EVALUATION OF ENTOMOPATHOGENIC ACTIVITY OF AN ENDOPHYTIC FUNGUS ISOLATED FROM POTATO

Mihaela Monica Dinu[1, 2,], Ana Cristina Fătu[1], Sorina Dinu[1], Narcisa Băbeanu[2]*

[1]Research and Development Institute for Plant Protection, Bucharest, Romania
[2]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Bucharest, Romania

* Correspondence address:
Research-Development Institute for Plant Protection
8 Ion Ionescu de la Brad, 013813, Bucharest, Romania
Phone: + 40 21269 32 31
Fax: + 40 21269 32 39
E-mail: mihaela.dinu@icdpp.ro

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Abstract: *This study aimed to evaluate the entomopathogenic activity of an endophytic fungal isolate from potato leaves for biological pest control. The isolate S1EnP2023, maintained in the RDIPP Bucharest Collection of entomopathogenic microorganisms, was identified as Beauveria bassiana (Bals. - Criv.) Vuill. Laboratory bioassays investigated its pathogenicity and virulence against the yellow mealworm (Tenebrio molitor, L). The influence of different conidial suspension concentrations on insect mortality was evaluated. The highest mortality of T. molitor was observed at a concentration of 10^8 conidia/ml. These results demonstrate the entomopathogenic potential of the B.bassiana. isolate and suggest 10^8 conidia/ml as a promising concentration for further investigation.*

Key words: *endophytic, entomopathogenic, Bassiana, potato, Tenebrio*

INTRODUCTION

Entomopathogenic fungi (EPF) belong to the order Hypocreales and Entomophthorales and are known for their ability to infect and kill insects. This characteristic has led to their extensive use as biocontrol agents in agriculture, offering a sustainable solution for managing insect pest populations. EPF also exhibit endophytic colonization of plants, establishing a symbiotic association.

Similar to how the human body harbors a diverse microbiota, with bacterial, archaeal, viral, and eukaryotic components inhabiting distinct anatomical niches, exceeding the number of our own somatic and germ cells by an order of magnitude (Turnbaugh et al., 2007, Eloe-Fadrosh & Rasko, 2013), plants also associate with endophytic microbial communities. An endophyte is a microorganism that establishes a non-pathogenic association with its host plant by colonizing internal tissues for a defined period during its life cycle, existing in a symbiotic relationship with their host. (Sharma & Singh, 2021).

Research has shown that endophytes can modulate a wide range of activities within their host plants, resulting in significant advantages for these colonized plants (Bamisile et al., 2021; Vega et al., 2008). However, the specific mechanisms underlying how these endophytic EPF suppress plant pathogens and enhance host resistance to disease remain poorly understood. Recent reports have shed light on their potential as biocontrol agents, but the in situ mechanisms employed by EPF within their plant hosts to control major plant diseases warrant further investigation (Sui et al., 2023).

Despite describing nearly 160,000 fungal species (http://www.speciesfungorum.org/; November 2023), a significant gap remains in our knowledge. Estimates suggest a potential range of 2.2 to 3.8 million species still waiting to be identified (Lücking et al., 2021), or as much as 11 million species (Phukhamsakda et al., 2022). Ubiquitous throughout diverse

ecosystems, some of these endophytes can exhibit biocontrol potential against plant pests and diseases. The endophytic community encompasses a broad range of taxa, including bacteria, archaea, fungi, and protists. However, research has primarily focused on bacterial and fungal endophytes due to their higher abundance and prevalence (Kumar & Dara, 2021). Characterized by narrow host range, entomopathogenic fungal endophytes selectively infect specific targets, minimizing adverse effects on non-target and beneficial arthropod populations (Bamisile et al., 2018).

Endophytic colonization of various plant species by *Beauveria bassiana* (Bals. Vuillemin) (Hypocreales: Cordycipitaceae) has been documented, demonstrating its ability to protect plants from pathogens (Quesada-Moraga et al., 2023).

This study presents the findings of insecticidal activity assessment of a fungus isolated from potato plants against the test insect *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), along with its identification using molecular biology techniques.

