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RESEARCH

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Alternaria alternata strain VLH1: a potential entomopathogenic fungus native to North Western Indian Himalayas

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Abstract

Background: The inadvertent observation of a substantial population reduction of greenhouse whiteflies infecting *Salvia divinorum* plants grown in a polyhouse sparked a flurry of inquiries on the cause of the population decline. The entomopathogenic fungus (EPF) (*Alternaria alternata* strain VLH1) infecting greenhouse whitefly on *S. divinorum* plants was isolated and morphologically and molecularly characterised using multilocus sequence typing.

Results: The fungus was found to be highly virulent against sucking pests; with LC_{50} values ranging from 1.7×10^4 to 2.5×10^6 spores per ml for the Mustard aphid (*Lipaphis erysimi* Kaltenbach) and soybean sucking bug (*Chauliops choprai* Sweet and Schaeffer), respectively. In the lepidopteran larvae treated with a concentration of 3×10^5 spores per ml, the fungus induced developmental abnormalities such as aberrant larval to pupal moulting, defective pupae, and deformed adults. Pathogenicity studies on the two beneficial insects (*Coccinella septempunctata* (Linn.) and *Apis mellifera* L.) and 11 host plants revealed no disease signs, indicating that it is safe for use in pest management in hill agriculture. The chitinolytic activity of the fungus and its crude protein extracts was reported in studies conducted against target insect pests, with the highest chitinase enzyme production (117.7 U/ml) on the fourth day of inoculation. Furthermore, over a 96-h period, third instar *Helicoverpa armigera* (Hubn.) larvae fed on a protein fraction-amended artificial diet showed a significant decrease in nutritional physiology indices such as relative growth rate, relative consumption rate, efficiency of ingested food conversion, efficiency of digested food conversion, and approximate digestibility. Moreover, the polyhouse and open-field studies against two sucking pests; *Myzus persicae* (Sulz.) infesting capsicum in polyhouse and *L. erysimi* infesting Indian rapeseed in open-field conditions showed, 81.14% and 63.14% mortality rates, respectively, at 3×10^7 spore/ml concentration.

Conclusions: Entomopathogenic fungus (EPF) was reported to be an effective biocontrol agent, which caused direct mortality in sucking pests to developmental abnormalities in lepidopteran insects. Despite positive findings in in vitro and in vivo bioassay investigations against various insect pests, the fungus still has to be inspected before it can be used on a broad scale for biological pest management.

Keywords: *Alternaria alternata*, Hemipteran insects, Chitinolytic activity, Nutritional physiology indices, Indian Himalayas

Background

The traditional form of crop-livestock agriculture and organic farming are the prima facie of hill agriculture in the Indian Himalayas from time immemorial (Behera et al. 2012). This type of traditional agriculture has not

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only increased pest population density over time, but has also resulted in significant economic losses in hill agriculture during pest outbreaks (Negi and Palni 2010). The growing demand for organic products grown in the hills among metropolitan centres has paved the way for Himalayan farmers to use environmentally friendly pest management measures that are compatible with organic farming systems (Stanley et al. 2022). It has become critical for researchers to develop fresh environmentally friendly pest management methods that are both socio-economically and culturally acceptable to Himalayan marginal and small farmers (Stanley et al. 2022). In the Himalayas, there is currently no recommended, effective, and widely accepted biological pest management solution. As a result, the hunt for a native, highly virulent, and self-sustaining biological control agents still persists (Paschapur et al. 2019).

Fungi, for example, are microbial biocontrol agents with the potential to be an effective pest control agent as well as a best alternative to traditional, hazardous insecticides (Quesada-Moraga et al. 2006). *Beauveria bassiana* (Bals.-Criv.) (Hypocreales: Clavicipitaceae), *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae), *Nomuraea rileyi* (Farlow) Kepler (Hypocreales: Clavicipitaceae), and others are some of the most well-known EPFs in the world (McKinnon et al. 2018). Apart from these, a few fungi, such as *Alternaria*, are known to be saprophytic, plant pathogenic, and in some circumstances, entomopathogenic (Poitevin et al. 2018).

Alternaria alternata (Fr.) Keissl (Pleosporales: Pleosporaceae) is a saprophytic and well-known plant pathogenic fungus that causes serious diseases in fruits and vegetables during their post-harvest shelf life. However, several authors, on the other hand, have reported *A. alternata*'s EPF activity against a variety of insect pests. Christias et al. (2001) isolated a new pathotype of *A. alternata* that is highly virulent against large number of aphids. In the studies of Mckinnon et al. (2018), they isolated the EPF *A. alternata* from the rhizosphere zone of maize crops, which was capable of infecting insect pests of maize under laboratory conditions. Mehrmoradi et al. (2020) found the EPF *A. alternata* in organic fruit orchard soils in Switzerland and Iran, respectively, and proved their toxicity against a range of insect pests infecting fruit crops.

From the previous studies carried out by several authors on isolation of *A. alternata* from the dead insect cadavers, qualitative assessment of toxicity of the fungus, laboratory bioassays, and ecological studies, cleared that the fungus was a potential biocontrol agent against range of insect pests belonging to Hemipteran, Coleopteran, and Lepidopteran orders. Furthermore, the fungal isolates were very specific to a specific group of insects, with a very little cross-infectivity (Christias et al. 2001). However, in recent years, the attention has switched to utilising *A. alternata* endophytic nature (Kaur et al. 2019), as well as the proteinaceous poisons produced by the fungus for infecting and killing insects (Yang et al. 2012). However, little emphasis was placed on isolating the fungi's protein component, testing its toxicity against insect pests, elucidating its mode of action, and exploiting the fungi's biocontrol potential in open-field conditions. Therefore, the present study was designed to morphologically and molecularly characterise (EPF *A. alternata* VLH1), study its ecological factors, test the fungus' cross-infectivity on a variety of insect pests, confirm its non-target effects on a variety of host plants and beneficial insects, test the fungus' chitinolytic activity and protein extracts to characterise its mode of action against insect pests, as well as examine the potential of *A. alternata* VLH1 for its exploitation under polyhouse and open-field conditions.

Methods

Isolation of the fungus and proving Koch's postulate

The greenhouse whitefly, *Trialeurodes vaporariorum* Westw. (Hemiptera: Aleyrodidae) infecting *Salvia divinorum* Epling & Jativa, 1962 (Lamiaceae: Lamiales) plants was found to be infected with an EPF at Experimental farm, Hawalbagh, ICAR-VPKAS (Vivekananda Parvatiya Krishi Anusandhan Sansthan), Almora, Uttarakhand, India (29.63° N and 79.63° E, 1250 amsl). Dead cadavers were collected from the field and transported to laboratory under aseptic conditions. To avoid bacterial contamination, the dead cadavers were surface sterilised with 70% ethyl alcohol and inoculated on potato dextrose agar (PDA) medium (Himedia labs Ltd. India) supplemented with 0.30 mg/l chloramphenicol. To obtain a pure single spore colony, a spore concentration of 1 spore/µl of double-distilled water was prepared by serial dilution (10^{-6}) and the spore suspension was inoculated on PDA media. After 24 h, single actively growing conidium was transferred to new PDA media and incubated at 27 ± 2 °C for 8–10 days. Furthermore, the pure conidia collected from the dead cadaver were analysed morphologically and molecularly (multilocus sequence typing). To test Koch's postulate, the pure culture was multiplied on potato dextrose broth and the conidia obtained were filtered through a double layered sterile muslin cloth. The conidial concentration of 1×10^8 conidia per ml was prepared by using Neubauer's haemocytometer and sprayed topically on tomato plants (VL tamatar-4 variety) infected with *T. vaporariorum* nymphs and adults under polyhouse condition. The dead cadavers of *T. vaporariorum* infected with the fungi were collected with a sterile forceps; surface sterilised with 70% ethyl alcohol and

inoculated on PDA medium. To confirm the species and prove Kochs' postulate, the fungal culture was morphologically and molecularly compared with the mother culture (Accession number MN704637, showed 100% similarity with mother culture of *A. alternata* VLH1 MN704636).

