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Effective biopesticides for sustainable management of *Plutella xylostella* L. (Lepidoptera: Plutellidae) in cauliflower: A comprehensive study on isolation, characterization and virulence assessment of highly virulent entomopathogenic fungi

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ABSTRACT: Cauliflower faces a significant threat from the notorious pest *Plutella xylostella* L., which has become challenging to manage using conventional insecticides due to the development of resistance. Seeking an environmentally sustainable solution, this study was conducted to explore the potential entomopathogenic fungi (EPF) for the management of *P. xylostella*. In the present study, soil samples were collected from the cauliflower growing regions of Tamil Nadu for the isolation of EPF, resulting in the isolation of 61 EPF isolates. Morphological and molecular characterization revealed that among the 61 isolates isolated, 42 were *Beauveria bassiana* and 19 were *Metarhizium anisopliae*. Preliminary bioassay was conducted for the virulent isolates, and the results revealed the remarkable virulence of *M. anisopliae* A11 (OR536962) and *B. bassiana* A10 (OR533688) isolates causing 87.50 ± 0.25 and 85.00 ± 0.40 percent mortality of *P. xylostella*, respectively at the conidial load of 1×10^8 conidia/ml. Dose and time mortality response revealed that the *M. anisopliae* A11 (OR536962) isolate has LC_{50} and LT_{50} values of 2.70×10^6 conidia/ml and 107.15 h, respectively. On the other hand, the most virulent *B. bassiana* A10 (OR533688) isolate has LC_{50} and LT_{50} values of 4.12×10^6 conidia/ml and 113.48 h, respectively against *P. xylostella*. The study concludes that these isolates demonstrate high virulence against *P. xylostella*, offering promising prospects for effective management of this pest in field conditions.

KEYWORDS: *B. bassiana*, characterization, ecofriendly management, isolation, *M. anisopliae*

INTRODUCTION

Cauliflower (*Brassica oleracea* var. *botrytis*), a winter season crop, is grown as an annual crop for vegetable purpose and as a biennial crop for seed production purpose. It is grown in an area of 14.36 lakh ha globally [1]. India ranks second in cauliflower production with a total area of 4.65 lakh ha; and in Tamil Nadu, it is cultivated in an area of 2180 ha with the production of 53.64 M tonnes [2]. Cauliflower cultivation is affected by various biotic and abiotic factors. Among the biotic factors, Diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) poses a serious threat [3].

P. xylostella is a globally important destructive pest of cauliflower, attacks all the crucifer crops, and is responsible for the decline in cauliflower production globally [4]. It can cause yield reduction up to 91.2% and it costs around US$ 4–5 million in terms of management of *P. xylostella* per year globally [5]. Farmers heavily depend on traditional insecticides for the management of *P. xylostella*, but their effectiveness is undermined by the pest's growing resistance to almost all classes of insecticides [6, 7]. Moreover, the excessive use of insecticides has adverse non-target effects, such as the loss of natural enemies, further complicating the management of *P. xylostella*. With the view of these negative effects of insecticides, biological control paves the way for efficient management of *P. xylostella*. Entomopathogenic fungi (EPF) are the major component of integrated pest management because of their host specific nature [8], serving as an eco-friendly option for managing various pests in agricultural and horticultural ecosystems.

Soil is an important reservoir of EPF, causing natural epizootics and, thus, controlling the outbreak of pests to a considerable extend at the months of high relative humidity [9]. Several EPF such as *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosorosea*, *Lecanicillium lecanii*, *Hirsutella thompsonii*, *Nomuraea rileyi*, and *Entomophthorales* were successfully employed for the management of various insect pests [8], and these entomopathogens were present naturally in the soil. EPF produce several cuticle-degrading enzymes and secondary metabolites that play a crucial role in the infection process of EPF on the target insect [10]. Soil borne entomopathogens particularly, *B. bassiana* and *M. anisopliae* were the well-studied

EPF species [11] that were successfully used for the management of *P. xylostella* in various regions of the world [12]. Hence, the current study focused on the isolation of EPF from soils of various cauliflower growing regions in Tamil Nadu and testing the virulence of these EPF against *P. xylostella*.

