Thermotolerant *Metarhizium robertsii* from Moroccan southeastern oases: Isolation, heat stress screening and pathogenicity to *Tenebrio molitor* larvae

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ABSTRACT

Entomopathogenic fungi of the genus *Metarhizium* are widely used as biological control agents; however, their performance in arid and semi-arid regions is strongly constrained by high temperatures. This study aimed to isolate and identify native thermotolerant *Metarhizium* strains from soils collected in four Moroccan southeastern oases (Erfoud, Rissani, Goulmima, and Aoufous) in the Drâa-Tafilalet region. A total of 82 isolates of *Metarhizium* were obtained, and 40 isolates were selected randomly and screened for thermotolerance by assessing conidial germination after heat exposure at 45 °C for 2 hours. Inter – and intra – population variability was observed, with germination rates ranging from 75% to 95.67%. Among all tested isolates, strains R2 and R4 from Rissani oasis exhibited the highest thermotolerance and were therefore selected for molecular identification. The identification of *Metarhizium* strains R2 and R4 was conducted phylogenetically based on a concatenated dataset of ribosomal internal transcribed spacer region (ITS) and the translation elongation factor 1-alpha (*TEF-1α*) sequences. Both strains were assigned to *Metarhizium robertsii* and clustered within the same terminal branch as three reference strains of *M. robertsii* (ARSEF 4739, ARSEF 727 and ARSEF 8020) supported by a high bootstrap value (92%). Pathogenicity assays against *Tenebrio molitor* larvae confirmed high virulence (86.67–100% mortality), further supporting their potential as effective biocontrol agents. The presence of highly thermotolerant *M. robertsii* in Moroccan oases suggests strong local adaptation to extreme thermal conditions and highlights promising candidates for developing climate-resilient biopesticides suitable for hot and arid agroecosystems. These results contribute to a better understanding of the ecological distribution and stress tolerance of *Metarhizium* spp. oasis habitat and support their integration into sustainable pest management strategies.

Keywords: *Metarhizium robertsii*, thermotolerance, oases ecosystem, Drâa-Tafilalet region

INTRODUCTION

The *Metarhizium* fungus (*Clavicipitaceae* family; Hypocreales order) is an important group of fungi used as biocontrol agents for agricultural pests (Goettel et al., 2005; Brunner-Mendoza et al., 2019). The *Metarhizium* species are environmentally safe alternatives to chemical pesticides (Vega et al., 2009; Zaki et al., 2020). *Metarhizium* fungi produce green conidia on insect cadavers (Vega et al., 2009). This genus was named by

Metchnikoff (1879), who described the first species, *M. anisopliae*, that had been collected from a wheat sulcus beetle (*Anisoplia austriaca*) in Russia (Vega et al., 2009). Reports indicate that *Metarhizium* can infect many different types of hosts and play several ecological roles. Some examples of where *Metarhizium* species have been found include soil and leaf endophytes (Bischoff et al., 2009; Clifton et al., 2018; Luz et al., 2019).

Historically, *Metarhizium* species were identified based primarily on morphological traits, including colony colour, conidial shape, and sporulation patterns (Bischoff et al., 2009). Yet, because of the high level of phenotypic variation in isolates, a morphological identification approach has many limitations (Bischoff et al., 2006 and 2009). Molecular systematics has had a significant effect on *Metarhizium* taxonomy; in particular, many cryptic *Metarhizium* species were discovered in the *M. anisopliae* and *M. flavoviride* groups using phylogenetic analysis of multiple genes (Mongkolsamrit et al., 2020).

Currently, the genus includes 82 accepted species worldwide, 26 were found within China ecosystems (Kepler et al., 2014; Chu et al., 2016; Montalva et al., 2016; Iwasaki et al., 2019; Mongkolsamrit et al., 2020 Fernández-Bravo et al., 2021; Chen et al., 2023; Li et al., 2023; Baró Robaina et al., 2024; Yang et al., 2025), reflecting a broad ecological distribution and remarkable genetic diversity (Roberts and St. Leger 2004; Yang et al., 2025).

Information about the diversity, distribution and ecology of the genus *Metarhizium* in Morocco is insufficient, though research worldwide has included a great deal of effort. Among all these ecosystems, the oases of Morocco contain the greatest potential for the establishment of new strains of *Metarhizium* spp.; they are unique examples of natural, traditional agroecosystems that represent some of the most significant and biologically valuable sites for preserving agricultural biodiversity and regional agricultural varieties (Arabi et al., 2024). These oases are also characterised by high solar radiation, low humidity, and extreme temperature fluctuations, which may select for fungal populations with enhanced thermotolerance and desiccation resistance. High temperatures have previously been shown to be extremely harmful to conidia germination, fungal growth and conidiogenesis (Yosri et al., 2018; Lee et al., 2018; Qiu et al., 2021; Muazu et al., 2024), consequently, limiting the development of effective biological control agents (Moore et al., 1996; Rangel et al., 2005;

Keyser et al., 2014). McCammon and Rath (1994) and Keyser et al. (2014) assumed that the ability of entomopathogenic fungi to germinate under various environmental stresses or temperature regimes is crucial for their effective use as biocontrol agents. Such adaptive traits are of great interest in the context of global climate change (Rangel et al., 2005; 2010; Muazu et al., 2024), as they could enable the development of *Metarhizium*-based bioinsecticides that remain effective under high-temperature conditions typical of arid and semi-arid agricultural systems. Exploring entomopathogenic fungi from these regions could thus provide valuable strains capable of maintaining virulence and persistence in challenging environments where conventional biocontrol agents may fail.

Generally, if an insect's body temperature exceeds that of the upper thermal threshold for a fungal pathogen, then it's unlikely to be able to infect that insect. However, once the ambient (or surrounding) temperature has become more favourable again, the pathogen usually continues with its infection (Rangel et al., 2010). Much of the research to date examining the heat stability of fungal pathogens have concentrated on two areas: survival of its spores (conidia) at elevated temperatures or relative germination rates following exposure (Arthurs and Thomas, 2001; Rangel et al., 2005).