Morphological and molecular characterisation of the fungus

The spore and colony characters were morphologically characterised with the help of several taxonomic references (Goettel and Inglis 1997; Tzean 1997; Woudenberg et al. 2015). For molecular characterisation, the DNA was isolated from both reproductive conidia and vegetative mycelium by modified CTAB method developed by Subbanna et al. (2019). The three gene fragments: ITS (internal transcribed spacer region), GAPDH (glyceraldehyde 3 phosphate dehydrogenase), and LSU (large subunit ribosomal RNA gene), were amplified using primer pairs ITS1/ITS4 (White et al. 1990), gpd1/gpd2 (Berbee et al. 1999), and LR5/LROR (Schoch et al. 2009), respectively, in a thermo-cycler (Eppendorf Mastercycler V ProTM). The PCR amplicon thus obtained was purified and sequenced with an automated DNA sequencer (ABI 377), using Big Dye terminator kit (Applied Biosystems) as per manufacturer's instructions. BLASTN was followed to compare the acquired sequences to the ITS, LSU, and GAPDH sequences in the NCBI GenBank database. Using the Molecular Evolutionary Genetics Analysis (MEGA X) sequence alignment software, the available sequences were assembled and aligned. The aligned DNA sequences of *A. alternata* VLH1 (ITS, LSU, and GAPDH) have been deposited in NCBI GenBank (http://www.ncbi.nlm.nih.gov). With 1,000 bootstrap repetitions, MEGA X software was utilised to perform phylogenetic analysis for concatenated genes (Kumar et al. 2018). The reference sequences for the ITS, GAPDH, and LSU genes were retrieved from NCBI GenBank and included in the tree. The node support and the confidence level of each branch were estimated, using 1000 bootstrap pseudo-replicates generated with random seed.

Laboratory bioassays and estimation of median lethal concentrations

A leaf dip bioassay was carried out with seven spore concentrations of *A. alternata* VLH1 (10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 spore/ml) and control. Ten individuals of each insect species (adult stage for hemipterans and larval stage for lepidopterans) were released in separate Petri dishes, with the experiment being replicated three times. The following insects were used for the study; cabbage aphid (*Brevicoryne brassicae* (Linnaeus, 1758) (Hemiptera: Aphididae)), nymphs and adults of greenhouse whitefly (*T. vaporariorum*), green peach aphid (*Myzus persicae* (Sulzer) (Hemiptera: Aphididae)), soybean seed bug (*Chauliops choprai* Banks (Hemiptera: Malcidae)), mustard aphid (*Lipaphis erysimi* (Kaltenbach, 1843) (Hemiptera: Aphididae)), wheat and barley aphid (*Sitobion avenae* (Fabricius, 1794) (Hemiptera: Aphididae)), soybean aphid (*Aphis craccivora* Koch, 1854 (Hemiptera: Aphididae)), greater wax moth (*Galleria mellonella* Linnaeus, 1758 (Lepidoptera: Pyralidae)) (Diet surface contamination technique), Bihar hairy caterpillar (*Spilarctia oblique* (Walker. 1855) (Lepidoptera: Arctiidae)), tomato fruit borers (*Helicoverpa armigera* (Hübner, (1808)) (Lepidoptera: Noctuidae) and *S. litura*). The non-target effect of the fungus was tested on seven spotted lady bird beetles (*Coccinella* septempunctata (Linnaeus, 1758) (Coleoptera: Coccinellidae)) and European honey bees (*Apis mellifera* Linnaeus, 1758 (Hymenoptera: Apidae)). The treated insects' mortality was recorded every 24 h up to 96 h and the mortality values were corrected using Abbott's (1925) formula. Hemipteran insects with brownish to blackish coloration and abundant mycelial development on deceased cadavers were deemed dead and the fungal mycelia and conidia were observed under a compound microscope and compared to mother culture of *A. alternata* VLH1. The collected data were subjected to probit analysis (Finney 1971) using the PoloPlus software package. Furthermore, the changes in the growth patterns and days required for consequent moulting of four lepidopteran pests throughout their life cycle (larvae, pupae, and adults) were also recorded and the data were subjected to a Tukey's-B test at 1% level of significance.

Testing chitinolytic activity of the fungus

Colloidal chitin was prepared from commercial chitin flakes using the procedure developed by Berger and Reynolds (1988). PDA media was prepared at half strength (50%) and supplemented with 1% colloidal chitin. *A. alternata* VLH1 pure fungal culture was inoculated on the plates and cultured for 4–5 days at 27 ± 2 °C and 65 ± 5% relative humidity. Following the incubation period, a fungal colony with a visible halo was observed on the plates, indicating that the fungus was capable of degrading chitin.

Infectivity of *A. alternata* VLH1 to host plants

The procedure developed by Sharma et al. (2012) was used in laboratory studies, while the procedure developed by Christias et al. (2001) was used for field level study for examining the infectivity of *A. alternata* VLH1 to various host plants (cabbage and cauliflower, capsicum, tomato, French bean, soybean, Indian rapeseed, maize, paddy, wheat, and Salvia were used as positive control). In laboratory assays, a total of 10 ml spore

suspension of 3×10^5 spores per ml of water was prepared with Neubauer's haemocytometer and four spots of 10 µl each of the suspension were inoculated equidistantly on individual leaf of 25 cm^2 (5 × 5 cm) area and the experiment was replicated five times. However, in field assays a total of 100 ml fungal spore suspension of 3×10^5 spores per ml water was sprayed over 20 host plants of 30 days old, grown in pots under polyhouse condition. Further, the plants were covered by polythene bags to create 100% relative humidity for 48 h. In both the studies, the treated leaves and plants were incubated at 25 ± 2 °C temperature, 75 ± 5% RH, and photoperiod of 16 h light and 8 h dark. In control pots, double-distilled water was sprayed up to runoff. In laboratory investigations, observations for disease development were made every 24 h for up to 7 days, while in field studies, observations were made after every 24 h for up to 21 days.

Isolation of crude proteins from *Alternaria* cultures

The crude protein extract was isolated from one-, two-, three-, four-, and five-day-old *Alternaria* cultures using the ammonium sulphate precipitation method established by Wallet and Provost Laboratory (DragonTech, a biotechnology services company). The fungal culture was inoculated with 100 µl of 3×10^5 spore suspension of *A. alternata* VLH1 on five consecutive days and incubated at 25 ± 2 °C on 100 ml potato dextrose broth (PDB) medium and at relative humidity of 65 ± 5% and photoperiod of 16 h light and 8 h dark. The protein was precipitated by stirring at room temperature for 15 min after adding solid ammonium sulphate to a final concentration of 85% (56.8 g of ammonium sulphate in 100 ml culture broth) estimated from the table. For 15 min, the precipitated protein was centrifuged at the highest speed. The supernatant was discarded, and the crude protein containing pellet was re-suspended in a 25 mM Tris–HCl buffer and dialyzed extensively at 4 °C against the same buffer. The protein content of the dialyzed crude extract was determined using the Bradford method and stored at − 80 °C for further investigation.

Assay for ascertaining chitinase activity of the crude protein extracts

The chitinase enzyme activity of dialyzed crude protein of *A. alternata* VLH1 was determined using colloidal chitin as a natural substrate at pH 6 in a 50 mM acetate buffer. Equal amounts (250 µl each) of adequately diluted crude protein extract and buffer containing 1% colloidal chitin made up the reaction mixture. After 30 min of incubation at 37 °C, the reaction was stopped by boiling for 10 min in a water bath. The residual colloidal chitin was precipitated by centrifugation at 10,000 rpm for 7 min, and the amount of liberated reducing sugars in the supernatant was calculated using a modified Schales reagent (0.5 g potassium ferricyanide in one litre of 0.5 M sodium carbonate) (Imoto and Yagishita 1971). In brief, 450 µl of supernatant was combined with 600 µl of Schales reagent and boiled for 15 min in a water bath. After cooling, absorbance was measured at 420 nm, and the reducing sugar was calculated using N-acetyl-glucosamine reference curve. The amount of enzyme that released 1 µmol of reducing sugar per min was defined as one unit of enzyme activity.

