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RESEARCH ARTICLE

Exploring a potential mycoherbicidal agent for eco-friendly management of *Trianthema portulacastrum* L.

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Abstract

Trianthema portulacastrum L. is one of the dominant weed species of the garden land ecosystem and poses a severe threat to crop cultivation. Chemical herbicides have the potential to manage the weed effectively, but their persistent residual nature negatively impacts the soil health and ecosystem. Therefore, biological approaches are being necessitated for weed management. Naturally, *T. portulacastrum* is highly susceptible to various fungal infections, resulting in severe defoliation and necrosis. Hence, a roving field survey was conducted at multiple locations in Tamil Nadu to collect the diseased *T. portulacastrum* samples and investigate the diversity of fungal pathogens associated with it. A total of 78 fungal isolates were isolated from the infected samples. A detached leaf assay was performed to screen the potential fungal isolates. Morpho-molecular analyses confirmed the potential isolates as *Gibbago trianthemae* and *Fusarium* spp. A polymorphism percentage of 68.88 % was observed among the potential *G. trianthemae* isolates, which were assessed using 10 different ISSR primers. Pot culture experiments revealed that *G. trianthemae* isolate TVM 2 recorded the maximum disease index of 34.4 %, 41.6 % and 49.3 % at 10, 20 and 30 DAI (days after inoculation), respectively, followed by isolate MDU 5. The potential fungal pathogen *G. trianthemae* also exhibited positive results for qualitative analysis of various extracellular and cell wall-degrading enzymes. Hence, there is a potential opportunity to exploit the infectious propagules of *G. trianthemae* for the development of a mycoherbicide formulation for environmentally friendly management of *T. portulacastrum*.

Keywords: bio-control; disease index; *Gibbago trianthemae*; horse purslane; mycopathogens; variability

Introduction

Weeds are a serious threat to crop production as they significantly interfere with the growth and development of various agricultural and horticultural crops, leading to a severe yield penalty. A huge economic loss of around 11 billion USD has been estimated due to severe weed infestation in 10 major crops of the Indian sub-continent (1). Among diverse biotic factors influencing crop growth and development, weeds are the dominant factor impacting the most. One such problematic weed is *Trianthema portulacastrum* L. (horse purslane), which widely dominates the garden land ecosystem. It is a broad-leaved weed that possesses various abilities, like the production of an enormous number of seeds per plant, a rapid growth rate and quick flowering. Moreover, it covers the entire surface of the soil and strongly competes with the crop plants for nutrients, moisture, space and sunlight, consequently reducing the yield of various crops. So, appropriate and timely implementation of weed management strategies has to be taken up to avoid the yield penalty.

The application of chemical herbicides is highly effective in controlling the weed flora. But in the long run, the residues of such chemicals negatively impact the soil health and microflora. An alternative strategy is therefore warranted. One such alternative strategy is the biological approach, which involves the use of various plant pathogens, insects, birds and fish for the management of weeds. Of those, the mycoherbicidal approach, i.e., exploiting the fungal pathogens, was highly successful for the sustainable and eco-friendly management of weeds. Various fungal pathogens were successfully utilized in managing a variety of weed flora, ensuring a fair to moderate level of weed control efficiency. Several bioherbicidal products based on fungi like Lubao, DeVine®, Collego™ (Lock Down®), Casst™, ABG-5003, Dr. Biosedge®, Velgo®, BioMal®, Stumpout™, BioChon™, Hakatak®, Woad Warrior®, MycoTech™, Chontrol™ (EcoClear™), Smoulder®, Sarritor™, Phoma®, Gibbatrianth and Di-Bak Parkinsonia® were used for the management of a specific weed or a group of weeds (2).

Diverse fungal pathogens, viz., *G. trianthemae*, *Phoma herbarum*, *Fusarium oxysporum*, *Cochliobolus australiensis*, *Cochliobolus spicifer*, *Colletotrichum gleosporioides*, *Alternaria alternata, Curvularia tuberculata, Curvularia lunata, Bipolaris maydis* and *Colletotrichum capsici* were associated with the weed, *T. portulacastrum,* with respect to the Indian context (3-8). This study aimed to explore the fungal pathogens associated with *T. portulacastrum* across different agro-climatic zones of Tamil Nadu and to identify a potential fungal pathogen for developing a mycoherbicide for eco-friendly management of *T. portulacastrum*.

Materials and Methods

Collection of diseased *T. portulacastrum*

To explore the diverse fungal pathogens associated with *T. portulacastrum*, a roving field survey was conducted during 2023 and 2024 to collect the diseased *T. portulacastrum* from different locations, covering all the agro-climatic zones of Tamil Nadu state except the hill and high-altitude zone, as *T. portulacastrum* does not thrive well in such conditions.

Isolation of fungal pathogens

The diseased plants collected from various locations were washed thoroughly in tap water to remove the soil debris. The infected leaves were cut into tiny bits in such a way that it has a portion of healthy tissue too. The tiny bits were surface sterilized with 70 % ethanol and 0.25 % sodium hypochlorite solution and they were rinsed well in the sterile distilled water and placed in the sterilized tissue paper to absorb the excess moisture. Then, these leaf bits were placed in the Petri dishes containing potato dextrose agar (PDA) medium supplemented with streptomycin sulphate and were incubated at 25 ± 2 °C. All the fungal isolates were characterized morphologically.

Detached leaf assay

Healthy leaves were collected from the *T. portulacastrum* plants grown in glasshouse conditions and they were washed in sterile distilled water and thereafter wiped with a cotton dipped in 70 % ethanol for surface sterilization. The leaves were then pinpricked slightly on the adaxial surface of the leaf using a sterilized needle and placed in the Petri dishes filled with moistened cotton. Fungal discs of 9 mm diameter were excised from a 7-day-old colony of fungal isolates using a cork borer and placed on the pinpricked portions of the leaf and covered with a piece of sterile, moistened cotton and incubated at 25 ± 2 °C. The leaves were observed for disease development at 5 days after inoculation (DAI).

Morphological characterization of potential fungal isolates

Morphological characteristics of the potential fungal isolates were examined using the compound microscope (Magnus MX21*i*LED equipped with MagVision software) and were cross-checked with the scientific literature for the confirmation of pathogens.

