STUDIES ON CYST NEMATODES IN HIMACHAL PRADESH AND THEIR MANAGEMENT USING NEMATOPHAGOUS FUNGI

Thesis Submitted For the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

(ZOOLOGY)

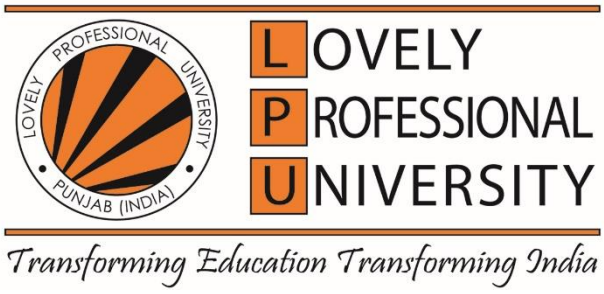
By

Ashvika Pathania

41600097

Supervised By

Dr. Joydeep Dutta



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PUNJAB

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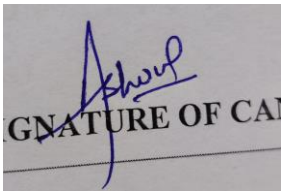
DECLARATION

I hereby declared that the presented work in the thesis entitled "Studies on cyst nematode in Himachal Pradesh and their management using nematophagous fungi" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision Dr. Joydeep Dutta, Professor and Head of Department, Department of Zoology and Botany, School of Bioengineering and Biosciences, Lovely Professional University Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



(Signature)

Ashvika Pathania

41600097

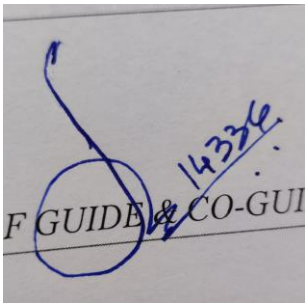
Department of Zoology

Lovely Professional University,

Punjab, India

CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Studies on cyst nematode in Himachal Pradesh and their management using nematophagous fungi" submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Zoology, School of Bioengineering and Biosciences, is a research work carried out by Ashvika Pathania, 41600097, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



(Signature of Supervisor)

Dr. Joydeep Dutta

Professor and Head of Department, Department of Zoology and Botany

School of Bioengineering and Biosciences

Lovely Professional University,

Punjab, India

Abstract

Cyst nematodes are considered an serious threat to agricultural and horticultural lands worldwide and pose an excessive limitation on the yield and production of numerous economically important crops, vegetables, and other important plants. One primary economic importance is the remarkable change in the host plant's roots induced by cyst nematodes. Cyst nematodes include six genera having the family Heteroderidae, i.e., *Heterodera*, *Globodera*, *Cactodera*, *Punctodera*, *Dolichodera* and *Afenestrata.* All cyst nematodes follow a unique life cycle; mature females can transform themselves into the form of rigid, resilient cysts after death. Females enclosed with their eggs inside the cyst can survive inside the soil for several years. A random survey was conducted from November 2018 to December 2021 in the agricultural fields of Himachal Pradesh to study the occurrence of cyst nematodes associated with crops. Soil samples were collected randomly, mainly from the wheat and potato cultivated areas. During this survey, 10 districts of Himachal Pradesh were covered: district Kangra, Mandi, Kullu, Chamba, Shimla, Una, Hamirpur, Bilaspur, Solan, and Sirmour. A minimum of four visits were conducted to collect soil samples from the particular area where cysts were noticed. Population density and percentage of occurrence of cyst nematodes isolated from the soil sample of each site were calculated. In addition, isolated cysts were subjected to morphological and molecular characterization. Once the morphological and molecular characterization was done, management of cyst nematodes was carried out. To manage the cyst population, the *in-vitro* effect of nematophagous fungi *Verticillium lecanii* after a

given interval of time was analyzed. The investigations were continued until the complete disintegration of cysts occurred.

Morphological and morphometrical studies revealed that the cysts of the district Una population were dark brown to moderately brown and lemon-shaped with distinct neck and vulval cone. Cysts length without neck was 701.7 ± 23.22 µm; neck length was 79.3 ± 8.43 µm, and width was 527.7 ± 41.79 µm, respectively. The fenestral length was 49.6 ± 2.66 µm, fenestral cone width was 21.8 ± 3.67 µm, vulval bridge width was 6.5 ± 0.58 µm, Vulval slit length was 6.3 ± 0.70 µm, and distance from the anus to fenestra were 28.4 ± 4.76 µm observed. Binfenstrate cone tops were observed under the light microscope. Morphological and morphometrical characteristics of Una population matched with genus *Heterodera*. The cysts of district Mandi (HPMD), Chamba (HPCH) and Shimla (HPSH) populations were light brown to dark brown and lemon-shaped with distinct neck and vulval cone. Morphometrics of district Mandi had cyst body length without a neck (N=10) were 556.93 ± 53.12 µm, neck length was 93.59± 3.71 µm, body width was 435.45± 51.05 µm, Length to width ratio were 1.21± 0.11 µm. Vulval cone fenestra diameter was 42.77 ± 4.31 µm. a typical circumfenestrate and abullate vulval cone were observed under the light microscope. Morphometrics of district Chamba had cyst length without neck measured 549.46± 65.42 µm, neck length measured 94.49± 4.21 µm, width measured 437.05±61.11 µm, and length to width ratio were 1.21± 0.11, and Vulval cone-diameter were 47.7 ± 4.33 µm. Abullate and circum-fenestrate cone top were present. Morphometrics of district Shimla had cyst length without neck measured 560.09 ± 65.92 µm, neck length measured 95 ± 4.45 µm, width measured 446.65 ± 54.30 µm, and length to width ratio were 1.2 ± 0.031, and Vulval cone-diameter were 45.68 ± 5.66 µm. Morphological and morphometrical characteristics of district Mandi, Chamba and Shimla populations matched with genus *Cactodera*. The cysts of district Mandi (HPPOMD), Chamba (HPPOCH), Kangra (HPPOPL), Kullu (HPPOKL) and Shimla (HPPOSH) populations were pale brown to golden brown and spheroid or globose in shape. Single circum-fenestrate vulval region were present. Morphometrics of district Mandi had cysts length without neck were 597.86 ± 77.97 µm, neck length was 141.39 ± 39.20 µm, and

body width was 502.54 ± 68.26 µm, and distance from vulval basin to anus were 62.94 ± 13.47 µm, and the number of cuticular ridges presents between vulval cone to anus were 19.2 ± 3.08. Morphometrics of district Chamba had cysts length without neck were 591.94 ± 79.42 µm, neck length was 132.8 ± 40.62 µm, and body width were 496.27 ± 86.92 µm. Distance from vulval basin to anus was 62.71 ± 13.53 µm, and the number of cuticular ridges presents between vulval cone to anus was 19.3 ± 3.23. Morphometrics of district Kangra had cysts length without neck were 609.8±63.06 µm, neck length was 143.93 ± 29.16 µm, and body width was 505.16 ± 67.87 µm, distance from vulval basin to anus were 65.71 ± 10.08 µm, and number of cuticular ridges present between vulval cone to anus were 19.9 ± 2.96. Morphometrics of district Kullu had cysts length without neck were 591.94 ± 79.42 µm, neck length was 132.8 ± 40.62 µm, and body width was 496.27 ± 86.92 µm, distance from vulval basin to anus were 62.71 ± 13.53 µm, and number of cuticular ridges present between vulval cone to anus was 19.3 ± 3.23. Morphometrics of district Shimla had cysts length without neck was 603.52 ± 48.18, neck length was 147.26 ± 40.56 and body width was 530.41 ± 71.10. The distance from the vulval basin to the anus was 62.72 ± 9.97 and number of cuticular ridges present between the vulval cone and anus was 20.8 ± 2.7. Morphological and morphometrical characteristics of district Mandi, Chamba, Kangra, Kullu and Shimla populations matched with genus *Globodera*.

According to molecular studies, the *Heterodera* population was identified as *Heterodera avenae* by amplifying the ITS region present in rDNA, including 18S and 28S genes. The resulted sequence was deposited to NCBI-GenBank, accession number OM049243.1. Based on ITS-rDNA, the *Cactodera* population were identified as *Cactodera estonica*. The resulted sequence was deposited to the NCBI-GenBank, accession numbers MN658364.1, MW821356 and MW821355. Finally, based on an amplified 18S small subunit of ribosomal RNA, the *Globodera* population was identified as *Globodera rostochiensis*. The resulted sequence was deposited to NCBI-GenBank, accession numbers MZ508280.1, MZ518783.1, MZ508279.1 and MW577347.1.

For cyst nematodes managment, the *in-vitro* efficiency of *Verticillium lecanii* on recovered cyst nematodes has been analyzed. The effect of different treatments *viz*.,

Treatment 1-10%, Treatment 2-20%, and Treatment 3-30% cell suspension of 1.7×10^5 CFUs / ml of *V. lecanii* was observed against cysts of *C. estonica*, *G. rostochiensis* and *H. avenae*. The *in-vitro* effect of *V. lecanii* was observed on the cysts of *C. estonica*. The results revealed that at 72 HAI, significant differences were noted in the number of cysts left (F = 253.808, df = 3, 20, P < 0.001). After 72 h, the entire cysts of *C. estonica* were disintegrated from Treatment 3 of *V. lecanii*. *In- vitro* effect of *V. lecanii* was observed on *G. rostochiensis* cysts; the results revealed that at 264 HAI significant difference in the number of cysts left was noted (*F=143.667, df= 3,20, P< 0.001*). After 264 h, the entire cysts of *G. rostochiensis* were disintegrated from the Treatment 3 of *V. lecanii*. *In-vitro* effect of *V. lecanii* was further observed on *H. avenae* cysts; the results revealed that at 168 HAI considerable difference in the number of cysts left was noted (*F= 201.852, df = 3,20, P< 0.001*). After 168 h, the entire cysts of *G. rostochiensis* were disintegrated from the Treatment 3 of *V. lecanii*. The *in-vitro* control study indicated that *V. lecanii* showed a dose and exposure time-dependent activity against cysts populations of *C. estonica, G. rostochiensis* and *H. avenae*. This survey confirmed the occurrence of *C. estonica, G. rostochiensis* and *H. avenae* in Himachal Pradesh. The present study provides the first report of *C. estonica* from Himachal Pradesh. This study suggested that few populations of *C. estonica* introduced into Himachal Pradesh have not been noticed yet. The present study concludes that among the three genera of cysts nematode, *G. rostochiensis* showed the highest occurrence in the soil. This study gives us knowledge about the distribution and identification of various cysts nematodes in Himachal Pradesh, which will help us make an appropriate management strategy.

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Table of contents

List of Tables

List of Figures

List of Appendices

Chapter-1

Introduction

Chapter-1

Introduction

1.1. Plant-parasitic nematodes (PPN): Plant-parasitic nematodes (PPN) are a significant threat to agricultural and horticultural land in developed and developing countries. PPNs are tiny worm-like multicellular, transparent microorganisms having bilateral symmetry, may act as endoparasite, ectoparasite and sedentary. They cause considerable yield loss to agricultural and horticultural plants such as potato, wheat, carrot, chickpea, lentil, sugar beet, alfa-alfa, cowpea, corn, eggplant, bean, sunflower and sunflower throughout the world. PPNs such as *Meloidogyne* spp. are obligate plant parasites; *Meloidogyne* spp. mainly parasitize vascular plant. *M. halpa, M. javanica, M. incognita, M. arenaria* are the most important species of the genus *Meloidogyne*. In the genus *Meloidogyne*, egg mass (protective gelatinous matrix) is formed by the eggs layed from mature females. This egg mass is generally found around the roots or plant tissue of the host plant(Eisenback and Triantaphyllou, 2020). Genus *Pratylenchus* is commonly known as root-lesion nematodes. The most important species are *Pratylenchus penetrans, Pratylenchus thornei*, *Pratylenchus neglectus*, *Pratylenchus zeae*, *Pratylenchus vulnus* and *Pratylenchus coffeae*. These are intercellular root endoparasites. Another economically significant PPN is *Radopholus similis,* commonly known as burrowing nematode. These are migratory endoparasitic nematodes that provide harm to citrus crops, pepper and banana. *Ditylenchus angus*, known as rice stem nematode, causes disease in rice(Jones *et al*., 2013). Among a root-knot, nematodes and cyst nematodes are economically most important PPNs worldwide.

1.2. Cyst nematodes: Cyst nematodes are considered as one of the economically important pests throughout the world for numerous crops, vegetables and other important plants. The significant economic importance of cyst nematodes is the notable change induce in their host plants. In most cases, damage or yield

loss caused by the nematodes is not that much exposed to sight due to other factors limiting the plant growth, such as biotic factors and abiotic factors.

Cyst nematode genera are particular among the plant-parasitic nematodes where females have the unique ability to transform themselves into resilient cysts that protect the eggs formed within the body. Eggs inside the cyst can survive up to 20 years without a host (Rehman, 2021). Cysts are tremendously resistant, durable, and spread quickly by biotic and abiotic factors, primarily associated with soil. Juveniles can disperse up to 1m after hatching and enter the plant through the root tip. After entering the root system, juveniles establish feeding sites by modifying plant cells that provide nutrients to them. Such infested plants have a underdeveloped root system, leading to low yield and plant death.

1.2.1. Life cycle: Cyst nematodes are sexually dimorphic. During the life cycle of cyst nematode, under favourable conditions, second-stage juveniles (J2) present inside the cyst hatch from the eggs into the soil (Fig.1). Hatching occurs in response to the root exudates secreted by the particular host plant. J2 synchronises their life cycle with the growth of their host plant, so that host plant roots secrete a gradient of stimuli to attract newly hatched J2 towards itself. The gradient of stimuli exists around the host plant's roots, consisting of amino acids, CO_2, pH and sugars. With the help of a protrusible, pointed stylet, J2 penetrates the root epidermis. Penetration occurs just above the root tip at the zone of elongation (Popeijus *et al*., 2000). Other sites of lateral root tip may also be selected for penetration. After penetration, J2 directed to the vacular tissue by crossing the cortical tissue intracellularly. A line of proliferation is produced in the cortical cell wall due to the mechanical action of the stylet and the action of the enzymes secreted by the nematodes. Sub-ventral pharyngeal glands secrete roots cell wall dissolving enzymes such as β-1,4-endoglucanases and pectate lyase through the stylet. After the invasion, syncytium is formed, which is highly metabolically active. The nematodes withdraw solutes from the feeding cells when the plant is refilled at regular intervals. After induced syncytial, juveniles undergo three moults and reach the adult stage(Toumi *et al.*,

2018). The adult male, after being motile, uses stylet to exit from the plant root into the soil and remain in the soil, whereas the female stay inside the roots of the host plant and keeps on feeding.

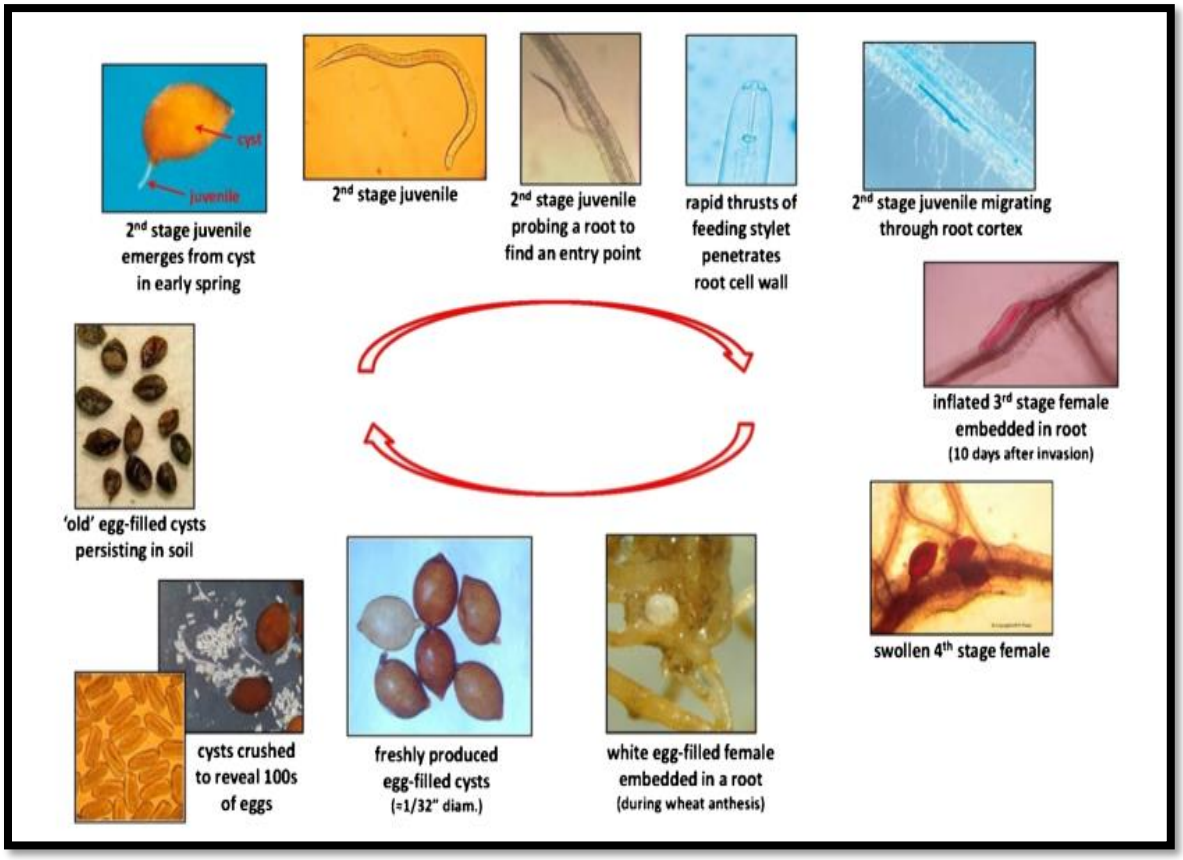

Figure 1: The complete life cycle of cereal cyst nematodes (Toumi *et al.*, 2018).

1.2.2. Genera: Cyst nematodes include six genera having the family Heteroderidae, i.e., *Heterodera*, *Globodera*, *Cactodera*, *Punctodera*, *Dolichodera* and *Afenestrata.* According to the shape of the cyst, all genera cysts consists of anterior protuberance represented as the neck and posterior protuberance represented as the vulval cone. Genus *Heterodera*, *Cactodera* and *Afenestrata* characteristically have a lemon-shaped cyst with slight variations in shape, size, and symmetry. Genus *Globodera* and *Punctodera* have a round or oval shape without a vulval cone. Genus *Dolichodera* has an elongated ovoid shape with the absence of posterior protuberance.

1.2.2.1. Genus *Globodera*: Genus *Globodera* is commonly called potato cyst nematode (PCN). *Globodera rostochiensis* (golden cyst nematode) and *Globodera pallida* (pale potato nematode) are PCN's two economically important species.

- **Morphology:** Morphologically, *Globodera* spp. can be differentiated from each other based on stylet knob shape, ridges between the anus and vulval basins and Granek's ratio (Wainer and Dinh, 2021). Mature dead female cysts are light brown to slightly dark brown, spherical to subspherical shape, and protruding short necks. In all specimens of *Globodera*, the anus is prominent, often with a V-shape mark. A single circumfenestrate opening is present in the vulval region occupying all the parts of the vulval basin. The number of cuticular ridges from the vulva to the anus is one of the important morphometrical characteristics. The Subcrystalline layer, vulval bridge, underbridge, and bullae are absent (Wainer and Dinh, 2021). Whereas in some species, bullae like structures are there. *Globodera* population morphologically varies from species to species.

  During maturation *G. rostochiensis* cyst changes from white to yellow into a brown cyst. In contrast, the *G. pallida* cyst is firstly seen as creamy white changes directly into brown colour. The body length of *G. pallida* cysts is slightly longer,

and the stylet of the second stage juveniles J2 stage is marginally shorter. The tail shape is sharply narrow, the posterior hyaline tail terminus has slight constrictions, and the tail is marked with annules. *G. rostochiensis* cysts have a small Granek ratio, from anus and vulva 12-31 cuticular ridges (Yu *et al*., 2010). Only 4-7 refractive bodies are present in the hyaline tail region. Second stage juvenile J2 stage tail termini are more bluntly pointed, stylet knobs are more anteriorly directed. *G. rostochiensis* and *G. pallida* are closely related species, and it isn't easy to differentiate between these species only on a morphological basis.

G. tabacum is another economically important species of PCN. Network like patterns is absent on the cyst wall. The tail terminus of the J-2 stage is distinctly narrow (Skantar *et al*., 2011). In *G. tabacum* anterior surface of the stylet, the knob is rounded, 10-14 ridges are present between the anus to the vulval basin and 1 to 4.2 Granek's ratio is there.

In *G. achilleae* cyst anterior surface of the stylet, the knob is rounded to anchor-shaped. In addition, 4-11 ridges are present between the anus to the vulval basin; Granek's ratio range is 1.3 to 1.9. Lastly, in *G. artemisiae,* cysts have rounded stylet knobs, anteriorly flattened, sometimes slightly indented. *G. artemisiae*, Granek's ratio range is 0.8 to 1.7 (Wainer and Dinh, 2021).

1.2.2.2. Genus *Cactodera*: consists of the lemon-shaped cyst, somewhat rounded or oval. The Colour of the cyst ranges within the species from creamy white (young stage cyst), golden yellow, light brown, dark brown to black. Therefore, the Colour of the cyst varies from species to species. There are approximately 11 species of the genus *Cactodera* has been studied yet.

- **Morphology:** All species are morphologically distinct from each other. The common structure of the cyst consists of the posterior protuberance, known to be the circumfenestrate cone top. No fenestration is present in the anus. The cuticle is present like a D-shape layer. Female cyst consists of vulval denticles, bullae and

underbridge are absent. Second stage juveniles i.e., J-2 stage have a lateral field with 4 incisures; phasmids are commonly punctiform. The labial disc is present, and six lip sectors are present. Eleven known species of the genus *Cactodera* is *C. cacti*, *C. estonica*, *C. thornii*, *C. chenopodiae*, *C. solani*, *C. torreyanae*, *C. evansi*, *C. salina*, *C. milleri*, *C. johanseni*, *C. galinsogae*. *C. cacti* cyst's shape varies from lemon shape to spherical. The Colour of the young cyst is pearly white, and then it turns to light brown in the matured stage. Both vulva and neck are distinct. Abullate and circumfenestrate cone top is seen in this species. Second stage juveniles have a vermiform body with both side tapering ends (Duan *et al*., 2012). *C. estonica* has a dark brown colour female cyst. Both neck and the vulval cone are distinct. *C. estonica* has a circumfenestrate vulval cone. Denticles and underbridges are also seen in this species (Yu and Sun, 2018). *C. thornii* feed particularly on cereal plants. Morphologically cyst shape is lemon or oval-shaped. *C. chenopodiae* cysts are round or subspherical in shape. The vulval cone size is small with small protruding lips. Outer cuticular have marked rugose pattern except in the vulval cone. Underbridge, vulval denticles and bullae are absent. Like other species, second stage juvenile J-2 is vermiform in shape with tapering anterior and posterior ends. Stylet knobs are spherical and projecting anteriorly. The tail consists of the hyaline region (Feng *et al*., 2018). *C. solani* cysts are lemon-shaped, matured cysts colour varies from brown to black. Circumfenestrate vulval cone is present.

C. torreyanae cysts are lemon-shaped and have a distinct vulval cone. The cyst is light brown or dark brown. In this species, a gelatinous sac is present beside the protruding cone. Eggs have a smooth surface without punctuation. J-2 body is cylindrical, with a tapering posterior end. J-2 stage has rounded stylet knobs which are slightly directed posteriorly. The stylet in juveniles and adults is slightly covered from the dorsal side. In the second stage, the juvenile minute pore is present at the beginning of the hyaline portion, known as phasmid (Evans, 2014). *C. evansi* cysts' shape ranges from subspherical to lemon-shaped. Comparatively small size female body. J-2 stage consist of labial disc. Juveniles have conical round shape

tails. Adult males have specialized structure cuticular blocks present in the lip region (Prado and Rowe, 2000). Morphologically cysts of *C. salina* have a unique structure. Cyst shape is rounded with reduced or no terminal cone. The size is tiny. The midbody cuticular pattern of the female and cyst is generally straight to wavy lines, showing morphologically distinct features from other species. The eggs of the genus *Cactodera* are covered with tubercles, but this feature is absent in *C. salina* (Baldwin *et al*., 1997). *C. milleri* consist of lemon-shaped cyst, dark brown, abullate and circumfenestrate vulval cone top. In the middle of the cyst wall, straight or wavy cuticular patterns have been seen. Juveniles have concave basal knobs, and the well-developed stylet is present (Skantar *et al*., 2021). *C. johanseni* also has lemon-shaped cysts. The Colour varies from dark brown to black. *C. johanseni* has a small cyst with large fenestra. Vulval cone top is circumfenestrate, abullate. J-2 stage has four lateral lines (Sharma *et al*., 2001). Among most of the species of the *Cactodera, C. galinsogae* has the smallest cysts. Spherical and sub-spherical in shape, circumfenestrate with a straight neck. The cyst is light brown or dark brown. An interlaced pattern has been observed on the mid-body of the cyst. Vulval denticles are absent. J-2 has six pseudo lips and four lateral lines (Tovar *et al*., 2003).

1.2.2.3. Genus *Heterodera*: Another genus of cyst forming nematodes is *Heterodera* belongs to the family Heteroderidae, one of the essential global agriculture pests. Some economically important and studied species are *H. avenae, H. glycines, H. latipons, H. filipjevi, H. cajani, H. schachtii, H. trifolii, H. zeae* and *H. cruciferae*. Each group of *Heterodera* spp. has several sub-species classified into species complex.

- **Morphology:** *H. avenae* consists of a light to dark brown, lemon-shaped cyst with a short, distinct protruding vulval cone. Bifenestrate vulval cone is present with narrow vulval bridge. Numerous bullae are found situated underneath of vulval slit. Underbridge is absent. Second stage juveniles are curved ventrally. The Lip region is rounded, and the labial disc is also present. The stylet is slightly forward from

the anterior region; strong and large knobs are present. Large phasmids are present. Tapering tail is present. *H. filipjevi* cysts are dark brown with a small distinct protuberance of the vulval cone. Sub crystalline layer is present. The vulval cone is bifenestrate, narrow vulval bridge is attached to it. A short vulval slit is present; numerous bullae are below the vulval slit. The under-bridge is moderately developed with bifurcated ends. Second stage juveniles have a cylindrical body with a tapering tail ended with a rounded tip. The rounded lip region is present with two annules. The labial disc is also present. The robust stylet is attached to the large and concave stylet knobs. A small vulval cone is present with *H. latipon.* Cysts are rounded or lemon-shaped. The Colour varies from pale to deep brown, and a sub crystalline layer is present. A bifenestrate vulval cone is present; both circular semifenestrae are separated by a vulval bridge. A strong underbridge is present; the middle portion is thick and bifurcated from the ends. At the level of underbridge ovoid bullae are present. The vulval slit is very short. Second-stage juveniles have a dome-shaped lip region with two annules and a labial disc. Large phasmids are present. A short tail with a rounded terminus is present (Maafi *et al*., 2007).

H. zeae is lemon-shaped and light brown. Cyst walls have zigzag patterns, and a thin wall cuticle is present. Vulval cone top is ambifenestrate having horseshoe shape semifenestra. Bullae are arranged in two levels in the vulval cone. The first one is below the underbridge, and the second is a long heavy mass like bullae randomly distributed below the underbridge. Second stage juveniles are cylindrical with a short tail having conical tapering and a rounded terminus. Hyaline terminus is one half of the tail end (Skantar *et al*., 2012). *Heterodera schachtii* is commonly known as sugar beet cyst nematodes having a close morphological resemblance with *H. trifolii*. The body structure of *H. trifolii* is straight or slightly curved, tapers posteriorly with conoid finely rounded tail terminus. Stylets have a deep concave anterior directed knob. Cysts are light brown to dark brown, lemon-shaped with distinct protruding neck and vulval regions, ambifenestrate cone top is present. The vulval bridge separates the semifenestrae; the beneath vulval bridge

underbridge is present, having bifurcated ends. Zigzag patterns are present on the wall. Whereas in *H. schachtii* cylindrical shape juveniles are present. The body tapers conically in the tail region with a rounded tail terminus. Phasmids are present. 4 incisures are present on the lateral area. Cysts are dark brown and lemon-shaped. Ambifenestrate cone top is present with a robust underbridge. Semifenestrae is separated by vulval bridge. Underneath of vulval bridge molar shaped bullae is present (Muwamula *et al*., 2018). It is challenging to quantify the yield loss caused by the cyst nematodes due to a absence of apparent symptoms. The occurrence of cyst nematodes remains unnoticed until the infestations caused by them are severe. Minnis *et al*. (2002) estimated that 64% of potato cyst nematodes occur in England and Wales potato fields. The same conditions exist in most countries globally, where various cysts nematode causing economic yield loss to many crops.

1.3. Control: Presently controlling the cyst nematode is the major challenge because all eggs remain in the dormant stage inside the tough protective layers for many years in the soil. Current control relies on various strategies employed by integrated pest management. Nematicides are popularly used but have already been withdrawn due to environmental concerns.

1.3.1. Chemical control: According to Lilley *et al*. (2005), some chemicals remain in use, such as oxamyl and foshiazate. Fumigants are also used to control nematodes such as metam-sodium, metam-potassium and dazomet. Metam sodium is a synthetic chemical product used in liquid formulation and acts as a fumigant. Trails showed higher efficiency against root-knot and cyst nematodes. Metam potassium has nematicidal, herbicidal, insecticidal and fungicidal activities. Dazomet is a granular product that acts as a fumigant. Some non-fumigants are also used against PPN, such as fenamiphos, fosthiazate, oxamyl and fluopyram.

1.3.2. Biological control: Biological control is more eco-friendly, sustainable, and cost-effective than chemical pesticides. Some bacterial and fungal derivatives are proved to be effective biocontrol agents (Fig 2). *Pasteuria penetrans* are an endosperm-forming bacterium used as an effective biocontrol agent against *Meloidogyne* spp. (Bishop *et al*., 2007). *Bacillus firmus* is also used as a biological nematicide against PPN. Abamectin is a mixture of macrocyclic lactones produced by *Streptomyces avermitilis*. It paralyses nematodes by causing an ionic imbalance in the nervous system of nematodes (Khalil, 2013).

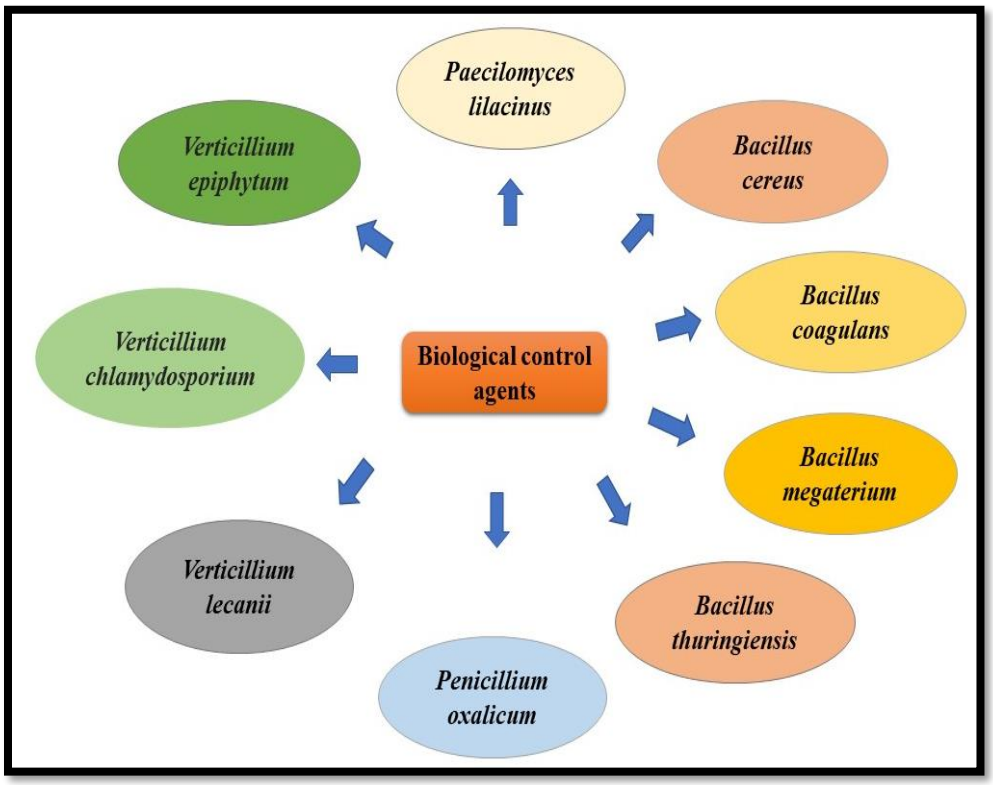

Figure 2: Various bacterial and fungal biocontrol agents.

Fungi is considered one of the important controlling agents against cyst nematodes. It helps regulate the population densities of cyst nematodes under natural and agricultural conditions. The phylum Mortierellomycota attack more on white females than in cysts, whereas Ascomycota attack mainly on cysts than in white females *Verticillium lecanii* infect the undifferentiated dormant eggs inside the cyst, matured eggs and any stage of the life cycle in cyst nematodes such as proved to be effective biocontrol agent for *G. pallida* and *H. schantii* (Uziel and Sikora, 1992; Shinya *et al*., 2008). *V. chlamydosporium* is also known to have an excellent nematicidal effect against *M. javaniva*. *V. chlamydosporium* reduces the number of eggs that hatch and also increases the mortality rate of root-knot nematodes. This fungus is known to have chitinolytic and proteolytic activities that cause alterations in the egg shell permeability and cuticular structure and cause perforations in the cuticle, which ultimately leads to perforations in egg structure and inhibit hatching (Mukhter and Pervaz, 2003). Other fungi are also proved as efficient biocontrol agents for different cyst nematodes, such as *Beauveria bassiana* is also a efficient biological control agent to control *H. filipjevi* population. Another *Verticillium* spp. i.e., *V. chlamydosporia* individually and in combination with *Aspergillus flavus* and *Penicillium chryosogenum* is a remarkably proven biocontrol against *M. incognita* (Naz *et al*., 2021). In addition to bacterial and fungal control of PPNs, plant extracts are also used as a biological control agent against PPNs, as garlic extracts are effective against *Meloidogyne* spp., *Tylenchus* spp., *Pratylenchus* spp., *Longidorus* spp., *Xiphinema* spp. *Trichodorus* spp., and the cyst nematodes (*Heterodera* spp., and *Globodera* spp.) (Andrés *et al.,* 2012). Clove oil has high nematicidal and nematostatic activity. It interferes with normal embryogenesis, reduces the hatching rate and is mainly used against eggs of *Meloidogyne* spp. (Djiwanti, 2019).

Table1: Various biocontrol agents used against plant-parasitic nematodes.

S. No	Controlling agent	Plant-parasiticNematodes	References
1.	*Bacillus cereus*	*Heterodera avenae*	Ahmed, 2019
2.	*Bacillus coagulans*	*Heterodera glycines, Meloidogyne incognita*	Xiang *et al.*, 2018
3.	*Bacillus megaterium*	*Heterodera glycines, Meloidogyne incognita, Meloidogyne graminicola.*	Mostafa *et al.*, 2018
4.	*Bacillus thuringiensis*	*Heterodera glycines, Meloidogyne incognita*	Mohammed *et al*., 2008
5.	*Pochonia chlamydosporia*	*Globodera pallida* and *Globodera rostochiensis*	Tobin *et al*., 2008
6.	*Penicillium oxalicum*	*Globodera pallida*	Martinez *et al*., 2013
7.	*Verticillium lecanii*	*Heterodera glycines*	Shinya *et al*., 2008
8.	*Verticillium chlamydosporium*	*Meloidogyne* spp.	Kerry, 2001
9.	*Verticillium epiphytum*	*Meloidogyne javanica*	Moosavi *et al*., 2011
10.	*Paecilomyces lilacinus*	*Globodera* spp. Potato cyst nematode	Jacobs and Crump, 2003

1.4. Economic loss due to PPNs: Plant parasitic nematodes cause high economic loss of crops (Table 2). The overall annual yield loss of 40 major crops occurs due to the infestation caused by plant-parasitic nematodes has been reported at approximately 12.3 %. In developing countries major crop yield loss (14.6%) caused by PPNs is more than in developed countries (8.8%). Alone *Meloidogyne* spp. cause losses in different crops constitutes about 75.83% of the total estimated losses (Kumar *et al*., 2020).

Table 2: Amount of loss caused by plant-parasitic nematodes on various crops.

S.No	Parasite	Affected crop	Amount of loss (rupees)	Reference
1.	*Meloidogyne graminicola*, *Heterodera oryzicola* and *Aphelenchodes besseyi*	Rice	4779.00 million	Jain *et al*., 2007
2.	*Heterodera cajani*	*Sesamum*	43.35 million	Jain *et al*., 2007
3.	*Heterodera avenae* and *Anguina tritici*	Wheat	97.28 million	Jain *et al*., 2007
4.	*Meloidogyne* spp.	Wheat, potato	77,373.87 million	Kumar *et al*., 2020
5.	*M. graminicola*	Rice	23,272.32 million	Kumar *et al*., 2020
6.	*H. avenae*	Wheat	8967.52 million	Kumar *et al*., 2020
7.	*Globodera* spp.	Potato	Rs 127.04 million	Kumar *et al*., 2020

Chapter- 2

Review of Literature

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Review of Literature

Plant-parasitic nematodes

Plant-parasitic nematodes (PPNs) are microscopic, small worm-like, transparent, bilaterally symmetrical, multicellular, pseudocoelomate, parasitic microorganisms (Shah and Mahamood, 2017). For plant parasitism, stylet and sub-ventral and dorsal oesophagus glands of PPN play a significant role in evolutionary adaptations. PPNs are unsegmented, but their body looks segmented on the cuticle due to numerous annulations. PPNs have well-developed feeding, digestive, nervous and reproductive organs, whereas they lack circulatory and respiratory organs (Goss, 2008). The damage caused by the nematodes to the crops is mostly not apparent and remains unseen due to many other limiting factors for plant growth (Schmitt and Sipes, 1998).

Characteristics of some important plant-parasitic nematodes

2.1 Root rot nematode (*Hrischmanniella* spp.)

- In the genus *Hrischmanniella, H. miticausa* is considered under the sedentary group and reported in Solomon Island and Papua New Guinea. It forms a knot-like structure in the host plant's roots (Subedi *et al*., 2020). Host ranges are rice, cotton, sugarcane and maize. There are 24 species reported in the genus *Hrischmanniella*. Twelve species are parasitic on rice plants only (Regmi *et al*., 2016). These species mainly enter through the host plant's lateral root, migrate to the aerenchyma region and cause necrotic traces. After the second invasion, necrosis leads to the browning of roots (Bauters *et al*., 2014). After that, a sexual mode of reproduction occurs. These are migratory endoparasitic. The life cycle is completed in up to 30 days.

2.2 Sting nematode (*Belonolaimus* spp.)

- *Belonolaimus* spp. found in Australia, Brazil and Venezuela. *Belonolaimus longicaudatus* was firstly reported in the southeastern United States. However, it is commonly found on the Atlantic coasts from Texas to Virginia and the Gulf of

Mexico (Gozel *et al*., 2006). Host ranges from turfgrasses, citrus, corn, peanuts and vegetables. Infected plants showed symptoms like a damaged root system, poor water and nutrient uptake, wilting, and chlorosis. These are ectoparasites. The life cycle completes up to 28 days (Singh *et al*., 2013).

2.3 Citrus Nematode (*Tylenchulus* spp.)

- Citrus nematodes are distributed worldwide. Commonly found in citrus plants, grapes, olive and persimmon. *T. semipenetrans* infection cause 10- 30 % yield loss. This infection caused slow plant growth, reducing the fruit yield and yellowing the foliage. In addition, the root system becomes thicker than in healthy plants (Lucas and Kenry, 2004). These nematodes are semi endoparasitic. The life cycle completes in 4 – 8 weeks (Singh *et al*., 2013).

2.4 Seed gall Nematode/Ear Cockle Nematode (*Anguina* spp.)

- The seed gall nematode is reported in India, Italy, Iraq, France, the USA, West Africa, North Africa, Australia, Brazil, and China (Tulek *et al*., 2015). Powers *et al.* (2001) reported 11 species of *Anguina*. Wheat barley and rye are the primary hosts of this genus. It causes 50-100% yield loss in wheat and barley (Mukhtar *et al*., 2018).

2.5 Lesion Nematode (*Pratylenchus* spp.)

- Root lesion nematodes are distributed worldwide. Genus *Pratylenchus* consists of 97 species. Cereals, legumes, vegetables, coffee, fruits, and ornamental plants are the main host of lesion nematodes. Out of 97 species, 12 species are causing severe damage in temperate areas, and 8 species are causing severe damage to cereal crops (Yu *et al*., 2012). Infected plants show symptoms such as necrosis, especially in vegetable plants' tumours and roots and foliage's yellowing. These are endoparasitic nematodes. The life cycle is completed in 3 weeks to 5 weeks, depending on environmental conditions. Genus *Pratylenchus* reproduces by parthenogenesis and anhydrobiosis. (Jones and Fosu-Nyarko, 2014; Esteves *et al*., 2015)

2.6 Root-Knot Nematode (*Meloidogyne* spp.)

- Root-knot nematodes are sedentary endoparasitic, distributed throughout the world. Genus *Meloidogyne* consists of 98 species causing severe damage to host plants and 5% total economic yield loss worldwide (Ralmi *et al*., 2016). Host ranges are crops, agronomic plants, vegetables, and fruit trees. Infected plants show knot formation in the roots due to the expansions in the root cells, and secondary symptoms are yellowing of leaves, stunted growth, nutrient deficiency, and wilting (Ralmi *et al*., 2016). These nematodes enter the root region and feed into it. Under suitable conditions, they complete their life cycle in 3-4 weeks. The favourable temperature for reproduction is 27-30ºC and reproduces by parthenogenesis and occasionally occurs by amphimixis (Singh *et al*., 2019)

2.7 Cyst nematodes

- Cyst nematodes are soil-borne parasites globally attacking numerous crops. Cyst nematodes are obligatory biotrophs. They are amphimictic species (Fig.3). They are highly specialized and economically significant. Cyst nematode infects a large group of plant parasites and forms a complex and specialized relationship with their hosts. Cyst nematodes infect the plant range of tropical, sub-tropical and temperate areas. A unique method to detect the cyst nematode infection is to check the occurrence of adult female nematodes attached to the roots of host plants after some time of parasitism. Cyst nematodes consist of hundred known species in six genera. Cyst nematodes have unique characteristic features during the life cycle; after their death, females convert their cuticle into the form of the hard layer within it encloses their eggs called a cyst. Almost all six genera of cyst nematodes follow a standard life cycle with slight modifications varies from species to species. Second-stage juveniles (J2) hatch in the soil from the eggs present inside the cyst under favourable environmental conditions. Hatching mainly occurs in response to the root exudates secreted from host roots. With the help of root exudates, J2 locate the host plant and synchronize its life cycle along with the growth of the host plant. (Tefft and Bone, 1985). J2 penetrate over the root epidermis, just above the root tip

in the zone of elongation J2 penetrates and migrates intracellularly from the cortical tissue towards the vascular cylinder. A line of perforations is produced by the mechanical action of nematode stylet and enzyme secretions. Cyst nematode forms the syncytium inside the roots, from where the nematodes take up plant nutrients.

Once the syncytium is induced, J2 undergoes three moults inside the root to form the adult stage. Male develop within the J4 cuticle from J3 after maturation; the male becomes motile and crosses the cuticle through its stylet and is released into the soil. A mature female remains intact to the roots and keeps on feeding throughout life. Dead females convert into cysts and cast into the soil (Popeijus *et al*., 2000; Von Mende *et al*., 1998). Presently cyst nematode includes six genera of the family Heteroderidae, i.e., *Heterodera*, *Globodera*, *Cactodera*, *Punctodera*, *Dolichodera* and *Afenestrata* (Golden, 1986). The cyst is the stage that persists after the female's death and is found frequently in the soil samples. All cysts have an anterior protuberance called the neck. Cyst of *Heterodera*, *Cactodera* and *Afenestrata* also have posterior protuberance called the vulval cone and are characteristically lemon-shaped but have many variations in exact shape and symmetry. But *Globodera* and *Punctodera* cysts are generally round, oval or pear-shaped and have no posterior protuberance. The cyst nematodes are globally causing substantial economic loss to the agricultural and horticultural fields every year (Nicol *et al*., 2011). These are known as effective plant parasites in temperate and tropical areas. Due to the family Heteroderidae, many morphological changes occur in the host plant. Genus *Heterodera* and *Globodera* are the most economically important genus of cyst nematodes.

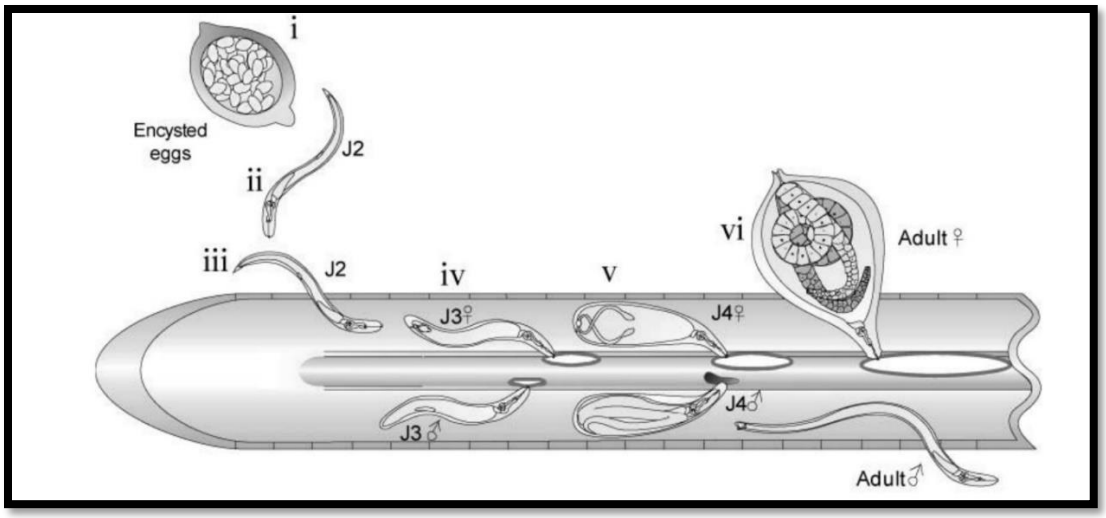

Figure 3: Common life cycle of amphimictic cyst nematode (Lilley *et al.*, 2005).

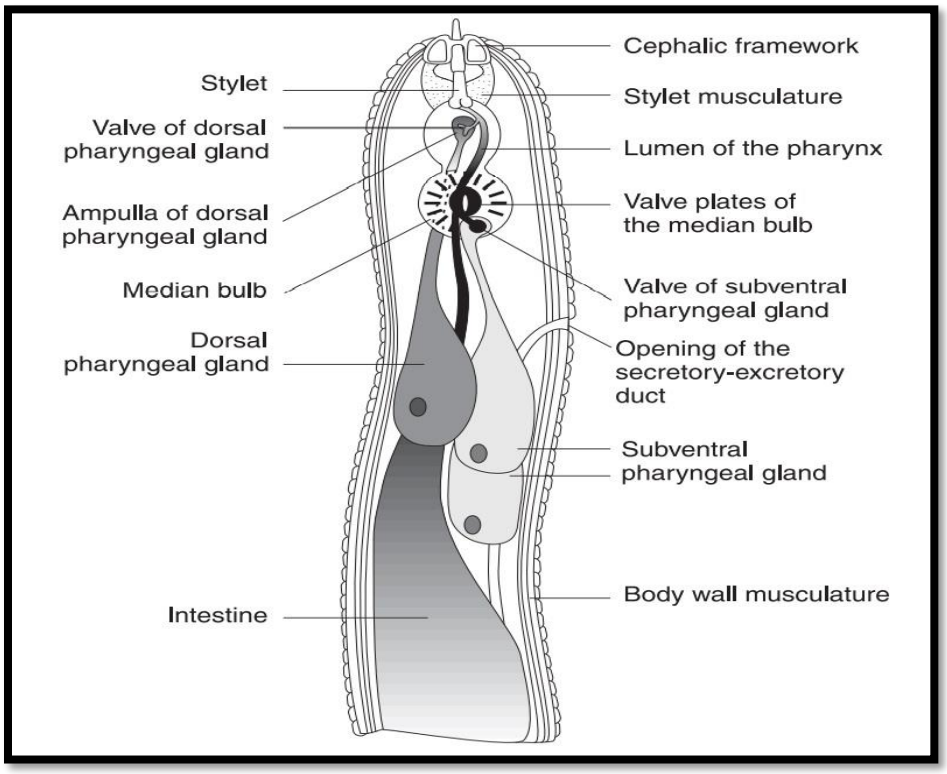

Figure 4: Systematic representation of J2 of cyst nematode anterior structure (Lilley *et al*., 2005).

