination percentage was observed after 18h because at 24h the germination percentage was similar at 25 and 30ºC. At 18 hours post inoculation, the germination was halted by transferring 0.5 ml of lactophenol cotton blue on to each plate and covered with a glass coverslip. Germination percentage was determined by counting 100 conidia for each plate at 400X magnification. Three replicates plates were prepared for each isolate/temperature combination.

Analysis of variance (ANOVA) was used to analyze the germination percentage, and the LSD test was used to compare means.

2.3.3.- Water activity effect on germination

In the natural environment, the ability of fungi to grow on a substrate with low availability of free water (a_w) is related to its ability to infect in water stress conditions. Under laboratory conditions, this fact is usually studied with the variation of the osmotic potential (ψ) of the culture medium using solutes such as KCl, NaCl or glycerol (Ma et al., 2001; Whiting y Rizzo, 1999).

In this case, the culture medium was performed with water-agar and glycerol. For each osmotic potential, half of the amount of water-agar indicated by the manufacturer was added, together with the amounts of glycerol that are shown in Table 3, each 1 liter flask contained 500 ml of distilled water. After that, it was sterilized in autoclave for 20 min and 1 overpressure atmosphere at 120 ºC.

Table 3.- Relationship between water activity (a_w) and glycerol concentration in the culture medium.

Potential(bar)	**a_w[(a)]**	**Glycerol[(b)] 20 ºC (moles/1.000 g H_2O)**	**Glycerol 20ºC (g/l H_2O)**	**0.5 l of medium**
1	0.999	0.05	4.61	2.30
5	0.996	0.21	19.34	9.63
20	0.985	0.80	73.68	36.84
40	0.970	1.57	161.18	80.59
60	0.995	2.30	211.83	105.92
100	0.928	3.71	341.69	170.85
150	0.895	5.42	499.18	249.6
200	0.862	7.14	657.59	328.8

[(a)] a_w was calculated in relation to osmotic potential by the following thermodynamic expression: $\psi=(RT/V_m)\ln a_w$; where ψ is the osmotic potential, R is universal gas constant (8.31×10^{-5} m^3 bar $mole^{-1}$ K^{-1}), V_m is the partial molal volume of water, and a_w is the water activity (Baver et al, 1972) [(b)] Molar mass of glycerol=92.10g/mol (Harris, 2006)

After sterilization, the flasks containing water-agar and glycerol medium were cooled at about 45°C. The medium was shaken and poured into Petri plates (60 mm diameter) and placed inside a biosafety chamber.

Conidial suspensions (obtained and adjusted as described in section 2.3.2) were spread on Petri plate (60mm diameter) of water-agar and glycerol medium and incubated at 25°C. Eight a_w regimes (0.999, 0.996, 0.985, 0.970, 0.995, 0.928, 0.895 and 0.862) and one control (free glycerol in the medium, with a_w=1) were assayed.

Three replicate plates were prepared for each strain and osmotic potential combination. Plates were sealed with Parafilm® and incubated at the referred temperature in darkness.

Twenty four hours post inoculation, the germination was halted by transferring 0.5 ml of lactophenol cotton blue on to each plate and covered with a glass coverslip. Germination percentage was determined by counting 100 conidia for each plate at 400X magnification. Three replicates plates were prepared for each isolate/temperature combination.

Analysis of variance (ANOVA) was used to analyze germination percentage, and the LSD test was used to compare means.

2.3.4- Ultraviolet radiation effect (UV-B) on fungal conidia

All isolates were grown on 150mm Petri plates with MA medium in darkness at 25 ºC for 15 days. The conidia were collected and suspended in 20ml distilled and sterilized water. This suspension was shaken, sonicated for 5 minutes, and filtered. The number of conidia in the suspension was counted with Malassez chamber (Blau Brand, Germany) at 400X magnification and finally all suspensions were adjusted to $1x10^3$ and $1x10^5$ conidia/ml. The suspensions were stored at 4ºC for no more than 24 hours.

Irradiation experiments were conducted in a temperature-controlled chamber (Fitoclima S600PL, ARALAB, Portugal). The temperature inside the chamber was maintained at 25±1 ºC. The irradiated material was covered with a 0.13 mm-thick cellulose diacetate film, which removed the radiation below 290nm. This allows the passage of most UV-B and UV-A, but prevents the UV-C (<280nm) exposure of samples Control plates were covered with aluminum foil and thus physically protected from radiation inside the UV chamber.

These spectral weighting functions were selected on the basis of the fungal response to the UVB-radiation that is closer to the action spectrum for DNA damage (Paul et al., 1997). The selected irradiances were 920 mW m^{-2} for low-irradiance, and 1200 mW m^{-2} for the high-irradiance. For our location, South of Spain, these irradiances corresponds with non sunlight for low-irradiance, and 30% ozone depletion for high irradiance. All UV measurements were made with the PMA2106 UVB detector that provides fast and accurate irradiance measurement in the UVB region.

3.3.4.1.- UV-B radiation effect on conidial germination

The experiment was performed using 60mm Petri plates containing MA medium with low concentration of dodine 60% (20µg/ml) that slow fungal growth and allowing, therefore, to monitor the germination for longer periods. Each plate was inoculated with 40µl of conidial suspension ($1x10^5$ conidia/ml) and also they were spread using a sterile disposable spreader. Tree replicates plates per exposure time and tree replicates control plates per exposure time were irradiated of 920 and 1200 mW m^{-2} for 2, 4 and 6 hours and immediately incubated at 25 ºC in the dark for 24 hours (modified Braga et al., 2001).

A total of 100 conidia were selected for each exposure time, and after 24 hours incubation, germination was observed with Leitz DMRB optical microscope (x400/0.65PH2). The relative germination percentage after each period of incubation was calculated by the following equation:

$$Relative\ germination\ (\%) = {}^{Wt}/_{Wc}\, x100$$

where W_t is the number of germ-lings at exposure time t per plate and W_c is the mean number of germ-lings mean of the control plate.

Analysis of variance (ANOVA) was used to analyze the relative germination percentage, and the LSD test was used to compare means.

2.3.4.2.- UV-B radiation effect on conidial culturability and on colony growth

The experiment was performed using 60mm Petri plates which contained MA medium. Each plate was inoculated with 40µl of conidial suspension ($1x10^3$ conidia/ml) and these were spread using a sterile disposable inoculation spreader. Three replicates plates per exposure time and three replicates control plates were prepared for each strain/treatment and irradiance combination.

Conidia were immediately exposed to irradiances of 920 and 1200 mW m^{-2} for 2, 4 and 6 hours and immediately incubated at 25 ºC in darkness for 48 hours (modified Braga et al., 2001).

Forty eight hours post inoculation, the growth on the plates was halted by transferring 0.5 ml of lactophenol cotton blue on to each plate.

Conidial **culturability** was evaluated counting colony forming units (CFUs), observed at 40X magnification and calculating the relative percentage culturability after each exposure time by the following equation:

$$Relative\ culturability\ (\%) = {}^{Tt}/_{Mc}\, x100$$

where Tt is the number of CFUs of each replicate at exposure time *t* and *Mc* is the mean number of CFUs for all control plates, regardless of exposure time.

