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RESEARCH ARTICLES

Biological control of papaya aphid (*Aphis gossypii* Glover) using entomopathogenic fungi

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Abstract

Papaya is economically important cultivated fruit crop grown in all tropical countries, having enormous nutritional values. Papaya Ring Spot Virus imposes a significant crop loss in terms of quality and quantity. To encounter the virus vector (*Aphis gossypii*), indiscriminate use of chemical pesticides creates severe environmental hazards whereas biological control is a perfect alternative to this problem. The objectives of our present study were isolation and characterization of indigenous fungi and their comparative analysis of entomopathogenic fungi against papaya aphid and finding its pathogenicity. Fungal isolates collected from natural sources were characterized and identified by the cultural and morphological study. Potential EPF genera were molecularly identified by PCR (ITS1-5.8S-ITS2) method. Entomopathogenic fungi were screened against *A. gossypii* for their pathogenecity by incised leaf disc method. LD_{50} (median lethal dose) and LT_{50} (median lethal time) were analyzed by regression analysis. Phylogenetic relationship among EPF was evaluated by MEGA software. Out of forty isolated entomopathogenic fungi, three (*Beauveria bassiana* deb4, *Penicillium verrucosum* Nlg1, and *Fusarium equiseti* khr4) were highly effective entomopathogen. The LD_{50} value of *B. bassiana*, *P. verrucosum* and *F. equiseti* were 1.4×10^4, 9.8×10^4, 1.0×10^6 spores ml^{-1}, and LT_{50} values were 32.14, 37.5, 32.14 h respectively. Their phylogenetic analysis indicates related closeness on the basis of their conserved internal transcribed spacer region. In conclusion, the indigenous isolated strain of *B. bassiana* (deb4) has shown highest biocontrol potentiality amongst three indigenous entomopatogenic fungi under lab condition against *A. gossypii* and can be applied in agrifields.

Keywords Vector control · Entomopathogenic fungi · Papaya Ring Spot Virus · LD_{50} · LT_{50}

Introduction

Papaya (*Carica papaya* Linn.) is one of the most cultivated fruit crops having abundant prophylactic, nutritional and therapeutic values, in almost all tropical countries. Despite taking several precautions, Papaya Ring Spot Virus (PRSV) causes huge destruction and crop loss (10–100%) every year (Tennant et al. 2007). Aphids; the insect vectors transmit this virus in a non-persistent manner (Chakrabarti and Raychaudhuri 1978). Several chemical pesticides are being used to control this disease, but they cause environmental pollution and health hazards (Kalleshwaraswamy et al. 2012). In the present scenario, biological control strategies are essential for increasing human awareness against the hazardous effects of chemical control of pest and disease (Fang et al. 2009; Garkoti et al. 2014). Insect management with the help of entomopathogenic fungi (EPF) is the modern approach as an alternative to chemical control (Han et al. 2014a, b; Verma et al. 2007). In the biological control approach, EPFs are applied in a lesser amount in pre-harvesting season, (in case of papaya, it is pre-monsoon season) expecting flourishment of growth whenever their natural enemy of pest appears in the field. This mycopesticide/mycoinsecticide is supposed to prevent the aphid pest to grow, multiply (ovipary or vivipary) (Ogawa and Miura 2014) and thereby preventing the capacity of carrying and transferring PRSV virus by means of primary (direct transmission by feeding) and secondary transfer method (flying

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to another healthy plant) (Ng and Perry 2004). Use of indigenous fungal isolate is very helpful against various pests, as EPFs are predominant in that particular geographical niche. Long-term control is the best strategy to prevent further pest attack. The pest population should be controlled below the economic threshold level, by using EPF as a bio-control tool.

The objectives of our present study are to isolate and characterize indigenous entomopathogenic fungi, to find out entomopathogenic potentiality, to compare antagonistic potentiality among isolates and to find out the mechanism of action against *A. gossypii* under lab condition.

Materials and methods

Collection of samples

Soil samples were collected by discarding the topsoil up to one inch in a randomized manner from different rhizospheric soils of agrifields of North 24 Parganas district during the pre-monsoon time mixed them well and then brought to the laboratory in a biodegradable polythene bag and shade dried, grinned and sieved (2 mm pore size) (Sharma et al. 2012). Insect cadavers collecting from different zones of this district were used for isolation of entomopathogenic fungi.