MATERIALS AND METHODS

Survey and collection of soil samples

Survey was conducted in the three major cauliflower growing areas of Tamil Nadu *viz*., Coimbatore, Nilgiris and Krishnagiri Districts during January to December 2023. A total of 168 soil samples (56 samples per district) were collected from the different cauliflower fields surveyed. Soil samples weighing around 1 kg were collected from the north side of the plot, where there were more humidity and little sunlight. In some cases, factors like crop canopy influence the sunlight and humidity. Therefore, in those cases, soil samples were collected from the shady areas of the plot, as higher shade increases the occurrence of entomopathogens [13]. Collected soil samples were placed in plastic bags and brought to the laboratory, shade dried, sieved, and stored at the temperature of 4 °C until further use.

'*Galleria*' bait method

The greater wax moth, *Galleria mellonella* L. larvae were collected from the infected bee hives and reared in the Biocontrol laboratory, Department of Agricultural Entomology, Tamil Nadu Agricultural University (TNAU), Coimbatore using artificial diet as per the protocol of Hickin et al [14]. '*Galleria*' bait method introduced by Zimmermann [15] was used for the isolation of EPF from soil. Second instar larvae of *G. mellonella* were immersed in hot water at 56 °C for 15 s to reduce the ability of the larvae to produce web in the soil. Following this, about ten larvae were introduced into one plastic container along with the soil and incubated at the temperature of 25 ± 1 °C. All containers were periodically agitated every 12 h to facilitate the movement of larvae from the upper to the lower section and vice versa. Subsequent to incubation, a thorough examination was conducted; and dead larvae were carefully removed. The experimental setup was replicated five times for each soil sample collected.

Isolation of EPF

Cadavers of *G. mellonella* were subjected to surface sterilization and transferred into a sterile petri dish containing potato dextrose agar (PDA) medium supplemented with 1% yeast extract. The petri dishes were incubated at the temperature of 25 ± 2 °C for sporulation. Subsequently, subculturing was done to achieve pure culture [16].

Morphological characterization of the EPF isolates

The EPF isolates of about 15 days old were used for morphological characterization. Using a Phase Contrast Microscope (Euromex, iScope, The Netherlands), the morphological characters such as colony morphology, spore shape, spore size, spore structure and mycelial character were studied according to the keys of Humber [17].

Molecular characterization of the EPF isolates

For further confirmation of the EPF isolates, molecular characterization was done, and genomic DNA of the EPF isolates was extracted using CTAB (Cetyl-trimethyl ammonium bromide) method [18]. For molecular characterization, the isolates were grown in potato dextrose liquid medium supplemented with 0.1% yeast for 5 days at 25 ± 2 °C. The mycelial mat was separated from the liquid medium, homogenized with 800 µl of CTAB (Himedia Laboratories, India) buffer, and then transferred to a sterile microfuge tube. Subsequently, it was incubated at 65 °C for 1 h. Chloroform:Isoamyl alcohol (24:1) was added to the microfuge tubes and centrifuged at 13,000 rpm for 10 min at 4 °C. The resulting supernatant was transferred to another sterile microfuge tube containing ice cold isopropyl alcohol and incubated at −20 °C overnight. After that, it was again centrifuged at 13,000 rpm for 10 min at 4 °C. and the pellet was washed with 70% ethanol, dried and then resuspended with 30 µl of TE buffer. The concentration of DNA was determined using Nano drop Spectrophotometer (Thermo Scientific, USA) [19].

Polymerase chain reaction (PCR)

The ITS1-5.8S-ITS4 genes were amplified using ITS-1 (5′-TCGGTAGGTAGGTGAACCTGCGG-3′) as forward primer and ITS-4 (5′-CAGGAGACTTGTACACGGTCCA G-3′) as reverse primer [20]. PCR amplifications were performed in a Gradient Master Cycler (Bio-Rad, USA) in a volume of 50 µl, containing 5 µl 10x Taq-DNA polymerase buffer, 1 µl each of the opposing amplification primer, 0.5 µl of Taq-DNA polymerase, 1 µl of dNTPs, and 50 ng of DNA. The PCR program consisted of 35 cycles after initial denaturation at 95 °C for 5 min, 95 °C for 1 min, 55 °C for 55 s, 72 °C for 2 min, with a final extension at 72 °C for 10 min. The PCR product was run in 1% agarose gel at 80 v for 40 min and visualized under UV transilluminator (Syngene G: Box, UK) to determine the presence of DNA and the quality of amplification. The PCR product was then sent to Eurofins Genomics, Bengaluru, India and sequenced using Sanger method. BLAST searches were performed using the NCBI database for the obtained sequences. The sequences were submitted to NCBI, and the accession number were obtained.