The effect of conidial irradiation on the **colony growth** was determined by evaluating the size of the 48 hours colonies after irradiation, recording the two orthogonal diameters of each colony and considering 10 colonies per treatment.

The analysis of colony growth was carried out by calculation of the growth index after each exposure time by the following equation:

$$Growth\ index = {Cc - Ct}/{Cc + Ct}\, x100$$

where C is the diameter mean of each replicate at exposure time t and Cc is the mean diameter for all control plates, regardless of exposure time. Values near to 100 represent that the isolate is very sensible to UV radiation and values near to 0, UV radiation no affect at colony growth isolate.

Analysis of variance (ANOVA) was used to analyze relative culturability and growth index, and the LSD test was used to compare means.

3. Results and Discussion

3.1.- Molecular characterization

Both primers (EF1-α and Bloc) were analyzed and compared with selected sequences available from GenBank (Rehner et al., 2011). The phylogenetic analysis combining EF1-α and Bloc showed that the 4 type sequences (TS) were grouped with the *Beauveria bassiana* species (Figure 1). Soil and phylloplane isolates may be found in ST 1, 2, 3, and 4 (Table 4). However, ST4 was detected to be phylloplane shaped as it included only three phylloplane isolates (Table 4).

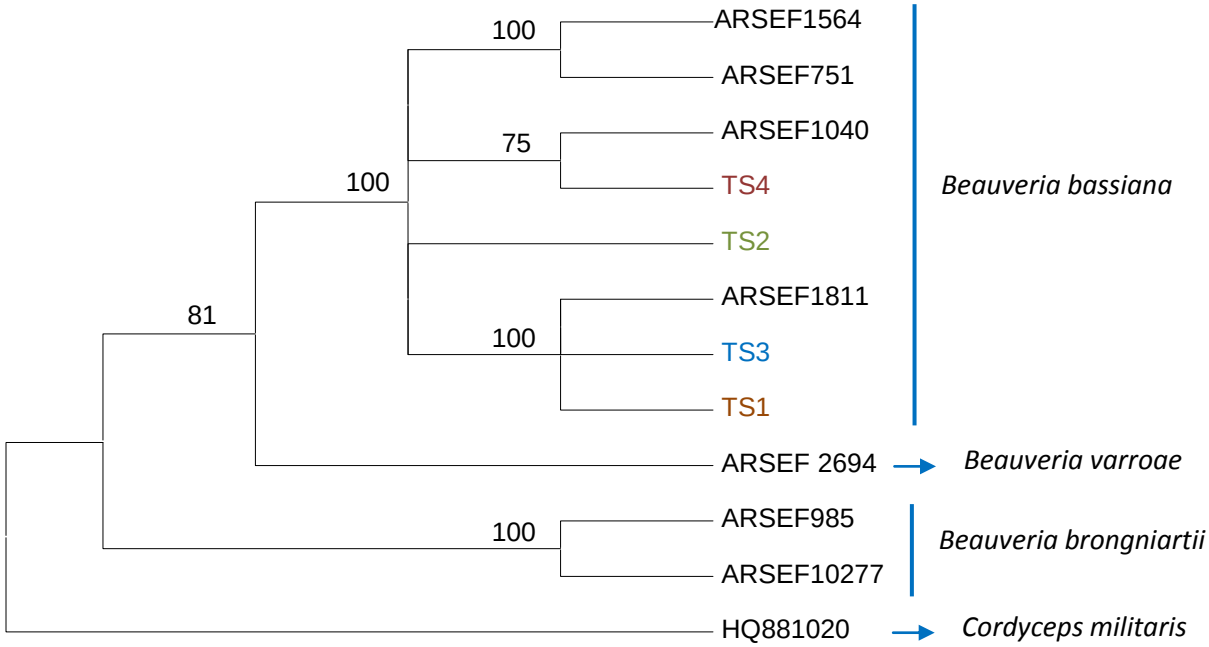

Figure 1.- Cladogram of four *Beauveria bassiana* type sequences illustrating species relationships inferred from joint Maximum Parsimony analysis of Bloc and TEF. Bootstrap values (based on 1,000 replicates) when above 50% are indicated on the branches. Species clades are indicated by vertical bars

Table 4.- Ecosystem, habitat and type sequence of the evaluated *Beauveria bassiana* isolates

Isolate	Ecosystem	Habitat	Sequence Type
EABb 10/126-Su	Holm oak dehesa	Soil	1
EABb 10/147-Su	Holm oak dehesa	Soil	1
EABb 10/275-Fil	Holm oak dehesa	*Quercus ilex* Phylloplane	1
EABb 10/150-Su	Holm oak dehesa	Soil	1
EABb 10/156-Su	Holm oak dehesa	Soil	2
EABb 10/225-Fil	Holm oak dehesa	*Quercus ilex* Phylloplane	2
EABb 10/282-Fil	Holm oak dehesa	*Quercus ilex* Phylloplane	2
EABb 10/129-Su	Holm oak dehesa	Soil	3
EABb 10/223-Fil	Holm oak dehesa	*Quercus ilex* Phylloplane	3
EABb 10/261-Fil	Holm oak dehesa	*Quercus ilex* Phylloplane	3
EABb 9/28-Fil	Holm oak dehesa	*Quercus ilex* Phylloplane	4
EABb 10/329-Fil	Holm oak dehesa	*Quercus ilex* Phylloplane	4
EABb 10/143-Fil	Holm oak reforestation	*Quercus ilex* Phylloplane	3
EABb 10/235-Fil	Holm oak reforestation	*Quercus ilex* Phylloplane	1
EABb 10/121-Su	Holm oak reforestation	Soil	2
EABb 9/20-Fil	Holm oak reforestation	*Quercus ilex* Phylloplane	2
EABb 10/133-Su	Holm oak reforestation	Soil	3
EABb 10/169-Su	Holm oak reforestation	Soil	3
EABb 9/29-Fil	Holm oak reforestation	*Quercus ilex* Phylloplane	3
EABb 09/16-Fil	Holm oak reforestation	*Quercus ilex* Phylloplane	4

Forty six loci alleles were detected with four microsatellite markers (ISSR primers). Phylogenetic analysis of these ISSR primers revealed polymorphism within isolates that had been phylogenetically and morphologically classified as *Beauveria bassiana*. The analysis revealed differences amongst all isolates, showing a relationship between microsatellites characterization and TEF/Bloc characterization (Figure 2). Of the 20 isolates studied, two large haplotypes were resolved: one containing isolates of TS1 and the second containing the remaining TS (Figure 2). The finding that the TS2, 3, and 4 are apparently polyphyletic suggests that temporal heterogeneity appeared between these three groups. ISSR analysis showed that particular groups of fungal isolates with similar genetic backgrounds are associated with particular TS.