Isolation and purification of fungi

Soil samples were then used for isolating fungi using serial dilution technique (Dhingra and Sinclair 1985) and by an insect bait method (Zimmermann 1986). Cadavers were surface sterilized with 75% ethanol for 30 s and then subjected to washing with sterilized water, followed by blotting the extra water in blotting paper, cut into small pieces, placed in Petri dishes containing Potato Dextrose Agar (PDA HiMedia-GM096) medium (pH 6.8). Plates were supplemented with 0.01% Rose Bengal and Streptomycin to prevent the growth of unwanted fastidious fungi and bacteria respectively. Purification of fungal isolates was done by transferring a hyphal tip from freshly growing single colony to a new PDA containing plate and incubated at 28 ± 2 °C. Purified fungal cultures were re-transferred in PDA slant and preserved at 4 °C for the further experimental purpose. Plates were kept in BOD incubator at 28 ± 2 °C for significant growth coverage.

Spore suspension preparation

All fungal genera isolated were applied for primary screening. Fungi were grown in Erlenmeyer flask containing wheat grain medium and kept in the incubator at 28 ± 2 °C for total medium coverage and sporulation. After relevant days of incubation 40 ml of sterilized distilled water was poured and vortex vigorously to detach the spores from hyphae/conidia. Water suspension containing spores were collected in a separate sterilized capped centrifuge tube (15 ml). Higher spore concentration was obtained by short spinning (1000 g for 3 min) in the centrifuge. Spore concentration was measured by haemocytometer and adjusted to 1×10^4, 10^5, 10^6, 10^7 and 10^8 spores ml^{-1}.

Screening of fungal isolates against papaya aphid

Incised Leaf Disk Method (ILDM) was adopted here (Ghosh et al. 2014). In brief, sterilized glass Petri dishes (9 cm diameter) were taken and sterilized Whatman filter paper (no. 1) soaked with sterile distilled water was placed. Fresh healthy papaya leaves were cut into pieces and surface sterilized with 3% Sodium hypochlorite (NaOCl) solution followed by twice of washing with sterilized distilled water. Leaves were placed on the filter paper (dorsal part down) and served as feed for test insect. *Aphis gossypii* Glover (3–4th instar), collected from fully grown infected papaya plant leaf (North 24 Parganas, West Bengal), were surface sterilized with 3% Sodium hypochlorite (NaOCl) solution followed by twice washing with sterilized distilled water and ten aphids were placed into each Petri dish containing papaya leaves and sterilized Whatman filter paper (no. 1).

One milliliter spore suspension (1×10^7 spore's ml^{-1} concentration) of each tested fungal isolates was spread uniformly on each Petri dish by a small glass chromatographic sprayer. The control sets were sprayed with sterile distilled water. The experiment was conducted at room temperature.

Aphid mortality was recorded by counting dead insect daily until total fatality. Dead aphids were further taken out to investigate fungal growth under the microscope. Only aphids which exhibited fungal sporulation were considered to have died from the fungus treatment. Entomopathogenecity of EPFs was graded on the basis of the mortality of the aphids. Best isolates were selected for further screening.

After primary screening an I–IV grade was constructed like Grade I = 70–100% mortality; Grade II = 40–50%; Grade III = 10–30% and Grade IV = non-effective.

Identification of selected entomopathogen by the cultural and morphological method

The fungal isolates were identified by cultural characteristics and microscopic morphological characters (Domsch et al. 1980; Nagamani et al. 2006; Humber 2005). For the microscopic study, fungal mycelium and spores were stained with a Lactophenol-Cotton-Blue solution (HiMedia-S016) on a glass slide and observed under bright field compound microscope (40X) (Olympus-CX31).

Determination of LD_{50} and LT_{50}

EPF treatment was done to test the mortality rate of aphid. The three top graded isolates which showed best entomopathogenic activity were cultured and their spore suspensions were prepared (1×10^4, 10^5, 10^6, 10^7, 10^8 spores ml^{-1}). Previous Incised Leaf Disc technique was followed here in triplicate along with control set and means value was taken. Comparative regression analysis was carried out using SPSS (v. 21). Different treatments of normally distributed data were compared by using one-way ANOVA. In addition, which isolates were shown the highest mortality in which time duration, was also evaluated.

Linear regression is a way to model the relationship between two variables. Finding a linear regression equation is to determine if there is a relationship between the two variables. Regression analysis was done by Probit method to determine the mortality, based on the formula of straight line equation [$Y = aX + b$]. Here X is the dependent variable (that's the mortality rate that plotted on the X-axis), Y is the independent variable (the spore concentration, plotted on the Y-axis), "a" is the slope of the line and "b" is the X-intercept (Abbott 1925, Gomez and Gomez 1984). Median Lethal Dose (LD_{50}) and Median Lethal Time (LT_{50}) (Han et al. 2014a, b) were estimated aftermath by using SPSS (v.21) and Microsoft Office Excel 2013. On the other hand, LT_{50} was determined using LD_{50} value as standard dosage. The regression equation was derived using Microsoft Office Excel 2007.