Molecular confirmation of potential fungal isolates

The genomic DNA of potential fungal isolates was extracted using the cetyltrimethylammonium bromide (CTAB) method (9). The concentration of extracted DNA was assessed using the NanoDrop equipment (NanoDrop Lite Plus, Thermo Fisher Scientific, Madison, WI, USA). The extracted DNA was subjected to polymerase chain reaction (PCR) with the universal primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) using the thermal cycler (Bio-Rad C1000 Touch thermal cycler). The PCR conditions involved: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec, extension at 70 °C for 2 min, 35 cycles, final extension at 72 °C for 7 min and an infinite hold at 4 °C. The obtained PCR products were loaded into 1 % agarose gel in the gel electrophoresis unit (Genei, Bangalore, India) to visualize the DNA amplicon using a gel imaging system (UVITEC, Cambridge, UK). After confirmation of the DNA amplicon, the PCR products were sequenced using the Sanger method (Biokart India Pvt. Ltd., Bangalore). The obtained sequences were analyzed in the BLASTN search algorithm of the National Center for Biotechnology Information (NCBI) database for the confirmation of fungal pathogens and they were submitted to GenBank to obtain the accession numbers. A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software version 11 by the neighbour-joining method to observe the evolutionary relationship among the isolates.

Assessing the molecular variability among *G. trianthemae* isolates using ISSR primers

A set of 10 ISSR (Inter Simple Sequence Repeat) primers (Table 1) were used to assess the molecular variability of *G. trianthemae* isolates. The PCR condition involved: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 sec, annealing at 51 °C for 45 sec, extension at 72 °C for 90 sec, number of cycles - 35, final extension at 72 °C for 7 min and infinite hold at 4 °C. The obtained PCR products were loaded into 1 % agarose gel in the gel electrophoresis unit to visualize the DNA amplicon.

Amplicon scoring and data analysis

The amplicon patterns were scored for all 10 ISSR primers. The presence of each amplicon in each isolate was coded as 1 and the absence of the amplicon was coded as 0. The unweighted pair group mean average (UPGMA) method was used to assess the genetic relationship and variability among the isolates. A dissimilarity matrix was formed using the R package 'poppr'. It was then employed to generate a UPGMA tree using the R package 'ape'. To determine the genetic variability among the isolates, diverse parameters, viz., number of monomorphic and polymorphic bands per primer, total number of bands, polymorphism percentage, polymorphism information content (PIC), marker index (MI) and effective multiplex ratio (EMR) were analyzed. PIC is a measure of the informativeness of a genetic marker, i.e., its ability to detect polymorphism among different isolates. A higher PIC value means the marker is more

Table 1. ISSR primers used in variability analysis and their sequence

Name of the primer	Sequence
ISSR 1	5′ - CTCTCTCTCTCTCTCTG - 3′
ISSR 2	5′ - GAGAGAGAGAGAGAGAC - 3′
ISSR 3	5′ - ACACACACACACACACCTT - 3′
ISSR 4	5′ - ACACACACACACACACCTTG - 3′
ISSR 5	5′ - CTCTCTCTCTCTCTCTAGG - 3′
ISSR 6	5′ - GTGTGTGTGTGTGTGTCTAG - 3′
ISSR 7	5′ - CTCTCTCTCTCTCTCTTG - 3′
ISSR 8	5′ - CTCTCTCTCTCTCTCTAC - 3′
ISSR 9	5′ - CTCTCTCTCTCTCTCTGC - 3′
ISSR 10	5′ - CACACACACACACAAC - 3′

ISSR: Inter Simple Sequence Repeat

informative in distinguishing genetic differences among isolates. EMR represents the number of polymorphic loci detected per primer, adjusted by the proportion of polymorphic bands. It indicates the effectiveness of a primer in generating useful polymorphic information. A higher EMR means the primer generates more polymorphic, informative bands. MI combines both the informativeness (PIC) of a primer and its multiplex ratio (EMR). It gives an overall measure of the utility of a primer system in genetic diversity studies. A higher MI indicates a primer system that is both informative and efficient in producing polymorphic markers. PIC, MI and EMR were calculated by using iMEC: Online Marker Efficiency Calculator (10).

Testing the pathogenicity of potential fungal isolates under glasshouse conditions

Seeds of *T. portulacastrum* were collected from the fields and were placed in 0.5 % sodium hypochlorite solution and rinsed thoroughly with sterile water. The seeds were sown in plastic pots filled with sterilized soil and regularly watered. The fungal inoculum was prepared by flooding the Petri dishes with sterile water; thereafter, the infective propagules, viz., mycelium and conidia of potential fungal isolates, were harvested and filtered through the cheesecloth. The inoculum concentration of 1×10^6 cfu mL^{-1} was sprayed on the test plants at the rate of 5 mL $plant^{-1}$ at the 2-3 leaf stage of the weed. The leaves were checked for disease development at 10, 20 and 30 DAI. The intensity of disease on the test plants was scored using a 0 to 5 scale rating technique where 0 = no symptoms, 1 = 1-10 %, 2 = 11-25 %, 3 = 26-50 %, 4 = 51-75 % and 5 = ≥75 % (11). Using this, the percent disease index was calculated using the following formula (12).

$$\text{Percent Disease Index (PDI)} = \frac{\text{Sum of ratings}}{\text{Number of leaves observed} \times \text{Maximum disease rating}} \times 100$$

The disease index values obtained were subjected to arcsine transformation and thereafter, statistical analysis was carried out (13).

Enzyme analysis

The potential fungal isolate screened was examined for the activity of extracellular and cell wall-degrading enzymes.

Cellulase

Cellulase activity was assessed by inoculating the fungal discs in Czapek Dox agar medium (comprising 30 g of sucrose L^{-1}) supplemented with CMC (carboxymethylcellulose) at the rate of 10 g L^{-1} and 0.3 g of Congo red L^{-1} . The pH of the medium was adjusted to 5.3. Five DAI, the plates were observed for a clear halo zone around the colony (14). The presence of a clear halo zone indicates a positive result.

Ligninase

The fungal discs were placed in PDA medium supplemented with Congo red at the rate of 200 mg L^{-1} and incubated at room temperature for 48 hr. After 2 days, the plates were visualized for the development of a clear halo around the fungal colony (15). The presence of a clear halo zone denotes a positive result.

Protease

The fungal isolate was grown in glucose-yeast-peptone (GYP) medium. The composition of the medium (per litre of distilled water) includes 1 g of glucose, 0.1 g of yeast extract, 0.5 g of peptone, 15 g of agar, which was supplemented with 1 % skim milk and the pH was adjusted to 6.5. The plates were observed after 7 DAI at 25 °C to visualize the clear zone around the fungal colony (16). The presence of a clear halo zone specifies a positive result.