Screening of the EPF isolates

The EPF isolates were grown in petri plates and incubated at a temperature of 24 ± 1 °C for sporulation. After completing sporulation (15 days old), the spores were scrapped using a sterile surgical knife and suspended in 10 ml sterile distilled water containing 0.05% Tween 80. The conidial concentration was assessed using an improved Neubauer haemocytometer, and the concentration was adjusted to 1×10^8 conidia/ml [21].

Preliminary bioassay was performed using leaf immersion method as per the protocol of Shehzad et al [12]. Cauliflower leaf discs of 9 cm diameter were dipped in the conidial suspension of 1×10^8 conidia/ml containing 0.05% Tween 80 for 1 min and air dried. About 30 s instar larvae of *P. xylostella* were released into each petri plate containing cauliflower leaves treated with conidial suspension, and the experiment was replicated four times. The experimental setup was maintained at the temperature of 25 ± 2 °C and the relative humidity of 70 ± 10% with the photoperiod of 12:12 (L:D). Percent larval mortality was observed at 3, 5, 7, and 9 days post inoculation. The dead larvae were kept in a humid chamber, only those larvae that were covered with mycelium were considered for calculating the mortality percentage [22].

Virulence of the EPF

Efficacy of the selected EPF isolates was performed by preparing the conidial suspensions with different loads of conidia *viz*., 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 conidia/ml containing 0.05% Tween 80. Cauliflower leaf discs (9 cm diameter) were dipped in the prepared different conidial suspensions for 1 min and air dried. About 30 s instar larvae of *P. xylostella* were released into each treatment, and the experiment was replicated four times. The experimental setup was incubated at the temperature of 25 ± 2 °C and the relative humidity of 70 ± 10% with the photoperiod of 12:12 (L:D). Mortality of the larvae was assessed up to 9 days after inoculation [22].

Statistical analysis

The percent larval mortality in different treatments was corrected using Abbott's formula [23] and, then, subjected to arc-sine transformation for obtaining the normal data sets. Analysis of variance was used to examine the differences in mortality between the fungal isolates and the control group. The Kenward-Rodger method was used to calculate degrees of freedom.

The relationship between the concentration of the EPF isolates and mortality of the larvae was studied using Probit analysis [24] and SAS (PROC PROBIT; SAS Institute, 2011) was used to determine the LC_{50} and the LT_{50} values.

The raw sequences obtained by the amplification of ITS region were assembled, edited and aligned with BioEdit and compared with the sequences from EPF isolates that were deposited in NCBI, GenBank. For phylogenetic analysis, multiple sequence alignments were done using MAFFT v6.833, and the phylogenetic tree was constructed by neighbour-joining (NJ) method using Molecular Evolutionary Genetics Analysis (MEGA) v7.0. Using the Kimura 2 parameter model, the distances for the neighbour joining tree were calculated. The reliability of the dendrograms was tested by bootstrap analysis with 1000 replicates [25].

RESULTS AND DISCUSSION

EPF, especially *Paecilomyces farinosus*, *B. bassiana*, *Conidiobolus* sp. and *M. anisopliae* var. *anisopliae*, were abundant in the soils [26, 27]. '*Galleria* bait' method introduced by Zimmerman [15] was a commonly used method for isolating EPF from soils. In *Galleria* bait, 92 mycosed *G. mellonella* cadavers were identified from the 168 soil samples surveyed. The EPF were isolated from the mycosed *G. mellonella* cadavers using PDA medium, and a total of 61 EPF isolates were isolated. *B. bassiana* and *M. anisopliae* were the most frequently isolated from the soils of several regions of the world [16].