Sex determination in cyst nematodes

Shortly before the moult to J3 or at the end of the J2 stage (Fig.4), the sexual fate of the cyst nematode becomes visible. The genital primordium begins to divide at this stage. Unbranched primordium led to the testis and branched primordium destined to develop into the ovary. Under sufficient nutrient supply and favourable conditions, most of the juveniles develop into Grundler *et al*. (1991) studied that 90% of the juveniles develop into females for *H. schachtii*. Under adverse conditions such as intraspecific competition and high infestation level, the adult population increases (Trudgill, 1967; Müller *et al*., 1981). According to Grundler *et al*. (1991) J2 has the potential to decide its fate to become either male or female. The initial stage of feeding influences the development pathway. The sexual fate of *H. schachtii* feeding on *Brassica rapa* roots is affected by the availability of sucrose concentration. The high concentration favoured the development of females due to an increase in the concentration of glutamine in the syncytium.

In contrast, tryptophan, methionine, lysine, and phenylalanine inhibited female development in favourable conditions. The structural difference was also studied between the syncytia or males and females of *H. schachtii*. Underdeveloped wall ingrowths and less hypertrophied roots are present in male syncytia (Betka *et al*., 1991; Sobczak *et al*., 1997). The physical factors that influence the hatching of cyst nematodes include temperature, humidity, moisture, aeration, and seasonal effect, especially concerning diapause.

2.7.1. Genus *Globodera* Potato cyst nematode (PCN) is one of the most important obligate sedentary soil-borne endoparasites of potato crops worldwide. Such nematode belongs to the genus *Globodera*, having two highly adaptive and most damaging species, i.e., *G. pallida* (pale cyst nematode) and *G. rostochiensis* (golden cyst nematode). It is highly susceptible to exploiting new environmental conditions and shows intimate association with the host plants, transported passively across the borders. One more *Globodera* spp. feeds on potato plants is *G. ellingtonae* only restricted in north-west USA and Argentina (Lax *et al*., 2014; Phillips *et al*., 2017). PCN can cause stunted plant growth, reduce crop yield and

sometimes completely damage the crop plants. In the fields where the PCN population is high, potato yield can be reduced compared to planted seeds. PCN can survive long term in the soil without a host; this highly specialized and adaptive nature of PCN can cause dreadful problems to farmers. PCN is considered the most difficult pest to control and is recognized all over the world's temperate regions. The host range of PCN is only in Solanaceae. Most importantly, feed on the host *Solanum tuberosum* (potato). It also feeds on other agronomic crops, such as *Solanum melongena* (eggplant) and *Solanum lycopersicum,* a few species of *Hyoscyamus*, *Salpiglossis*, *Lycopersicon*, *Datura*, *Physalis*, *Saracha* and *Physoclaina* (Sullivan *et al*., 2007). Adult cysts and juveniles are mostly attached to the roots and tubers or can be found in the soil. Whereas males are restricted to soil only. Like cyst nematodes, *Globodera* spp. also follows the unique life cycle where dead females hardens their bodies, convert themselves into a cyst, and protect the eggs within the cyst. Cyst shape is spheroid with a reduced neck. During maturation *G. rostochiensis* female cyst colour changes from white to yellow, then into the brown cyst, and *G. pallida* female cyst colour changes directly from creamy to dark brown (Pylypenko *et al*., 2005). After hatching, infective second stage juveniles (J-2) diffuse up to 1m inside the soil. Like other cysts nematodes, *Globodera* spp. enters inside the roots of the host plant by puncturing the root tip and infects the host plant. Inside the roots, PCN forms feeding sites by modifying the root cells after that the nematode becomes sedentary. This infection cause poor or undergrowth of root system and shoot system of host plant, poor yield or plant death may also occurs (Mimee *et al*., 2015; Cotton *et al*., 2014). The life cycle of PCN is completed in 56-63 days in autumn and spring (Ebrahimi *et al*., 2014). The egg hatching depends on temperature is confirmed by Fournet *et al*. (2018). PCN reproduce by sexual reproduction. Pheromones (sex attractants) are secreted by adult females to attract males for mating, and mating occurs several times in their life span. Each female during their life time produces 200-500 eggs, after the death of female cuticle converts in the form of cyst enclosed their eggs inside the cyst.

Under unfavourable conditions, eggs remain in the dormant stage inside the cyst. Under favourable conditions hatching occurs, hosts roots releases root exudates in the form of chemicals which act as a hatching stimulus. Second stage juveniles are released from the cyst. Eggs can remain in dormant stage (under unfavourable conditions) inside the cyst for 30 years and both eggs and cyst are resistant to many nematicides (Roach *et al*., 1968). Hatching of *Globodera* generally occurs at 10^{o}C or above. *G. rostochiensis* eggs hatching condition range from 5-29^{o}C temperature, whereas *G. pallida* eggs hatching temperature conditions range from 13-25^{o}C. In favourable temperature conditions, second-stage juveniles hatch from the cyst and escape from the cyst, moving towards the host plant's roots (Clarke *et al*., 1984; Zauhar *et al*., 2006; Kaczmarek *et al*., 2014). All eggs inside the cyst do not hatch during hatching; 60-80% of eggs hatch in comparison to 5% of egg hatching (Moens *et al*., 2018). After hatching from the eggs, second stage juveniles emerge into the soil and start penetrating the host root for feeding. When J2 starts feeding, root cortex cells are stimulated and forms specialized cells called syncytia. Through syncytia, plant cell nutrients are transferred to the nematodes. After the second stage, juveniles undergo three more moults and become an adult. The adult female becomes round, breaks the roots, and exposes its body's posterior portion to the external environment. Male juveniles keep on feeding inside the roots and remain active until the maturity attained. After attaining maturity adult males stop feeding, become vermiform, and release in to the soil. Adult males start looking for females for mating (Ebrahimi *et al*., 2014; Mimee *et al*., 2015). Even if the crop yield is significantly reduced PCN does not show specific apparent symptoms of infestation in plants. At high densities of PCN some plants have poor patches of plant growth, yellowing, wilting and necrosis. These symptoms shouldn’t be considered the proof symptoms for the presence of PCN, because other plant-parasitic nematodes also have the same symptoms. The mature female cyst can be easily detected after lifting the plant roots. If the crop shows stunted and poor growth in patches, the soil should undergo a visual check by carefully lifting the plant. Cysts are attached to the roots

and appear like beads that naked eyes can easily. Cysts can also be seen on tubers during heavy infestations (Trayanov *et al*., 2020).

2.7.1.1. Taxonomic Descriptions

Globodera pallida

- **Female**: Female body is subspherical in shape with a projecting neck bearing head, pharynx, isthmus and anterior part of pharyngeal glands. Live females are white or creamy and become brown when dead. Amalgamated lips are present in the labial region. The Neck region has irregular annulations. The most body surface is covered with reticulate patterns a weakly developed head framework with hexaradiate symmetry. Stylet knobs are inclined backwards. The median pharyngeal bulb is present in large, circular crescentic valve plates. The pharyngeal gland lobe is large with attached three gland nuclei. At the base of the neck, a prominent excretory pore is present. Hyaline secretions occur on the cuticle surface, due to which the internal structure found in the neck region is unclear. A transverse slit is present in the posterior area known as the vulva present inside the vulval basin. In addition, 12 parallel ridges are present between the anus and vulval basins on the cuticle surface. Irregularly arranged subsurface punctuations are present on much of the surface (Cooper *et al*., 1993).
- **Cyst**: young females convert themselves into cyst when it dies. Initially, it appears white or creamy on the root surface, slowly converted into dark brown colour at a mature stage. It is sub spherical in shape with a slightly protruding neck (Ganguly *et al*., 2010). Single circumfenestration is present in the vulval region. Sometimes, small darkened and thickened vulval bodies are present in the vulval region. The anus is visible in most species. Sub crystalline layer is absent in the cysts (Dauda *et al*., 2014; Hajjaji *et al*., 2021).
- **Male**: Regular annulations are present in the cuticle. The terminating tail is present with four incisors in the lateral field. The labial region consists of a

large and rounded oral disc. Six irregular lips and six annuli are present. The Head is heavily sclerotized, having hexaradiate framework. Well, develop stylet with posteriorly facing stylet knobs present. A prominent ellipsoid pharyngeal median bulb is present with attached crescent valve plates. The pharyngeal gland lobe is situated ventrally from that point attached to a broad nerve ring. A short tail with having bluntly rounded terminus is present (Jones *et al*., 2003; Zouhar *et al*., 2003; Narabu *et al*., 2015; 2016).

- **The juvenile (J2)** lateral field has four incisures. Up to the first seven annuli cuticle is thick. The rounded labial region is presently attached with slightly 4-6 annuli. The sub-rectangular oral disc is boarded by two lateral lips bearing amphibian apertures, adjoined with fused dorsal and ventral sub medial lips. The Head is highly sclerotized with hexaradiate framework. In 60% of specimens, dorsal and ventral radii are bifurcated at the tips. Well develop stylet is present, and basal knobs are projected anteriorly. Excretory pore present at the anterior end. A uniformly tapering tail is present with a finely rounded point. About half of the tail region forms a hyaline region (Jones *et al*., 2009; Blok and Phillips, 2012).

***Globodera rostochiensis*:**

- **Female**: subspherical or ovate, protruding neck and creamy or pearly white. The colour of cysts changes from white to yellow to light golden when the female transforms into the cyst stage. A thick cuticle with a superficial rugose pattern is present. D-layer is present, and punctuations are resolved beneath the surface. The labial region bears two annuli, and the labial framework is weakly developed. A reasonably strong stylet is present, straight to slightly curved, basal knobs are rounded and well developed, directed to posterior side. The median bulb is large, spherical, well-developed valve are present. Pharyngeal glands give a clustered appearance. The excretory pore is conspicuous and always appears at the base of the neck. Terminal, ellipsoid, and circumfenestrate vulva is present and the vulval slit is medium in length. Anal

fenestration is absent. Both anus and vulva lie together in the vulval basin. Cuticular rings do not encircle the vulval basin. The anus is shorter than the vulva. Eggs remain inside the body without egg mass (Camacho *et al*., 2017).

- **Cyst**: yellow colour appears when first seen in the roots and then turns brown with age. Spherical or ovate with protruding neck, circumfenestrate and abullate. A circular fenestra is present, and V shape subsurface cuticular mark is present at the apex of the anus. A wavy lines-like pattern is present on the cyst's wall, especially at the midbody surface. Sub crystalline layer absent. Punctations arrangements and intensity vary (Jones *et al*., 2009).
- **Male**: vermiform body, tapering from posterior and anterior regions. Prominent annulation is present in the cuticle. The hemispherical labial region has six annuli, and the labial framework is highly sclerotized. A strong stylet is present with prominent knobs. Lateral fields consist of equally spaced lines. Anterior and posterior cephalids are present. An ellipsoid median bulb is present. One testis is present. Slightly arcuate with rounded tips, spicules are present. Tail length and size variable, generally short tail is present (Jones *et al*., 2009).
- **Juveniles (J2)**: Have tapering body from the posterior and anterior-posterior but slightly more at the posterior region. Well defined cuticular annulations are present. The labial region bears 4-6 annuli, is rounded or flattened from anterior side. Highly sclerotized labial framework is present. Well-developed stylet with prominent rounded stylet knobs presented laterally. Anterior and posterior cephalids both are present. A prominent and ellipsoid medial bulb valve is present. A typical characteristic of genus *Globodera* is the isthmus and pharyngeal glands. The excretory pore is situated adjacent to or slightly posterior to hemizonid. Tapering tail with rounded terminus is present. Phasmids are difficult to see in the juvenile stage and generally present about halfway along the tail (Jones *et al*., 2009).

Genus *Globodera* completes its life cycle ranges from 55 to 60 days. Females are present inside the roots, whereas males are recovered from the soil. Cysts are attached to the roots of the host plants.

2.7.1.2. Identification features

Morphologically and morphometrically *G. rostochiensis* and *G. pallida* are very much similar. Therefore, the identification process should be performed by combining morphological and molecular techniques. After soil sampling, the collected nematode cyst should be carefully observed under the stereomicroscope. Globose cyst and the non-globose cyst should be separate. Non-globose cyst includes *Cactodera, Betulodera, Dolichodera, Heterodera and Punctodera*.

- **Morphological identification**

  The most important consideration is to differentiate the cyst of the genus *Globodera* from other cyst forming genera. The common characteristic of all cyst forming nematodes is that all the females convert themselves into hard wall cysts. This particular character can confuse the cyst of *Globodera* with all six other genera. Cyst shape is the vital characteristic that helps differentiate between the genus *Globodera* to another cyst forming genus. Globose or spheroid in *Globodera*, elongate-ovoid in *Dolichodera* and *Punctodera*, lemon-shaped in *Heterodera*, *Betulodera* and *Cactodera*. Occasionally cyst of the genus *Betulodera* and *Cactodera* shape tends to be globose. These specimens can be separated with the help of posterior protrusion, i.e., terminal cone top encompassing the anus and vulva. This characteristic is not present in the genus *Globodera*.

  Some species of the genus *Punctodera* have globose cysts similar to *Globodera*. But all the cysts can be differentiated with the help of fenestra found in the anal region called vulval fenestra. A terminal cyst region from which the juveniles are permitted to emerge by rupturing the wall. The terminal region wall is thin compared to the cyst body wall. In genus *Punctodera*, very short vulval slit is present, i.e., <5µm, whereas in *Globodera*, it's about 11-12 µm in *G. pallida* and

9-10µm *G. rostochiensis*. A conspicuous V-shape sub surface cuticular mark is seen in genus *Globodera* but absent in genus *Punctodera*. Monocot plants are said to be host plants of the genus *Punctodera*.

In comparison with another non-cyst nematode such as *Meloidogyne* spp. adult females, like *Globodera* spp. adult females are swollen and sedentary root feeders. *Meloidogyne* adult females lack cuticle thickening, and the formation of cysts can be distinguished from *Globodera* (González *et al*., 1992). *Meloidogyne* have annulation like fingerprint (whorls), whereas in case of *Globodera* annulation are lost in adults. *Meloidogyne* adult females do not form cysts but form egg mass (Eisenback *et al*., 1980). Egg mass is in the form of gelatinous matrix with extruded eggs. J-2 are more robust in *Globodera* and have more conspicuous thicker and longer stylet (Kaur and Attri, 2013). The tail terminus is hyaline in *Globodera* and non-hyaline in *Meloidogyne*. Phasmids are small and pore-like in *Meloidogyne,* large and lens like in *Globodera* (Salalia *et al*., 2017; Visagie *et al*., 2018).

Once it is confirmed that the genus belongs to *Globodera*, identify the species. Both cyst and J-2 stage is commonly present in the soil infested with PCN. However, methods for extracting cysts from the soil are different for both cysts and juveniles. Cysts can be removed from the soil by the floatation method. Cysts need to be broken and eggs suspended freely into the water to obtain larva. The most important characteristics for identifying the juveniles are length, a knob shaped stylet, and knob width (Mulvey, 1973). *G. rostochiensis* <23 µm, J-2 stylet length, stylet knobs are flattened anteriorly and rounded, whereas in *G. pallida,* stylet length is >23 µm, and stylet knobs are distinctly anteriorly directed (Mawangi *et al*., 2015; Hajjaji *et al*., 2021).

Cyst populations were observed under a stereomicroscope, dissecting microscope and compound microscope for morphological identification. There is no such evident difference in the size, colour and shape of the cyst of *G. pallida* and *G. rostochiensis*. For species identification, the perineal portion of the cyst consists of the vulval cone top. It should be observed under the 40X lens in a

compound microscope. The pattern of fenestration and the number of cuticular ridges between the vulval basin and anus gives a clear idea about the species. Granek's ratio is one of the essential characteristics of morphological characterization, the distance from the anus to the nearest edge of the vulval basin divided by vulval basin diameter. Whereas in some cases, the cuticular ridges are tough to count. Then species diagnosis can be made by combining the granek's ratio of juveniles. For further confirmation of species identification, the molecular analysis must be done. The process of identification should involve both morphological and molecular methods. Molecular characterization consists of the extraction of DNA from the cyst, which ultimately leads to identifying nematodes through nucleotide sequencing.

G. rostochiensis and *G. pallida* are considered the most economically important pest of potatoes throughout the world. Recently *G. ellingtonae* has been reported from Oregon (USA) with an unknown host plant (Lax *et al*., 2014). In Europe, PCN was reported during the 1850s and spread throughout the world during the export of seed tubers and breeding materials (Evans and Stone, 1977).

2.7.2.Genus *Heterodera*

2.7.2.1 Distribution

The cereal cyst nematode (CCN) is distributed worldwide. The genus *Heterodera* is one of the oldest genera among PPNs. Closely related species of CCN for a complex, which is widely distributed in the family Poaceae. *H. avenae*, *H. filipjevi* and *H. latipons* are considered the most important species economically among the genus *Heterodera* in cereals (Nicol and Rivoal, 2008). *H. avenae* is the most widely distributed species and was firstly reported in Germany. CCN occurs in almost all the European countries such as Germany, Belgium, Greece, France, Germany, the Netherlands, Poland, Norway, Spain, Portugal, Sweden, UK; Asia: India, Iran, Iraq, China, Japan, Pakistan, Turkey; Africa: South Africa, Morocco, Algeria, Tunisia; North America: Canada, USA, South America; Peru (Smiley, 2009; Smiley and

Nicol, 2009; Rilay and McKay, 2009). Kang *et al*., (2021) reported *H. sojae* in soyabean fields in Korea. *H. sojae* parasitizing soyabean field along *H. glycines*. *H. trifolii* is reported in Costa Rica on white clover and Rumex obtusifolius (Rodríguez *et al*., 2021). Hop cyst nematode *H. humuli* was reported in Yakima valley region USA (Darling *et al*., 2021).

In India: Mainly wheat growing states in India such as Rajasthan, Himachal Pradesh, Punjab, Jammu and Kashmir, Haryana, Delhi, Gujarat, Uttar Pradesh, Uttarakhand and Madhya Pradesh are mainly infected by *H. avenae*. Some parts of Punjab and Haryana is also infected with *H. filipjevi*. *H. iri* have been reported in the village Tihri, Kangra Himachal Pradesh (Bishnoi and Bajaj, 2004). The pigeon cyst nematode *H. cajani* is known as the second most widespread cyst forming nematode in India have been reported from Delhi (Koshy, 1967) and other states also, i.e., Jammu and Kashmir, Himachal Pradesh, Punjab, Haryana, Gujarat, Rajasthan, Uttarakhand, Uttar Pradesh, Madhya Pradesh, Bihar, West Bengal, Andhra Pradesh and Tamil Nadu (Kaushal *et al.*, 2007). *H. zeae* was reported firstly by Koshy *et al*. (1970) from village Chapli, Udaipur (Rajasthan) in maize. Rest all maize growing areas of J&K, Himachal Pradesh, Punjab, Haryana, Delhi, Gujarat, Rajasthan, Uttarakhand, Uttar Pradesh, Madhya Pradesh, Bihar, Maharashtra, Tamil Nadu, and Karnataka also infected with *H. zeae* (Kaushal *et al*., 2007). *H. sorghi* (sorghum cyst nematode) was first reported from Ghaziabad (U.P) by Jain *et al*. 1982 and observed in many other states also J&K, Himachal Pradesh, Punjab, Haryana, Delhi, Uttar Pradesh, Uttarakhand, Andhra Pradesh, Madhya Pradesh and Maharashtra. *H. sorghi* is very similar to *H. gambiensis*. Another cyst nematode found from turf glass is known as turf glass cyst nematode *H. graminis*. In India, *H. graminis* is reported by Sharma *et al*. (1984) in Delhi and Jaipur (Rajasthan) at the same time. Further, it is also found in Himachal Pradesh, Uttarakhand, Uttar Pradesh and Haryana, having host turf glass. The rice cyst nematode *H. oryzicola* (Rao and Jayaprakash, 1978) was described from the roots of rice in Pattambi area of Kerela. After that, Goa, Madhya Pradesh and Haryana

have also been reported on rice. Kaushal *et al*. (2000) reported a new cyst nematode *H. skohensis* in a rice field from village Skoh, Dharamshala, Himachal Pradesh. Further, it is also recovered from Palampur, Himachal Pradesh. *H. mothi* is firstly reported in Uttar Pradesh, India, infesting nut grass, i.e., *Cyprus rotundus* (Khax and Husain, 1965). Further, it is present in north-western areas, including Jammu and Kashmir, Himachal Pradesh, Punjab, Haryana, Delhi, and Uttarakhand. *H. raskii* was firstly reported from Hyderabad (Andhra Pradesh), infesting roots of bulb grass (*Cyperus Bulbosus*). But it has not been reported from another place of India (Basnet and Jayaprakash, 1984).

2.7.2.2. Life cycle

CCN, like other cyst nematodes, generally shares a familiar life cycle. Eggs present inside the cyst undergo moulting up to four successive juvenile stages, ultimately leading to mature adult males and females. Under favourable conditions, soil temperature, moisture, and roots exudates trigger the cyst. The first juvenile (J1) Inside the eggs moults into the second-stage juvenile (J2). Not every J1 inside the egg hatch some retain inside the cyst. Once the J2 hatch into the soil, they initiate to find out the host. With the help of root exudates such as amino acids, sugars and CO_2, J-2 seek host roots (Smiley *et al.,* 2009; Hajihasani *et al.,* 2010). Glycinoeclepin is the first characterized cyst nematode hatching factor. This is the terpenoid compound purified from the roots of a kidney bean. It induces the hatching of *H. glycines* at very low concentrations. (Masamune *et al.,* 1982) J2 enters into the meristematic tissue behind the root tip. With the help of stylet and its secretions, J2 migrates intracellularly up to the stelar region. By day four after the invasion, J2 selects capable root cells to form syncytium to feed on root secretions with the help of pharyngeal glands. The cluster of feeding cells are called syncytium (Vanholme *et al.,* 2004). Enlarged, multinucleate and metabolically active neighbouring cell walls, (syncytium) are formed, which act as a source of nutrition (Seah *et al.,* 2000).

After syncytium formation, J3 start feeding on roots, and in the third stage, juvenile J3 moult occurs. J3 moult keeps feeding inside the root with the help of syncytium development of the fourth juvenile stage (J4). Adult males and females develop at the same rate and same roots. Male are non-feeding; they leave the roots and enter the soil. They can survive for a very short period. With the help of sex pheromones, females attract males, and several males can mate with one female. After mating, eggs remain inside the female body unless J-2 hatch in the soil under suitable conditions (Moens *et al.,* 2018). After the death of the adult female, its cuticle becomes a hard protective layer called a cyst. Cyst, the dormant stage, consists of hundreds of embryonated eggs and unhatched J2. The cyst can remain in the soil in dormant condition for several years. The feeding sites in the roots may vary from species to species (Scholz and Sikora, 2004).

2.7.2.3. Morphology

The principal structure to identify the cyst is associated with the vulva and vagina. Mainly vulva is internally situated but persists after death. Identification of cyst nematode by vulva and vagina was previously described in detail by Mulvey, (1972). Morphologically, all the species are examined by positioning the vulva on the top of the cone. The radius of curvature of the cone top is considerably less from the rest of the body. Mulvey, (1972) arranged 39 different species of *Heterodera* into five groups.

i) The pear or round-shaped cyst, circumfenestrate, reduced vulval slit and rear presence of under bridge and bullae.
ii) Round to lemon-shaped, circumfenestrate, strong protuberance is present under bridge absent and short vulval slit.
iii) Lemon shaped, ambifenestrate or bifenestrate, bullae and under the bridge are present with a small vulval slit.
iv) Globose to lemon-shaped with the posterior protuberance, ambifenestrate, bullae and under bridge prominently developed with a long vulval slit.

v) Spherical or lemon-shaped cyst, bullae are present in a row around fenestrae or scattered, having a long vulval slit.

The study of vulval cone and internal or external characters has been chiefly used to identify cyst nematodes and their intraspecific variations. The vulval cone mainly consists of a vulval slit under the bridge, fenestrae, primary and secondary bullae and anus (Abdollahi *et al.*, 2007). In the case of *H. zeae* and *H. cajani*, the bullae are finger-shaped. Vulval slits are boarded with vulval lips, which further form parapets of the vulval bridge (Mulvey, 1974). This vulval bridge divides the fenestra into semifenestrae. The shape and size of the semifenestrae are helpful in species diagnosis. For the diagnosis of cyst nematodes, the type of fenestration, length and width of semifenestrae, the position of the anus and the distance between the anus and the edge of fenestra are essential and valuable (Bishnoi and Bajaj, 2004). Apart from these characteristics, one more morphological character of the vulval cone is necessary i.e., the vulval basin. Basin is the band of thick cuticles around the fenestra. Basin is prominent in *H. zeae, H. sorghi*, *H. skohensis*. *H. sorghi* and *H. mothi* have strong under the bridge. The number of bullae is present in *H. avenae* and is absent under the bridge. Weak underbridge is present in *H. filipjevi*. In the case of *H. zeae* and *H. cajani* both consist of strong and finger-like bullae. Sometimes, a medium-sized underbridge is present, equal to the length of the vulval bridge. *H. avenae* consist of short vulval slit and well separated fenestrae is present (Mulvey, 1974). In *Heterodera* spp. most commonly 'D' shaped fenestrae are present. Just below the fenestra one more bridge like structure is present which crosses the vulval cone. It is the hardened muscle tissue found in the right angle to the under bridge (Handoo, 2002).

H. avenae female cysts are lemon shaped, with protruding neck and vulva. Colour varies from brown to black. Young cysts are white in colour. On cuticle zigzag patterns appears. Bifenestrate vulval cone is present having short vulval slit. Highly packed protruding bullae are also present. During transformation of female

in to cyst, sub crystalline layer shed off. Female consist of cylindrical shape stylet with posteriorly sloping basal knobs. Under bridge is absent in vulval cone. Second stage juvenile J-2 are cylindrical in shape. Somewhat balanced head is present and tail tip are tapering and rounded. A strong stylet with slightly anteriorly concave basal knobs is present. *H. zeae* have light brown lemon shaped cysts. Thin wall cuticle is present and cyst wall is covered with zig zag patterns. Ambifenestrate with horse-shoe shaped fenestra is present. Bullae are located at two level of vulval cone; one level is immediately below the under bridge and level two is mass like structure randomly located long and heavy bullae (Kaushal *et al*., 2007). Second stage juveniles are identified on the basis of body and stylet length, shape and stylet of knobs, shape and length of tail, hyaline, number of lines in the lateral fields. The lateral fields consist of four distinct lines. Tail is short with conical tapering and acutely rounded terminus is present. In *H. zeae* stylet knobs are consistent (Skantar *et al*., 2012). The morphological features for *H. avenae, H. filipjevi* and *H. latipons* are very much identical. The cysts of all three species are lemon shaped, light brown to dark brown in colour and body consist of ridges running in zigzag patterns. No under bridge found in the vulval cone in these three populations. The vulval cone is bifenestrate in *H. avenae* and *H. filipjevi* and *H. latipons* consists of two distinct semifenestrae. A strong under bridge with bifurcated extremities is present. *H. avenae*, *H. filipjevi* and *H. latipons* have cylindrical shaped second stage juveniles with tapering round tail tip and slightly offset head is present. A strong stylet is present with shallow anteriorly concaved basal knobs are present. In *H. filipjevi* the stylet knobs are moderately anteriorly concaved (Imren *et al*., 2015). *H. oryzae* consist of either oval or lemon shaped cysts colour varies from light brown to dark brown. Ambifenestrate vulval cone with bullae and strong under bridge is present (Mwesige *et al*., 2020).

2.7.2.4. Effect of temperature:

Heterodera avenae generally hatch at constant temperature between 5^{o} to 20^{o} C but maximum hatching occurs at 15^{0}C. For prehatch development optimum

temperature 10^0C is required and 20^0C is required for eclosion. Second stage juveniles show marked response to root diffusate at 10^0C- 15^0C. Hatching and root invasion occurs during spring and autumn. *H. cajani* shows little different temperature conditions for hatching, they are adapted to tropical conditions. 29^0C is the optimum temperature required for hatching and temperature range for hatching is between 15^0C- 37^0C (Fisher, 1981). For *H. glycines* the optimum temperature for hatching in root diffusates is 25^0C and in water also its almost similar i.e., 24 ^{0}C. First stage juvenile proceeds to develop at 10^0C and second stage juvenile developed at 15-30 ^{0}C overall hatch occurs between 20-30 ^{0}C (Alston and Schmitt, 1988). *H. filipjevi* hatch greater at lower temperature 5, 10 and 15^0C as compared to higher temperature 20-25 ^{0}C (Sahin *et al*., 2010). The optimum temperature range between 15-20 ^{0}C for *H. schachtii* and 15-30 ^{0}C for *H. betae* (Vandenbossche *et al*., 2015). The life cycle of *H. zeae* (corn cyst nematode) takes 15-18 days to complete at high temperature 33^0C for optimal growth (Hutzell and Krusberg, 1990). In contrast *Globodera* takes around 3 months to complete its life cycle. *G. pallida* hatches freely at higher temperature than *G. rostochiensis*, possibly prefer lower temperature to hatch. Possibly *G. pallida* hatches at lower temperature. *G. pallida* is more insistent to due to competition. *G. pallida* hatch freely at 23^0C whereas *G. rostochiensis* population adapted to hatch at somewhat lower temperature. But in glass house conditions *G. rostochiensis* do not hatch below 10^0C (Kaczmarek *et al*., 2014).

2.7.3. Genus *Cactodera*

Mulvey and Golden, (1983) given the description of genus *Cactodera*. Siddiqui, (1986, 2000), Woults and Baldwin, (1998) further diagnosed the description about genus *Cactodera*. Like other cyst nematodes genus *Cactodera* shows common morphological features. Cyst shape varies from lemon shaped, somewhat rounded or oval. Colour ranges from light brown to dark brown. Body consists of distinct neck and vulval cone. Abulate and circumfenestrate cone top were observed in this genus. In some species fenestra is surrounded by a thin cuticle like semitransparent material. Vulval denticles were also

observed. Graney and Bird, (1990) discussed that Straight to wavy patterns covered the middle body of the cyst.

- **Female:** female consists of pearly white body. Subcrystalline layer is absent. Stylet and knobs are well developed. Excretory pore is located posteriorly at the level as end of isthmus. Vulval cone shows slightly protruding lips (Escobar *et al*., 2020).
- **Cyst:** rounded to lemon shaped, light brown to dark brown in colour. Small vulval cone is present. zigzag patterns appear on the surface of cyst except area around vulval cone. Circumfenestrate vulval cone is present sometimes denticles may also present. Mostly bullae are absent.
- **Male:** male body is vermiform with tapering anterior end. Slightly offset labial region is present with oval shaped labial disc. Six lip sector is present in labial disc. Well-developed stylet is present with rounded knobs. Lateral field consists of four lines. Spicules are curved and having slightly notched tips.
- **Second stage juveniles**: vermiform body is present, tapering at both ends. Offset head region. Well-developed stylet is present with rounded stylet knobs slightly bulging anteriorly. Pharyngeal glands are present at ventral side on overlapping on each other. Lateral field consists of four lines with partially areolated two ridges. Tapering tail is present with prominent hyaline region demarcated with strong U-shaped outline (Escobar *et al*., 2020).

Genus *Cactodera* completes its life cycle ranges from 40-55 days. Females are present inside the roots whereas males are recovered from the soil. Cysts are attached with the roots of the host plants.

2.7.3.1. Morphological identification of genus *Cactodera*

Genus *Cactodera* shows distinct morphological characters in different species. Cyst shape varies from rounded, spherical to lemon shaped. Circumfenestrate vulval cone is present. Vulval denticles are mostly present. Bullae and under bridge

are absent. Second stage juveniles consist of four incisures along with lateral field. Phasmids are punctiform. Each species consists of labial disc with six lip sectors.

- ***C. cacti*:** Cyst shape is spherical to lemon shaped and initial colour of the cyst is pearly white latterly changes in to golden yellow to light brown with protruding neck and vulva. Prominent vulva is present. Circumfenestrate cone top is present. Bullae are absent. Vulval denticles are present. Punctations on the shell surface of the eggs are present (Duan *et al*., 2012). Second stage juveniles have vermiform body with tapering ends. Dorsoventral lip region with elongated labial disc is present. Five lip annuli are present. lip region also consists of two small lateral and four large sub medial lips. Cephalids are not observed in second stage juveniles. Six lip annuli are in adults. Stylet consist of dorsal knob with concave anterior surface. 1-3 annuli (hemizonid) anteriorly lead to excretory pore. Adult male head region is continuous with body. Adult male contains single testis (Skantar *et al*., 2019; Duan *et al*., 2012).
- ***C. chenopodiae:*** Consists of round, subspherical or lemon shaped cyst with small protruding vulval cone. Vulval cone consists of slight protruding lips. Except vulval cone outer layer of cyst consists of rugose pattern. Circumfenestrate cone top is present. Vulval denticles, bullae and underbridge absent. Distinct anus is present, encircled with disc like cuticular region. Eggs consists of heavy punctations. Second stage juveniles consist of vermiform body, tapering from anterior and posterior ends. Lip region consists of four annuli. Elongated labial disc with surrounded by four sub-medial and two lateral lips. Rounded stylet knobs, projecting anteriorly. Excretory pore is present at the level of gland lobe (Feng *et al*., 2018).
- ***C. torreyanae*:** Cysts are lemon shaped, light brown to dark brown in colour. Distinct vulval cone is present. protruding vulval cone and lips are observed. Young cysts are oval in shape and pearly white in colour. From protruding vulval cone a gelatinous sac is observed. Stylet is slightly covered from

dorsal side. Stylet knobs are slightly directed posteriorly. Lateral field consists of four incisures with incomplete areolation on outer ridges. Eggs have smooth surface with no punctuations. Second stage juveniles are cylindrical in shape, tapering from posterior ends. Four annuli are present in the lip region. An oval rectangular slip disc is present with six rectangular lip sector and present themselves in such a way look like equal dorsal lip, ventral lip and lateral lip. Stylet knobs are rounded. In second stage juveniles a minute pore is present at the level of hyaline beginning called phasmid (Evans *et al*., 2015; Evans, 2014).

- ***C. estonica***: Cysts are dark brown in colour and lemon shaped. Protruding vulval cone is present. Circumfenestate vulval cone is observed. Vulval slit is short. Under bridge and bullae are present. vulval denticles are present. Second stage juveniles are vermiform. Short hyaline portion is present with rounded tail. lateral field consist of four incisures (Yu and Sun, 2018; Saranya *et al*., 2017).
- ***C. milleri***: Another species obtained from the host *Chenopodium album***.** *C. milleri* is differentiated from other species due to one different characteristic is the presence of punctuated egg shells. The potential host of *C. milleri* include 11 weed speices,18 agronomic crop species and 5 cactus species (Graney and Bind, 1990).
- ***C. solani***: Consist of light brown to black lemon shaped cyst. Circumfenestrate small vulval cone present. Morphologically *C. solani* resembled to *C. milleri* (Escobar *et al*., 2020)**.**
- ***C. johanseni*:** Consists of lemon shaped cysts, brown to black in colour. Have circumfenestrate vulval cone top and bullae are absent. The second stage juveniles have four lateral lines. This is the new species related to *C. milleri*.

2.7.3.2. Host range

Host range of genus *Cactodera* all belongs to subclass Caryophyllidae. Which includes two orders i.e., Caryophyllales and Polygonales. Order Caryophyllales further includes 5 families; Caryophyllaceae having host plant dianthus for *C. evansi*, Cactaceae having host plant phyllocactus for *C. cacti*, Chenopodiaceae have host plant *Antriplex, Chenopodium* and *Salicornia* for *C. eremica*, *C. milleri* and *C. salina*. Amaranthaceae with host plant Acnida and Amaranthus, Portulacaceae having host plant Montia for *C. thornei*. Second order Polygonales include family Polygonaceae having host plant *Polygonum* for *C. estonica* and *C. weissi*. The mature female cyst of *C. johanseni* is firstly recovered from the plant rhizosphere of radish. Radish is considered as the host of *C. johanseni* (Sharma *et al*., 2001). The main host for *C. cacti* is cactus plant. Common lambsquarters are considered as the excellent host for the *C. milleri* and *C. chenopodiae* (Schroender *et al*., 2008).

2.7.3.3. Distribution

The first report of genus *Cactodera* was from China. *C. thornii* found in the cereal field of China (Peng, 1994). Cactus cyst nematode *C. cacti* has firstly recorded from Northern China from cactus plant (Duan, 2012). *C. cacti* geographically recorded from Kowa parasitizing *Zygocactus truncatus* and *Hylocerers trigonus*. Infection leads to the yellowing of stems, late blooming, retarded growth and wilting (Cho *et al*., 1995). *C. cacti* was reported from the cactus garden in median, Ada County, Idaho. Cysts, white females, second stage juveniles, and eggs recovered from the rhizosphere of cactus plant (Skantar *et al*., 2019). *C. milleri* had lemon shaped cysts, reported from Mattawan, Michigan (Graney *et al*., 1990). Schroender *et al*. (2008) reported *C. milleri* from Wisconsin, lemon shaped cyst and second stage juveniles was recovered from the soil at Wisconsin Agriculture Research station. *C. estonica* is reported from the Nilgiri Hills, India (Saranya *et al*., 2017; Pathania *et al*., 2019). *C. estonica* was also

reported from Canada. Mature female cyst and second stage juveniles was recovered from the rhizosphere of *Polygonum avicular* (Yu and Sun, 2017). *C. chenopodiae* recorded from the host plant Lambsquarter, *Chenopodium album L*., in Liaoning, China (Feng *et al*., 2018). *C. torreyanae* is detected from the soil around the succulent plant *Suaeda torreyana* in Mexico. *Suaeda torreyana* commonly known as 'romeritos' used for the preparation of common Christmas dish (Vera *et al*., 2014). Evans *et al*., (2015) studied the interaction *C. torreyanae* with *Suaeda edulis*. This nematode can survive in extreme saline conditions in the soil with the host plant *S. edulis*. *C. galinsogae* found in Hidalgo, Mexico as a parasite in the roots of barley (*Hordeum vulgare*, L.), wheat (*Triticum aestivum*), maize (*Zea mays* L.) and some weeds growing in the barley field (Tovar *et al*., 2003; 2007; 2008). *C. galinsogae* induced irregular syncytia which lodge up to vascular bundle, in the cortex of secondary and territory barley roots which leads to the breaking of xylem and phloem. Mexico recently reported *C. solani* species, parasitizing tomato and common Lambsquarter (Escobar, 2020).

2.7.4. Molecular characterization

For the confirmed identification up to species level molecular studies has been done. Many researchers have been used a set of primers for the amplification of 18S small subunit, ITS-1 region, 5.8S, ITS-2 region and 28S large subunit of ribosomal DNA. Skantar *et al*. (2012) and Subbotin, (2021a) described a set of primers 18S (59-TTGATTACGTCCCTGCCCTTT-39) and 28S (59-TTTCACTCGCCGTTACTAAGG-39) for the amplification of 18S and 28S conserved regions for identification and phylogenetic study. For molecular analysis of cereal cyst nematode *Heterodera* spp. many researchers have been used mainly ITS regions of rDNA for molecular sequencing. Based on ITS-rDNA sequencing *Heterodera* species identified up to species level. Ferris *et al*. (1994) used forward primer(5'-CGTAACAAGGTAGCTGTAG-3') and reverse primer(5'-TCCTCCGCTAAATGATATG-3'). The ITS region of rDNA was amplified by PCR with the forward TW81 primer (3'-GTTTCCGTAGGTGAACCTGC-5') and

reverse AB28 primer (3'-ATATGCTTAAGTTCAGCGGGT-5') (Madani *et al.*, 2004). For *H. oryzae* universal primers TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-A TTGCTTAAGTTCAGCGGGT-3′) has been used (Mwesige *et al*., 2020). The primers sequences mentioned above are the universal primers used by Due to too much similarity in morphological structures within the species of *Globodera*, specimens must be studied on molecular basis. The molecular characterization using mitochondrial cytochrome b gene (cyt b) PCR product of *G. pallida* is 850bp in size (Ohki *et al*., 2018). According to Djebroune *et al.* (2021), *G. pallida* and for *G. rostochiensis* PCR produces single fragment of 265bp and 434 bp using species-specific primers. *G. tabacum* has 265 bp amplicon size.

2.7.5. Yield loss and economic importance

Annually Plant-parasitic nematodes cause 21.3% crop losses up to Rs. 102,039.79 million. Total 19 horticultural crops at Rs. 50,224.98 million and 11 field crops were assessed at Rs. 51,814.81 million (Kumar *et al*., 2020). Genus *Heterodera* can cause considerable yield loss, especially in areas where monoculture cultivation exists, semiarid conditions, and temperate climatic conditions. The yield loss caused by CCN can be calculated by finding the relationship between the Pi (population density) of CCN and the wheat yield (Ibrahim *et al*., 1999). Meagher, (1972) reported that *H. avenae* caused 50% of yield loss in wheat and 20% in barley in Australia. 10-30% yield loss in wheat occurred due to *H. avenae* in China (Peng *et al*., 2015). In Saudi Arabia total 40-92% yield loss in wheat and 17-77% in barley was caused by *H. avenae* (Ibrahim *et al*., 1999). In wheat fields, 24% of yield loss occurred in USA, 15-20% in Pakistan (Dababat *et al*., 2015). Fard *et al*. (2019) conclude that under trial fields, *H. filipjevi* caused up to 20 % yield loss in cereal growing areas in Iran. 4-26% yield loss in wheat occurred in Turkey (Imren *et al*., 2012) and 40–50% loss in India (Mathur *et al*., 1986). *H. avenae* and *H. filipjevi* showed 50% of yield loss in Norway. In Syria Hassan *et al*. (2010) analysed the effect of *H. avenae* on the plant growth, yield, and CCN reproduction in durum and

bread wheat cultivations under field conditions and conclude that under field conditions *H. avenae* and *H. filipjevi* caused yield loss 44%-56% respectively in cereals. In the USA, *H. avenae* reduced 50% wheat yield loss (Smiley *et al*., 1994). *Heterodera filipjevi* providing yield losses up to 40.5% and 8.54% to wheat and barley areas (Imren *et al*., 2020). *G. rostochiensis* causes up to 85% yield loss of potato (Greco *et al*., 1984) and *G. pallida* cause up to 80% yield loss of potato (Talavera *et al*., 1998). *G. tabacum* is the tobacco pest reported 40-60% yield loss of tobacco (Mondia, 2002). Ibrahim *et al*. (1999) proved that *H. avenae* could reduce wheat's 40-92% grain yield production. Wheat and barley plants are the principal hosts of *H. avenae*. *G. rostochiensis* is one of the severe pests of potatoes present in the soil. Hajihassani *et al*. (2013) estimated the yield loss to potato tubers caused by *Globodera rostochiensis*, the maximum tuber yield loss was>50%.

2.8. Control of plant-parasitic nematodes (PPNs)

PPNs attack mainly cereals, vegetables and fruit plants. On average PPNs provide up to $80 billion estimated losses occurs worldwide annually due to the nematodes (Bernard *et al*., 2017). Complete elimination of the nematode is impossible, but its management is essential by reducing its population below the damaging level. One of the best ways to control its population is a microbial control of these pest, a variety of microorganisms, such as fungi, bacteria, and actinomycetes have been successfully act as a effective biocontrol agents against PPNs on different crops.

2.8.1 Management

Various methods are followed for the management of nematodes such as

i. **Cultural method:** Various cultural methods have been used to manage the population of PPN including crop rotation, cooperation of soil amendments, planting of resistant crops or genetically modified plants, and soil solarization (Schmitt and Sipes, 2000). In integrated pest management, crop rotation and use of cover crops

help decrease the population density of PPN in the soil, such as Mucuna pruriens and Crotaralia spectabilis, showing the resistance against *Meloidogyne arenaria*, *Meloidogyne javanica*, *Meloidogyne incognita*. Rotational crops such as onion, garlic, corn, and asparagus are beneficial in reducing the infestation of Meloidogyne spp. also help prevent the diseases in plants (Bernard *et al*., 2017). Resistant crops for example, rye is resistant to *Meloidogyne* spp. (root knot nematodes). Sudan grass and sorghum releases 'Dhurrin' (allele chemical). Dhurrin is converted in to hydrogen cyanide which act as a powerful nematicidal agent. Marigold is also act as an antagonist against *Meloidogyne* spp. it efficiently decreases the population density of almost 14 genera of PPNs (Kafle, 2013).

ii. **Biotechnology methods:** Modern Biotechnological method to control PPN population help to show the crops resistance against PPN. Resistant plants destroy the feeding cells of the PPNs and insert the toxic compounds inside the invading cells (Nyarko and Jones, 2015). This method of nematode control targets either the exploitation of resistant genes present in the gene pool of a particular crop plant or to apply the synthetic form of disease resistance, such as gene silencing (RNAi technology) used to disrupt the specific pathogenesis-related proteins. RNAi technology controls PPN; infected plants produce double-stranded RNA that targets the parasitic genes in the nematode and silences their activity. For example, Flp gene is present in potatoes used to disrupt CN parasitic gene in *G. pallida*. On the other hand, gene silencing initiates motor disruption and unusual sensitivity in *G. pallida* (Kimber *et al*., 2007).

iii. **Micro RNA (mi RNA) technology:** It is the alternative gene silencing method. Plant mi RNA are non-coding RNA sequences post act by transcriptional gene method. This mi RNA initiates the changes in the root cells of the plants in response to the PPN infection. MIR produces miRNA precursors which is helpful in gene silencing (Yu, 2017).

iv. **Chemical control:** The main aim of chemical control is to generate a toxic barrier between host and pathogen (Table 3). Chemical nematicide are divided into two

categories; fumigants and non-fumigants. Fumigants are a kind of liquid for formulations vaporized after getting in contact with air. In vaporized form, the molecules of the fumigants move into the soil and finally decompose into the product, which penetrates into the cuticle of the nematode, hindering metabolic functions. Non-fumigants such as carbamates and organophosphates have synthetic action on PPN considered more effective at low doses. Chemical nematicides are toxic, affects human and animal health as well as environment also (Ebone *et al*., 2019; Hussain *et al*., 2017).

Table 3: Different types of chemicals used to control Plant-parasiticNematodes (Ebone *et al*., 2019; Hussain *et al*., 2017).

Organophosphates (ops)	
Cadusafos	Potato, Cotton, Sugarcane
Ethoproph	Potato
Fenamiphos	Banana, Cotton, Coffee, Tomato
Phorate	Corn, Tomatoes, Wheat
Fosthiazate	Potato, Banana, Carrot
Thiodicarb	Cotton, Oats, Peanuts, Barley, Beans
Iso thiocyanates	
Metam sodium	Potato, Carrot, Tobacco, Strawberry and Tomato
Abamectin	Cotton, Garlic, Corn and Soyabean
Fluensulfone	Cotton, Potato, Coffee, Sugarcane, Citrus, Guava, Chilli, Pepper

v. **Biological control:** The action of a pathogen, parasite of predators to control the control the population density of any pest is known as biological control (Table.4). Stirling, (1991) states, "A reduction of nematode population density by the action of any living organisms occurs naturally or by introducing antagonists or manipulating the environment". In sustainable agriculture, biocontrol agents is one of the attractive option to control plant-parasitic nematodes. It is the involvement of some other

efficient organism's genes/products to manage the population of PPNs. Term biopesticides mean use of chemical-free or biological substances as a pesticide to control the population of soil-borne plant parasites. Biocontrol is efficient, effective, eco-friendly approach for the management of pest. *Meloidogyne* spp. are important pest of rice in temperate and tropical areas. Infected plants show knots in roots, stunted growth, and poor reproduction (Pokhrel *et al.*, 2007; Kyndt *et al*., 2014).

