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Isolation, Identification and Evaluation of Media for *Metarhizium anisopliae* Growth and Sporulation

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ABSTRACT : The entomopathogenic fungal species *Metarhizium anisopliae* is widely distributed in agricultural soil and it is popular for their used as a biocontrol agent against many insect pests. *Metarhizium anisopliae* have been studied widely in agriculture sector. Now a day, the demand of *Metarhizium anisopliae* is produced by its valuable biomass used against many insects. However temperature is the key factor which limits their effective use. In present study six strains of *Metarhizium anisopliae* isolated from different vegetable field from Nashik region of Maharashtra state of India, identified on the basis of morphological characters. *Metarhizium anisopliae* produce dark herbage green and olivaceous colonies with white mycelial margin and then tested for growth in Potato Dextrose Broth (PDB) and Sabouraud's Dextrose Broth (SDB) in terms of development of conidia. Then isolates were tested for their temperature tolerance. The result revealed that all isolates were faster grown in PDB than SDB media. Isolate PCPL-Ma-13 showed maximum number of conidia formation in PDB medium at 30ºC.The production media of *Metarhizium anisopliae* and the effect of temperature on *Metarhizium anisopliae* is discussed.

KEYWORDS: Biocontrol agent, Insecticide, *Metarhizium anisopliae*, PDB, Pesticide

I. INTRODUCTION

The population has been increasing rapidly, due to highly developed maternity and healthcare factors. Due to this the number of challenges also expanded around the global sustainability, including the demand for more food. As an essential resource, the supply of food is major concern. It is depend on growers, supplier and distributors to bring it to market. According to Statista report, the global vegetable production was approximately 816.8 million metric tons in 2019 and India produced approximately 189 million metric tons. Vegetable farming business is profitable business. On the other hand, vegetable damages from pests and disease cause significant losses for producers. To control the pest high load of chemical pesticides used to kill the pests and insects which can attack on crops and harm them. Chemical pesticides benefit the crops but they have affected the environment. Excessive use of pesticides may lead to the destruction of biodiversity, aquatic organisms, animals, birds and natural enemies of agricultural pests [1], [2], [3], [4]. In current scenario, for the sustainable vegetable production cost effectively and ecofriendly pesticide management is necessary due to the side effect of chemical pesticides. To overcome this situation we need to produce biopesticides or biological control agent using beneficial antagonist is environmentally sustainable alternative to chemical pesticides [5]. Biopesticides have several advantages viz. safety, friendly to non-target species, targeted activity to the desired pests, cost effective, effective in lower quantities hence they offering lower exposure and they decompose quickly, largely avoiding the pollution problems. Several microorganisms viz., bacteria [6], [7], [8], [9], [10], [11]; fungi [13], [13], [14], [15], [16]; virus [17], [18], [19], [20]; protozoan [21], [22], [23], [24], [25], [26] and nematodes [27], [28], [29], [30] have been systematic studied for their effectively beneficial characters as a biocontrol agents.

Among the studied microorganisms entomopathogenic fungi have considerable scope as plant protection agent against insects [31]. Last few decades, biocontrol potential entomopathogenic fungi are use as biological control agents for insects. Among these fungi *Metarhizium anisopliae* have been most intensively investigated as insecticide in the crop pest control [32]. *Metarhizium anisopliae* being facultative fungal parasites, which grow on non-living media, can be mass produced (solid state), can infect and grow on insect host. It is control more than 200 species of insects belonging to orders Homoptera, Lepidopter, Coleoptera, Orthoptera and Dermoptera [33]. For this reason our research interest in expansion and application of biopesticides for commercial production and sustainable utilization of the biopesticides. *Metarhizium anisopliae* has a potential for development into biopesticides and occurs in soils of different agro ecosystem.

The present study undertaken to isolate and identify the entomopathogenic *Metarhizium anisopliae* from different vegetable field of Nashik region of Maharashtra state of India, furthermore check growth media for mycelial and sporulation production of isolates then to evaluate their temperature tolerance for conidial yield.

II. MATERIALS AND METHODS

Collection of soil sample: Soil samples were collected from different villages of Nashik district, Maharashtra, India. This covered major area of vegetable crops shown in Table 1.Total 58 soil samples each containing 100 g in depth 0-20 cm were taken using cylindrical sampler and stored in sterile polythene bags from vegetables field sealed with a rubber band followed the method of [34] and brought to the R&D laboratory, Poorva Chemtech Pvt. Ltd. Pimpalgaon (B), Tal- Niphad, Dist- Nashik (MS) India. The samples passed through a sieve 2mm mesh and stored at 4ºC until culture as described by [13], [35].