The isolated EPF species were subjected to morphological and molecular characterization. Colony characters such as shape and size of the conidia were the commonly used traits for the morphological identification of fungi [17]. Morphological characterization revealed that 42 isolates produced white or yellowish white coloured cottony or powdery colonies. Similar variations in the colony colour of *B. bassiana*, such as white or yellowish-white, were reported by Affandi et al [28]. The spherical conidia were borne on flask shaped conidiogenous cells with zig-zag rachis indicating that these isolates were *B. bassiana*. The spore size was found to be in the range of 2.33 to 3.19 µm. Similar variation in spore size of *B. bassiana* from 2.4 to 3.6 µm was reported by Talaei-Hassanloui et al [29]. Another 19 isolates produced bright green to yellowish green colonies with aseptate, cylindrical or ovoid conidia. This type of variation in spore morphology of *M. anisopliae* with cylindrical or ovoid conidia was reported by Sapna Bai et al [30] (Table 1).

Identification of the EPF species based on morphological characters is not always reliable due to the large heterogeneity of spherical and cylindrical conidia [31]. Therefore, for the precise identification of the fungal species, DNA based molecular identification was widely used. The internal transcribed spacer (ITS), a conserved region, has the highest probability of successful identification of a broad range of fungal species [32]. Therefore, ITS region of rDNA has been used widely in the fungal systematics [20]. In the current study, ITS region was amplified using the primers ITS 1 and ITS 4, recommended as the universal primers

Table 1 Morphological characters of the isolated virulent EPF.

Isolate	Morphological character							
	Growth pattern	Colony color	Reverse colony color	Surface texture	Elevation	Mycelial structure	Conidial shape	Conidial size (µm)
A1	Flat and dense	Yellowish white	White	Smooth, powdery	Levelled	Hyaline and septate	Globose	2.45
A2	Disperse and dense	White	White	Smooth, powdery	Levelled			2.51
A3	Disperse and dense	White	White	Smooth, cottony	Slightly elevated			2.33
A4	Disperse and dense	Yellowish white	White	Smooth, powdery	Flattened			2.65
A5	Disperse and dense	Yellowish white	White	Smooth, cottony	Slightly elevated			2.99
A6	Disperse and dense	Yellowish white	White	Smooth, powdery	Levelled			2.49
A7	Flat and dense	White	Yellowish white	Smooth, powdery	Levelled			2.11
A8	Flat and dense	White	White	Smooth, powdery	Slightly elevated			2.32
A9	Flat and disperse	White	Yellowish white	Smooth, powdery	Flattened			3.19
A10	Flat and dense	White	White	Smooth, powdery	Flattened			2.77
A11	Flat and distributed	Dark green	Brown	Dense powdery and firm	Levelled		Cylindrical	6.34

Table 2 Identification of the EPF isolates by partial sequencing of ITS1-5.8S-ITS4 genes, where the E value was found to be "0" for all the isolates.

Sample no.	Isolate	Matching organism in NCBI database	% Similarity with other isolates in NCBI	Accession no. obtained
1	A1	*B. bassiana*	97.11	OR537630
2	A2	*B. bassiana*	98.78	OR541489
3	A3	*B. bassiana*	99.34	OR687301
4	A4	*B. bassiana*	98.53	OR537694
5	A5	*B. bassiana*	97.55	OR687249
6	A6	*B. bassiana*	99.12	OR537575
7	A7	*B. bassiana*	96.65	OR670027
8	A8	*B. bassiana*	97.38	OR689231
9	A9	*B. bassiana*	97.22	OR536597
10	A10	*B. bassiana*	98.61	OR533688
11	A11	*M. anisopliae*	98.78	OR536962

[20]. Amplification of the ITS region of the *B. bassiana* and *M. anisopliae* isolates resulted in the amplicon size of approximately 560 bp (Fig. 1 and Fig. 2), and the amplified PCR products were sent for sanger sequencing. The obtained sequences were subjected to BLAST analysis for similarity searches, and results indicates that the EPF isolates A1, A2, A3, A4, A5, A6, A7, A8, A9, and A10 had 97.11%, 98.78%, 99.34%, 98.53%, 97.55%, 99.12%, 96.65%, 97.38%, 97.22%, and 98.61% homology with *B. bassiana* isolates that

Table 3 Dose mortality response of the virulent EPF isolates against *P. xylostella*.