Table 4: Different type of biocontrol agents used to control plant-parasitic nematodes (Trainer *et al*., 2014).

Bio control agents	**Pathogens**
Paecilomyces lilacinus	Severely attack on the egg of different plant parasite nematodes species
Pasteuria penetrans	Decrease the infection as well as the fertility rate of the plant parasite nematodes species.
Rhizosphere bacteria (*Bacillus subtilis*)	Have an effect on nematode multiplication
Green manure	crop residue plant-parasitic nematodes (PPNs)
Cow dung/ poultry manure	Plant-parasitic nematodes (PPNs)
Neem seed powder	Plant-parasitic nematodes (PPNs)

- **Nematophagous bacteria:** Nematophagous bacteria also shows various modes of action against PPNs such as parasitising, toxins production, enzymes/antibiotics, competition for nutrients; and promoting plant health. They are helpful to control of PPNs, helps to promote plant growth, and facilitate the rhizosphere colonization. The nematophagous bacteria include parasitic bacteria, opportunistic parasitic bacteria, rhizobacteria, Cry protein-forming bacteria, endophytic bacteria and symbiotic bacteria. Nematophagous Bacterial parasite species such as *Bacillus thuringiensis, Pasteuria penetrans, and Pseudomonas fluorescens are known as*

*cyst nematodes' potential biological control agent*s (Zhang *et al.,* 2016). *Achromobacter xylosoxidans* (09X01), and *Bacillus cereus* (09B18), culture filtrate of the two strains *in-vitro* causes high mortality of the J-2 and also decreased the egg hatch of cyst nematode population. *B. cereus* strain 09B18, is distributed widely in the soil, these are the well-known plant growth-promoting bacterium considered as a parasite for *Heterodera filipjevi* (Tian *et al.,* 2007; Elgawad *et al.,* 2018). *Rhizobacteria* as well as the members of the genera *Actinomycetes, Agrobacterium, Arthrobacter, Azotobacter, Chromobacterium, Clavibacter, Clostridium, Enterobacter, Flavobacterium* and *Rhizobium* have been studied for the biocontrol of cyst nematodes (Tian and Riggs, 2000; Li *et al.,* 2015; Hallmann *et al*.,1997; Meyer, 2003). *Bacillus* isolates have also been used to inhibit the activity of second stage juveniles *in-vitro* and cyst wall of *G. rostochiensis*, the eggshell and cyst wall of the *G. rostochiensis* consists of protein, chitin, and lipid. These can be used by parasitic bacteria as energy and carbon sources. (Kurniasari *et al.,* 2019). *Rhizobacteria* generally reduce nematode populations by regulating nematode behavior interfering with plant–nematode recognition, promoting plant growth, competing for essential nutrients, induce systemic resistance by producing toxins, enzymes and other metabolic products (Mhatre *et al*., 2018). Also, the presence of rhizosphere bacteria, such as *Enterobacter cloacae* and *Pseudomonas mendocina*, spores get attached to the nematode cuticle and destroy the cuticle layer of the cyst (Kerry, 2000). *Pasteuria* species have been reported for infecting nematode, but only *Pasteuria nishizawae*, is parasitic to *Heterodera* spp. (Atibalentja *et al.*, 2004). As a parasite, *Brevibacillus laterosporus* is also considered as a potential biocontrol agent having wide spectrum of biological activities against PPNs reported for *Heterodera glycines*. The egg shell of the nematode *Globodera rostochiensis* contains chitin which provides rigidity and toughness to the body, invitro study shows that chitinase-producing bacteria have ability to interfere with the hatching process of *Globodera rostochiensis* (Cronin *et al*., 1997). Chitin modifications release ammonia which suppresses nematode populations, by the stimulation of

chitinolytic organisms such as bacteria and actinomycetes which attack nematode eggshells (Culbreath *et al*., 1986; Spiegel *et al.*, 1987, 1988).

Achromobacter xylosoxidans isolate 09X01 and *Bacillus cereus* isolate 09B18, *Pseudomonas fluorescens* have potential to act as effective biocontrol agents for cyst nematodes (Saxena and Stotzky, 2001; Siddiqui *et al*., 2003). The biocontrol bacteria produce secondary metabolites such as enzymes and toxins which decrease the population of plant-parasitic nematodes, which inhibits reproduction in nematodes, egg hatch or survival of juveniles. Some *Bacillus* sp. produce serine protease which hydrolyzes collagen and cuticle of cyst nematode (Zhang *et al*.,2016). Also, the endospores of *Pasteuria* spp. parasitized *H. avenae H. cajani* cysts, body of J2 (Davies *et al*., 1991). For *H. cajani* (pigeon pea cyst nematode), the presence of bacteria *B. subtilis* reduce the multiplication (Siddiqui and Mahmood, 1999). Racke and Sikora, (1992) studied the Agrobacterium sp. such as *Agrobacterium radiobacter* reduced the nematode *Globodera pallida* infection by 40% when sprayed on seed pieces of potato. Zhao *et al*. (2019) studies demonstrated that more than 70% juvenile populations inside the roots can be reduced by Sneb517, and more than 60% cyst population can be reduced in the soil without effecting the growth and yield of soybean (host) plant.

Further studies also analysed that by the addition of organic matter into the soil may act as a nutrient source for nematode parasites such as fungi (Godoy *et al*., 1983). Nematode-trapping fungi amended with organic matter is considered as one of the important biocontrol agents for controlling plant-parasitic nematode population in soil. Addition crustacean chitin into soil may effectively control the population of soybean cyst nematode (Rodriguez *et al*., 1984) and Akhtar and Malik, (2000) reported that, by adding farmyard and composted manures into the potato field may reduced the population densities of *Globodera rostochiensis*.

a. ***Pasteuria:*** It is a gram-positive bacterium. Endospores spores of *Pasteuria* attached to the cuticle of second stage juveniles J2 and germinate on it when juveniles enter into the roots of the host plant and start feeding on it. The germ

tubes penetrate on the cuticle and form vegetative colonies, proliferate throughout the body. First step of attack is the attachment of spores to the nematode cuticle. *Pasteuria penetrans* is a potential biocontrol agent against root knot nematode Meloidogyne spp. (Davies *et al*., 2006). Similarly, *Pasteria nishizawae* infects *Heterodera* spp. and *Globodera* spp. (Gives *et al*., 1999; Atibalentja *et al*., 2000). According to monoclonal antibody studies high degree of heterogeneity is present within and among the different populations of *Pasteria penetrans*. The spores are distributed of particular epitope are involved in the process of adhesion (Davies and Redden, 1997).

b. Rhizobacteria: Rhizobacteria is act as a biocontrol agent for plant-parasitic nematodes (PPNs). Mainly *Bacillus* spp. and *Pseudomonas* spp. act as a parasite for PPNs (Krebs *et al.,* 1998). *Bacillus* spp. show antagonism towards PPNs especially *Meloidogyne* spp. and *Heterodera* spp. (Li *et al*., 2005; Oostendorp and Sikora, 1990). Other rhizobacteria such as members of genera *Actinomycetes, Agrobacterium*, *Arthrobacte*r, *Alcaligenes*, *Aureobacterium*, *Azotobacter*, *Chromobacterium*, *Clavibacter*, *Clostridium*, *Corynebacterium*, *Enterobacter*, *Flavobacterium*, *Methylobacterium*, *Phyllobacterium*, *Rhizobium*, *Serratia*, *Stenotrotrophomonas* and *Variovorax* as a antagonist of nematodes (Tian *et al*., 2007). *Rhizobium etli* G12 can suppress the early infection of potato cyst nematode *Globodera pallida* and the root-knot nematode *Meloidogyne incognita* (Hallmann *et al*., 2001).

c. Plant growth promoting rhizobacteria (PGPR): Plant roots surrounded with soil is known as rhizosphere. Plant rhizosphere includes many bacterial species which promotes the release of plant growth regulators, plant growth regulators speed up the growth of the plants by increasing the nutrient availability such kind of bacteria is known as plant growth promoting rhizobacteria (PGPR) (Table 5). PGPR improves the yield of agricultural crops. Genera Rhizobacteria is commercially colonized such as *Bacillus*, *Azospirillum*, or *Pseudomonas*

(Subedi *et al.,* 2020). *Pseudomonas* spp. and *Bacillus* spp. are belonging to endospore bacteria

d. Cry protein forming bacteria: *Bacillus thuringiensis* (Bt) produces Cry proteins or d-endotoxins. These cry proteins/endotoxins are toxic to many insect species such as order Coleoptera, Diptera, Lepidoptera and Hymenoptera (Maagd *et al.,* 2001). Some cry proteins are act as a toxic agents against some invertebrates such as parasitic nematodes and parasitic protozoans. Cry5, Cry6, Cry12, Cry13, Cry14, Cry21 are known to be toxic for parasitic nematodes (Kotze *et al*., 2005; Wei *et al.,* 2003). Cry5, Cry12, Cry13, Cry14 and Cry21 are nematode-specific proteins. Cry proteins forms lytic pores in the cell membranes of gut epithelium. Nematode larvae ingest toxin, the ingested crystals dissolve inside the gut of the nematodes, leads to proteolytic activation (Crickmore, 2005). Cry5B protein is act as a antagonist of root knot nematodes. Cry proteins have excellent potential to act as a controlling agents of plant-parasitic nematode population (Li *et al*., 2008). *Bacillus thuringiensis* delta-endotoxin, Cry14Ab, is produced in the roots of transgenic soyabean act as antagonist for soyabean cyst nematode, used to control cyst population (Kahn *et al*., 2021).

Table 5: Different PGPR strains used to control plant-parasitic nematodes and their mode of action.

PGPR strains	Plant-parasitic nematode	Mechanism of action	Reference
Bacillus cereus	*H. avenae*	Chitinase, Sphingosine, protease, secondary metabolites and antibiotic production	Ahmed *et al*., 2019
Bacillus coagulans	*H. glycines* and *M. incognita*	Hydrolytic enzymes	Xiang *et al*., 2018
Bacillus subtilis	*Meloidogyne graminicola*, *Meloidogyne incognita*, *Meloidogyne javanica*, *Helicotylenchus multicinctus* and *Rotylenchulus reniformis*	Hydrolytic enzymes, lipopeptide antibiotics and secondary metabolites	Basyony and Abo-Zaid, 2018
Bacillus megaterium	*Heterodera glycines*, *Meloidogyne incognita*, *Meloidogyne graminicola*	Secondary metabolites and protease	Mostafa *et al*., 2018
Bacillus pumilus L1	*Heterodera glycines* and *Meloidogyne arenaria*	Chitinase and protease	Forghani and Hajihassani, 2020
Bacillus thuringiensis	*Heterodera glycines* and *Meloidogyne incognita*	Bt crystal protein (toxin protein) and Thuringiensin (exotoxin)	Mohammed *et al*., 2008

- **Fungal biocontrol of plant-parasitic nematodes:**

  Nematophagous fungi is prevalent across the soil, can grow around the plant rhizosphere. The Nematophagous fungi are known as natural enemies of these pest and have considerable potential to act biological control agents for cyst nematodes. They are one of the best alternatives to nematicides, are environmentally beneficial and inexpensive. They have ultimate capacity to parasitize nematodes and to kill them at any stage of their life cycle such as eggs, juvenile, adults and cysts. They are of 4 categories:

a) Nematode-trapping fungi: Produces adhesive and non-adhesive trapping agents to parasitize and kill the nematodes. They have efficient saprophytic ability. Adhesive agents include hyphae, branches, knobs and nets, coated with adhesives. These adhesive agents lead to the penetration and colonization of fungus by firmly holding the nematode. Non-adhesive trapping agents are responsible for the formation of constricting and non-constricting rings.
b) Endo parasitic fungi: Enters in to the nematode body with the help of adhesive conidia. They are obligate parasites, in expense of body content fungi grow and finally kill the nematode. To parasitize nematodes, Oomycota and Chytridiomycota produces uniflagellate and biflagellate zoospores.
c) Females, eggs or cysts act as a food source for the parasites of cyst nematodes and root-knot nematode, the growth of somatic hyphae fungus colonize over the nematode body, eggs or cyst and cause enzymatic dissolution of egg shell, body wall and larval cuticle. Over time such fungi are also responsible for the degradation of cysts in soil. These fungi have the capacity to control its host population in soil.
d) Toxin-producing fungi: Secretes toxins to immobilize nematodes, e.g., *Verticillium chlamydosporium, Paecilomyces lilacinus, Microdochium bolleyi, Cylindrocarpon* spp., *Arthrobotrys oligospora*, *Nematophthora gynophila* fungus are known to attack on female cyst nematodes. These are zoosporic fungi. Such fungi used to parasitize females present on or near the root surface, breaking the nematode cuticle and preventing the formation of cyst. Fungal parasites decrease the population of female cyst nematodes in the soil (Kerry *et al.,* 1988).

There are two types of parasitic fungi i.e., opportunistic and obligate fungi. Some fungi release protease enzymes that cause egg shell disintegration whereas another form appressoria to infect eggs (Kerry, 1995). *Lecanicillium lecanii* and *Pochonia chlamydosporia* infects females and eggs with the help of appressoria

and zoospores, extracellular enzymes such as chitinases and proteases promote penetration (Yang *et al.*, 2007). Segers *et al.* (1994) demonstrate that nematophagous fungus in situ *Verticillium chlamydosporium* produces extracellular enzymes such as chymoelastase-protease like, such enzymes hydrolyze proteins present in the cell wall of nematodes. were by Morton *et al.* (2004) identified one of the important proteases from nematophagous fungi which belong to the proteinase K family of subtilases. In the infection of ***Heterodera and Globodera*** eggs, production of lipases also been suspected, lipase activity degrades inner lipid layer of nematode eggs. The fungus proliferates ingeniously, ultimately destroys the larval contents is another important group of natural enemies of genus ***Heterodera*** (Yu *et al*.,1998; Saxena, 2018).

Some important genera of fungi can reduce the population of PPN and reduce its multiplication by the process of parasitism, antagonism or predation. Nematophagous fungi have a special in-built feature to act as an antagonist against various genera of economically important plant-parasitic nematodes such as the genus *Meloidogyne*, *Heterodera* and *Globodera* (Brand *et al*., 2004). Nematode egg wall and cuticle of juveniles or adults played a significant role in a fungal infestation. Cuticle walls consist of protein such as fiber, chitin, and collagen act as precursors for nematode invasion of nematophagous fungi (Huang *et al*., 2004). Nematophagous fungi are classified based on the mode of their inhibitory actions on plant-parasitic nematodes.

1. **Predatory fungi:** They grow on the epidermis and can feed on microorganisms by trapping them; this type of fungi is called predatory fungi. Some predatory fungi have unique trapping systems, such as *Arthrobrotrys oligospora, A. superb*. It also includes **nematode feeding fungi** which feed on nematodes by forming constructive rings, such as *A. anchonia, A. dactyloides, Dactylaria brochopaga*. Another is adhesive knob forming fungi such *Monacrosporium cionopagum* and *Dactylella lobata (*Rahman and Mohamed, 2014; Huang *et al*., 2004).

2. **Egg parasitic fungi:** Egg parasitic fungi can infect and destroy the nematode eggs. Some examples are fungi belonging to *Verticillium*, *Paecilomyces* and *Pochonia* (Van Damme *et al*., 2005; Haseeb and Kumar, 2006).

   Several management strategies were implemented to control cysts' nematodes, such as host plant resistance (Dababat *et al*., 2015). To maintain the cyst nematode population, crop rotation with the cultivation of non-cereals was also opted. Singh *et al*., (2009) demonstrated that crop rotation such as rice- wheat, cotton- wheat, and maize- wheat reduced the population of *H. avenae* (CCN). This can decline the rate of spontaneous hatching of juveniles. But in many cases, the crop rotation method was insufficient, use of chemicals was opted to control the CCN population. In India, Australia and Israel, soil and seeds were treated with low nematicides (Rivoal and Nicol, 2009). As a result, the use of aldicarb during the plantation was reduced 24% of the infestation of *H. avenae* on wheat (Smiley *et al*., 2005). Among all the biocontrol methods such as crop rotation, use of resistant cultivars, use of nematophagous bacteria and use of nematophagous fungi. Nematophagous fungi showed some promising results to effectively control the cysts, juveniles and adults of plant parasitic nematodes.(0

Verticillium lecanii is considered as an effective biocontrol agent against arthropods and nematodes. It is biologically active against arthropods (Askary *et al*., 1998). *V. lecanii* can grow on living and dead materials (Aiavo, 2015). *V. lecanii* is non-fastidious and can grow on any media. Chitin is a source of carbon and nitrogen, which helps grow *V. lecanii* (Aiavo, 2015). *V. lecanii* is considered the most common and important entomophagous fungi against the order Diptera, Hymenoptera, Homoptera, Lepidoptera under all climatic conditions. This fungus is effective against several species of nematodes as after 60 hours of inoculation *V. lecanii* mycelium attack the cyst wall of *Heterodera schachtii.* Fungal mycelium passes inside the cyst cavity and colonizes over the cyst wall, egg shells, and larvae.

V. lecanii secretes some enzymes that degrade the cyst wall and eggshells (Aiavo, 2015).

The optimum temperature required for the spore growth of *V. lecanii* is between 15^0C- 25^0C. At 5^0C, spore growth begins and ceases above 30^0C (Aiavo, 2015). Shinya *et al.* (2008) used the hybrid strain of *V. lecanii* to control the cysts and eggs of soyabean cyst nematode (SCN) *Heterodera glycines*. The protoplasm fusion technique was used for the development of nematode control agents. AaF42 hybrid strain reduces nematode egg density by 93.2% compared with control. A wide variety of hybrid strains arises by protoplast fusion in *V. lecanii*. Some of the resulting hybrid strains exhibit appropriate characteristics as a biocontrol agents. Meyer, (1999) studied the efficiency of two strains of *V. lecanii* against root-knot nematode *Meloidogyne incognita*. Studies revealed that both strains of *V. lecanii* suppress the nematode population and eggs present in the soil outside the root tissue. Meyer and Meyer, (1996) compared the effect of different strains of *V. lecanii* against *Heterodera glycines*. They concluded that all mutant strains effectively reduced the number of cysts present inside the soil.

V. lecanii is considered one of the important biopesticides for insects. *V. lecanii* use hydrolytic enzymes and mechanical force to penetrate the insect or hosts integument directly(Fig. 5). Goettel *et al.* (2008) stated that *V. lecanii* have parasitic, antagonistic and disease-resistant inducing characteristics. *Therefore, V. lecanii* has the potential to act as a single microbial control agent. By protoplast fusion resulted, hybrids have improved efficiency and broad host range. Combination of *V. lecanii* plus vanillic acid and *V. lecanii* plus syringic acid was used in microplot tests and in field trials. In microplot experiments, the number of cysts observed was lower than the control. In the field trials, the number of cysts was reduced (Meyer *et al*., 1997). Within 16 hours of inoculation of *V. lecanii*, its hyphae grow externally on gelatinous matrics of *H. glycines* females. Hyphae start shallow penetration on gelatinous matrics of females. Due to fungal infection, holes have been seen on the cyst's wall and females after 3days of infection. More cyst

colonization than females occurred by the infection of *V. lecanii* (Meyer and Wergin, 1998). Shinya *et al.* (2008) studied the effect of culture filtrates of *V. lecanii* hybrid strains on *H. glycines*. Meyer and Wergin, (1998) studied the effect of *V. lecanii* on *H. glycines* cysts and young females. They stated that within 16 hours of *V. lecanii* colonize over the surface of cysts and females. Cysts tended to be colonized more quickly than adult females. Fungal hyphae penetrated directed from the body wall into it. *V. lecanii* efficiently lowers the cyst number invitro and microplots (Meyer *et al*., 1997; Meyer and Wergin, 1998). Shinya *et al*. (2008) proved that culture filtrates of *V. lecanii* inhibit egg hatch of soyabean cyst nematode, *Heterodera glycines*, and these filtrates are highly toxic against embryonated eggs. Infection of *V. lecanii* on embryonated eggs is responsible for converting normal embryonated eggs into abnormal ones after the 3rd day.

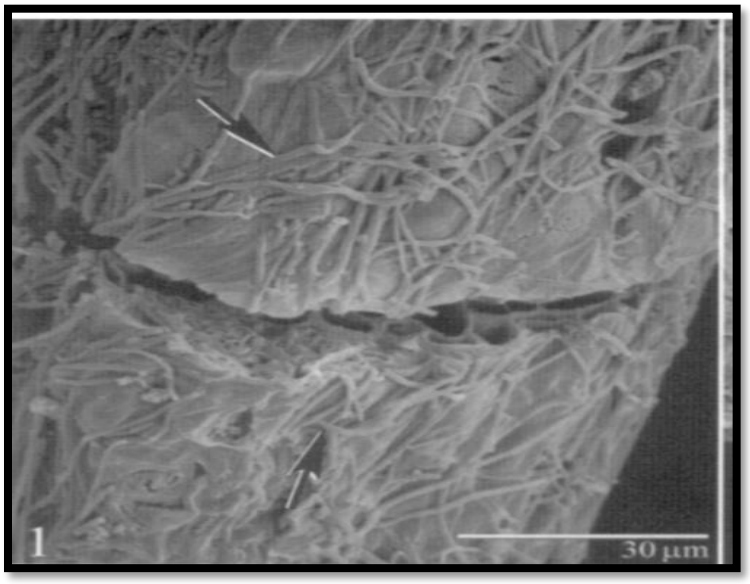

Figure 5: Scanning electron micrographs of hyphae of *Verticillium lecanii* growing in close association with soyabean root tip (Meyer *et al.*, 1998).

Chapter-3

Hypothesis

Chapter-3

Hypothesis

Cyst nematodes are sedentary, and obligatory plants root parasitic nematodes. Cyst nematodes induce feeding sites in the host plant's roots by forming syncytium. Cyst nematodes are amphimictic. They follow a unique life cycle; after death, the female body converts into a protective cyst enclosed with its eggs inside it. At favourable conditions, eggs inside the cysts hatch, and juveniles hatch out searching for the host. Cyst nematodes include six genera having the family Heteroderidae, i.e., *Heterodera*, *Globodera, Cactodera*, *Punctodera*, *Dolichodera* and *Afenestrata.* Cyst nematodes directly damage the root of the various important crop plants. In India, cysts and nematodes show their economic importance by causing a considerable loss in crop yields such as wheat, potato, corn, and pulses, by infecting the roots of the plants. Studies have reported the availability of cysts nematodes in the soil of Himachal Pradesh, so the **study would be focused on understanding the specific level occurrence of such pests in the agricultural lands of Himachal Pradesh.**

The morphological and molecular characteristics are involved in the project to decipher the specific diversity of cyst nematodes. The study of morphological and molecular characteristics of isolated cyst nematodes from the agricultural fields is the primary motive of this project. This project focuses on the occurrence and population density of various cysts' nematodes in the agricultural fields of Himachal Pradesh.

Parasite resistant plants, crop rotation methods, and chemical nematicides have not been used yet to control cysts populations. Chemical pesticides are effective but not environmentally safe and reduce soil fertility. To facilitate a load of chemical pesticides in the soil, various other methodologies have been explored to date. Control of pests by their natural enemies such as parasites, predators, and competing organisms is called biological control. It is an alternative to using **broad-**

spectrum pesticides. Biological pest control creates **no chemical** run-off in waterways or soil pollution. Biological control of a plant-parasitic nematode concerns the decline of populations of the cyst nematodes. Another motive of this project is the eco-friendly control of isolated cyst nematodes. The use of the biocontrol method is one of the safe methods to reduce the parasite concentration in the soil and help. The use of fungi is well exemplified for controlling various insects and pests of crops**.** This study focuses on the *in-vitro* control of cyst nematodes with the application of parasitic nematode fungi. **Therefore, the analysis incorporates such fungi, especially *Verticillium* spp., to control nematode, which may help reduce the loss of food or crop economics. *Verticillium lecanii*** is considered as an effective biocontrol agent against arthropods and nematodes. *V. lecanii* can grow on living and dead materials. *V. lecanii* is considered one of the important biopesticides for insects. *V. lecanii* use hydrolytic enzymes and mechanical force to penetrate the insect integument or nematode body wall directly and effectly kill the parasite effectively. Use of *V. lecanii* could be act as complementary strategy because when *V.lecanii* applied simoulanously, it can reduce the efficiency of other biocontrol agents such as natural predators and parasitoids.

Chapter-4

Objectives

Chapter-4

Objectives

The brief objectives of our research are

1. To study the morphology of cyst nematode of Himachal Pradesh.

2. Molecular characterization of cyst nematode of Himachal Pradesh.

3. To study the biocontrol of cyst nematode by nematophagous fungus *Verticillium* spp.

Chapter- 5

Material and methods

Chapter- 5

Material and methods

5.1.Collection of soil samples

5.1.1. Soil sampling

A survey was performed on agricultural land. Various agricultural fields were inspected, and soil samples were collected from 10 districts of Himachal Pradesh throughout the year (2018-2021), especially during the grain filling period to harvest time (from mid-February to late May and September – December). Several sub-samples were collected from each site and mixed to make a composite bulk of each sample (Wainer and Dinh, 2021) and kept in properly labelled polythene zip lock bags and preserved in the refrigerator. Samples were carefully washed within a short time as soon as possible. Cyst nematode population density and percentage of occurrence were determined in all collected samples. Population density (P.D) and percentage of occurrence were calculated (Bakr *et al*., 2011)

P.D. = No. of nematodes / Total no. of samples.

% occurrence = No. of positive samples / Total no. of samples ×100

The population density of cyst nematode was evaluated as:

1) Zero, for no infestation
2) Very low for less than 10
3) Low for less than 20
4) Medium for 21-50
5) High for 51-100
6) Very high for more than 100 cysts/250g of soil

5.1.2. Soil sample processing

Each composite sample was thoroughly mixed in a plastic tub to break soil lumps (if any). Then 250g of soil was taken into the beaker mixed with equal water to get an excellent suspension. Decanting and sieving methods were followed to

separate the cyst from the soil. First, 60 and 100 mesh size sieves (250 microns and 149micronsn) were set over another, and the soil suspensions were sieved. Next, the residue in the 60 mesh (250 microns) sieve was collected in a beaker and was used for cyst examination. Next, a Small Portion of residue in Petri dishes was examined under the stereo zoom microscope. Finally, the cysts were hand-picked with the help of a brush or forceps and separately collected in distilled water (Fenwick 1940).

5.1.3. Sterilization of soil for nematode culture

The soil was collected from the non-agricultural land, and sand was collected from the river bed. First, both soil and sand were mixed in 3:1 ratio. Then the mixture was autoclaved. The autoclaved mixtures were exposed to open air for 24 hours before filling the pots.

5.1.4. Pure culture of nematode

Ten to fifteen cysts were placed along with one-week-old host plant (family Polygonaceae, Solanaceae, Poaceae) seedlings were grown in 15cm and 30 cm pots.

5.1.5. Material used for soil sampling

- **a.** Khurpi.
- **b.** Zip lock bags/ polythene bags.
- **c.** Refrigerator.
- **d.** Gloves.

5.1.6. Material used for soil sample processing

- **a.** 60 and 100 mesh size sieves (250 microns and 149microns).
- **b.** Plastic tank.
- **c.** 100ml, 250ml, 500ml and 1L beakers.
- **d.** Stereomicroscope.
- **e.** Petri dishes.

f. Forceps.

5.1.7. Material used for nematode culture

a. Autoclave.

b. 15 c.m and 30 c.m pots

5.2. To study the morphology of the Cyst nematode of Himachal Pradesh.

- Cysts were isolated from 250g of the soil by decanting and sieving method (Fenwick, 1940).
- 60 and 100 mesh sieves (250 microns and 149microns) were used for washing soil.
- Each sample was examined under a stereomicroscope.
- Cysts were separated from the soil, and collected cysts were further processed for morphological identification.
- **Cyst cone top study:** With the help of a surgical blade, cyst vulval cones were cut. The content of the cone was cleaned up with the use of a bamboo bristle. The clean cone was trimmed carefully. The cone was washed with 70% ethanol for 5 minutes and mounted in glycerin jelly on a glass slide (Mulvey, 1972). The section was studied under a compound microscope 40X and 100X lens.
- **Morphological characteristics of cyst**: Cyst colour, shape, length, width, length/width, fenestral length, fenestral width, vulval bridge, vulval slit length and fenestra to anus distance were observed under the compound microscope 40X and 100X lens (Mulvey, 1972).
- Vulval cones of mature cysts were mounted in glycerin jelly for each population. The underbridge structure, the shape of semi fenestra in the fenestral area, and the development of bullae were observed under a microscope. The morphological identifications were made (Handoo, 2002).

5.2.1 Material used

a. Compound microscope.

b. Stereomicroscope.

c. Stage micrometer.

d. Glass slides.

e. Cover slips.

f. Glycerin jelly.

g. Distilled water.

h. Petri dishes.

i. Forceps.

j. Needles.

k. Glass slides.

l. Brushes.

5.3. Molecular characterization of Cyst nematode of Himachal Pradesh.

5.3.1. DNA extraction

- Cysts (100) were crushed using mortar and pestle in 10ml of double-distilled water (ddH2O) to isolate genomic DNA from each population. Next, the squashed cyst content was crushed carefully in 10 ml of nematode lysis buffer (125mMKCL, 25 mM Tris-HCL pH 8.3, 3.75 mM $MgCl_2$, 2.5 DTT, 1.125 % Tween 20, 0.025% gelatin) (Qiagen Kit) (Rao *et al*., 2013).
- Complete suspensions were transferred in an Eppendorf tube. 2ml of proteinase K (600g/ml) was added to the homogenate. The tubes were frozen at –80 °C for at least 10 minutes and then incubated at 65 °C for 1 h and 95 °C for 10 minutes consecutively in a thermocycler.
- The Eppendorf tubes were subjected to centrifugation for 1min at 16000 rpm. Then, the supernatants were removed without disturbing the pellet in another Eppendorf tube and kept at –20 °C until use (Imren *et al.,* 2015).
- After centrifugation of the squashed cyst content, 40 µL of the mix was transferred to a PCR tube (0.2 mL) containing 50 µL of worm lysis buffer and 10 µL of proteinase K (20 mg/mL). After incubation, the tubes were centrifuged for 1 min at 14,000 rpm (Imren *et al.,* 2015).

5.4.1. Amplification of genomic DNA

- Isolated genomic DNA was amplified by using PCR thermocycler using primers. Amplification was done in a 50-µL reaction volume containing 22 µL of ddH2 O and 25 µL of 2X Dream Taq PCR Master Mix.
- Amplification of the ITS region present in rDNA, including 18S and 28S genes was carried out by using 1µM each of forward primer-V5367 (5'-TTGATTACGTCCCTGCCCTTT-3') and 26S (5'-TTTCACTCGCCGTTACTAAGG-3') (Vrain *et al*., 1992), forward primer-TW81 (3'-GTTTCCGTAGGTGAACCTGC-5') and reverse primer-AB28(3'-ATATGCTTAAGTTCAGCGGGT-5') (Subbotin *et al*., 2003) and 1 µL of DNA extract.
- Amplification of the 18S small subunit (SSU) of rDNA was carried out by using 1µM each of forward primer-SSU18A (AAAGATTAAGCCATGCATG) and reverse primer-SSU26R (CATTCTTGGCAAATGCTTTCG) (Floyd *et al.*, 2002) and 1 µL of DNA extract.

5.4.2. Polymerase Chain Reaction

- The PCR reaction conditions for V5367 and 26S were as follows: 95 ^{0}C for 3 min, followed by 35 cycles consisting of denaturation at 95 ^{0}C for 10 s, annealing at 55 ^{0}C for 20 s, and extension at 72 ^{0}C for 30 s, with a final extension at 72 ^{0}C for 10 min.
- SSU18A and SSU26R: 94 ^{0}C for 5 min; 35 cycles of 94 ^{0}C for 1 minute; 52 ^{0}C for 1 minute 30 s; 68 ^{0}C for 2 min; 68 ^{0}C for 10 min.
- TW81 and AB28: 4 min at 94 ^{0}C; 44 cycles of 94 ^{0}C for 1min, 55 ^{0}C for 1min 30 sec, 2min in 72 ^{0}C and 10 min 72 ^{0}C.
- After amplification, 5 µL of each PCR product were mixed with 1 µL of 6X loading buffer and loaded on a 1.5% standard TAE buffered agarose gel.

- After electrophoresis (100 V for 40 min), the gel was stained with ethidium bromide (0.1 µg/mL) for 15 min and visualized and photographed under UV light.
- The leftover PCR product was stored at –20 ^{0}C (Subbotin *et al*., 2003; Maafi *et al*., 2003).
- The clear PCR product band were cut and eluted from the gel and purified using a PCR purification kit from Qiagen. For genomic sequencing, the gel-purified products were sent to a commercial company (Eurofins Pvt. Ltd Bangalore).
- Phylogenetic analysis for identification purposes, an alignment of all our obtained sequences together with representatives of all species of respective genus available in GenBank, was constructed using Mega X (Tamura *et al.*, 2011).

5.4.3. Material used

a. Double distilled water.
b. Eppendorf tubes.
c. Mortar and pestle.
d. PCR tubes.
e. Worm lysis buffer.
f. Proteinase K.
g. Centrifuge.
h. 2X Dream Taq PCR Master Mix.
i. TAE buffered agarose gel.
j. Ethidium bromide.
k. Electrophoretic unit.
l. UV chamber.
m. Agarose gel.
n. PCR product purification kit.

5.5. To study the biocontrol of Cyst nematode by nematophagous fungus *Verticillum* spp.

5.4.1. Nematode inoculum

- Cysts are isolated from soil samples from a single agricultural field by using the standard Fenwick can method (Fenwick, 1940). After sieving, cysts were handpicked with the help of brush/forceps under a stereo zoom microscope and collected in separate Petri plates.
- Collected cysts were surface sterilized by exposing them to 0.1% NaOCl (sodium hypochlorite) solution for 30 minutes, then rinsed with distilled water to remove the traces of the NaOCl (Ayatollahy *et al*., 2008).
- The cysts included in the study were firstly identified morphologically and molecularly. The cysts were free from fungal or bacterial growth. After sterilization, the extracted cysts were used for further studies.

5.4.2. Fungal culture (biocontrol agent)

- The fungal isolate *Verticillium lecanii* (ITCC 7084), was obtained from the Indian Agricultural Research Institute, New Delhi, India. *V. lecanii* were grown in the Potato Dextrose Agar (PDA) media for 14 days at a temperature $25\pm1^{o}C$.
- After full growth of the fungus culture, the conidia were scraped from the culture plates with the sterile glass rod and then suspended in 10 ml of sterilized distilled water (Illathur and Sridhar, 2021).
- The mycelium was removed from conidial suspensions using gauze and then centrifuged at 3000 rpm for 5 minute. The resulted pellet was resuspended in 10 ml of sterilized distilled water.
- As per the procedure given by Hashem and Elyousr, (2010), the conidial count was maintained at 1.7×10^5 CFUs per ml by using a haemocytometer.

- The conidial suspensions were mixed in the required amount of distilled water for setting up different concentrations for *in-vitro* experiments (Mukhtar and Pervaz, 2003; Illathur and Sridhar, 2021).

5.4.3. *In-vitro* effect of *V. lecanii* on cysts nematodes

- For testing the efficacy of conidial suspension of *V. lecanii* on the cysts of *C. estonica*, *G. rostochiensis* and *H. avenae*, three concentrations were tested (Treatments) (T1-10%, T2-20% and T3-30%) along with untreated control (T4).
- Three concentrations *viz*., 10%, 20% and 30% of *V. lecanii* were prepared by mixing 1 ml, 2 ml and 3 ml conidial suspensions of *V. lecanii*, (1.7×10^5 CFUs per ml) (Mukhtar and Pervaz, 2003).
- The experiment was conducted using five cysts per replication, and there were six replicates per treatment. These presoaked cysts were inoculated in the 90 mm petri dish containing water agar media and different concentrations of fungal conidial suspensions. Each set was placed in biological oxygen demand incubator at 25 ± 1^oC.
- Due to the destructive sampling protocol, different sets were maintained for each time of observation, for each time interval hours after inoculation (HAI).
- The observations on the damaged cyst were recorded after each time interval and expressed in percentage. The experiments were repeated twice.

5.4.4. Material used

- Stereo zoom microscope
- Compound microscope
- Autoclave
- Petri dishes
- Micropipette
- Tips

- 0.1 % NaOH
- Absolute alcohol
- Incubator
- Potato Dextrose Agar
- Antibiotics
- Water agar
- Haemocytometer
- Spectrophotometer

5.5. Statistical Analysis

Data were normalized by calculating the mean, standard error and standard deviation for the morphological and morphometrical analysis of cysts nematodes. Bio efficacy percentage data were pooled from repetitive experiments and subjected to analysis of variance (ANOVA). When ANOVA were significant, comparisons of relevant means (effect of *V. lecanii* on nematode cysts) were made using Tukey's significance test values at a 5% level of significance. The statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) statistics (IBM Corp, 2012).

Chapter- 6

Results and Discussions

Chapter- 6

Results and Discussions

6.1. A random survey was conducted for three years, from November 2018 to December 2021, in the agricultural fields of Himachal Pradesh to study the occurrence and population density of cyst nematodes associated with crops. Soil samples were collected randomly, mainly from the wheat and potato cultivated areas. During this survey, 10 districts of Himachal Pradesh were covered: district Kangra, Mandi, Kullu, Chamba, Shimla, Una, Hamirpur, Bilaspur, Solan and Sirmour.

Table 6: District-wise coordinates and elevation from sea level of visited districts during the survey.

S. No	District	Latitude	Longitude	Elevation from sea level
1.	Kangra	32˚13’0” N	76˚19’0” E	785.79 m
2.	Mandi	31˚72’0” N	76˚92’0” E	769.65m
3.	Kullu	31° 57' 28.2636" N	77° 6' 34.0524"E	1229.27m
4.	Chamba	32˚33’12.11” N	76˚7’32.91”E	930.39m
5.	Shimla	31° 6' 17.33" N	77°10'24.25"E	2196.89m
6.	Una	31°28'6.4" N	76°16'14.79"E	381.63m
7.	Hamirpur	31°41'10.23"N	76°31'16.7"E	774.75m
8.	Bilaspur	31°20'34.59"N	76°45'45.03"E	572.58m
9.	Solan	30° 54' 16.15" N	77° 5' 48.25" E	1451.96m
10.	Sirmour	30°33'46.25" N	77°28' 12.71" E	996.18m

All these districts mention above mainly cultivate wheat and potato. Various agriculture farms, private agriculture lands and Krishi Vigyan Kendra (KVK) have also been covered

along with private agriculture lands. Each district's climatic condition slightly varies from the other due to height above sea level. Table 6 represents the soil sample collected from different districts of Himachal Pradesh. Soil samples were collected from different areas of each district from 2019-to 2022 (Fig. 6,7,8,9,10,11 and 12). Soil samples were collected from district Kangra, 13 different areas, i.e., KVK Kangra, agriculture farm Malan, Shahpur, Tang, Pantehar, Pathiar, Skoh, Bagora, Birta, Rehan, Nurpur, Nagrota and Jiya from district Kangra. At least three times, sampling was done at each site to ensure the presence of cyst nematodes in the soil. In the district, Kangra *Globodera* (globose shaped) cysts were found in potato fields, i.e., Jiya and Pathiar. *Globodera* cyst recovered from Jiya. In the district, Mandi soil samples were collected from 23 areas, i.e., Phulladhar farm, Basunti Barot, Rewalsar, Joginder Nagar, Sunder Nagar, Sarkaghat, Dhanotu, Near Chownk, Anu, Althu, Dadoh, Chawri, Kangru, Kasarla, Sakroha, Trawai, Pandoh, Nasloh, Tikar Kalan, Nagdhar, Tihri, Ratti, Tanda. *Cactodera* cysts and *Globodera* cysts were recovered from Phulladhar farm, potato field, and *Globodera* cysts from the Barot area. Kullu soil samples were collected from sites from the district, i.e., Bandrol, Anni, Chowaii, Shoja, Bhuntar, Sultanpur, Kasol, Manikaran, Balh Bhalyani, Bhullang, Biasar, Dawara, Dobhi, Diar, Hat, Jari, Mandaldarh and Manjhli. *Cactodera* (lemon-shaped) cysts and *Globodera* (globose shaped) cysts were recovered from the potato field of KVK Kullu. Soil samples were collected from 5 areas of district Chamba, i.e., Ahla, KVK Chamba, Salooni, Sihunta and Dalhousie. *Globodera* cyst was recovered from Potato Development Center Ahla, and genus *Globodera* cysts (globose shaped) were retrieved from KVK Chamba. From the district, Shimla soil samples were collected from 3 areas i.e., Totu pani, Khadrala, Chail. *Globodera* cysts (globose shaped) were isolated from the soil collected from potato farm Totu pani, and *Cactodera* (lemon-shaped) cysts were isolated from Khadrala. Soil samples were collected from 5 areas of district Una i.e., Akrot farm, KVK Jhalera, Jhalera, Amb, Mubarikpur. Out of these five areas, *Heterodera* (lemon-shaped) cysts were recovered from Akrot farm and Jhalera. Soil samples were collected from district Hamirpur in 14 different regions i.e., Tira Sujanpur, Chakothi, Bir Khera, Rithari, Terha, Shekhoopur, Misripur, Todarpur, Sikrohi, Danda, Vidokhar, Kangu, Nadaun and

KVK Hamirpur. No cyst nematode was recovered in the soil samples collected from district Hamirpur. In district Bilaspur, soil samples were collected from 3 different areas, i.e., KVK Berthin, Chamlog and Khatehr. No cyst nematode was recovered from the soil sample collected from district Bilaspur. Soil samples were collected from district Solan from 4 different areas, i.e., Kandaghat, Nalagarh, Waknaghat and Kasauli. No cyst nematode was recovered in district Solan. Soil samples were collected from district Sirmour in 5 different areas, i.e., Kamrau, Nahan, Renuka, Dadahu and Ponta Sahib. No cyst nematode was recovered in district Sirmour. This survey confirmed the occurrence of three genera of cyst nematode found in the agricultural fields of Himachal Pradesh, i.e., *Globodera*, *Cactodera* and *Heterodera*. *Globodera* cysts were found in the potato field of Phulladhar farm and Barot district Mandi, Chowaii district Kullu, Potato Development Center Ahla and KVK Saru district Chamba, Totu pani district Shimla, Jiya and Pathiar district Kangra. *Cactodera* cysts were found in the potato field Phulladhar farm district Mandi, Potato Development Center Ahla district Chamba and Khadrala district Shimla. *Heterodera* cysts were found in the wheat field of Akrot farm and Jhalera district Una.

Figure 6: a. Soil sample collected from wheat field district Una. **b.**Soil sample collected from potato field district Chamba. **c.** Soil sample collected from potato field district Mandi. **d.** Soil sample collected from potato field district Kangra.

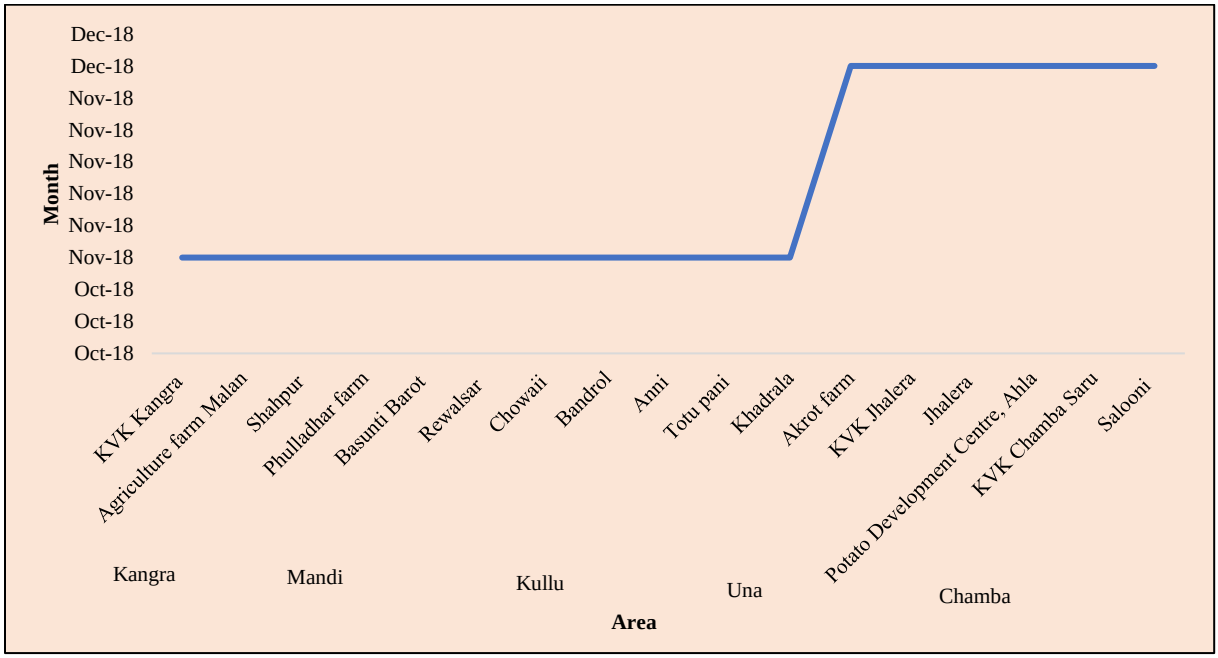

Figure 7: District-wise areas visited for soil sample collection from November 2018 to December 2018.

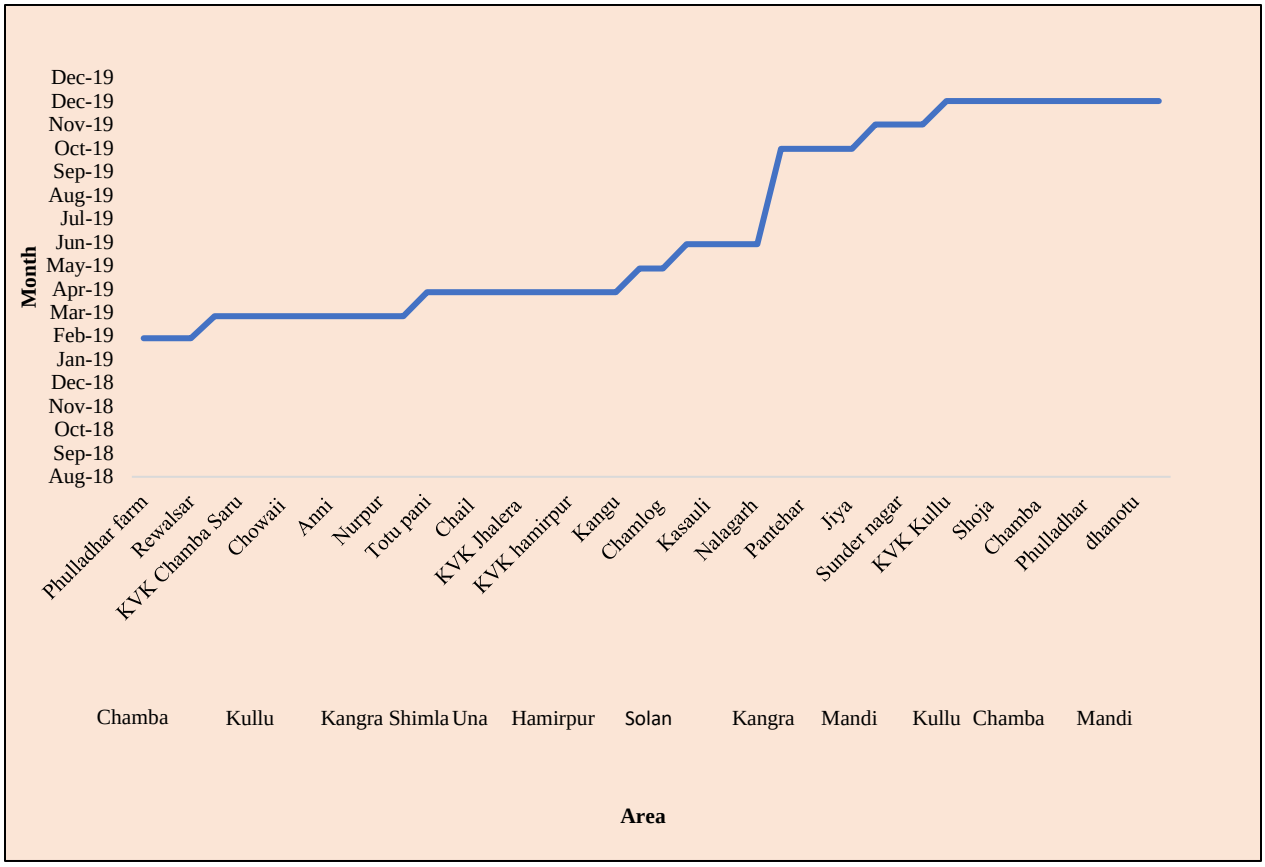

Figure 8: District-wise areas visited for soil sample collection from February 2019 to December 2019.