MATERIALS AND METHODS

Location and sampling

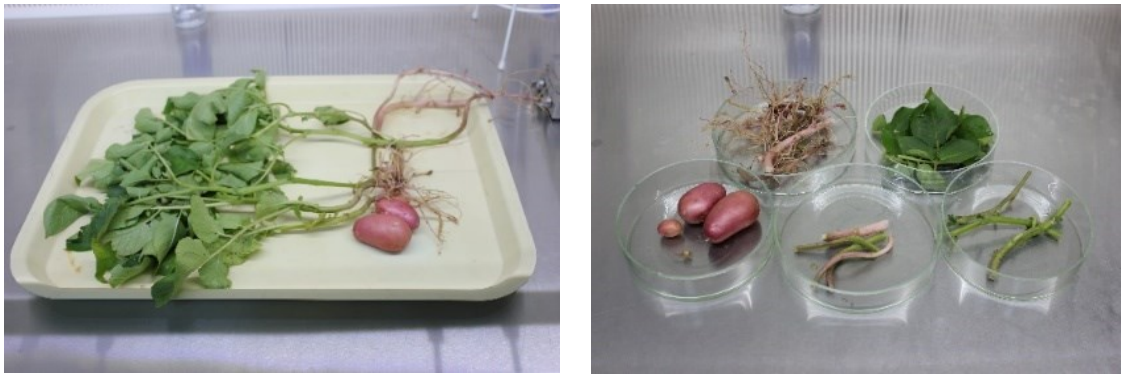
In July 2023, three healthy plants of *Solanum tuberosum* L. (Solanales: Solanaceae) were collected from an untreated field in northern Bucharest, Romania. These were not cultivated potatoes, but rather new plants that grew naturally from leftover tubers of the previous year's crop. The potato plants were transported to the laboratory in paper bags and processed for endophytic fungi within 2 hours of collection.

Surface sterilization and isolation

The isolation of endophytic fungi was carried out using a modified protocol based on the method of Arnold et al. (2001). Three leaves were collected from each plant: one from the top, one from the middle, and one from the base of the plant. Similarly, stem and roots sections were collected from the top, middle, and base. After washing under running tap water to remove soil debris, all samples were transferred to absorbent paper to dry. Once dry, the stem sections were cut into small pieces. The plant segments underwent a surface sterilization process. First, they were submerged in 70% ethanol for 1 minute, followed by 0.5 % sodium hypochlorite (NaOCl) for 1 minutes, 70% ethanol for 1 minute and then rinsed three times with sterile distilled water. Finally, they were dried on sterile filter paper.

This study focused on the detection of endophytic EPF within the selected potato plants, rather than conducting a comprehensive survey of the resident microbiota (Berg et al., 2020). Consequently, a selective medium was chosen for the cultivation of plant fragments (fig.1).

The plates were incubated at 22-24°C in darkness. Fungal growth emerging from the plant segments was monitored for three weeks.



