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Comparative study of fungal stability between *Metarhizium* strains after successive subculture

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Abstract

Background: *Metarhizium* species are considered one of the most outstanding powerful biological control agents that have been commercialized as biopesticides against various agricultural pests. Fungal stability with successive in vitro cultivation is a desirable trait for a large-scale production of fungal biopesticide.

Main body: The new Egyptian strain *Metarhizium anisopliae* AUMC 3262 exhibited auspicious results when compared to *Metarhizium brunneum* ARSEF 4556 and *M. brunneum* V275 based on the variations of fungal characteristics, and essential quality control parameters (radial growth rate, conidial yield, viability, and virulence) after repeated in vitro subculturing. Changes in morphological characteristics were noted at both AUMC 3262 and ARSEF 4556. Following the 5th subculture, decreased conidial yield was noted, though radial growth remained stable, confirming that there is a non-positive correlation between conidial yield and radial growth rate for these species. In contrast, V275 showed a high morphological stability, conidial yield, and radial growth rate after repeated subculture. The three tested strains manifested high viability up to 100% and displayed the same pattern of Pr1 production. A slight variation was recorded in the median lethal time (LT₅₀) values against the great wax moth, *Galleria mellonella* (L.), larvae between different subcultures of the tested *Metarhizium* strains.

Conclusion: The new Egyptian strain AUMC 3262 showed a high stability with a slight difference in some parameters after the successive subculture compared to both ARSEF4556 and V275.

Keywords: Fungal stability, *Metarhizium* strains, Pr1, Virulence

Background

Application of the entomopathogenic fungi (EPF), as an alternative to conventional chemical pesticides, represents a safe sustainable crop protection method for integrated pest management (IPM) strategies. More than 700 species of EPF are used as biological control agents, with particular emphasis on *Metarhizium* spp. (Humber, 2008). Approximately 39% of mycoinsecticides based on genus *Metarhizium* as an active ingredient (Faria and Wriaght, 2007), probably due to their high host

specificity, non-persistence, and eco-friendly with a unique mode of action against target pests. *M. anisopliae* showed high mortality rates against the stored grain pests *Sitophilus granarius* and *S. oryza* depending on conidial concentrations and the absence or presence of food (Ak, 2019), while evaluation of different strains of *M. anisopliae* against *Spodoptera exigua* and *S. littoralis* showed a high accumulative mortality rate (100%) against 2nd and 4th larval instars of *S. littoralis* and 1st and 3rd instars of *S. exigua* larvae (Anand and Tiwary, 2009; Han et al., 2014).

EPF used as biological control agents are characterized by critical factors to be used effectively in IPM programs, one key aspect concerning propagule stability after repetitive subculture or mass production. Genus

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Metarhizium has the ability to be mass produced using a biphasic production system on a wide variety of relatively inexpensive organic substrates (Jenkins et al., 1998). Moreover, *M. anisopliae* AUMC 3262 could be mass produced on cheap agriculture byproducts using biphasic production technique (Ezzat et al., 2019).

Sector formation is an important sign of fungal instability resulting from cultural degeneration. Sectors can differ from the parent culture in a range of morphological and physiological characteristics, including a decline in infective propagule production and certain metabolites, resulting in decreased fungal pathogenicity (Shah and Butt, 2005). The sterile sectors cause reduced spore yield and increased production costs, making the end product less cost-effective; in addition, a virulent inoculum could decrease the end product efficacy when compared with competing agents (Rayan et al., 2002).