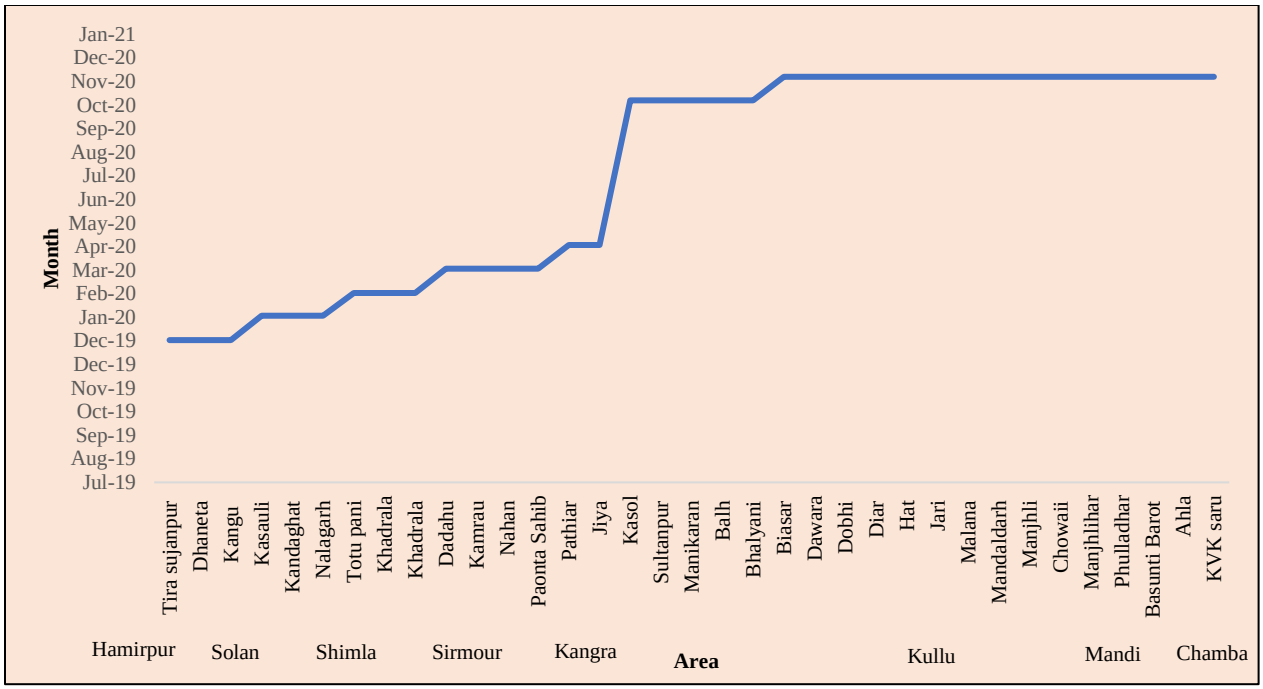

Figure 9: District-wise areas visited for soil sample collection from January 2020 to December 2020.

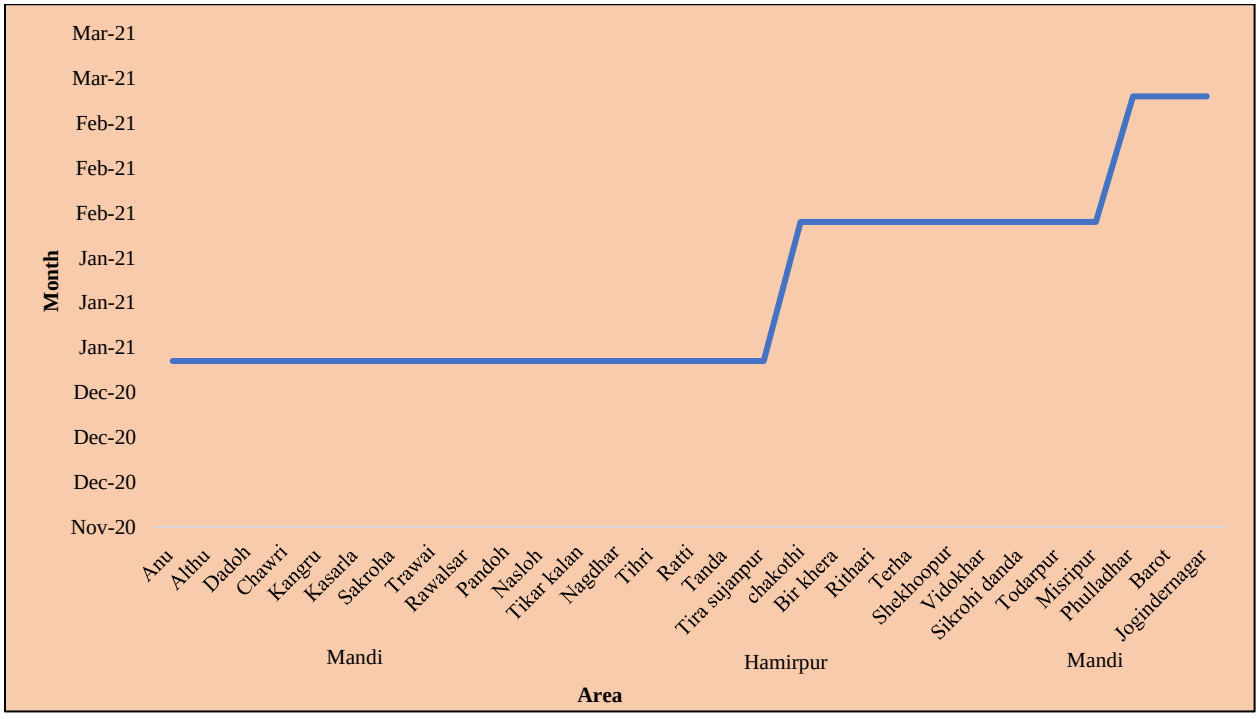

Figure 10: District-wise areas visited for soil sample collection from January 2021 to March 2021.

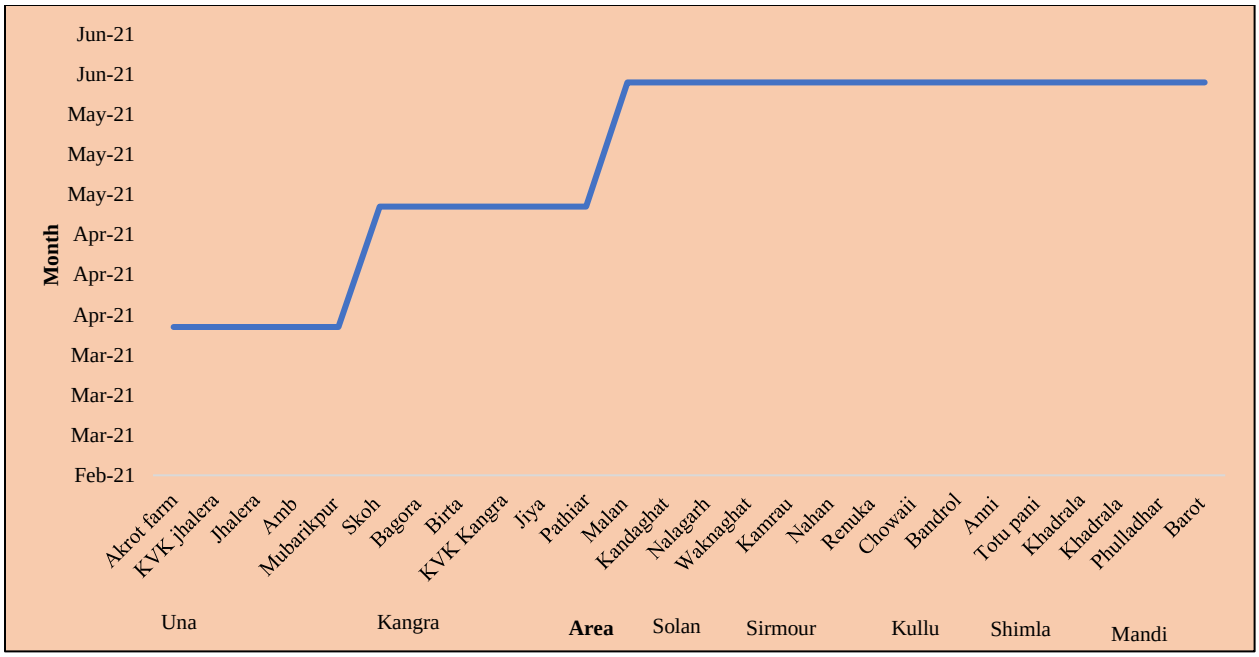

Figure 11: District-wise areas visited for soil sample collection from April 2021 to June 2021.

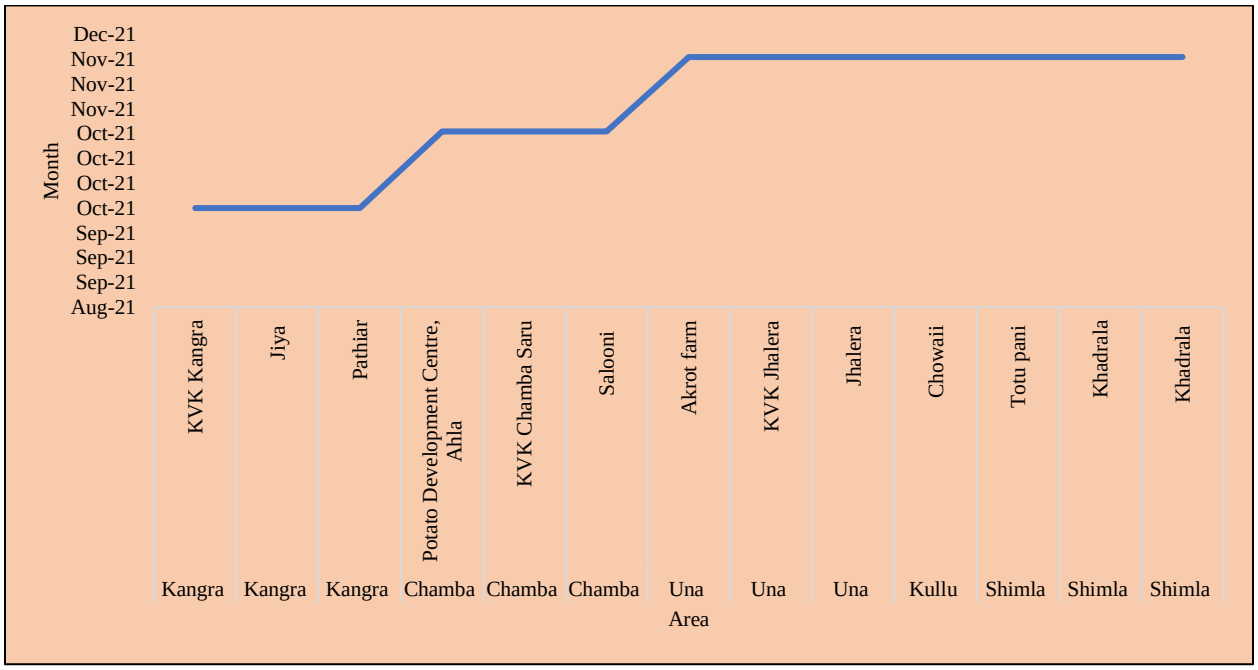

Figure 12: District-wise areas visited for soil sample collection from October 2021 to December 2021.

Table 7: Occurrence of *Globodera* in different districts during 2018.

S. No	DISTRICT	AREAS	No. OF CYST/ 250 g SOIL
1.	Mandi	Phulladhar farm	48
2.	Mandi	Basunti Barot	45
3.	Shimla	Totu pani	19
4.	Shimla	Khadrala	5
5.	Kullu	Chowaii	48
6.	Chamba	Potato Development Centre, Ahla	52
7.	Chamba	KVK Chamba Saru	4

Table 8: Occurrence of *Globodera* in different districts during 2019.

S. No	DISTRICT	AREAS	No. OF CYST/ 250 g SOIL
1.	Mandi	Phulladhar farm	49
2.	Mandi	Basunti Barot	48
3.	Chamba	Potato Development Centre, Ahla	50
4.	Chamba	KVK Chamba Saru	2
5.	Mandi	Dhanotu	5
6.	Kullu	Chowaii	48
7.	Shimla	Totu pani	18
8.	Kangra	Pathiar	4
9.	Kangra	Jiya	42

Table 9: Occurrence of *Globodera* in different districts during 2020.

S. No	DISTRICT	AREAS	No. OF CYST/ 250 g SOIL
1.	Shimla	Totu pani	17
2.	Shimla	Khadrala	3
3.	Kangra	Pathiar	4
4.	Kangra	Jiya	40
5.	Chamba	KVK Chamba Saru	2
6.	Chamba	Potato Development Centre, Ahla	54
7.	Kullu	Chowaii	45
8.	Mandi	Basunti Barot	50
9.	Mandi	Phulladhar	55

Table 10: Occurrence of *Globodera* in different districts during 2021.

S. No	DISTRICT	AREAS	No. OF CYST/ 250 g SOIL
1.	Mandi	Phulladhar	48
2.	Mandi	Basunti Barot	30
3.	Mandi	Rewalsar	10
4.	Chamba	Potato Development Centre, Ahla	41
5.	Chamba	KVK Saru	4
6.	Kangra	Jiya	32
7.	Kangra	Pathiar	2
8.	Kullu	Chowaii	38
9.	Shimla	Totu pani	15
10.	Shimla	Khadrala	3
11.	Mandi	Phulladhar	51
12.	Mandi	Basunti Barot	10
13.	Kangra	Jiya	40
14.	Kangra	Pathiar	2
15.	Chamba	Potato Development Centre, Ahla	48
16.	Chamba	KVK Chamba Saru	0
17.	Kullu	Chowaii	50
18.	Shimla	Totu pani	16
19.	Shimla	Khadrala	5

From district Mandi (Phulladhar, Barot) minimum of 48 cysts / 250g soil and a maximum of 55cysts/ 250g soil *Globodera* cysts were collected from soil samples. From district Chamba (Ahla farm, KVK Saru) minimum of 2 cysts/ 250g soil and a maximum of 54 cysts/ 250g soil *Globodera* cysts were collected from soil samples. From district Kullu (Chowaii) minimum of 38 cysts/ 250g soil and a maximum of 50 cysts/ 250g soil *Globodera* cysts were collected from soil samples. From district Shimla (Totu pani) minimum of 5cysts/ 250g soil and a maximum of 19 cysts/ 250g soil *Globodera* cysts were collected from soil samples. From district Kangra (Jiya, Pathiar) minimum 2cysts/ 250g soil and a maximum of 42 cysts/ 250g soil *Globodera* cysts were collected from soil

samples mentioned in Table 7,8,9 and 10. In *Cactodera*, from district Mandi (Phulladhar), a minimum of 18 cysts/ 250g soil and a maximum of 20cysts/ 250g soil *Cactodera* cysts were collected from soil samples. From district Chamba (Ahla farm) minimum of 20 cysts/ 250g soil and a maximum of 22 cysts/ 250g soil *Cactodera* cysts were collected from soil samples. From district Shimla (Khadrala) minimum of 7 cysts/ 250g soil and a maximum of 17 cysts/ 250g soil *Cactodera* cysts were collected from soil samples mentioned in Tables. 11, 12,13 and 14. In the case of *Heterodera*, from district Una (Akrot farm, Jhalera), a minimum of 0 cysts/ 250g soil and a maximum of 15 cysts/ 250g soil *Heterodera* cysts were collected from soil samples mentioned in Table 15,16 and 17.

Table 11: Occurrence of *Cactodera* in different districts during 2018.

S. No	DISTRICT	AREAS	No. OF CYST/ 250 g SOIL
1.	Mandi	Phulladhar farm	18
2.	Shimla	Khadrala	17
3.	Chamba	Potato Development Centre, Ahla	22

Table 12: Occurrence of *Cactodera* in different districts during 2019.

S. No	DISTRICT	AREAS	No. OF CYST/ 250 g SOIL
1. 2	Mandi	Phulladhar farm	20
2.	Chamba	Potato Development Centre, Ahla	25
3.	Shimla	Khadrala	16

Table 13: Occurrence of *Cactodera* in different districts during 2020.

S. No	DISTRICT	AREAS	No. OF CYST/ 250 g SOIL
1.	Shimla	Khadrala	15

Table 14: Occurrence of *Cactodera* in different districts during 2021.

S. No	DISTRICT	AREAS	No. OF CYST/ 250 g SOIL
1.	Mandi	Phulladhar	18
2.	Chamba	Alha potato farm	20
3.	Shimla	Khadrala	16
4.	Mandi	Phulladhar	20
5.	Chamba	Potato Development Centre, Ahla	22

Table 15: Occurrence of *Heterodera* in different districts during 2018.

S. No	DISTRICT	AREAS	No. OF CYST/ 250 g SOIL
1.	Una	Akrot farm	15
2.	Una	Jhalera	4

Table 16: Occurrence of *Heterodera* in different districts during 2019.

S. No	DISTRICT	AREAS	No. OF CYST/ 250 g SOIL
1.	Una	Akrot farm	8
2.	Una	Jhalera	1

Table 17: Occurrence of *Heterodera* in different districts during 2021.

S. No	DISTRICT	AREAS	No. OF CYST/ 250 g SOIL
1.	Una	Akrot farm	14
2.	Una	Jhalera	2
3.	Una	Akrot farm	10
4.	Una	Jhalera	0

Table 18: Population density (P.D) and percentage of occurrence of *Globodera* during survey.

S. No	**District**	**Location**	**Mean no. of cyst/ 250 g soil**					**Population density (P.D)**	**%Occurrence *Globodera***
			1	2	3	4	5		
1.	Mandi	Phulladhar farm	48	49	55	48	51	50.2	100%
2.	Mandi	Basunti Barot	45	48	50	30	10	36.6	100%
3.	Shimla	Totu pani	19	18	17	15	16	17	100%
4.	Kullu	Chowaii	48	48	38	45	50	45.8	100%
5.	Chamba	Potato Development Centre, Ahla	52	50	54	41	48	49	100%
6.	Chamba	KVK Chamba Saru	4	2	2	4	0	2.4	80%
7.	Kangra	Pathiar	4	4	2	2	4	3	100%
8.	Kangra	Jiya	42	40	32	40	40	38.8	100%

Table 19: Population density (P.D) and percentage of occurrence of *Cactodera* during survey.

S. No	District	Location	Mean no. of cyst/ 250 g soil				Population density (P.D)	%Occurrence *Cactodera*
			1	2	3	4		
1.	Mandi	Phulladhar farm	18	20	18	20	19	100%
2.	Shimla	Khadrala	17	16	15	16	16	100%
3.	Chamba	Potato Development Centre, Ahla	22	25	20	22	22.25	100%

Table 20: Population density (P.D) and percentage of occurrence of *Heterodera* during survey.

S. No	District	Location	Mean no. of cyst/ 250 g soil				Population density (P.D)	% Occurrence *Heterodera*
			1	2	3	4		
1.	Una	Akrot farm	15	8	14	10	11.75	100%
2.	Una	Jhalera	4	1	2	0	2.5	75%

As mentioned in Table 7, the *Globodera* cysts were recovered from the soil sample collected from districts Mandi, Shimla, Kullu and Chamba in 2018. In 2019 soil samples collected from districts Mandi, Shimla, Kullu, Chamba, and Kangra showed the occurrence of *Globodera* in it shown in Table 8. Tables 9 and 10 show the continuous occurrence of *Globodera* cyst in 2020 in districts Shimla and Kangra and 2021 districts Chamba, Kangra, Mandi, Shimla and Kullu. *Cactodera* cysts recovered from Himachal Pradesh. The soil samples collected from districts Mandi, Kullu, Shimla, and Chamba during 2018 and 2019 showed the occurrence of *Cactodera* demonstrated in Tables 11 and 12. From 2020 to 2021, soil samples collected from districts Mandi, Kullu, Shimla and Chamba showed the occurrence of *Cactodera* mentioned in Tables 13 and 14. During the survey (2018 to 2021), *Heterodera* cysts were recovered from district Una demonstrated in Tables 15, 16 and 17. After completing the study, the population density (P.D) and percentage of occurrence of each genus were determined in Tables 18, 19 and 20. Phulladhar farm in district Mandi showed the highest population density of *Globodera,* i.e., 50.2. KVK Saru and Pathiar area from district Chamba and Kangra showed the lowest population density *Globodera,* i.e., 3. Whereas the percentage of occurrence of *Globodera* in districts Mandi, Shimla, Kullu, Chamba and Kangra was 100% shown in table 18. The population density of *Cactodera* was highest in Ahla district Chamba, i.e., 22.5 and was lowest in Khadrala district Shimla, i.e., 16 mentioned in table 19. Whereas the percentage of occurrence of *Cactodera* in

districts Mandi, Shimla, Kullu, Chamba was 100%. Among all the soil samples collected from 10 different districts, cysts of *Heterodera* were recovered from Akrot farm and Jhalera in district Una. Akrot farm showed the highest population density, i.e., 11.75 with 100% occurrence mentioned in Table 20.

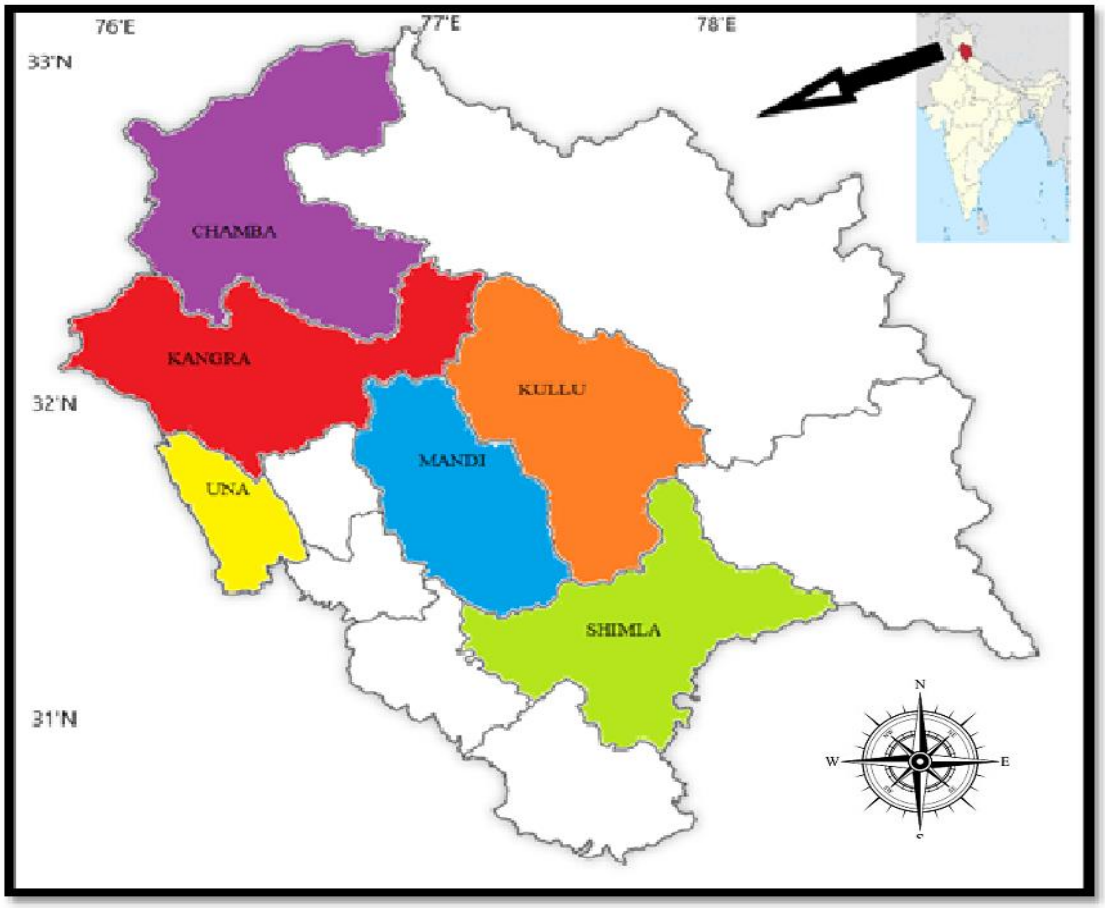

Figure13: Map representing highlighted districts of Himachal Pradesh infected with Cyst nematodes.

This survey was conducted for three years, from November 2018 to December 2021, in the agricultural fields of Himachal Pradesh. This study aimed to cover maximum districts to confirm the presence of cyst nematodes in the soil. During this survey, at least 3 samples were collected from the agricultural areas, especially from the roots of the crop plants. In addition, soil samples were collected from the agricultural sites of 10 districts- Kangra, Mandi, Kullu, Chamba, Shimla, Una, Hamirpur, Bilaspur, Solan and Sirmour Himachal Pradesh. To calculate the population density and per cent occurrence of cyst nematodes, soil samples were collected at least 4 times from the areas where cysts were found. Various Agriculture farms, KVK and crops cultivating lands were covered in each district: Kangra, Mandi, Kullu, Chamba, Shimla, Una, Hamirpur, Bilaspur, Solan and Sirmour. Cyst nematode populations are affected by different factors such as temperature, soil type, soil moisture, and location. Ten districts were covered in this survey; out of ten districts, six districts, namely Chamba, Mandi, Kullu, Kangra, Shimla and Una are infected with cysts nematodes (Fig. 13).

Globodera cysts were found in districts Mandi (Phulladhar farm), Shimla (Totu pani), Kullu (Chowaii), Chamba (Ahla farm), and Kangra (Jia) from 2018 to 2021(Tables 4,5 and 6). *Cactodera* cysts were found in the soil samples collected from district Mandi (Phulladhar farm), Shimla (Khadrala), and Chamba (Ahla farm) from 2018 to 2021 (Tables 7,8,9 and 10). *Heterodera* cysts were found in district Una during the survey from 2018 to 2021 (Tables 11,12 and 13).

Globodera cysts were found in the soil samples of district Mandi (Phulladhar, Barot) minimum of 48 cysts to a maximum of 55 cysts. From the soil samples of district Chamba (Ahla farm, KVK Saru) minimum of 2 cysts to a maximum of 54 cysts. From district Kullu's (Chowaii) soil samples, *Globodera* cysts were found in at least 38 cysts to a maximum of 50 cysts. From the soil samples of district Shimla (Totu Pani, Khadrala), a minimum 5cysts to a maximum of 19 cysts. From the soil samples of district Kangra (Jiya, Pathiar) minimum 2cysts to maximum 42cysts per 250 gm soil Table (7,8,9 and 10). During this survey more cyst population was obtained at pre-harvest stage of crops than post harvest.

Table 21: Cyst nematodes report from various host plants in different states of India.

S.No	Nematode	Host plant	State	Reference
1.	*Globodera rostochiensis* and *Globodera pallida*	Potato	Himachal Pradesh	Ganguly *et al*. 2010
2.	*Globodera* spp.	Potato	Himachal Pradesh, Jammu and Kashmir and Uttarakhand.	Chandel *et al*., 2020
3.	*Globodera* spp.	Potato	Tamil Nadu (Kodaikanal hills).	Seenivasan *et al.*, 2017
4.	*G. pallida*	Potato	Kerala	Ramana *et al*. (1988)
5.	*C. estonica*	Potato	Tamil Nadu Nilgiri Hills	Saranya *et al*. (2018)
6.	*H. aveane*	Wheat	Himachal Pradesh, Punjab, Haryana, Uttar Pradesh, Delhi and Jammu & Kashmir	Swarup *et al*. (1982)
7.	*H. avenae*	Wheat	Rajasthan, Punjab, Haryana and Himachal Pradesh.	Koshy and Swarup, (1971)
8.	*Heterodera* spp.	Wheat	Himachal Pradesh, Leh and Delhi.	Devindrappa and Gowda, (2020)

Ganguly *et al*. (2010) reported two species of *Globodera,* namely *Globodera rostochiensis* and *Globodera pallida,* from potato breeding Farm ICAR-Central Potato Research Institute (CPRI) Shimla, Himachal Pradesh. PCN is a severe threat to the domestic and international trade of potatoes. CPRI-Shimla is a well-known institution for developing Potato varieties, germplasm and hybrid. Chandel *et al*. (2020) discussed PCN distribution in three hilly regions of North India, i.e., Himachal Pradesh, Jammu and Kashmir and Uttarakhand. According to Chandel *et al*. (2020), among 11 surveyed districts, PCN presence was positive in 9 districts, i.e., Shimla, Sirmour, Mandi, Chamba, Kullu, Una, Kangra and Lahaul & Spiti. Chandel *et al*. (2020) agreed with our survey

findings; we collected soil samples from 10 districts during the survey. Out of 10 districts, 5 districts showed the presence of PCN, i.e., Shimla, Mandi, Kullu, Kangra, and Chamba.

Among potato cyst nematodes, *G. rostochiensis* is most likely to occur in potato fields in India than Ganguly *et al*. (2010) observed the distribution of *Globodera* spp. in the district Shimla Himachal Pradesh. Chandel *et al*. (2020) discussed the distribution of *Globodera spp*. in Himachal Pradesh, Uttarakhand, Jammu & Kashmir (Table 21). Seenivasan *et al.* (2017) observed the status of *Globodera* spp. on potatoes in Tamil Nadu (Kodaikanal hills). Soil samples were collected from the 75 different potato fields, and their results revealed that 36% of potato fields were infested with *G. rostochiensis* and *G. pallida*. The potato fields of Kodaikanal hills were infected with a mixed population of *G. rostochiensis* and *G. pallida.* Their field study revealed that due to the natural infestation of *Globodera* spp. 33% of potato tuber yield loss was observed. Ramana *et al*. (1988) discussed the occurrence of *G. pallida* in Kerala. Devrajan *et al*. (2011) stated that up to 80% of potato yield loss was found in Nilgiri Hills, Tamil Nadu, India. The spread and infestation of *Globodera spp*. is worldwide. Hafez *et al*. (2007) firstly reported the occurrence of *G. pallida* in the United States. Madani *et al*. (2010) demonstrated about infestation of *G. rostochiensis* and *G. pallida* in Canada. The amount of yield loss due to *Globodera* spp. varies from several factors such as nematode density and nutrient status of soil, and soil type (Elston *et al*., 1991). Hence it is proved that the population density of cyst's nematodes also prevailed due to the influence of environmental factors.

Cactodera cysts were found in the soil samples of district Mandi (Phulladhar), minimum of 18 cysts to a maximum of 20 cysts. From district Chamba (Ahla farm), a minimum of 20 cysts to a maximum of 22 *Cactodera* cysts were collected from soil samples. From the district, Shimla (Khadrala) minimum 7cysts to a maximum of 17cysts per 250g of *Cactodera* cysts were collected from soil samples mentioned in Tables 7,8,9 and 10. *C. estonica* was first reported from Nilgiri Hills, India, by Saranya *et al*. (2018), who discussed that *Cactodera* is found in Polygonaceae. Kaushal *et al*. (2007) examined the report of three species of *Cactodera*, namely *C. Cacti*, *C. Chaubattia* and *C. jahanseni* from India. *C. cacti* and *C. Chaubattia* was reported from the hills of Uttar Pradesh. Our

study is agreed with the report of all the scientists mentioned above. The present study firstly reported *C. estonica* from Himachal Pradesh agreed with the report of Saranya *et al*. (2018). These reports discussed that *C. estonica* hosts range belongs to the family Polygonaceae. *Cactodera* spp. is distributed worldwide. Duan, (2012) firstly recorded *Cactodera cacti* from Northern China from the cactus plant. *C. cacti* recorded in Kowa parasitized *Zygocactus truncatus* and *Hylocerers trigonus*. This infection leads to the yellowing of stems, late-blooming, retarded growth and wilting (Cho *et al*., 1995). Yu and Sun, (2017) reported *C. estonica* from Canada. Mature female cyst and second-stage juveniles are recovered from the rhizosphere of *Polygonum avicular*. *C. chenopodiae* is recorded from the host plant Lambs quarter, *Chenopodium album* in Liaoning, China (Feng *et al*., 2018). *C. galinsogae* was recorded in Hidalgo, Mexico, as a parasite in the roots of barley, wheat, maize, and some weeds growing in the barley field (Tovar *et al*., 2003; 2007; 2008). Saranya *et al*. (2018) supported our study, revealing the initial prevalence of *C. estonica* in India.

Heterodera cysts were found in the soil sample of district Una (Akrot farm, Jhalera) with a minimum of 0 cysts to a maximum of 15 cysts per 250g soil (Table 11, 12 and 13). *Heterodera avenae* (CCN) was reported by Vasudeva, (1958) from Rajasthan on the roots of the wheat plant. Swarup *et al*. (1982) reported *H. aveane* from Himachal Pradesh, Punjab, Haryana, Uttar Pradesh, Delhi and Jammu & Kashmir. Koshy and Swarup, (1971) discussed the distribution of *H. avenae* in Rajasthan, Punjab, Haryana and Himachal Pradesh. In Syria and Turkey, *H. avenae* infested wheat fields greatly in Syria and Turkey Abidou *et al*. (2005) discussed that 69.9% of wheat fields were infested by *H. avenae* in Syria, and 80% of wheat fields were infested *H. avenae* in Turkey. Ahmadi and Maafi, (2014) studied the incidence of *H. avenae* and *H. filipjevi* in Iran. Their results stated that from 2008 to 2011, *H. avenae* and *H. filipjevi* were widely distributed in wheat and barley fields of Iran. Out of 200 collected samples from the field, 37% and 35% of *H. avenae* and *H. filipjevi* were isolated. *H. avenae* is commonly known as cereal cyst nematode widespread in India, mainly in wheat-growing areas, reported from many states, namely

Rajasthan, Jammu and Kashmir, Himachal Pradesh, Punjab, Haryana, Gujarat, Delhi, Uttar Pradesh, Madhya Pradesh, and Uttarakhand discussed by Kaushal *et al*. (2007)

This survey results stated the percentage of occurrence of *H. avenae* was 100% in Akrot farm and 75% in Jhalera. In contrast, the population density of *H. avenae* was 11.75 on Akrot farm and 2.5 on Jhalera farm from district Una. Therefore, the population density of *H. avenae* was highest at Akrot farm compared to Jhalera. The percentage of occurrence of *C. estonica* was 100 % in all three districts, namely district Mandi (Phulladhar farm), district Shimla (Khadrala) and district Chamba (Ahla farm); in contrast, to 19, 16 and 22.25 in districts Mandi, Shimla and Chamba. The population density was highest at Ahla Farm district Chamba compared to districts Mandi and Shimla (Tables 13, 14 and 15); in contrast, to 50.20 and 43.25 in district Mandi (Phulladhar and Barot), 17 in Shimla (Totu pani), 45.8 in Kullu (Chowaii), 52 and 3 in Chamba (Ahla and KVK Saru), 3 and 38.8 in Kangra (Pathiar and Jia). The population density of *G. rostochiensis* was highest in Ahla district Chamba and Phulladhar farm district Mandi compared to district Kangra and Shimla. Survey was carried out in Himachal Pradesh. The survey was undertaken from November 2018 to December 2021 to give a small contribution to filling the research gap.

The present study reveals the occurrence of three genera of cysts nematodes, i.e., *Globodera*, *Cactodera* and *Heterodera*, in the agricultural field of Himachal Pradesh. *Globodera* spp. specialized in Solanaceae, *Cactodera* spp. in Polygonaceae and *Heterodera* spp. in Poaceae (Himachal Pradesh). This survey study states that among three genera, namely *Cactodera*, *Globodera* and *Heterodera*. The highest population were found in the *Globodera* than *Cactodera* and *Heterodera* in Himachal Pradesh.

This survey's findings follow the investigations of earlier researchers who showed the spread of various cysts' nematodes all over India and different other countries. Genus *Cactodera* is the major pest of the Polygonaceae family. Kaushal *et al*. (2007) discussed cysts' nematode distribution in India. They stated that genus *Heterodera*, *Globodera* and *Cactodera* are reported in India. *H. avenae* was reported from Rajasthan, Jammu & Kashmir, Himachal Pradesh, Punjab, Haryana, Gujarat, Delhi and Himachal Pradesh. *C.*

cacti were reported from Uttar Pradesh, and *C. johanseni* from Uttarakhand at the same time, *G. rostochiensis* from Tamil Nadu, Karnataka and Kerela. This study indicates the distribution of cyst nematodes, namely genus *Heterodera*, *Globodera* and *Cactodera*, associated with different crops in different districts of Himachal Pradesh.

6.2. Morphological characterization

The morphological characters and morphometrics were studied for the morphological characterization of cyst nematodes, and the perineal portion was examined under the microscope. Morphological characters include cyst colour, shape and type of fenestra present in the cone top, and morphometrical studies have cyst length, neck length, length/width ratio, fenestra length width and distance from the anus to the fenestra. These are the essential characteristics of cysts for the morphological characterization of cyst nematodes. Therefore, vulval cone studies or perineal pattern of cyst is the main characteristic to distinguish cyst nematodes.

6.2.1. Morphological characteristics of genus *Heterodera*

The morphological studies of cysts isolated from the wheat field of district Una, Himachal Pradesh (HPWHUN) reveal the presence of *Heterodera*. The cyst colour, shape, length without neck, neck length, width, length/width ratio, fenestral length, width, bridge width, vulval slit length, and distance from the anus to a fenestra were recorded for morphological characterization. Lemon-shaped cysts with protruding neck and vulval cone were observed. All cysts were dark brown to moderate brown shown in Fig 14(a). Cysts were examined through cone top mounting. Bifenestrate cone tops were observed under a light microscope, Fig 14(b). The morphological and morphometrical characters were studied; cysts length, width, fenestral length, fenestral width, vulval bridge width, vulval slit length and distance from the anus to fenestra were measured. Cysts length without neck was 701.7 ± 23.22 µm, neck length was 79.3 ± 8.43 µm, and width was 527.7 ± 41.79 µm, respectively. The fenestral length was 49.6 ± 2.66 µm, fenestral cone width was 21.8 ± 3.67 µm, vulval bridge width was 6.5 ± 0.58 µm, and vulval slit

length was 6.3 ± 0.70 µm presented in Table 22. Zigzag patterns were also observed on the cyst body cysts and circular cuticular ridges continued after fenestra. Soft underbridge was seen in some populations. After puncturing the cyst, J-1 enclosed inside the eggs were released. Based on these morphological characteristics, the collected population were belonged to genus *Heterodera.*

Table 22: Morphometrics of cyst and cone top structure (mean in µm, ± standard deviation, range) of *Heterodera* population from Himachal Pradesh.

S. No	**Characters**	**Measurements (N=10)**
1.	Length without neck	701.7 ± 23.22 (650-728)
2.	Neck length	79.3 ± 8.43 (62-90)
3.	Width	527.7 ± 41.79 (448-589)
4.	Fenestral length	49.6 ± 2.66 (43.1-52.2)
5.	Fenestral width	21.8 ± 3.67 (15.1-26)
6.	Vulval bridge width	6.5 ± 0.58 (5.4-7.2)
7.	Vulval slit length	6.3 ± 0.70 (7.3-5.1)

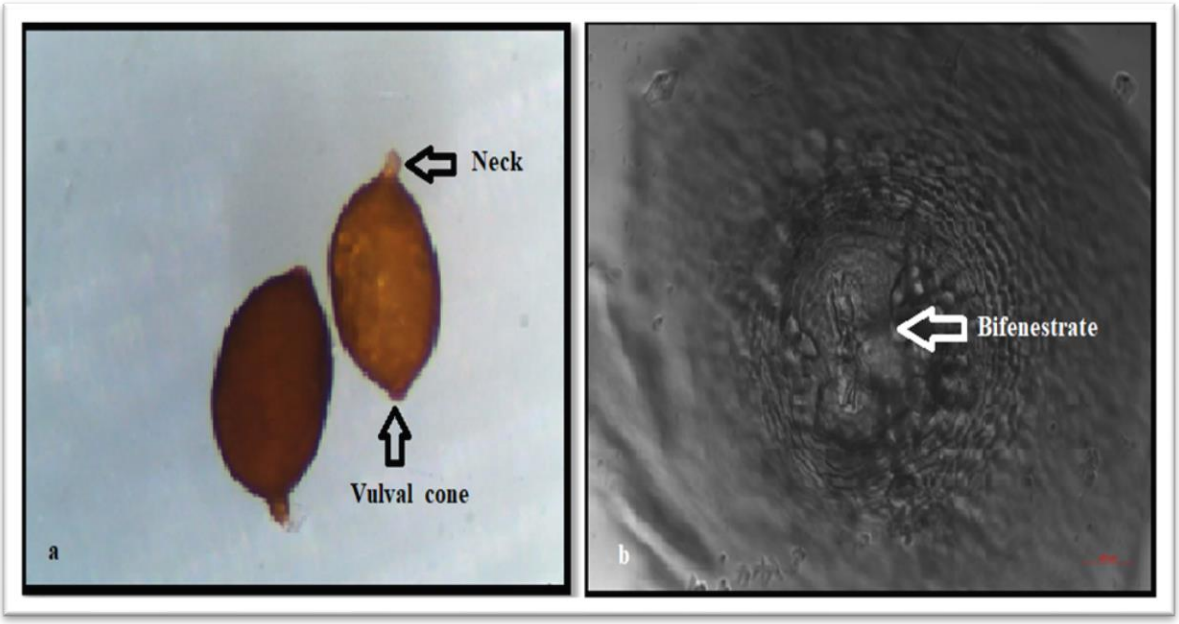

Figure14: Morphological characters of genus *Heterodera* **a)** Matured *Heterodera* cysts with distinct neck and vulval cone. **b)** Bifenestrate vulval cone top of *Heterodera* (20 µm scale bar).

6.2.2. Morphological characteristics of genus *Cactodera*

6.2.2.1. The morphological studies of cysts isolated from the potato field of district Mandi, Himachal Pradesh (HPMD) reveal the presence of *Cactodera*. Collected cysts were subjected to morphological characterization, and morphometrical studies of cyst colour, shape, body length without neck, neck length, width, length to width ratio and vulval cone were studied. Cysts were lemon-shaped and dark brown. Distinct neck and vulval cone were observed under stereomicroscope shown in Fig. 15(a). In addition, wavy or zig-zag patterns of cuticular ridges were seen on the middle part of the cyst wall. Cysts' body length without a neck (N=10) was 556.93 ± 53.12 µm, neck length was 93.59± 3.71 µm, body width was 435.45± 51.05 µm, Length to width ratio was 1.21± 0.11 µm (Table 23). Cysts were examined through cone tope mounting, a typical circumfenestrate and abullate vulval cone were observed under a light microscope, shown Fig.15 (b). No denticles were observed. Vulval cone fenestra diameter measured as 42.77 ± 4.31 µm.

Along with circum-fenestration circular ridges continued over the body in zigzag patterns. After puncturing the cyst, hundreds of eggs were released. Cylindrical shaped eggs enclosing the J-1 stage were seen. In addition, eggs were covered with small protuberances. Based on these morphological characteristics, the collected population belonged to the genus *Cactodera.*

Table 23: Morphometrics of cyst and cone top structure (mean in µm, ± standard deviation, range) of *Cactodera* population from district Mandi, Himachal Pradesh.

S. No	Characters	Measurements (N=10)
1.	Length without neck	556.93 ± 53.12 (466.83 -635.25)
2.	Neck length	93.59± 3.71 (88.55-99.82)
3.	Width	435.45± 51.05 (350.91-515.10)
4.	Length to width ratio	1.21± 0.11 (1.1-1.4)
5.	Vulval cone diameter	42.77 ± 4.31 (39.10-52.63)

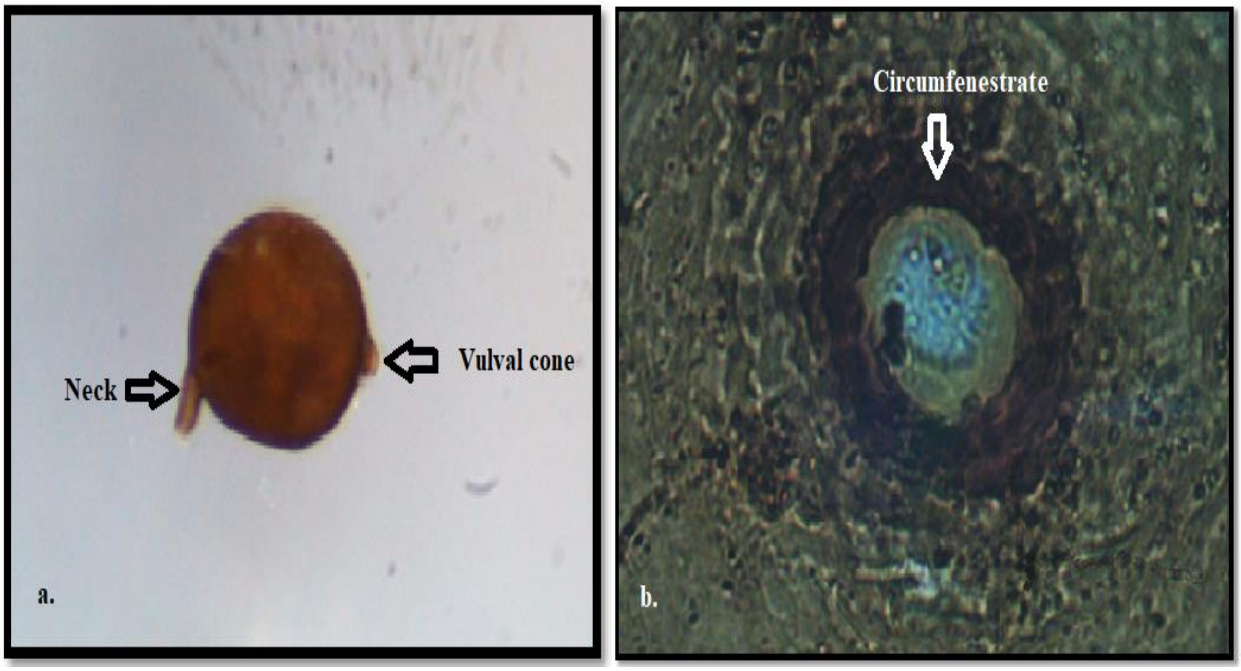

Figure 15: Morphological characters of genus *Cactodera* from district Mandi **a)** Matured *Cactodera* cysts with distinct neck and vulval cone. **b)** Circumfenestrate vulval cone top of *Cactodera.* (20 µm scale bar).