Table 1: Collection of soil sample for the study

Sl. No.	Vegetables field	Name of the villages	Collected soil samples	Taluka
1	Tomato	Pimpalgaon	3	Niphad
		Mukhed	2	
		Vadner Bhairav	2	
2	Cabbage	Meshi	4	Deola
		Bahur	4	
		Vithevadi	6	
3	Cauliflower	Malwadi	5	Deola
		Lohner	3	
4	Chilli	Kalwan	4	Kalwan
		Manur	6	
		Bej	3	
5	Cucumber	Bramhangaoan	5	Satana
		Arai	3	
6	Brinjal	Khakurdi	4	Malegaon
		Dahiwad	4	

Isolation of *Metarhizium anisoliae*: The isolation of *Metarhizium anisoliae* from soil was carried out using selective agar media. Selective agar media was prepared by using Potato Dextrose Agar (PDA) (composition g/L: Infusion from potatoes – 200, Dextrose – 20, Agar – 15) for normally occur in the soil, as described by [36] with a serial dilution method. 10 g of soil sample from each site was added to 250 ml Erlenmeyer flask containing 90 ml of sterile distilled water and placed on rotary shaker (Omkar instruments) for 15 min for proper mixing and diluted to (10^{-3} and 10^{-4}). Volume of 100 µl of each dilutions were then spread on culture petri dishes S-line (90 x 15 mm, Borosil) containing PDA media and incubated at 25 ± 1ºC in BOD incubator (REMI) for seven days. For pure culture single colonies were transferred and sub cultured on PDA medium and incubated at 25 ± 1ºC for seven days.

Identification of *Metarhizium anisoliae*: During seven days incubation period Petri dishes were evaluated daily for the existence of colonies of *Metarhizium anisoliae*. Identifications were made by observing characteristics under microscope (Olympus CX 21i) at 100X magnification. Colonies were identified by morphological characteristics as describe by [37], [38]. The characteristics such as colour, shape, surface, edge, consistency and elevation and hyphae arrangement, conidia and conidiophores were considered for identification.

Evaluation of growth media: For the growth and sporulation of *Metarhizium anisopliae* Potato Dextrose Broth (PDB) and Sabouraud's Dextrose Broth (SDB) these two media were used. PDB and SDB were prepared by using broth powder of Hi Media. 100 ml of each media was dispensed in to 250 ml Erlenmeyer flasks and autoclaved at 15 psi pressure for 30 min. Three flask of each medium was inoculated with 1 ml of spore suspension of culture separately and incubated at 25 ± 1ºC in BOD incubator for 7 – 10 day. Counting of spore was made by serial dilution of the suspension using Neubauer haemocytometer.

Determination of optimal temperature: To quantify the effects of temperature on the growth of isolated strains were analyzed in PDB and SDB media at various temperatures of 20, 25, 30 and 35 ºC by following the method by [39]. Growth measured after 7 day incubation period. Spore concentration was determined by using Neubauer haemocytometer [40].

III. RESULTS

Strain isolation: *Metarhizium* spp. occurred naturally in the agricultural soil. Total 18 filamentous fungal isolates obtained from 15 sample collected sites on different Petri dishes. The isolate from each Petri dish was considered as a strain. All these strains were transferred to PDA agar Petri dish and strains were purified for further identification.

Strain identification: As a result, six isolates were identified as *Metarhizium anisopliae* by using morphological and colony characteristics. The origin and place of the isolates are shown in Table 2. The strain of *Metarhizium anisopliae* produced dark herbage green, olivaceous colonies after 7 days of incubation when it grown on PDA agar petridish. The strain produces a white mycelial margin with clumps (Fig. 1 A and B); colonies were highly branched with cylindrical conidiophores closely packed. These conidiophores become colored with development of the spores. Conidial chains were round and columnar phialides in a dense parallel arrangement and conidia were cylindrical to oval were seen (Fig. 2 A and B) when observed under microscope.

Table 2: Origin of isolated *Metarhizium* species

Sr. No.	*Code No.*	*Organism*	*Origin of isolate (vegetable field)*	*Place Name of village*	*State*
1	PCPL-Ma-07	*Metarhizium anisopliae*	Tomato	Vadner Bhairav, Niphad	Maharashtra
2	PCPL-Ma-13	*Metarhizium anisopliae*	Cabbage	Bahur, Deola	Maharashtra
3	PCPL-Ma-29	*Metarhizium anisopliae*	Cauliflower	Loner, Deola	Maharashtra
4	PCPL-Ma-35	*Metarhizium anisopliae*	Chilli	Manur, Kalwan	Maharashtra
5	PCPL-Ma-48	*Metarhizium anisopliae*	Cucumber	Arai, Satana	Maharashtra
6	PCPL-Ma-52	*Metarhizium anisopliae*	Brinjal	Khakurdi, Malegaon	Maharashtra

***Metarhizium anisopliae* growth:** The success production of biocontrol agent depends on screening, isolation and characterization in the laboratory. To find out the appropriate media for naturally available *Metarhizium anisopliae* growth and sporulation two liquid media PDB and SDB were tested in R & D laboratory. In this study, it was seen that *Metarhizium anisopliae* isolated strains had well grown in both PDB media and SDB media. *Metarhizium anisopliae* formed olivegreen mycelial mat on the surface of both medium. The results proved that PDB was the optimal culture medium for colony growth and conidia yield for *Metarhizium anisopliae.* Significant difference in conidial count was observed on tested medium after seven days on isolated strain growth shown in Table 3. The highest conidial count was observed of PCPL-Ma-13 in Potato Dextrose Broth (PDB) media (24.8×10^5) followed by Sabouraud's Dextrose Broth media (86.7×10^4).