Isolate	Regression equation	R^2	LC_{50} (Conidia/ml)	Fiducial limit (95% confidence interval)
A11 (OR536962)	$y = 0.6013x + 1.3561$	0.9377	2.70×10^6	1.58×10^5–4.65×10^7
A10 (OR533688)	$y = 0.5672x + 1.446$	0.9525	4.12×10^6	1.94×10^5–8.75×10^7
A2 (OR541489)	$y = 0.5085x + 1.5723$	0.9722	6.06×10^6	3.40×10^5–1.07×10^8
A9 (OR536597)	$y = 0.5057x + 1.542$	0.9653	6.33×10^6	3.73×10^5–1.07×10^8

Table 4 Time mortality response of the virulent EPF isolates against *P. xylostella*.

Isolate	Regression equation	R^2	LT_{50} (h)	Fiducial limit (95% confidence interval)
A11 (OR536962)	$y = 3.8148x - 2.7396$	0.9772	107.15	80.69–142.28
A10 (OR533688)	$y = 3.5264x - 2.2489$	0.9892	113.48	84.98–151.54
A2 (OR541489)	$y = 3.4159x - 2.1072$	0.9989	120.28	90.09–160.58
A9 (OR536597)	$y = 3.2566x - 1.8002$	0.9981	122.23	90.52–165.05

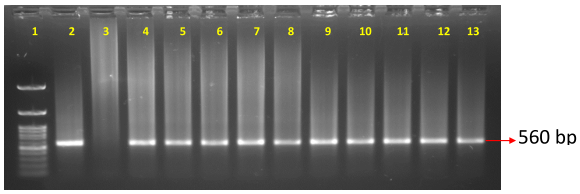

Fig. 1 Amplification of ITS region of the *B. bassiana* isolates using ITS 1 and ITS 4 primers, where L, Ladder (100 bp); 1, Positive control; 2, Negative control; 3, A1; 4, A2; 5, A3; 6, A4; 7, A5; 8, A6; 9, A7; 10, A8; 11, A9; and 12, A10.

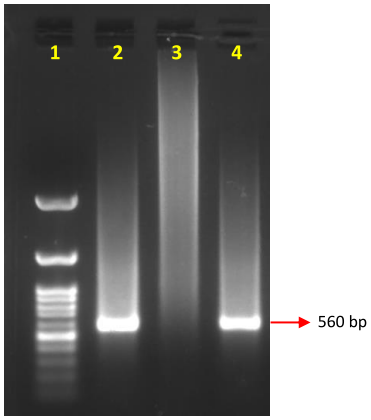

Fig. 2 Amplification of ITS region of the *M. anisopliae* isolate using ITS 1 and ITS 4 primers, where L, adder (100 bp); 1, Positive control; 2, Negative control; 3, A11.

were deposited in NCBI, respectively. The other isolate, A11 had 98.78% homology with the other *M. anisopliae* isolates deposited in NCBI. Similar amplification was noted by Gebremariam et al [33], who amplified the ITS region of *B. bassiana* and *M. anisopliae* isolates, and they found that the PCR amplification of the ITS region of rDNA resulted in a single product that produced approximately 545 bp fragment size for all isolates of *B. bassiana* and *M. anisopliae*. After BLAST analysis, the sequences were submitted to NCBI, and the accession numbers were obtained *viz*., OR537630 (A1), OR541489 (A2), OR687301 (A3), OR537694 (A4), OR687249 (A5), OR537575 (A6), OR670027 (A7), OR689231 (A8), OR536597 (A9), OR533688 (A10), and OR536962 (A11) (Table 2).