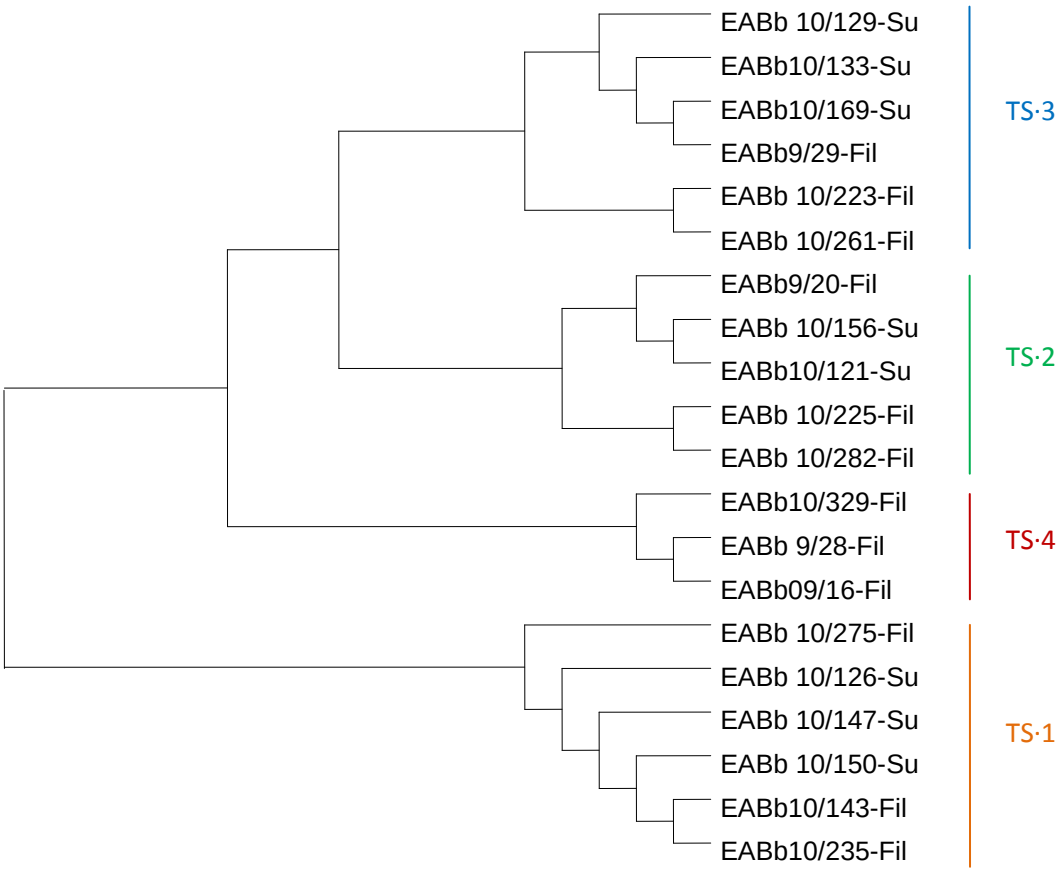

Figure 2.-Dendrogram of genetic relationship between *Beauveria bassiana* isolates from different natural habitat (Fil=Phylloplane; Su=Soil) and their relationship with type sequences obtained with Bloc and TEF molecular markers.

The combined analysis of EF1-α and Bloc and ISSR analysis allow highly sensitive detection of diversity, so they are well suited to differentiate closely related *B. bassiana* isolates. Concordance of genetic similarity among *B. bassiana* isolates and geographic origin or host affiliation has been previously studied but it is yet ambiguous (Wang et al., 2003; Rehner, 2005). In our study, 20 isolates has been selected in the holm oak dehesa and holm oak reforestation ecosystems in two habitat, soil and phylloplane, to determine the genetic structure of these populations, and as starting point to select isolates with different environmental requirements. As it known *B. bassiana* have a saprophytic phase, which may be more important than the insect-pathogenic phase in

determining the population genetic structure (Bidochka et al., 2002), because the fungus is subject to many environmental factors in this phase of its life cycle. These operating changes could resulting in heterogenous populations might facilitate the occurrence and persistence of the fungus in the environment (Luan et al., 2013). Our results seem to indicate that the presence of *B. bassiana* in the phylloplane could be the consequence of the dispersion of fungal propagules from the soil to the plant by the wind, insects etc., whereas the phylloplane shaped ST4 could reveal the occurrence of particular epiphyte-like genotypes.

3.2.- Ecological characterization

3.2.1.- Temperature effect on the fungal growth

Temperature had a significant effect on *in vitro* radial colony growth of all fungal isolates (Table 5). The radial measurements from the 2^{nd} to the 10^{th} day fitted a linear model. All fungal isolates grew at 15, 20, 25 and 30ºC, whereas none grew at 35ºC. The examination of the fixed regression lines indicates that maximum growth rate was reached at 25ºC. In general, incubation temperature higher or lower than 25ºC, were established to be suboptimal for the fungal growth of all the isolates.

There was no apparent relationship between the fungal growth rate and the habitat or ecosystem of isolation and molecular analysis. The growth rate became maximum when optimal temperature (T_{opt}) ranged between 23.8 and 28.9ºC. Similar results were obtained by other authors, which established as an optimal range of growth between 20 and 30ºC (Roberts and Campbell, 1977; Fargues et al, 1992; 1997; Quesada-Moraga et al., 2006). Fungal growth rate (TY_{opt}), which was estimated at the optimal temperature, varied between 2.1 and 3.9 mm/day (Table 5). The maximum temperature for fungal growth (T_{max}) did not reach 35ºC for any strain.

Table 5.- Estimated parameters (±SE) of the generalized β function modified according to Bassanezi et al. (1998) fitted to data of the vegetative growth of different *Beauveria bassiana* isolates. Vegetative growth was not observed for any isolate at 35ºC.

Isolate	Estimated parameters [(a)(b)]		
	T_{opt}	T_{yopt}	Tb3
EABb 10/126-Su	24.7±0.2acg	3.2±0.1ah	1.3±0.1acek
EABb 10/147-Su	28.7±2.0ab	2.6±0.1bc	0.3±0.2b
EABb 10/275-Fil	25.0±0.2ad	3.2±0.0a	1.3±0.1a
EABb 10/150-Su	24.6±0.3ae	2.6±0.0bd	0.91±0.1cd
EABb 10/156-Su	25.5±0.2af	2.4±0.0be	1.2±0.1ad
EABb 10/225-Fil	25.0±0.3ah	2.1±0.0fj	1.1±0.1ad
EABb 10/282-Fil	25.7±0.3bdfhi	2.3±0.0bfg	0.9±0.1adf
EABb 10/129-Su	25.3±0.2a	3.1±0.0a	1.3±0.1ag
EABb 10/223-Fil	24.8±0,1gh	3.9±0.0m	1.9±0.0h
EABb 10/261-Fil	24.9±0.2aij	3.1±0.0a	1.2±0.1degj
EABb 09/28-Fil	25.0±0.1aik	2.3±0.0b	1.2±0.0ad
EABb 10/329-Fil	27.3±0.5b	2.7±0.1gi	0.3±0.2bi
EABb 10/143-Fil	24.4±0.4al	2.9±0.1aci	1.0±0.2ad
EABb 10/235-Fil	26.0±0.4bfhi	2.0±0.0f	0.4±0.0bf
EABb 10/121-Su	23.8±0.5cehjl	2.5±0.1bij	1.8±0.3ahj
EABb 09/20-Fil	24.4±0.2cdehjkl	2.3±0.0bjk	1.3±0.1ad
EABb 10/133-Su	25.6±0.1bfl	2.6±0.0ci	1,2±0.0ad
EABb 10/169-Su	24.8±0.2ah	2.1±0.0fl	1.2±0.1ad
EABb 09/29-Fil	24.1±0.4cdejkl	2.1±0.0fk	1.1±0.2dkl
EABb 09/16-Fil	25.5±0.2ah	3.3±0.1degh	1.5±0.1kl