Molecular characterisation by (ITS1-5.8S-ITS2) PCR based technique

Genomic DNA of these three fungi was extracted by a modified CTAB method (Chutima et al. 2011), purified and visualized under gel electrophoresis (1% agarose). These internal transcribed spacers (ITS1-5.8S-ITS2) regions were amplified by PCR technology using DNA amplification reagent kit (GeNei) with the help of fungus-specific forward primer ITS-1(5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns 1993) and the reverse primer ITS-2(5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The amplified PCR product was purified by DNA purification kit (HiMedia), and run on 1.2% agarose gel Sequencing of PCR products was carried out in Sci Genome Labs Pvt. Ltd., Kerala, India. These sequences were analyzed by homology searching (BLAST) against the Gene Bank nucleotide database (http://blast.ncbi.nlm.nih.gov) and the most similar sequences were selected to compare with reference sequences from the online bioinformatics tools of Gene Bank from NCBI. The sequences obtained were submitted to NCBI Gene Bank to obtain the Accession numbers.

Establishment of a phylogenetic relationship

Sequence homology searched by putting nucleotide sequences (considered as FASTA format) in n-BLAST within NCBI online search tool for each isolate. Fungal species were determined by the highest similarity of ITS sequences to known strains. For each fungal isolate, highest similar strain (with accession number) was used as a reference sequence to our subject strain to determine evolutionary likeliness (Lv et al. 2011; Kumar et al. 2016) the maximum alike sequence has been taken as a reference organism and their accession number was noted (Nei and Kumar 2000).

Sequence alignment was done with the help of MEGA 7 software by Muscle alignment method. Maximum parsimony phylogenetic tree of these genera was constructed using the UPGMA method with the help of MEGA 7 software (Tamura et al. 2013). Tajima's relative rate test (Tajima 1993) was done to establish the divergence of sequence distribution among these isolates.

Interaction of entomopathogenecity

To find out the interaction of entomopathogenecity, Incised Leaf Disk Method (ILDM) as described earlier was performed and critically examined under light compound microscopy (Olympus-CX31) for host-parasite interaction.

Results and discussion

Total 40 isolates have been isolated from the rhizospheric soil (15 cm below soil surface, near the root zone) and insect cadaver. Out of 40 isolates, we have found 12 genera as per our phenotypical identification. Out of them, *Trichoderma* has 11 isolates, *Aspergillus* 9 isolates and *Penicillium* 8 isolates (Table 1). Out of 40 isolates, 20 isolates have shown entomopathogenic property against papaya aphid (3–4th instar) under laboratory condition. Out of them, three fungal isolates (ded4, Nlg1 and khr4) showed potent entomopathogenic activity (Table 1).

The detail cultural and morphological characteristics of the three profound fungal isolates of entomopathogens against aphid were presented in Table 2 and Fig. 1 and they were phenotypically identified as *Beauveria* sp (deb4), *Penicillium* sp. (Nlg1) and *Fusarium* sp. (khr4). These three isolates were identified molecularly.

Molecular characterization

Figure 2a showed the DNA bands under UV-transilluminator. DNA band of each EPFs were near 3 kb comparing with DNA ladder. The PCR product showed band near 650 bp under UV-transilluminator (Fig. 2b).