The production of stable conidial yield with high viability is also an imperative parameter for commercial purposes as insect mortality is dose-dependent; the more conidia that adhere to the body of the insect, the faster the fungus will kill the host insect (Butt, 2002). Following conidial attachment to the insect cuticle, the conidia germinate and exert a combination of passive hydrophobic and electrostatic forces, followed by secretion of cuticle-degrading enzymes. Cell wall-bound subtilisin protease Pr1 plays a pivotal role in insect cuticle penetration. Furthermore, Pr1 is classified as a pathogenicity determinant (St. Leger et al., 1987). Small and Bidochka (2005) found that *M. anisopliae* Pr1 genes are upregulated from the point of initial infection until the mycelia emerge and produce conidia on the surface of the cadaver, indicating a potential link between conidial virulence and Pr1 production. Consequently, the ability of *M. anisopliae* conidia to produce a high amount of Pr1 qualifies its use as a biocontrol agent.

Virulence maintained over several generations is the most important factor in choosing the fungi as effective biocontrol agents. Several reports have stated that EPF, including *M. anisopliae*, tended to experience a loss or reduction in sporulation and virulence (attenuation), following successive subculture on artificial media (Santoro et al., 2014). These studies also confirmed that the virulence was improved or restored when the pathogen was passed through a suitable insect host or particular culture media (Wang and St. Leger, 2005).

In this study, a new Egyptian isolate, *M. anisopliae* AUMC 3262, the promising *M. brunneum* ARSEF 4556, and the commercial *M. brunneum* V275 strains were evaluated for their fungal stability and efficacy that could help in developing mass production strategy and commercialization process.

Main text

Materials and methods

Fungal isolates

The *Metarhizium* strains used in this comparison study were *M. anisopliae* AUMC 3262 isolated from the great wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), Egypt; *M. brunneum* ARSEF 4556 isolated from *Boophilus* sp. (Acari: Ixodidae), the USA; and *M. brunneum* V275 isolated from *Cydia Pomonella* L. (Lepidoptera: Tortricidae), Austria. Prior to their use in these studies, all 3 fungal strains were first passed through the insect host, *G. mellonella*, in order to restore virulence, before being re-isolated on Sabouraud dextrose yeast agar (SDAY), composed of 40 g glucose, 10 neopeptone, 2 yeast extract, and 15 agar in 1 l dist. H₂O (Sigma-Aldrich, UK) incubated in total darkness at 25 ± 2 °C for 15 days. Subcultures were maintained on SDAY at 4 °C for subsequent studies.

Insect rearing

Galleria mellonella cultures were maintained in 2-l glass jars covered with a muslin cloth at 27 ± 2 °C and 8:16 (L:D) h photoperiod, provided with honey and wheat germ as nutrient. All individuals used in this study were last larval instars, with the size range of 15–25 mm.

Effect of successive subculture on quality control parameters

Fungal plugs (8 mm) were taken from the edge of the colony of a 15-day-old culture and used to inoculate Petri dishes of SDAY incubated at 25 ± 2 °C for 7 days. This culture represented the first subculture. Successive subcultures were prepared by inoculating fresh SDAY plates with an inoculum of the previous subculture, with each subculture grown on SDAY for 7 days at 25 ± 2 °C. Following incubation, subcultures were stored at 4 °C until the 9th subculture had been produced. After the production of the 9th subculture, 1st, 3rd, 5th, 7th, and 9th subcultures were grown on new SDAY for 7 days. This procedure allowed the simultaneous generation of these subcultures under similar growth conditions (5 replicates per subculture). These cultures were then used for subsequent studies.

Monitoring fungal growth and sector formation

After the simultaneous growth, conidia of 7-day-old cultures were scraped from the simultaneously cultivated plates, spread on to 90 mm SDAY Petri dish, and incubated in darkness for 3 days at 25 ± 2 °C. Individual plugs (8 mm diameter) of un-sporulated mycelium from the 3-day-old cultures were placed upside down in the center of SDAY medium in 90-mm Petri dishes and incubated in darkness for 15 days at 25 ± 2 °C. Three replicates were prepared from each replicate of the five

replicates previously prepared (total no. 15). Morphological changes and sector formation were observed daily, and surface radial growth was recorded from the 3rd to 15th days and was calculated following the equation:

$$\frac{\text{Colony diameter at the end of incubation period} - \text{Fungal disc diameter}}{\text{Total incubation days}}$$