[(a)] The generalized β function is given by: $V(T) = TY_{opt} * ((T - T_{min}) / (T_{opt} - T_{min}))^{\wedge} (TB3 * ((T_{opt} - T_{min}) / (T_{max} - T_{opt}))) * ((T_{max} - :T) / (T_{max} - T_{opt}))^{\wedge} TB3$, where V(T) is the fungal growth in mm per day (dependent variable) and **T** is the incubation temperature (independent variable). $\mathbf{T_{min}}$, $\mathbf{T_{max}}$ and $\mathbf{T_{opt}}$ are, respectively the lowest, the highest, and the optimal temperature for fungal growth. $\mathbf{TY_{opt}}$ is the fungal growth at the optimal temperature T_{opt} and **TB3** is the shape parameter. (b) Means within columns with the same letter are not significantly different according to the least significant difference Student's t test.

A relationship was not found between lower and upper threshold temperatures or growth rates at different temperatures and their origin as reported by Fargues et al. (1997) using isolates from insects and soils from different latitudes. Nonetheless, it has speculated that response of fungal isolates to environmental factors could be related to their geographical origin, with possible adaptation of *B. bassiana* populations to specific habitats inside the climatic zones (Roberts and Campell, 1977; Vidal et al., 1997;

Fernandes et al. 2008). Besides, Kryukov et al. (2012) found a correlation between *B. bassiana* ISSR haplotypes and temperature response, even if the isolates were obtained from highly distant habitat. In our study, such a relationship among temperature, haplotype and habitat has not been detected.

3.2.2.- Temperature effect on germination

There were significant effect of temperature on germination of conidia at 18h post-inoculation at 15, 20, 25 and 30ºC (Table 6).

The range of conidial germination percentage at different temperatures is recorded in Table 6. The effect of temperature on germination was highly significant at all temperatures ($F_{19,59}$=89.28; $F_{19,59}$=29.52, $F_{19,59}$=11.28 and $F_{19,59}$=12.43 at 15, 20, 25 and 30ºC respectively; P<0.001). The maximum value of germination was observed at 25ºC and ranged between 64.7 and 94.3%. None of the isolates germinated at 35ºC, however only four of them germinated at 15ºC, even if they belonged to different ST.

There were differences among the isolates in the effect of temperature on conidial germination. Spore germination occurred at 20-30ºC, although the optimum temperature for all the isolates was 25ºC. Similar results were reported by other studies about *B. bassiana* thermal requirements (Devi et al., 2005; Luz and Fargues, 1997), in which there was no apparent relationship between the fungal growth rate and the habitat, ecosystem, or genetic characteristics of the isolates. It appeared that tolerance to environmental stresses, such as high temperature or UV irradiation depends on enzyme regulation as superoxide dismutases (SOD), mitochondrial isoenzymes, or different catalases (Xie et al., 2010; 2012; Wang et al., 2013). These reactive oxygen species (ROS) are involved in the response of the entomopathogenic fungi to abiotic stresses

and provide target genes to be genetically manipulated for improved performance (Wang and Feng, 2013).

Table 6.- Conidial germination (%) of *Beauveria bassiana* isolates at different temperatures

Isolate	Temperature °C[(a)]			
	15	20	25	30
EABb 10/126-Su	0.0±0.0d	43.0±1.1ef	66.6±3.1hi	51.0±2.8ghij
EABb 10/147-Su	0.0±0.0d	45.6±2.7ef	82.3±0.6bcde	58.0±5.0defg
EABb 10/275-Fil	0.0±0.0d	32.6±1.2g	73.0±2.0gh	54.3±2.0fghi
EABb 10/150-Su	0.0±0.0d	42.3±2.1f	74.0±1.0fgh	44.6±3.3hijk
EABb 10/156-Su	0.0±0.0d	51.0±2.6def	90.0±1.0ab	70.6±6.1abcd
EABb 10/225-Fil	0.0±0.0d	25.3±2.3gh	72.0±3.0ghi	23.3±2.9l
EABb 10/282-Fil	0.0±0.0d	27.0±3.0gh	84.0±2.0bcd	61.0±6.5cdefg
EABb 10/129-Su	0.0±0.0d	44.0±1.1ef	93.3±3.4a	79.0±2.5a
EABb 10/223-Fil	0.0±0.0d	22.0±1.7h	67.3±2.9hi	41.3±2.0jk
EABb 10/261-Fil	0.0±0.0d	62.6±5.1a	77.6±6.1defg	56.0±6.0efgh
EABb 09/28-Fil	0.0±0.0d	19.3±3.5h	76.0±2.5efg	44.0±5.2hijk
EABb 10/329-Fil	13.6±3.3b	51.3±5.0cde	92.3±4.4a	69.0±5.7abcd
EABb 10/143-Fil	0.0±0.0d	57.6±3.6bcd	64.6±3.3i	32.6±2.4kl
EABb 10/235-Fil	6.3±1.6c	45.0±2.5ef	84.0±2.3bcd	43.0±4.3ijk
EABb 10/121-Su	0.0±0.0d	42.6±4.2ef	78.3±3.1defg	55.0±3.5efghi
EABb 09/20-Fil	42.3±2.6a	65.0±1.7b	87.3±2.0abc	76.6±3.4ab
EABb 10/133-Su	0.0±0.0d	9.3±1.6i	81.3±2.3cdef	64.3±1.4bcdef
EABb 10/169-Su	15.6±1.4b	60.0±3.6bc	83.0±0.5bcde	73.6±9.0abc
EABb 09/29-Fil	0.0±0.0d	45.6±4.3ef	94.3±1.8a	79.0±4.5a
EABb 09/16-Fil	0.0±0.0d	26.6±0.3gh	94.0±1.0a	67.6±4.9abcde

[(a)]Standard errors are after each mean. Means in the same column followed by different letters are significantly different ($P<0.05$, LSD test)

3.2.3.- Water activity effect on germination

The conidia germination percentages at 24 hours post-inoculation at different water activities were statistically different ($F_{19,59}=2.19$; $F_{19,59}=2.23$, $F_{19,59}=7.90$, $F_{19,59}=35.54$, $F_{19,59}=137.45$, $F_{19,59}=50.68$ and $F_{19,59}=26.55$ at 1, 0.998, 0.996, 0.985, 0.970, 0.955 and 0.928a_w respectively; $P<0.05$) (Table 7).