Table 1 Screening of EPF isolates against 3–4th instar papaya aphid

Serial number	Isolate number	Fungus genera	Mortality percentage of aphid at 10^7 spores/ml (48 h)	Entomopathogenic effect against aphid nymph	Grade of entomopathogenecity
1.	A 1	*Cunninghamella* sp.	10	+	Grade III
2.	A 2	*Aspergillus niger*	0	−	Grade IV
3.	A 3	*Penicillium* sp.	0	−	Grade IV
4.	A 4	*Mucor* sp.	0	−	Grade IV
5.	A 5	*Penicillium simplicissimum*	0	−	Grade IV
6.	A 6	*Aspergillus* sp.	0	−	Grade IV
7.	A 7	*Trichoderma asperellum*	30	+	Grade III
8.	A 9	*Aspergillus* sp.	0	−	Grade IV
9.	A 10	*Penicillium chrysogenum*	0	−	Grade IV
10.	A 11	*Trichoderma* sp.	0	−	Grade IV
11.	A 14	*Trichoderma harzianum*	50	++	Grade II
12.	A 15	*Cladosporium tenuissimum*	40	++	Grade II
13.	A 16	*Paecilomyces liacinous*	50	++	Grade II
14.	A 17	*Verticillium sp.*	30	+	Grade III
15.	A 18	*Trichoderma asperellum*	0	−	Grade IV
16.	A 20	*Verticillium* sp.	50	++	Grade II
17.	A 21	*Aspergillus heteromorphus*	50	++	Grade II
18.	Nlg1	*Penicillium* sp.	70	+++	Grade I
19.	A 25	*Penicillium pulvillorum*	40	++	Grade II
20.	A 26	*Trichoderma* sp.	0	−	Grade IV
21.	A 27	*Penicilliumcitrinum*	40	++	Grade II
22.	A 28	*Aspergillus* sp.	0	−	Grade IV
23.	A 29	*Aspergillus* sp.	0	−	Grade IV
24.	A F1	*Fusarium oxysporium*	0	−	Grade IV
25.	A Ver	*Verticillium* sp.	20	+	Grade III
26.	M3	*Trichoderma* sp.	20	+	Grade III
27.	A M8	*Metarhizium* sp.	30	+	Grade III
28.	A T1	*Trichoderma* sp.	0	−	Grade IV
29.	deb4	*Beauveria* sp.	80	+++	Grade I
30.	T13C	*Trichoderma harzianum*	50	++	Grade II
31.	khr4	*Fusarium* sp.	60	+++	Grade I
32.	A Met	*Metarhizium* sp.	30	+	Grade III
33.	A H1	*Aspergillus tamarii.*	0	−	Grade IV
34.	A H2	*Trichoderma asperellum*	0	−	Grade IV
35.	A H3	*Aspergillus* sp.	0	−	Grade IV
36.	A K1	*Penicillium camemberti*	40	++	Grade II
37.	A K2	*Trichoderma* sp.	0	−	Grade IV
38.	A K3	*Penicillium simplicissimum*	50	++	Grade II
39.	A K4	*Aspergillus* sp.	0	−	Grade IV
40.	A K5	*Trichoderma asperellum*	0	−	Grade IV

"−" indicates not effective (Grade IV), "+" = less effective (Grade III), "++" = more effective (Grade II)) and "+++" = most effective (Grade I)

PCR amplified products were sent for sequencing in Eurofins Genomics India Private Limited, Bengaluru, India. Obtained FASTA sequences were searched for homology by BLAST in NCBI nucleotide database. *B. bassiana* isolate deb4 (567 nucleotides) showed the highest similarity with *B. bassiana* (Accn. No. AJ560690.1) and identified as same species. Similarly, *P. verrucosum* Nlg1 isolate (607 nucleotides) and *F. equiseti* khr4 isolate (548 nucleotides) showed the highest similarity with *P. verruculosum* (Accn. No. HM469420.1) and *F. equiseti* (Accn. No. KY523100.1)

Table 2 Cultural and microscopical characteristics of isolates

Serial no.	Isolate name	Cultural characteristics	Microscopical characteristics	Identified genus
1	deb4	White radiating colony, no exudates forms (Fig. 1 a).	Hyphae thin, much branched, measured 3–6 µm. Grouped on conidiogenous cells. The conidiogenous cells are short and ovoid, terminate in a narrow apical extension called a rachis, measured 4–5 µm. The rachis elongates and results in a long zig-zag extension. Conidia are single-celled, round to elliptic, 2–3 µm in diameter (Fig. 1d)	*Beauveria*
3	Nlg1	Bluish green compressed colony (Fig. 1b)	Conidiophores borne singly from substrate, hyphae measured 3–5 µm, the wall is thin and smooth, typically bearing irregular penicillus. Penicillus contains rami, metulae phialide and conidia. The rami is smooth, vary in 5–7 µm; metulae is in verticals of 2–3 and ranges 5–6 µm; phialide measures 3–4 µm and each is cylindrical; Conidia are born in distorted chain spherical to sub spherical ranging 3–5 µm (Fig. 1e)	*Penicillium*
3	khr4	Grayish white colony (Fig. 1c)	Conidiophores branched; 3–4 µm. Conidia are of two types. Microconidia usually abundant, mostly aseptate, oval, produced on simple lateral phialides, free from conidiophores never form in chains 4–6 µm; Macroconidia are 2–4 septate, slightly curved ranging 2–3 µm (Fig. 1f)	*Fusarium*