The present study aims to isolate and identify native thermotolerant *Metarhizium* isolates from soil samples collected across four oases in the southeastern region of Morocco. This work contributes to a better understanding of the natural diversity of *Metarhizium* within the Moroccan oases ecosystem and provides a foundation for the development of locally adapted, climate-resilient biological control agents.

MATERIAL AND METHODS

Soil sampling and isolation of *Metarhizium* spp.

Soil samples were collected from different oasis ecosystems located in the Errachidia region of southeastern Morocco (Figure 1), an area characterised by an arid to semi-arid climate with hot summers and large temperature fluctuations between day and night. Sampling sites included four representative oases, Arfoud, Rissani, Goulmima, and Aoufous (Figure 2). Ten soil samples were collected from each site; approximately 500 grams of soil sample was collected from the

rhizosphere at depths of 5 to 10 centimetres, as outlined by Imoulan et al. (2016). Samples were collected after clearing away surface litter. A total of 40 soil samples were collected and placed in sterile polyethylene bags with labels, then taken to the lab for processing in a cooler.

After sieving soil samples to completely remove the presence of soil debris and/or rocks, we used extreme caution in order to prevent any cross-contamination of the various isolates. Isolation of *Metarhizium* spp. was performed using Potato Dextrose Agar (PDA) + Chloramphenicol (500 mg·L⁻¹) + Cycloheximide (200 mg·L⁻¹) per the methods outlined in Goettel and Inglis (1997) and Imoulan et al. (2011). PDA plates for isolation were incubated in 25 °C. The plates were checked for any new growth on a daily basis. Any fungal growth that had developed green conidial masses that were characteristic of *Metarhizium* species were isolated from these PDA plates and the fungus was transferred to new PDA plates and subsequently incubated in 25 °C for 7–10 days. Pure cultures were obtained via single spore isolation as per Imoulan et al. (2016) and maintained at 4 °C for both morphological and molecular identification. To avoid sampling bias associated with clonal repetition within individual soil samples, a single representative isolate was randomly selected from each soil sample for all subsequent experimental.

Entomopathogenicity assessment of *Metarhizium* isolates

Given that all isolates were recovered from soil, the entomopathogenic potential of 40 randomly selected isolates (one isolate per soil sample) was evaluated against the last instar of *Tenebrio molitor* larvae (Coleoptera: *Tenebrionidae*), a standard model to evaluate fungal virulence in larvae bioassay. Conidial suspensions were prepared from 10-day-old fungal cultures grown on PDA. Conidia were harvested by scraping the surface of sporulating colonies in a sterile 0.01% Tween 80 solution. The suspension was vortexed, filtered through sterile cheesecloth to remove mycelial fragments, and the concentration was adjusted to 1×10^7 conidia·mL⁻¹ using a hemocytometer. Conidial viability was determined prior to bioassay by plating a 100 µl aliquot on PDA and calculating germination rates after 24 h of incubation at 25 °C. Only suspensions of isolates exhibiting germination rates greater than 95% were selected for subsequent assays.

Ten healthy last instar larvae of *T. molitor* were placed together per plate and treated with 10 µL of conidial suspension (1×10^7 conidia·mL in 0.01% Tween 80). For the control groups, an equal volume of sterile water containing 0.01% Tween 80 was applied to the larval surfaces. Each experiment was replicated three times using ten

Figure 1. Location map of the sampled soil samples within the southeastern Morocco oases in Drâa-Tafilalet region
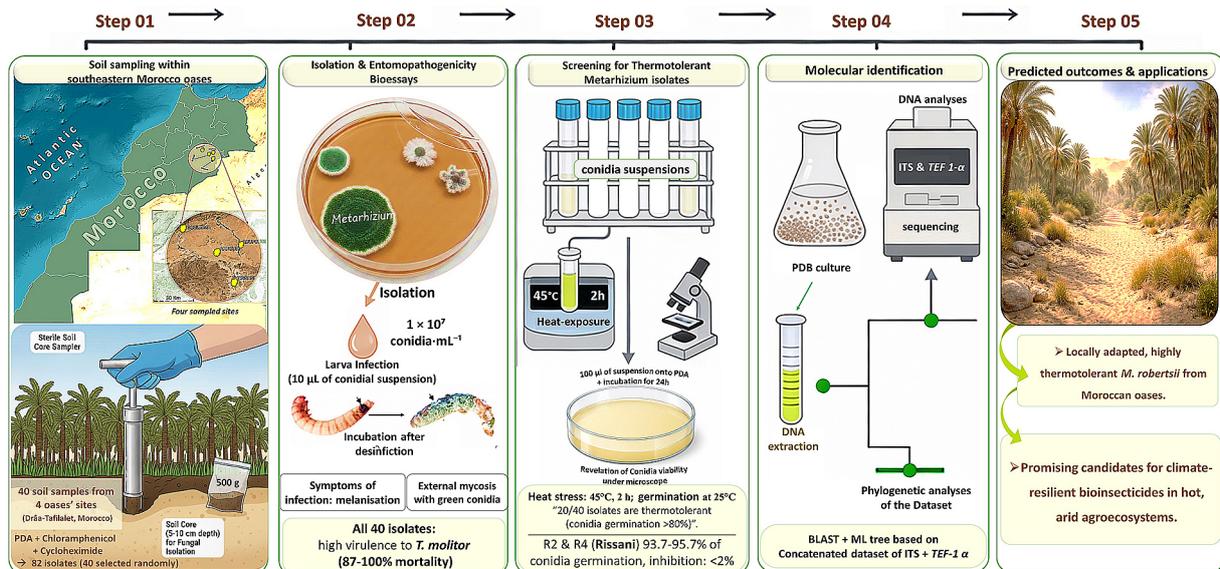
Figure 2. Conceptual workflow for isolating, screening, and identifying thermotolerant *Metarhizium robertsii* from Moroccan oases soils, illustrating each experimental step from field sampling to anticipated applications in hot, arid agroecosystems

larvae for each replicate. All experiments were run under 25 °C. Each plate was checked for dead larvae as soon as they died, at least for a period of a week, and the cadavers were removed and disinfected, then placed individually in a plate and checked for any fungus growth or contamination as described by Imoulan et al. (2011).