Determination of optimal temperature: Results for the growth of isolated colonies of *Metarhizium anisopliae* in PDB media with all test temperatures are presented in Fig 3. The isolate PCPL-Ma-13 showed maximum mycelial growth at 30°C. The number of conidia found to be 46.92×10^5 after seven day. Minimum mycelial growth was observed at 20°C of isolate PCPL-Ma-52 and at 35°C of isolate PCPL-Ma-07. The minimum number of conidia found to be (6.08×10^5) and (2.08×10^5) respectively. Also results on the growth of isolated colonies of *Metarhizium anisopliae* in SDB media with all test temperatures are presented in Fig 4. The isolate PCPL-Ma-13 showed maximum mycelial growth at 30°C. The number of conidia found to be 84.63×10^4 after seven day. Minimum mycelial growth was observed at 20 and 35°C of isolate PCPL-Ma-07. The minimum number of conidia found to be (14.66×10^4) and (3.29×10^4) respectively.

Figure 1**.** A) Seven days-old culture of *Metarhizium anisopliae* on PDA Petri dish B) Mycelia

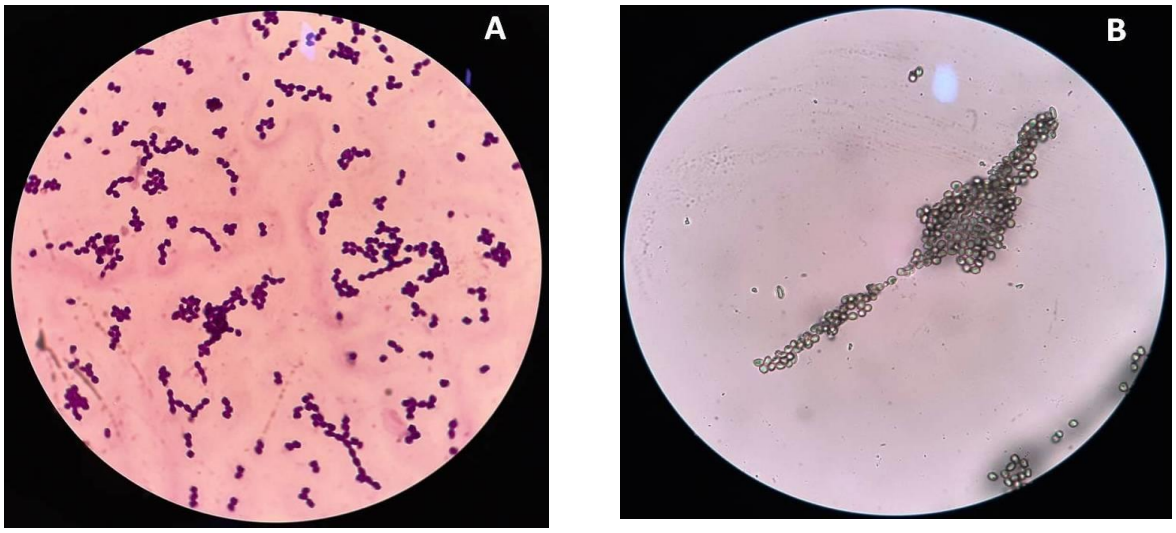

Figure 2. A) *Metarhizium anisopliae* Conidia, phialides and B) spore

Table 3: Conidial count of isolated *Metarhizium anisopliae* strain with PDB and SDB media.

Sr. No.	*Metarhizium anisopliae isolates*	*Growth Media*	
		Potato Dextrose Broth (PDB)	*Sabouraud's Dextrose Broth (SDB)*
1	PCPL-Ma-07	08.60×10^5	33.3×10^4
2	PCPL-Ma-13	24.8×10^5	86.7×10^4
3	PCPL-Ma-29	10.2×10^5	64.5×10^4
4	PCPL-Ma-35	16.3×10^5	52.8×10^4
5	PCPL-Ma-48	16.25×10^5	74.2×10^4
6	PCPL-Ma-52	09.70×10^5	38.3×10^4