6.2.2.2. The morphological studies of cysts isolated from the potato field of district Chamba, Himachal Pradesh (HPCH) reveal the presence of *Cactodera.* Collected cysts were subjected to morphological, and morphometrical studies; cyst colour, shape, body length without neck, neck length, width, length to width ratio and vulval cone were measured. Cysts were morphologically characterized by colour ranges from dark brown to light brown, as shown in Fig. 16(a). Lemon shaped cysts were observed with distinct neck and vulva under the stereomicroscope. Zig-zag cuticular ridges were seen on the middle part of the body of the cyst. According to cysts morphometrics presented in Table 24, cyst length without neck measured 549.46± 65.42 µm, and neck length measured 94.49± 4.21 µm, width measured 437.05±61.11 µm and length to width ratio was 1.21± 0.11. cysts were undergone cone top examination under a 40X lens compound microscope. Cysts were examined through cone tope mounting, Abullate and circum-fenestrate cone top were present, shown in Fig. 16(b). Circum-fenestration outwards leads to a circular ridges pattern. Vulval cone diameter were 47.7 ± 4.33 µm. Eggs were present inside the cysts and were covered with small protuberances. Based on these morphological characteristics, the collected population belongs to the genus *Cactodera.* Compared with the sample collected from district Mandi, district Chamba had slightly larger cysts.

Table 24: Morphometrics of cyst and cone top structure (mean in µm, ± standard deviation, range) of *Cactodera* population from district Chamba, Himachal Pradesh.

S. No	Characters	Measurements (N=10)
1.	Length without neck	549.46± 65.42 (468.1-650.4)
2.	Neck length	94.49± 4.21 (88.9-101.2)
3.	Width	437.05±61.11 (361.9-525.3)
4.	Length to width ratio	1.2 ± 0.031 (1.2-1.3)
5.	Vulval cone diameter	47.7 ± 4.33 (39.9-52.7)

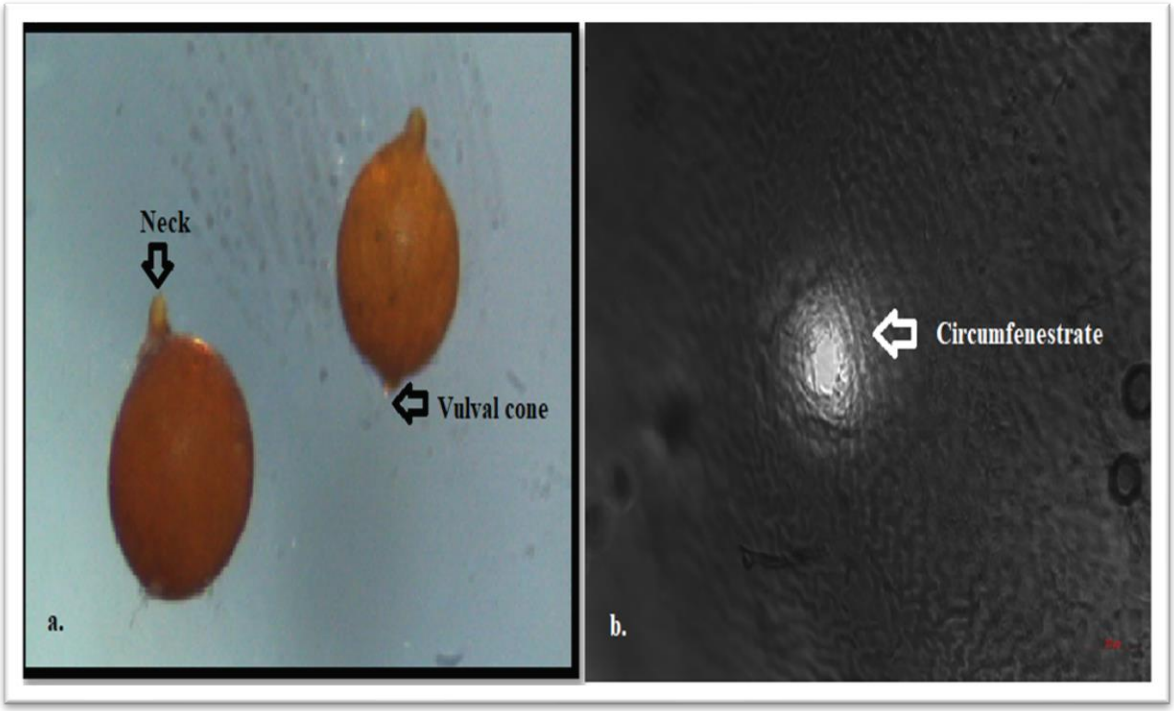

Figure 16: Morphological characters of genus *Cactodera* from district Chamba a) Matured *Cactodera* cysts with distinct neck and vulval cone. b) Circumfenestrate vulval cone top of *Cactodera.* (20 µm scale bar).

6.2.2.3. The morphological studies of cysts isolated from the potato field of district Shimla, Himachal Pradesh (HPSH) reveal the presence of *Cactodera*. Collected cysts were subjected to morphological and morphometrical studies; cyst colour, shape, body length without neck, neck length, width, length to width ratio and vulval cone were measured. Cysts were morphologically characterized by colour ranges from dark brown to light brown. Lemon shaped cysts were observed with distinct neck and vulva under a stereo microscope, shown in Fig. 17(a). On the middle part of the body of the cyst zig-zag, cuticular ridges were seen. According to cysts morphometrics, cyst length without neck measured 560.09 ± 65.92 µm, neck length measured 95 ± 4.45 µm, width measured 446.65 ± 54.30 µm, and length to width ratio was 1.2 ± 0.031 (Table 25). cysts were undergone cone top examination under a 40X lens compound microscope. Cysts were examined through cone tope mounting. Abullate and circumfenestrate cone top were present in Fig. 17(b). Circumfenestration outwards leads to a circular ridges pattern. Vulval cone diameter were 45.68 ± 5.66 µm. Eggs were present inside the cysts and were covered with small protuberances. Based on these morphological characteristics, the collected population belongs to the genus *Cactodera*. Compared with the sample collected from district Mandi and district Chamba, the average length and width of district Shimla cysts were slightly larger. Overall *Cactodera* cysts from districts Mandi, Chamba, and Shimla were similar in shape and colour only the measurements in size slightly differ from each other. Cyst cone top of all samples were similar i.e., circumfenestrate and abullate.

Table 25: Morphometrics of cyst and cone top structure (mean in µm, ± standard deviation, range) of *Cactodera* population from district Shimla, Himachal Pradesh.

S. No	Characters	Measurements (N=10)
1.	Length without neck	560.09 ± 65.92 (464.4- 641.7)
2.	Neck length	95 ± 4.45 (89.4 – 101.1)
3.	Width	446.65 ± 54.30 (352.9- 509.6)
4.	Length to width ratio	1.2 ± 0.11 (1.1-1.4)
5.	Vulval cone diameter	45.68 ± 5.66 (38.4- 52.5)

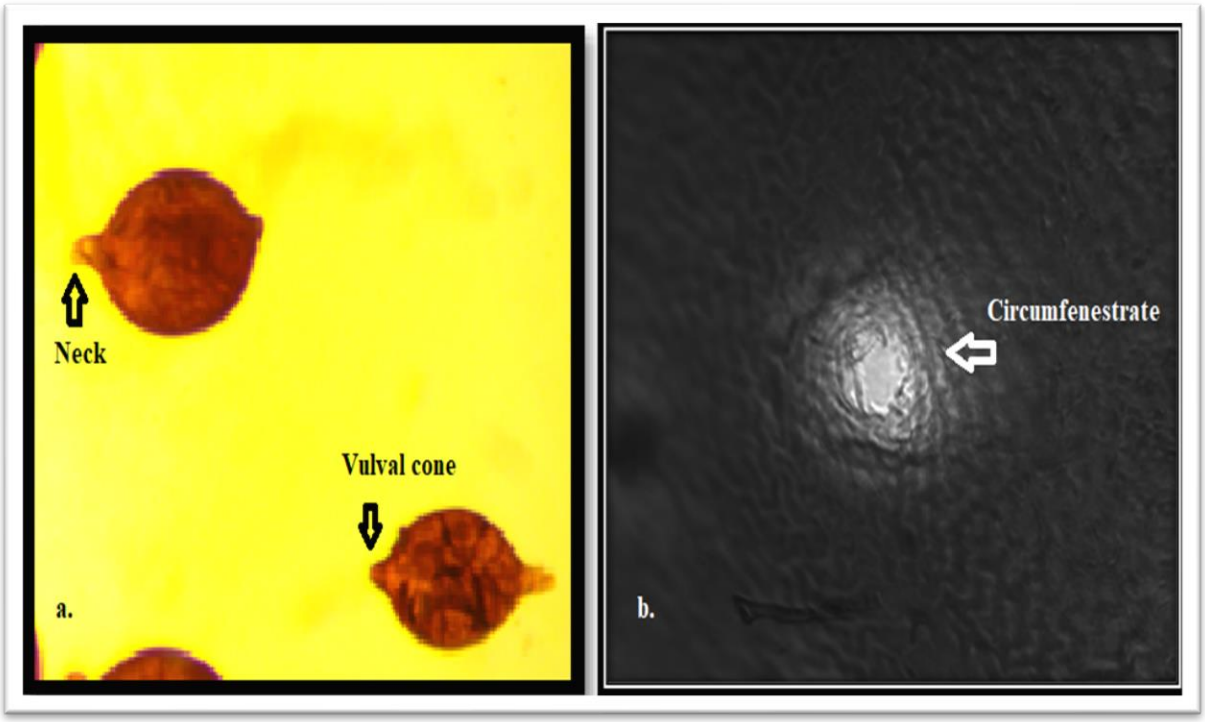

Figure 17: Morphological characters of genus *Cactodera* from district Shimla **a)** Matured *Cactodera* cysts with distinct neck and vulval cone. **b)** Circumfenestrate vulval cone top of *Cactodera.* (20 µm scale bar).

6.2.3. Morphological characters of genus *Globodera*:

Soil samples were collected from potato breeding farms and potato cultivated agricultural lands from various districts, namely district Kangra (Palampur region) named as HPPOPL, district Mandi (Phulladhar region) named as HPPOMD, district Kullu (Chowaii region) named as HPPOKL, district Chamba (Alha farm) named as HPPOCH, district Shimla (Totu pani area) called as HPPOSH.

6.2.3.1 The morphological studies of cysts isolated from the potato field of district Kangra, Palampur region Himachal Pradesh (HPPOPL) reveal the presence of *Globodera*. Collected cysts were subjected to morphological and morphometrical studies cyst colour, shape, body length without neck, neck length, width, distance from vulval basin to anus and number of cuticular ridges present between the vulval basin and anus were measured. Undergone for morphometrical studies. Cysts were morphologically characterized by having pale brown or golden brown in colour, sub-spherical or globose in shape, with protruding necks shown in Fig. 18 (a). A reticulate pattern was present on the cuticle. Our results shown in Table 26 indicates that cysts length without neck was 609.8±63.06 with ranges 500.1-679.1, neck length was 143.93 ± 29.16 ranges 98.7-188.7, and body width was 505.16 ± 67.87 ranges 391.1-598. Cysts were examined through cone top studies, and single circum-fenestrate vulval regions were present. The anus was seen at varying distances outside the vulval basin. Distance from vulval basin to anus was 65.71 ± 10.08 ranges 42.3-79.1, and the number of cuticular ridges present between vulval cone to anus was 19.9 ± 2.96 ranges 14-23 shown in Fig. 18 (b). Based on these morphological characteristics, the collected population belongs to the genus *Globodera.*

Table 26: Morphometrics of cyst and cone top structure (mean in µm, ± standard deviation, range) of *Globodera* population from district Kangra, Himachal Pradesh.

S. No	Characters	Measurements (N=10)
1.	Body length without neck	609.8±63.06 (500.1-679.1)
2.	Neck length	143.93 ± 29.16 (98.7-188.7)
3.	Body width	505.16 ± 67.87 (391.1-598)
4.	Distance from vulval basin to anus	65.71 ± 10.08 (42.3-79.1)
5.	No. of cuticular ridges present between vulval cone to anus	19.9 ± 2.96 (14-23)

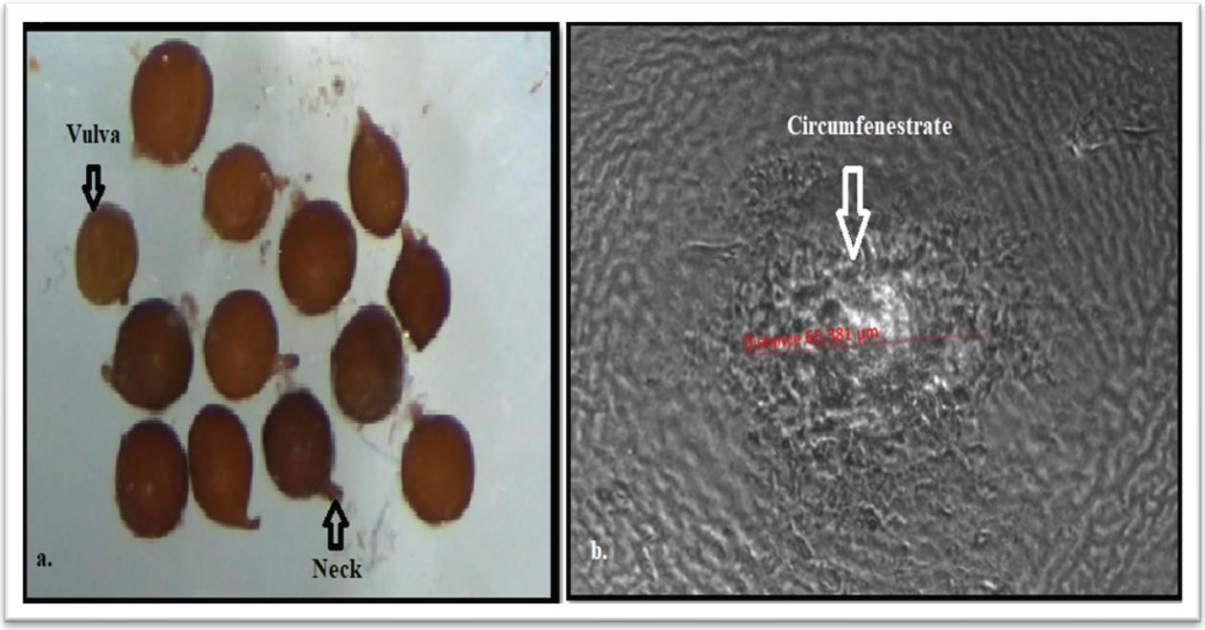

Figure 18: Morphological characters of genus *Globodera* from district Kangra **a)** matured globose *Globodera* cysts with distinct neck. **b)** Circumfenestrate vulval cone top of *Globodera* and V shaped anus. (20 µm scale bar).

6.2.3.2. The morphological studies of cysts isolated from the potato field of district Mandi, Phulladhar region Himachal Pradesh (HPPOMD) reveal the presence of *Globodera*. Collected cysts were subjected to morphological and morphometrical studies; cyst colour, shape, body length without neck, neck length, width, distance from vulval basin to anus and number of cuticular ridges present between the vulval basin and anus were measured. Cysts were morphologically characterized by having pale brown or golden brown in colour, Sub-spherical or globose in shape with protruding necks shown in Fig. 19(a). Reticulate pattern present on the cuticle. Our results shown in Table 27 indicates that cysts length without neck was 597.86 ± 77.97, ranges 467.1-671.9, neck length was 141.39 ± 39.20 ranges 84.1-196.8, and body width was 502.54 ± 68.26 ranges 387.2-589.7. Cysts were examined through cone top studies, and a single circum-fenestrate vulval region was presently shown in Fig. 19(b). The anus was seen at varying distances outside the vulval basin. The distance from the vulval basin to the anus was 62.94 ± 13.47, with ranges 39.2-79.3. The cuticular ridges between the vulval cone and anus were 19.2 ± 3.08, 14-24. Based on these morphological characteristics, the collected population belongs to the genus *Globodera*. Compared with the *Globodera* population of district Kangra, both the population morphometrics showed almost similar morphometrics.

Table 27: Morphometrics of cyst and cone top structure (mean in µm, ± standard deviation, range) of *Globodera* population from district Mandi, Himachal Pradesh.

S.NO	Characters	Measurements (N=10)
1.	Body length without neck	597.86 ± 77.97 (467.1-671.9)
2.	Neck length	141.39 ± 39.20 (84.1-196.8)
3.	Body width	502.54 ± 68.26 (387.2-589.7)
4.	Distance from vulval basin to anus	62.94 ± 13.47 (39.2-79.3)
5.	No. of cuticular ridges present between vulval cone to anus	19.2 ± 3.08 (14-24)

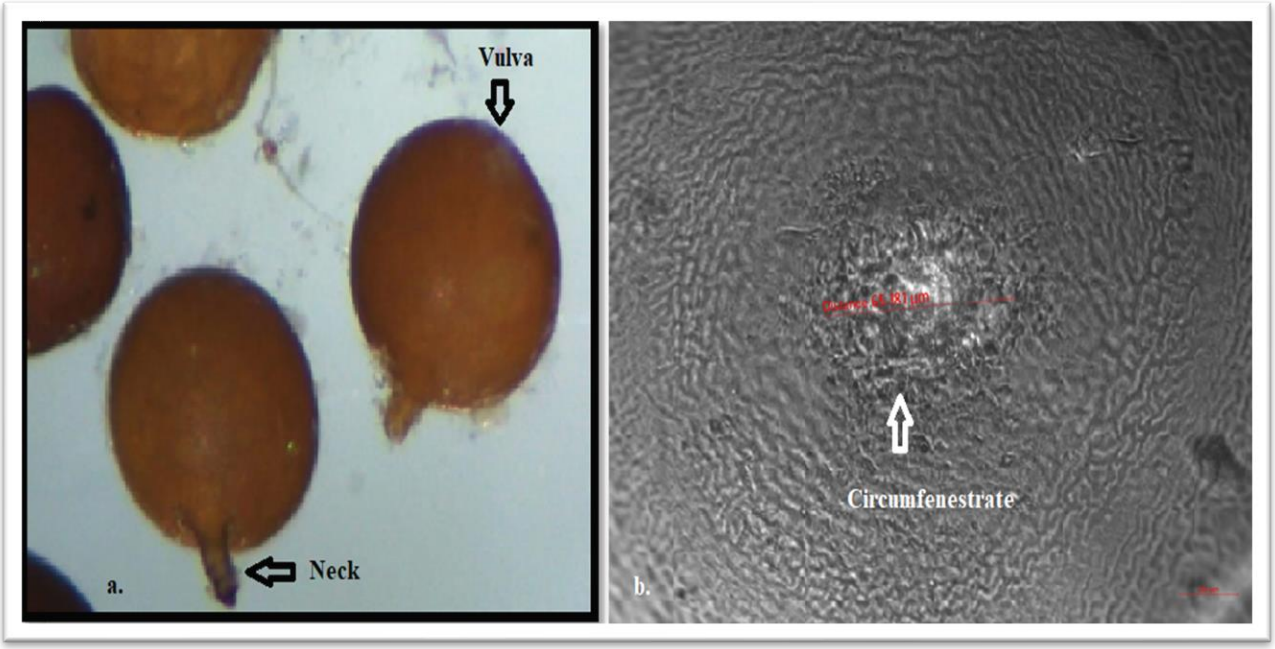

Figure 19: Morphological characters of genus *Globodera* from district Mandi **a)** matured globose *Globodera* cysts with distinct neck. **b)** Circumfenestrate vulval cone top of *Globodera* and V shaped anus. (20 µm scale bar).

6.2.3.3 The morphological studies of cysts isolated from the potato field of district Kullu, Himachal Pradesh (HPPOKL) reveal the presence of *Globodera*. Collected cysts were subjected to morphological, and morphometrical studies; cyst colour, shape, body length without neck, neck length, width, distance from vulval basin to anus and number of cuticular ridges present between the vulval basin and anus were measured. Cysts were morphologically characterized by having pale brown or golden brown in colour, sub-spherical or globose in shape, with protruding necks shown in Fig.20(a). A reticulate pattern was present on the cuticle. Our results shown in Table 28 indicate that cysts length without neck were 591.94 ± 79.42, 463.2-680.3, neck length was 132.8 ± 40.62 ranges 81.9-190.1 body width was 496.27 ± 86.92 range 356.7 – 590.2. Cysts were subjected to cone top studies, and a single circum-fenestrate vulval region was presently shown in Fig. 20(b). The anus was seen at varying distances outside the vulval basin. The distance from the vulval basin to the anus was 62.71 ± 13.53 ranges 39.8-to 78.6. The cuticular ridges present between the vulval cone and anus were 19.3 ± 3.23, 14-25. Based on these morphological characteristics, the collected population belongs to the genus *Globodera.* In comparison with district Kangra and district Mandi, the district showed more cuticular ridges, i.e., 14-25.

Table 28: Morphometrics of cyst and cone top structure (mean in µm, ± standard deviation, range) of *Globodera* population from district Kullu, Himachal Pradesh.

S.NO	Characters	Measurements (N=10)
1.	Body length without neck	591.94 ± 79.42 (463.2-680.3)
2.	Neck length	132.8 ± 40.62 (81.9-190.1)
3.	Body width	496.27 ± 86.92 (356.7 – 590.2)
4.	Distance from vulval basin to anus	62.71 ± 13.53 (39.8-78.6)
5.	No. of cuticular ridges present between vulval cone to anus	19.3 ± 3.23 (14-25)

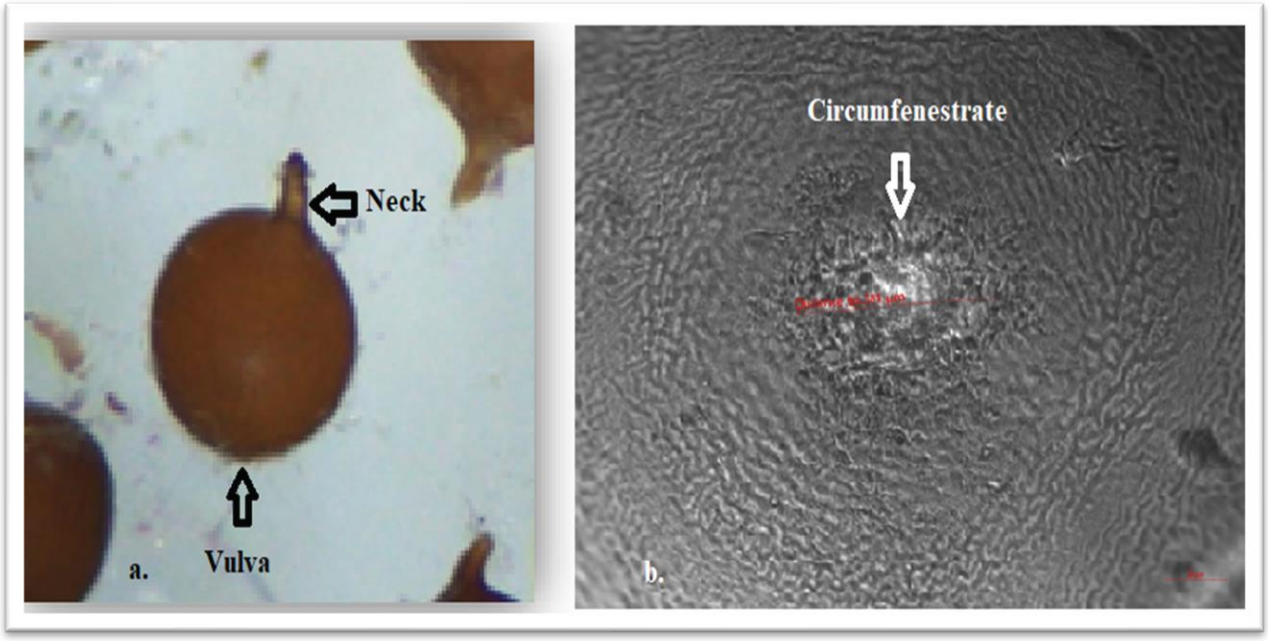

Figure 20: Morphological characters of genus *Globodera* from district Kullu **a)** Matured globose *Globodera* cysts with distinct neck. **b)** Circumfenestrate vulval cone top of *Globodera* and V shaped anus. (20 µm scale bar).

6.2.3.4 The morphological studies of cysts isolated from the potato field of district Chamba in Ahla region Himachal Pradesh (HPPOCH) reveal the presence of *Globodera*. Collected cysts were subjected to morphological, and morphometrical studies cyst colour, shape, body length without neck, neck length, width, distance from vulval basin to anus and number of cuticular ridges present between the vulval basin and anus were measured. Cysts were morphologically characterized by having pale brown or golden brown. The shape of sub-spherical or globose with protruding neck is shown in Fig. 21(a). Reticulate pattern present on the cuticle. Our results shown in Table 29 indicates that cysts length without neck were 615.82 ± 72.49 ranges 476.9 -679.5, neck length were 127.74 ± 30.20 ranges 80.2-175.9 and body width was 517.07 ± 62.29 ranges 376.1-578.5. Cysts were examined through cone top mounting; a single circum-fenestrate vulval region was present, shown in Fig. 21(b). The anus was seen at varying distances outside the vulval basin. The distance from the vulval basin to the anus was 62.6 ± 13.04 ranges 38.9-78.1. The number of cuticular ridges present between the vulval cone and anus was 20 ± 2.7 ranging from 15-24. Based on these morphological characteristics, the collected population belongs to the genus *Globodera*. Morphometrical studies of district Chamba showed similarities with district Kangra, Mandi and Kullu.

Table 29: Morphometrics of cyst and cone top structure (mean in µm, ± standard deviation, range) of *Globodera* population from district Chamba, Himachal Pradesh.

S. No	Characters	Measurements (N=10)
1.	Body length without neck	615.82 ± 72.49 (476.9 -679.5)
2.	Neck length	127.74 ± 30.20 (80.2-175.9)
3.	Body width	517.07 ± 62.29 (376.1-578.5)
4.	Distance from vulval basin to anus	62.6 ± 13.04 (38.9-78.1)
5.	No. of cuticular ridges present between vulval cone to anus	20 ± 2.7 (15-24)

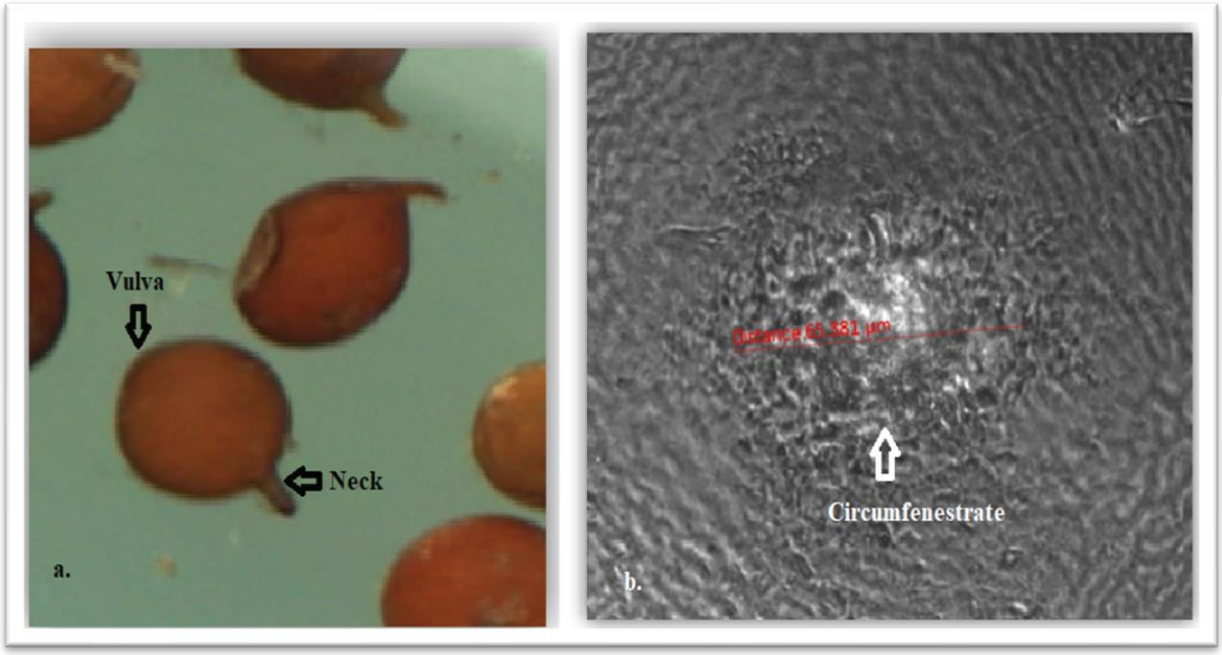

Figure 21: Morphological characters of genus *Globodera* from district Chamba **a)** matured globose *Globodera* cysts with distinct neck. **b)** Circumfenestrate vulval cone top of *Globodera* and V shaped anus (20 µm scale bar).

6.2.3.5. The morphological studies of cysts isolated from the potato field of district Shimla, Himachal Pradesh (HPPOSH) reveal the presence of *Globodera*. Collected cysts were subjected to morphological and morphometrical studies cyst colour, shape, body length without neck, neck length, width, distance from vulval basin to anus and number of cuticular ridges present between the vulval basin and anus were measured. Cysts were morphologically characterized by having pale brown or golden brown. The shape of sub-spherical or globose with protruding neck is shown in Fig. 22(a). Reticulate pattern present on the cuticle. Our results shown in Table 30 indicates that cysts length without neck was 603.52 ± 48.18 ranges 480.2-658, neck length was 147.26 ± 40.56 ranges 82.1- 201.9 and body width was 530.41 ± 71.10 ranges 355.1-592.4. Cysts were examined through cone top mounting; a single circum-fenestrate vulval region was present, shown in Fig.22(b). The anus was seen at varying distances outside the vulval basin. The distance from the vulval basin to the anus was 62.72 ± 9.97, range 38.1-76.9.1and number of cuticular ridges present between the vulval cone and anus was 20.8 ± 2.7, ranges 16-25. Based on these morphological characteristics, the collected population belongs to the genus *Globodera*. District Shimla morphometrics showed more similarity to district Kullu in both districts number of cuticular ridges were reached up to 25.

Table 30: Morphometrics of cyst and cone top structure (mean in µm, ± standard deviation, range) of *Globodera* population from district Shimla, Himachal Pradesh.

S.NO	Characters	Measurements (N=10)
1.	Body length without neck	603.52 ± 48.18 (480.2-658)
2.	Neck length	147.26 ± 40.56 (82.1- 201.9)
3.	Body width	530.41 ± 71.10 (355.1-592.4)
4.	Distance from vulval basin to anus	62.72 ± 9.97 (38.1-76.9)
5.	No. of cuticular ridges present between vulval cone to anus	20.8 ± 2.7 (16-25)

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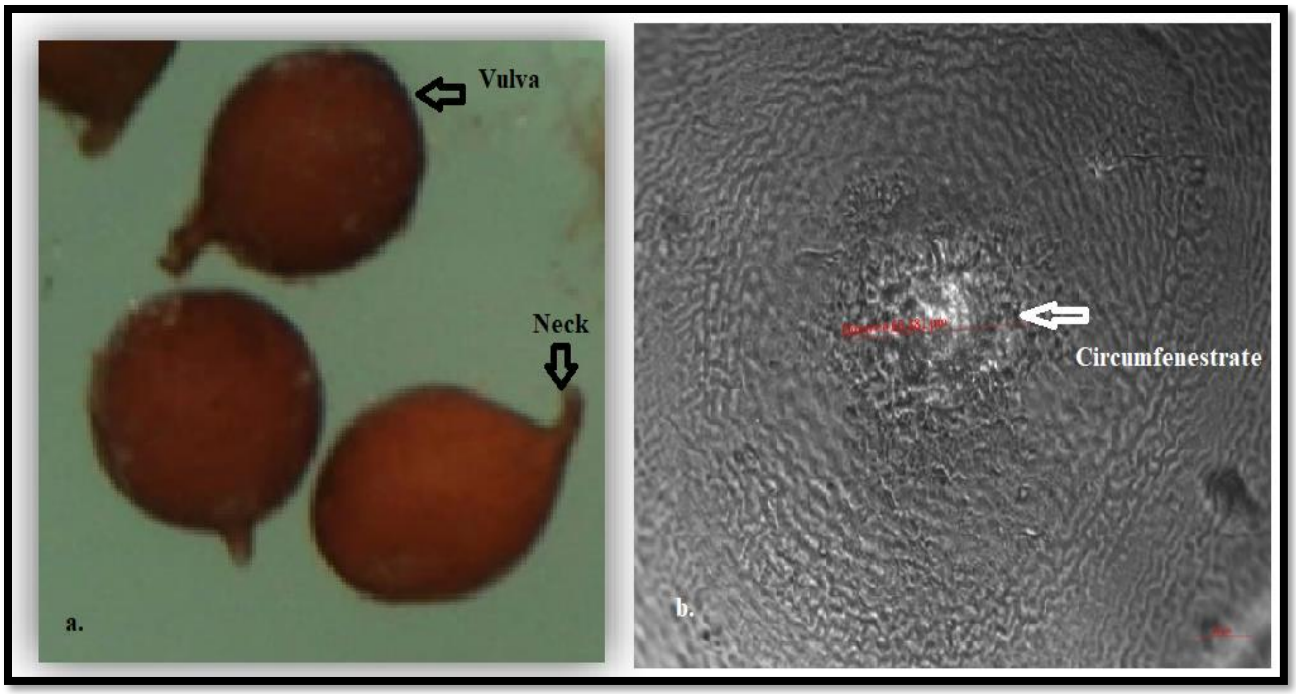

Figure 22: Morphological characters of genus *Globodera* from district Shimla **a)** Matured globose *Globodera* cysts with distinct neck. **b)** Circumfenestrate vulval cone top of *Globodera* and V shaped anus (20 µm scale bar).

Morphology and morphometrics of cysts *Heterodera, Globodera* and *Cactodera,* were studied. Soil samples were collected from different agricultural lands from district Kangra, Mandi, Kullu, Chamba, Shimla and Una Himachal Pradesh. Each genus was morphologically identified separately, and morphometrics was noted.

6.2.4. Morphology and morphometrics of *Heterodera* cysts

Cysts were lemon-shaped, light brown to dark brown. Heads were distinctly seen from the rest of the body. A prominent vulval cone was present in the whole population studied. Fenestral organization on the cone top were Bifenestrate. Two distinct fenestra was noticed with a soft underbridge. External cuticular patterns were also observed. The study of the vulval cone by cone top mounting is the principal structure used to identify cyst nematodes. Morphometric characteristics of the cyst population (mean n=10) were, cyst length without neck ranges from 650µm to 728µm, cyst width ranges from 448µm to 589µm, neck length ranges from 62µm to 90µm, fenestral length ranges from 43.1µm to 52.2µm, fenestral width ranges from 15.1µm to 26µm, vulval bridge width ranges from 5.4µm to 7.2µm, vulval slit length was 7.3µm to 5.1µm (Table 20). According to the morphological characteristics discussed above, the results revealed that the collected cysts population belongs to the genus *Heterodera*. The morphological and morphometrical study discussed above confirmed the occurrence of *Heterodera* in district Una.

Devindrappa and Gowda, (2020) discussed the morphometrics of *H. avenae* populations from Leh reveals that cyst length without neck was 669.70µm ± 34.75, neck length was 78.52µm ± 8.61, the width was 500.58µm ± 53.08, fenestral Length was 47.41µm ± 2.88, fenestral width was 21.33µm ± 3.81, vulval bridge width was 5.97µm ± 0.69, and vulval slit length was 6.0µm ± 0.71. Morphometrics of *H. avenae* Leh populations exposed a similarity with the present work. Devindrappa and Gowda, (2020) studied the morphometrics of *H. filipjevi* population from Himachal Pradesh reveals that cyst length was 742µm ± 38.81, neck length was 74.5µm ± 7.61, the width was 537µm ± 21.10, fenestral Length was 45.86µm ±

7.42, fenestral width was 21.43µm ± 3.97, vulval bridge width was 5.70µm ± 1.08, and vulval slit length was 7.50µm. If we compare the morphometrics of *H. filipjevi* populations from Himachal Pradesh reported by Devindrappa and Gowda, (2020) with the *H. avenae* population of Himachal Pradesh, morphometrics negligible difference was observed. The study conducted by Devindrappa and Gowda, (2020) supports the present study, revealing that the *H. avenae* population of Leh and Himachal Pradesh shows that the Leh population has comparatively slightly shorter cysts with almost similar neck length than Himachal Pradesh Populations. This study reveals that *H. avenae* population of Northern hilly region morphometrically showed similarity. *H. avenae* and *H. filipjevi* are both included in *H. avenae* group. The comparison revealed that *H. filipjevi* have slighter, longer and broader cysts than *H. avenae*. But the noticeable difference was not observed during the comparison. *H. avenae* populations isolated from the soil samples from Himachal Pradesh indicate that the morphometrics was within the range of *H. avenae* population present in Saudi Arabia and Czech Republic (Dawabah *et al*., 2012; Kumari, 2017). Bishnoi and Bajaj, (2004) carried out taxonomic studies of 8 populations of cereal cyst nematode *H. avenae* complex from Delhi, Haryana, Punjab, Himachal Pradesh and Rajasthan. In cysts characters, the major difference was observed in Rajasthan, Punjab and Himachal Pradesh. Mulvey, (1974) and Subbotin *et al*. (2010) have formed distinct vulval cone groups based on the structure of vulval cone. There are 12 species included in *Heterodera* "*avenae* group" all species are grouped based on the vulval cone (terminal cone) especially having a short vulval slit and bifenestration (Handoo, 2002). With the increasing number of *Heterodera* species, identification based on morphology alone becomes difficult. To separate genus *Heterodera* from *Globodera*, Wollenweber, (1923) studied and used vulval cone and cyst shape (lemon shape and spherical shape).

6.2.5. Morphology and morphometrics of *Cactodera* cysts

Cysts were recovered from the samples collected from the potato breeding agriculture farms, potato agriculture private fields, and other agricultural lands from three different districts, namely district Mandi (Phulladhar region) named as HPMD, district Chamba (Alha farm) named as HPCH and district Shimla (Khadrala) named as HPSH. Lemon-shaped cysts were noticed in each population, light brown to dark brown. The neck was distinctly seen from the rest of the body. Protruding vulval cone was observed. Fenestral organization on the cone top were circumfenestrate. Wavy or zig-zag patterns of cuticular ridges were seen on the cyst wall's middle part. Each population was morphologically and morphometrically studied separately. Cysts external characters of cysts, such as cyst colour, shape, and fenestration were similar in all four populations. The study of the vulval cone by cone top mounting is the principal structure used to identify cyst nematodes.

All four populations were followed by circumfenestration. Morphometrics for each population was calculated. Morphometrical characteristics of district Mandi (HPMD) (n=10) were, cysts length without neck ranges from 466.83µm to 635.25µm, neck length ranges from 88.55µm -99.82µm, body width ranges from 350.91µm to 515.10µm, Length to width ratio ranges 1.1µm to 1.4µm and vulval cone fenestra diameter ranges from 39.10µm to 52.63µm (Table 23). Morphometrical characteristics of district Chamba (HPCH) (n=10) were, cysts length without neck ranges from 468.1µm to 650.4µm, neck length ranges from 88.9µm to 101.2µm, body width ranges from 361.9µm to 525.3µm, Length to width ratio ranges from 1.2µm to 1.3µm and vulval cone-diameter ranges from 39.9µm to 52.7µm (Table 24). Morphometrical characteristics of district Shimla (HPSH) (n=10) were, cysts length without neck ranges from 464.4 to 641.7µm, neck length ranges from 89.4µm to 101.1µm, body width ranges from 352.9µm to 509.6µm, Length to width ratio ranges from 1.1µm to 1.4µm and vulval cone-diameter ranges from 38.4µm to 52.5µm (Table 25). A comparison of morphology and morphometrics of three populations of isolated cysts revealed that all three

populations had dark brown to light brown, lemon-shaped cysts. Distinct neck and vulva are present in each population. According to the morphological and morphometrical characteristics discussed above, the results revealed that collected cysts populations belong to the genus *Cactodera*. The morphological and morphometrical study discussed above confirmed the occurrence of *Cactodera* in district Mandi (Phulladhar region), district Chamba (Alha farm) and district Shimla (Khadrala). According to morphometrical studies, slightly longer and broader cysts were recovered from district Shimla compared to district Mandi and Chamba. But if we compare the range of length and width, all three district cysts were in the same range. The vulva was circumfenestrate maximum vulva diameter seen in district Chamba cysts (47.7 ± 4.33µm). The morphological and morphometrical studies indicate no such evident variations found in all three districts' cysts.

Saranya *et al*. (2018) discussed a morphometrical study of cysts reveals that body length without neck was (n=5) 550.45 ± 12µm, width 454.41 ± 9µm, neck length 97.76 ± 4.1µm, length-to-width ratio 1.4 ± 0.16µm, and vulval cone length 47.44 ± 3.9µm. A comparison of morphology and morphometrics of *C. estonica* populations from Nilgiri Hills, Tamil Nadu and *C. estonica* populations from Himachal Pradesh reveals that both the populations are in similar range hence study conducted by Saranya *et al*. (2018) supports to present work. Yu and Sun, (2018) firstly reported *C. estonica* from Canada. Their study reveals *C. estonica* cysts was lemon shaped with distinct neck and vulval cone, had circumfenestrate cone top and vulval fenestra was circumfenestrate 20–48µm in diameter whereas Himachal Population *Cactodera* vulva diameter was over all between 38 -53µm and Nilgiri Hills population ranges between 40.73–53.63µm. Canadian populations showed more variations in the vulval diameter, and a short vulval slit was also noticed. Indian populations showed more similarity in the morphology and morphometrics than Canadian populations, but in the case of range, Indian are in the range of Canadian populations. Hence findings support the present study.

According to Krall & Krall, (1979) genus, *Cactodera* has multiple species with distinct morphological characters. Duan, (2012) studied the morphological characteristics of *C. cacti*. female cyst body varies from lemon-shaped to spheroid with distinct neck and vulva. Circumfenestrate cone top observed. Our results mentioned that *C. estonica* cysts were lemon-shaped with distinct neck and vulval cone, and circumfenestrate cone top were present. These findings also indicate that genus *Cactodera* also shared some common features for different species i.e., cyst body shape varies from lemon shape to spherical, consists of distinct neck and vulva and circumfenestrate cone top were present in genus *Cactodera*. Even if different species have distinct morphological features, the genus also shared some common morphological features.

6.2.6. Morphology and morphometrics of *Globodera* cysts

Cysts were recovered from the soil samples collected from the potato agriculture farms and private potato agriculture fields from four different districts, namely district Kangra (Jia) named as HPPOPL, district Mandi (Phulladhar region) called as HPPOMD, district Kullu (Chowaii region) named as HPPOKL, district Chamba (Alha farm) named as HPPOCH and district Shimla (Totu Pani) HPPOSH. Each population of cysts was globose or spherical shaped cysts was noticed, light brown to a golden brown. The neck was distinctly seen from the rest of the body. Fenestral organization on the cone top were circumfenestrate. External cuticular patterns were also observed. A reticulate pattern was present on the cuticle. All four cyst populations were morphologically characterized separately. External characteristics of cysts such as cyst colour, shape and fenestration were similar in all four populations. The study of the vulval cone by cone top mounting is the principal structure used to identify cyst nematodes. All four populations are followed by circumfenestration pattern on the vulva. Morphometrics for each population was calculated. Morphometrical characteristics of cyst population isolated from potato field of district Kangra, Palampur region Himachal Pradesh (HPPOPL) (n=10) were, body length without neck was ranged from 500.1µm to

679.1µm, neck length was ranged from 98.7µm to 188.7µm, body width was ranged from 391.1µm to 598µm, distance from vulval basin to anus was ranged from 42.3µm to 79.1µm and number of cuticular ridges present between vulval cone to anus was ranged from 14 to 23(Table 26). The morphometric study of cyst populations isolated from district Mandi, Phulladhar region Himachal Pradesh (HPMD) were, body length without neck was ranged from 467.1µm to 671.9µm, neck length was ranged from 84.1µm to 196.8µm, body width was ranged from 387.2µm to 589.7µm, distance from vulval basin to anus was ranged from 39.2µm to 79.3µm and number of cuticular ridges present between vulval cone to anus was ranged from 14 to 24 (Table 27). The morphometric study of cyst populations isolated from district Kullu, Himachal Pradesh (HPPOKL) were, body length without neck was ranged from 463.2µm to 680.3µm, neck length was ranged from 81.9µm to 190.1µm, body width ranged from 356.7µm to 590.2µm, distance from vulval basin to anus was ranged from 39.8µm to 78.6µm and number of cuticular ridges present between vulval cone to anus was ranged from 14 to 25 (Table 28). The morphometric study of cyst populations district Chamba from Ahla region Himachal Pradesh (HPPOCH) were, body length without neck was ranged from 476.9µm to 679.5µm, neck length ranged from 80.2µm to 175.9µm, body width ranged from 376.1µm to 578.5µm, distance from vulval basin to anus was ranged from 38.9µm to 78.1µm and number of cuticular ridges present between vulval cone to anus was ranged from 15- 24 (Table 29). The morphometric study of cyst populations district Shimla, Totu Pani region Himachal Pradesh (HPPOSH) were, body length without neck was ranged from 480.2µm to 658µm, and neck length was ranged from 82.1 to 201.9 µm, body width ranged from 355.1µm to 592.4µm, distance from vulval basin to anus was ranged from 38.1µm to 76.9µm and number of cuticular ridges present between vulval cone to anus was ranged from 16- 25 (Table 30). According to the morphological and morphometrical characteristics discussed above, the results revealed that collected cysts populations belong to genus *Globodera*. The morphological and morphometrical study discussed above

confirmed the occurrence of *Globodera* in Kangra (Jia), district Mandi (Phulladhar region), district Kullu (Chowaii region) and district Chamba (Alha farm). A comparison of all four cyst populations revealed that cysts collected from district Chamba had maximum Length and width as compared to the rest of the four districts. Surprisingly among four populations, district Chamba cysts had the shortest neck. The other three populations from Kullu, Mandi, and Kangra showed similar neck lengths. District Kangra's body length and width are slightly shorter and thinner than district Chamba. All four cyst populations showed equal distances from vulval basin to anus ranging from 65.71µm to 62.60µm. The number of cuticular ridges ranges from 19-to 20 and district Chamba showed a maximum no of cuticular ridges present between the vulval cone and anus. This comparison states that there are very few variations noticed in all four populations' morphometrics. All four populations showed morphologically and morphometrically similar to each other.