Treated larvae were transferred to sterile Petri dishes lined with moist filter paper and incubated in the dark at 25 ±1 °C (Figure 3). Larval mortality was recorded daily for 7 days.

Dead larvae were surface-sterilised with 0.5% sodium hypochlorite (v·v⁻¹), rinsed three times with sterile distilled water, and incubated on plates with moist filter paper to allow fungal outgrowth (Imoulan et al., 2011). The appearance of characteristic green sporulation confirmed the infection by *Metarhizium* spp., thereby validating the pathogenicity of *Metarhizium* isolates.

Screening of thermoresistant isolates of *Metarhizium* spp.

The thermotolerance of *Metarhizium* isolates was evaluated by assessing conidial germination rate after heat exposure at 45 °C, according to modified protocols of Fernandes et al. (2008) and Imoulan et al. (2011). Aliquots (1 mL) of each suspension (1×10^7 conidia·mL⁻¹) were transferred into sterile Eppendorf tubes and incubated in a water bath at 45 °C for 2 hours. Control

samples were maintained at 25 °C. After heat exposure, 100 µl of each suspension was spread onto PDA plates and incubated at 25 ± 1 °C for 24 hours in darkness.

The percentage of germinated conidia was determined under a light microscope at 400× magnification. A conidium was considered germinated when the germ tube length equalled or exceeded half the conidial diameter (Fernandes et al., 2008; Imoulan et al., 2011).

Isolates exhibiting high post-stress germination (>80% after exposure to 45 °C for 2) were considered thermotolerant and potentially well adapted to hot and arid environments such as Moroccan oases. These high-performing isolates were subsequently selected for molecular identification.

Molecular Identification of high thermotolerant *Metarhizium* isolates

Conidia from the most thermotolerant *Metarhizium* isolates were cultured using pure culture methodology, by inoculating 250 mL of potato dextrose broth (PDB) with 500 mg·L⁻¹ of chloramphenicol, and by incubating at 150 rpm, 25 °C in darkness for 7 days to stimulate mycelium growth. Following the incubation period, the mycelium was separated from the PDB by centrifugation at 3,500 RPM for 10 minutes using an Eppendorf Centrifuge (Eppendorf, Hamburg,
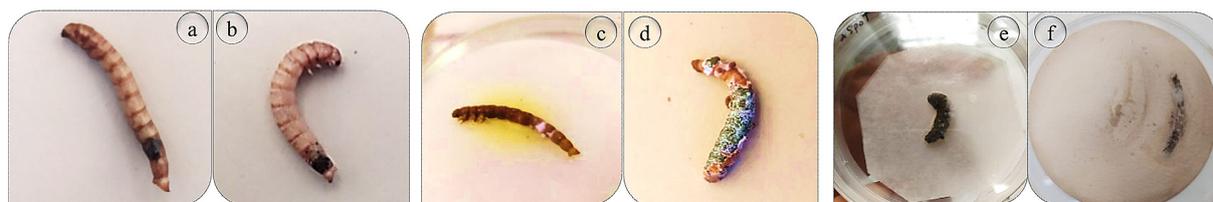
Figure 3. Illustration of some larvae of *T. molitor* showing treated larvae incubated individually in Petri dishes lined with moist filter paper at 25 ± 1 °C in the dark; (a+b) infection spot after treatment with conidia ; (c+d) Emergence of fungi and sporulation on cadavers after disinfection and incubation; and (d+e) Emergence of fungi and sporulation on cadavers after disinfection and incubation

Germany). Mycelial pellets were washed twice with sterile distilled water to remove residual PDB (Imoulan et al., 2016). Approximately 300 mg of mycelial biomass from each isolate was frozen in liquid nitrogen and ground up in sterile mortars using pestles. The genomic DNA from each was extracted following a modified CTAB method based on the work of Yao et al. (1999).

The incubation of the DNA Sample Workups mixtures was performed at 65 °C for a total of 2 hours. The samples were then extracted using two extractions of chloroform:isoamyl alcohol (24:1, v:v), followed by centrifugation to collect the supernatant phase at 10,000 RPM for 15 minutes at 4 °C. The supernatant phase was placed into a new tube with one-tenth the volume of Sodium Acetate (3M, pH 5.2) added to it. Precipitated total genomic DNA was subsequently separated from the supernatant phase by adding two-thirds of the volume of cold isopropanol and placing in the freezer overnight (−20 °C). After removing the total genomic DNA from the isopropanol, the total genomic DNA was washed consecutively with two 70% ethanol washes, allowed to dry at room temperature, and then re-suspended in TE Buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). All purified total genomic DNA samples were stored at −20 °C for further analysis. The concentration of total genomic DNA was determined using the NanoDrop One Device (Thermo Scientific, Waltham, MA, USA). Thus, before their use as diagnostic PCR templates, total genomic DNA samples were diluted into sterile TE Buffer (Imoulan et al., 2011; 2016).

Purified genomic DNA was utilized as a template in amplification of the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) using primer pair ITS1F (5′—TCCG-TAGGTGAACCTGCGG—3′) and ITS4R (5′—TCCTCCGCTTATTGATATGC—3′) (White et al. 1990), and the translation elongation factor-1α

(*TEF*-1α) gene using primer pair EF1α-EF (5′—GCTCCYGGHCAYCGTGAYTTYAT—3′) and EF1α-ER (5′—ATGACACCRA CRGCRACRG-TYTG—3′) (Bischoff et al., 2005). Marker sequences were amplified from the DNA of fungus samples with standard Taq polymerase (Promega). The PCR was conducted in a T-One thermocycler (Biometra, Göttingen, Germany) with the cycling schedule of: 95 °C for two minutes, 35 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds, and 72 °C for one minute, and a final 2-minute extension step at 72 °C.