Germination was not observed in any isolate at 0.895 and 0.862a_w. Only two isolates germinated at 0.928a_w, particularly EABb 10/126-Su isolate with a 32.0%

germination, which could be present an adaptive advantage in dry climatic conditions of arid areas in southern Spain. Gillespie and Crawford (1986) noted that the *B. bassiana* development ceased at 0.92 $a_{w,}$ which was inhibitory for growth (Luz and Fargues, 1997; Lazzarini, et al., 2006). Conidia germination is marked by an increase in oxygen consumption during the germ tube growth (Braga et al., 1999). It is possible that the hydrogen peroxide formed during the germination produce other ROS (Wang and Feng, 2013). Indeed, water activity below 0.93 had a considerable negative effect on the germination kinetics (Lazzarini et al., 2006). However, in our study an important decrease in conidial germination was observed between 0.985 and 0.970, which is remarkable for two phylloplane isolates (EABb 10/275-Fil and EABb 10/282-Fil). The optimal water activity for conidia germination ranged between 1 and 0.985 a_w, in agreement with Lazzarini et al. (2006) that reported 0.99 a_w as the optimal water activity for *B. bassiana* conidial germination.

Table 7.- Conidial germination (%) of *Beauveria bassiana* isolates at different water activities

Isolate	a_w [(a)]						
	1	**0.999**	**0.996**	**0.985**	**0.970**	**0.955**	**0.928**
EABb 10/126-Su	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	91.3±2.9ab	32.0±6.1a
EABb 10/147-Su	100.0±0.0a	100.0±0.0a	100.0±0.0a	98.6±1.3a	91.3±2.4b	72.6±1.7cd	0.0±0.0c
EABb 10/275-Fil	100.0±0.0a	96.6±2.4bc	98.6±2.3a	77.3±3.7d	12.6±1.7h	0.0±0.0i	0.0±0.0c
EABb 10/150-Su	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	99.3±0.6a	92.0±3.0a	0.0±0.0c
EABb 10/156-Su	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	92.0±1.1b	74.0±3.0cd	0.0±0.0c
EABb 10/225-Fil	100.00±0.0a	100.0±0.0a	100.0±0.0a	71.3±4.6e	64.0±1.1f	35.3±2.9h	0.0±0.0c
EABb 10/282-Fil	98.0±1.1abc	99.3±0.6ab	96.0±1.1b	70.6±2.9e	32.6±2.9g	35.3±2.9h	0.0±0.0c
EABb 10/129-Su	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	82.6±0.6cd	41.3±2.4gh	0.0±0.0c
EABb 10/223-Fil	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	99.3±0.6a	82.0±2.0bc	0.0±0.0c
EABb 10/261-Fil	99.3±0.6ab	100.0±0.0a	100.0±0.0a	100.0±0.0a	85.3±3.5c	55.3±1.7e	0.0±0.0c
EABb 09/28-Fil	99.3±0.6ab	100.0±0.0a	100.0±0.0a	100.0±0.0a	85.3±3.5c	55.3±1.7e	0.0±0.0c
EABb 10/329-Fil	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	76.0±1.1e	54.6±5.8ef	0.0±0.0c
EABb 10/143-Fil	97.3±1.3bc	99.3±0.6ab	100.0±0.0a	100.0±0.0a	99.3±0.6a	77.3±2.4cd	0.0±0.0c
EABb 10/235-Fil	98.0±1.1abc	94.6±1.7c	92.6±1.3c	88.6±2.4b	66.6±2.6f	32.0±2.3h	0.0±0.0c
EABb 10/121-Su	100.0±0.0a	100.0±0.0a	98.6±1.3a	99.3±0.6a	77.3±3.7de	45.3±8.1fg	0.0±0.0c
EABb 09/20-Fil	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	96.0±1.1ab	58.6±3.5e	4.0±1.1b
EABb 10/133-Su	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	91.3±2.9ab	0.0±0.0c
EABb 10/169-Su	99.3±0.6ab	100.0±0.0a	100.0±0.0a	100.0±0.0a	82.6±1.7cd	70.0±4.6d	0.0±0.0c
EABb 09/29-Fil	96.0±2.3c	97.3±2.6abc	100.0±0.0a	82.6±1.7c	68.6±0.6f	34.6±1.7h	0.0±0.0c
EABb 09/16-Fil	99.3±0.6ab	98.6±1.3ab	98.6±1.3a	92.6±1.7b	92.6±1.7b	40.0±4.0gh	0.0±0.0c

[(a)]Standard errors are after each mean. Means in the same column followed by different letters are significantly different (P<0.05, LSD test)

3.2.4- Ultraviolet radiation effect (UV-B) on fungal conidia

3.2.4.1.- UV-B radiation effect on conidial germination

UV-B exposure time had a significant effect on the relative germination percentage for the twenty isolates at 920 mW m^{-1} ($F_{19,59}$=11.32; $F_{19,59}$=20.41 and $F_{19,59}$=38.35 for 2, 4 and 6 hours of exposure time respectively; P<0.001) and 1200 mW m^{-1} ($F_{19,59}$=22.51; $F_{19,59}$=36.87 and $F_{19,59}$=32.55 for 2, 4 and 6 hours of exposure respectively; P<0.001). Be aware of the germ tube was shorter after 6 hours of exposure time than in the control for 920 and 1200 mW m^{-1} (Figure 3).

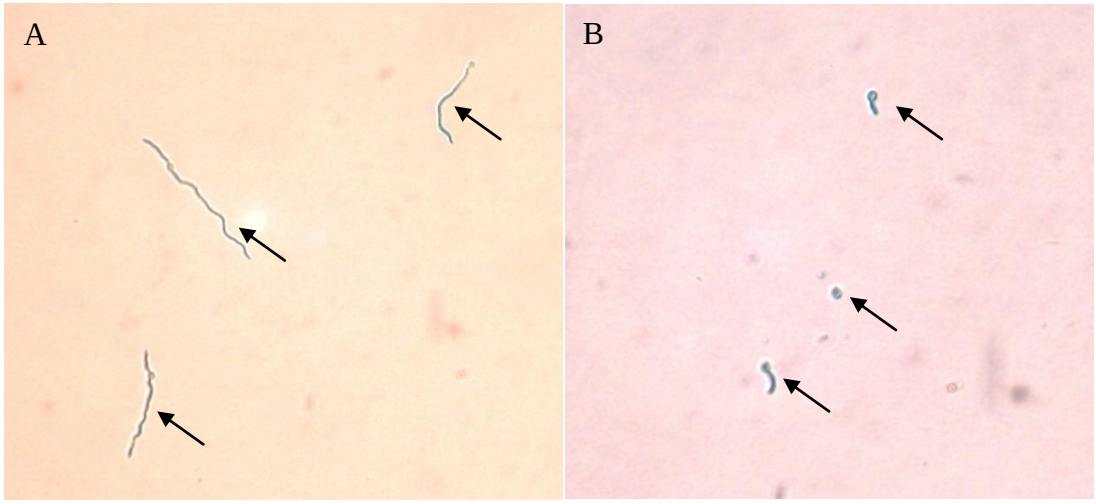

Figure 3.- Germination of EABb09/16-Fil isolate. **A**.- Non irradiated control at 24 hours post-inoculation. **B**.- Conidial germination after 6 hours of exposure time to 1200 mW m^{-1} and 24 hours post-inoculation.