Jenifer *et al*. (2020) demonstrated the morphological study of *G. rostochiensis* and *G. pallida* from Nilgiri Hills, Tamil Nadu, India. According to their research *G. rostochiensis* cysts Body length excluding neck 615.5 ± 58.8µm, Body width 557.7 ± 30.13µm, Neck length 143.5 ± 35.1µm, distance from vulval basin to anus 65.3 ± 24.6µm, Number of cuticular ridges between vulval basin to anus 18.4 ± 2.7 and the number of cuticular ridges between vulval basin to anus ranges from 14-21. By comparing the morphology and morphometrics of Nilgiri Hills, *G. rostochiensis* population, with our isolated Himachal Pradesh population, *G. rostochiensis* populations - very few variations are found in the morphometrics of both populations; there is a slight difference in the range (n=10) of morphometrical readings. The length and the width are slightly high in the Nilgiri Hills, than in Himachal Pradesh. Jenifer *et al*. (2020) also reveal a mixed population of *G. rostochiensis* and *G. pallida* present in the Nilgiri Hills. Ganguly *et al*. (2010) recorded the occurrence of *G. rostochiensis* and *G. pallida* from the potato breeding farm Kufri, Shimla. The morphometric study of *G. rostochiensis*

reveals that (n=28) cysts' body length without neck was between 300-680µm in range, body width was between 210-600µm, neck length was between 60-200µm, fenestral Length was between 14-18µm, distance from the anus to fenestra was between 42-56µm, and no of cuticular ridges from anus to fenestra was 18-25. The morphometrical study conducted by Ganguly *et al*. (2010) supports the present study, the reported study was in agreement with the morphometrical study of *G. rostochiensis* mentioned in the present study. Golden, (1972) demonstrated the morphological identification of *Globodera* and concluded that the vulval slit in *G. rostochiensis* was about 9 -10 µm. According to Gitty and Maafi, (2010) morphometric studies of *G. rostochiensis* from Iran reveal that fenestral diameter was between 10-17 µm and distance from the anus to vulval region ranges from 32–68 µm, and the number of cuticular ridges present between vulval basin to anus ranges from 15-24. This study reveals that the morphometrical study of *G. rostochiensis* population from Iran showed similarities with the present study. Potato cyst nematode (PCN) *Globodera* spp. Worldwide damaging potato and other solanaceous crops. Morphologically *Globodera* population is variable from different genera. *G. rostochiensis* and *G. pallida* are the most important damaging species. They distinguish *Globodera* cysts from other genera cysts. Genus *Globodera* cysts were spheroid or globose in shape. Cysts can be separated genus wise according to the formation of fenestra in the terminal (anal) region. The shape and size of fenestra played an essential role in morphological identification. The perineal pattern is one of the important characteristics of distinguishing the potato cyst nematode (PCN). Still, sometimes among the numerous populations, the morphological diagnostic characteristics of different species overlap (Baldwin and Mundo, 1991). *G. rostochiensis* populations isolated from the soil samples of different districts of Himachal Pradesh indicate that the morphometrics was within the range of *G. rostochiensis* population present in Serbia (Oro *et al*., 2010). The present study also reveals that *G. rostochiensis* population occurred at the areas where potato was grown mostly twice or thrice in a year. The temperature remains

between 15-25 ^{o}C in the summers and below 12 ^{o}C during winters. Potato cyst nematodes are mostly seen in the cooler areas. The information on the distribution and identification of potato cyst nematode is valuable in designing effective strategies for the control and can help reduce its spread. Therefore, **molecular characterization can identify the organism up to species level.**

The present study states that morphological characterization of the cysts isolated from the soil samples of different districts in Himachal Pradesh reveals the occurrence of 3 genera- *Heterodera, Globodera* and *Cactodera*. Morphological comparison of all 3 genera reveals that the cysts *Globodera* showed a distinct feature than *Heterodera* and *Cactodera*. *Globodera* had globose-shaped cysts with a distinct short neck and lack of vulval cone, whereas *Heterodera* and *Cactodera* had lemon-shaped cysts with distinct neck and vulval cone. Further, cone top mounting reveals that bifenestrate vulval cone was present in *Heterodera,* whereas the circumfenestrate cone top was present in *Cactodera* and *Globodera*. Morphological studies observed that each genus showed different morphological characteristics. In the case of Morphometrical studies, the genus *Heterodera* had larger and wider cysts than the genus *Globodera* and *Cactodera*. At the same time, genus *Cactodera* and *Globodera* had almost similar body lengths and width. Present cyst reported the occurrence of *C. estonica* from district Mandi, Himachal Pradesh firstly.

Further survey reveals that two more districts, namely district Chamba and district Shimla also showed the occurrence of *C. estonica*. Saranya *et al*. (2018) reported *C. estonica* firstly from Nilgiri Hills, Tamil Nadu India. The information on the distribution and identification of Estonian cyst nematode is valuable to design effective strategies for the control and help reduce its spread.

6.3. Molecular characterization of cyst nematodes

6.3.1. Molecular characteristics of *Heterodera* population

Heterodera population isolated from the soil sample collected from the wheat field of district Una was named HPWHUN were sequenced. PCR analysis was carried out using Subbotin *et al*. (2003) set of primers. Amplified products were obtained from the crude lysate of 100 cysts. *Heterodera* population was identified by amplifying the ITS region present in rDNA, including 18S and 28S genes. This yielded a single fragment of 960 bp mentioned in Fig. 23. Based on ITS-rDNA, the isolated population were identified as *Heterodera avenae*. The resulted sequence was deposited into a gene bank with accession number OM049243.1. According to the phylogenetic analysis presented in Fig. 23 based on ITS1-5.8S-ITS2, only one group1 was found, the *Heterodera avenae* group. *H. avenae* sequences submitted in GenBank were compared with our resulted sequence were highly similar. Group includes *H. avenae* population i.e KC736872.1(Morocco), KM199828.1, OL828553.1, OL828552.1, OL828551.1, OL828550.1, OL828549.1 and OL828548.1 (Turkey).

6.3.1.1. Consensus sequence: 960 bp DNA Accession: OM049243.1

>GGAAATTAAACCCGGACAGCCTACCAGGGAAGCACTTCCACCCA
GCACACATGGCGAGCAGTTGCGTCCTAGTTACGGACCGTAAAACG
CGAAAGAACCTGACAGTTTCGCGCCAACACAACCACACTCCACGG
CCGAGCATTTTCAAGAATAGCGCAAGAACACGTCATGCCGACATG
ATCTCACCAACATAACAGGCACAGCAGTGCCTTTTATGATAACGAC
CCTGAACCAGGCGTGCCAATGGATGTAACTCCAATGGCGCAATGT
GCATTCGAAAGTTTTGTGTTCAAGGTTTCTGCAGTTCGCACTAATTA
TCGCAGTTGGCTGCGTTCTTCATCGATCCACGAGCCGAGTGATCCA
CCGATAAGACTAGAATTTCTTTCTTCAACAAAGAAGTGGTCAAAAA
AATAAATGAAAAAAAACACTGGCGGTGCCTAGCTGACACTCATTG
AGTCTTAAGCCACGTGCAGAAGCATAGCACGGGCCCTACCACGTC

GTCTCATCACGGCCACGGACGTAGCACACAGGCACAGACACACCG
CTAGTTACGGTTCGTAAGGCCAGGAAAGAACCACTTTCCCAGGCC
AGTGTGTCACATGTGCCAGGCACAACAACCGCTCAACGACGCACA
GACACCAGCACAGCCGTTGGGTTACCCAACGAGCGTGCTCGTCCA
ACGACGGTACAGCAAACCAACCAGCAAGGCGGTCCGCCACCAACT
CGATTGCTTCGCAGCCAAACCTCAGGCTTTTCACTTTGCTCAGCAA
CGAGTCAGTATATACCAAGCACCATCGTCGGAGAACACCCCAAAG
CACAGAGTGCTCGCAGGGTAGCCGTCCACTCAGTGTTGTCCCGTGC
CCACAGACCATCTCGACCGGTCCGTGTGCCCGTCAGCAGACGGGG
GCATGTGGTGGTGCCAAGCAACGTTTCAACTGGACAGCACAGGTA
GCATGGTG

6.3.1.2. Phylogenetic analysis: Phylogenetic analysis was carried out based on a pairwise alignment score. The distance matrix was calculated to prepare the phylogenetic tree based on the neighbour-joining method. The phylogenetic tree represents the closest relationship or similarities of the present study with various cereal cyst nematodes. OM049243.1 showed 99.27% similarity with KC736872.1 *Heterodera avenae* population from Morocco, Berchid. Showed 99.27% similarity with KM199828.1, KM199828.1, OL828553.1, OL828552.1, OL828551.1, OL828550.1, OL828549.1, and OL828548.1 *Heterodera avenae* population from Turkey, showed 92.27% similarity with KF225719.1, HM560755.1 *Heterodera avenae* population from Germany. OM049243.1 showed the highest similarity with *Heterodera avenae* population from Morocco, Turkey and Germany (Fig. 24).

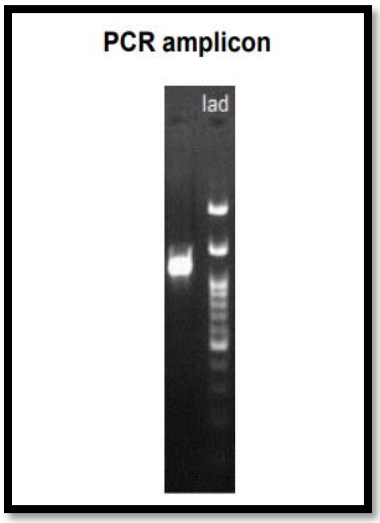

Figure 23: Amplified ITS region in agarose gel electrophoresis of *Heterodera avenae* from district Una, Himachal Pradesh.

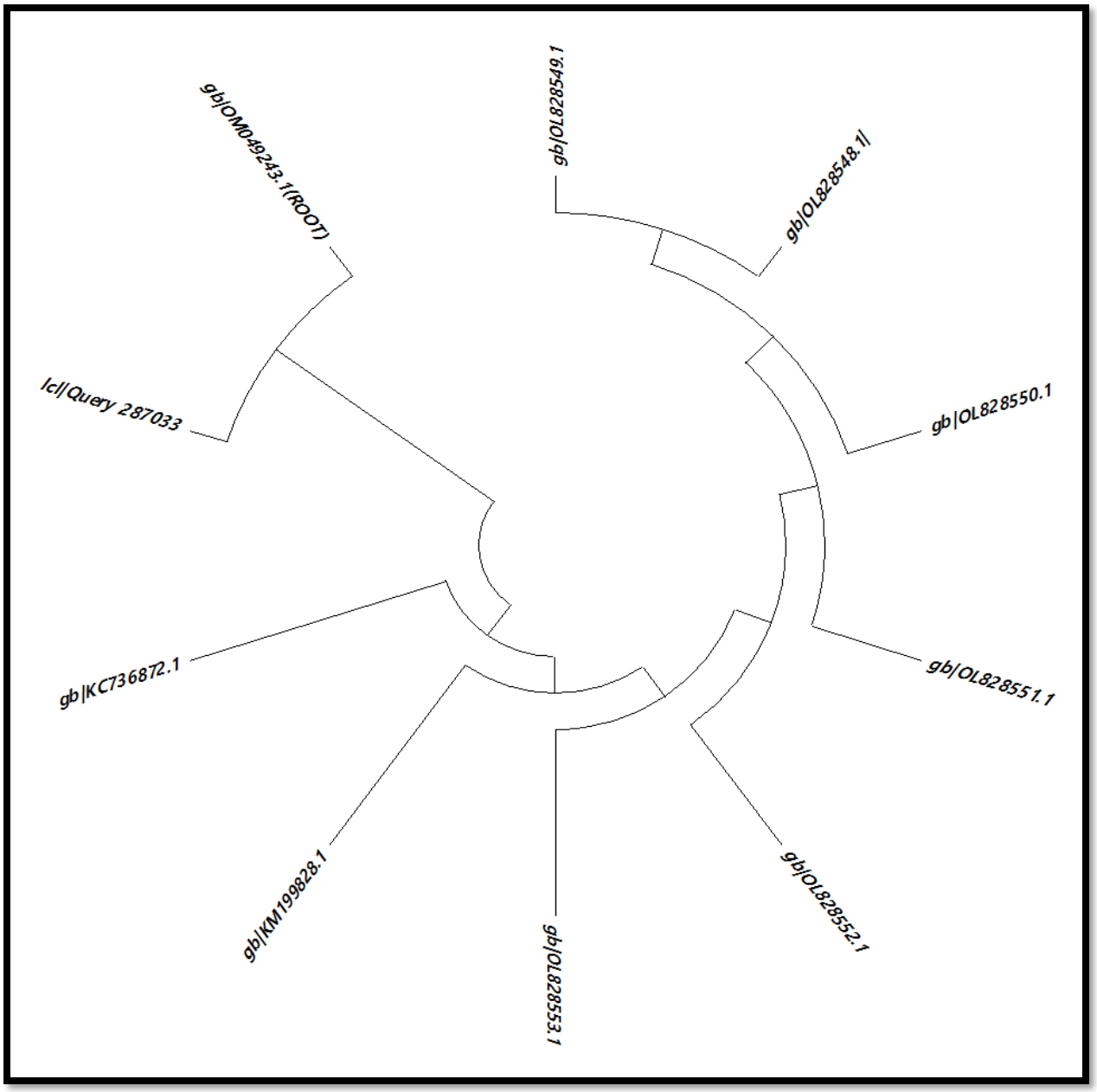

Figure 24: Phylogenetic tree showing phylogentic relationship between *Heterodera avenae* population collected from district Una Himachal Pradesh.

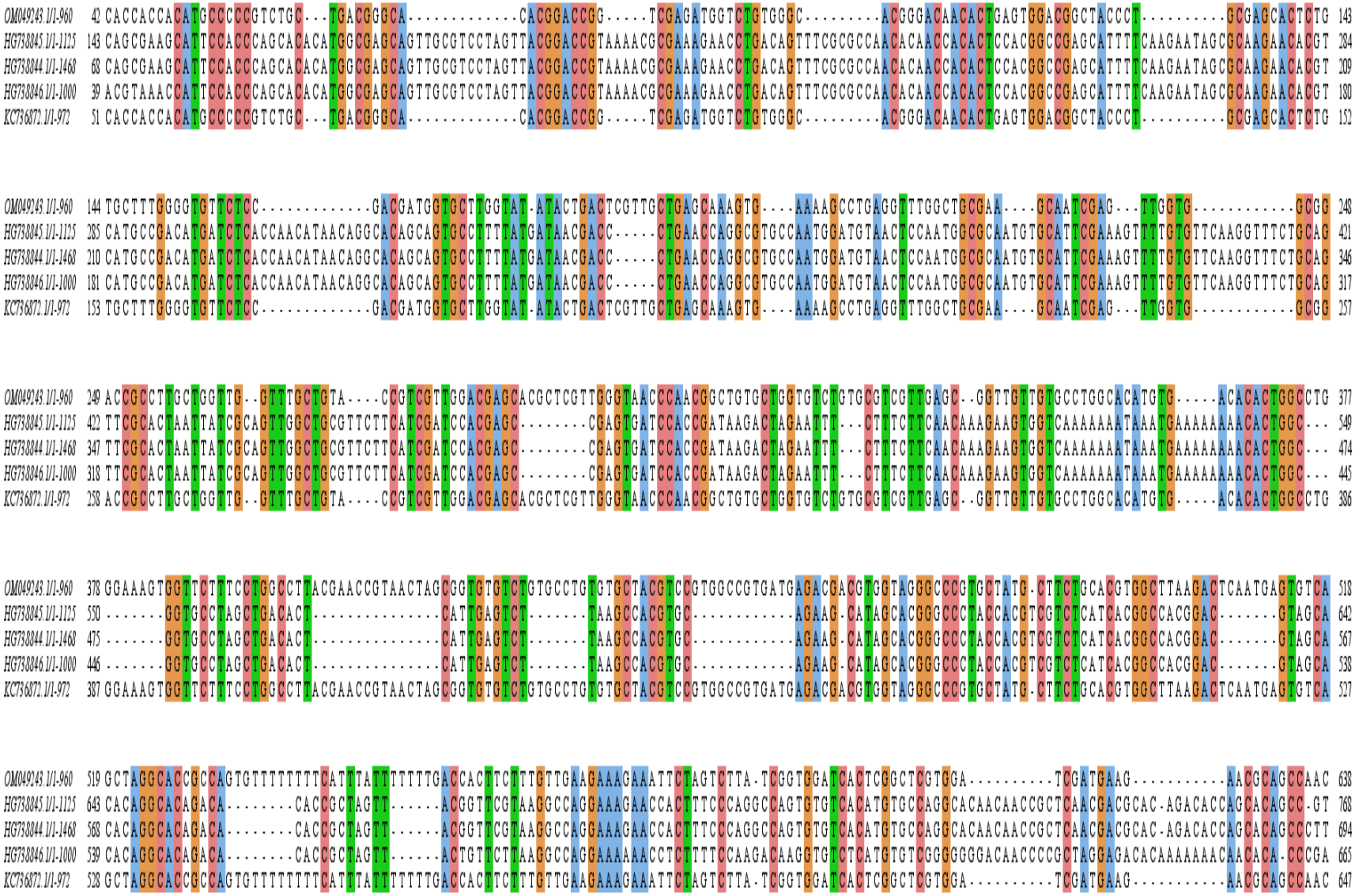
OM049243.1/1-960
HG738845.1/1-1125
HG738844.1/1-1468
HG738846.1/1-1000
KC736872.1/1-972

Figure 25: Multiple Sequence Alignment of 5 sequences of *Heterodera* spp. containing ITS1, 5.8S and ITS2 and partial sequences of 18S and 28S gene highly conserved regions of *Heterodera avenae* OM049243.1(Himachal Pradesh), *Heterodera filipjevi* HG738845.1 (Himachal Pradesh), *Heterodera avenae* HG738844.1 (Delhi), *Heterodera avenae* HG738846.1(Leh) and *Heterodera avenae* KC736872.1(Morocco) coloured sequences showing highly conserved regions between Himachal Pradesh, Delhi, Leh and from Morocco.

6.3.2. Molecular characteristics of *Cactodera* population

Cactodera population isolated from the soil sample collected from the potato field district Mandi, Chamba and Shimla was named as HPMD, HPCH and HPSH were sequenced. PCR analysis was carried out using forward and reverse primers of Vrain *et al*. (1992). Crude lysate of 100 cysts was amplified. *Cactodera* population was identified by amplifying the ITS region present in rDNA, including 18S and 28S genes. The amplified product of district Mandi yielded a single fragment of 1050 bp mentioned in Fig. 26(a), and the amplified product of district Chamba yielded a single fragment of 1067 bp mentioned in Fig. 26(b). The amplified product of district Shimla yielded a single fragment of 1065 bp, approximately mentioned in Fig. 26(c). There is a difference of 15-17 bp approximately in yielded fragments between the districts. Based on ITS-rDNA, the isolated population were identified as *Cactodera estonica*. The resulting sequence was deposited to the gene bank with accession numbers MN658364.1, MW821356.1 and MW821355.1.

6.3.2.1. Resulted consensus sequences of cyst collected from district Mandi (HPMD) MN658364.1, district Chamba (HPCH) MW821356, district Shimla (HPSH) MW821355 given below:

- >TTCATTGTGGGCAACACCACATGCCTCCGTTTGCTGCTAAC GGACACAGACCCTACGGAAAGGGCTGACACATTGACCAAC

ATTGAAGGGACGGCGGTCCCCGTGAGCACAAGTTTGGGGTG
CTACCTGAGTTGGTGGTTCGATGGGTGAGCCGGCTGCTGCC
GTCGGTTCATTGTACCAACAAAGGTAGCACGCTCACAGGGG
GCACCCAACGGCTGTGCTGGCGTCTGTGCGTTGTTGAGCGGT
TGTTGCGCCTTGCGTGGACATACTGACGTGGTGAGTTGGTCG
TACCTTCCACGTCGTACCAACGGTACCCAGCGGTATGTCTGT
GCTTGTGTGCTACGTCCGTGACCGTGATGAGACGACGTGTTA
GGACTCGTGCCTGGCATTTGGCATGTGGTTTAAGACTTAATG
AGTGCCCGACAGGCACCGCCAGTGTTTTTTCATTTTCAATAA
TTTTTTTTATGCAACGTTGTTGCTAAATATTCTAGTCTTATCG
GTGGATCACTCGGCTCGTGGATCGATGAAGAACGCAGCCAA
CTGCGATAATTAGTGTGAACTGCAGAAACTTTGAACACAGA
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CACGCCTGGTTCAGGGTCGTAACCAAAAAATGCACTGCATG
TGCGTGTTTTGACTGTTAAGATCACGCCTGGTCGTGTTCTTG
CATAAAGCTATGGCTACGCTGTGTAGCGTTGGACGTGCTGG
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TTTAGGCACGTCCCTCGGTGCACATGCGATAGCTAAATGCCT
CGCCAATAGGCATTTGTACTTGACTGCTTCGACCTGAACTCA
GACGTGAGTACCCGCTGAACTTAAGCAT

- >AACTCGGGGACGATTGTGCGCGTCGGCTTCGGTCGTCGCGT
TGATTGAAACCGATTTAATCGCAGTGGCTTGAACCGGGCAA
AAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCTGCTGGAT
CATTACCCAAGTGACTCCTATTCACCAGCTACCTGCTGTCTA
GTTGGTTCATTGTGGGCAACACCACATGCCTCCGTTTGCTGC
TAACGGACACAGACCCTACGGAAAGGGCTGACACATTGACC
AACATTGAAGGGACGGCGGTCCCCGTGAGCACAAGTTTGGG
GTGCTACTGAGTTGGTGGTTCGATGGGTGAGCCGGCTGCTG

CCGTCGGTTCATTGTACCAACAAAGGTAGCACGCTCACAGG
GGCACCCAACGGCTGTGCTGGCGTCTGTGCGTTGTTGAGCG
GTTGTTGCGCCTTGCGTGGACATACTGACGTGGGAGTTACCT
TCCACGTCGTACCAACGGTACCCAGCGGTATGTCTGTGCTTG
TGTGCTACGTCCGTGACCGTGATGAGACGACGTGTTAGGAC
TCGTGCCTGGCATTTGGCATGTGGTTTAAGACTTAATGAGTG
CCCGACAGGCACCGCCAGTGTTTTTTCATTTTCAATAATTTT
TTTTATGCAACGTTGTTGCTAAATATTCTAGTCTTATCGGTG
GATCACTCGGCTCGTGGATCGATGAAGAACGCAGCCAACTG
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TTCGAATGCACATTGCGCCATTGGAGTAATATCCTTTGGCAC
GCCTGGTTCAGGGTCGTAACCAAAAAATGCACTGCATGTGC
GTGTTTTGACTGTTAAGATCACGCCTGGTCGTGTTCTTGCAT
AAAGCTATGGCTACGCTGTGTAGCGTTGGACGTGCTGGTGC
GGAAATGTGTTGTTCTCTTCCGTGCTTTACAGACCGTAATTT
AGGCACGTCCCTCGGTGCACATGCGATAGCTAAATGCCTCG
CCAATAGGCATTTGTACTTGACTGGGTTCGACCTGAACTCAG
ACGTGAGTACCCCGCTGAACTTAAGCATATC

- >GGGGACGATTGTGCGCGTCGGCTTCGGTCGTCGCGTTGATT
GAAACCGATTTAATCGCAGTGGCTTGAACCGGGCAAAAGTC
GTAACAAGGTAGCTGTAGGTGAACCTGCTGCTGGATCATTA
CCCAAGTGACTCCTATTCACCAGCTACCTGCTGTCTAGTTGG
TTCATTGTGGGCAACACCACATGCCTCCGTTTGCTGCTAACG
GACACAGACCCTACGGAAAGGGCTGACACATTGACCAACAT
TGAAGGGACGGCGGTCCCCGTGAGCACAAGTTTGGGGTGCT
ACTGAGTTGGTGGTTCGATGGGTGAGCCGGCTGCTGCCGTC
GGTTCATTGTACCAACAAAGGTAGCACGCTCACAGGGGCAC
CCAACGGCTGTGCTGGCGTCTGTGCGTTGTTGAGCGGTTGTT
GCGCCTTGCGTGGACATACTGACGTGGGAGTTACCTTCCAC

GTCGTACCAACGGTACCCAGCGGTATGTCTGTGCTTGTGTGC
TACGTCCGTGACCGTGATGAGACGACGTGTTAGGACTCGTG
CCTGGCATTTGGCATGTGGTTTAAGACTTAATGAGTGCCCGA
CAGGCACCGCCAGTGTTTTTTCATTTTCAATAATTTTTTTTAT
GCAACGTTGTTGCTAAATATTCTAGTCTTATCGGTGGATCAC
TCGGCTCGTGGATCGATGAAGAACGCAGCCAACTGCGATAA
TTAGTGTGAACTGCAGAAACTTTGAACACAGAACTTTCGAA
TGCACATTGCGCCATTGGAGTAATATCCTTTGGCACGCCTGG
TTCAGGGTCGTAACCAAAAAATGCACTGCATGTGCGTGTTTT
GACTGTTAAGATCACGCCTGGTCGTGTTCTTGCATAAAGCTA
TGGCTACGCTGTGTAGCGTTGGACGTGCTGGTGCGGAAATG
TGTTGTTCTCTTCCGTGCTTTACAGACCGTAATTTAGGCACG
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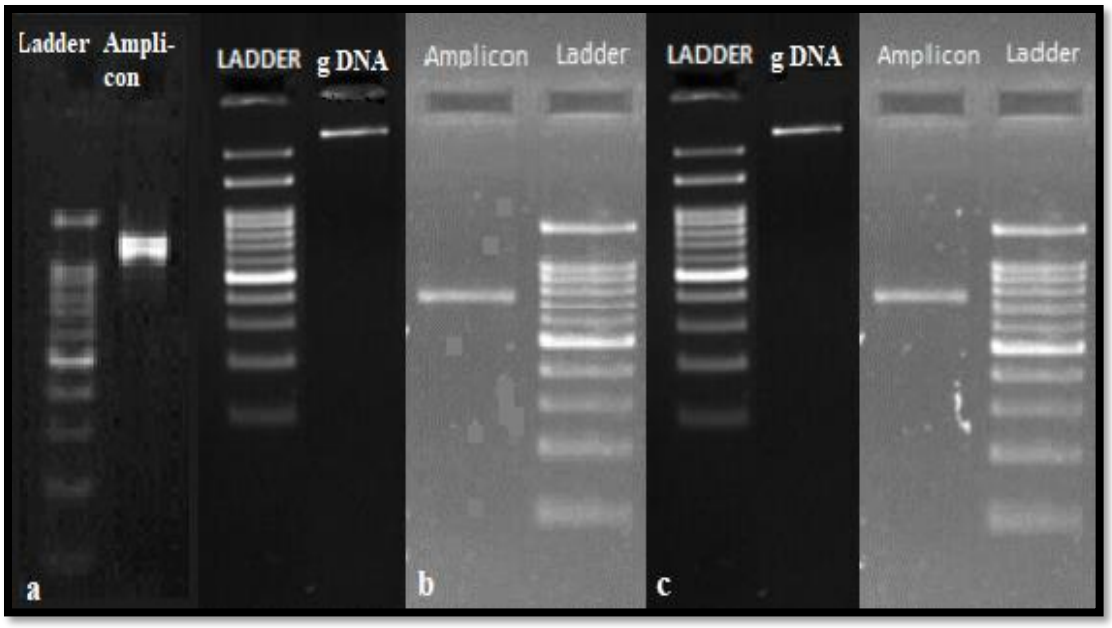


Figure 26: Amplified ITS region in agarose gel electrophoresis of Estonian cyst nematode **a)** *Cactodera estonica* from district Mandi, Himachal Pradesh. **b)** *Cactodera estonica* from district Chamba, Himachal Pradesh. **c)** *Cactodera estonica* from district Shimla, Himachal Pradesh.

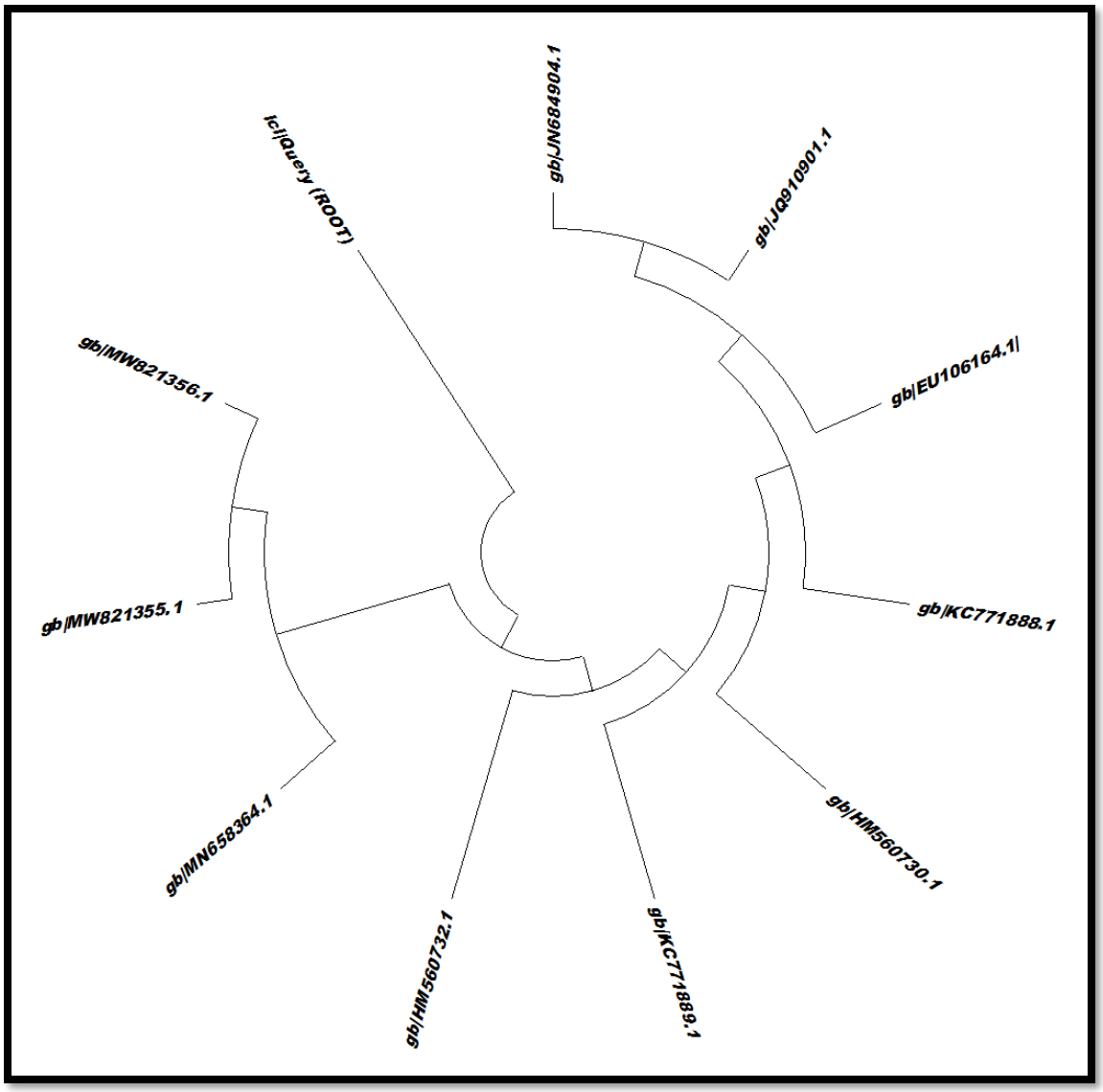


Figure 27: Phylogenetic tree showing the closest relationship of *Cactodera estonica* (MN658364.1) from district Mandi (HPMD), Himachal Pradesh with other species.

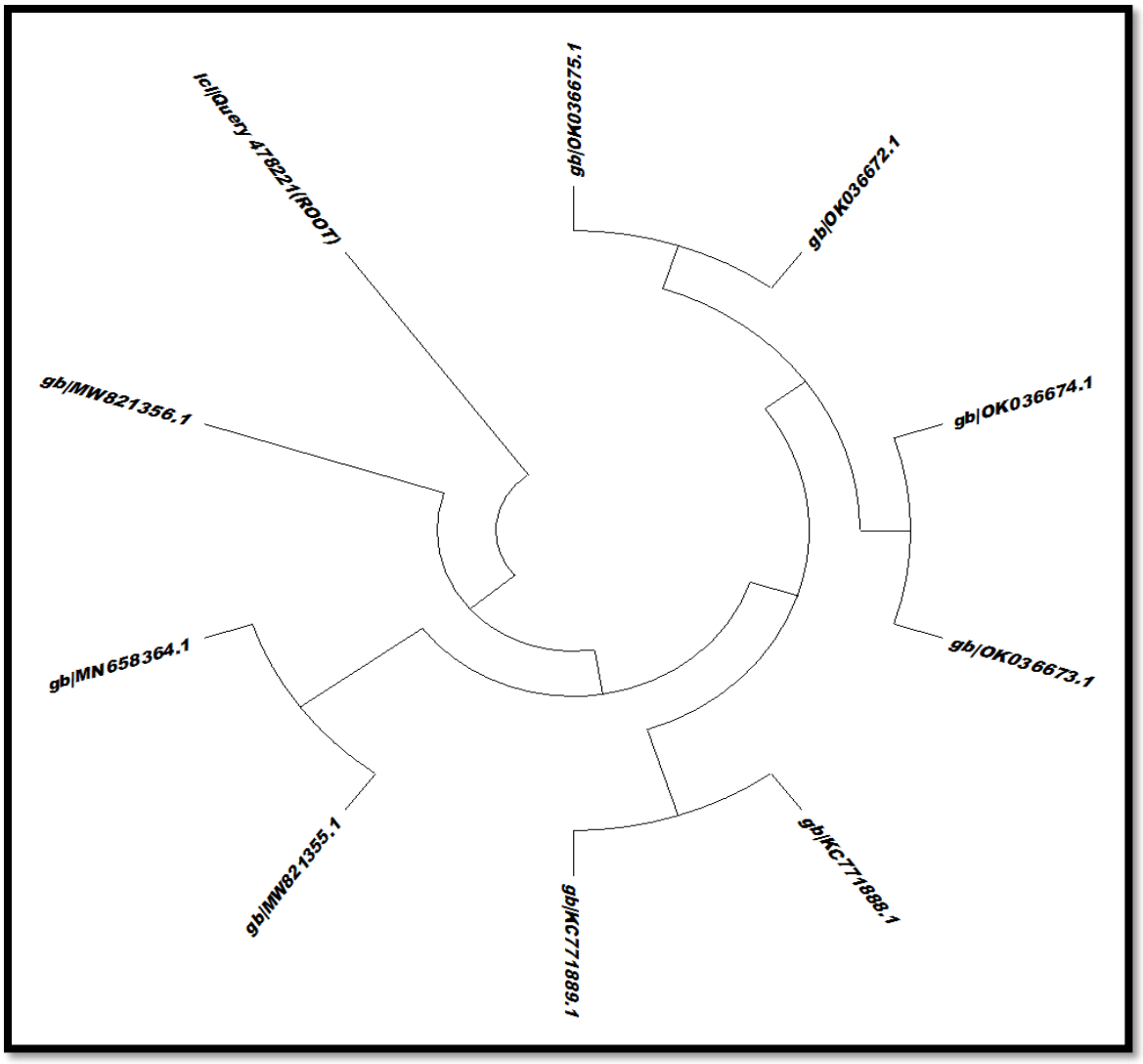


Figure 28: Phylogenetic tree showing a closest relationship of *Cactodera estonica* (MW821356) from district Chamba (HPCH), Himachal Pradesh with other species.

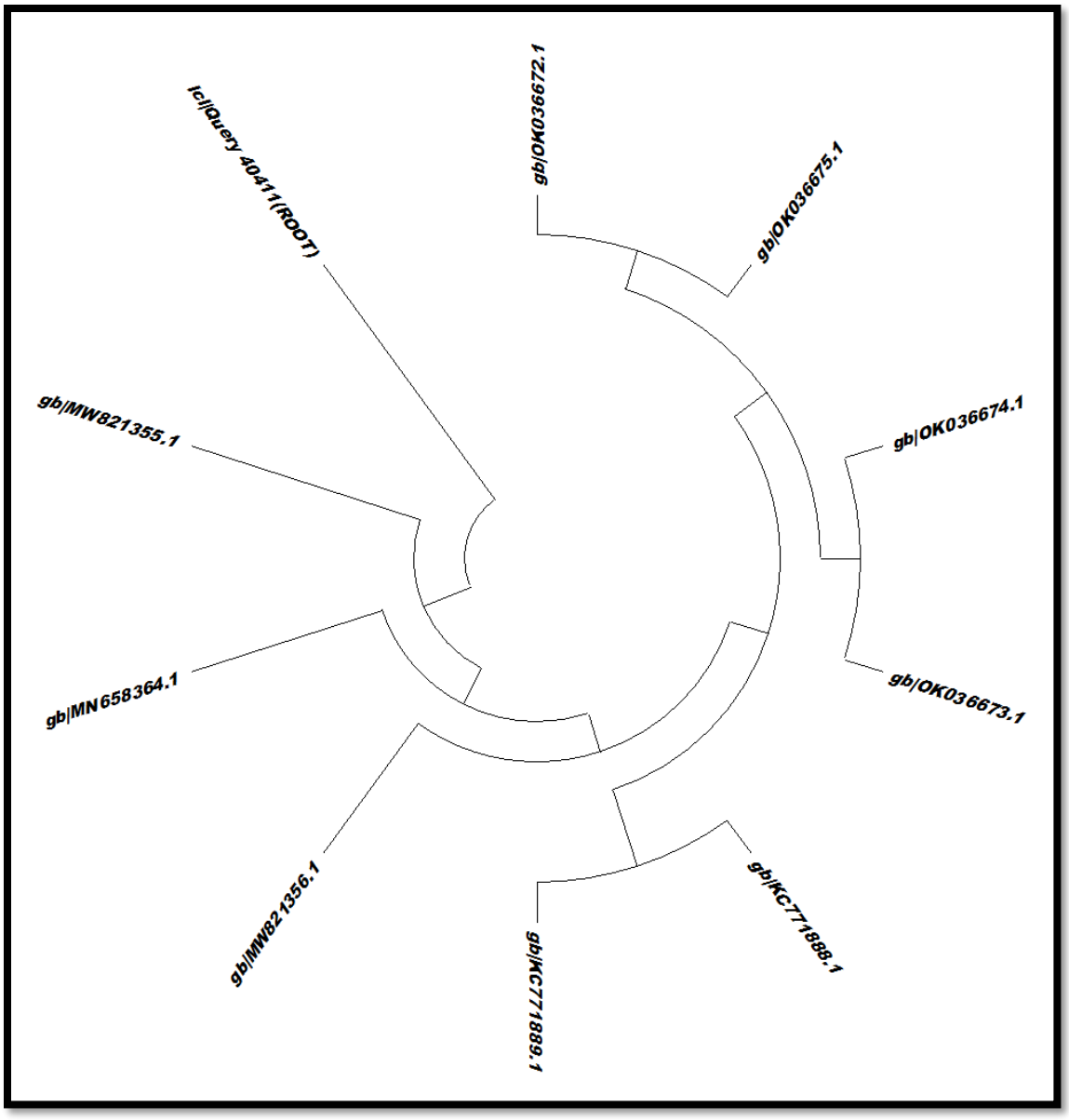


Figure 29: Phylogenetic tree showing the closest relationship of *Cactodera estonica* (MW821355) from district Shimla (HPSH), Himachal Pradesh with other species.

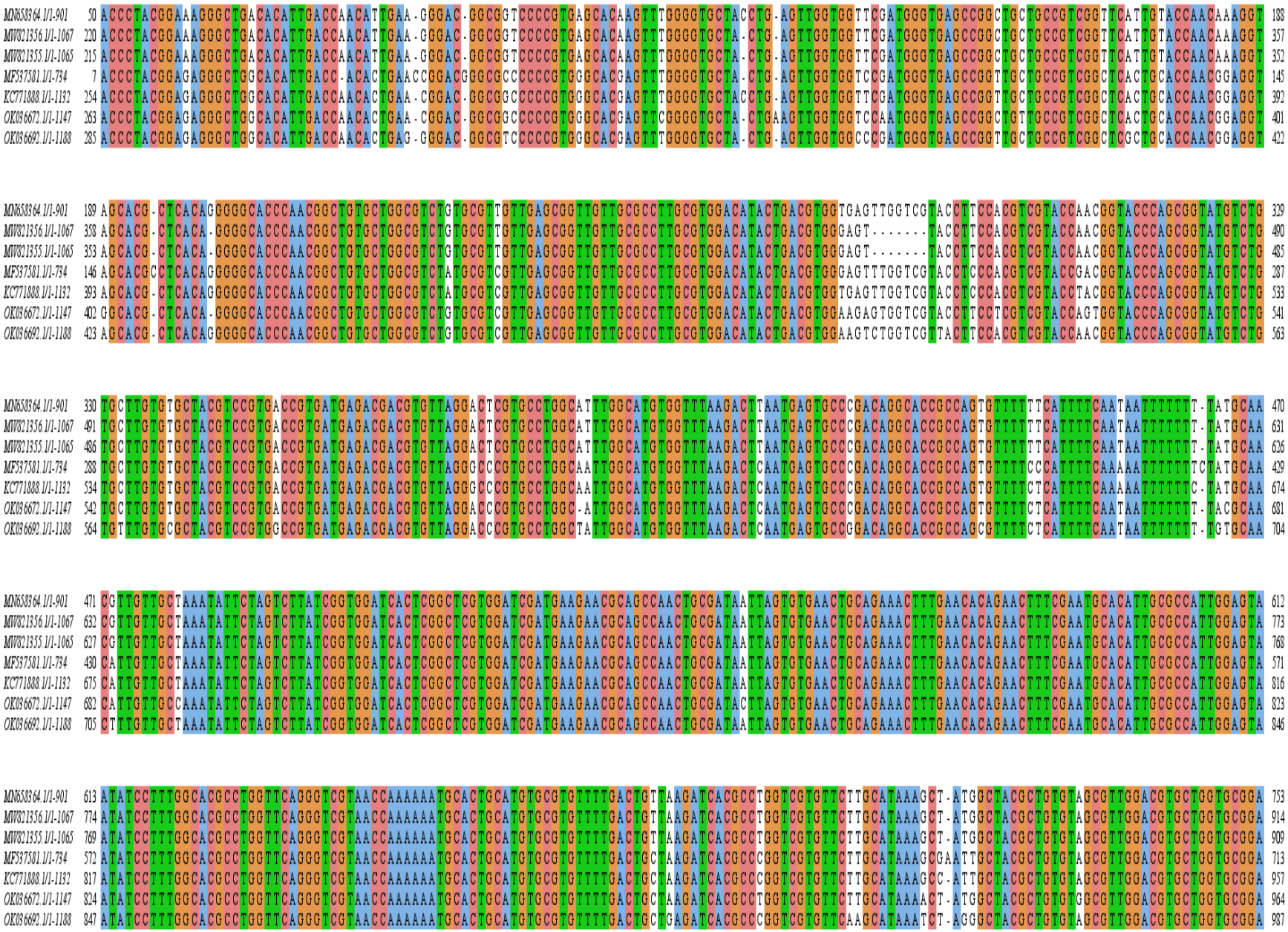

MN658364.1/1-901 50 ACCCTACGGAAAGGGCTGACACATTGACCAACATTGAA-GGGAC-GGCGGTCCCCGTGAGCACAAGTTTGGGGTGCTACCTG-AGTTGGTGGTTCGATGGGTGAGCCGGCTGCTGCCGTCGGTTCATTGTACCAACAAAGGT 188
MW821356.1/1-1067 220 ACCCTACGGAAAGGGCTGACACATTGACCAACATTGAA-GGGAC-GGCGGTCCCCGTGAGCACAAGTTTGGGGTGCTA-CTG-AGTTGGTGGTTCGATGGGTGAGCCGGCTGCTGCCGTCGGTTCATTGTACCAACAAAGGT 357
MW821355.1/1-1065 215 ACCCTACGGAAAGGGCTGACACATTGACCAACATTGAA-GGGAC-GGCGGTCCCCGTGAGCACAAGTTTGGGGTGCTA-CTG-AGTTGGTGGTTCGATGGGTGAGCCGGCTGCTGCCGTCGGTTCATTGTACCAACAAAGGT 352
MF537581.1/1-734 7 ACCCTACGGAGAGGGCTGGCACATTGACC-ACACTGAACCGGACGGGCGCCCCCGTGGGCACGAGTTTGGGGTGCTA-CTG-AGTTGGTGGTCCGATGGGTGAGCCGGTTGCTGCCGTCGGCTCACTGCACCAACGGAGGT 145
KC771888.1/1-1132 254 ACCCTACGGAGAGGGCTGGCACATTGACCAACACTGAA-CGGAC-GGCGGCCCCCGTGGGCACGAGTTTGGGGTGCTACCTG-AGTTGGTGGTTCGATGGGTGAGCCGGTTGCTGCCGTCGGCTCACTGCACCAACGGAGGT 392
OK086672.1/1-1147 263 ACCCTACGGAGAGGGCTGGCACATTGACCAACACTGAA-CGGAC-GGCGGCCCCCGTGGGCACGAGTTCGGGGTGCTA-CTGAAGTTGGTGGTCCAATGGGTGAGCCGGCTGTTGCCGTCGGCTCACTGCACCAACGGAGGT 401
OK086692.1/1-1188 285 ACCCTACGGAGAGGGCTGGCACATTGACCAACACTGAG-GGGAC-GGCGTCCCCCGTGGGCACGAGTTTGGGGTGCTA-CTG-AGTTGGTGGCCCGATGGGTGAGCCGGTTGCTGCCGTCGGCTCGCTGCACCAACGGAGGT 422
MN658364.1/1-901 189 AGCACG-GTCACAGGGGGCACCCAACGGCTGTGCTGGCGTCTGTGCGGTTGTTGAGCGGTTGTTGCGCCTTGCGTGGACATACTGACGTGGTGAGTTGGTCGTACCTTCCACGTCGTACCAACGGTACCCAGCGGTATGTCTG 329
MW821356.1/1-1067 358 AGCACG-GTCACA-GGGGCACCCAACGGCTGTGCTGGCGTCTGTGCGGTTGTTGAGCGGTTGTTGCGCCTTGCGTGGACATACTGACGTGGGAGT-------TACCTTCCACGTCGTACCAACGGTACCCAGCGGTATGTCTG 490
MW821355.1/1-1065 353 AGCACG-GTCACA-GGGGCACCCAACGGCTGTGCTGGCGTCTGTGCGGTTGTTGAGCGGTTGTTGCGCCTTGCGTGGACATACTGACGTGGGAGT-------TACCTTCCACGTCGTACCAACGGTACCCAGCGGTATGTCTG 485
MF537581.1/1-734 146 AGCACGCCTCACAGGGGGCACCCAACGGCTGTGCTGGCGTCTATGCGTCCGTTGAGCGGTTGTTGCGCCTTGCGTGGACATACTGACGTGGGAGTTTGGTCGTACCTCCCACGTCGTACCGACGGTACCCAGCGGTATGTCTG 287
KC771888.1/1-1132 393 AGCACG-GTCACAGGGGGCACCCAACGGCTGTGCTGGCGTCTATGCGTCCGTTGAGCGGTTGTTGCGCCTTGCGTGGACATACTGACGTGGTGAGTTGGTCGTACCTCCCACGTCGTACCTACGGTACCCAGCGGTATGTCTG 533
OK086672.1/1-1147 402 GGCACG-GTCACA-GGGGCACCCAACGGCTGTGCTGGCGTCTGTGCGGTCCGTTGAGCGGTTGTTGCGCCTTGCGTGGACATACTGACGTGGAAGAGTGGTCGTACCTTCCTCGTCGTACCAGTGGTACCCAGCGGTATGTCTG 541
OK086692.1/1-1188 423 AGCACG-GTCACAGGGGGCACCCAACGGCTGTGCTGGCGTCTGTGCGGTCCGTTGAGCGGTTGTTGCGCCTTGCGTGGACATACTGACGTGGAAGTCTGGTCGTTACTTCCACGTCGTACCAACGGTACCCAGCGGTATGTCTG 563
MN658364.1/1-901 330 TGCTTGTGTGCTACGTCCGTGACCGTGATGAGACGACGTGTTAGGACTCCGTGCCTGGCATTGGCATGTGGTTTAAGACTTAATGAGTGCCCGACAGGCACCGCCAGTGTTTTTTCATTTTCAATTAATTTTTTT-TATGCAA 470
MW821356.1/1-1067 491 TGCTTGTGTGCTACGTCCGTGACCGTGATGAGACGACGTGTTAGGACTCCGTGCCTGGCATTGGCATGTGGTTTAAGACTTAATGAGTGCCCGACAGGCACCGCCAGTGTTTTTTCATTTTCAATTAATTTTTTT-TATGCAA 631
MW821355.1/1-1065 486 TGCTTGTGTGCTACGTCCGTGACCGTGATGAGACGACGTGTTAGGACTCCGTGCCTGGCATTGGCATGTGGTTTAAGACTTAATGAGTGCCCGACAGGCACCGCCAGTGTTTTTTCATTTTCAATTAATTTTTTT-TATGCAA 626
MF537581.1/1-734 288 TGCTTGTGTGCTACGTCCGTGACCGTGATGAGACGACGTGTTAGGGCCCGTGCCTGGCAATTGGCATGTGGTTTAAGACTCAATGAGTGCCCGACAGGCACCGCCAGTGTTTTCCCATTTTCAAAAATTTTTTTCTATGCAA 429
KC771888.1/1-1132 534 TGCTTGTGTGCTACGTCCGTGACCGTGATGAGACGACGTGTTAGGGCCCGTGCCTGGCAATTGGCATGTGGTTTAAGACTCAATGAGTGCCCGACAGGCACCGCCAGTGTTTTCTCATTTTCAAAAATTTTTTTC-TATGCAA 674
OK086672.1/1-1147 542 TGCTTGTGTGCTACGTCCGTGACCGTGATGAGACGACGTGTTAGGACCCGTGCCTGGC-ATTGGCATGTGGTTTAAGACTCAATGAGTGCCCGACAGGCACCGCCAGTGTTTTCTCATTTTCAATTAATTTTTTT-TACGCAA 681
OK086692.1/1-1188 564 TGTTTGTGCGCTACGTCCGTGGCCGTGATGAGACGACGTGTTAGGACCCGTGCCTGGCTATTGGCATGTGGTTTAAGACTCAATGAGTGCCGGACAGGCACCGCCAGCGTTTTCTCATTTTCAATTAATTTTTTT-TGTGCAA 704
MN658364.1/1-901 471 CGTTGTTGCTAAATATTCTAGTCTTATCGGTGGATCACTCGGCTCGTGGATCGATGAAGAACGCAGCCAACTGCGATAATTAGTGTGAACTGCAGAAACTTTGAACACAGAACTTTCGAATGCACATTGCGCCATTGGAGTA 612
MW821356.1/1-1067 632 CGTTGTTGCTAAATATTCTAGTCTTATCGGTGGATCACTCGGCTCGTGGATCGATGAAGAACGCAGCCAACTGCGATAATTAGTGTGAACTGCAGAAACTTTGAACACAGAACTTTCGAATGCACATTGCGCCATTGGAGTA 773
MW821355.1/1-1065 627 CGTTGTTGCTAAATATTCTAGTCTTATCGGTGGATCACTCGGCTCGTGGATCGATGAAGAACGCAGCCAACTGCGATAATTAGTGTGAACTGCAGAAACTTTGAACACAGAACTTTCGAATGCACATTGCGCCATTGGAGTA 768
MF537581.1/1-734 430 CATTGTTGCTAAATATTCTAGTCTTATCGGTGGATCACTCGGCTCGTGGATCGATGAAGAACGCAGCCAACTGCGATAATTAGTGTGAACTGCAGAAACTTTGAACACAGAACTTTCGAATGCACATTGCGCCATTGGAGTA 571
KC771888.1/1-1132 675 CATTGTTGCTAAATATTCTAGTCTTATCGGTGGATCACTCGGCTCGTGGATCGATGAAGAACGCAGCCAACTGCGATAATTAGTGTGAACTGCAGAAACTTTGAACACAGAACTTTCGAATGCACATTGCGCCATTGGAGTA 816
OK086672.1/1-1147 682 CATTGTTGCCAAATATTCTAGTCTTATCGGTGGATCACTCGGCTCGTGGATCGATGAAGAACGCAGCCAACTGCGATACTTAGTGTGAACTGCAGAAACTTTGAACACAGAACTTTCGAATGCACATTGCGCCATTGGAGTA 823
OK086692.1/1-1188 705 CTTTGTTGCTAAATATTCTAGTCTTATCGGTGGATCACTCGGCTCGTGGATCGATGAAGAACGCAGCCAACTGCGATAATTAGTGTGAACTGCAGAAACTTTGAACACAGAACTTTCGAATGCACATTGCGCCATTGGAGTA 846
MN658364.1/1-901 613 ATATCCTTTGGCACGCCTGGTTCAGGGTCGTAACCAAAAAATGCACTGCATGTGCGTGTTTTGACTGTTAAGATCACGCCTGGTCGTGTTCTTGCATAAAGCT-ATGGCTACGCTGTGTAGCGTTGGACGTGCTGGTGCGGA 753
MW821356.1/1-1067 774 ATATCCTTTGGCACGCCTGGTTCAGGGTCGTAACCAAAAAATGCACTGCATGTGCGTGTTTTGACTGTTAAGATCACGCCTGGTCGTGTTCTTGCATAAAGCT-ATGGCTACGCTGTGTAGCGTTGGACGTGCTGGTGCGGA 914
MW821355.1/1-1065 769 ATATCCTTTGGCACGCCTGGTTCAGGGTCGTAACCAAAAAATGCACTGCATGTGCGTGTTTTGACTGTTAAGATCACGCCTGGTCGTGTTCTTGCATAAAGCT-ATGGCTACGCTGTGTAGCGTTGGACGTGCTGGTGCGGA 909
MF537581.1/1-734 572 ATATCCTTTGGCACGCCTGGTTCAGGGTCGTAACCAAAAAATGCACTGCATGTGCGTGTTTTGACTGCTAAGATCACGCCCGGTCGTGTTCTTGCATAAAGCGAATTGCTACGCTGTGTAGCGTTGGACGTGCTGGTGCGGA 713
KC771888.1/1-1132 817 ATATCCTTTGGCACGCCTGGTTCAGGGTCGTAACCAAAAAATGCACTGCATGTGCGTGTTTTGACTGCTAAGATCACGCCCGGTCGTGTTCTTGCATAAAGCC-ATTGCTACGCTGTGTAGCGTTGGACGTGCTGGTGCGGA 957
OK086672.1/1-1147 824 ATATCCTTTGGCACGCCTGGTTCAGGGTCGTAACCAAAAAATGCACTGCATGTGCGTGTTTTGACTGCTAAGATCACGCCTGGTCGTGTTCTTGCATAAAACT-ATGGCTACGCTGTGTGGCGTTGGACGTGCTGGTGCGGA 964
OK086692.1/1-1188 847 ATATCCTTTGGCACGCCTGGTTCAGGGTCGTAACCAAAAAATGCACTGCATGTGCGTGTTTTGACTGCTGAGATCACGCCCGGTCGTGTTCAAGCATAAATCT-AGGGCTACGCTGTGTAGCGTTGGACGTGCTGGTGCGGA 987

Figure 30: Multiple Sequence Alignment of 5 sequences of *Cactodera* spp. containing ITS1, 5.8S and ITS2 and partial sequences of 18S and 28S gene highly conserved regions of *Cactodera estonica* MN658364.1 (Himachal Pradesh), *Cactodera estonica* MW821356.1(Himachal Pradesh), *Cactodera estonica* MW821355.1 (Himachal Pradesh), *Cactodera estonica* MF537581.1 (Tamil Nadu), *Cactodera estonica* KC771888.1 (China), *Cactodera milleri* OK036672.1 (Canada), *Cactodera torreyanae* OK036692.1 (Canada). Coloured sequences show highly conserved regions between Himachal Pradesh, Tamil Nadu, China and Canada.