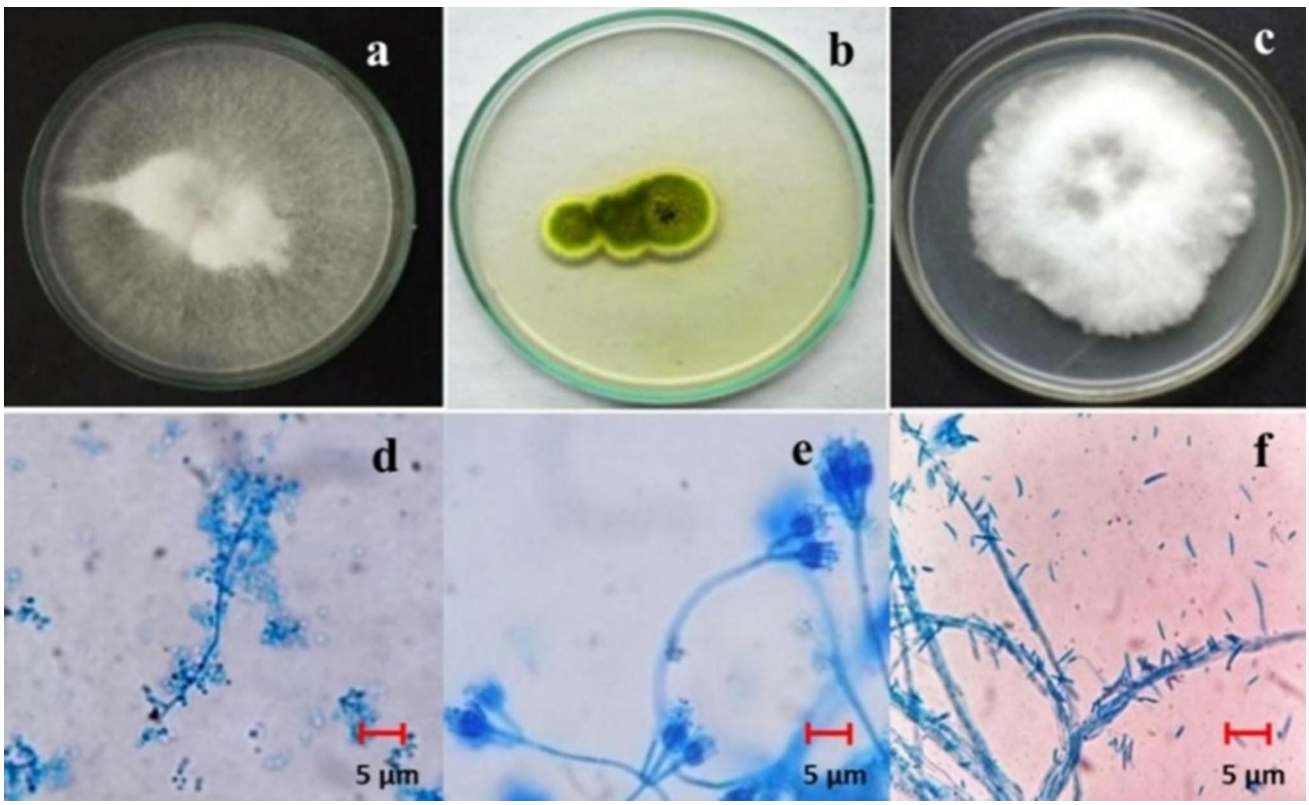

Fig. 1 Colony morphology and their corresponding microscopic view of isolates under compound microscope. *Beauveria bassiana* (**a**, **d**), *Penicillium verrucosum* (**b**, **e**) and *Fusarium equiseti* (**c**, **f**) (× 40 magnification). Scale bar 5 µm

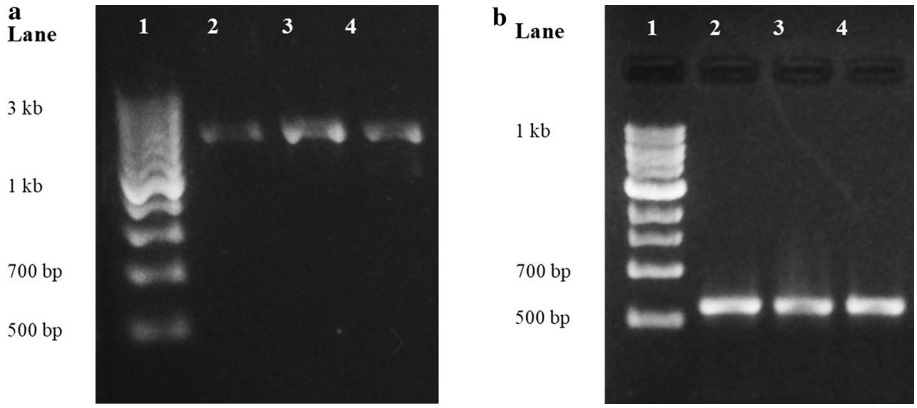

Fig. 2 Visualization of DNA bands by agarose gel electrophoresis. **a** Band of interest under UV-transilluminator. From the left 3 kb Ladder (Lane 1) and genomic DNA of *Beauveria* sp. (Lane 2), *Penicillium* sp. (Lane 3), *Fusarium* sp. (Lane 4). **b** Banding pattern of PCR product from ITS regions. From the left 1 kb ladder (Lane 1) and PCR product from *Beauveria* sp. (Lane 2), *Penicillium* sp. (Lane 3), *Fusarium* sp. (Lane 4)

respectively. These sequences were submitted to NCBI to get the accession number and *P. verrucosum* has got the Accession number KY966028.1 so far.