The PCR products were purified and identified by performing agarose gel electrophoresis using the Qiagen QIAquick® purification system. The sequencing of the PCR products was conducted by StarSEQ in Germany (Mainz). The sequences generated from the sequencing were aligned and assembled into one consensus sequence using MEGA 11 software (Tamura et al., 2020). The assembled sequences were compared with reference sequences downloaded from the NCBI GenBank Database (Zhou et al., 2018) using the BLAST search function. Table 1 provides a list of the *Metarhizium* species that were included in our phylogenetic analysis, along with their voucher information, as well as the GenBank accession numbers for both the internal transcribed spacer (ITS) and the Translation Elongation Factor 1 alpha (*TEF-1α*) gene.

The research utilised representative sequences of *Metarhizium* for comparative analysis, along with one representative sequence of *Pochonia boninensis* JCM18598, which served as an outgroup. Sequences were obtained from the National Centre for Biotechnology Information's online database (GenBank) (Table 1) and were aligned using the ClustalW Multiple alignment software program (Thompson et al., 1997) by way of the MEGA 11 software package (Tamura et al., 2021). The datasets created

Table 1. Phylogenetic analysis of the *Metarhizium* species by use of GenBank Accession Number Data and Collection/Accession Voucher Numbers

Species	Voucher information	GenBank accession number		Reference
		TEF-1α	ITS	
Metarhizium robertsii	Strain R2	Under process*	PX587909*	Present study
	Strain R4		PX587910*	
Metarhizium bibionidarum	CBS 64867	LC126075	–	(Nishi et al., 2017)
Metarhizium argentinense	CEP424	MF966624	–	(Gutierrez et al., 2019)
Metarhizium blattodeae	ARSEF 12850	KU182917	KU182915	(Montalva et al., 2016)
Metarhizium brachyspermum	CM 1	LC469751	LC469747	(Yamamoto et al., 2020)
Metarhizium brittlebankisoides	Hn 1	AB778556	–	(Nishi et al., 2015)
Metarhizium gryllidicola	BCC37918	MN781691	MN781897	(Thanakitpipattana et al., 2020)
Metarhizium album	ARSEF 2082	DQ522352	AY375446	(Sung et al., 2018)
Metarhizium dianzhongense	KUNCC10809	PP328484	PP256143	(Wei et al., 2024)
Metarhizium frigidum	ARSEF 4124	DQ464002	NR132012	(Bischoff et al., 2006)
Metarhizium phasmatodeae	BCC 2841	MN781681	MN781911	(Thanakitpipattana et al., 2020)
Metarhizium rileyi	CBS 806.71	EF468787	AY624205	(Sung et al., 2007)
Metarhizium alvesii	CG 1123	KY007614	–	(Lopes et al., 2018)
Metarhizium humberi	IP 46	MH837574	–	(Luz et al., 2019)
Metarhizium campsosterni	BUM 10	MH143849	–	(Chen et al., 2018)
Metarhizium gaoligongense	BUM 3.5	KY087818	KY087806	
Metarhizium guniujiangense	RCEF 880	MW723106	–	
Metarhizium macrosemiae	RCEF 6696	MW723113	MW718317	
Metarhizium baoshanense	CCTCC M2016588	KY264169	KY264172	
Metarhizium granulomatis	UAMH 11028	KJ398781	NR132013	(Sigler et al., 2010)
Metarhizium kalasinense	BCC 53582	KC011189	KC011179	(Luangsa-Ard et al., 2016)
Metarhizium reniforme	ARSEF 429	HQ165690	DQ069284	
Metarhizium purpureogenum	ARSEF 12571	LC126078	–	
Metarhizium reniforme	ARSEF 429	HQ165690	DQ069284	
Metarhizium samlanense	BCC 17091	HQ165686	HQ165707	
Metarhizium takense	BCC 30934	HQ165679	HQ165698	
Metarhizium indigoticum	TNS-F 18553	JF416010	JN049874	(Kepler et al., 2012)
Metarhizium pseudoatrovirens	TNS-F16380	KJ398785	KJ398785	
Metarhizium owariense	NBRC 33258	JF416017	JN049883	
Metarhizium cylindrosporum	CBS 256.90	KJ398783	MH862209	(Kepler et al., 2014)
Metarhizium koreanum	ARSEF 2038	KJ398805	HM055431	
Metarhizium pemphigi	ARSEF 6569	KJ398813	–	
Metarhizium brasiliense	ARSEF 2948	KJ398809	–	
Metarhizium novozealandicum	ARSEF 4661	KJ398811	–	
Metarhizium candelabrum	BCC 29224	MN781708	MN781881	(Mongkolsamrit et al., 2020)
Metarhizium cicadae	BCC 48696	MN781703	MN781885	
Metarhizium culicidarum	BCC 2673	MN781706	MN781887	
Metarhizium fusoideum	BCC 28246	MN781699	MN781893	
Metarhizium eburneum	BCC 79252	MN781682	MN781914	
Metarhizium ellipsoideum	BCC12847	MN781715	MN781925	
Metarhizium anisopliae	CBS130.71	MT078845	MT078884	
Metarhizium flavoviride	CBS 125.65	MT078846	MT078885	
Metarhizium flavum	BCC 90870	MN781731	–	
Metarhizium huainamdangense	BCC32190	MN781710	MN781899	