Conidial yield and viability

From the previous prepared 15 plates, 5-mm agar plugs were taken randomly after 15-day incubation from each plate and placed in 1 ml of 0.03% (v/v), Tween 80 then vortexed to suspend the spores. Spore yield was determined, using an Improved Neubauer hemocytometer, Germany. Germination and viability were assessed according to Inglis et al. (2012). 15 SDAY plates were inoculated with 50 μ l of the previous conidial suspension (4×10^7 conidia/ml), then incubated in darkness at $25 \pm 2^\circ\text{C}$, after 20–24 h. Plates were examined for germination at $\times 40$ (100 conidia per microscopic field). A spore was considered to have germinated if it had formed a germ tube that was as long as the spore width.

Determination of spore bound Pr1

Conidia-bound Pr1 activity was determined according to Shah et al. (2005) as follows: 10 mg of conidia was harvested from 15-day-old culture and washed once with 1 ml of 0.03% (v/v) aq. Tween 80 and twice with 1 ml distilled water, then incubated in 1 ml of 0.1 M Tris-HCl (pH 7.95) containing 1 mM Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma-Aldrich UK) for 5 min at room temperature. After incubation, conidia were pelleted by centrifugation at 12,000 rpm for 5 min (Sanyo, Harrier 18/80 centrifuge). The supernatant (200 μ l) was transferred to a 96-well flat-bottom plate, optical grade (Greiner Bio-One) and absorbance measured, using a BioTek Synergy H1 multi-mode reader at 405 nm. Buffered substrate was used as a control.

Determination of fungal virulence

Assessment of conidial virulence for each subculture was carried out against the last larval instar of *G. mellonella*. Batches of 10 *G. mellonella* larvae were immersed for 10 s in 10 ml of 0.03% (v/v) aqueous Tween 80 conidial suspension (4×10^7 conidia/ml) prepared from each subculture. Excess conidial suspension was removed by transferring the inoculated insects to filter paper before moving them to a 90-mm Petri dish lined with filter paper moistened with distilled water and sealed with paradigm tape. No food was provided. Control insects were immersed in

0.03% (v/v) aqueous Tween 80 only. Treatments were carried out in triplicate. All dishes were incubated at $25 \pm 2^\circ\text{C}$ in darkness and monitored daily for 5 days.

Statistical analysis

LT₅₀ was calculated using Bio-Stat V5 (analyst soft inc. V.5.9.33). Other results were expressed as the mean \pm standard error (SE). Statistical significance was determined by the way of analysis of variance (one-way ANOVA, SPSS software version 16), followed by the least significant difference LSD test.

Results and discussion

Effect of successive subculture on quality control parameters

Morphological characteristics

Specific criteria must be met by EPF to be used as biocontrol alternatives to chemical pesticides. Sector formation and changes in morphological and physiological characteristics could cause significant commercial problems including a decline in the production of certain metabolites and virulence as well as conidial yield. All of the tested strains shared almost the same morphological appearance of a circular, dark olivaceous-green colony. ARSEF 4556 was characterized by a very good sporulation pattern with the 1st subculture, as the conidia were arranged as central layers. Following the 5th subculture, yellow sectors appeared and increased gradually in size with the following subcultures, while AUMC 3262 was found to have small, central white mycelial growth, which increased in size from the 7th subculture until full coverage of the Petri dish was observed at the 9th subculture. The changes in fungal morphology and sector appearance were noted in both AUMC 3262 and ARSEF 4556 after the 5th and 7th subcultures and increased with the successive subcultures were often observed in fungal cultures maintained on artificial media as a result of cultural degradation, caused by age of the culture, method of propagation or nature of the culture media itself (Shah and Butt 2005 and Wang et al., 2005a). This is attributed to mutation, transposons, or genomic rearrangement (Firon et al., 2002). In contrast, V275 maintained its morphology until the 9th subculture. A small mycelial growth appeared over the colony center (Fig. 1).