Construction of phylogenetic tree and determination of the evolutionary relationship

B. bassiana (deb4) query sequence was compared with the most related isolate having Accession number AJ560690.1. *P. verrucosum* (Nlg1) has reference isolate having accession number HM469420.1. *F. equiseti* (khr4) was compared with isolate accession number KY523100.1.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 1.16130728 is created. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to conclude the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 6 nucleotide sequences. All positions comprising gaps and missing data were rejected. There were a total of 446 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013) (Fig. 3). Result specifies that, regardless of their genus distinction, the conserved ITS1-5.8s-ITS2 region of these three fungi have significant sequence similarity.

The equality of evolutionary rate between sequences A (*B. bassiana* deb4) and (*P. verrucosum* Nlg1), with sequence C (*F. equiseti* khr4) used as an outgroup in Tajima's relative rate test (Table 3) (Tajima 1993). The χ^2 test statistic was 92.47 (P = 0.00 with 1 degree of freedom). The P value less than 0.05 used to reject the null hypothesis of equal rates between lineages. A total of 480 positions calculated in the final dataset. These analyses were conducted in MEGA7 (Kumar et al. 2016).

Bio-assay and mortality of aphid by EPF

Data obtained from Fig. 4 showed the different concentrations of spore suspension (1×10^4 to 1×10^8) of three EPFs, have different effects on aphids. Ten aphids were tasted

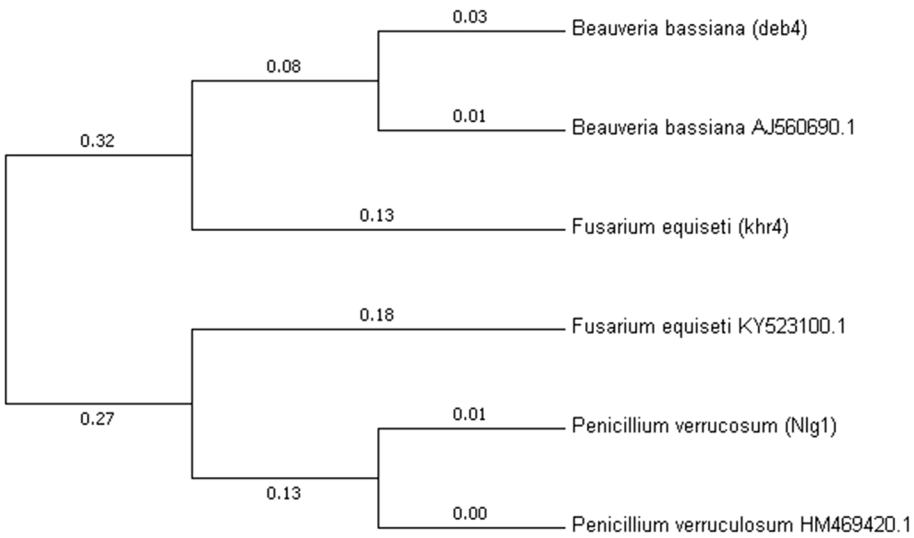

Fig. 3 Evolutionary relationships of taxa

Table 3 Tajima's relative test for 3 Sequences

Serial no.	Sites/differences	Number of nucleotide
1	Identical sites in all three sequences	194
2	Divergent sites in all three sequences	32
3	Unique differences in sequence A	37
4	Unique differences in sequence B	178
5	Unique differences in sequence C	39

under lab condition for each isolate and their respective spore concentration. Figure 4 clearly showed that *B. bassiana* had the highest effectiveness followed by *P. verrucosum* and *F. equiseti*. Some of the isolates showed high fatality against papaya aphid, others showed minimal to null effect (Martinuz et al. 2012; Mishra et al. 2013).

Regression analysis and evaluation of LD_{50}

The results presented in Table 4 clearly explained that *B. bassiana* was diagnosed with lowest LD_{50} value (1.4×10^4 spores ml^{-1}) followed by *P. verrucosum* (9.8×10^4 spores ml^{-1}) and *F. equiseti* ($1.0 \times 10^6 ml^{-1}$). Similarly, LT_{50} values were also evaluated (Table 5) and the lowest LT_{50} (32.14 h at 1×10^4 spores ml^{-1}) indicated *B. bassiana* as best EPF followed by *P. verrucosum* (37.5 h at $1 \times 10^5 ml^{-1}$) and *F. equiseti* (32.14 h at 1×10^6 spores ml^{-1}).