Metarhizium megapomponiae	BCC 25100	MN781702	MN781906	(Mongkolsamrit et al., 2020)
Metarhizium niveum	BCC 52400	MN781685	MN781907	
Metarhizium nornnoi	BCC 19364	MN781696	MN781891	
Metarhizium phuwiangense	BCC 85068	MN781720	MN781912	
Metarhizium purpureum	BCC 82173	MN781722	MN781919	
Metarhizium ovoidosporum	BCC 29223	MN781716	MN781909	
Metarhizium viridulum	BCC 36261	MN781680	MT078878	
Metarhizium purpureonigrum	BCC89248	MN781728	–	
Metarhizium sulphureum	BCC 36592	MN781687	–	
Metarhizium biotecense	BCC 51812	MN781693	MN781878	
Metarhizium clavatum	BCC84543	MN781689	MN781886	
Metarhizium guizhouense	CBS 258.90	EU248862	HQ331448	(Bischoff et al.,2009)
Metarhizium globosum	ARSEF 2596	EU248846	NR132020	
Metarhizium pingshaense	CBS 257.90	EU248850	HQ331450	
Metarhizium brunneum	ARSEF2107	EU248855	KC178691	
Metarhizium lepidiotae	ARSEF 7488	EU248865	HQ331456	
Metarhizium majus	ARSEF 1015	EU248866	HQ331444	
Metarhizium acridum	ARSEF 7486	EU248845	HQ331458	
Metarhizium robertsii	ARSEF 4739	EU248848	–	
	ARSEF 727	–	–	
	ARSEF 8820	–	–	
Pochonia boninensis	JCM 18597	AB758463	AB709858	(Nonaka et al., 2013)

Note: * The accession numbers in bold font refer to newly generated sequences in this study.

from the ITS and *TEF-1α* were combined into one sequence file using SequenceMatrix (Vaidya et al., 2011) to form a phylogenetic tree based on maximum likelihood (ML) as determined by the MEGA 11 software package (Tamura et al., 2021)

Data analyses

The germination percentage was calculated according to the formula developed by Vega et al. (2008) (Equation 1):

$$Germination\ (\%) =$$
$$= \frac{germinated\ conidia}{total\ conidia} \times 100 \quad (1)$$

As for the inhibition of conidial germination, it was determined as follow (Equation 2):

$$GI\ (\%) = \frac{(GerC - GerT)}{GerC} \times 100 \quad (2)$$

where: *GI* is the germination inhibition, *GerT* is the germination rate in the treatment, and *GerC* is the germination rate in the control. The one-way analysis of variance (ANOVA) test was used to perform a statistical analysis of the findings from the current study, while Tukey's Studentized Range Test (Tukey's Test) at a $P = 0.05$ was used for comparisons between the means of groups. These analyses were completed using SPSS for Windows (SPSS Inc., 2013). The tests for both conidial germination and pathogenic potential were each repeated three times for validation.

RESULTS

Isolation and morphological identification

Metarhizium spp. were isolated from all soil samples, and a total of 82 isolates were obtained. Morphological characteristics of all obtained *Metarhizium* isolates were examined to confirm their identification at the genus level. On PDA medium, the colonies of *Metarhizium* isolates developed the typical green pigmentation associated with sporulating cultures (Figure 4).

Several negative plates (Figure 4a). did not yield any *Metarhizium* growth (negative plates),

while the positive plates developed one or more *Metarhizium* colonies (Figure 4b). Microscopic examination further revealed that all isolates produce elongated green-shaped conidia, a key diagnostic feature of the genus *Metarhizium*. Representative isolates were purified by serial single-spore purification (Figure 4c,d).

These observations are consistent with previously described morphological traits for *Metarhizium* species and support their preliminary classification within this genus.

Entomopathogenicity

All 40 of the *Metarhizium* isolates tested against *T. molitor* larvae display a consistently high level of virulence based on the assessment of pathogenicity. Conidial viability of those same isolates, utilised in the bioassays, ranged from 86.67% to 100%. Larvae began to die three days after inoculation, and throughout all sampling sites, there were consistently high levels of pathogenicity seen by the tested isolates on *T. molitor* larvae. Isolates taken from both Rissani and Goulmima oases produced mortality levels between 93.34% and 96.67%. *Metarhizium* isolates taken from Arfoud oasis showed the greatest virulence potential, with mortality reaching between 90% and 100%. Isolates from Aoufous oasis caused mortality levels of between 86.67% and 93.34%. Statistical analysis revealed no significant differences ($P > 0.05$) among the different isolates of a given population group in terms of determining their respective levels of pathogenicity apart from the Aoufous population group, where there was a significant difference between isolates (F = 4.069, df = 2, $P < 0.05$). Additionally, a statistical difference ($P < 0.05$) was noted when comparing pathogenicity levels between different populations. These results collectively indicate that the
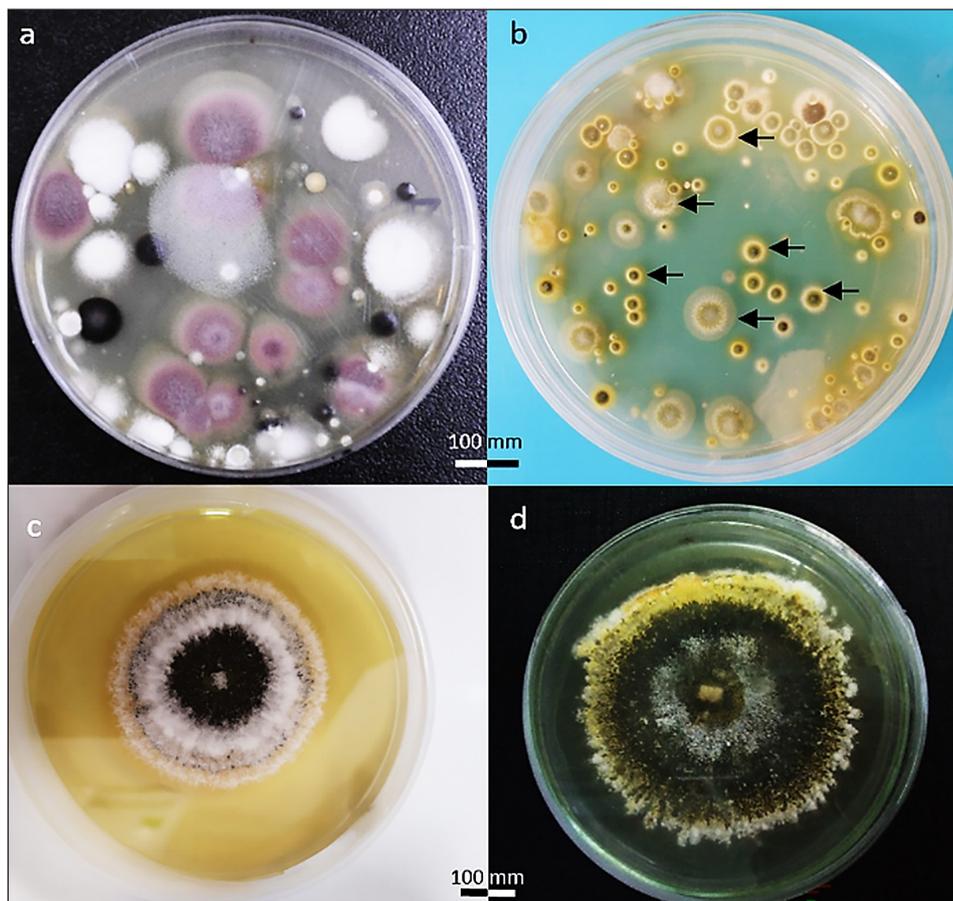