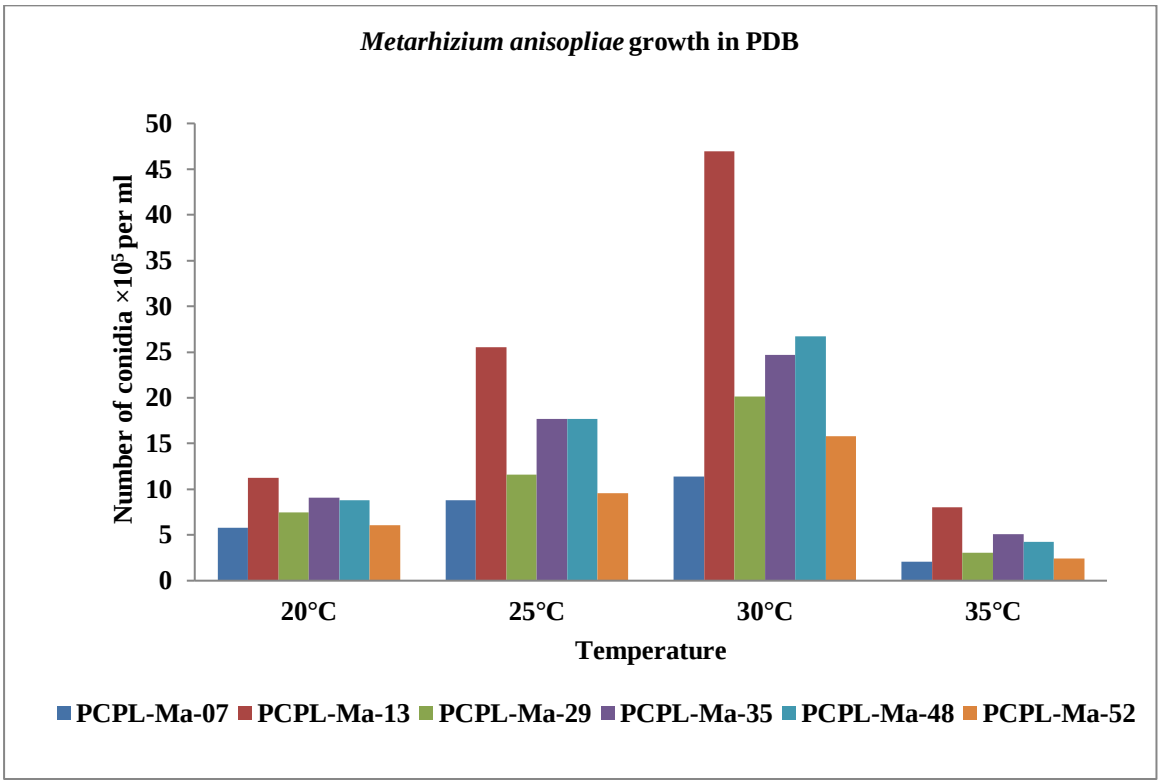

Figure 3. Effect of different temperatures on growth of isolated strains *Metarhizium anisopliae* in PDB media