Monitoring of radial growth rate, conidial yield, and viability

Effective EPF must be characterized by the production of a highly stable conidial yield with high viability along with frequent subcultures. The growth rate and conidial yield varied across the tested *Metarhizium* isolates, and the radial growth rate of AUMC 3262 was found to be

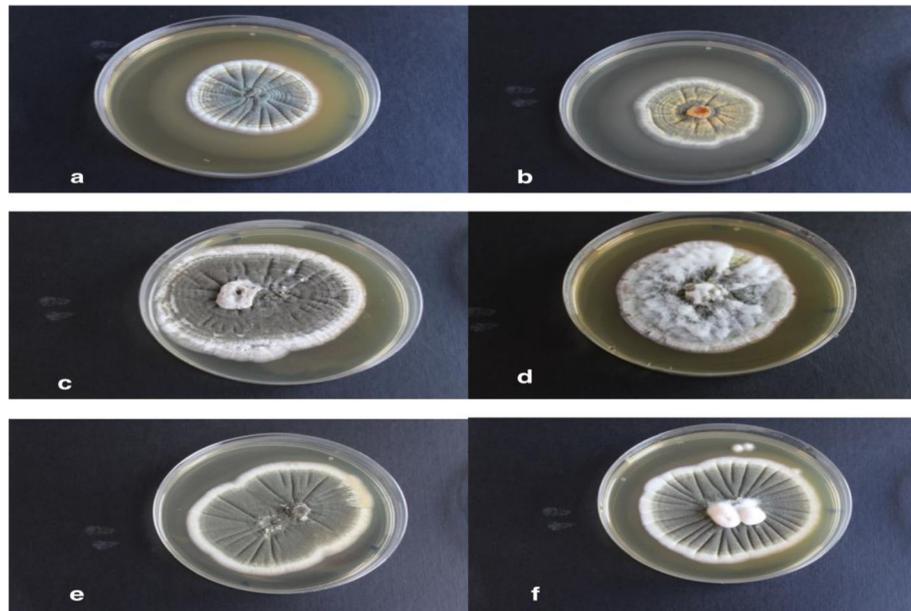


Fig. 1 Influence of successive subculture on morphology and sector formation after 15 days growth on SDAY at $25 \pm 2^\circ\text{C}$. **a** The first subculture of ARSEF 4556. **b** The ninth subculture of ARSEF 4556. **c** The first subculture of AUMC 3262. **d** The ninth subculture of AUMC 3262. **e** The first subculture of V275. **f** The ninth subculture of V275

different between different subcultures, gradually decreasing in size from 4.2 ± 0.11 mm/day at 1st subculture to 3.7 ± 0.13 mm/day at 9th subculture. Furthermore, the conidial yield obtained with AUMC 3262 showed some instability, ranging from $4.43 \pm 0.69 \times 10^7$ conidia/ml at the 1st subculture to $1.93 \pm 0.40 \times 10^7$ conidia/ml at 5th subculture, which was found to be the lowest conidial yield observed. On the other hand, V275 and ARSEF 4556 offered a relatively stable radial growth pattern between subcultures, ranging from 3.4 ± 0.54 to 3.9 ± 0.11 mm/day for V275 and 2.75 ± 0.08 to 2.60 ± 0.06 mm/day for ARSEF 4556, which represent the lowest linear growth rate through different subcultures. Although the 1st subculture of ARSEF 4556 showed a superior production of conidia with a value of $6.23 \pm 0.23 \times 10^7$ conidia/ml, a significant decrease ($P < 0.05$) in conidial yield observed through successive subculture, dropped to $3.36 \pm 0.18 \times 10^7$ conidia/ml at 9th subculture, whereas V275 displayed a high stability in conidia production through successive subcultures, ranging from 4.46 ± 0.38 to $3.80 \pm 0.55 \times 10^7$ conidia/ml with a non-significant difference through successive subcultures ($P > 0.05$) as shown in Table 1. This study revealed that the radial growth rate varied between fungal species and strains, with no relationship between conidial yield and radial growth rate. Similarly, Safavi et al. (2007) reported that *M. anisopliae* strains showed a high radial growth rate with a low conidial yield in contrast to 3 tested