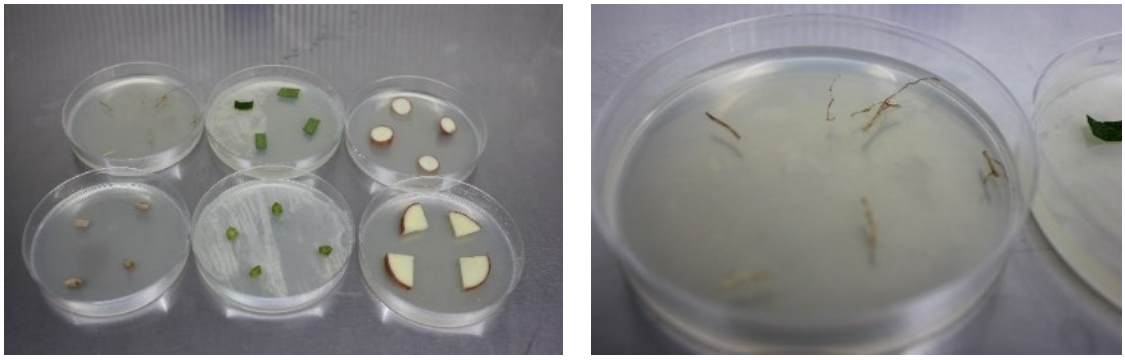


Figure 1. Preparing potato plants for endophytic fungal isolation

Identification of fungal isolate

Monosporal cultures of the fungal isolate were obtained. The initial identification of the isolate relied on a comparison of its morphological characteristics with those described by Barnett (1962). Confirmation of the initial morphological identification for the isolate was achieved through BIOLOG System identification and subsequent molecular characterization.

The Biolog FF MicroPlate assay involved incubating the fungal isolate on YMA agar for 14 days. After this, a cell suspension (plaque inoculum) was prepared by transferring the culture to inoculation media and then pipetting it onto the FF plate. To ensure optimal results, the cell density of was adjusted to an optical density of 75% using a turbidimeter. The inoculated plate was then incubated, and the colorimetric response was subsequently measured using a MicroStation Reader. The resulting data was analyzed with Biolog software (Bochner 1989ab; Garland & Mills, 1991; Strong-Gunderson et al., 1992; Bochner, 2003).

Molecular identification of the fungal isolate was achieved by sequencing the internal transcribed spacer (ITS) region (ITS1, 5.8S rRNA, and ITS2). Amplification of the marker sequence, the ITS region (ITS1, 5.8S rRNA gene, and ITS2), was performed using specific primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990).

The obtained sequences were edited, aligned, and compared to reference sequences from international databases (Altschul et al.,1997; Zhang et al., 2000; Tamura et al., 2021). Reference sequences included in the phylogenetic analysis of the *Beauveria* genus were obtained from the studies of Rehner & Buckley (2005) and Rehner et al. (2011).

Fungal isolate

Before use, the isolate was incubated for 12-16 days on PDA media in the dark at 25°C ± 2°C. Spores from well-sporulating colonies were harvested with a sterile loop for suspension preparation. This involved suspending the spores in a 0.02% Tween 80® water solution and homogenizing the mixture with a vortex mixer. Spores' concentration was determined using a Burker hemocytometer. Finally, the suspension was diluted with water to achieve the desired final concentration of spores/ml.

Insect rearing

Adults of *T.molitor* were reared in a controlled laboratory environment on sterilized wheat bran and were all hatched on the same day to ensure age uniformity (fig.2).

Treatment

To confirm the pathogenicity and to evaluate the virulence of the isolate, laboratory bioassays were carried out using adult beetles were immersed in conidial suspensions adjusted to a titer of 1 x 10^7, 1 x 10^8, 1 x 10^9, conidia spores/ml + 0.1% Tween 80. In the control group, insects were dipped in sterile distilled water + 0.1% Tween 80. For each treatment, three replicates of 20 insects each were set up. The insects were housed individually in plastic boxes (Fig.2). These were maintained in an incubator at 25 ± 2 °C, 16:8 L:D, 50-60% RH for 14 days after treatment application.