To deepen the molecular identification of the EPF isolates and for studying the evolutionary relationship, phylogenetic analysis can be done [34]. In the study, all the ten *B. bassiana* isolates were clustered together in a single clade along with the reference *B. bassiana* sequence (MH233319). Similarly, the *M. anisopliae* isolate (OR536962) clustered together with the *M. anisopliae* reference sequence (AB524408) in a single clade (Fig. 3) which was in accordance with the results of Bich et al [35], where all the *B. bassiana* isolates clustered together with the reference *B. bassiana* sequence. Similar results of *B. bassiana* and *M. anisopliae* clustering together in different clades were noted by Kutalmis et al [36].

In the present study, efficacy of the EPF isolates were tested against the second instar larvae of *P. xylostella*. Both *B. bassiana* and *M. anisopliae* caused significant mortality of *P. xylostella*. The *M. anisopliae* isolate OR536962 resulted in 87.50% mortality of the second instar larvae of *P. xylostella* at the conidial load of 1×10^8 conidia/ml (Fig. 4). This was in relation to the results of Loc and Chi [37] as the *M. anisopliae* isolate *M.a* (OM3-STO) caused 87.3% mortality of the second instar larvae of *P. xylostella* in laboratory conditions at the conidial load of 1×10^8 conidia/ml. The *B. bassiana* isolate OR533688 caused 85.00% mortality of *P. xylostella* at the conidial load of 1×10^8 conidia/ml. This was supported by Nithya et al [22], as the *B. bassiana* isolate MH590235 resulted in 82.00% mortality of *P. xylostella* at the conidial load of 1×10^8 conidia/ml.

Dose and time mortality response was calculated for the four virulent isolates, *viz*., OR536962 (*M. anisopliae*), OR533688 (*B. bassiana*), OR541489 (*B. bassiana*) and OR536597 (*B. bassiana*) as these four isolates resulted in more than 60 percent mortality in the preliminary screening. All the four isolates were found to be pathogenic to *P. xylostella* with varying degree of virulence. The percent mortality was dose dependent and increased with an increase in concentration. The LC_{50} values of the isolates were in the range of 2.70×10^6 to 6.33×10^6 conidia/ml (Table 3). This was supported by the results of Chui-Chai et al [38] as the *B. bassiana* isolate Bb5335 resulted in the LC_{50} value of 2.66×10^6 conidia/ml against *P. xylostella*.

LT_{50} values of the four virulent isolates *viz*., OR536962, OR533688, OR541489, and OR536597 were calculated against *P. xylostella*. The results revealed that the LT_{50} values of these four isolates were in the range of 107.15 to 122.23 h (Table 4). The current results were supported by Nithya et al [22], as *B. bassiana* MK918495 isolate resulted in the LC_{50} value of 110.16 h against the second instar larvae of *P. xylostella*. Similarly, Atrchian and Mahdian [39] also supported our findings.

CONCLUSION

EPF appear to be the important component of Integrated pest management (IPM). These mycoinsecticides are safer to the environment and can be used as a safer alternative to the synthetic insecticides. In the present study, eleven virulent EPF isolates were isolated from the soils of cauliflower fields in Tamil Nadu. The study concluded that the *M. anisopliae* isolate (A11) and the *B. bassiana* isolate (A10) were highly virulent against *P. xylostella*. Therefore, these