isolates of *B. bassiana*, which provided a high radial growth rate with a high conidial yield, depending on the provided nutrition. This suggests that there is no correlation between increased radial growth rate and conidial yield. The speed of germination was dictated by the protein content of the growth media as well as the size of the conidia, and this may explain why all of the tested strains displayed high stability and maximum germination up to 100% throughout successive subcultures. Notably, a positive relationship between the rate of conidial germination and virulence of the strain against the target pest was recorded by Dimbi et al. (2004) and Ansari and Butt (2011).

Effect of successive subculturing on the activity of conidial Pr1 enzyme

Following attachment to the insect cuticle, the conidia begin to germinate, exerting a combination of passive hydrophobic and electrostatic forces, followed by secretion of cuticle-degrading enzymes. One of the most important cuticle-degrading enzymes contribute to fungal pathogenicity is the subtilisin-like Pr1. Although Pr1 is not the only pathogenicity determinant for *Metarhizium* species, mutant *M. anisopliae* lacking the Pr1 gene was found to be less pathogenic (Wang et al., 2002). A slight fluctuation was reported in Pr1 production between ARSEF 4556, AUMC 3262, and V275 with non-significant differences through successive subcultures of

Table 1 Effect of successive subculture on radial growth rate and conidial yield of ARSEF 4556, AUMC 3262, and V275 after 15 days growth on SDAY at $25 \pm 2^\circ\text{C}$

Subcultures	Radial growth rate (mm/day)			Conidial yield ($\times 10^7$)		
	ARSEF4556	AUMC 3262	V275	ARSEF4556	AUMC 3262	V275
1st	2.71 ± 0.04^a	4.22 ± 0.11^a	3.39 ± 0.54^a	6.23 ± 0.23^a	4.43 ± 0.69^a	4.00 ± 0.45^a
3rd	2.60 ± 0.21^a	4.13 ± 0.0^{ab}	3.66 ± 0.7^a	5.26 ± 1.17^b	2.46 ± 0.38^b	4.03 ± 0.60^a
5th	2.75 ± 0.08^a	4.08 ± 0.04^{abc}	3.62 ± 0.07^a	5.03 ± 0.42^b	1.93 ± 0.40^b	3.80 ± 0.55^a
7th	2.66 ± 0.11^a	3.82 ± 0.17^{bc}	3.77 ± 0.02^a	3.70 ± 0.15^c	2.50 ± 0.23^b	4.46 ± 0.38^a
9th	2.60 ± 0.06^a	3.73 ± 0.13^c	3.95 ± 0.11^a	3.36 ± 0.18^c	2.70 ± 0.15^b	4.34 ± 0.72^a

Each value represents the mean of three replicates. The means within a column followed by the same letter are not significantly different (LSD test, $P > 0.05$)

the 3 *Metarhizium* strains ($P > 0.05$) (Table 2). Production of Pr1 by *M. anisopliae* and *B. bassiana* conidia is affected by different nutritional conditions as well as repeated subcultures (Dhar and Kaur, 2010 and Wanida and Poonsuk, 2012). In the same context, Shah et al. (2007) reported that spore adhesion, hydrophobicity, and spore-bound Pr1 declined, following the first subculture, as the spore-bound Pr1 was directly correlated with a decline in virulence, and so is used as a quality control marker to monitor changes in virulence. A slight fluctuation in Pr1 production between the examined strains and subcultures was noted, but this did not affect the virulence of the tested fungi. This is likely due to relative Pr1 production still being high enough to maintain a high virulence.