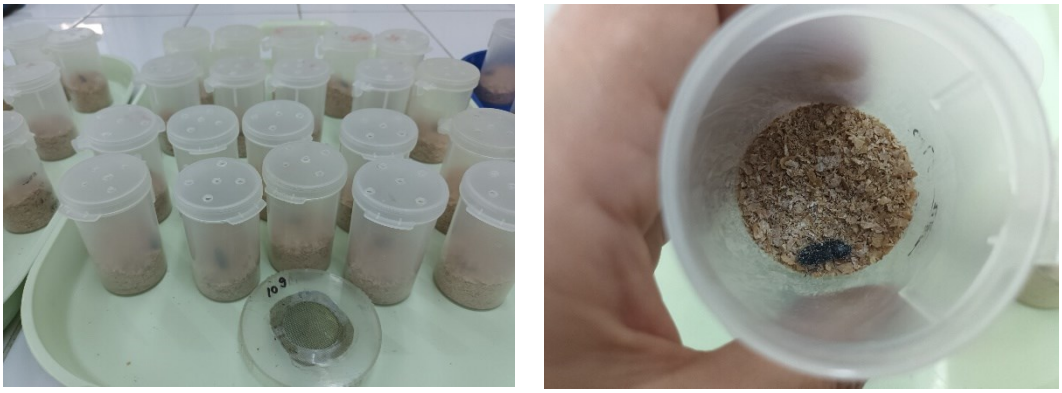


Figure 2. Individual housing of *T. molitor* adults in plastic containers

Dead insects were collected daily (fig.3) and transferred to wet chambers to monitor for fungal growth. After 14 days, the number of insects exhibiting signs of white muscardine infection was counted to evaluate the efficacy of the tested concentrations (fig.4).

Figure 3. Transfer of a dead *T. molitor* beetle to a wet chamber.

Figure 4. The sporulated (green arrow) vs. unsporulated (red arrow) fungus on *T. molitor* adults

Statistical analysis

Kaplan-Meier survival curves were employed to evaluate the effect of conidial suspension concentration on insect survival. Pairwise comparisons were conducted to determine significant differences between treatment groups, using Log-Rank (Mantel-Cox). All statistical analyses were carried out using GraphPad Prism V.7 software for Windows.

RESULTS AND DISCUSSIONS

Isolation and identification of fungal isolate

The entomopathogen was isolated from potato leaves. Based on morphological characteristics, both macro and microscopic, the isolate was identified as belonging to the genus *Beauveria* Vuill. (Hypocreales: Cordycipitaceae) and was deposited in the Collection of Entomopathogenic Fungi of the Research-Development Institute for Plant Protection (ICDPP) with code S1EnP2023.

Biolog FF MicroPlate

The isolate S1EnP2023 was identified as *B. bassiana*, at 168 hours, with a high probability (1) and a similarity higher than 0.5 (0.670).

*Molecular identification*The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1978). The optimal tree is shown (fig.5). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches (Felsenstein, 1985). The evolutionary distances were computed using the p-distance method (Nei & Kumar, 2000) and are in the units of the number of base differences per site. This analysis involved 38 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 525 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

Strains AY532050 and AY531997 cluster with the *B. bassiana* type strain from Clade A (Ghikas et al., 2010), confirming the identity of strain S1EnP2023 as belonging to the B. bassiana species. Internal transcribed spacer (ITS) analysis of the isolate revealed a 99.4% identity to *B. bassiana* ARSEF 1564, which is considered a type strain. This is supported by the isolate's origin from a region in northern Italy and a lepidopteran host phylogenetically related to silkworms, on which *B. bassiana* was initially observed (Rehner et al., 2011).

Bioassay

The pathogenicity of the *B. bassiana* isolate S1EnP2023 was confirmed in bioassays against *T. molitor.* A sporulation of the fungus was observed on the bodies of nearly all dead insects beginning on the third day post-mortem. Only individuals displaying infection signs were taken into consideration for assessing the efficacy of treatments (fig. 6). Conidial concentration significantly impacted *T. molitor* adult mortality. By day 14 post-treatment, the control group exhibited a cumulative mortality of 15%. Treatments with varying conidial suspension concentrations resulted in a range of cumulative mortality at the experiment's end, from 50% (at $1x10^7$ spores/mL) to complete mortality (100%) observed at the concentration of $1x10^8$ spores/mL.