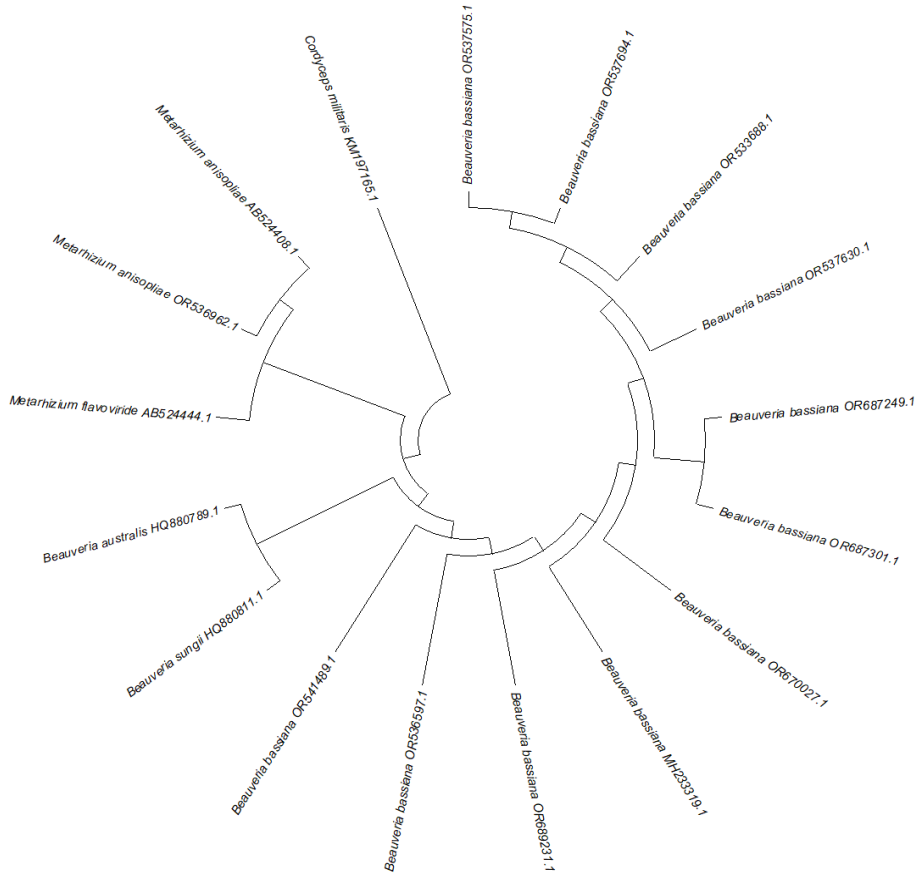

Fig. 3 Phylogenetic tree obtained by NJ analysis of Internal Transcribed Spacer (ITS) sequences of the 11 isolated isolates along with six isolates as reference where *Cordyceps militaris* serving as an outgroup.

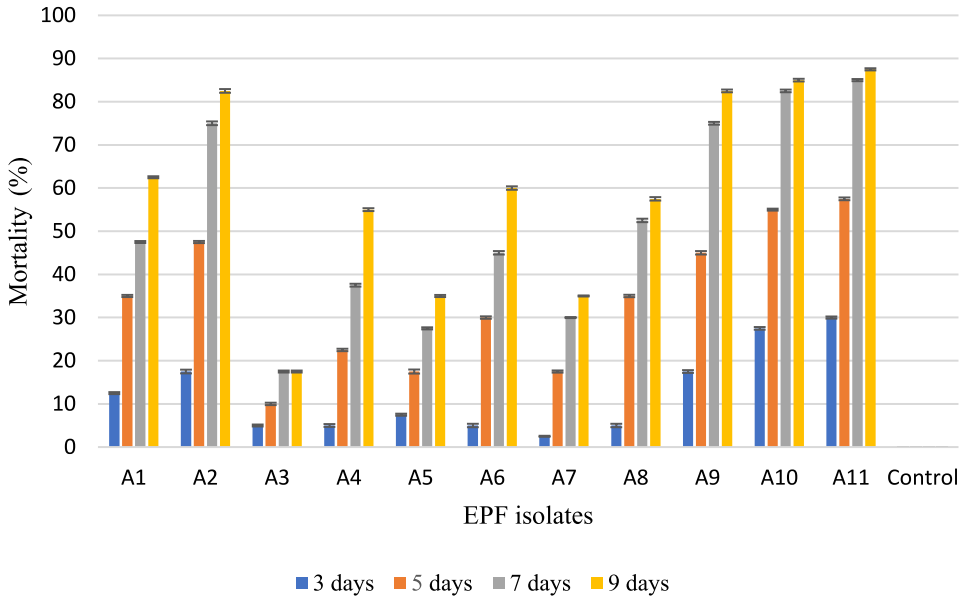

Fig. 4 Pathogenicity of the EPF isolates against *P. xylostella* at the conidial load of 1×10^8 conidia/ml.

isolates were promising against *P. xylostella* and could be essential to be formulated efficiently using an appropriate carrier. The toxicity, persistence and safety of these isolates to other non-target pests in cauliflower ecosystem must be further evaluated in future studies.

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