A group of scientists (Kim et al. 2013) recorded that *B. bassiana* isolate Bb08 showed LT_{50} in 3.0 ± 0.3 days against green peach aphid. In our laboratory, previously isolated *B. bassiana* (BB1) strain showed ED_{50} value at 10^6 spores ml at 3.2 days after inoculation (Ghosh et al. 2014). Supporting that information, in our present study, *B. bassiana* (deb4) isolate showed the lowest LD_{50} value (1.4×10^4 spores ml^{-1}) on the 2nd day after inoculation. *P. verruculosum* can produce cellulase enzyme that in turn could be used as a potential biocontrol agent against aphids (Morozova et al. 2010). Some scientist (Nicoletti and De Stefano 2000) reported *P. verruculosum*to have potential killing effect against aphids. Some other *Penicillium*. spp. are also capable of killing several pests (Shah and Iqbal 2017; Patil and Jadhav 2015). In our present study, *P. verrucosum* showed significant LD_{50} value i.e., 9.8×10^4 spores ml^{-1} on the 2nd day after inoculation. *Fusarium semitectum* was also used against *Aphis gossypii* as a biocontrol agent (Jayasimha et al. 2013). Our *F. equiseti* isolate has also successfully controlled the *A. gossypii* insect having the LD_{50} value of 1.0×10^6 spores ml^{-1} on 2nd day after inoculation (Table 4).

Host-parasitic interaction

These fungi harbour entomopathogenecity through adhesion, germination, ramification and penetration as shown in Fig. 5. For the mechanical study, EPFs are well known for their capacity to produce several extracellular enzymes and biologically active molecules (Freimoser et al. 2003, Ganassi et al. 2001). Fungal spores first adhere to the cuticle of *Aphis gossypii* and then penetrate the cuticle layer by secreting chitinase, hydrolase etc. (Quesada-Moraga et al. 2006). Thereafter entering the host, they ramify and secrete secondary metabolites (Ortiz-Urquiza and Keyhani

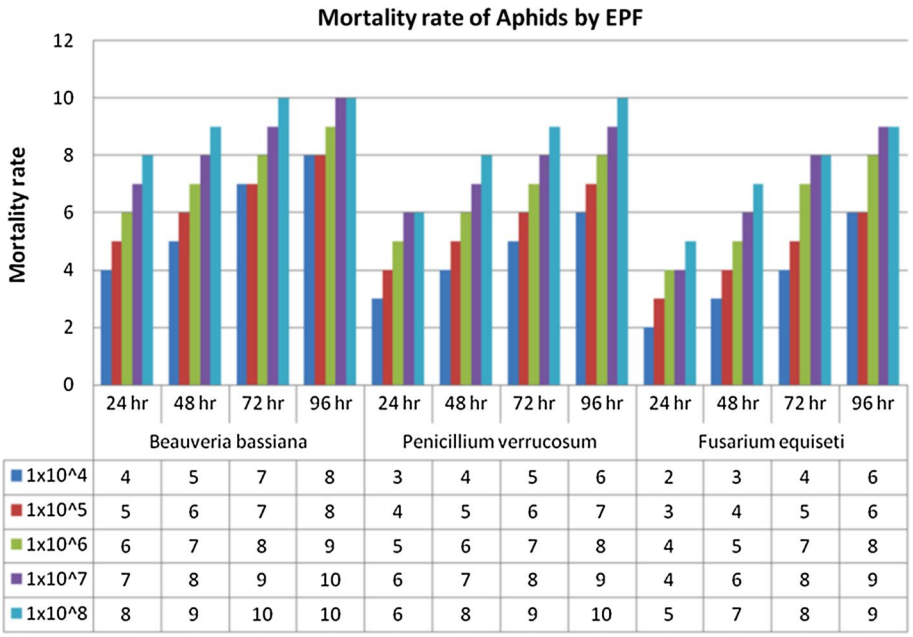

Fig. 4 Mortality of aphids at different spore concentrations and time by different EPF

Table 4 LD_{50} value of *B. bassiana*, *P. verrucosum* and *F. equiseti* on papaya aphid (3–4th instar) at 48 h

Sl no.	Organism name	Spore conc. (spore/ml)	Mortality percentage after 48 h	Log of spore conc. (Y)	Probit mortality (X)	Regression statistics b = X intercept a = slope	Regression equation Y = aX + b	In LD_{50} calculation, Y = 5, LD_{50} = anti-$\log_{10}$X
1	*Beauveria bassiana*	10^4	50	4	5.00	a = 0.315 b = 3.668	Y = 0.315X + 3.688	$\mathbf{1.4 \times 10^4}$
		10^5	60	5	5.25			
		10^6	70	6	5.52			
		10^7	80	7	5.84			
		10^8	90	8	6.28			
2	*Penicillium verrucosum*	10^4	40	4	4.75	a = 0.270 b = 3.652	Y = 0.27X + 3.652	$\mathbf{9.8 \times 10^4}$
		10^5	50	5	5.00			
		10^6	60	6	5.25			
		10^7	70	7	5.52			
		10^8	80	8	5.84			
3	*Fusarium equiseti*	10^4	30	4	4.48	a = 0.258 b = 3.452	Y = 0.258X + 3.452	$\mathbf{1.0 \times 10^6}$
		10^5	40	5	4.75			
		10^6	50	6	5.00			
		10^7	60	7	5.25			
		10^8	70	8	5.52			