Figure 6. Fungal infection (mycosis) progression in *T. molitor* adults

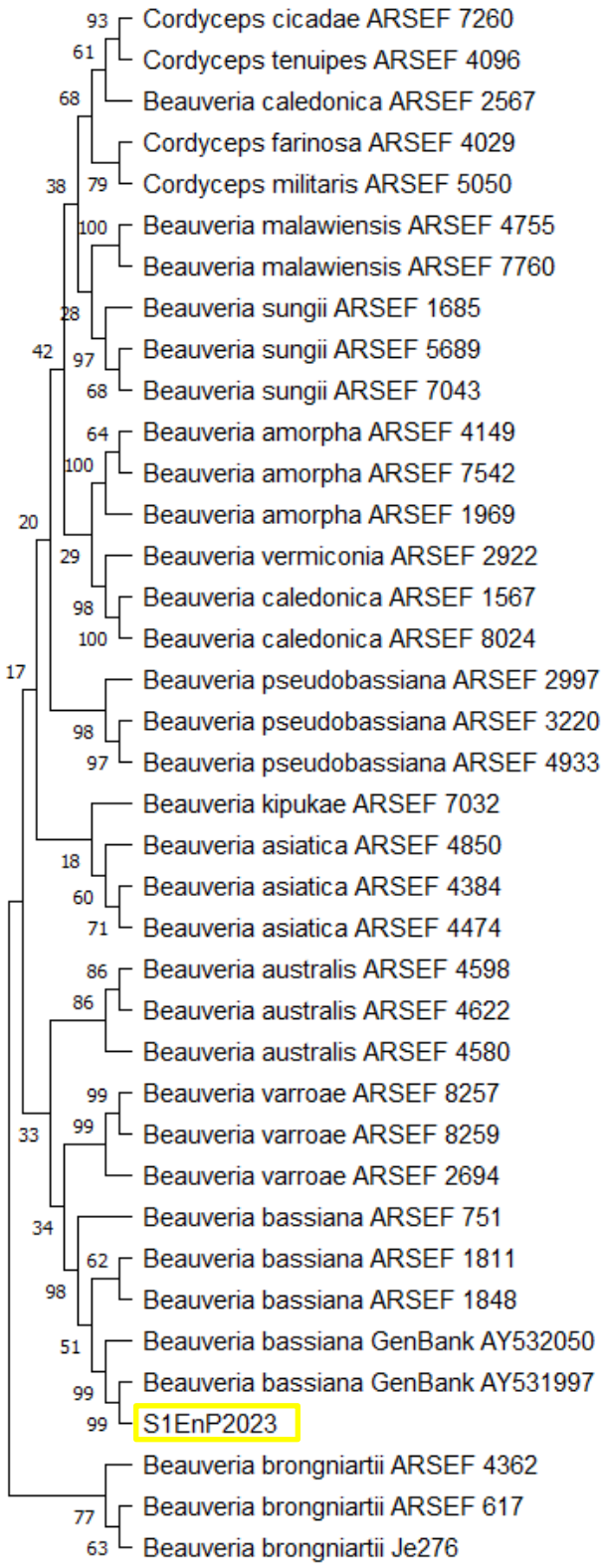


Figure 5. Overall branching pattern of the phylogenetic tree

constructed for isolate S1EnP2023

Kaplan-Meier survival curves show that all spores treatments have a significant effect on the mortality of *T. molitor* adults, reducing survival compared to the control. Concentrations of $1x10^8$ spores/ml and $1x10^9$ spores/ml had the greatest effects (fig. 7).