Amylase

The fungal isolate was inoculated in the GYP medium enriched with soluble starch at the rate of 1 % concentration as a substrate. After 7 DAI at 25 °C, an aqueous solution of 1 % iodine (w/v) in 2 % potassium iodide (w/v) was poured into Petri plates and after 10 min, the plates were decanted to remove the solution and observed for the presence of a clear halo around the fungal colony (17). The presence of a clear halo zone implies a positive result.

Endoglucanase

The fungal isolate was inoculated in Czapek-agar medium comprising (per litre of distilled water) 2 g of $NaNO_3$(sodium nitrate), 1 g of KH_2PO_4 (potassium dihydrogen phosphate), 0.5 g of KCl (potassium chloride), 0.5 g of $MgSO_4.7H_2O$ (magnesium sulfate heptahydrate), 0.01 g of $FeSO_4.7H_2O$ (ferrous sulfate heptahydrate), 20 g of agar, supplemented with CMC at a rate of 1 % concentration as the sole carbon source. The pH of the medium was adjusted to 4.5 with 100 % glacial acetic acid. Seven DAI, the plates were immersed in 0.1 % Congo red solution (w/v). After 5 min, the solution was poured out and the Petri plates were washed with 5 M NaCl (sodium chloride) to observe the halo zone (18). The presence of a clear halo zone signifies a positive result.

Results and Discussion

Collection of diseased *T. portulacastrum* samples

A roving field survey was taken up at 20 different locations covering diverse agro-climatic zones of Tamil Nadu for collecting the diseased *T. portulacastrum* plants. Highly infected plants with severe necrotic lesions on leaves and stems were collected. The diseased samples were collected from the geographical locations displayed in Fig. 1. The details on the survey and collection of infected *T. portulacastrum* are provided in Table 2.

Isolation of fungal pathogens

A total of 78 fungal isolates were isolated from the infected *T. portulacastrum* samples, where each fungal isolate exhibits diverse colony characteristics like fluffy mycelium, compact mycelium, wavy margin and varied colour pattern (Fig. 2).

Detached leaf assay

The results of the detached leaf assay revealed that out of 78 fungal isolates, only 46 isolates (approximately 59 % of the fungal isolates) exhibited disease symptoms, while the other fungal isolates were not pathogenic to *T. portulacastrum*. Based on the lesion length and diameter of the necrotic spot, ten potential pathogenic fungal isolates were selected for further studies. The lesion length and diameter of the necrotic spot were maximum in the leaf inoculated with the fungal isolate TVM 2, followed by ARI 3, MDU 5, MDU 8, TEN 4, KKI 3, MDU 7, KKI 4, PER 7 and CBE 3 (Fig. 3, Table 3).

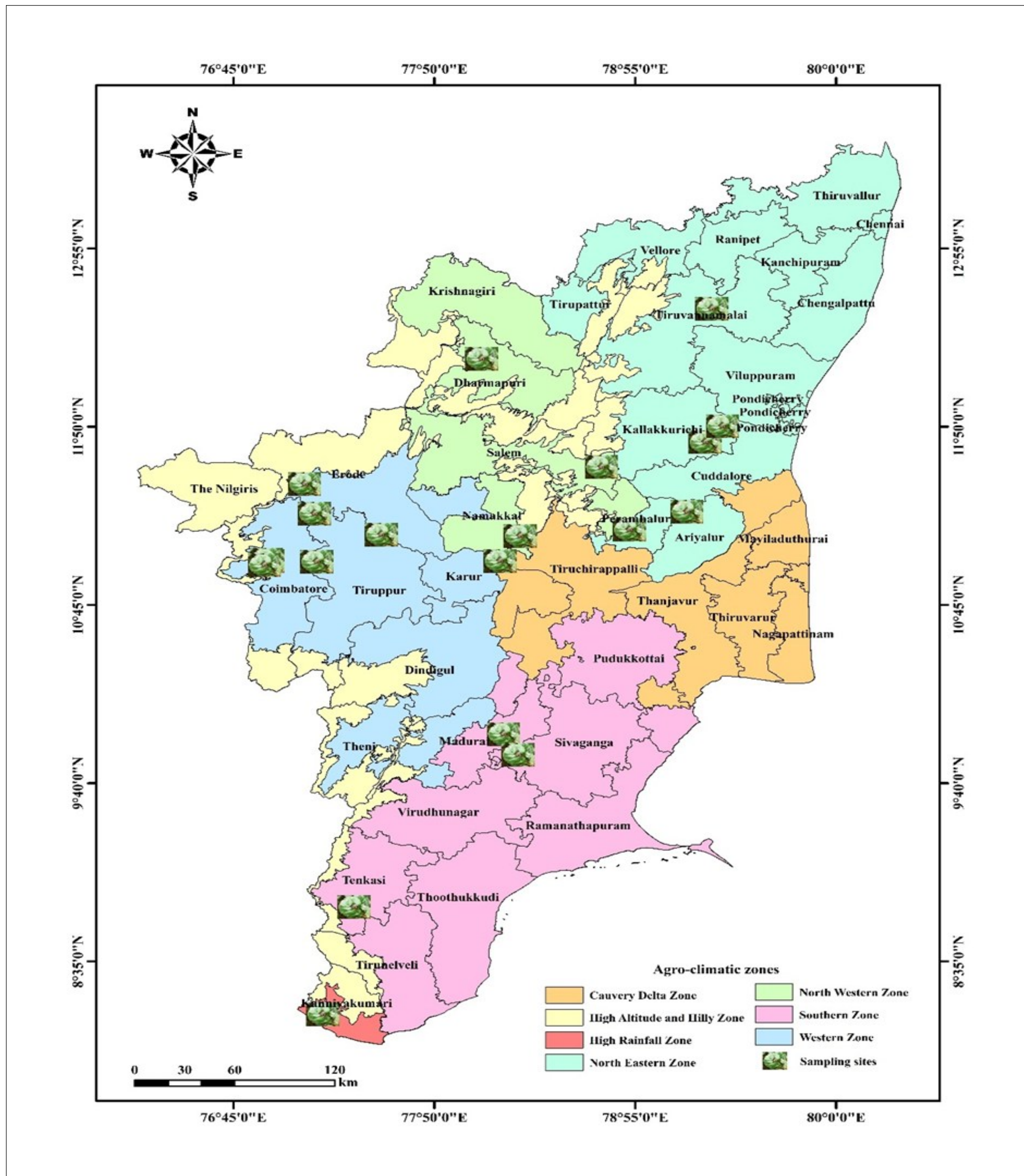

Fig. 1. Sampling locations of infected *T. portulacastrum* across different agro-climatic zones of Tamil Nadu.