Identification of chitinolytic activity through protein electrophoresis

To verify the chitinolytic activity of *A. alternata* VLH1 crude protein extract, electrophoresis of crude protein supernatant on a native PAGE gel at 4 °C was performed. In a Bio-Rad mini-protean II cell assembly, 10 g of protein was electrophoresed through a 5% stacking gel and a 10% separating gel at a constant voltage of 100 V for 3–4 h. To visualise the protein bands, the gel was stained for one hour with Coomassie brilliant blue R-250 dye and then de-stained for two hours using a 3% NaCl buffer. The gel was superimposed on a substrate gel composed of 2% agarose and 0.2% colloidal chitin after the clear and evident bands were noticed in the gel. To allow for enzymatic activity, the assemblage was incubated overnight at 37 °C. After incubation, the agarose gel was stained for 15 min with chitin binding fluorescent dye solution (0.01% Calcofluor white M2R in 50 mM Tri HCl (pH 8)) and then de-stained with distilled water for two hours. Under a UV illuminator, the zones of chitinolytic activity were seen and compared to duplicate protein banding patterns that were run simultaneously.

Effect of crude protein extracts on nutritional physiology of *H. armigera*

The influence of protein extracts from *A. alternata* VLH1 on dietary physiology of *H. armigera* third instar larvae (8 days old) was studied using Waldbauer's (1968) gravimetric approach. The experiment was replicated four times with five treatments (four with protein extract amended diets of 5, 10, 20, and 40 ppm, and a control with an un-amended diet). A total of 200 larvae were employed in the investigation, with 10 third instar larvae per treatment were pre-starved for 2–3 h and fed with a known amount of artificial diet (5 g). Larvae were kept in plastic Petri plates (5 cm diameter) and incubated in a BOD incubator for 96 h at 25 ± 2° C and 65 ± 5% relative humidity. The weight of larvae, faeces, and leftover feed were recorded after every 24 h till 96 h and the final values were recorded once after the experiment was completed by drying the materials at 65 °C for 3 days. The

nutritional indicators were calculated using Wheeler and Isman's (2001) formula.

$$\mathrm{RGR} = \frac{\text{Change in larval dry weight/day}}{\text{Initial larval dry weight}}$$

$$\mathrm{RCR} = \frac{\text{Change in diet dry weight/day}}{\text{Initial larval dry weight}}$$

$$\mathrm{ECI} = \frac{\text{Dry weight gain of insect} \times 100}{\text{Dry weight of food ingested}}$$

$$\mathrm{ECD} = \frac{\text{Dry weight gain of insect} \times 100}{\text{Dry weight of food ingested Dry weight of frass}}$$

$$\mathrm{AD} = \frac{\text{Dry weight of food ingested Dry weight of frass} \times 100}{\text{Dry weight of food ingested}}$$

RGR—Relative growth rate, RCR—relative consumption rate, ECI—efficiency of conversion of ingested food, ECD—efficiency of conversion of digested food, AD—approximate digestibility.

Laboratory bioassay of fungal crude protein extracts against aphids

The dialyzed protein treatments with following concentration; 25, 50, 100, 150, and 200 ppm and a control (double-distilled water) was imposed against adults of two aphid pests, mustard aphid (*L. erysimi*), and wheat aphid (*S. avenae*), using a four-day-old *Alternaria* culture. Every 24 h, the treated insects' mortality was reported and corrected using Abbott's (1925) formula. Aphid cadavers with brownish to blackish coloration were deemed dead.

Testing virulence of the fungus under polyhouse conditions

The green peach aphid (*M. persicae*) infecting capsicum plants were chosen as the target insect pest to investigate the infectivity and virulence of *A. alternata* VLH1 under polyhouse conditions. The capsicum (VL Shimla Mirch-3) plants were grown in a fully automated polyhouse and the inoculum of *M. persicae* was artificially inoculated on the plants and the infestation was allowed to grow by creating favourable environmental conditions in the polyhouse (temperature 25–28 °C, RH > 80%, and a photoperiod of 16 h of light and 8 h of darkness). Once the pest population crossed ETL (average of > 50 aphids in one top, one middle, and one lower leaf), four spore suspensions of five litres each of *A. alternata* VLH1 at the rate of 3×10^4, 3×10^5, 3×10^6, and 3×10^7 spores per ml were prepared for foliar spray. The field was divided into 50 plots of 1 m^2 each containing 4 plants per plot and five treatments (four with 3×10^4, 3×10^5, 3×10^6, and 3×10^7 spores per ml of *A. alternata* VLH1 and control with Triton-X-100 at 0.02% concentration) were induced with 10 replications (Triton-X 100, 0.02% was used as surfactant with every fungal conidial spray). Every plant in each plot was counted for the number of aphids infesting the plants before and after spray (96 h after spray) and aphid mortality was determined by counting the number of aphids on one top, one middle, and one bottom leaf, respectively, to obtain the data regarding per cent pest reduction. SPSS software for WINDOWS version 16.0 was used to calculate the average per cent mortality and SE(m) values for various treatments (SPSS Inc, Chicago).

Virulence of the fungus under open-field conditions

The mustard aphid (*L. erysimi*) was chosen as the target insect pest to investigate the infectivity and virulence of *A. alternata* VLH1 under open-field conditions. The Indian rapeseed variety (VLT-3) was grown and allowed to naturally infest with *L. erysimi*. Twenty-five plots of 1 m^2 each containing 82.5 ± 3.87 plants per plot were demarcated in the field. Four spore suspensions of *A. alternata* VLH1 at a concentration of 3×10^4, 3×10^5, 3×10^6, and 3×10^7 spores per ml were prepared with one control treatment with Triton-X-100 at 0.02% concentration. The treatments were replicated five times and total 5 l spore suspensions of each concentration were prepared for foliar spray. Once the pest population density crossed ETL (> 50 aphids per top 5 cm plant), the treatments were imposed. For calculating the per cent pest reduction, ten plants were selected randomly from each plot and numbers of aphids in the top 5 cm of plant were counted before and after spray (96 h after spray).

Statistical analysis

Aphid cadavers' data collected were subjected to probit analysis (Finney 1971) using the PoloPlus software package (LeOra Software 2013). SPSS software for WINDOWS version 16.0 was used to calculate the average per cent mortality for various treatments of fungus virulence (SPSS Inc, Chicago).

Results

Morphological characterisations of *A. alternata* VLH1

The spore clearly belongs to the *Alternaria* species based on preliminary stereomicroscope examinations. The conidial measurements like conidia length, conidia breadth, number of transverse and longitudinal septa, and presence or absence of beak were recorded (Table 1). All measurements were taken with a fluorescent microscope at 40X magnification on freshly isolated conidia (Olympic BX 61). The colony characteristics and sporulation pattern of *A. alternata* VLH1 were observed (Fig. 1a, b). The pure fungal culture first formed buff-coloured colonies, which gradually turned brownish and blackish in colour. The colony generated pluffy aerial mycelia with many branches and brownish septate mycelium. It had somewhat long, branched conidiophores with ellipsoid

Table 1 Spore characters of *Alternaria alternata* VLH1 (Average of minimum 30 spores)

Character	Measurements (µm)
Length of spore	19.96 ± 1.09
Breadth of spore	8.08 ± 0.39
Number of septa	
Transverse	2.13 ± 0.24
Longitudinal	1.03 ± 0.15
Beak	Present (very short)

The values in the table are presented as mean ± SD

to ovoid conidia that tapered to a very short beak at one end. The length and width of the conidial septa ranged from 11.8 to 31.6 m, with 1–3 transverse septa and 0–2 longitudinal septa. The fungal culture showed high sporulation capacity but moderate ability multiply on artificial culture medium (9 cm in 18 days on PDA). On the basis of morphological characteristics and comparison with available studies of Goettel and Inglis (1997), Tzean (1997) and Woudenberg et al. (2015), the fungus was identified as *Alternaria alternata* (Fr.).