6.3.2.2. Phylogenetic analysis: Phylogenetic analysis was carried out based on a pairwise alignment score. The distance matrix was calculated to prepare the phylogenetic tree based on the neighbour-joining method. The phylogenetic tree represents the closest relationship or similarities of the present study with various cyst nematodes. District Mandi (HPMD) sequence MN658364.1 were showing 99% similarity with *C. estonica* our submitted sequence, i.e., MW821355 from Shimla, 98.67% similarity with *C. estonica* our submitted sequence, i.e., MW821356 from Chamba, 96.12% similarity with *C. estonica* KC771888.1, 96% similarity with HM560730.1, 95.89% similarity with JN684904.1, EU106164.1 and 95.78% similarity with KC771889.1from China. Fig.27 represents a phylogenetic tree of *Cactodera estonica* (MN658364.1) from district Mandi, clearly showing that all the sequences of *C. estonica* from Himachal Pradesh are highly similar to each other. In comparison with other countries *C. estonica* population (MN658364.1) is highly similar to the *C. estonica* population from China. District Chamba (HPCH) MW821356 showed 99.72 % similarity with *C. estonica* in our submitted sequence, i.e., MW821355.1from Himachal Pradesh, 95.08 % similarity with KC771888.1 and 94.98% similarity with KC771889.1 *C. estonica* population from China. 94.52% similarity with OK036675.1 and OK036672.1 *C. estonica* population from Canada. District Chamba (HPCH) MW821356.1 showed the highest similarity with *C. estonica* population from

Himachal Pradesh. Compared with other countries, MW821356.1 showed high similarity with *C. estonica* population from China and Canada (Fig. 28).

Resulted sequence from district Shimla (MW821355.1) showed 99.72 % similarity with MW577347.1 and 99% similarity with MN658364.1 *C. estonica* population from Himachal Pradesh. In addition, 95.35 % similarity was shown with KC771888.1and 95.25% similarity with KC771889, 95.35% similarity was shown with HM560732 and 95.16 % similarity with HM560730 *C. estonica* populations from China (Fig. 29). According to NCBI nucleotide BLAST studies, all the submitted sequences of *C. estonica* in this study, i.e., MN658364.1, MW821356 and MW821355, showed the highest similarity with each other. After that, all the resulted sequences were highly similar to *C. estonica* populations from China. Apart from China, *C. estonica* Himachal Pradesh populations also showed similarities with Canadian populations of *C. estonica*.

6.3.3. Molecular characteristics of *Globodera* population

Globodera cysts population were recovered from the soil samples collected from the potato fields in five different districts, namely Mandi, Chamba, Kangra, Kullu and Shimla. All the soil samples were coded based on districts HPPOMD, HPPOCH, HPPOPL, HPPOKL, HPPOSH. A set of forward and reverse primers of Floyd *et al*. (2002) PCR analysis was carried out. Crude lysate of 100-150 cysts was amplified. *Globodera* population was identified by amplifying 18 S small subunit (SSU) of rDNA. The amplified product of crude lysate yielded a single fragment of 638 bp for district Kangra (HPPOPL) represented in Fig. 31(a), 843 bp for district Mandi (HPPOMD) mentioned in Fig. 31(b), 860bp for district Kullu (HPPOKL) illustrated in Fig. 31(c), 843 bp for district Chamba (HPPOCH) depicted in Fig. 31(d). The identified sequence was *Globodera rostochiensis* in all districts. The resulted sequences were submitted to NCBI-GenBank accession numbers MZ508280.1, MZ518783.1, MZ508279.1 and MW577347.1.

6.3.3.1. Resulted consensus sequences of cyst collected from district Kangra (MZ508280.1), district Mandi (MZ518783.1), district Kullu (MZ508279.1) and district Chamba (MW577347.1) are given below:

- >TTACTTGGATAACTGTGGTAATTCTAGAGCTAATACATGCACC
  AAAGCTTCAATCTTCCCAGAGCGGAGCGCATTTGTTCGTCACAA
  AAACTAGCGCCTTCGGGCGTCCAGTGTTGACTCAGAACAACTTA
  GCTGATTGCACCGTCTTGTACCGGCGACGTGTCTTTCAAGTGTC
  TGCCTTATCAACTTTCGATGGTAGTGTACCTGACTACCATGGTG
  ATGACGGGTAACGGAGGATAAGGGTTCGACTCCGGAGAAGGG
  GCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCGCG
  CAAATTACCCACTCTCAACATGAGGAGGTAGTGACGAGAAATA
  ACGAGACCGATCTCTTATGAGGCCGGTCATCGGAATGGGTACA
  ATTTAAACCCTTTAACGAGTATCTATGAGAGGGCAAGTCTGGTG
  CCAGCAGCCGCGGTAATTCCAGCTCTCAAAATGCATAGAATTAT
  TGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGTGCTAGCCGGCC
  GGTCCACCCACTGGGTGTGCACTGGTTCGGTTGGCTTTTCTGCC
  GGTCTTTCCCCGGCGTTGGCCTTCACGGGTCGGCGTCGGTGGGC
  TGGCGAGTTTACTTTGAACAAATCAGAG
- >AGGGAAACTGCGTACGGCTCATTACACCAGCTATAATTTACTT
  GACCTTGACTTCTTACTTGGATAACTGTGGTAATTCTAGAGCTA
  ATACATGCACCAAAGCTCCAATCTCCCCAGAGCGGAGCGCATT
  TGTTCGCCACAAAACCAAGCGCCTTCGGGCGTCCAGTGTTGACT
  CAGAACAACTAAGCTGATCGCACGGTCTTGTACCGGCGACGTG
  TCTTTCAAGTGTCTGCCTTATCAACTTTCGATGGTAGTGTACCTG
  ACTACCATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACT
  CCGGAGAAGGGGCCTGAGAAATGGCCACTACGTCTAAGGATGG
  CAGCAGGCGCGCAAATTACCCACTCTCAACATGAGGAGGTAGT
  GACGAGAAATAACGAGACCGATCTCTTATGAGGCCGGTCATCG
  GAATGGGTACAATTTAAACCCTTTAACGAGTATCTATGAGAGG

GCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAAA
TGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCT
GTGCTAGCCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGT
TGGCTTTTCTGCCGGTCTTTCCCCGGCGTTGGCCTTCACCGGTCG
GCGTCGGTGGGCTGGCAAGTTTACTTTGAACAAATCAAAGTGCT
TCAAACAGGCGTTTCGCTTGAATGTTCGTGCATGGAATATTAGA
GGAGGATTTCGGTCCGATTTTATGGGTTTTGCTGACCGAGATAT
GGTTAAACAAAGACAAACGGGGCCATTCGTATTGCTACGTGAG
AGGTGAAATTCTTGG

- >TACGGGCTCATTACACCACGGCTATAACTTTACTTGGACACTT
GACTTCTTACTTGGATAACTGTGGTAATTCTAGAGCTAATACAT
GCACCAAAGCTCCAATCTCCCCAGAGCGGATCGCATTTGTTCGC
CACAAAACCAAGCGCCTTCGGGCGTCCAGTGTTGACTCAGAAC
AACTAAGCTGATCGCACGGTCTTGTACCGGCGACGTGTCTTTCA
AGTGTCTGCCTTATCAACTTTCGATGGTAGTGTACCTGACTACC
ATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACTCCGGAG
AAGGGGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAG
GCGCGCAAATTACCCACTCTCAACATGAGGAGGTACTGACGAG
AAATAACGAGACCGATCTCTTATGAGGCCGGTCATCGGAATGG
GTACAATTTAAACCCTTTAACGAGTATCTATGAGAGGGCAAGTC
TGGTGCCAGCAGCCGCGGTAATTCCCGCTCTCAAAATGCATAG
AATTATTGCTGCGGTTAAAAAGCTCGTAATTGGATCTGTGCTAT
CCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGTTGGCTTT
TCTGCGGGTCTTTCCCCGGCTTTGGCCTTCACCGGTCGGCATCG
GTGGGCTGGCAATTTAACTTTGAACAATTCAAAGTGCTGCAAAC
AGGCGTTTCGCTTGAATGTTCTTGCATGAAATAGTACGAGGAGG
ATTTCGGTCCGATTTTATTGGTTTTGCCTGACCGAAATATGGGT
AAACAGAGACGAACGGGGGCCTTTCTTATTGCTACGTGAAAAG
GTGAAATTCTTGGACCGTACCAGGACGGACTA

- >AGGGAAACTGCGTACGGCTCATTACACCAGCTATAATTTACTT
  GACCTTGACTTCTTACTTGGATAACTGTGGTAATTCTAGAGCTA
  ATACATGCACCAAAGCTCCAATCTCCCCAGAGCGGAGCGCATT
  TGTTCGCCACAAAACCAAGCGCCTTCGGGCGTCCAGTGTTGACT
  CAGAACAACTAAGCTGATCGCACGGTCTTGTACCGGCGACGTG
  TCTTTCAAGTGTCTGCCTTATCAACTTTCGATGGTAGTGTACCTG
  ACTACCATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACT
  CCGGAGAAGGGGCCTGAGAAATGGCCACTACGTCTAAGGATGG
  CAGCAGGCGCGCAAATTACCCACTCTCAACATGAGGAGGTAGT
  GACGAGAAATAACGAGACCGATCTCTTATGAGGCCGGTCATCG
  GAATGGGTACAATTTAAACCCTTTAACGAGTATCTATGAGAGG
  GCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAAA
  TGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCT
  GTGCTAGCCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGT
  TGGCTTTTCTGCCGGTCTTTCCCCGGCGTTGGCCTTCACCGGTCG
  GCGTCGGTGGGCTGGCAAGTTTACTTTGAACAAATCAAAGTGCT
  TCAAACAGGCGTTTCGCTTGAATGTTCGTGCATGGAATATTAGA
  GGAGGATTTCGGTCCGATTTTATGGGTTTTGCTGACCGAGATAT
  GGTTAAACAAAGACAAACGGGGCCATTCGTATTGCTACGTGAG
  AGGTGAAATTCTTGG

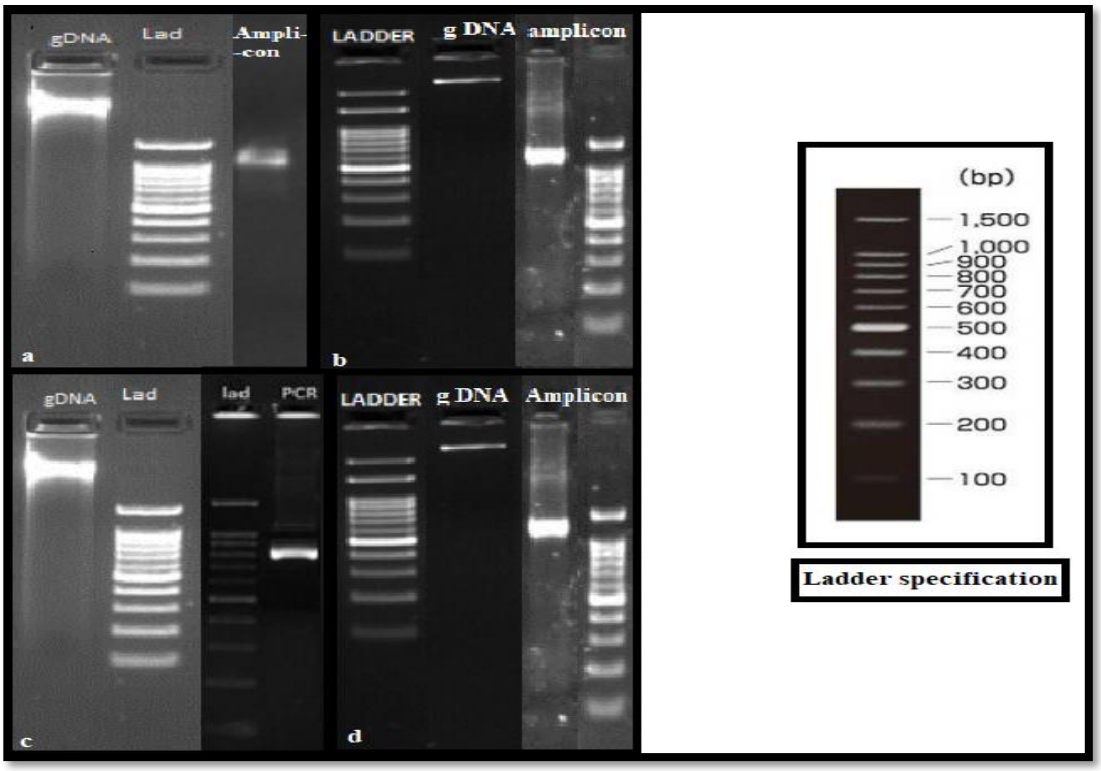


Figure 31: Amplified 18S region in agarose gel electrophoresis of potato cyst nematode **a)** *Globodera rostochiensis* from district Kangra, Himachal Pradesh. **b)** *Globodera rostochiensis* from district Mandi, Himachal Pradesh. **c)** *Globodera rostochiensis* from district Kullu, Himachal Pradesh. **d)** *Globodera rostochiensis* from district Chamba, Himachal Pradesh.

6.3.3.2. Phylogenetic analysis: The resulted sequences were submitted to NCBI-GenBank accession numbers MZ508280.1, MZ518783.1, MZ508279.1 and MW577347.1. Phylogenetic analysis was carried out based on a pairwise alignment score. The distance matrix was calculated to prepare the phylogenetic tree based on the neighbour-joining method. The phylogenetic tree represents the closest relationship or similarities of the present study with various potato cyst nematodes. According to the phylogenetic tree, the resulted sequence of district Kangra MZ508280.1 showed 98.59% similarity with the KJ636272.1 *Globodera rostochiensis* population from the Netherlands, 98.59% similarity MZ613180.1

Globodera rostochiensis population from China (Fig. 32). Our resulted sequence (MZ508280.1) showed equal similarity with the *G. rostochiensis* population in both Netherlands and China. The resulted sequence from district Mandi MZ518783.1 showed 100% similarity with MW577347.1 from Himachal Pradesh, India. MZ518783.1 showed 99.05% similarity with KJ636272.1 *G. rostochiensis* population from the Netherlands, 99.05% similarity with MZ613180.1 *G. rostochiensis* population from China (Fig. 33). The phylogenetic tree of resulting sequences of district Kullu MZ508279.1 showed 96.08% similarity with KJ636272.1 *G. rostochiensis* population from the Netherlands, 96.08% similarity with MW577347.1 *G. rostochiensis* populations from China. MZ508279.1 showed 95.58% similarity with KJ636272.1 *G. rostochiensis* populations from the Netherlands, 95.58% similarity with MZ613180.1 *G. rostochiensis* populations from China. MZ508279.1 showed equal similarity with Netherlands and China but was more nearly related to Netherlands populations based on the phylogenetic tree (Fig. 34). The phylogenetic tree of the resulting sequence of district Chamba MW577347.1 showed 100% similarity with MZ518783.1 from Himachal Pradesh, India. 99.05% similarity with KJ636272.1 *G. rostochiensis* population from the Netherlands, 99.05 % similarity with MZ613180.1 *G. rostochiensis* population from China. 99.05% similarity with AY284619.1 Netherlands. 98.93 % similarity with KJ636271.1, AY593879.1 *G. rostochiensis* population from the Netherlands. MZ518783.1 also showed 98.82 % similarity with the EU855120.1*G. rostochiensis* population from Poland (Fig. 35). Comparing all the four *G. rostochiensis* resulted in sequences MZ508280.1, MZ518783.1, MZ508279.1 and MW577347.1 all the sequences firstly showed the highest similarity with each other (sequences reported from Himachal Pradesh). Secondly, the Netherlands and China *G. rostochiensis* populations are closely related to our sequences. Only MW577347.1 showed a similarity with the *G. rostochiensis* population from Poland.

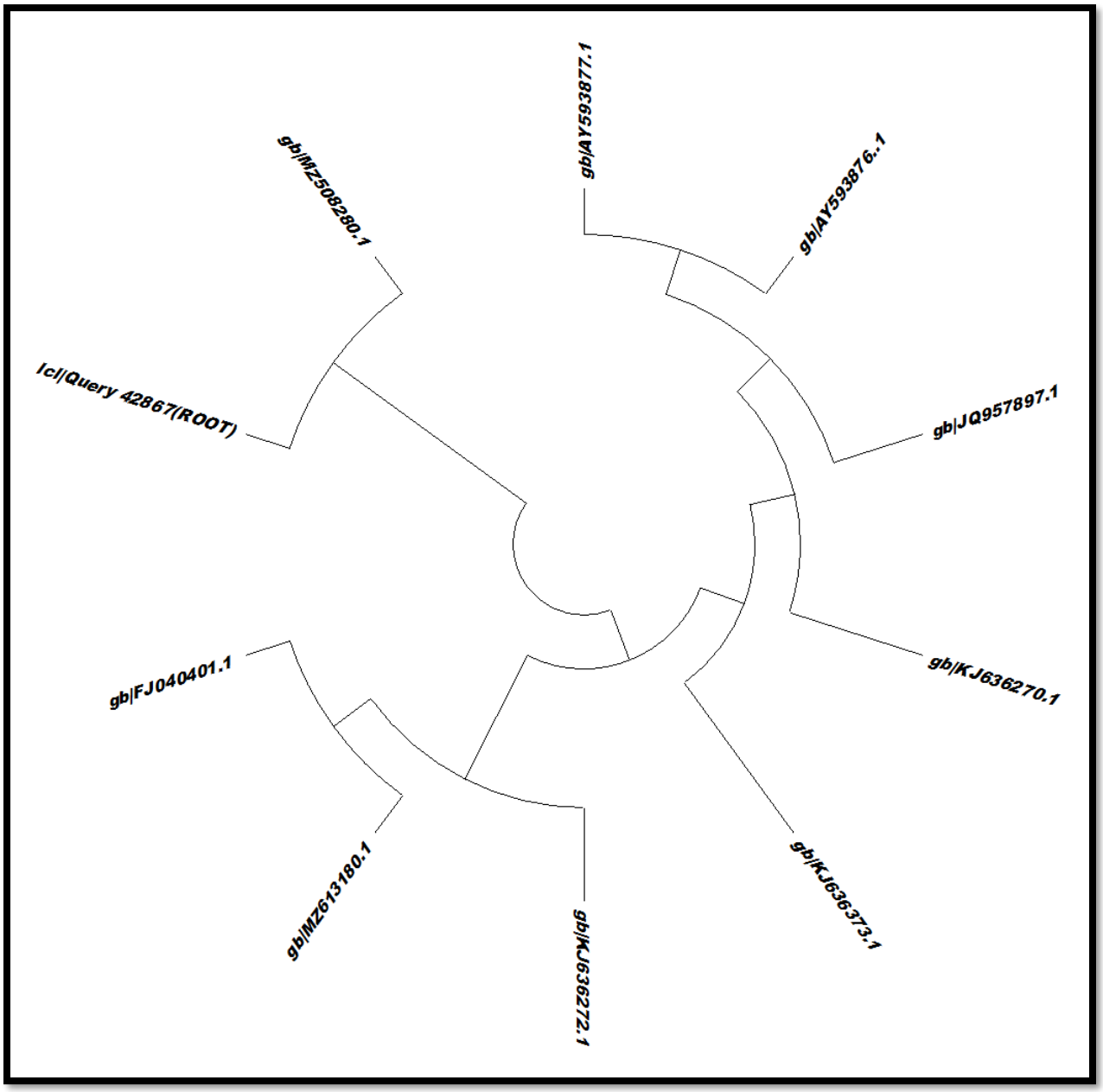


Figure 32: Phylogenetic tree showing the closest relationship of *Globodera rostochiensis* population collected from district Kangra, Himachal Pradesh (MZ508280.1).

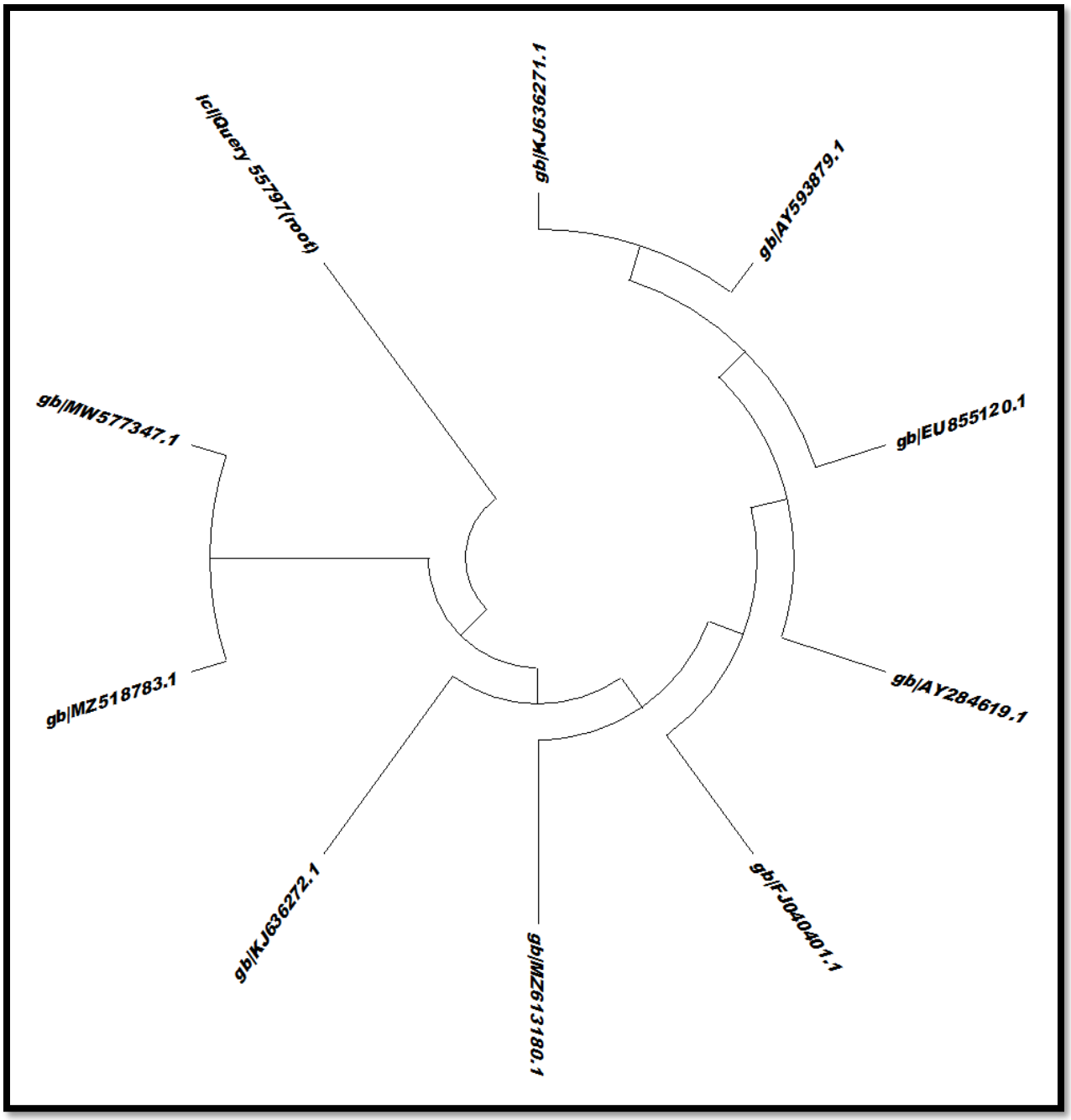


Figure 33: Phylogenetic tree showing the closest relationship of *Globodera rostochiensis* population collected from Himachal district Mandi, Himachal Pradesh (MZ518783.1).

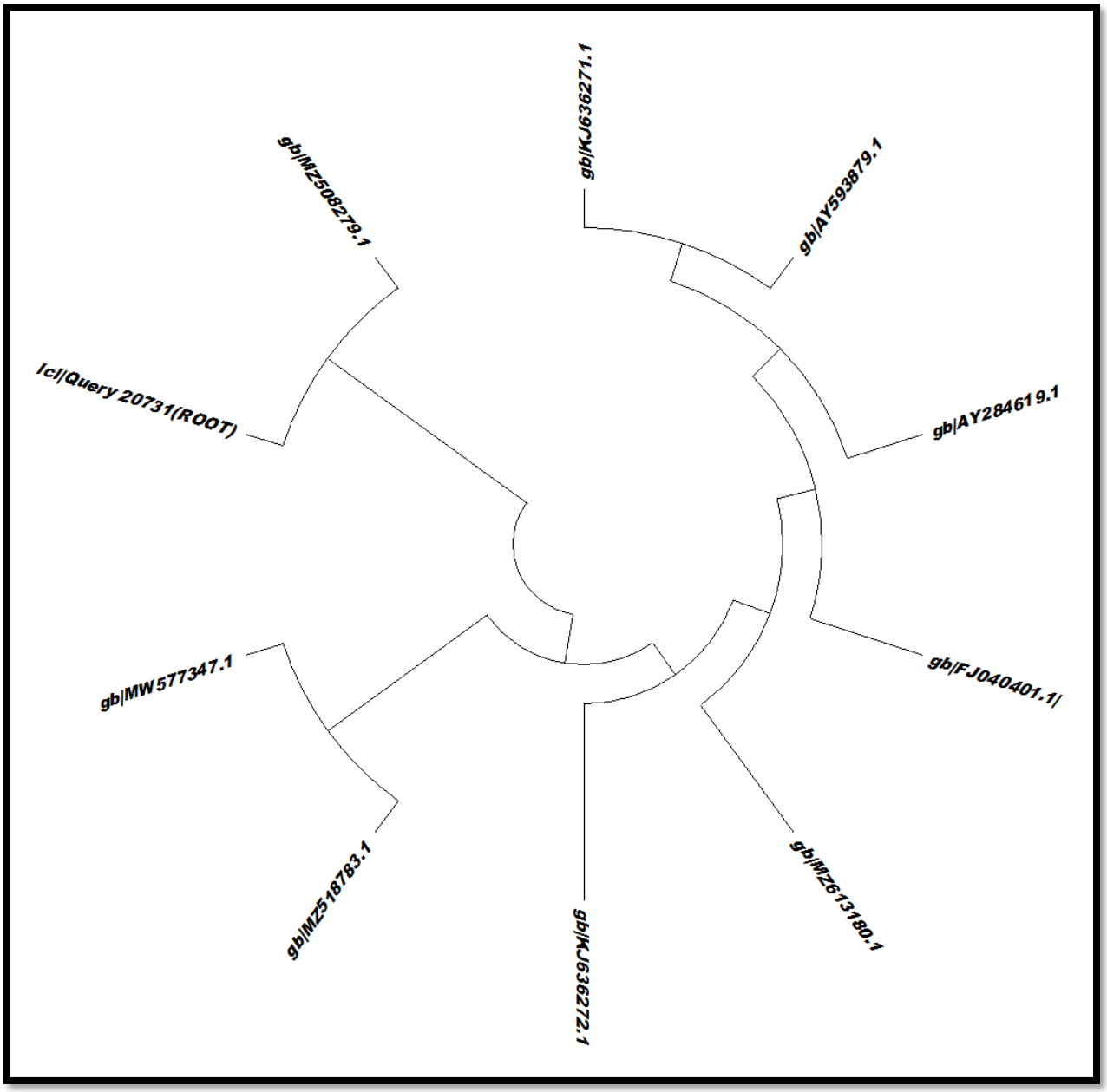


Figure 34: Phylogenetic tree showing the closest relationship of *Globodera rostochiensis*, population collected from district Kullu, Himachal Pradesh (MZ508279.1).

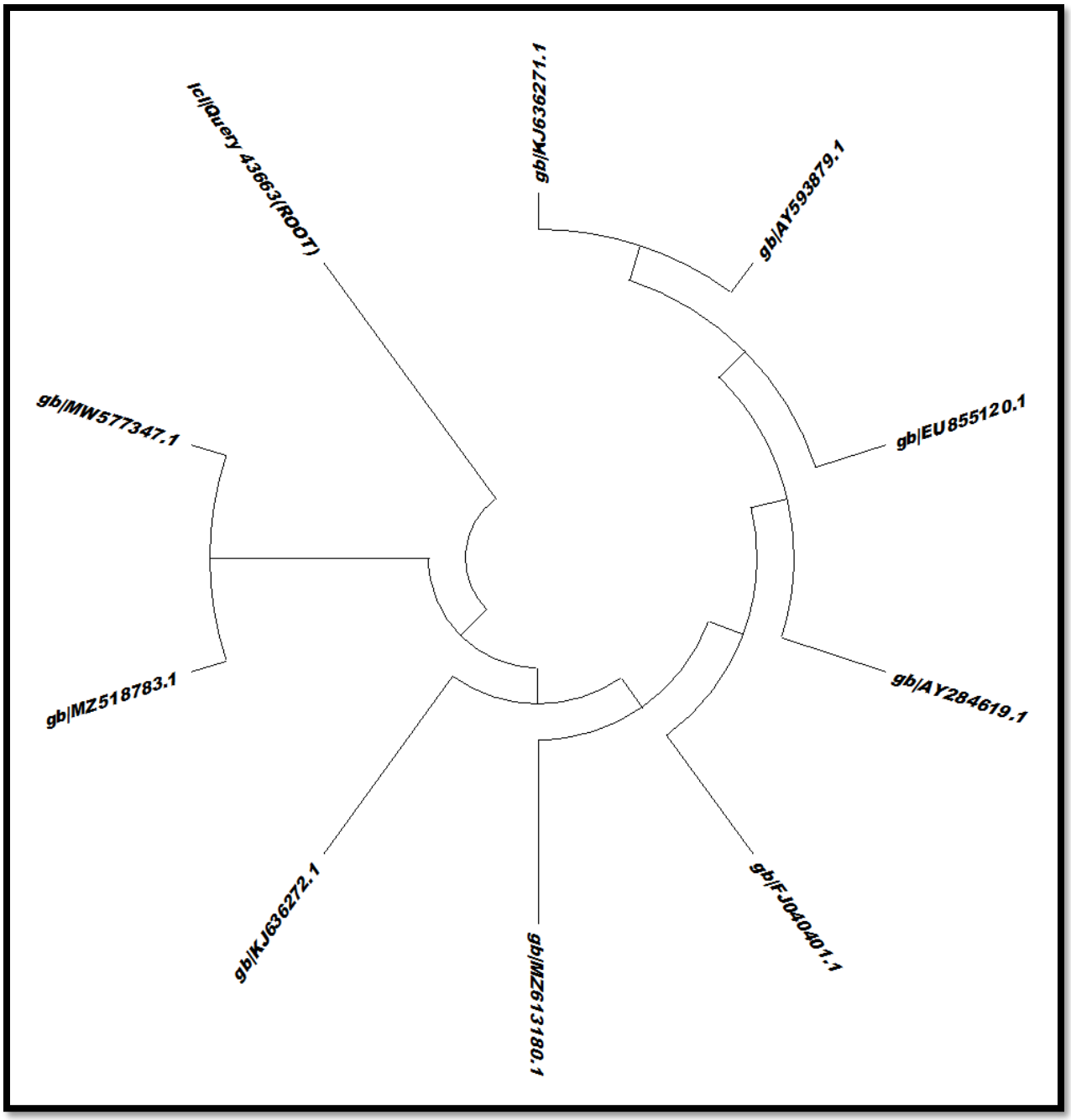


Figure 35: Phylogenetic tree showing the closest relationship of *Globodera rostochiensis*, population collected from district Chamba, Himachal Pradesh designated (MW577347.1).

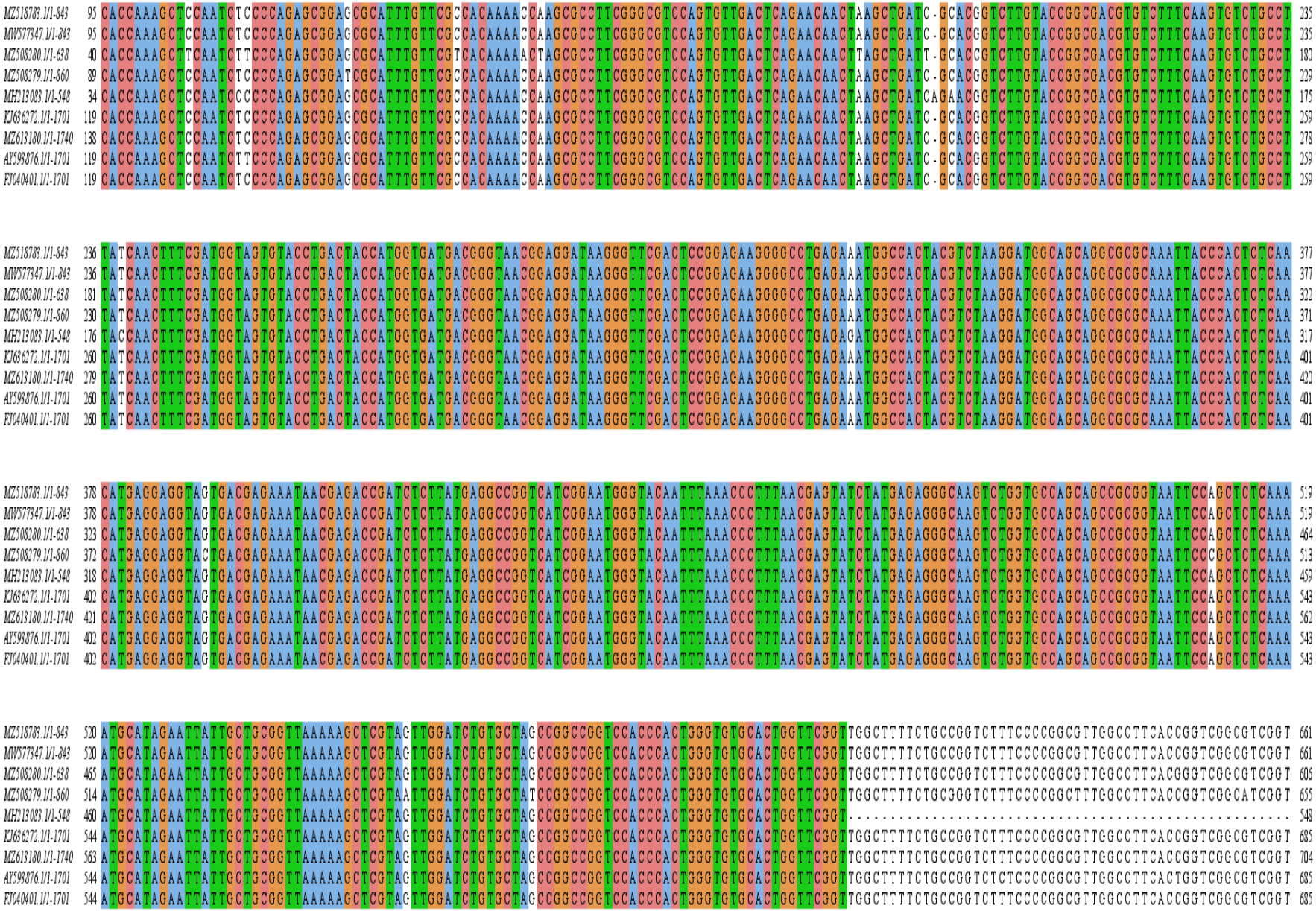

MZ518783.1/1-843 95 CACCAAAGCTCCAATCTCCCCAGAGCGGAGCGCATTTGTTCGCCACAAAACCAAGCGCCTTCGGGCGTCCAGTGTTGACTCAGAACAACTAAGCTGATC-GCACGGTCTTGTACCGGCGACGTGTCTTTCAAGTGTCTGCCT 235
MW577347.1/1-843 95 CACCAAAGCTCCAATCTCCCCAGAGCGGAGCGCATTTGTTCGCCACAAAACCAAGCGCCTTCGGGCGTCCAGTGTTGACTCAGAACAACTAAGCTGATC-GCACGGTCTTGTACCGGCGACGTGTCTTTCAAGTGTCTGCCT 235
MZ508280.1/1-638 40 CACCAAAGCTTCAATCTTCCCAGAGCGGAGCGCATTTGTTCGTCACAAAAACTAGCGCCTTCGGGCGTCCAGTGTTGACTCAGAACAACTTAGCTGATT-GCACCGTCTTGTACCGGCGACGTGTCTTTCAAGTGTCTGCCT 180
MZ508279.1/1-860 89 CACCAAAGCTCCAATCTCCCCAGAGCGGATCGCATTTGTTCGCCACAAAACCAAGCGCCTTCGGGCGTCCAGTGTTGACTCAGAACAACTAAGCTGATC-GCACGGTCTTGTACCGGCGACGTGTCTTTCAAGTGTCTGCCT 229
MH213083.1/1-548 34 CACCAAAGCTCCAATCCCCCCAGAGCGGAGCGCATTTGTTCGCCACAAAACCAAGCGCCTTCGGGCGTCCAGTGTTGACTCAGAACAACTAAGCTGATCAGAACGGTCTTGTACCGGCGACGTGTCTTTCAAGTGTCTGCCT 175
KJ696272.1/1-1701 119 CACCAAAGCTCCAATCTCCCCAGAGCGGAGCGCATTTGTTCGCCACAAAACCAAGCGCCTTCGGGCGTCCAGTGTTGACTCAGAACAACTAAGCTGATC-GCACGGTCTTGTACCGGCGACGTGTCTTTCAAGTGTCTGCCT 259
MZ613180.1/1-1740 138 CACCAAAGCTCCAATCTCCCCAGAGCGGAGCGCATTTGTTCGCCACAAAACCAAGCGCCTTCGGGCGTCCAGTGTTGACTCAGAACAACTAAGCTGATC-GCACGGTCTTGTACCGGCGACGTGTCTTTCAAGTGTCTGCCT 278
AY593876.1/1-1701 119 CACCAAAGCTCCAATCTTCCCAGAGCGGAGCGCATTTGTTCGCCACAAAACCAAGCGCCTTCGGGCGTCCAGTGTTGACTCAGAACAACTAAGCTGATC-GCACGGTCTTGTACCGGCGACGTGTCTTTCAAGTGTCTGCCT 259
FJ040401.1/1-1701 119 CACCAAAGCTCCAATCTCCCCAGAGCGGAGCGCATTTGTTCGCCACAAAACCAAGCGCCTTCGGGCGTCCAGTGTTGACTCAGAACAACTAAGCTGATC-GCACGGTCTTGTACCGGCGACGTGTCTTTCAAGTGTCTGCCT 259
MZ518783.1/1-843 236 TATCAACTTTCGATGGTAGTGTACCTGACTACCATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACTCCGGAGAAGGGGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTCAA 377
MW577347.1/1-843 236 TATCAACTTTCGATGGTAGTGTACCTGACTACCATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACTCCGGAGAAGGGGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTCAA 377
MZ508280.1/1-638 181 TATCAACTTTCGATGGTAGTGTACCTGACTACCATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACTCCGGAGAAGGGGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTCAA 322
MZ508279.1/1-860 230 TATCAACTTTCGATGGTAGTGTACCTGACTACCATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACTCCGGAGAAGGGGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTCAA 371
MH213083.1/1-548 176 TACCAACTTTCGATGGTAGTGTACCTGACTACCATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACTCCGGAGAAGGGGCCTGAGAGATGGCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTCAA 317
KJ696272.1/1-1701 260 TATCAACTTTCGATGGTAGTGTACCTGACTACCATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACTCCGGAGAAGGGGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTCAA 401
MZ613180.1/1-1740 279 TATCAACTTTCGATGGTAGTGTACCTGACTACCATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACTCCGGAGAAGGGGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTCAA 420
AY593876.1/1-1701 260 TATCAACTTTCGATGGTAGTGTACCTGACTACCATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACTCCGGAGAAGGGGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTCAA 401
FJ040401.1/1-1701 260 TATCAACTTTCGATGGTAGTGTACCTGACTACCATGGTGATGACGGGTAACGGAGGATAAGGGTTCGACTCCGGAGAAGGGGCCTGAGAAATGGCCACTACGTCTAAGGATGGCAGCAGGCGCGCAAATTACCCACTCTCAA 401
MZ518783.1/1-843 378 CATGAGGAGGTAGTGACGAGAAATAACGAGACCGATCTCTTATGAGGCCGGTCATCGGAATGGGTACAATTTAAACCCTTTAACGAGTATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAA 519
MW577347.1/1-843 378 CATGAGGAGGTAGTGACGAGAAATAACGAGACCGATCTCTTATGAGGCCGGTCATCGGAATGGGTACAATTTAAACCCTTTAACGAGTATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAA 519
MZ508280.1/1-638 323 CATGAGGAGGTAGTGACGAGAAATAACGAGACCGATCTCTTATGAGGCCGGTCATCGGAATGGGTACAATTTAAACCCTTTAACGAGTATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAA 464
MZ508279.1/1-860 372 CATGAGGAGGTACTGACGAGAAATAACGAGACCGATCTCTTATGAGGCCGGTCATCGGAATGGGTACAATTTAAACCCTTTAACGAGTATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCCGCTCTCAAA 513
MH213083.1/1-548 318 CATGAGGAGGTAGTGACGAGAAATAACGAGACCGATCTCTTATGAGGCCGGTCATCGGAATGGGTACAATTTAAACCCTTTAACGAGTATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAA 459
KJ696272.1/1-1701 402 CATGAGGAGGTAGTGACGAGAAATAACGAGACCGATCTCTTATGAGGCCGGTCATCGGAATGGGTACAATTTAAACCCTTTAACGAGTATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAA 543
MZ613180.1/1-1740 421 CATGAGGAGGTAGTGACGAGAAATAACGAGACCGATCTCTTATGAGGCCGGTCATCGGAATGGGTACAATTTAAACCCTTTAACGAGTATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAA 562
AY593876.1/1-1701 402 CATGAGGAGGTAGTGACGAGAAATAACGAGACCGATCTCTTATGAGGCCGGTCATCGGAATGGGTACAATTTAAACCCTTTAACGAGTATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAA 543
FJ040401.1/1-1701 402 CATGAGGAGGTAGTGACGAGAAATAACGAGACCGATCTCTTATGAGGCCGGTCATCGGAATGGGTACAATTTAAACCCTTTAACGAGTATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAA 543
MZ518783.1/1-843 520 ATGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGTGCTAGCCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGTTGGCTTTTCTGCCGGTCTTTCCCCGGCGTTGGCCTTCACCGGTCGGCGTCGGT 661
MW577347.1/1-843 520 ATGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGTGCTAGCCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGTTGGCTTTTCTGCCGGTCTTTCCCCGGCGTTGGCCTTCACCGGTCGGCGTCGGT 661
MZ508280.1/1-638 465 ATGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGTGCTAGCCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGTTGGCTTTTCTGCCGGTCTTTCCCCGGCGTTGGCCTTCACGGGTCGGCGTCGGT 606
MZ508279.1/1-860 514 ATGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTAATTGGATCTGTGCTATCCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGTTGGCTTTTCTGCGGGTCTTTCCCCGGCTTTGGCCTTCACCGGTCGGCATCGGT 655
MH213083.1/1-548 460 ATGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGTGCTAGCCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGT---------- 548
KJ696272.1/1-1701 544 ATGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGTGCTAGCCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGTTGGCTTTTCTGCCGGTCTTTCCCCGGCGTTGGCCTTCACCGGTCGGCGTCGGT 685
MZ613180.1/1-1740 563 ATGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGTGCTAGCCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGTTGGCTTTTCTGCCGGTCTTTCCCCGGCGTTGGCCTTCACCGGTCGGCGTCGGT 704
AY593876.1/1-1701 544 ATGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGTGCTAGCCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGTTGGCTTTTCTGCCGGTCTCTCCCCGGCGTTGGCCTTCACTGGTCGGCGTCGGT 685
FJ040401.1/1-1701 544 ATGCATAGAATTATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGTGCTAGCCGGCCGGTCCACCCACTGGGTGTGCACTGGTTCGGTTGGCTTTTCTGCCGGTCTTTCCCCGGCGTTGGCCTTCACCGGTCGGCGTCGGT 685

Figure 36: Multiple Sequence Alignment of 5 sequences of *Globodera* spp. containing small subunit 18S gene highly conserved regions of *Globodera rostochiensis* MZ518783.1 (Himachal Pradesh), *Globodera rostochiensis* MW577347.1 (Himachal Pradesh), *Globodera rostochiensis* MZ508280.1 (Himachal Pradesh), *Globodera rostochiensis* MZ508279.1 (Himachal Pradesh), *Globodera rostochiensis* MH213083.1 (Tamil Nadu), *Globodera rostochiensis* KJ636272.1 (Netherlands), *Globodera rostochiensis* MZ613180.1 (China), *Globodera pallida* AY593876.1 (China), *Globodera tabacum* FJ040401.1 (China). Coloured sequences show highly conserved regions between Himachal Pradesh, Tamil Nadu, Netherlands and China.