Table 2. Details on the survey and collection of infected *T. portulacastrum* across different agro-climatic zones of Tamil Nadu

S. No.	Location	Month of collection	Latitude (N) & Longitude (E)	Crop	Count of isolates
1	Ariyalur (ARI)	April, 2024	11.316507°; 79.194562°	Pearl millet	5
2	Bhavanisagar (BSR)	December, 2023	11.483896°; 77.133856°	Maize	4
3	Coimbatore (CBE)	January, 2024	11.015344°; 76.937884°	Maize	4
4	Deviyakurichi (DEV)	November, 2023	11.588202°; 78.733688°	Greengram	2
5	Dharmapuri (DPI)	January, 2024	12.245934°; 78.088191°	Arecanut plantation	1
6	Kanyakumari (KAN)	July, 2024	08.238414°; 77.201173°	Fallow land	1
7	Kallakurichi (KKI)	April, 2024	11.739239°; 79.292173°	Maize	3
8	Kodangipalayam (KOD)	April, 2024	11.013109°; 77.198791°	Black nightshade	6
9	Madurai (MDU)	April, 2024	09.965506°; 78.206023°	Groundnut	8
10	New Area Millets (NAM)	November, 2023	11.025148°; 76.927034°	Sorghum	2
11	Namakkal (NKL)	April, 2024	11.169512°; 78.296119°	Fallow land	3
12	Paddy Breeding Station (PBS)	November, 2023	10.993086°; 76.916442°	Fallow land	10
13	Perambalur (PER)	April, 2024	11.206741°; 78.884139°	Maize	4
14	Perundurai (PRD)	July, 2024	11.177141°; 77.546764°	Fallow land	5
15	Sivagangai (SIV)	April, 2024	09.839826°; 78.284574°	Fallow land	7
16	Tenkasi (TEN)	November, 2023	08.912583°; 77.399279°	Guava orchard	6
17	Tirupur (TIR)	December, 2023	11.303923°; 77.186451°	Coconut plantation	1
18	Trichy (TRI)	January, 2024	11.017781°; 78.187661°	Watermelon	2
19	Tiruvannamalai (TVM)	February, 2024	12.549933°; 79.327903°	Groundnut	2
20	Villupuram (VIL)	April, 2024	11.835568°; 79.385534°	Brinjal	2

Fig. 2. Diverse fungal isolates isolated from the infected *T. portulacastrum* samples.

Table 3. Lesion length and diameter of necrotic spot induced by potential fungal isolates under *in vitro* detached leaf assay

Fungal isolate	Lesion length (cm)	Diameter of necrotic spot (cm)
TVM 2	1.3	1.2
ARI 3	1.3	0.9
MDU 5	1.2	0.8
MDU 8	1.1	0.8
TEN 4	1.1	0.8
KKI 3	1.0	0.8
MDU 7	1.0	0.8
KKI 4	1.0	0.8
PER 7	1.0	0.8
CBE 3	1.0	0.8

Morphological characterization of potential fungal isolates

Microscopic observations of the potential fungal colonies revealed that out of 10 isolates, 7 were *G. trianthemae* (CBE 3, KKI 3, KKI 4, MDU 5, MDU 8, PER 7 and TVM 2) and the rest were *Fusarium* spp. (ARI 3, MDU 7 and TEN 4) (Fig. 4). Mycelium and conidial characteristics of *G. trianthemae* observed under the microscope revealed that the conidia were pale yellow to dark brown, ellipsoidal, approximately 20-25 µm × 40-45 µm in size, having transverse and longitudinal septa. Grey to brown-coloured mycelia were observed. A septate, long, brown-coloured conidiophore was observed. Regarding *Fusarium* spp., hyaline, single-celled microconidia and macroconidia with 3-4 septation were observed. Hyaline mycelium with septation was observed.

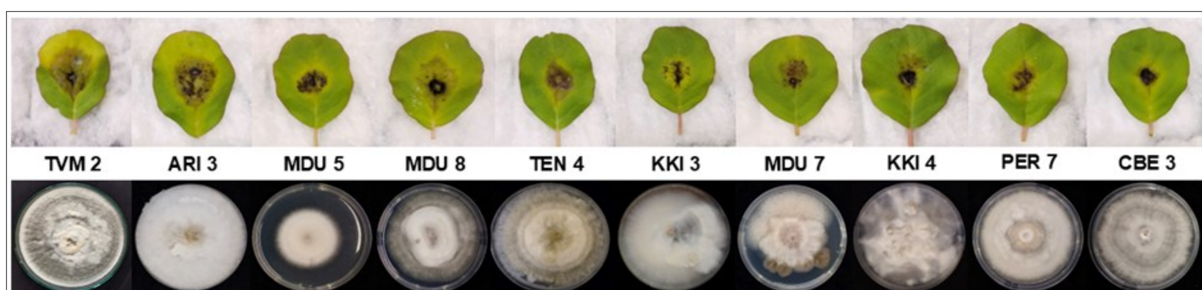

Fig. 3. Infected leaf and colony of potential fungal isolates assessed *in vitro* by detached leaf assay.