Effect of successive subculture on conidial virulence

Significant differences ($P < 0.05$) were noted among subcultures in the median lethal time (LT_{50}) against *G. mellonella* larvae. Table 2 shows the fluctuations in LT_{50} between the tested isolates, with conidia of V275 representing the most aggressive isolate with an LT_{50} of 1.69 ± 0.12 days with the conidia of the 1st subculture. Conidia produced by AUMC 3262 subcultures displayed a high mortality in the range 1.92 ± 0.03 to 2.34 ± 0.09 days, with 5th and 9th subcultures, respectively. Conidia of ARSEF 4556 were found to be less virulent than other strains with the highest

LT_{50} of 2.71 ± 0.01 days obtained with conidia produced by the 7th subculture. Nahar et al. (2008) and Santoro et al. (2014) recorded that *M. anisopliae* displayed a decreased virulence against *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae) after 20–40 subcultures on nutrient-rich media. There is however little information about this phenomenon and how EPF stability changes when cultured on artificial media, but Brownbridge et al. (2001) believed the attenuation of virulence may be due to random mutation or caused by subculture conditions. Wang et al. (2005b) and Wang and St Leger (2005) studies on *M. anisopliae* returned this to the pathogenicity-related genes, which are upregulated differently when the fungus grows on different artificial media or media containing insect hemolymph.

Conclusion

EPF efficacy depends on several factors including fungal strain and the species of tested insects. Successive subculture affects greatly on the fungal quality control parameters, as appeared with both ARSEF4556 and AUMC3262, which showed changes in morphological characters, conidial yields and radial growth rates, and stability of both fungal viability and Pr1 production, while V275 displayed high stability with all examined parameters after repeated subculture.

Table 2 Influence of successive subculture on the activity of Pr1 enzyme and the LT_{50} values of ARSEF4556, AUMC 3262, and V275 after 15 days growth on SDAY at $25 \pm 2^\circ\text{C}$

Subcultures	Pr1 ($\mu\text{mol ml}^{-1} \text{min}^{-1}$)			LT_{50}		
	ARSEF4556	AUMC 2362	V275	ARSEF 4556	AUMC3262	V275
1st	0.73 ± 0.015^a	0.68 ± 0.056^a	0.77 ± 0.025^a	2.27 ± 0.07^b	2.14 ± 0.04^b	1.69 ± 0.12^c
3rd	0.72 ± 0.013^a	0.73 ± 0.030^a	0.77 ± 0.016^a	2.34 ± 0.09^b	2.07 ± 0.02^{bc}	2.41 ± 0.02^a
5th	0.72 ± 0.014^a	0.71 ± 0.11^a	0.73 ± 0.036^a	2.21 ± 0.05^b	1.92 ± 0.03^c	2.05 ± 0.03^b
7th	0.73 ± 0.002^a	0.66 ± 0.10^a	0.79 ± 0.010^a	2.71 ± 0.01^a	2.21 ± 0.05^{ab}	2.05 ± 0.03^b
9th	0.73 ± 0.014^a	0.70 ± 0.021^a	0.74 ± 0.027^a	2.36 ± 0.02^b	2.34 ± 0.09^a	2.27 ± 0.07^{ab}

Each value represents the mean of three replicates. The means within a column followed by the same letter are not significantly different (LSD test, $P > 0.05$). LT_{50} median lethal time. Pr1 activity is expressed as $\mu\text{mol ml}^{-1} \text{min}^{-1}$ released from succinyl-ala-ala-pro-phe-p-nitroanilide

Abbreviations

AUMC: Assiut University Mycological Center; ARSEF: The Agricultural Research Service Collection of Entomopathogenic Fungal Cultures; IPM: Integrated pest management programs; Pr1: Protease enzyme; EPF: Entomopathogenic fungi; LT₅₀: Median lethal time

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Authors' contributions

TB suggested the aim of study and designed the experimental work. SE and AE designed the research and performed the statistical analysis. RH and JT conducted the study and wrote and revised the manuscript. All the authors read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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