However, EABb 10/129-Su and EABb 10/223-Fil isolates showed a particularly higher susceptibility to UV-B, both of them grouped in the ST 4.

Table.8- Relative percentage germination of *Beauveria bassiana* isolates observed at 24 hours after exposure to 920 and 1200 mW m^{-2} and irradianced for 2, 4 and 6 hours.

Isolate	920 mW m^{-1}			1200 mW m^{-1}		
	2h	4h	6h	2h	4h	6h
EABb 10/126-Su	98.9±0.8abA	89.9±1.7cA'	74.7±1.7cdeA"	82.5±2.3efB	25.6±6.4jB'	5.4±2.4hB"
EABb 10/147-Su	97.3±0.6bcdA	87.3±1.7cdA'	64.7±2.4efgA"	80.9±5.6efgB	68.4±8.3efA'	33.3±3.5eB"
EABb 10/275-Fil	100.0±0.0aA	85.3±2.9cdA'	81.2±2.9cA"	73.8±5.7fgB	72.9±4.6cdeA'	63.7±2.4bcB"
EABb 10/150-Su	97.1±2.9abA	80.6±1.1cdefA'	50.6±1.3hiA"	94.6±2.1abcB	80.0±1.1bcdeA'	40.6±7.7deA"
EABb 10/156-Su	98.6±1.3abA	86.6±3.5cdA'	72.0±4.7cdefA"	97.1±1.6abA	80.8±3.6bcdA'	59.5±8.8deA"
EABb 10/225-Fil	100.0±0.0aA	97.3±0.6bA'	78.0±2.0cdA"	83.2±1.8efB	82.3±3.8bcB'	49.6±7.2cdB"
EABb 10/282-Fil	87.8±3.5eA	70.9±1.2fA'	44.9±2.3hiA"	84.3±1.8defA	70.2±3.2deA'	39.3±1.2deA"
EABb 10/129-Su	51.8±6.6gA	42.1±1.9hA'	13.8±1.3lA"	16.1±2.2iB	3.2±1.8kB'	0.5±0.3iB"
EABb 10/223-Fil	72.0±6.1fA	56.2±3.3gA'	23.4±3.2kA"	59.1±1.3hA	22.9±4.7jB'	0.7±0.1iB"
EABb 10/261-Fil	92.0±4.2deA	72.0±6.1efA'	44.3±2.3iA"	53.5±9.1hB	40.3±3.1hiB'	12.9±5.0ghB"
EABb 09/28-Fil	85.1±3.1eA	72.3±2.4efA'	30.1±7.9jkA"	68.4±2.3ghB	33.3±5.2ijB'	18.8±4.6fgA"
EABb 10/329-Fil	100.0±0.0aA	100.0±0.0aA'	90.5±3.5bA"	93.2±2.9bcdB	77.7±2.4cdeB'	62.8±1.2bcB"
EABb 10/143-Fil	92.0±1.2deA	77.3±4.6defA'	41.3±4.8ijA"	89.8±2.3cdeA	45.4±2.5ghiB'	31.1±3.6efA"
EABb 10/235-Fil	86.5±1.7eA	80.1±1.3defA'	56.4±4.0ghA"	78.1±1.5fgB	47.5±1.9ghB'	31.8±3.8eB"
EABb 10/121-Su	98.0±1.1abcA	96.7±2.4bA'	62.6±5.7fgA"	90.5±4.9bcdeA	89.2±2.0abA'	55.9±4.3bcA"
EABb 09/20-Fil	98.0±1.1abcA	77.9±8.6defA'	67.3±4.2defgA"	96.6±1.8abA	78.2±3.2deA'	66.1±3.6bA"
EABb 10/133-Su	92.1±4.2cdeA	82.7±2.3cdeA'	46.1±1.2hiA"	77.8±1.6fgB	48.2±3.2ghB'	11.8±1.4ghB"
EABb 10/169-Su	87.3±0.6eA	72.4±2.3efA'	56.6±2.4ghA"	73.3±5.8fgA	56.2±7.2fgA'	48.9±8.2cdA"
EABb 09/29-Fil	100.0±0.0aA	100.0±0.0aA'	98.6±1.3aA"	98.6±1.3aA	94.0±1.1aB'	88.0±3.0aB"
EABb 09/16-Fil	98.7±1.2abA	98.6±1.3abA'	90.0±3.4bA"	99.1±0.4aA	80.7±0.6bcdeB'	35.3±3.7deA"

The percentage of germination after each period was calculated in relation to the non-irradiated controls. Standard errors are after each mean. Means in the same column followed by different small letters are significantly different (P<0.05, LSD test). Means in the same row and exposure time (2, 4 or 6h) followed by different capital letter are significantly different (P<0.05, LSD test).

3.2.4.2.- UV-B radiation effect on conidial culturability and on colony growth

The colonies of most of the isolates originating from irradiated conidia shoed delayed development and high heterogeneity both in shape and size as compared to colonies from non-irradiated conidia (Fig. 4). The was a significant effect of UV-B exposure on colony size (as revealed by the growth index) both at 920 mW m^{-1} ($F_{19,59}$=6.16; $F_{19,59}$=9.71 and $F_{19,59}$=10.51 for 2, 4 and 6 hours of exposure respectively; P<0.001) and 1200 mW m^{-1} ($F_{19,59}$=6.82; $F_{19,59}$=8.40 and $F_{19,59}$=13.17 for 2, 4 and 6 hours of exposure respectively; P<0.001) (Table 9).

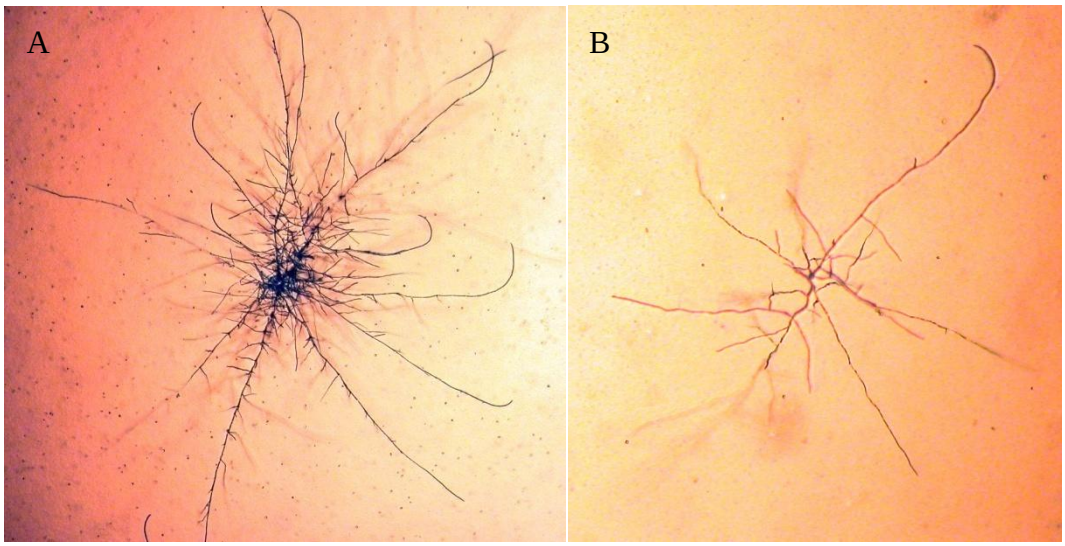

Figure 4.- Effect of 1200mW m^{-1} exposure on the colony growth of EABb09/16-Fil isolate **A**.- Non-irradiated control at 48 hours post-inoculation. **B**.- Exposure for 6 hours to 1200 mW m^{-1} and 48 hours post-inoculation.