Molecular characterisations

The fungus was identified up to species level using molecular markers, which was used to back up the morphological findings. Multilocus sequence typing (MLST), which integrates several loci of conserved genes to improve the detection of phylogenetic signals among taxa, to construct a high resolution phylogenetic tree, was employed. In an MLST-based phylogenetic analysis, a sequence dataset from the NCBI database that showed ITS, GAPDH, and LSU markers that were comparable to *A. alternata* VLH1 (MN704636) was acquired. The homologous sequence dataset was aligned with ClustalW, and then, comparative data were reconstructed to a tree with MEGA X, using the widely used maximum likelihood technique (Saitou and Nei 1987). Using ten sequences, the ITS marker-based MLST identified *A. alternata* isolate E20 (MT524319.1) as the closest relative in the form of a monophyletic group (Fig. 2). This close relative was found in the rhizosphere zone of maize crops and was shown to be capable of infecting insects (Mckinnon et al. 2018). In this research, the isolate *Beauveria bassiana* JALBB1 (MF187104.1) was used as an out-group. Monophyly with *A. alternata* KUC21222 (KT207688.1) was inferred from the LSU gene-based phylogeny, which was capable of hyper-parasitizing *Puccinia striiformis f. sp. tritici* (Pucciniales: Pucciniaceae), the cause of wheat stripe rust (Zheng et al. 2017). Due to a different diverging branch, the GAPDH marker-based analysis revealed the independent origin of *A. alternata* VLH1. The *A. alternata* VLH1 isolate's similarity to other known EPF *A. alternata* isolates was confirmed by a categorised phylogenetic reconstruction using MLST. Sequences of nine entomopathogenic and 21 plant pathogenic strains of *A. alternata* in the NCBI database were found and included in alignment and phylogenetic tree inference, our evaluation of phylogenetic tree suggests the existence of strains of *A. alternata* in nature with diversified traits infecting plants and insects. The ability to conduct numerous downstream analyses, contextual phenotypic differences, and identify possible metabolites requires the identification of fungal bio-agent at the species level. The major obstacles in species delineation are evolutionary events such as horizontal gene transfer, recombination, and independent assortment. DNA sequencing of a taxon's highly conserved region provides a dependable method of overcoming the aforementioned difficulties.

Proving Koch's postulate

The Koch's postulate was proved for seven insect pests: *T. vaporariorum* nymphs and adults, *C. choprai* adults, *B. brassicae* adults, *A. craccivora* adults, *M. persicae* adults, *L. erysimi* adults, and larvae of *Pieris brassicae* (Linnaeus, 1758) (Lepidoptera: Pieridae) (Additional file 1: Fig. S1). The colony and spore characteristics of the fungal spores isolated from the dead cadavers of treated insects were comparable to those of the mother culture.

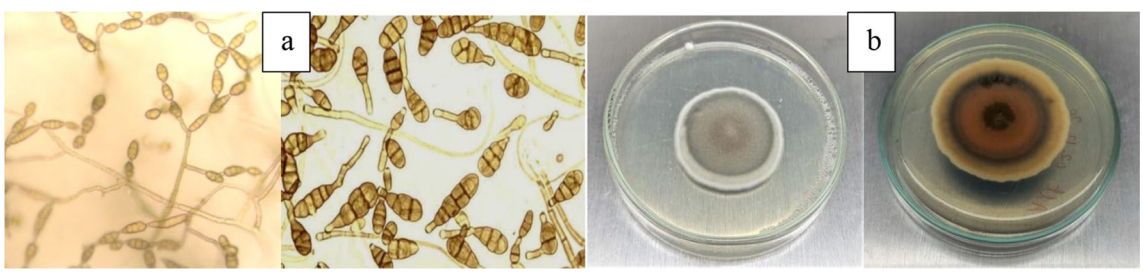

Fig. 1 **a** Spores of *Alternaria alternata* VLH1, **b** fungal colony on PDA media

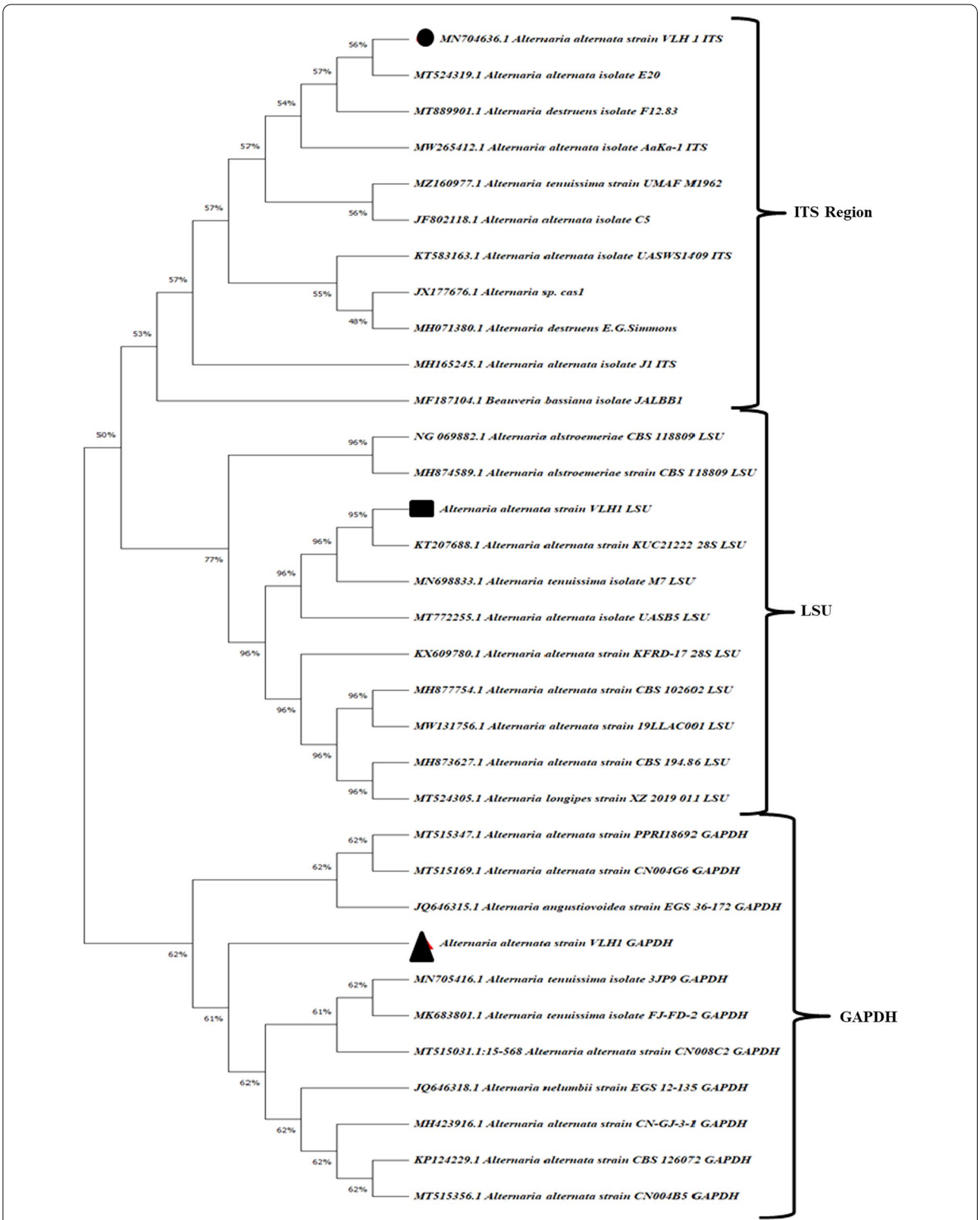

Fig. 2 Phylogenetic analysis of *Alternaria alternata* VLH1 along with 33 closely associated *Alternaria* species sequences (*Beauveria bassiana* isolate JALBB1 is taken as out-group). Filled circle: *A. alternata* VLH1 ITS region, filled square: *A. alternata* VLH1 LSU region, filled triangle: *A. alternata* VLH1 GAPDH region

Testing the infectivity of *A. alternata* VLH1 against target insect pests

The EPF *A. alternata* VLH1 had a variety of impacts on the insect pests it was tested on. For important sucking pests, the median fatal concentration (LC_{50}) was determined, and the results are presented in Table 2 along with upper and lower fiducially limits, p and χ^2 values at 1% level of significance. *L. erysimi* had the lowest LC_{50} value of 1.7×10^4 spores per ml, followed by *M. persicae* (4.8×10^4 spores per ml), *A. craccivora* (8.5×10^4 spores per ml), *S. avenae* and *B. brassicae* (1.2×10^5 spores per ml). Despite the fact that the fungus was identified against *T. vaporariorum*, the LC_{50} values against the nymphs (4.3×10^5 spores per ml) and adults (2.0×10^5 spores per ml) were higher than those of the aphid pests. Even though *C. choprai* adults were susceptible to the fungus, it had the highest LC_{50} value of 2.5×10^6 spores per ml. Except for *C. choprai,* which demonstrated mortality only after 96 h, other insect species showed mortality after 72 h.