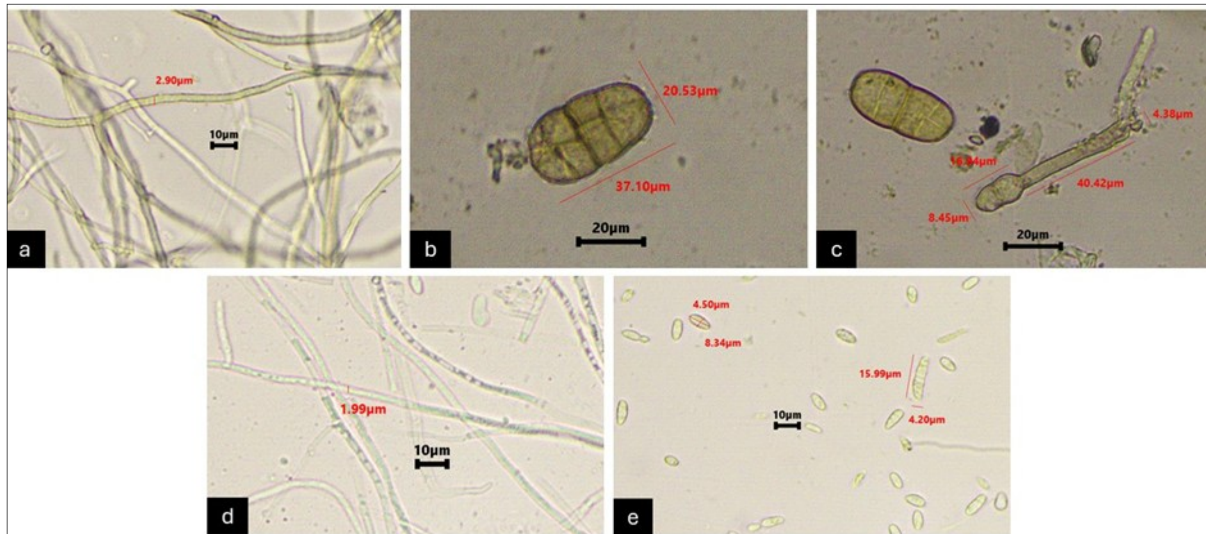

Fig. 4. Morphological characteristics of *G. trianthemae* and *Fusarium* spp. observed under the microscope at 40x. a: mycelium of *G. trianthemae*, b: conidia of *G. trianthemae*, c: conidiophore of *G. trianthemae*, d: mycelium of *Fusarium* spp., e: micro and macroconidia of *Fusarium* spp.

Molecular confirmation of potential fungal isolates

The ITS region amplification of the genomic DNA was observed at ≈550 bp amplicon size, confirming the fungal pathogens (Fig. 5). The Sanger sequencing of the amplified PCR product of ten potential isolates confirmed them as *G. trianthemae* and *Fusarium* spp. Among the 10 isolates, the isolates ARI 3, MDU 7 and TEN 4 were *Fusarium incarnatum*, *F. fujikuroi,* and *F. equiseti,* respectively, while the remaining isolates were confirmed as *G. trianthemae*. The nucleotide sequences were submitted to GenBank and the accession numbers were obtained (Table 4). The phylogenetic tree constructed using MEGA11 software revealed that the *G. trianthemae* isolates, viz., CBE 3, KKI 3, KKI 4, MDU 5, MDU 8, PER 7 and TVM 2, were closely aligned with the previously deposited *G. trianthemae* isolates in GenBank (Fig. 6).

Variability among *G. trianthemae* isolates as assessed by ISSR primers

A total of 47 amplicons were observed in ISSR analysis, of which 36 were polymorphic with an average polymorphism of 68.88 %. Maximum polymorphism was observed in the primers ISSR 8, 9 and 10, whereas no amplification was observed in the primer ISSR 5 (Table 5, Fig. 7). The dendrogram was divided into 3 clades, viz., clade I - KKI 4, CBE 3, clade II - TVM 2, PER 7, MDU 8 and clade III - MDU 5, KKI 3 (Fig. 8). The maximum similarity is between the isolates TVM 2 and PER 7, while the maximum dissimilarity is between the isolates TVM 2 and KKI 3 (Table 6).

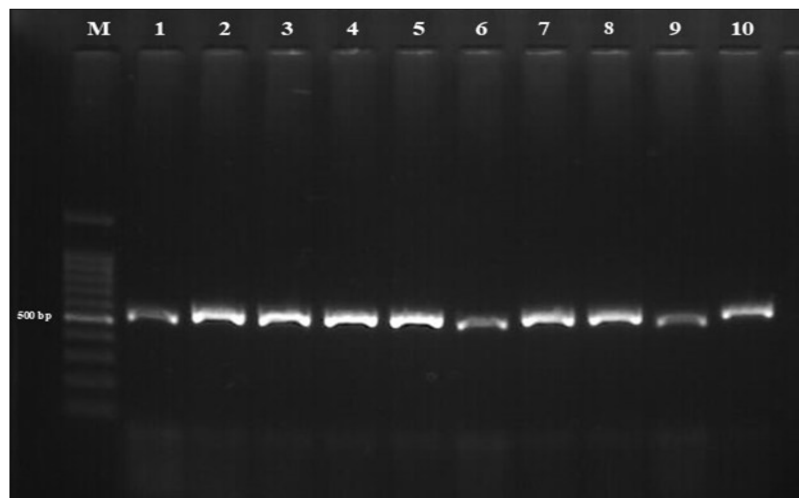

Fig. 5. Agarose gel image revealing the ITS region amplification of the fungal isolates. M: 100 bp ladder, 1: ARI 3, 2: CBE 3, 3: KKI 3, 4: KKI 4, 5: MDU 5, 6: MDU 7,7: MDU 8, 8: PER 7, 9: TEN 4, 10: TVM 2.

Table 4. Accession number of fungal pathogens screened from the detached leaf assay

Isolate code	Name of the pathogen	Accession number	Percent identity
ARI 3	*F. incarnatum*	PQ412789	99.59
CBE 3	*G. trianthemae*	PQ412791	99.60
KKI 3	*G. trianthemae*	PQ412800	99.60
KKI 4	*G. trianthemae*	PQ412807	99.60
MDU 5	*G. trianthemae*	PQ412809	99.60
MDU 7	*F. fujikuroi*	PQ412812	99.17
MDU 8	*G. trianthemae*	PQ412816	98.61
PER 7	*G. trianthemae*	PQ412817	99.21
TEN 4	*F. equiseti*	PQ412821	99.59
TVM 2	*G. trianthemae*	PQ412822	99.80

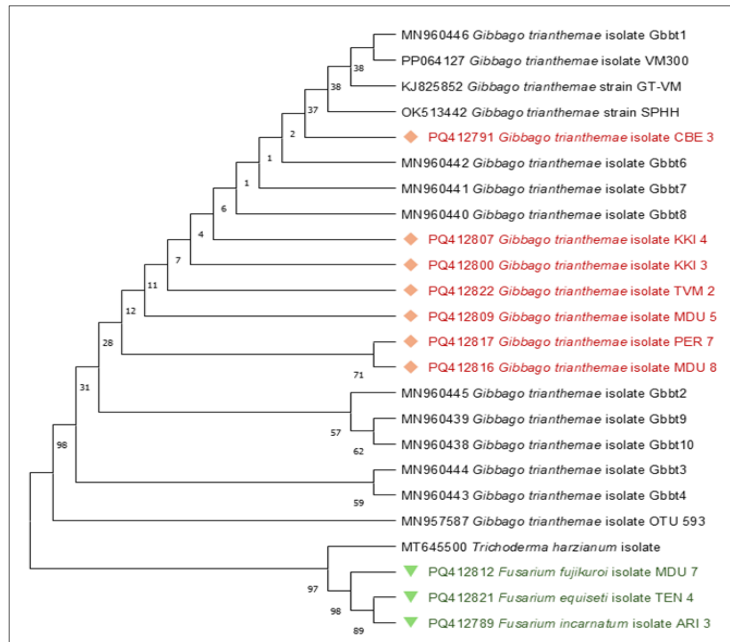

Fig. 6. Phylogenetic relationship of potential fungal isolates with the previously deposited isolates of *G. trianthemae*.