Once again UV-B had a significant effect on culturability of the twenty isolates (Table 10) at 920 mW m^{-1} ($F_{19,59}$=5.01; $F_{19,59}$=20.62 and $F_{19,59}$=9.11 for 2, 4 and 6 hours of exposure respectively; P<0.001) and 1200 mW m^{-1} ($F_{19,59}$=4.54; $F_{19,59}$=19.02 and $F_{19,59}$=12.82 for 2, 4 and 6 hours of exposure respectively; P<0.001). In general, culturability decreased with increasing exposure time to UV-B even if such an effect was more acute for germination.

Table.9- Growth index observed at 48 hours after the exposition to 920 and 1200 mW m^{-2} irradiances for 2, 4 and 6 hours of exposure time.

Isolate	920 mW m^{-1}			1200 mW m^{-1}		
	2h	4h	6h	2h	4h	6h
EABb 10/126-Su	3.3±0.5efgA	15.2±2.1defA'	15.9±2.5hiA"	6.8±2.52efghiA	8.2±2.9hiA'	15.7±3.2hA"
EABb 10/147-Su	13.5±3.1abA	18.1±0.6cdeA'	17.7±2.0jA"	11.2±1.8defgA	30.2±2.9aB'	47.6±0.7abB"
EABb 10/275-Fil	14.4±0.7aA	12.9±1.5efgA'	19.6±0.6efghiA"	12.1±1.1defA	14.5±1.5fghA'	21.1±2.9ghA"
EABb 10/150-Su	5.5±1.6cdefgA	15.8±2.3defA'	23.9±7.8defgA"	10.2±2.2defgA	16.7±1.7defgA'	22.5±1.6ghA"
EABb 10/156-Su	1.4±0.7gA	8.6±1.8ghA'	20.1±3.4efghiA"	2.3±0.7hiA	22.8±3.2abcdeB'	39.7±2.4cdeB"
EABb 10/225-Fil	9.0±0.2cA	26.1±1.2abA'	16.9±0.1ghiA"	5.5±0.2fghiB	18.0±4.4cdefgA'	25.0±0.7fghB"
EABb 10/282-Fil	4.0±1.8defgA	17.8±2.9cdeA'	14.7±1.1ijA"	2.4±1.1hiA	13.0±2.3ghA'	24.2±2.1fghB"
EABb 10/129-Su	9.2±0.8bcA	13.0±0.4efgA'	27.0±2.3cdeA"	1.1±0.3iB	24.7±2.1abcB'	27.1±2.3fgA"
EABb 10/223-Fil	10.4±2.7aA	14.4±2.6efA'	39.4±0.6aA"	4.2±2.4ghiB	15.8±0.9efghA'	49.3±1.5abB"
EABb 10/261-Fil	5.3±1.0cdefgA	16.7±3.2deA'	35.4±1.8abA"	25.7±7.5aB	26.6±6.1abA'	53.1±9.9aB"
EABb 09/28-Fil	8.4±0.8cdA	11.2±2.7fghA'	37.7±2.8abA"	11.1±2.4defgA"	25.1±3.5abcB'	45.8±3.5abcdB"
EABb 10/329-Fil	4.1±1.3defgA	7.1±0.3hA'	20.1±1.7efghiA"	11.9±2.7defB	13.4±0.7iB'	20.1±3.5ghA"
EABb 10/143-Fil	3.5±1.6efgA	16.1±2.2defA'	31.5±2.3bcdA"	7.9±0.8efghiB	28.5±2.6abB'	37.2±2.2deA"
EABb 10/235-Fil	3.2±1.5efgA	28.2±0.1aA'	33.5±0.6abcA"	19.9±4.9abcB	20.7±4.5bcdefgA'	43.1±2.8bcdB"
EABb 10/121-Su	6.4±1.7cdefA	22.8±0.8bcA'	23.7±3.2defgA"	9.4±1.7defghA	24.1±2.4abcdA'	32.2±2.7efA"
EABb 9/20-Fil	4.4±2.4cdefgA	16.6±0.6deA'	23.2±3.2efghA"	2.3±0.5hiA	2.4±0.3iB'	44.4±0.9abcdB"
EABb 10/133-Su	5.7±1.5cdefgA	17.8±1.8cdeA'	13.7±2.6ijA"	20.5±0.9abB	20.9±1.0bcdefA'	26.1±1.8fgB"
EABb 10/169-Su	7.6±0.6cdeA	26.8±1.4abA'	34.9±2.6abA"	16.3±0.6bcdB	20.8±0.9bcdefgB'	45.2±2.8abcdA"
EABb 09/29-Fil	7.6±1.2cdeA	12.9±1.5efgA'	25.1±2.1defA"	11.3±2.4defgA	28.6±1.4abB'	47.6±5.2abcB"
EABb 09/16-Fil	3.0±1.2fgA	20.4±1.2cdA'	28.7±1.4fghiA"	13.2±0.8cdeB	24.4±0.7abcdA'	50.0±2.1abB"

The grow index after each period was calculated in relation to the nonirradiated controls. Standard errors are after each mean. Means in the same column followed by different small letters are significantly different (P<0.05, LSD test). Means in the same row and exposure time (2, 4 or 6h) followed by different capital letter are significantly different (P<0.05, LSD test).

Table.10- Relative culturability observed as 48 hours after the exposition to 920 and 1200 mW m^{-2} irradiances for 2, 4 and 6 hours of exposure time.