With respect to effect of *A. alternata* VLH1 on larval, pupal, and adult periods of *H. armigera, S. litura, S. obliqua*, and *G. mellonella*, the data are presented in Table 3 (Additional file 1: Fig. S2). In paired sample data, the Student's T test was used to compare difference of means at < 0.01% (1% level of significance) (*Alternaria* treated vs untreated insects). When *H. armigera, S. litura*, and *G. mellonella* were treated with spore suspension of *A. alternata* VLH1, there was a substantial change in larval, pupal, and adult lifespan periods, with reduced larval duration, increased pupation period, and lowered adult longevity. The fungus had the least effect on *S. obliqua* larvae, pupae, and adults, and there was non-significant alteration in the life cycle.

Non-target effect of *A. alternata* VLH1 on host plants

In the laboratory studies, the leaves treated with *A. alternata* VLH1 spore suspension showed no effect on detached leaves of 11 host plants in laboratory trials. In field trials where plants were sprayed with the fungal spore suspension up until run off, no impacts or symptoms were found on 11 host plants. The results indicate that the native EPF *A. alternata* VLH1 was less toxic and had no pathogenicity for important field crops grown in hill agriculture in the Indian Himalayas.

Ascertaining chitinase activity and characterisation of chitinolytic protein of *A. alternata* VLH1

The rates of protein production from the fungus *A. alternata* VLH1 (Additional file 1: Fig. S3) revealed that the most protein was produced on day 4, 7.90 µg/100 µl, followed by day 3, 2, and 1 (7.27, 5.42, 4.94 µg/100 µl), respectively. On day 5, however, the amount of protein dropped dramatically, with a mean value of

Table 2 Lethal concentrations of *A. alternata* VLH1 against major sucking pests of hill agriculture

Insect species	Linear equation ($Y = ax + c$)	LC_{50} (Spores/ml)	Lower fiducial limits (5%)	Upper fiducial limits (95%)	χ^2 value	p values	df
B. brassicae	$Y = 0.31x + 3.37$	1.2×10^5	1.9×10^1	1.7×10^8	0.88	0.00011	7
T. vaporariorum (Adults)	$Y = 0.48x + 3.01$	2.0×10^5	5.4×10^1	3.6×10^8	0.96	0.00006	7
T. vaporariorum (Nymphs)	$Y = 0.18x + 3.72$	4.3×10^5	6.5×10^1	1.7×10^{10}	0.98	0.000011	7
M. persicae	$Y = 0.47x + 3.27$	4.8×10^4	1.7×10^1	1.5×10^7	0.94	0.00021	7
C. choprai	$Y = 0.3x + 3.38$	2.5×10^6	8.5×10^1	7.4×10^{11}	0.98	0.00003	7
A. craccivora	$Y = 0.42x + 3.35$	8.5×10^4	1.9×10^1	6.8×10^7	0.97	0.00007	
L. erysimi	$Y = 0.485x + 2.94$	1.7×10^4	1.1×10^1	9.5×10^7	0.98	0.00016	7
S. avenae	$Y = 0.42x + 2.87$	1.2×10^5	7.3×10^1	4.3×10^8	0.91	0.00005	7

Table 3 Changes in growth pattern of larvae, pupae and adults of four Lepidopteran pests (data presented as number of days required to transform to next instar)

Target insects	Larval period (in days)		Pupal period (in days)		Adult longevity (in days)	
	Treated	Control	Treated	Control	Treated	Control
Helicoverpa armigera	11.57 ± 0.30	16.42 ± 0.37*	13.14 ± 0.69*	6.57 ± 0.75	2.85 ± 0.34	6.57 ± 0.43*
Spodoptera litura	13.42 ± 0.48	18.14 ± 0.26*	12.14 ± 2.21*	7.14 ± 0.90	2.14 ± 0.51	7.29 ± 0.42*
Spilarctia obliqua	17.71 ± 0.68	19.29 ± 0.52	7.71 ± 0.69	7.57 ± 0.50	5.71 ± 0.29	6.14 ± 0.51
Galleria mellonella	8.86 ± 0.99	17.95 ± 0.72*	11.43 ± 2.72*	7.43 ± 0.96	2.14 ± 0.77	5.14 ± 0.40*

*Indicates the significant difference in number of days required to complete one instar, calculated by Student's *T* test at 1% level of significance (mean ± SD values are presented in the table)

4.71 µg/100 µl (OD 0.157). Moreover, a study that evaluated the chitinase enzyme activity in the protein extract revealed a gradual rise in enzymatic activity over time (Fig. 3). The greatest protein concentration was 117.78 U/ml four days after inoculation, followed by 104.72 U/ml and 91.83 U/ml on the third and fifth days, respectively. The protein extract thus obtained was electrophoresed, and the resulting band on a PAGE gel was compared to a molecular weight marker (Fig. 4). The existence of an enzyme with a size of 75–90 kDa was confirmed by the band. To confirm the band's chitinolytic activity, colloidal chitin 0.2 per cent was utilised as a substrate, and the superimposition of PAGE gels generated a distinct chitin degradation zone on a 2% agarose gel, indicating that the protein fraction isolated from *A. alternata* VLH1 had chitinolytic activity. This crude protein extract showcasing chitinolytic activity was tested against two aphid pests, *L. erysimi* and *S. avenae* and Table 4 shows the median lethal concentrations. *L. erysimi* had the lowest LC_{50} value of 74.47 ppm, whereas *S. avenae* had a higher LC_{50} value of 127.06 ppm, indicating that it was less susceptible to crude proteins. These findings suggested that proteinaceous substances are involved in *A. alternata* VLH1's entomopathogenic activity against insect pests. Additionally, the effect of protein extracts on the nutritional physiology of *H. armigera* third instar larvae revealed a considerable decrease in the larval food utilisation efficiency (Table 5). RGR fell from 7.09 to 2.18 mg/mg/day 96 HAT ($F=32.65$, $p<0.001$), RCR decreased from 18.56 to 3.81 mg/mg/day 96 HAT

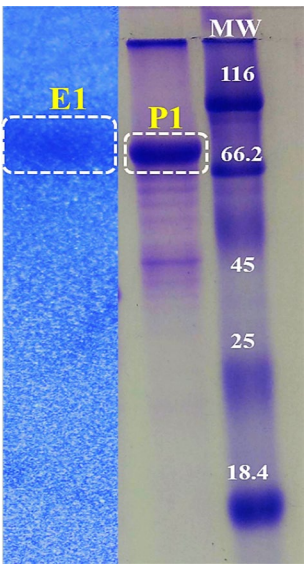

Fig. 4 Characterisation of chitinolytic activity of protein extract of *Alternaria alternata* VLH1 (Lane 1. (E1) chitinolytic activity on 2% agarose gel with 0.2% colloidal chitin as substrate, Lane 2. Chitinase enzyme band isolated from crude protein extracts of *A. alternata* VLH1, Lane 3. Molecular weight marker)