Figure 4. Isolation technique of *Metarhizium* spp. on PDA supplemented with Chloramphenicol and Cycloheximide. (a): negative plate without development of any *Metarhizium* colonies, (b): positive plate developing different aspects of *Metarhizium* colonies, arrows indicate some colonies of *Metarhizium* ssp. (c) and (d): spot cultures of monospored isolates of *Metarhizium* after 10 and 17 days of incubation at 25 °C, respectively

tested isolates possess strong entomopathogenic potential. In all cases, infected larvae developed characteristic symptoms of *Metarhizium* infection, including progressive melanisation followed by external mycosis and the emergence of green conidial masses (Figure 5). The number of mortalities was recorded in the control group.

Screening for thermotolerant *Metarhizium* isolates

Metarhizium isolates were screened for thermotolerance by assessing conidial germination after exposure to 45 °C for 2 hours (Figure 6). The gemination rate was 75–95.67% (F = 0.840, df = 2, *P > 0.05*), 78–83.67% (F = 0.617, df = 2, *P >* 0.05), 77.67–87.67% (F =0.623, df = 2, *P* > 0.05) and 76.67–84.67% (F = 0.704, df = 2, *P* > 0.05) for isolated obtained from Rissani, Aoufous, Arfoud and Goulmima, respectively. Although there was no significant (*P*> 0.05) intraspecific variation in heat tolerance within *Metarhizium* populations (Figure 6).

Thermotolerance was recorded in 50% (20/40) of the heat-treated isolates, which displayed conidia germination rates exceeding 80% and inhibition rates from 0.35% to 16.38%. However, two isolates (R2 and R4) from Rissani oasis habitat displayed the highest post-heat-treatment germination rates, reaching 93.67% and 95.67%, respectively. For both isolates, the reduction in conidia germination relative to the untreated controls did not exceed 1.75%, indicating a remarkably low inhibition rate and a strong intrinsic tolerance to thermal stress, making them particularly promising candidates for the development of thermoresistant bioinsecticides suited to the hot and arid conditions characteristic of Moroccan oasis agroecosystems.

Phylogenetic Identification of the most thermoresistant strains

The two most thermotolerant *Metarhizium* strains, R2 and R4, were subjected to molecular identification. PCR amplification of the ITS and *TEF-1α* gene was successfully achieved for both isolates, providing high-quality sequences suitable for subsequent phylogenetic analyses.

After the exclusion of ambiguous positions from both ends, the ITS amplicons measured 550 bp and 545 bp, respectively, while the *TEF-1α* fragments were about 950 bp for both. BLAST searches against the GenBank database revealed that both ITS and *TEF-1α* sequences from these isolates showed high sequence identity to *M. anisopliae* and *M. robertsii*, with 100% similarity at 100% query coverage. The concatenated dataset of ITS and *TEF-1α* alignment included 67 ingroup taxa and 1431 characters, including gaps. The phylogenetic relationship inferred from a concatenated dataset of ribosomal internal transcribed spacer region (ITS) and the transcribed elongation factor 1-alpha (*TEF-1α*) sequences revealed that *Metarhizium* strains R2 and R4 cluster within the *Metarhizium robertsii* lineage. Both strains were grouped in the same terminal branch, supported by a 92% bootstrap value, within reference strains of *M. robertsii* ARSEF 4739, ARSEF 8820 and ARSEF 727 isolated from soil samples in Australia, Orthoptera in Brazil and Coleoptera (*Curculionidae*) in the USA, respectively (Figure 7).

DISCUSSION

Metarhizium is regarded as a globally effective entomopathogenic fungus for biological
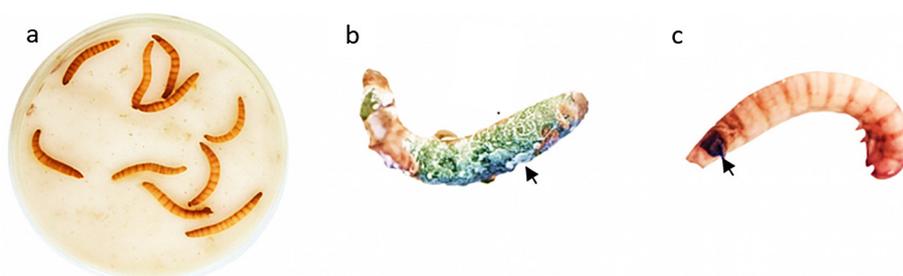