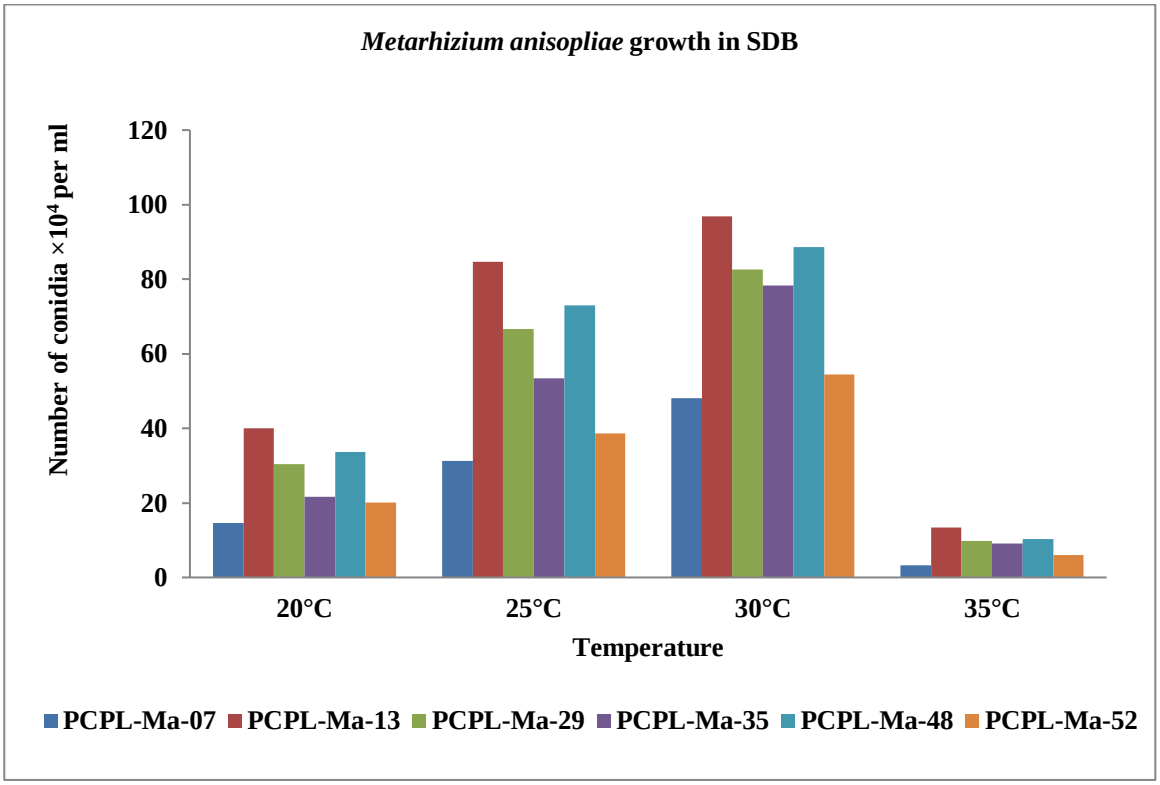

Figure 4. Effect of different temperatures on growth of isolated strains *Metarhizium anisopliae* in SDB media

IV. DISCUSSION

Selection of these isolates is the first priority to commercialize as biological control agent. For this purpose we isolated, identified and evaluated the strains biological control agents in our R&D laboratory. *Metarhizium anisopliae* are ubiquitous in microbial community [41]. They are closely associated with agricultural crops and maintained outer rhizosphere level in the vegetable field. Finding from the present research work showed that *Metarhizium anisopliae* occurred naturally in all collected samples sites. It may be due to the producers using this fungal as biocontrol agent in their field or due to climatic conditions of this region. In the present study we obtained six isolates of *Metarhizium anisopliae* from different vegetable field from Nashik District, Maharashtra, India as mentioned in Table 1.

This is the first study report that, strains of *Metarhizium anisopliae* were isolated from vegetable field from Nashik area as mentioned in Table 2. Based on the observations the isolated strains morphology, size and shape of mycelia, conidia the isolated strains were identified as *Metarhizium anisopliae*. On PDA Petri dish colonies of this fungus well grow. After seven day white mycelial margin was observed outer side of dark green color. Fig. 1A. This is also observed by [35], [42], [43] and morphological characters are similar to described by [44], [45], [46], [47], [48], [49].

To evaluate isolated strains of *Metarhizium anisopliae* cultivation the suitable media is needed to determine for the growth. In present study for growth and sporulation of isolates two liquid media PDB and SDB were tested. We found that all six isolates of *Metarhizium anisopliae* were grown and developed spore after 7 day incubation from application. Microscopic observations were the evidence. This is in agreement to reports of [33], [43], [50], [51], [52], [53], [54] who reported the *Metarhizium anisopliae* growth in Potato Dextrose Agar and Potato Dextrose Broth and Sabouraud's Dextrose Agar and Sabouraud's Dextrose Broth. We found that in PDB media all isolates growth was faster than SDB Table 3. The isolate PCPL-Ma-13 have shown maximum number of conidia 24.8×10^5 in PDB media than in SDB media 86.7×10^4.

All isolates of *Metarhizium anisopliae* showed growth at all tested temperature. Highest mycelial growth was obtained of all isolate at 30°C in PDB media Fig. 3 and the number of conidia found maximum 46.92×10^5 in isolate PCPL-Ma-13. When all isolates grown in SDB media, PCPL-Ma-13 shown maximum growth at 30°C and the conidia number found to be 84.63×10^4 Fig 4. After 7 days of incubation. Similar growth was observed in the study conducted by [36], [55], [56], [57], [58], [59], [60].

V. CONCLUSION

Our findings of isolated *Metarhizium anisopliae* strains from vegetable field, Nashik region increase the possibility for developing a commercial biopesticides. The strategy could reduce the chemical pesticides. From our finding it was clear that the *Metarhizium anisopliae* strains is able to grow on solid and liquid medium which can be useful to farmers for culture these strains easily. Our work may be providing an overview for development of an effective biocontrol agent for ecofriendly control system and sustainable development.

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REFERENCES

[1] M. W. Aktar, D. Sengupta and A. Chowdhury, Impact of pesticides in agriculture: their benefits and hazards. *Interdiscip Toxicol*, 2 (1), 2009, 1-12.

[2] C. A. Damalas and I. G. Eleftherohorinos, Pesticide exposure, safety issues and risk assessment indicators. *Int J Environ Res Public Health*, 8(5), 2011, 1402-1419.

[3] I. Mahmood, S. R. Imadi, K. Shazadi, A. Gul, and K. R. Hakeem, Effects of Pesticides on Environment. In, K. Hakeem, M. Akhtar, S. Abdullah (Ed.), Plant, Soil and Microbes. (Switzerland: Springer, Cham, 2016) 253-269.