($F=136.96$, $p<0.001$), per cent ECI decreased from 9.47 to 3.19 after 96 h. ($F=41.94$, $p<0.001$), and per cent ECI decreased from 9.47 to 3.19 after 96 h. ($F=41.94$), in control and 40 ppm protein concentrations. Moreover, ECD decreased from 8.02 to 1.96 after 96 h ($F=39.79$,

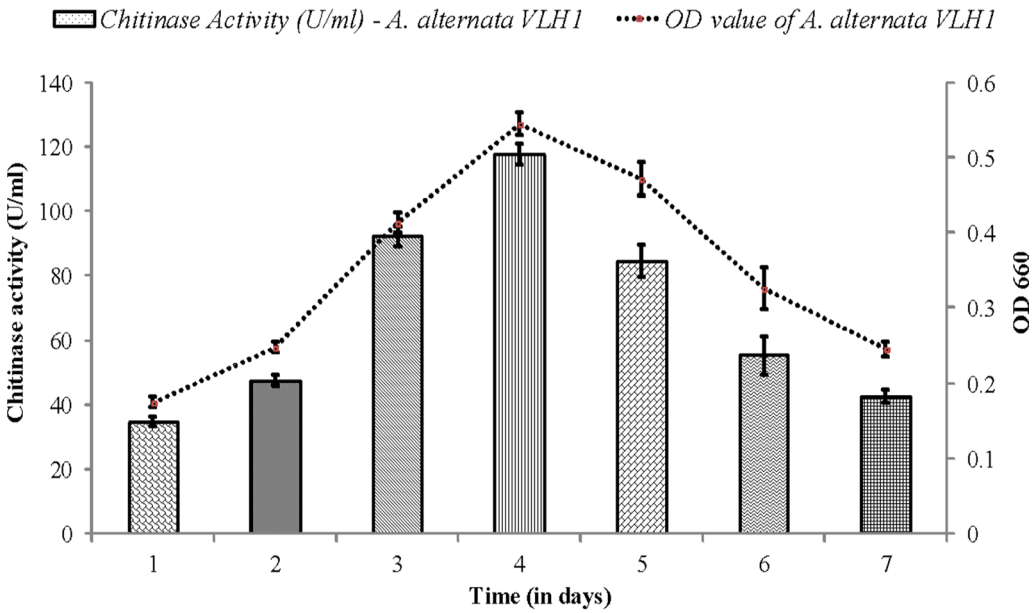

Fig. 3 Kinetics of chitinase production by protein extracts of *Alternaria alternata* VLH1

$p < 0.001$), and AD decreased from 99.65 to 96.70% after 96 h ($F = 6.72$, $p < 0.001$). The highest concentration of protein extract (40 ppm) resulted in abnormal pupal moulting and the formation of deformed adults (Fig. 5). RGR, RCR, ECI, ECD, and AD all decreased 3.25, 4.87, 2.96, 4.09, and 1.03 times over control at the highest protein concentration. Furthermore, substantial reduction in all nutritional indices was recorded at 1% level of significance.

Polyhouse condition

Figure 6 shows the per cent mortality of green peach aphid (*M. persicae*) infecting capsicum following treatment with *A. alternata* VLH1 spore suspension 96 HAT. Among all the treatments, T4 (3×10^7 spores per ml) recorded the highest mortality of 81.14%, which was followed by 3×10^6 (68.64%), 3×10^5 (54.89%), and 3×10^4 (37.80%) spores per ml. Based on results, it can be concluded that the increase in spore concentration resulted into concurrent increase in the per cent pest mortality. However, the population of aphids in control plots was unaltered even after spraying with Triton-X-100 at a concentration of 0.02% and the decrease by 2.86% only was reported, following treatment. The polyhouse bioassays open the way for the safe and effective application of *A. alternata* VLH1 to control important sucking pests that infect a variety of crops under polyhouse conditions.

Table 4 Laboratory bioassay with crude protein extracts of *Alternaria alternata* VLH1 against major aphid pests of *Rabi* crops

Insect species	Linear equation ($Y = ax + c$)	LC_{50} Protein concentration (in ppm)	Lower Fiducial limits (5%)	Upper Fiducial limits (95%)	X^2 value	*p* values	*df*
Lipaphis erysimi	$Y = 0.94x + 3.24$	74.47	1.34	4139.99	0.87	0.00012	7
Sitobion avenae	$Y = 1.25x + 2.37$	127.06	6.19	2606.15	0.89	0.00004	7

Table 5 Effect of chitinolytic protein extracts of *Alternaria alternata* VLH1 on nutritional physiology of third instar larvae of *Helicoverpa armigera* (96 HAT)

Concentration of protein (in ppm)	RGR (mg/mg/day)	RCR (mg/mg/day)	ECI (%)	ECD (%)	AD (%)
Control	7.09 ± 0.14^{e}	18.56 ± 0.76^{e}	9.47^{d}	8.02^{d}	99.65^{c}
5 ppm	5.12 ± 0.43^{d}	10.83 ± 0.44^{d}	6.01^{c}	3.39^{c}	97.27^{b}
10 ppm	4.81 ± 0.35^{c}	8.31 ± 0.38^{c}	4.90^{b}	3.23^{c}	97.21^{b}
20 ppm	3.45 ± 0.45^{b}	5.25 ± 0.50^{b}	4.41^{b}	2.45^{b}	97.04^{ab}
40 ppm	2.18 ± 0.12^{a}	3.81 ± 0.23^{a}	3.19^{a}	1.96^{a}	96.70^{a}
F	32.65**	136.96**	41.94**	39.79**	6.72**

The differences in SE (m) values are compared for different nutritional physiology parameters and the significant difference in these parameters is mentioned by the superscripts on the numerical values

RGR relative growth rate, *RCR* relative consumption rate, *ECI* efficiency of conservation of ingested food, *ECD* efficiency of conservation of digested food, *AD* approximate digestibility

**Tukey's-B test conducted at 1% level of significance

Fig. 5 Abnormal pupal moulting and formation of deformed adults in *Helicoverpa armigera* treated with 40 ppm protein concentration of *Alternaria alternata* VLH1

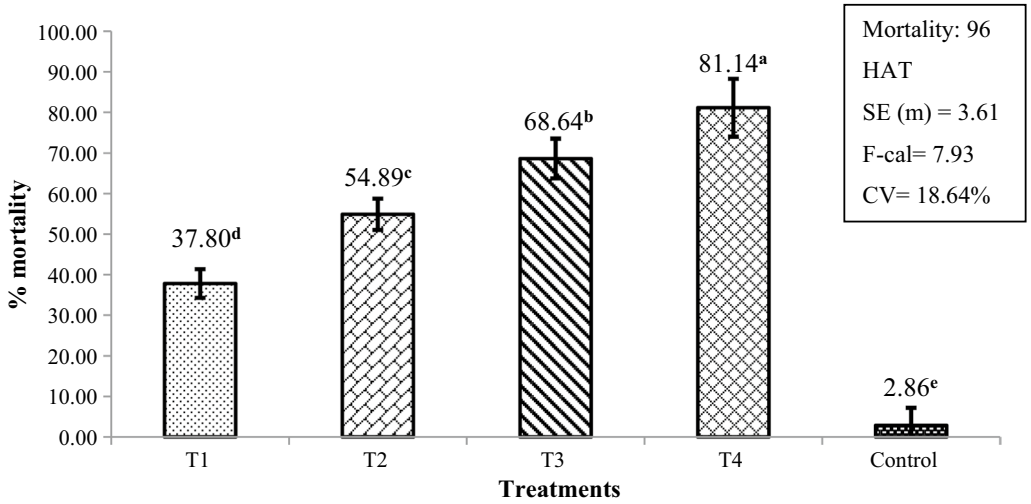

Fig. 6 Per cent mortality of aphids in controlled polyhouse conditions after treatment with spore suspension of *Alternaria alternata* VLH1

Open-field conditions

Figure 7 shows the per cent mortality of mustard aphid (*L. erysimi*) infecting Indian rape 96 HAT with *A. alternata* VLH1 spore suspension. Among all the treatments, T4 (3×10^7 spores per ml) recorded the highest mortality of 63.14%, which was followed by 3×10^6 (48.64%), 3×10^5 (38.89%), and 3×10^4 (23.80%) spores per ml. However, more than 50% mortality of pests was recorded only in spore concentration of 3×10^7 spores per ml, indicating the need for high concentration of biocontrol agent for managing the pests under open-field conditions. On the other hand, aphid population in control plots was unaltered, with a mere reduction of 1.46%. The field circumstances differed from one site to the next, and the findings may differ as well. Before proposing *A. alternata* VLH1 for large-scale open-field application, more testing against other sucking pests in diverse locations and climate regimes is required.