Figure 5. Bioassay assessing the pathogenicity of isolated *Metarhizium* strains against last-instar of *T. molitor* larvae: (a) infected larvae at 0 days post-inoculation; (b) and (c) the arrows highlight fungal sporulation on the larval cadzaver and visible infection sites on the larval cuticle
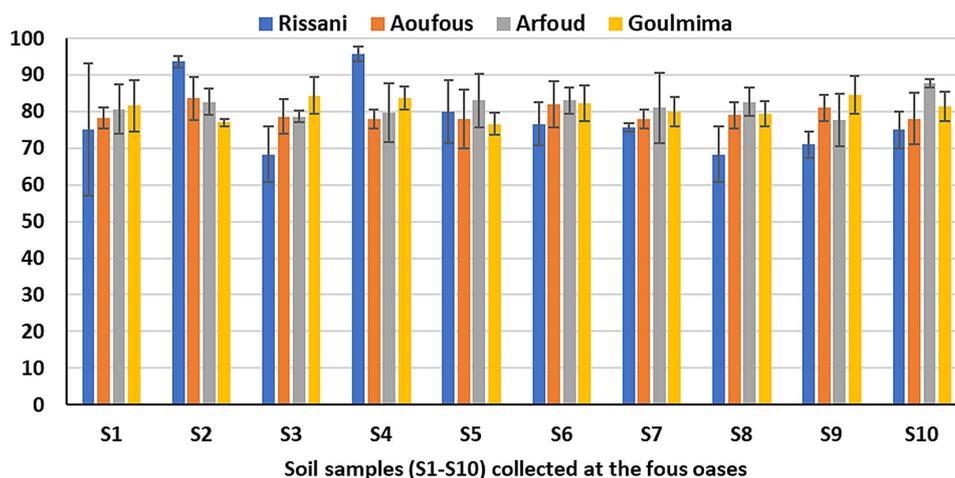
Figure 6. Conidial germination rate (%) of forty *Metarhizium* isolates after heat-exposure for 2 hours at 45 °C

control applications (Roberts and St. Leger 2004; Zimmermann 2007). Nevertheless, the increasing frequency of high-temperature episodes associated with climate change underscores the necessity of isolating thermotolerant strains to ensure sustained performance under elevated thermal regimes. In Morocco, oases of the southeastern region constitute ecologically distinctive environments that may serve as natural reservoirs for the thermotolerant *Metarhizium* genotype.

In the current study, 82 *Metarhizium* isolates were recovered from 40 soil samples collected across four Moroccan oases. The virulence of 40 isolates was assessed through an evaluation of *T. molitor* larvae conidia germination following a 2-hour exposure to 45 °C or higher temperatures. Although many of these isolates were recovered from soil habitats, they appear to be highly pathogenic to *T. molitor* larvae and possess significant potential for entomopathogenic activities. These observations are in agreement with prior studies that showed rapid mortality in coleopteran hosts and that *Metarhizium* spp. produced cuticle-degrading enzymes and strong secondary metabolites involved in the pathogenesis of these insects (Vivekanandhan et al., 2024).

Entomopathogenic fungi grow at all developmental stages due to temperature. Temperature has a significant impact on the infection process and on host-fungi relationships (Zimmermann, 1982; Cabanillas and Jones, 2009; Khan and Ahmad, 2019). In addition, it has been suggested that a fungal isolate cannot infect an insect host if the temperature is greater than its upper growth limit (Rangel et al., 2010). Therefore, the ability of conidia to endure temperature

fluctuations is a crucial characteristic of effective Mycoinsecticide production (Li and Feng, 2009; Zhao et al., 2016), thus demonstrating the need for developing mycoinsecticides that will perform at high temperatures, particularly with the increasing incidence and severity of heatwaves as a consequence of climate change. In oases ecosystems, temperatures regularly rise over 40 °C at the soil surface in the spring and summer months, leading to an environment that favours the survival of heat-tolerant fungi over time, while also putting intense selection pressure on their genotypic variability. In order to provide effective biocontrol solutions for insect hosts in the hotter conditions anticipated over the coming decades, it will be important to isolate thermotolerant strains of *Metarhizium* such that they will remain infective in future climatic situations (Chu et al., 2015).