[4] K. Gyawali, Pesticide Uses and its Effects on Public Health and Environment, Journal *of Heath Promotion*, 6, 2018, 28-36.

[5] P. V. Pandav, R. S. Ahire, S. R. Patil and S. B. Pawar, Process Development for High Density Cultivation Yield for *Bacillus subtilis*, *International Journal of Recent Advances in Multidisciplinary Topics*, 2(7), 2021, 335-339.

[6] A. K. Saxena, K. K. Pal, and K. V. B. R. Tilak, Bacterial Biocontrol Agents and their Role in Plant Disease Management. In, R. K. Upadhyay, K. G. Mukerji, B. P. Chamola (Ed.), Biocontrol Potential and its Exploitation in Sustainable Agriculture. (Baston, MA: Springer, 2000) 25-37.
[7] J. M. Whipps, Microbial interactions and biocontrol in the rhizosphere, *Journal of Experimental Botany*, 52(1), 2001, 487-511.
[8] S. Compant, B. Duffy, J. Nowak, C. Clement and E. A. Barka, Use of plant-growth promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl Environ Microbio*l 71(9), 2005, 4951-4959.
[9] M. S. Mota, C. B. Gomes, I. T. Souza and A. B. Moura, Bacterial selection for biological control of plant disease: criterion determination and validation. *Braz JMicrobiol*, 48(1), 2017, 62-70.
[10] J. Kohl, R. Kolnaar and W. J. Ravensberg, Mode of action of microbial control agents against plant disease: Relevance beyond efficacy, *Frontiers in Plant Sciences*, 10, 2019, 845.
[11] A. M. Pirttila, H. M. P. Tabas, N. Baruah and J. J. Koskimaki, Biofertilizers and Biocontrol Agents for Agriculture: How to Identify and Develop New Potent Microbial Strains and Traits, *Microorganisms*, 9(4), 2021, 817.
[12] G. Zimmermann, Review on safety of the entomopathogenic fungus *Beauveria bassiana* and *Beauveria brongniaetti*, *Biocontrol Science and Technology*, 17(6), 2007, 533-596.
[13] G. Zimmermann, Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*, *Biocontrol Science and Technology*, 17(9), 2007, 879-920.
[14] B. W. Narladkar, P. R. Shivpuje, and P. C. Harke, Fungal biological control agents for integrated management of *Culicoides* spp. (Diptera: Ceratopogonidae) of livestock. *Vet World*, 8(2), 2015, 156-163.
[15] S. Singh, S. Bhatnagar, S. Choudhary, B. Nirwan and K. Sharma, Fungi as Biocontrol Agent: An Alternate to Chemicals. In, P. Gehlot, J. Singh (Ed), Fungi and their Role in Sustainable Development: Current Perspectives (Singapore: Springer, 2018) 23-33.
[16] K. M. Thambugala, D. A. Daranagama, A. J. L. Phillips, S. D. Kannangara and I. Prompottha, Fungi vs. Fungi in Biocontrol: An Overview of Fungal Antagonists Applied Against Fungal Plant Pathogens. *Front Cell Infect Microbiol*, 10, 2020, 604923.
[17] C. Payne, Insect viruses as control agents, *Parasitology*, 84(4), 1982, 35-77.
[18] D. P. Harding and M. N. Raizada, Controlling weeds with fungi, bacteria and viruses: a review, *Frontiers in Plant Sciences*, 6, 2015, 659.
[19] M. Ramzan, B. Tabassum, I. A. Nasir, A. Khan, M. Tariq, M. F. Awan, N. Shahid, A. Q. Rao, M. U. Bhatti, N. Toufiq and T. Husnain, Identification and application of biocontrol agents against Cotton leaf curl virus disease in *Gossypium hirsutum* under greenhouse conditions, *Biotechnology and Biotechnological Equipment*, 30(3), 2016, 469-478.
[20] A. M. M. Abd-Alla, I. K. Meki and G. Demirbas-Uzel, Isect Virus as Biocontrol Agents: Challenges and Opportunities, In N. El-Wakeil, M. Saleh, M. Abu-hashim (Ed), Cottage Industry of Biocontrol Agents and Their Applications (Switzerland: Springer, Cham, 2020), 277-295.
[21] J. E. Henry, Natural and Applied Control of Insects by Protozoa, *Annual Review of Entomology*, 26(1), 1981, 49-73.
[22] M. J. Bidochka and G. G. Khachatourians, Microbial and protozoan pathogens of grasshoppers and locusts as potential biocontrol agents, *Biocontrol Science and Technology*, 1(4), 1991, 243-259.
[23] J. Gronvold, S. Aa. Henriksen, M. Larsen, P. Nansen and J. Wolstrup, Biological control aspects of biological control-with special reference to arthropods, protozoans and helminthes of domesticated animals, *Veternary Parasitology*, 64(1-2), 1996, 47-64.
[24] D. Johnson, Nosematidae and other Protozoa as agent for control of Grasshoppers and Locusts: Current Status and Prospects, *Memoirs of the Entomological Society of Canada*, 129(S 171), 1997, 375-389.
[25] H. D. Burges and K. A. Jones, Formulation of Bacteria, Viruses and Protozoa to Control Insects, In H. D. Burges (Ed), Formulation of Microbial Biopesticides. (Dordrecht: Springer, 1998) 87-109.
[26] M. S. Muller, S. Scheu and A. Jousset, Protozoa Drive the Dynamics of Culturable Biocontrol Bacterial Communities, *PLos One*, 8(6), 2013, e66200.
[27] R. Mankau, Biocontrol: fungi as nematode control agents, *J Nematol*, 12(4), 1980, 244-252.
[28] T. H. Askary, Nematodes as Biocontrol Agents, In E. Lichtfouse (Ed), Sociology, Organic Farming, Climatic Change and Soil Sciences. *Sustainable Agriculture Reviews* 3, (Dordrecht: Springer, 2007), 347-378.
[29] M. S. Tranier, J. Pognant-Gros, R. C. Quiroz, C. N. A. Gonzalez, T. Mateille and S. Roussos, Commercial Biological Control Agents Targeted Against Plant-Parasitic Root-knot Nematodes, *Braz Arch Bio Technol*, 57(6), 2014, 831-841.