Discussion

The inadvertent observation of a substantial population reduction of greenhouse whiteflies infecting *S. divinorum* plants grown in a polyhouse sparked a flurry of inquiries on the cause of the population decline. Whitefly dead

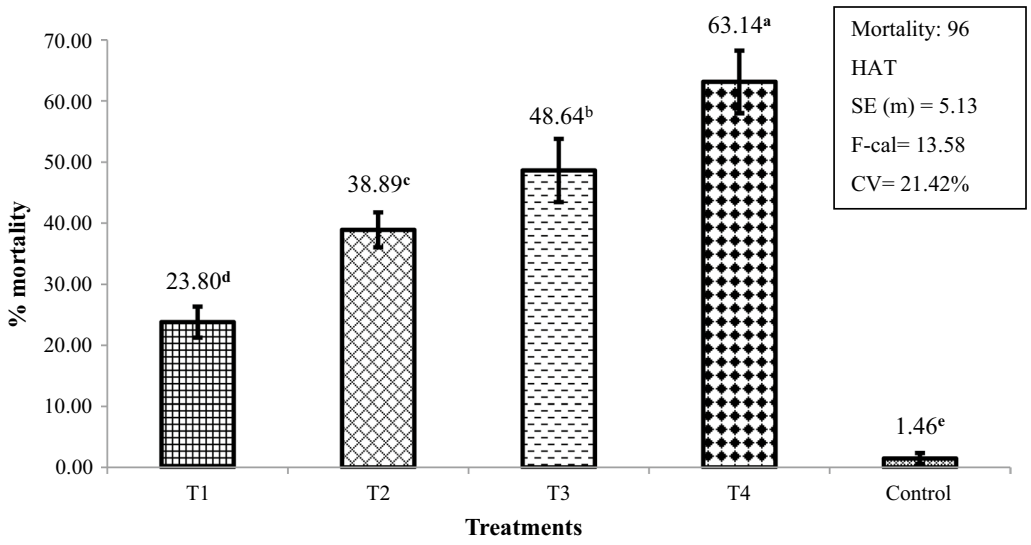

Fig. 7 Per cent mortality of aphids in open fields after treatment with spore suspension of *Alternaria alternata* VLH1

cadavers with abundant fungal development were collected from the host plants and dissected to examine the conidia/spores under a stereo microscope. The fungus species was verified as *Alternaria* through ocular observation. Furthermore, the literature search found that *Alternaria* fungi are saprophytic and might be used as a biocontrol agent against a variety of insect pests (Mehrmoradi et al. 2020).

Kochs' postulate was tested by infecting *T. vaporariorum* nymphs and adults with pure culture of the fungus isolated using the single spore colony method before determining native *Alternaria* species to be EPF infecting *T. vaporariorum*. The mycelia obtained had colony and spore characteristics that were identical to those of the mother culture.

Alternaria alternata VLH1, was found to be monophyletic with *A. alternata* isolate E20 (MT524319.1) based on phylogenetic analysis and multilocus sequence typing. This close relative was found in the rhizosphere zone of maize crops and had the ability to infect insects (Mckinnon et al. 2018). Furthermore, our findings showed the presence of a diverse characteristic in *A. alternata* strains found in nature, infecting both plants and insects. Based on a phylogenetic study of sequences from nine entomopathogenic and 21 plant pathogenic strains of *A. alternata* in the NCBI database, a small mutation or transformation in SNPs may have produced entomopathogenic behaviour in *A. alternata* VLH1. However, it is worth noting that utilising the ITS marker to trace the phylogeny of *A. alternata* was only the tip of the iceberg. It was difficult inferring the exact picture of the evolutionary events that lead to distinct strains of *A. alternata* featuring entomopathogenic activity due to close similarity in ITS sequences across insect and plant pathogenic *A. alternata* strains. However, if additional molecular data becomes available, such as genome sequencing of strains to find single copy orthologues, a phylogenetic tree based on effector genes may provide a clear picture of the evolutionary relevance in achieving adequate virulence on plants and insects. Furthermore, a gene tree employing effectors and single copy orthologues will provide a better understanding of the specific/key genetic alteration that favours *A. alternata* strains that parasitize insects.

The sucking insects were the first priority targets of the fungus, according to laboratory assays done to examine the infectivity and pathogenicity of *A. alternata* VLH1 against insect pests. *L. erysimi* and *M. persicae* had the lowest LC_{50} values of the seven sucking pests studied. Except for *C. choprai*, most insects died after 72 h of exposure (96 HAT). These findings were similar to those of Christias et al. (2001), who found that the fungus *A. alternata* completed its life cycle in 48–72 h and that the insects were sluggish, stopped feeding, and became brownish to blackish in colour with abundant mycelial development. In the present research, similar changes in body colour and mycelial development on the carcass of a dead insect were noticed.

Furthermore, lepidopteran pests are most prevalent insect pests in the Indian Himalayas, making biocontrol approaches challenging. To investigate the virulence and infectivity of *A. alternata* VLH1, four lepidopteran insects (*H. armigera, S. litura, S. obliqua,* and *G. mellonella*) were chosen. The treated insects were compared with untreated ones for time required to complete larval, pupal and adult stages. In three insects, *H. armigera, S. litura*, and *G. mellonella*, the pupal stage was extended further; however in *S. obliqua*, non-significant difference in larval, pupal, and adult periods was seen. Furthermore, few *H. armigera, S. litura*, and *G. mellonella* individuals treated with *A. alternata* VLH1 were unable to complete their life cycle. Pupae were found to have developmental abnormalities, adults emerged prematurely, and the emerged adults died within a short time of emergence. Obtained findings were similar to those of Kaur et al. (2019) who found that when an ethyl acetate extract of *A. alternata* strain NL23 was tested against *S. litura* larvae, it resulted in a 28–81% reduction in relative growth rate (RGR) and a 47–55% reduction in relative consumption rate (RCR) compared to control, lowering the larvae's growth efficiency and increasing premature mortality. Sharma et al. (2012), on the other hand, found results that were diametrically opposed to ours. The larval, pupal, and adult developmental periods of *H. vigintioctopunctata* increased after feeding on the fungal pathogen *A. alternata*, which infects *Withania somnifera* (L.) Dunal, (Solanales: Solanaceae) plants, compared to the control.

In a bioassay trial with two non-target organisms, *C. septempunctata* and *A. mellifera*, treated with 3×10^6 spore/ml concentrations, non-significant effect on insects was seen. The above findings demonstrated the efficacy of local EPF against a variety of insect pests in hill agriculture, as well as their safety against non-target insects.

Despite the fact that the fungus was effective at controlling insect pests, data on its non-target impacts on host plants were crucial before embarking on any open-field experiments. The findings of this study showed that *A. alternata* VLH1 did not develop any disease symptoms on the treated host plants.