Table 5 LT_{50} value of *B. bassiana, P. verrucosum* and *F. equiseti* on papaya aphid (3–4th instar) at their LD_{50} concentration

Serial no.	Organism name	Aphid mortality time (h) (X)	Mortality percentage (Y)	Regression statistics b = X intercept a = slope	Regression equation Y = aX + b	LT_{50} calculation, Y = 50 X = LT_{50} (h)
1	*Beauveria bassiana*	24	4	a = 0.58 b = 32	Y = 0.58X + 32	32.14
		48	5			
		72	7			
		96	8			
2	*Penicillium verrucosum*	24	4	a = 0.4 b = 35	Y = 0.4X + 35	37.5
		48	5			
		72	6			
		96	7			
3	*Fusarium equiseti*	24	4	a = 0.58 b = 32	Y = 0.56X + 32	32.14
		48	5			
		72	7			
		96	8			

2013; Han et al. 2014a). Surface interaction and subsequent penetration, germination of these fungi on aphid cuticle have also established by the microscopical study as revealed in an earlier study (Ortiz-Urquiza and Keyhani 2013; Ghosh et al. 2014). Our mechanical studies by microscopical observations are assertive as par the other workers. In brief, these three EPF isolates also exhibited attachment and penetration of aphid cuticle, observed under a compound microscope. The details mechanical studies on respect to fungus insect interaction were done by few workers and a correlation between toxin secretion and reduction of PPO, which is responsible for insect immunity, has been recorded by some authors (Binggeli et al. 2014; Karthikeyan and Selvanarayanan 2011; Podder and Ghosh 2019).

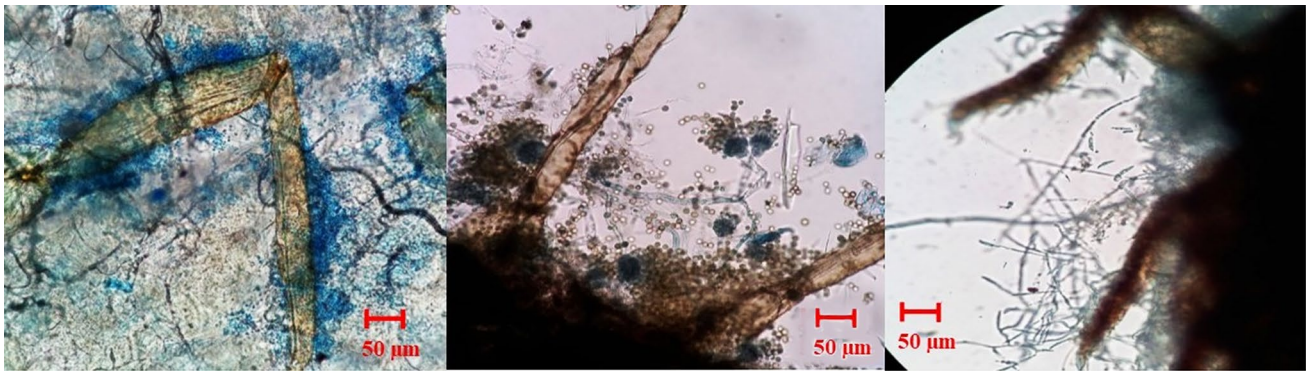

Fig. 5 Interaction of four EPF *Beauveria bassiana* (**a**), *Penicillium verrucosum* (**b**), *Fusarium equiseti* (**c**) with *Aphis gossypii* under compound microscope (× 10 magnification). Scale bar 50 µm

Conclusions

Among 40 isolates of different fungi, 3 isolates (*B. bassiana* deb4, *P. verrucosum* Nlg1 and *F. equiseti* khr4) were best effective against papaya aphid. *B. bassiana* (deb4) isolate was most pathogenic against *A. gossypii* on the 2nd day after inoculation and this may be tested under field condition for biological control of this pest. The mechanistic study showed that these fungal EPFs produced spores and these spores germinated on aphid surface and by penetration directly to kill the host. Moreover, as per phylogenetic tree analysis, it showed that these three EPFs had a close ancestral relationship, whereas Tajima's relative rate test of sequences showed significant diverseness among them.

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