Isolate	920 mW m^{-1}			1200 mW m^{-1}		
	2h	4h	6h	2h	4h	6h
EABb 10/126-Su	89.2±5.2abcdeA	92.9±1.8abA'	68.0±3.2defA"	73.6±5.4deA	69.0±3.9efgB'	66.6±2.8bcdeA"
EABb 10/147-Su	93.2±2.8abcA	80.5±4.2defA'	51.1±3.4hiA"	86.9±1.6abcA	63.2±3.1fghB'	53.3±2.5ghiB"
EABb 10/275-Fil	97.1±0.4aA	69.4±1.2ghA'	69.2±5.6defA"	93.3±5.0aA	56.4±5.1hijA'	50.3±5.5hijA"
EABb 10/150-Su	90.2±4.3abcdA	72.6±1.0fgA'	76.8±5.9abcdA"	85.2±4.3abcA	71.8±2.9defA'	42.9±1.8jB"
EABb 10/156-Su	87.2±1.2bcdeA	78.3±1.1efA'	81.9±4.1abcA"	84.6±3.9abcA	62.7±1.9ghiB'	64.2±4.1cdefB"
EABb 10/225-Fil	94.8±4.5abA	92.6±4.0abcA'	85.2±2.7abA"	84.3±1.1abcA	87.7±1.9abA'	85.0±2.7aA"
EABb 10/282-Fil	94.3±2.7abcA	93.0±4.5abA'	74.3±6.3bcdeA"	87.2±1.4abcA	88.4±1.6aA'	59.0±3.8efghA"
EABb 10/129-Su	85.5±2.2cdeA	63.1±2.2hA'	64.5±1.4efgA"	84.8±2.2abcA	53.9±1.8ijB'	66.5±2.2bcdeA"
EABb 10/223-Fil	82.7±3.5deA	68.9±2.1ghA'	63.9±4.6efgA"	78.4±2.5cdA	63.7±2.4fghA'	61.5±2.1defgA"
EABb 10/261-Fil	91.3±0.9abcdA	82.5±1.6deA'	71.5±3.9cdeA"	85.4±4.4abcA	79.2±1.3bcdA'	76.1±1.9abA"
EABb 09/28-Fil	66.3±2.2fA	46.9±3.3iA'	46.2±3.0iA"	65.8±4.1eA	44.7±0.5kA'	30.8±1.1kB"
EABb 10/329-Fil	96.7±3.2bA	96.3±3.1abA'	86.6±7.1aA"	92.0±2.6aA	62.7±4.7ghi	60.4±7.1defgA"
EABb 10/143-Fil	91.0±4.8abcdA	79.5±4.1defA'	65.5±4.2defgA"	91.1±1.6aA	77.4±3.6cdeB'	49.5±3.3hijB"
EABb 10/235-Fil	94.8±2.1abA	77.5±4.1efgA'	66.8±3.3defgA"	78.9±5.7bcdA	82.4±4.3abcA'	57.3±4.1efghA"
EABb 10/121-Su	89.5±3.7abcdeA	99.8±0.0aA'	68.1±3.4defA"	90.2±2.7aA	81.5±2.8abcB'	65.6±2.9cdeA"
EABb 09/20-Fil	93.0±2.4abcA	84.0±1.2cdeA'	46.0±1.2iA"	88.0±2.2abA	65.6±1.8fgB'	63.1±3.6cdefgB"
EABb 10/133-Su	91.6±4.7abcdA	88.2±5.4bcdA'	85.0±2.9abA"	91.0±1.5aA	88.5±1.2aA'	55.2±3.5fghB"
EABb 10/169-Su	93.8±2.3abcA	92.0±2.6abcA'	68.6±2.0defA"	90.2±1.8aA	88.5±1.9aA'	69.3±4.3bcdA"
EABb 09/29-Fil	94.4±1.1abcA	96.3±3.1abA'	55.8±1.6ghiA"	91.6±3.1aA	84.6±6.6abcA'	72.4±2.4bcB"
EABb 09/16-Fil	80.8±1.2eA	61.9±2.6hA'	58.6±2.3fghA"	78.5±0.9cdA	51.5±0.9jkB'	44.9±0.4ijB"

The percentage of culturability after each period was calculated in relation to the nonirradiated controls. Standard errors are after each mean. Means in the same column followed by different small letters are significantly different (P<0.05, LSD test). Means in the same row and exposure time (2, 4 or 6h) followed by different capital letter are significantly different (P<0.05, LSD test).

Our results indicate that exposition to UV-B radiation resulted in a delay of germination of *B. bassiana*, whereas this effect was linked neither to ST nor isolate origin, soil or phylloplane. This is supported by the fact that the effect of UV-B exposure on germination was more acute than the one observed for culturability. In general, fungal colonies from irradiated conidia were more heterogeneous in shape than the controls and their growth was delayed. It could be concluded that a "recovery" of the fungal propagules could occur after exposition to UV-B, even if such recovery is lower for longer exposure times (6h) and irradiance (1200 mW m^{-1}), which highlights both the key role of the changes operating in the environment in determining the efficacy of microbial control with entomopathogenic fungi, and the need for selecting isolates adapted to this particular environment ("environmental competence"). This is even more important under climatic change scenarios, with a reduction in the ozone layer.

UV-B, which is the most harmful radiation to biological systems (Quaite et al, 1992; Paul et al., 1997), is increasing due to the reduction in the ozone layer (Caldmell and Flint, 1997; Sola and Lorente, 2011). Differences in UV-B exposure throughout Spain national territory are evident both at season and geographical level (Martinez-Lozano et al., 2012). The higher values of UV-B irradiance reach 1800 mW m^{-1} at solar noon in July in Badajoz, while the lower are found in December reaching 200 mW m^{-1} in La Coruña (Martinez-Lozano et al., 2012). These differences could explain the isolation frequency of the EF. Quesada-Moraga et al. (2007) showed that *Beauveria* sp. is more frequently isolated in natural and cultivated habitats in southern Spain, whereas *Metarhizium* sp. is the more abundant fungal species in northern Spain. This could be due to the fact that this second fungal species is more susceptible to UV-B inactivation than the former, with only few hours of exposure to 920 and 1200 mW m^{-1} need to

steadily decrease the germination rate and culturability (Braga et al., 2001a, 2001b, Rangel et al., 2004).

On the other side, our study shows that there was no apparent relationship between the response of the fungal isolates to UV-B and their habitat (soil or phylloplane), ecosystem, ST or haplotype. In spite of, its remarkable that some isolates showed desirable response to the abiotic environmental factors evaluated in this work. Thus, isolate EABb 09/20-Fil showed: (1) high resistance to inactivation by UV-B, (2) high percentage of germination at 15-30ºC and (3) optimum germination at lower moisture levels, making it an excellent candidate to be developed for pest control in Mediterranean conditions. Even if it could be argued that isolates of entomopathogenic fungi from the phylloplane that are most exposed to abiotic environmental factors could have evolved to resist more unfavorable environmental conditions, the present work results clearly shows that such habitat not always provide the more environmentally competent genotypes.

As a **conclusion,** this work shows that combined analysis of TEF/Bloc and ISSR allows highly sensitive detection of *Beauveria bassiana* isolate diversity. Besides, similar patterns of isolate grouping were observed by using microsatellites (ISSR) and EF1-α/Bloc. Twenty *B. bassiana* isolates was grouped in two haplotypes, one of them with possible polyphyletic origin suggesting temporal heterogeneity among isolates of this group. However, the patterns of isolate grouping were linked neither to habitat (soil or phylloplane) nor to origin, holm oak dehesa or reforestation. On the other hand, thermal requirements for germination and growth of most isolates were around 25 ºC, while optimum water activity was detected at 0.999-0,985a_w. High tolerance to UV-B

exposure for all *B. bassiana* isolates after an initial depletion of the germination was found. Isolates of entomopathogenic fungi from the phylloplane not always provided the more environmentally competent genotypes, despite of this entomopathogenic fungi are most exposed to abiotic environmental factors.

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