Table 5. Variability among *G. trianthemae* isolates as identified by ISSR primers

S. No.	Primer	No. of amplicons	No. of monomorphic amplicons	No. of polymorphic amplicons	Polymorphism (%)	PIC	MI	EMR
1	ISSR 1	3	1	2	66.67	0.346	0.042	2.000
2	ISSR 2	6	3	3	50.00	0.325	0.042	4.286
3	ISSR 3	5	3	2	40.00	0.269	0.037	4.000
4	ISSR 4	8	2	6	75.00	0.374	0.033	3.714
5	ISSR 5	0	0	0	0.00	0.000	0.000	0.000
6	ISSR 6	8	1	7	87.50	0.367	0.028	3.286
7	ISSR 7	3	1	2	66.67	0.374	0.034	1.429
8	ISSR 8	1	0	1	100.00	0.370	0.030	0.429
9	ISSR 9	5	0	5	100.00	0.370	0.030	2.143
10	ISSR 10	8	0	8	100.00	0.374	0.033	3.714
Total		47	11	36	-	-	-	-
Average		4.7	1.1	3.6	68.88	0.317	0.031	2.500

ISSR- Inter Simple Sequence Repeat, PIC - Polymorphism Information Content, MI - Marker Index, EMR - Effective Multiplex Ratio

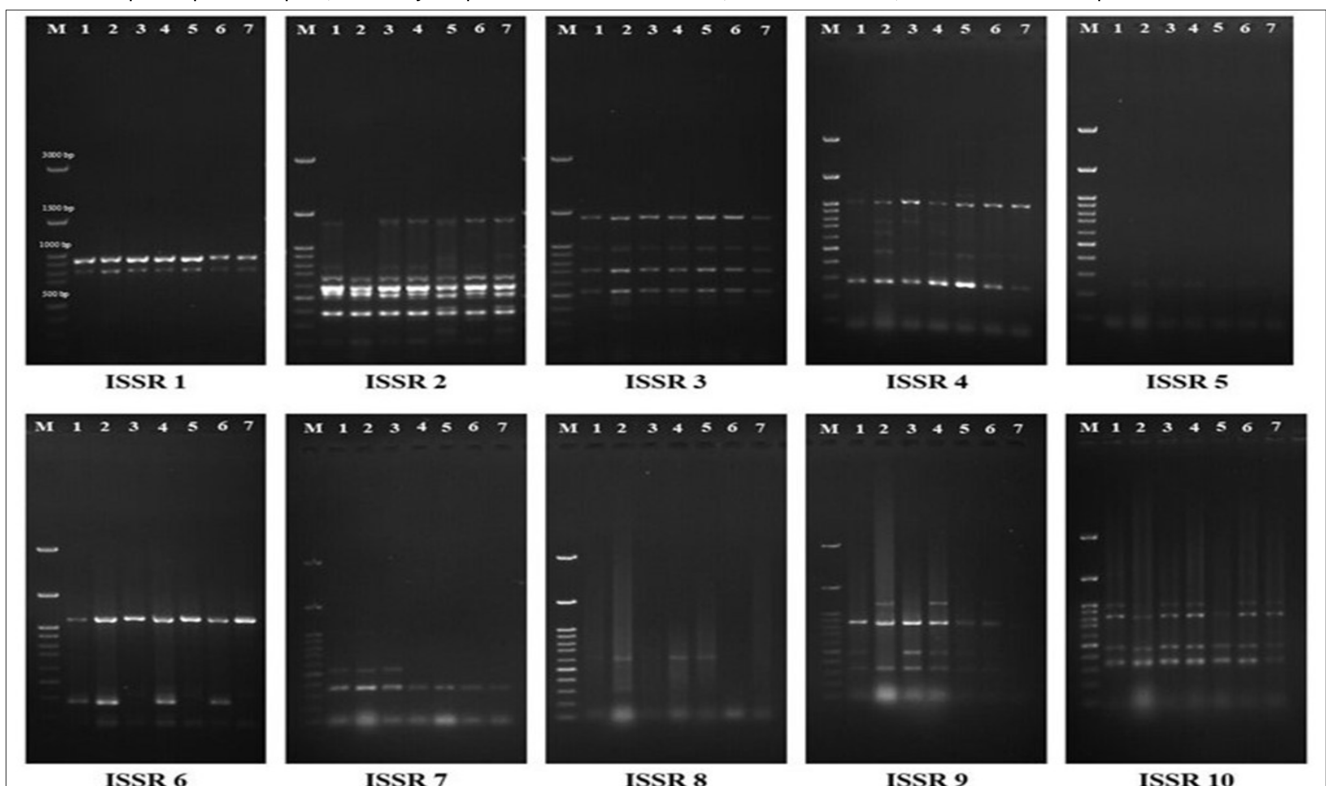

Fig. 7. Amplicon pattern of *G. trianthemae* isolates assessed using ISSR primers. M: 100 bp ladder, 1: CBE 3, 2: KKI 3, 3: KKI 4, 4: MDU 5, 5: MDU 8, 6: PER 7, 7: TVM 2.

Table 6. Dissimilarity matrix of *G. trianthemae* isolates assessed using ISSR primers

	CBE 3	KKI 3	KKI 4	MDU 5	MDU 8	PER 7
KKI 3	0.565					
KKI 4	0.293	0.559				
MDU 5	0.365	0.259	0.269			
MDU 8	0.559	0.520	0.262	0.280		
PER 7	0.339	0.641	0.337	0.298	0.298	
TVM 2	0.472	0.808	0.280	0.416	0.187	0.154

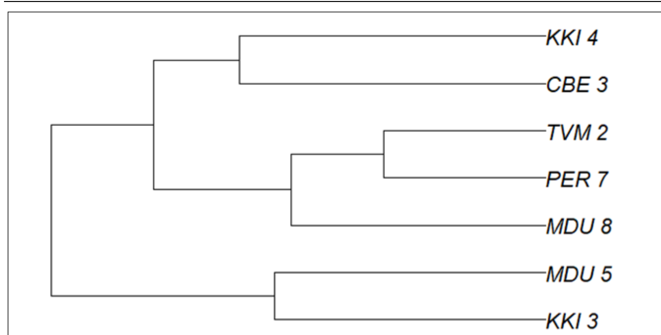

Fig. 8. Dendrogram constructed using the UPGMA method.