Alternaria alternata is an entomopathogenic fungus that produces appressorium, which allows it to penetrate plant surfaces or insect cuticles. The fungus is known to produce more than 70 types of toxins (EFSA 2011) that may have entomopathogenic activity on insects, in

addition to exerting mechanical forces to infect the target insect or plant. A laboratory trial was done to investigate the entomopathogenic activity of crude protein extracts of fungi against two aphid pests, yielding LC_{50} values of 74.47 ppm and 127.06 ppm for mustard and wheat aphids, respectively. Samuels and Paterson (1995) reported that the combined action of mechanical force exerted by infection pegs formed from appressoria and enzymatic digestion of the insect epicuticle through production of proteinaceous toxins is required for fungal pathogen penetration of insect hosts. Furthermore, Green et al. (2001) believed that entomopathogenic *Alternaria* species produce destruxins (A to E) and the chitinase enzyme, which are a primary feature related with fungi's virulence against insects. As a consequence, the methods for isolating proteinaceous toxins and identifying fungal toxins in order to examine their insecticidal properties and non-target effects were standardised. The fungus was cultivated on a colloidal chitin-based substrate containing 1% colloidal chitin, and the presence of a distinct halo around the colony validated the strain VLH1's chitinolytic activity. Further research into quantifying the chitinase enzyme produced by *A. alternata* VLH1 at various time intervals revealed that the highest enzymatic activity of 117.78 U/ml was found on the fourth day after inoculation. Our findings were similar to those of Christias et al. (2001), who found that *A. alternata* chitinolytic activity was the predominant method of action for causing mortality in the oleander aphid, *Anuraphis nerii* Kaltenbach, 1843 (Homoptera: Aphididae).

The nutritional physiology of *H. armigera* third instar larvae given a protein extract-added artificial diet demonstrated significant alterations. Over control, the RGR, RCR, ECI, ECD, and AD decreased 3.25, 4.87, 2.96, 4.09, and 1.03 times, respectively. When *S. litura* larvae were fed on protein fractions from *Alternaria destruens* AKL-3 (Fr.) Keissl (Pleosporales: Pleosporaceae), Kaur et al. (2019) reported a 31.96–53.94% decrease in RGR, 19.24–72.93% decrease in RCR, and adverse effects in ECI and ECD. Furthermore, Quesada-Moraga et al. (2006) found that protein isolates from multiple EPFs (*M. anisopliae, B. bassiana, Beauveria brongniartii* (Bals.-Criv.) (Hypocreales: Clavicipitaceae), and *Scopulariopsis brevicaulis* Saccardo (1881) (Microascales: Microascaceae)) induced significant growth abnormalities and adult mortality in *Scopulariopsis littoralis* Saccardo (1881) (Microascales: Microascaceae). The degradation of the chitin layer found in the stomach of insects, as well as concomitant toxicities produced by other insecticidal protein fractions, could be the source of growth deformity and mortality in insects caused by *A. alternata* VLH1. The EPF potential of *A. alternata* VLH1 may have been conferred by the destruction of the chitinolytic cuticular layer during appressoria production and fungal penetration.

The next stage was to investigate the activity of fungi in both polyhouse and open-field environments after obtaining successful bioassay results against a variety of insect pests in vitro. *M. persicae*, a major polyphagous aphid that infects vegetable crops in polyhouses, was treated with *A. alternata* VLH1 spore suspension. The average per cent mortality in different treatments was compared using one-way analysis of variance (ANOVA) at a 5% level of significance. The mortality ranged from 37.80% at lowest conidial concentration (3×10^4) to 81.14% at the highest conidial concentration (3×10^7 spores/ml), and the per cent mortality between *Alternaria* treated and a control plot was highly significant; moreover, the per cent mortality among the treated plots was also statistically significant with each other. The findings of field sprays of *A. alternata* VLH1 spore suspension against *L. erysimi* infecting Indian rapeseed were almost identical to those of polyhouse investigations. In four separate treatments, the per cent mortality ranged from 23.80% at lowest conidial concentration (3×10^4) to 63.14% at highest conidial concentration (3×10^7 spores/ml). The ANOVA test used to compare the means revealed that the per cent mortality differences between the treated and control plots were statistically significant, moreover, the per cent mortality among the treated plots were also highly significant. The population of aphids dropped to a very low level of 2.86 and 1.46% in the control plots of both polyhouse and open-field environments, respectively. Based on results, it can be concluded that the increase in spore concentration resulted into concurrent increase in the per cent pest mortality. To our knowledge, this is the first study to look at *A. alternata* as a possible biocontrol agent for insect pests in polyhouses and open fields under Indian Himalayan conditions.

Conclusion

The entomopathogenic fungus (EPF), *A. alternata* VLH1 was found to be an effective a biocontrol agent against wide range of insect pests that infest hill crops. From direct mortality in sucking pests to developmental abnormalities in lepidopteran insects, the insects treated with the fungal strain showed a range of responses. Despite positive findings in in vitro and in vivo bioassay investigations against various insect pests, the fungus still has to be inspected before it can be used on a broad scale for biological pest management. Insect infection requires a temperature of 25–30 °C, according to ecological research. However, the average annual temperature in the Himalayan region is well below 25 °C. This flaw must be rectified by

conducting comprehensive field surveys in the Indian Himalayas to isolate different strains of *Alternaria* species and other EPF. The methods used to isolate proteinaceous toxins from the fungus strain resulted in a very poor toxin recovery. As a consequence, processes for isolating toxins and characterising them down to the molecular level must be standardised by culturing on various artificial culture medium. Other biosafety procedures, such as the toxicity of proteinaceous toxins to humans, animals, fish, and birds, must also be investigated before *A. alternata* VLH1 is recommended for field use.

Abbreviations

AD: Approximate digestibility; ANOVA: Analysis of variance; BLASTN: Basic local alignment search tool-nucleotide; BOD: Biochemical oxygen demand; CTAB: Cetyltrimethylammonium bromide; DNA: Deoxy-ribose nucleic acid; ECD: Efficiency of digested food conversion; ECI: Efficiency of ingested food conversion; EFSA: European Food Safety Authority; ETL: Economic threshold level; GAPDH: Glyceraldehyde 3 phosphate dehydrogenase; HAT: Hours after treatment; ITS: Internal transcribed spacer; kDa: Kilo Daltons; LC50: Lethal concentration 50; LSU: Large subunit ribosomal RNA gene; MEGA: Molecular evolutionary genetics analysis; MLST: Multilocus sequence typing; NCBI: National centre for biotechnology information; OD: Optical density; PAGE: Polyacrylamide gel electrophoresis; PBD: Potato dextrose broth; PDA: Potato dextrose agar; RCR: Relative consumption rate; RGR: Relative growth rate; SE(m): Standard error of mean; SPSS: Statistical package for social sciences; UV: Ultra violet radiation; VL: Vivekananda Laboratory; VLH1: Vivekananda Laboratory Hawalbagh 1.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-022-00637-0.

Additional file 1. Supplementary Fig 1. Insect cadavers infected with *A. alternata* strain VLH1, four days after treatment, **a** *T. vaporariorum* nymph, **b** *T. vaporariorum* deformed adult, **c** Chauliops choprai adult, **d** Brevicoryne brassicae, **e** Aphis craccivora, **f** Myzus persicae, **g** Lipaphis erysimi, **h** Pieris brassicae larvae. **Supplementary Fig 2**. Changes in growth pattern of larvae, pupae and adults of four Lepidopteran pests (data presented in number of days required to transform to next instar). **Supplementary Fig 3**. Kinetics of proteinaceous toxin production by A. alternata strain VLH1 Laboratory bioassay of crude proteins against aphid pests.

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Author contributions

AUP carried out laboratory and field studies, drafting and designing of the experiment, ARNSS was involved in scientific guidance, support during laboratory, and field studies, AKS helped in molecular data analysis, JB contributed to morphological characterisation and mass multiplication of the fungi, JS was involved in technical guidance and support for conducting laboratory experiments, RH helped in data analysis and revising the manuscript, KKM contributed to language editing and revision of the manuscript, PSK was involved in language editing and English improvement, LK provided facility and scientific guidance, AP provided facility and guidance, final reviewing and revision of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data are available within the article or its supplementary materials. The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with animals performed by any of the authors. This article does not contain any studies with human participants or animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

Consent for publication

All the authors have received the funding from ICAR, New Delhi, and therefore have no conflict of interest for the submission and publication of the article to "Egyptian Journal of Biological Pest control".

Competing interests

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