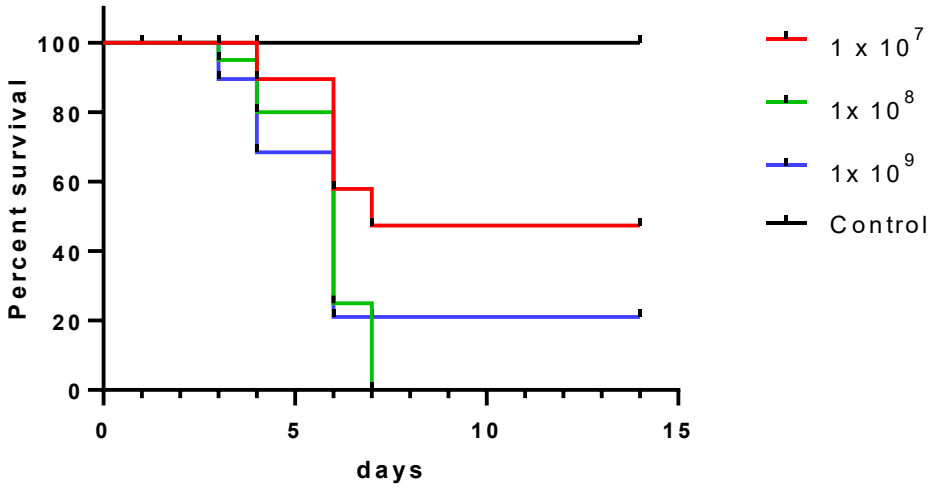


Figure 7. Kaplan-Meier survival curves of *T. molitor* adults treated with conidial suspensions of different concentrations ($1x10^7$ spores/ml, $1x10^8$ spores/ml and $1x10^9$ spores/ml)

The difference in survival between the $1x10^8$spores/ml and $1x10^9$spores/ml concentration of conidial suspensions was not significant, suggesting a threshold effect were increasing the spore's concentration above $1x10^8$ spores/ml does not significantly increase mortality, reaching a plateau. This confirms subsequent statistical results showing no significant difference between these two concentrations (Table 1).

Table 1. Pairwise treatment comparisons using Log-Rank (Mantel-Cox) test based on Kaplan Meyer survival analysis of *T. molitor* adults

Spores concentration	1 x 10^7		1 x 10^8		1 x 10^9		Control	
	X^2	P	X^2	P	X^2	P	X^2	P
1 x 10^7	-	-						
1 x 10^8	9.832	0.0017	-	-				
1 x 10^9	4.285	0.0384	0.209	0.6476	-	-		
Control	12.13	0.0005	36.95	<0.0001	22.05	<0.0001	-	-

Lethal effects were first observed on the second day. Median survival times indicate that treatments with $1x10^8$ spores/ml and $1x10^9$spores/ml increase mortality, reducing MST to 6 days, while $1x10^7$ spores/ml sporess increase mortality to 7 days compared to the control group where survival was high (Table 2).

Table 2. Median survival time (MST) of the *T. molitor* adults treated with aqueous conidial suspensions of different concentrations after 15 days

Isolate	MST (days)
1 x 10^7	7
1 x 10^8	6
1 x 10^9	6
Control	Undefined

During the laboratory bioassays, it was observed that insect mortality was closely related to the conidial suspension concentration, with the highest mortality recorded at a concentration of 10^8 spores/ml. This observation is consistent with other studies that have reported a positive correlation between conidial density and entomopathogenic efficacy. For example, Ozdemir et al. (2020) compared the efficacy of two concentrations (1 x 10^6 and 1 x 10^8 conidia/ml) of *B. bassiana* against adult of *Callosobruchus maculatus*, and the results indicated that a concentration of 1 x 10^8 spores/ml was much more effective, producing nearly 100% mortality within 8 days at 26°C. Additionally, Deca et al. (2021) tested different concentrations of conidial suspensions of the BKN20 isolate of *B. bassiana* against *Helopeltis theivora*. Higher concentrations demonstrated increased pathogenicity, underscoring the importance of conidial density for entomopathogenic efficacy.

CONCLUSIONS

This study presents the first documentation of entomopathogenic fungus *B. bassiana* as an endophyte colonizing potato plants in Romania.

The pathogenicity and virulence of an entomopathogenic strain of *B. bassiana*, isolated as an endophyte from potato plants, were evaluated against the coleopteran *T. molitor*, a storage pest. The results reveal a dose-dependent relationship between fungal concentration and insect survival. This means that insect mortality increased steadily as the concentration of the fungus increased, up to a concentration of 1x10^8 spores/ml. Beyond this point, the mortality rate did not increase. Further studies are warranted to validate its efficacy against target insect pests under field conditions.

REFERENCES

ALTSCHUL, S.F., MADDEN, T.L., SCHÄFFER, A.A., ZHANG, J., ZHANG, Z., MILLER, W., LIPMAN, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* Sep 1;25(17):3389-402. doi: 10.1093/nar/25.17.3389. PMID: 9254694; PMCID: PMC146917.