[30] M. M. M. Abd-Elgawad, Biological control agents in the integrated nematode management of pepper in Egypt, *Egypt J Biol Pest Control*, 30, 2020, 70.
[31] S. K. R. Namasivayam and R. S. A. Bharani, Biocontrol potential of entomopathogenic fungi *Nomuraea rieyi* (f.) Samson against major groundnut defoliator *Spodoptera litura* (fab.) *Lepidoptera; Noctuidae*, *Adv Plants Agric Res*, 2(5), 2015, 221-225.
[32] K. M. S. Aw and S. M. Hue, Mode of infection of *Metarhizium* spp. Fungus and Their Potential as Biological Control Agents, *J Fungi (Basel)*, 3(2), 2017, 30.
[33] S. V. Agale, S. Gopalkrishnan, K. G. Ambhure, H. Chandravanshi, R. Gupta and S. P. Vani, Mass Production of Entomopathogenic Fungi (*Metarhizium anisopliae*) using Different Grains as a Substrate, *Int J Curr Microbiol App Sci*, 7(1), 2018, 2227-2232.
[34] T. Castro, J. Mayerhofer, J. Enkerli, J. Eilenberg, N. V. Meyling, R. A. Moral, C. G. B. Demetriod, and I. Delalibera, Persistence of Brazilian isolates of the entomopathogenic fungi *Metarhizium anisopliae* and *M. robertsii* in strawberry crop soil after soil drench application, *Agriculture, Ecosystems and Environment*, 233, 2016, 361-369.
[35] G. D. Inglis, J. Enkerli and M. S. Goettel, Laboratory techniques used for entomopathogenic fungi: hypocreales, In L. A. Lacey (Ed), Manual of techniques in invertebrate Pathology Second Edition (Dordrecht: Springer, 2012) 189-253.
[36] S. Ghayedi and M. Abdollahi, Biocontrol Potential of *Metarhizium anisopliae* (hypocreales:Clavicipitaceae), isolated from suppressive soils of Boyer-Ahmad region, Iran, against J2s of *Heterodera avenae*, *Journal of Plant Protection Research*, 53(2), 2013, 165-171.
[37] M. Tulloch, The genus Metarhizium, *Transactions of the British Mycological Society*, 66(3), 1976, 407-411.
[38] R. A. Humber, Fungi: Identification, In A manual of Techniques in insect Pathology, Academic Press, New York, 1997, 153-167.
[39] E. Kerry, Effect of temperature on growth rates of fungi from sub-Antarctic Macquarie Island and Casey, Antarctica, *Polar Biol*, 10(4), 1990, 293-299.
[40] U. Mustafa and G. Kaur, Effects of corbon and nitrogen sources and ratio on the germination, growth and sporulation characteristics of *Metarhizium anisopliae* and *Beauveria bassiana* isolates, *African journal of Agricultural Research*, 3(10), 2009, 922-930.
[41] C. Brunner-Mendoza, M. R. Reyes-Montes, S. Moonjely, M. J. Bidochka and C. Toriello, A review on the genus *Metarhizium* as an entomopathogenic microbial control agent with emphasis on its use and utility in Mexico, *Biocontrol Science and Technology*, 29(1), 2019, 83-102.
[42] P. D. Bridge, M. A. J. Williams, C. Prior and R. R. M. Paterson, Morphological, biochemical and molecular characteristics of *Metarhizium anisopliae* and *Metarhizium flavoviridae*, *Journal of General Microbiology*, 139, 1993, 1163-1169.
[43] P. Mythili, S. Gomathinayagam, C. Balachandran, D. Raj and L. John, Isolation and mass production of entomopathogenic fungi for biocontrol assays, *Tamilnadu J Veternary and Animal Sciences*, 6(6), 2010, 271-274.
[44] B. L. K. Brady, *Metarhizium anisopliae* CMI Descriptions of Pathogenic Fungi and Bacteria No. 609. Commonwealth Agricultural Bureaux : Kew, Srrey, UK, 1979, 2.
[45] Z. L. Pu and Z. Z. Li, Insect Mycology, *Anhui Sci Technol*, Press, Hefei, Anhui, China, 95-97, 1996, 360-368.
[46] R. A. Humber, Fungi: identification, In Manual of Techniques in insect Pathology, L. A. Lacy (Ed), Biological Technique Series, Academic Press. New York, USA, 1997, 153-185.
[47] G. Hu and R. J. St Leger, Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent, *Appl Environ Microbiol*, 68(12), 2002, 6383-6387.
[48] M. S. H. Elham, P. K. Kin, G. L. E. Lin, I. Ishak and W. A. Azmi, Occurance of entomopathogenic fungus, *Metarhizium anisopliae* isolated from Island, Bris and Coastal soils of Terengganu, Malaysia, *Journal of Sustainability Science and Management*, 13(5), 2018, 179-189.
[49] S. Mongkolsamrit, A. Khonsanit, D. Thanakitpipattana, K. Tasanathai, W. Noisripoom, S. Lamlertthon, W. Himaman, J. Houbraken, R. A. Samson and J. Luangsa-ard, Revisiting *Metarhizium* and the description of new species from Thiland, *Studies in Mycology*, 95, 2020, 171-251.
[50] R. K. Campbell abd G. L. Arnes, B. O. Cartwright, R. D. Eikenbary, Growth and sporulation of *Beauveria bassiana* and *Metarhizium anisopliae* in a basal medium containing various carbohydrate source. *Journal of Incertebrate Pathology*, 41, 1983, 117-121.
[51] S. P. Sharma, R. B. Gupta and C. P. Yadava, Selection of suitable medium for mass multiplication of entomofungal pathogens, *Indian J Entomol*, 14, 2002, 255-261.