Testing the pathogenicity of potential fungal isolates under glasshouse conditions

Since *Fusarium* spp. were known to cause severe infection in diverse agricultural and horticultural crops, the isolates of *G. trianthemae* alone were selected for further studies. The test plants sprayed with the infective propagules viz., mycelium and spores of *G. trianthemae* were observed for disease development at 10, 20 and 30 DAI and the results revealed that the isolate TVM 2 had registered the maximum disease index of 34.4 % at 10 DAI, 41.6 % at 20 DAI and 49.3 % at 30 DAI followed by the isolate MDU 5 which recorded the disease index of 28.9 % at 10 DAI, 38.4 % at 20 DAI and 46.7 % at 30 DAI (Table 7). The inoculation of fungal isolate TVM 2 produced enlarged lesions on leaves and stems of *T. portulacastrum*, resulting in drying and wilting of some leaves. In addition, the collar region of the plant also gets infected (Fig. 9).

Qualitative test for assessing enzyme activity

The results of the qualitative tests confirmed the activity of various extracellular and cell wall-degrading enzymes investigated. The appearance of a clear and degraded halo around the fungal colony indicated the positive activity of enzyme production (Fig. 10). The diameter of the halo zone was higher in the amylase assay among all the hydrolytic enzymes tested.

Table 7. Percent disease index of potential *G. trianthemae* isolates on *T. portulacastrum* under glasshouse conditions

Isolates of *G. trianthemae*	Percent disease index (%)		
	10 DAI	20 DAI	30 DAI
CBE 3	19.4^{f} (26.2)	30.7^{f} (33.6)	39.2^{d} (38.7)
KKI 3	25.9^{cd} (30.6)	35.2^{d} (36.4)	44.0^{bc} (41.6)
KKI 4	24.1^{de} (29.4)	34.4^{de} (35.9)	42.9^{bcd} (40.3)
MDU 5	28.9^{b} (32.5)	38.4^{b} (38.3)	46.7^{ab} (43.1)
MDU 8	27.8^{bc} (31.8)	36.8^{c} (37.3)	45.3^{ab} (42.3)
PER 7	22.2^{e} (28.1)	33.0^{e} (35.1)	40.7^{cd} (39.6)
TVM 2	34.4^{a} (35.9)	41.6^{a} (40.2)	49.3^{a} (44.6)
Uninoculated control	0.0^{g} (0.0)	0.0^{g} (0.0)	0.0^{e} (0.0)
SEd	0.8	0.4	1.2
CD (0.05)	1.7	0.8	2.5

Data in the parentheses are arcsine-transformed values

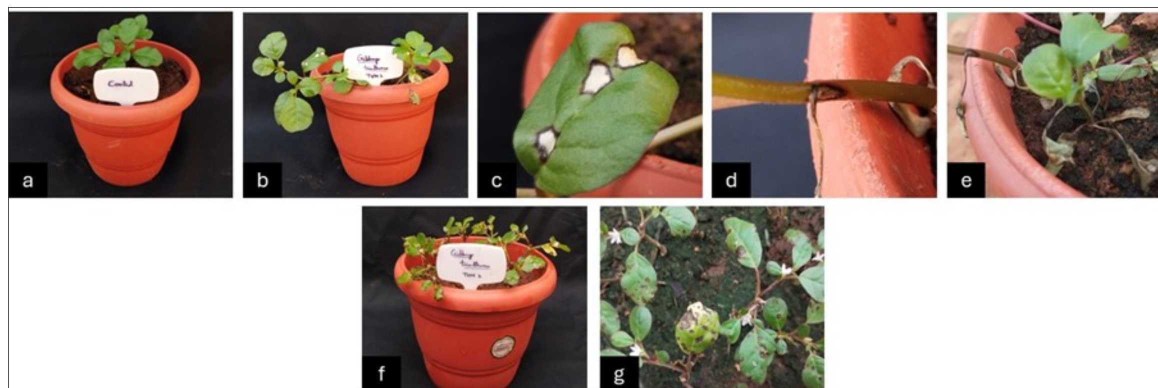

Fig. 9. Results of pathogenicity test under glasshouse conditions. a: control plant with no symptoms, b: plant inoculated with *G. trianthemae* (TVM 2) showing initial symptoms of disease development, c: enlarged leaf spot, d: lesions on the stem region, e: drying and wilting of infected leaves, f: Infection of *G. trianthemae* on *T. portulacastrum* at 20 DAI and g: severe lesions on leaves at 30 DAI.

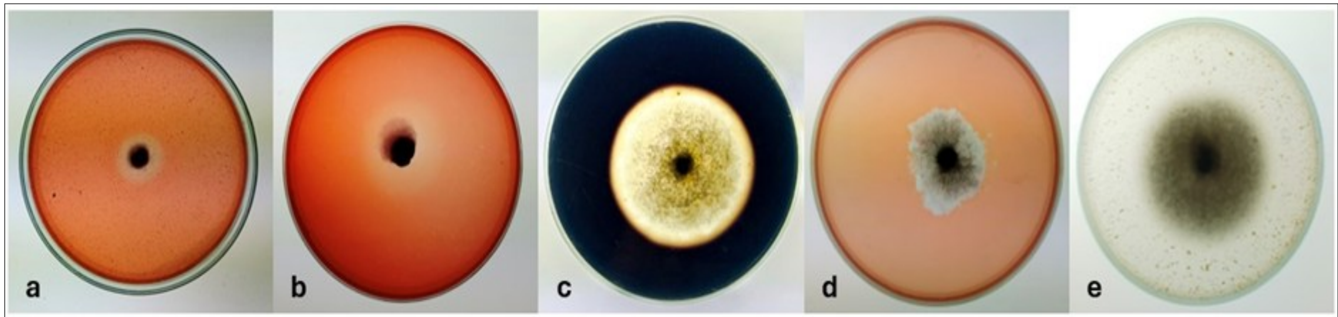

Fig. 10. Qualitative analysis of enzyme activity. a: ligninase, b: cellulase, c: amylase, d: endoglucanase, e: protease.