ARNOLD A.E., MAYNARD, Z., GILBERT, G.S. (2001). Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity* *Paper presented at the Asian Mycological Congress 2000 (AMC 2000), incorporating the 2nd Asia-Pacific Mycological Congress on Biodiversity and Biotechnology, and held at the University of Hong Kong on 9-13 July 2000. *Mycological Research*, Volume 105, Issue 12, 2001, Pages 1502-1507, ISSN 0953-7562, https://doi.org/10.1017/S0953756201004956.

BAMISILE, B.S., DASH, C.K., AKUTSE, K.S., KEPPANAN, R., WANG, L. (2018). Fungal Endophytes: Beyond Herbivore Management. *Frontiers in microbiology*, 9, 544. https://doi.org/10.3389/fmicb.2018.00544.

BAMISILE, B. S., SIDDIQUI, J. A., AKUTSE, K. S., RAMOS AGUILA, L. C., XU, Y. (2021). General Limitations to Endophytic Entomopathogenic Fungi Use as Plant Growth Promoters, Pests and Pathogens Biocontrol Agents. *Plants (Basel, Switzerland)*, 10 (10), 2119. https://doi.org/10.3390/plants10102119.

BARNETT, H.L. (1962). Moniliales. In *Illustrated Genera of Imperfect Fungi* (2 nd. ed), Burgess Publishing Co., Minneapolis, 56-71.

BERG, G., RYBAKOVA D., FISCHER, D., CERNAVA, T., CHAMPOMIER VERGÈS, M.C., CHARLES, T., XIAOYULONG C., et al. (2020). "Microbiome Definition Re-Visited: Old Concepts and New Challenges." *Microbiome* 8: 103. https://doi.org/10.1186/s40168-020-00875-0

BOCHNER, B.R. (1989a). Sleuthing out bacterial identities. *Nature*, 339, 157–158.

BOCHNER, B.R. (2003). New technologies to assess genotype – phenotype relationships. *Nature Reviews Genetics*, 4, 309–314.
BOCHNER, B.R. (1989b). "Breathprints" at the Microbial Level. *American Society for Microbiology News* 55:536-539.
DEKA, B., BABU, A., PETER, A.J. et al. (2021). Beauveria bassiana: as a potential microbial biocontrol agent for tea mosquito bug, Helopeltis theivora Waterhouse (Hemiptera: Miridae) in Dooars and Darjeeling, India. Egypt J Biol Pest Control 31, 134. https://doi.org/10.1186/s41938-021-00478-3
ELOE-FADROSH, E.A., RASKO, D.A. (2013). The human microbiome: from symbiosis to pathogenesis. *Annual Review of Medicine,* Volume 64 :145-63. doi: 10.1146/annurev-med-010312-133513. PMID: 23327521; PMCID: PMC3731629.
FELSENSTEIN J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
GARLAND, J.L., MILLS, A.L. (1991). Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology Journal*, 57:2351-2359
GHIKAS, D.V., KOUVELIS, V.N., TYPAS, M.A. (2010). Phylogenetic and biogeographic implications inferred by mitochondrial intergenic region analyses and ITS1-5.8S-ITS2 of the entomopathogenic fungi *Beauveria bassiana* and B. *brongniartii*. *BMC Microbiol* 10, 174 (2010). https://doi.org/10.1186/1471-2180-10-174.
KUMAR, K.K., DARA, S.K. (2021). Fungal and Bacterial Endophytes as Microbial Control Agents for Plant-Parasitic Nematodes. *International journal of environmental research and public health*, 18(8), 4269. https://doi.org/10.3390/ijerph18084269
LÜCKING, R., AIME, M.C., ROBBERTSE, B., MILLER, A.N., AOKI, T., ARIYAWANSA, H.A., CARDINALI, G., CROUS, P.W., DRUZHININA, I.S., GEISER, D.M., HAWKSWORTH, D.L., HYDE, K.D., IRINYI, L., JEEWON, R., JOHNSTON, P.R., KIRK, P.M., MALOSSO, E., MAY, T.W., MEYER, W., NILSSON, H.R., SCHOCH, C.L. (2021). Fungal taxonomy and sequence-based nomenclature. *Nature microbiology*, 6(5), 540–548. https://doi.org/10.1038/s41564-021-00888-x
NEI M., KUMAR S. (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.
OZDEMIR, I.O., TUNCER, C., ERPER, I. et al. (2020). Efficacy of the entomopathogenic fungi; Beauveria bassiana and Metarhizium anisopliae against the cowpea weevil, Callosobruchus maculatus F. (Coleoptera: Chrysomelidae: Bruchinae). Egyptian Journal of Biological Pest Control 30, 24. https://doi.org/10.1186/s41938-020-00219-y.
PHUKHAMSAKDA, C., NILSSON, R.H., BHUNJUN, C.S. et al. (2022). The numbers of fungi: contributions from traditional taxonomic studies and challenges of metabarcoding. *Fungal Diversity* 114, 327–386 (2022). https://doi.org/10.1007/s13225-022-00502-3
QUESADA-MORAGA, E., GARRIDO-JURADO, I., GONZÁLEZ-MAS, N., YOUSEF-YOUSEF, M. (2023). Ecosystem services of entomopathogenic ascomycetes. *Journal of Invertebrate Pathology*, 201, 108015. https://doi.org/10.1016/j.jip.2023.108015
REHNER, S. A., BUCKLEY, E. (2005). A *Beauveria* phylogeny inferred from nuclear ITS and EF1-sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia*, 97(1), 84–98. doi:10.3852/mycologia.97.1.84
REHNER, S. A., MINNIS, A. M., SUNG, G. H., LUANGSA-ARD, J. J., DEVOTTO, L., HUMBER, R. A. (2011). Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. *Mycologia*, 103(5), 1055–1073. https://doi.org/10.3852/10-302
SAITOU, N., NEI, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4:406-425.
SHARMA, P., SINGH, S.P. (2021). Chapter 11 - Role of the endogenous fungal metabolites in the plant growth improvement and stress tolerance, Editor(s): Vijay Kumar Sharma, Maulin P. Shah, Shobhika Parmar, Ajay Kumar, *Fungi Bio-Prospects in Sustainable Agriculture, Environment and Nano-technology,* Academic Press, Pages 381-401, ISBN 9780128217344, https://doi.org/10.1016/B978-0-12-821734-4.00002-2.