[52] P. K. Hoe, C. F. J. Bong, K Jugah and A. Rajan, Evaluation of *Metarhizium anisopliae* var. *anisopliae* (Deuteromycotina: Hypomycete) isolates and their effect on subterranean termite *Coptotermes curvignathus* (Isoptera: Rhinotermitidae), *Am J Agric Biol Sci*, 4(4), 2009, 289-297.
[53] N. Orduno-Cruz, A. W. Guzman-Franco, E. Rodriguez-Leyva, R. Alatorre-Rosas, H. Gonzalez-Hernandez and G. Mora-Aguilera, In vivo selection of entomopathogenic fungal isolates for control *Diphorina citri* (Hemiptera: Liviidae), *Biological Control*, 90, 2015, 1-5.
[54] H. Prabowo, T. Yulianti, J. Damaiyani and A. Fudholi, Effect of Temperature and Medium Composition on Growth of *Metarhizium anisopliae*, Entomopathogenic Fungi of Sugarcane White Grubs, *International Journal of Advanced Science and Technology*, 29(06), 2020, 4490-4499.
[55] S. Arthurs and M. B. Thomas, Effects of temperature and relative humidity on sporulation of *Metarhizium anisopliae* var. acridum in mycosed cadavers of *Schistocerca gregaria*, *J Invertebr Pathol*, 78(2), 2001, 59-65.
[56] S. Dimbi, N. K. Maniania, S. A. Lux and J. M. Mueke, Effect of constant temperature on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies, *BioControl*, 49, 2004, 83-94.
[57] D. M. Bugeme, N. K. Maniania, M. Knapp, and H. I. Boga, Effect of temperature on virulence of *Beauveria bassiana* and *Metarhizium anisopliae* isolates to *Tetranychus evansi*. *Exp Appl Acarol*, 46(1-4), 2008, 275-285.
[58] J. Medo and L. Cagan, Factors affecting the occurrence of entomopathogenic fungi in soils of Slovakia as revealed using two methods. *Biological Control*, 59(2), 2011, 200-208.
[59] K. N. P. Chandra Teja and S. J. Rahman, Characterisation and evaluation of *Metarhizium anisopliae* (Metsch.) Sorokin strains for their temperature tolerance, *Mycology*, 7(4), 2016, 171-179.
[60] D. G. P. Oliveira, R. B. Lopes, J. M. Rezende and I. Delalibera, Increased tolerance of *Beauveria bassiana* and *Metarhizium anisopliae* conidia to high temperature provided by oil-based formulations, *Journal of Invertebrate Pathology*, 151, 2018, 151-157.