Among all tested isolates, R2 and R4 from the Rissani oasis exhibited the highest thermal resistance, as germination conidia rates were > 92% after thermal stress exposure, and the inhibition of conidia germination was 1.75% and 0.35%, respectively, making them particularly promising candidates for the development of thermoresistant bioinsecticides suited to the hot and arid conditions characteristic of Moroccan oasis agroecosystems. Such a result is consistent with reports indicating that *Metarhizium* populations exposed to strong abiotic stress tend to harbour high adaptive potential to thermal extremes (Rangel et al., 2005; Fernandes et al., 2008; Muazu et al., 2024). Similar investigations found that some strains of *Metarhizium* spp. were resistant to heat stress up to 40 °C
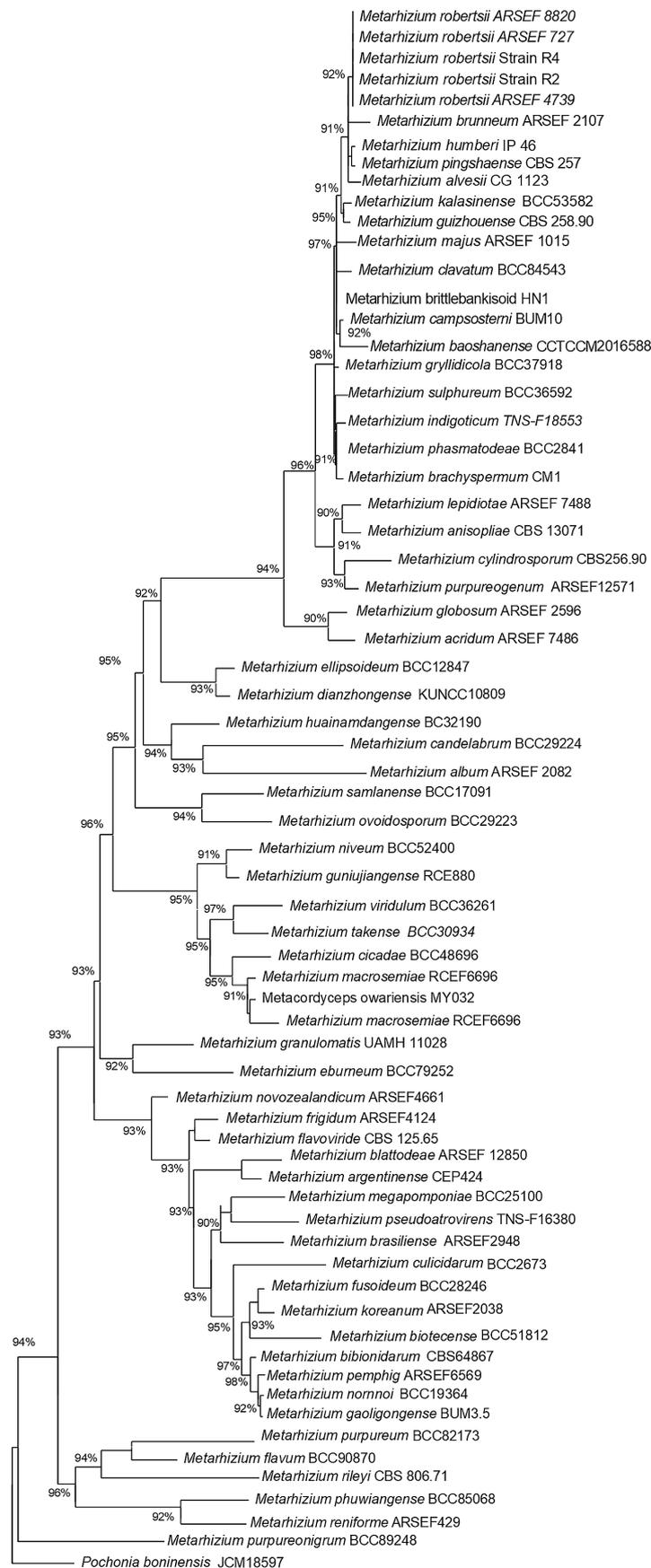
Figure 7. Maximum likelihood phylogeny analysis of *Metarhizium* fungi as reconstructed from concatenated dataset of combined ITS and *TEF-1α* genes of *Metarhizium* species: Values on terminal branches indicate bootstrap support percentage

(Liu et al., 2009; Keyser et al., 2014; Muazu et al., 2024). Additionally, Ferreira et al. (2021) reported a significant reduction in the germination rate of *M. robertsii* ARSEF 2575 after 4, 5, and 6 h of exposure to 45 °C. However, Velavan et al. (2022) observed an increase in conidial germination of *M. robertsii* after exposure to 45 and 48 °C, with maximum germination rates at the longest incubation periods (60 h) of exposure, demonstrating a notable ability to maintain viability under prolonged heat stress.

Phylogenetic analysis of the dataset of ITS and *TEF-1α* resolved the two thermotolerant strains R2 and R4 to *M. robertsii* species as confirmed by the high bootstrap support value (92%) of maximum likelihood (Figure 7). The combined dataset based on internal transcribed spacer (ITS) region of rDNA and the elongation factor 1-alpha (*TEF-1α*) gene was also successfully employed to delimit *M. robertsii* (Zheng et al., 2024) and *M. majus* (Kwak et al., 2021). Now, the revolution of molecular markers and advanced sequencing methods, species-level identification has become more accurate and reliable, overcoming the limitations of traditional approaches that could not clearly resolve closely related *Metarhizium* species (Bischoff et al., 2009, Luangsa-ard et al., 2017; Chen et al., 2019). There have been many studies showing an association between geographic origin and climate and habitat and thermotolerance (Rangel et al., 2005). Several studies show that fungi from the equator have thermotolerance abilities higher than their counterparts collected from regions further from the equator (Vidal et al., 1997; HoFmann and Parsons, 1991). In addition, the study shows that fungus from the southern and northern hemispheres has a greater ability to survive cold temperatures than fungus from the equator (McCammon and Rath, 1994; Vänninen, 1995).

Hence, in *M. robertsii*, conidial tolerance to heat stress is enhanced through multiple mechanisms, including the upregulation of the small heat-shock protein HSP25 under extreme temperatures (Liao et al., 2014; Xie et al., 2019), the expression of the Mrsvp gene (Xie et al., 2019), and the overexpression of a pyruvate kinase gene, which increases intracellular pyruvate levels and consequently boosts thermotolerance (Wu et al., 2019).

The greatest temperature tolerance reported in isolates recovered from this experiment appears to correlate with other studies that have shown that species from this lineage of *M. robertsii* exhibit significant ecological flexibility and have increased abiotic stress tolerance (heat and drought) (Wu et al., 2019; Velavan et al., 2022). These characteristics may enable them to survive in soils that are inhospitable, and they may arise through different physiological processes. For example, the ability to produce heat-shock proteins and stabilize cell membranes or, alternatively, enhance the production of cellular repair mechanisms.

CONCLUSIONS

The first example of the characterisation of thermotolerant *Metarhizium* from the oases of Morocco was created using the *M. robertsii* native strains. The results of the molecular analysis showed that the two thermotolerant isolates (R2 and R4) are members of *M. robertsii*, a species that has demonstrated an affinity for adapting to stressors and for showing an ability to reproduce in the environment. In addition, the germination rates following heat shock were high, indicating that the two strains can withstand the high temperatures common to oases in the environment. Thus, this work has contributed to our knowledge of the ecological distribution of *Metarhizium* in Morocco, especially in the areas of southeastern Morocco within the Drâa-Tafilalet area. Finally, it has provided opportunities for the development of biopesticides that will be able to withstand climate change in hot and dry locations. It will require additional research on the stress and survival mechanisms employed by the strains to further support the eventual use of these organisms in sustainable pest management systems.

Acknowledgements

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