STRONG-GUNDERSON, J. M., PALUMBO, A. V., SCARBOROUGH, S. O. (1992). New Method for Rapidly Determining Utilization of Volatile Contaminants. *Abstracts of the 92nd Meeting of the American Society for Microbiology*, 371.

SUI, L., LU, Y., ZHOU, L., LI, N., LI, Q., ZHANG, Z. (2023) Endophytic *Beauveria bassiana* promotes plant biomass growth and suppresses pathogen damage by directional recruitment. *Frontiers in Microbiology*, 14:1227269. doi: 10.3389/fmicb.2023.1227269

TAMURA K., STECHER G., KUMAR S. (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution* https://doi.org/10.1093/molbev/msab120.

TURNBAUGH, P.J., LEY, R.E., HAMADY, M., FRASER-LIGGETT, C.M., KNIGHT, R. GORDON, J.I. (2007). The human microbiome project. *Nature*, 449(7164), 804–810. https://doi.org/10.1038/nature06244.

VEGA, F. E, POSADA, F., AIME, M. C., PAVA-RIPOLL, M., INFANTE, F., REHNER, S.A. (2008). Entomopathogenic fungal endophytes, *Biological Control*, Volume 46, Issue 1, Pages 72-82, ISSN 1049-9644, https://doi.org/10.1016/j.biocontrol.2008.01.008.

WHITE, T. J., BRUNS, T., LEE, S., TAYLOR, J., (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*, pp. 315–322. Edited by N. Innis, D. Gelfand, J. Sninsky & T. White. London: Academic Press.

ZHANG, Z., SCHWARTZ, S., WAGNER, L. MILLER, W. (2000). *A* greedy algorithm for aligning DNA sequences, *Journal of Computational Biology*; 7(1-2):203-14.