A roving field survey was undertaken at different locations of Tamil Nadu to collect the diseased *T. portulacastrum* and to explore the diversity of fungal pathogens associated with it. As each agroclimatic zone possesses distinct geographical and climatic conditions, correspondingly, *T. portulacastrum* might also display varied morphological characters for its adaptation. Similar to weeds, there is a possibility that various fungal pathogens associated with *T. portulacastrum* may also exhibit varied growth patterns and sporulation. Previously, extensive surveys were conducted in various districts of Punjab, Haryana and Uttar Pradesh to explore the extent of *T. portulacastrum* infestation and the diversity of fungal pathogens associated with *T. portulacastrum* in different cropped fields like maize, pearl millet, sugarcane, greengram, blackgram, redgram, groundnut, soybean, cotton and onion (19). Similarly, field surveys to observe the infestation of *T. portulacastrum* in various crops in the district of Vishakapatnam, Andhra Pradesh were conducted and explored the diverse mycopathogens associated with it (20). Likewise, 45 *T. portulacastrum* infested fields in the states of Rajasthan, Uttar Pradesh and Madhya Pradesh were surveyed to collect and examine the diseased *T. portulacastrum* plants (6).

Diverse fungal pathogens, *G. trianthemae*, *Alternaria* sp., *Curvularia* sp., *Colletotrichum* sp. and *Fusarium* sp. were isolated from the diseased *T. portulacastrum* samples. Earlier reports revealed that *Alternaria* sp., *Bipolaris maydis, Cladosporium* sp., *Cochliobolus* sp., *Colletotrichum* sp., *Curvularia* sp., *Fusarium* sp., *G. trianthemae, Phoma* sp. and *Septoria* sp. were associated with *T. portulacastrum*. Among them, *G. trianthemae* was highly infectious, causing severe defoliation (3-6). The results of the detached leaf assay revealed that there was no disease development in nearly 40 % of the isolates. This is because not all the strains of the fungi may be virulent to *T. portulacastrum*; some will be non-pathogenic to it, and they might be the secondary pathogens that were observed in the nearby cropped areas. Previously, a study involving the detached leaf assay revealed that disease severity of around 35.0 %, 46.3 %, 56.0 % and 61.0 % was observed on 5, 10, 15 and 20 DAI of *G. trianthemae,* respectively (21).

Observations on morphological characteristics of *G. trianthemae* conformed with the earlier reports (19, 22-24). Most of the characteristics associated with *G. trianthemae* are similar to those of the genera *Alternaria* and *Stemphylium* (25). Similarly, the microscopic observation of the morphological characteristics of *Fusarium* spp. corroborated with the previous reports (26, 27). The evolutionary relationship tree clearly revealed that *G. trianthemae* isolates were closely aligned with the earlier *G. trianthemae* isolates deposited in GenBank. The host specificity studies of *G. trianthemae* conducted earlier (8, 28, 29), revealed that *G. trianthemae* is highly specific to *T. portulacastrum* and is non-pathogenic to various agricultural and horticultural crops as well as to other weed flora. With this perspective, only the *G. trianthemae* isolates were considered for the pot culture studies. Various factors influenced the growth of the pathogen as well as the development of the disease.

The maximum disease index (34.4, 41.6 and 49.3 % at 10, 20 and 30 DAI, respectively) was observed in the plants sprayed with the inoculum of isolate TVM 2. This might be due to its rapid growth and better sporulation than the other *G. trianthemae* isolates, which enabled the quick penetration of the germ tube into the leaves, causing necrotic spots and lesions. Previously, a pathogenicity test revealed that disease severity of 58.0 % (14 DAI) was observed in *T. portulacastrum* due to the application of *G. trianthemae* at the concentration of $5×10^5$ conidia mL^{-1} (22). The post-emergence spraying of liquid formulation of *G. trianthemae* at the rate of $10×10^6$ spores mL^{-1} effectively controlled *T. portulacastrum* (3). Spraying of *G. trianthemae* spores at the concentration of $5×10^4$ mL^{-1} in combination with 0.02 % Tween-20 at different growth stages of *T. portulacastrum* revealed that DI was maximum (95.5 ± 1.1) during the stage-1 (3-5 foliage stage), followed by stage-2, i.e., 6-10 foliage stage (87.04 ± 2.0). Also, the disease intensity gradually increased from 69.12 ± 2.94 % at 20 DAI to 94.56 ± 2.82 % at 50 DAI (20). Application of conidial suspension of *G. trianthemae* at the rate of $2.2×10^5$ conidia mL^{-1} on *T. portulacastrum* resulted in severe defoliation (8). Likewise, a study showed that inoculation of *G. trianthemae* at the concentration of $5×10^4$ spores mL^{-1} effectively controlled *T. portulacastrum* (30).

Fungi secrete diverse enzymes responsible for the breakdown of the plant cell wall (31). *Gibbago trianthemae* screened for enzyme activity exhibited positive results for the activity of cellulase, protease, ligninase, amylase and endoglucanase. Similarly, *Alternaria macrospora,* a potential mycoherbicidal candidate used for managing *Parthenium hysterophorus,* was assessed for enzymatic activities, viz., cellulase, amylase, pectinase, laccase, LiP (lignin peroxidase) and Mn peroxidase and the fungal pathogen displayed positive results (32, 33). All these extracellular and cell wall-degrading enzymes have been reported and proven to assist in facilitating the entry, colonization and pathogenicity of diverse plant pathogenic fungi. From the qualitative analysis of various enzyme activities, it can be suggested that all these enzymes produced by *G. trianthemae* might have a direct or indirect effect on plant-pathogen interaction and disease development, which has to be studied in detail to ascertain the relationship between the specific enzyme and disease development.

Conclusion

Owing to the harmful hazards caused by synthetic herbicides on soil and the environment, the scientists have turned towards the use of various biological agents for the management of specific weed species or a group of weeds. From this study, it is evident that the infestation of *T. portulacastrum* was observed in the fields of various agricultural and horticultural crops as well as in diverse agroclimatic zones of Tamil Nadu. In search of a potential mycopathogen associated with *T. portulacastrum*, *G. trianthemae* (TVM 2 isolate) has been identified to cause the maximum disease infection. Hence, there is a potential possibility for exploiting the fungal pathogen for formulating a mycoherbicide for eco-friendly management of *T. portulacastrum*. Further research has to be taken up to optimize the growing conditions for enhanced growth and sporulation of the fungi. In addition, the use of mycometabolites and omics approaches can also be considered in the future for the effective management of weeds.

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Authors' contributions

VS[1] carried out the experiments and drafted the manuscript. KT assisted in formulating the study, providing resources and reviewing and editing the manuscript. VS[2] participated in formulating the study, providing resources, supervision and reviewing and editing the manuscript. RK, MKK, SV, SN and KN participated in reviewing and editing the manuscript. All authors read and approved the final manuscript. (VS[1] stands for Vaddi Saitheja and VS[2] stands for Vaithiyanathan Sendhilvel)

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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