6.3.4. Molecular analysis of *Heterodera avenae*

From the isolates of *Heterodera* cysts, PCR analysis was performed by using a set of primers Subbotin *et al*. (2003). ITS1-5.8S-ITS2 was used for the sequence analysis. Amplification of ITS region yielded approximately 960bp single fragment for the cyst population collected from the wheat field of district Una. Our studies found that ITS1-5.8S-ITS2 (ITS region) was 960 characters in the district Una population. The resulted nucleotide sequence was submitted to GenBank accession number OM049243.1. OM049243.1 was BLAST in NCBI and showed the highest similarity with *H. avenae* population from Morocco and Turkey. Therefore, these results indicate that the cysts populations collected from district Una were *Heterodera avenae*. Phylogenetic analysis revealed that OM049243.1 were more similar to KC736872.1, KM199828.1, KM199828.1, OL828553.1, OL828552.1, OL828551.1, OL828550.1, OL828549.1, and OL828548.1 *Heterodera avenae* population from Morocco and Turkey (Fig. 24). OM049243.1 showed some similarities with H. *filipjevi*. Multiple sequence alignment was performed on Jalview, to study the conserved sequence in the ITS1-5.8S-ITS2 and partial 18S and 28S regions present between *Heterodera avenae* OM049243.1(Himachal Pradesh), *Heterodera filipjevi* HG738845.1 (Himachal Pradesh), *Heterodera avenae* HG738844.1 (Delhi), *Heterodera avenae* HG738846.1(Leh) and *Heterodera avenae* KC736872.1(Morocco). Multiple sequence alignment analysis

revealed that *H. avenae* Himachal Pradesh populations shared more conserved ITS regions with Morocco populations. However, in the case of Indian populations, Himachal Pradesh *H. avenae* populations shared more conserved ITS regions with Delhi populations compared to Leh populations. Multiple sequence alignment of *H. avenae* populations and *H. filipjevi* populations showed some conserved regions present in ITS1- 5.8S- ITS2 regions of both species (Fig. 25).

For *Heterodera avenae* group Krall and Krall, (1978) gave a generic name called Bidera. Whereas Mulvey and Golden, (1983) differentiated the *Heterodera avenae* group with other *Heterodera* species. Bishnoi and Bajaj, (2004) compared taxonomically 8 cereal cyst nematode (CCN) populations from 3 different states, namely Himachal Pradesh, Rajasthan and Punjab, observed significant differences in morphological characters. One species of *H. avenae* group, namely *H. filipjevi* was reported in the wheat field of Himachal Pradesh. This report supports our findings that genus *Heterodera* has already infested the wheat fields. Our study found that *Heterodera avenae* from district Una Himachal Pradesh had a full length ITS region of 960 characters. Devindrappa *et al*. (2020) reported the data of ITS region 971 characters from district Kangra. However, we obtained 960 characters from district Una using the same primers. This may be due to the variation within the ingroup taxa. Subbotin *et al*. (2003) reported 998 characters in full length ITS region in *Heterodera avenae* group. Rao *et al*. (2013) used PCR-RFLP r-DNA sequencing to study the homogeneity of Indian populations of *H. avenae.* They conclude that the Indian population of *H. avenae* was genetically homogeneous, and it is appropriate for next-generation sequencing. At the same time, Subbotin *et al*. (1999) reported intraspecific polymorphism in ITS region within *H. avenae*. They compared the Indian population with the Spain population.

Subbotin *et al*. (1999) identified two ITS-regions types: European populations type A, Indian populations type B, and French populations combination of type A+B. They also stated that Indian populations of *H. avenae* group differed from other populations. In our studies, a slight difference in the ITS characters was

observed. Subbotin *et al*. (2003) discussed that CCN population is morphologically distributed in 2 main groups within 4 clades. The first group consists of 3 clades, and the second group consists of the single clade. The first main group represents the populations of Asia and Africa, France and Europe. At the same time, the second group represents populations from China. The second group indicates another evolutional branch of cereal cyst nematodes.

6.3.5. Molecular analysis of *Cactodera estonica*

PCR analysis was carried out from the isolates of *Cactodera* cysts using a set of primers Vrain *et al*. (1992). ITS1-5.8S-ITS2 was used for the sequence analysis. Amplified ITS region yielded approximately 1050 bp single fragment for the cyst population collected from district Mandi. Our studies found that ITS1-5.8S-ITS2 (ITS region) was 1050 characters in district Mandi. The resulted nucleotide sequence was submitted to GenBank accession number MN658364.1. MN658364.1 was BLAST in NCBI and showed the highest similarity with *C. estonica* populations from China. Cyst populations collected from district Chamba amplified ITS region yielded a single fragment of 1067 bp. Our studies found that ITS1-5.8S-ITS2 (ITS region) was 1067 characters in district Chamba. The obtained sequence was submitted to GenBank accession number MW821356.1. The resulted nucleotide sequence (MW821356.1) was BLAST in NCBI and showed the highest similarity with *C. estonica* population from Himachal Pradesh and China. The amplified ITS region yielded a single fragment of approximately 1065 bp for district Shimla. Our studies found that ITS1-5.8S-ITS2 (ITS region) was 1067 characters in district Shimla. The obtained sequence was submitted to GenBank accession number MW821355.1. The resulted nucleotide sequence (MW821355.1) was BLAST in NCBI and showed the highest similarity with *C. estonica* population from China. Multiple sequence alignment was performed on Jalview, to study the conserved sequence in the ITS1-5.8S-ITS2 and partial 18S and 28S regions present.

Therefore, the results discussed above indicate that the lemon-shaped cyst population collected from district Mandi (HPMD), Chamba (HPCH) and district Shimla (HPSH) was *Cactodera estonica*. Phylogenetic studies indicate that *C. estonica* collected from district Mandi, Chamba and Shimla showed the highest similarity with each other. Individually MN658364.1 district Mandi (HPMD) sequence showed high similarity with KC771888.1, HM560730.1, JN684904.1, EU106164.1 and KC771889.1 *C. estonica* populations from China (Fig. 27). District Chamba (HPCH) MW821356 showed high similarity with KC771888.1 and KC771889.1 *C. estonica* population from China, OK036675.1 and OK036672.1 *C. estonica* population from Canada (Fig. 28). District Shimla sequence MW821355.1 showed high similarity with KC771888.1, KC771889.1, HM560732 and HM560730 *C. estonica* populations from China (Fig. 29). MN658364.1, MW821356.1 and MW821355.1 were similar to *C. milleri* and *C. torreyanae*. Multiple sequence alignment was performed on Jalview to study the conserved sequence in the ITS1-5.8S-ITS2 and partial 18S and 28S regions present between *Cactodera estonica* MN658364.1 (Himachal Pradesh), *Cactodera estonica* MW821356.1(Himachal Pradesh), *Cactodera estonica* MW821355.1 (Himachal Pradesh), *Cactodera estonica* MF537581.1 (Tamil Nadu), *Cactodera estonica* KC771888.1 (China), *Cactodera milleri* OK036672.1 (Canada), *Cactodera torreyanae* OK036692.1 (Canada). Multiple sequence alignment analysis revealed that *C. estonica* Himachal Pradesh populations shared more conserved regions with Tamil Nadu populations than other populations. However, Himachal Pradesh *Cactodera* populations shared more conserved regions with *C. estonica* from China populations than Canadian populations. Multiple Sequence Alignment between *C. estonica*, *C. milleri* and C. *torreyanae* showed some conserved regions present between all three *Cactodera* species at ITS1-5.8S- ITS2 regions (Fig. 30).

Studies conducted by Saranya *et al*. (2018) revealed that amplified ITS-rDNA yielded approximately a single fragment of 735bp. Amplified ITS-rDNA of

C. estonica population yielded a single fragment of 1050bp approximately from district Mandi, 1065 bp from district Chamba and 1067 bp from district Shimla. Hence, the study conducted by Saranya *et al*. (2018) supports agreed with our work. Multiple sequence alignment revealed some similarity in the ITS-rDNA sequence of *C. estonica* MN658364.1, MW821356.1 and MW821355.1 with *C. milleri* and *C. torreyanae*. The present study reported the first time occurrence of *C. estonica* in Himachal Pradesh. The first report of *C. estonica* in India was given by Saranya *et al*. (2018). They discussed that there is an occurrence of *C. estonica* in India, which supports our study. Yu and Sun, (2018) also used ITS-rDNA sequences of *C. estonica*, to study the conserved sequences in *C. estonica*. They revealed that the amplified ITS region of *C. estonica* was 1480 bp in size. Hence, Yu and Sun, (2018) support the present study.

6.3.6. Molecular analysis of *Globodera rostochiensis*

From the isolates of *Globodera* cysts PCR analysis was carried out by using a set of primers Floyd *et al*. (2002), 18S small subunit was used for the sequence analysis. A single fragment of 638 bp was yielded from district Kangra. Our studies found that 18S small subunit was 638 characters in district Kangra. The obtained sequence was submitted to GenBank accession number MZ508280.1. The resulted nucleotide sequence (MZ508280.1) was BLAST in NCBI and showed the highest similarity with *G. rostochiensis* populations from the Netherlands. For district Mandi isolates, a single fragment of 843 bp was yielded. Our studies found that 18S small subunit was 843 characters in district Mandi. The obtained sequence was submitted to GenBank accession number MZ518783.1. The resulted nucleotide sequence (MZ518783.1) was BLAST in NCBI and showed the highest similarity with *G. rostochiensis* populations from the Netherlands. For district Kullu isolates, a single fragment of 860 bp was yielded. Our studies found that 18S small subunit was 860 characters in district Kullu. The obtained sequence was submitted to GenBank accession number MZ508279.1. The resulted nucleotide sequence (MZ508279.1) was BLAST in NCBI and showed the highest similarity with *G.*

rostochiensis populations from the Netherlands. For district Chamba isolates, a single fragment of 843 bp was yielded. Our studies found that 18S small subunit was 843 characters in district Chamba. The obtained sequence was submitted to GenBank accession number MW577347.1. The resulted nucleotide sequence (MW577347.1) was BLAST in NCBI, and showed the highest similarity with *G. rostochiensis* populations from Netherlands.

Therefore, the results discussed above indicate that the globose shaped cyst population collected from district Kangra (HPPOPL), Mandi (HPPOMD), Chamba (HPPOCH) and district Kullu (HPPOKL) was *Globodera rostochiensis*. Phylogenetic studies indicate that G. rostochiensis collected from districts Kangra, Mandi, Chamba and Kullu showed the highest similarity. MZ508280.1, MZ518783.1, MZ508279.1 and MW577347.1 showed similarities with the KJ636272.1 *G. rostochiensis* population from the Netherlands and *G. rostochiensis* population from China (Fig.32,33,34and35). MZ508280.1, MZ518783.1, MZ508279.1 and MW577347.1 are similar to *G. pallida* and *G. tabacum.* Multiple sequence alignment was performed on Jalview, to study the conserved regions in 18S small subunit of *G. rostochiensis* MZ518783.1 (Himachal Pradesh), *G. rostochiensis* MW577347.1 (Himachal Pradesh), *G. rostochiensis* MZ508280.1 (Himachal Pradesh), *G. rostochiensis* MZ508279.1 (Himachal Pradesh), *G. rostochiensis* MH213083.1 (Tamil Nadu), *G. rostochiensis* KJ636272.1 (Netherlands), *G. rostochiensis* MZ613180.1 (China), *G. pallida* AY593876.1 (China), *G. tabacum* FJ040401.1 (China).

Multiple sequence alignment analysis revealed that *G. rostochiensis* Himachal Pradesh populations shared more conserved regions at small subunit 18S with *G. rostochiensis* populations from the Netherlands, *G. rostochiensis* populations from China and *G. rostochiensis* populations from Tamil Nadu than *G. pallida* and *G. tabacum*. Furthermore, multiple sequence alignment showed that in *G. rostochiensis, G. pallida* and *G. tabacum* populations, some conserved regions

were also present in all three *Globodera* species at small subunit 18S r-RNA (Fig. 36).

This study explored the sequences of 5' segments of the small subunit rRNA gene (SSU-rRNA) repeats. Small subunit rRNA/ 18S data set for plant-parasitic nematodes is unique in a particular species, to explore the phylogenetic diversity of a large number of identified specimen's various sequences are available as discussed by Blaxter *et al*. (1998). Hence Blaxter *et al*. (1998) support our molecular work. Phylogenetic analysis based on 18S rRNA gene, isolates from *Globodera* cysts were closely related to *G. rostochiensis* reported from Netherlands. These primers were used for the amplification of an internal fragment of 18S gene. At 5' end approximate 100 bp inward, the forward primer binds, and the reverse primer binds at 700bp towards inside from 3' end. Almost half of the 18S gene has covered by 5' fragment, making it a functional region for barcoding. Floyd *et al*. (2005) also mentioned the accurate amplification of 18S gene by nematode primers which completely agreed with our molecular analysis for *G. rostochiensis*. The present study reveals that the amplified 18S rRNA gene, a single fragment of 638 bp *G. rostochiensis* population from district Kangra were obtained, 843 bp *G. rostochiensis* population from district Mandi were obtained, 860 bp *G. rostochiensis* population from district Kullu were obtained, 843 bp *G. rostochiensis* population for district Chamba were obtained.

Our data suggest that the genus *Heterodera* is well differentiated from *Globodera* and *Cactodera*. ITS-rDNA data differentiated *H. avenae* from other species and *C. estonica* from other genera *Cactodera*. Molecular characterization of cysts nematodes by ITS-rDNA and 18S rRNA small ribosomal subunit SSU helps us understand the range of taxa. The results sequences help us with phylogenetic analysis. The above findings confirm the usefulness of gene and internal transcribed spacer region r-DNA for the systemic identification of different genera in cyst nematodes. If we have to conclude this study, we conclude that SSU and ITS regions are too conserved and useful for nematodes' phylogenetic analyses. The technique used in this study provides us with the best

knowledge to molecular approach for identifying cysts' nematodes. Molecular characterization is a reliable alternative to morphological characterization, especially for cysts nematodes whose identification through morphological characters is more complicated and complex.

Reliable identification of species based only on morphological characteristics is difficult due to the many variations in the group of species. Sequencing is the best way to identify and find out the relationships at the species level to resolve this problem. The present study used internal transcribed spacers ITS1 & ITS2 regions and small nuclear ribosomal unit 18S gene for molecular sequencing. Subbotin *et al*. (2001) proved that ITS regions and 18S small subunit were beneficial and practical for the phylogenetic study of cyst nematodes. Once the morphological characterization was done.

6.4. *In-vitro* control of cysts nematodes

In-vitro efficiency of *Verticillium lecanii* was evaluated against cyst nematodes. Different treatments *viz*., (Treatment) T1-10%, T2-20%, and T3-30% cell suspension of 1.7×10^5 CFUs / ml of *V. lecanii* was observed at particular hours after inoculation (HAI) against cysts of *C. estonica*, *G. rostochiensis* and *H. avenae*. The experiment was continued until the complete disintegration of cysts occurred. Then, the number and percentage of cysts leftover after each time interval were calculated. The experiment was terminated when complete disintegration of cysts was observed. Statistical analysis was done by univariate, Tukey test.

6.4.1. *In-vitro* effect of *Verticillium lecanii* on the cysts of *Cactodera estonica*:

In-vitro effect of *V. lecanii* was observed on the cysts of *C. estonica* after the given intervals of time, i.e., 8, 24, 48, and 72 HAI (Hours after inoculation). The attack of *V. lecanii* on the cyst wall of *C. estonica* was noticed within 8 hours (Fig. 38a), followed by heavy colonization of fungus on the cyst wall of the cyst within 24 hours (Fig. 38b). However, no cyst wall changes were observed at 8 or 24 hours.

Moreover, according to univariate Tukey test, no significant differences were observed in the number of cysts left after 8 and 24 HAI. However, at 48 HAI, significant differences were noted in the number of cysts left (F = 45.427, df = 3, 20, $P < 0.001$) compared to untreated control. In untreated control, no changes were observed on the cyst of *C. estonica* after the given time intervals, i.e., 8, 24, 48, and 72 HAI. The damage to the cyst wall was evident in the *C. estonica* cyst treated with *V. lecanii* treatments compared to the untreated control (Fig. 38c). Similarly, at 72 HAI the significant differences were noted in number of cysts left (F = 253.808, df = 3, 20, $P < 0.001$). The observations were terminated after 72 hours, as the entire cysts were disintegrated from the higher concentration (Treatment 3) of *V. lecanii*. Only the debris was seen at the bottom of the Petri dishes (Fig. 38d). At 72 HAI, the minimum number of the cyst was noted in 30% *V. lecanii* suspension (00.00%), followed by 20% *V. lecanii* suspension (6.89%) and 10% *V. lecanii* suspension (13.44%) compared to untreated control (100% normal cysts) (Table 31). The percentages of leftover cysts were found inversely proportional to the concentration of cell suspension of *V. lecanii* and the duration of the exposure (Fig. 37).

Table 31. Effect of different concentrations of *Verticillium lecanii* on the percentage of leftover cysts of *Cactodera estonica* at different time intervals, i.e., 8, 24, 48 and 72 hours after inoculation (HAI).

S. No	Treatments	Leftover cysts (%)			
		8 HAI	**24 HAI**	**48 HAI**	**72 HAI**
1.	**T1**	100.00±00.00^{a}	100.00±0.40^{a}	70.00±0.83^{c}	13.44±0.51^{b}
2.	**T2**	100.00±00.00^{a}	100.00±00.00^{a}	50.00±0.52^{b}	6.89±0.52^{ab}
3.	**T3**	100.00±00.00^{a}	100.00±00.00^{a}	26.67±0.68^{a}	0.00±00.00^{a}
4.	**T4**	100.00±00.00^{a}	100.00±00.00^{a}	100.00±00.00^{d}	100.00±00.00^{c}

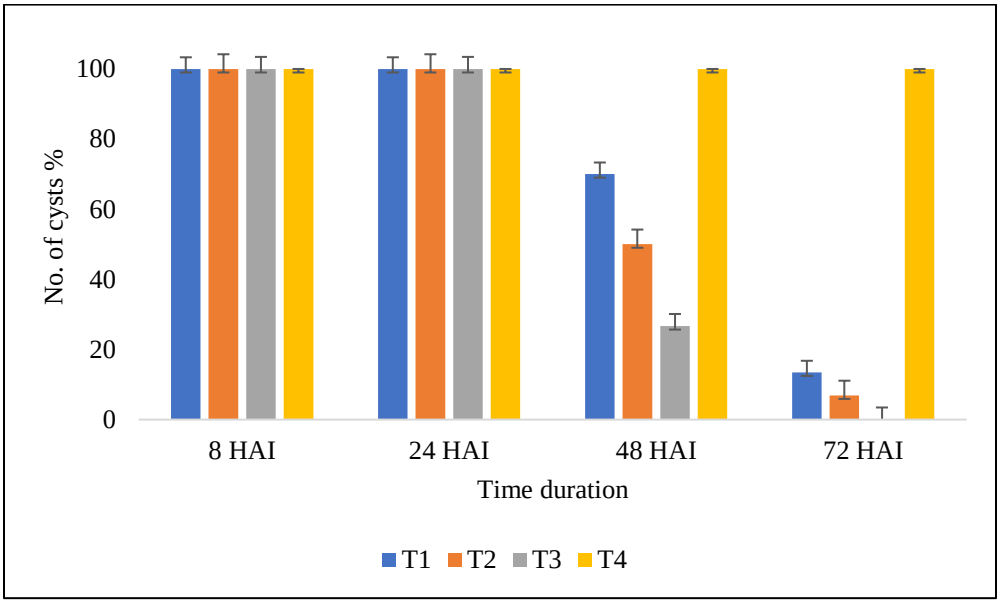


Figure 37: Graphical representation of the effect of different concentrations of *Verticillium lecanii* on the percentage of leftover cysts *Cactodera estonica* at different time intervals, i.e., 8, 24, 48 and 72 hours after inoculation (HAI)

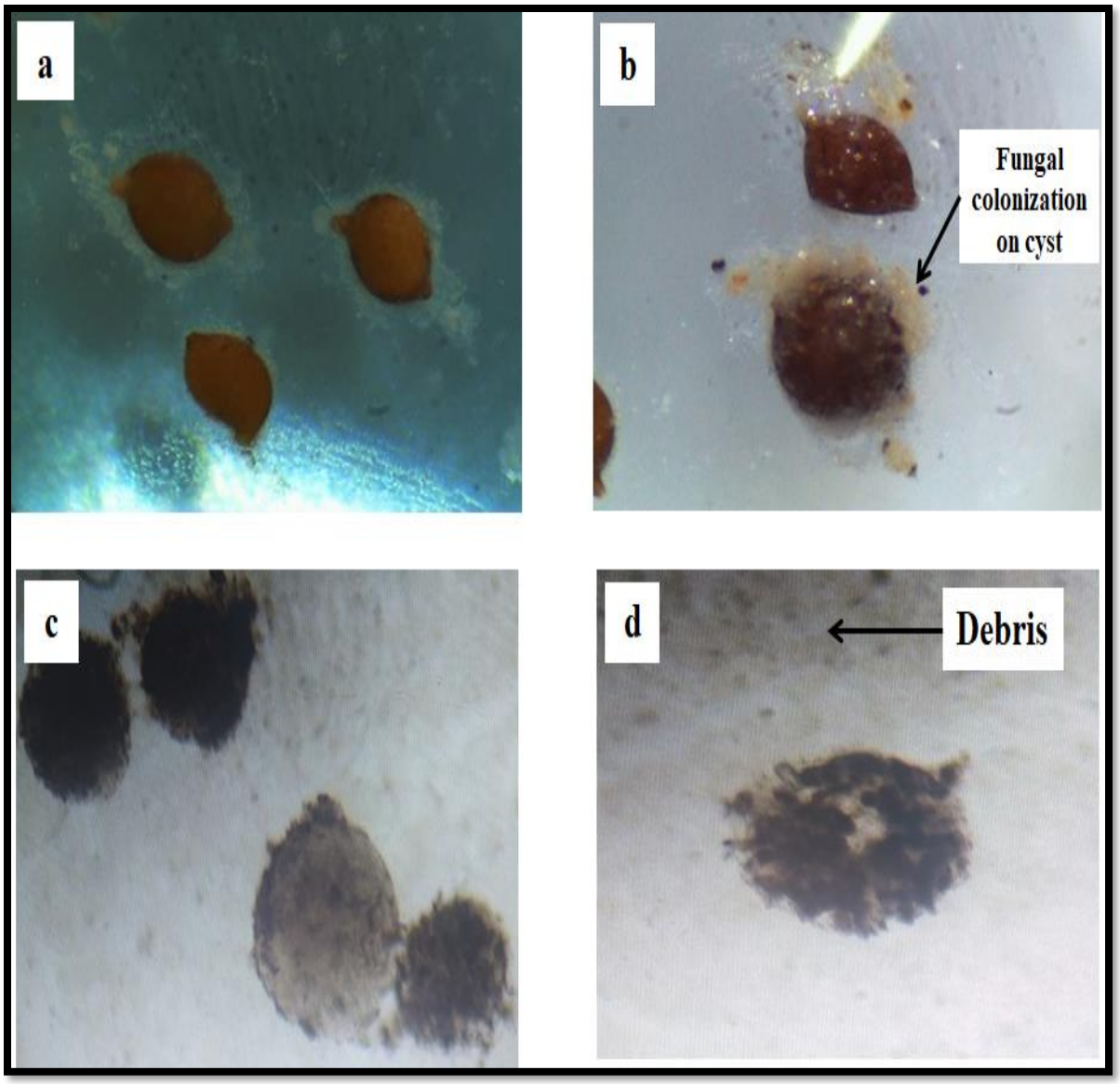


Figure 38: Effect of 30% concentrations of *V. lecanii* (Treatment 3) against the cyst of *Cactodera estonica* at different time intervals. **a.** attack of fungus on cyst at 8 HAI; **b.** heavy colonization of fungus on cyst at 24 HAI; **c.** disintegration of cyst wall at 48 HAI and **d.** the disintegration of cyst wall along with the debris of cyst body content at 72 HAI.

6.4.2. *In-vitro* effect of *Verticillium lecanii* on the cysts of *Globodera rostochiensis*

In vitro V. lecanii was observed on the *G. rostochiensis* cysts after a given time interval, i.e., 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 HAI. The attack of *V. lecanii* on the cyst wall of *G. rostochiensis* after 24 HAI (Fig. 40a). Heavy colonization of *V. lecanii* on the cyst wall was observed between 72 and 96 HAI (Fig. 40c and 40d). However, no change was seen on the cyst wall at 96 HAI. Moreover, no significant difference was observed in the number of cysts after 24, 48, 72, 96 and 120 HAI. At 144 HAI, no significant difference was observed (*P=0.413*) in the number of cysts left (Fig. 40f). However, at 168 HAI (Fig. 40g) univariate Tukey test indicates that in Treatment 3, there was a significant decrease in the number of cysts left (*F=15.000, df=3,20, P<0.001*) as compared to untreated control. According to univariate Tukey test at 192, 216, 240 HAI (Fig. 40h, 40i and 40j) significant difference in the number of cysts left was noted in T3 (*F=121.000, df=3,20, P<0.001*), (*F=80.667, df=3,20, P<0.001*), (*F=52.059, df = 3,20, P< 0.001*) compared to the untreated control. Univariate Tukey test at 192, 216, 240 HAI indicates no significant decrease in Treatment 1, Treatment 2 and Treatment 4. Still, Treatment 3 showed a significant decrease in the number of cysts left compared to Treatment 1, Treatment 2 and Treatment 4. The damage to the cyst wall was evident in the *G. rostochiensis* cyst treated with *V. lecanii* treatments compared to the untreated control. Similarly, at 264 HAI (Fig. 40k) significant difference in the number of cysts left was noted (*F=143.667, df= 3,20, P< 0.001*). Univariate Tukey test at 264 HAI indicates that in treatment 3, there is a significant difference in the number of cysts left. There is no significant difference between Treatment 2 and Treatment 1. The observations were terminated after 264 HAI as the entire cysts were disintegrated from the higher concentration (Treatment 3) of *V. lecanii.* Only the debris was seen at the bottom of the Petri dishes. At 264 HAI, the minimum number of cysts were noted in 30% *V. lecanii* suspension (3.34%), followed by 20% *V. lecanii* suspension (80%) and 10% *V. lecanii* suspension (93.34%) compared to untreated control (100% normal cysts) (Table 32). The

percentages of leftover cysts were found inversely proportional to the concentration of cell suspension of *V. lecanii* and the duration of the exposure (Fig. 39).

Table 32. Effect of different concentrations of *Verticillium lecanii* on the percentage of leftover cysts of *Globodera rostochiensis* at different time intervals, i.e., 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 hours after inoculation (HAI).

S.no	Treatments	Leftover cysts (%)											
		8 HAI	**24 HAI**	**48 HAI**	**72 HAI**	**96 HAI**	**120 HAI**	**144 HAI**	**168 HAI**	**192 HAI**	**216 HAI**	**240 HAI**	**264 HAI**
1.	**T1**	100.00 ±00.00[a]	100. 00±00. 00[a]	100.00 ±00.00[a]	100.00 ±00.00[a]	100. 00±00. 00[a]	100.00± **00.00**[a]	100. 00±00.0 0[b]	100.00± 00.00[b]	100.00± 00.00[b]	100.00± 00.00[b]	100.00± 00.00[b]	93.34±1 0.33 [c]
2.	**T2**	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00± 00.00[a]	100.00± 00.00[b]	100.00± 00.00[b]	100.00± 00.00[b]	96.66±8. 16[ab]	90±10.9 5[ab]	80±12.6 4 [b]
3.	**T3**	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00± 00.00[a]	96.67±8. 16[a]	80±12.6 4[a]	63.34±8. 16[a]	56.67±8. 16[a]	53.34±1 0.32[a]	3.34±8.1 7[a]
4.	**T4**	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00 ±00.00[a]	100.00± 00.00[a]	100.00± 00.00[b]	100.00± 00.00[b]	100.00± 00.00[b]	100.00± 00.00[b]	100.00± 00.00[b]	100.00± 00.00 [d]

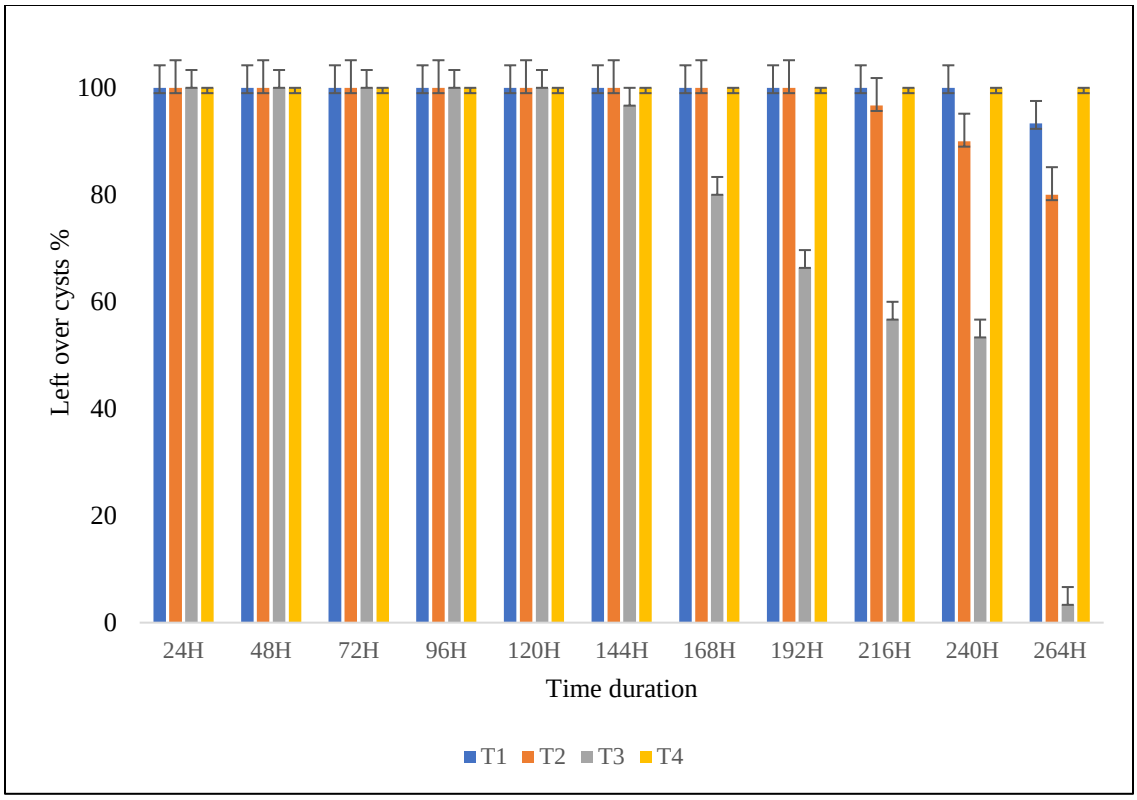
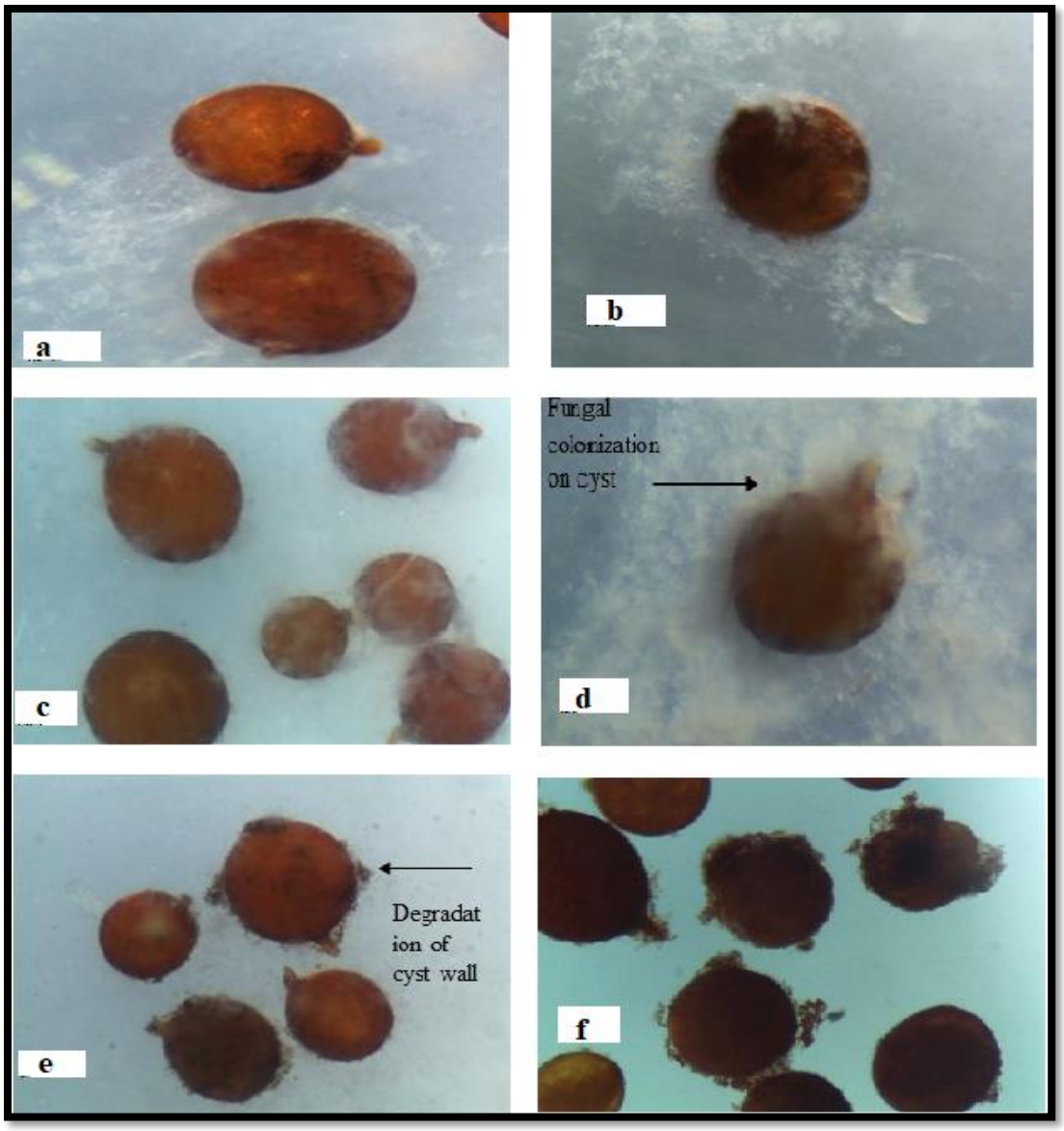


Figure 39: Graphical representation of the effect of different concentrations of *Verticillium lecanii* on the percentage of leftover cysts *Globodera rostochiensis* after different time intervals, i.e. 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 hours after inoculation (HAI).


a
b
c
Fungal colonization on cyst
d
Degradation of cyst wall
e
f

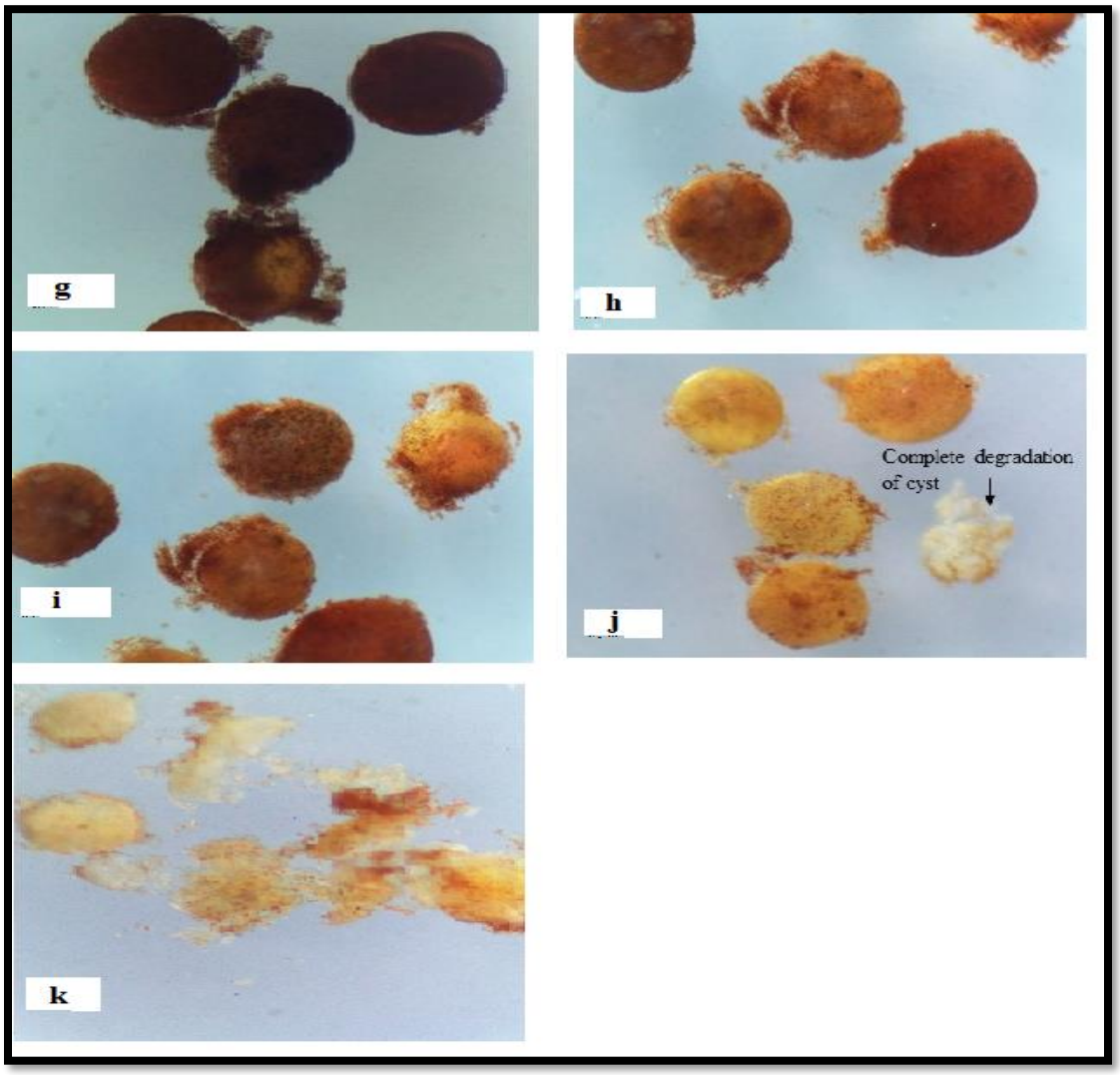


Figure 40: Effect of 30% concentrations of *V. lecanii* (Treatment 3) against the cyst of *Globodera rostochiensis* at different time intervals. **a**. fungal attack on cyst at 24 HAI; **b.** colonization of fungus on cyst at 48 HAI; **c.** heavy colonization of fungus on cyst at 72 HAI; **d.** degradation of cyst wall at 96 HAI; **e.** degradation of cysts wall at 120HAI; **f.** degradation of cysts wall at 144 HAI; **g.** degradation of cysts wall at 168 HAI; **h.** degradation of cysts walls at 192 HAI, **i.** degradation of cysts walls at 216 HAI, **j.** disintegration of cyst wall along with the debris of cyst body content at 240 HAI, **k.** complete degradation of the cyst at 264 HAI.

6.4.3. *In-vitro* effect of *Verticillium lecanii* on the cysts of *Heterodera avenae*

In- vitro effect of *V. lecanii* was observed on the *H. avenae* cysts after a given interval of time, i.e., 8, 24, 48, 72, 96, 120, 144 and 168 HAI. The attack of *V. lecanii* on the cyst wall of *H. avenae* after 24 HAI (Fig. 42a & 42b). Heavy colonization of *V. lecanii* on the cyst wall was observed at 48 HAI (Fig 42c). However, no change was seen on the cyst wall till 48 HAI. Moreover, no significant difference was observed in the number of cysts after 8 and 24 HAI. At 48 and 72 HAI, no significant difference was observed (*P=0.413*) (*P=0.089*) in the number of cysts left in each treatment (Fig. 42c & 42d). However, at 96 and 120 HAI (Fig. 42e and 42f) univariate Tukey test indicates that in treatment 3, there was a significant decrease in the number of cysts left (*F=40.000, df=3,20, P<0.001*) (*F=33.000, df=3,20, P<0.001*) as compared to untreated control. There was a significant difference in the number of cysts shown in Treatment 3 compared to the rest. The damage to the cyst wall was evident in the *H. avenae* cyst treated with *V. lecanii* treatments compared to the untreated control. Similarly, at 144 HAI (Fig. 42g) significant difference in the number of cysts left was noted (*F= 53.857, df = 3,20, P< 0.001*). Univariate Tukey test at 144 HAI indicates that in Treatments 3 and 2, there was a significant difference in the number of cysts left. There is no significant difference in the number of cysts in Treatment 1 and 4. At 168 HAI (Fig. 42h) considerable difference in the number of cysts left was noted (*F= 201.852, df = 3,20, P< 0.001*). The observations were terminated after 168 HAI as the entire cysts were disintegrated from the higher concentration (Treatment 3) of *V. lecanii.* Only the debris was seen at the bottom of the Petri dishes. At 168 HAI, the minimum number of the cyst was noted in 30% *V. lecanii* suspension (3.34%), followed by 20% *V. lecanii* suspension (73.34%) and 10% *V. lecanii* suspension (96.67%) compared to untreated control (100% normal cysts) (Table 33). The percentages of leftover cysts were found inversely proportional to the concentration of cell suspension of *V. lecanii* and the duration of the exposure (Fig.41).

Table 33. Effect of different concentrations of *Verticillium lecanii* on the percentage of leftover cysts of *Heterodera avenae* at different time intervals, i.e., 24, 48, 72, 96, 120, 144 and 168 hours after inoculation (HAI).

S.no	Treatments	Leftover cysts (%)							
		8 HAI	**24 HAI**	**48 HAI**	**72 HAI**	**96 HAI**	**120 HAI**	**144 HAI**	**168 HAI**
1.	**T1**	100±00.00^{a}	100±00.00^{a}	100±00.00^{b}	100±00.00^{b}	100±00.00^{b}	100±00.00^{b}	96.67±8.1^{c}	96.67±8.1^{bc}
2.	**T2**	100±00.00^{a}	100±00.00^{a}	100±00.00^{b}	100±00.00^{b}	100±00.00^{b}	96.67±8.1^{a}	73.34±10.32^{b}	73.34±10.32^{b}
3.	**T3**	100±00.00^{a}	100±00.00^{a}	96.67±8.1^{a}	93.34±10.32^{a}	83.34±15.05^{a}	76.67±8.1^{ab}	30±16.7^{a}	3.33±10.32^{a}
4.	**T4**	100±00.00^{a}	100±00.00^{a}	100±00.00^{b}	100±00.00^{b}	100±00.00^{b}	100±00.00^{b}	100±00.00^{d}	100±00.00^{c}

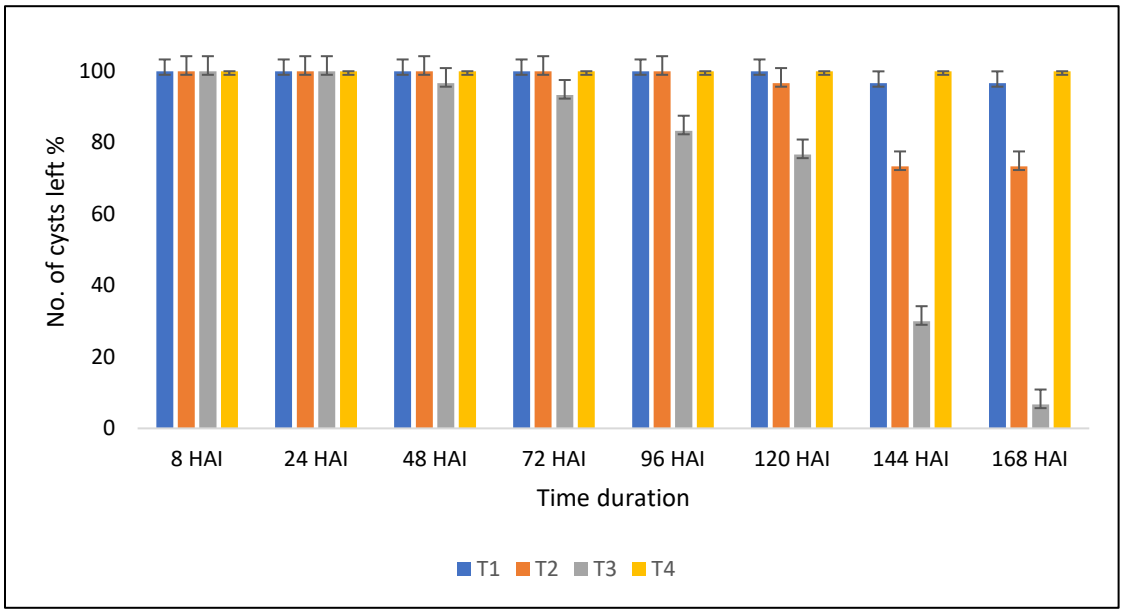
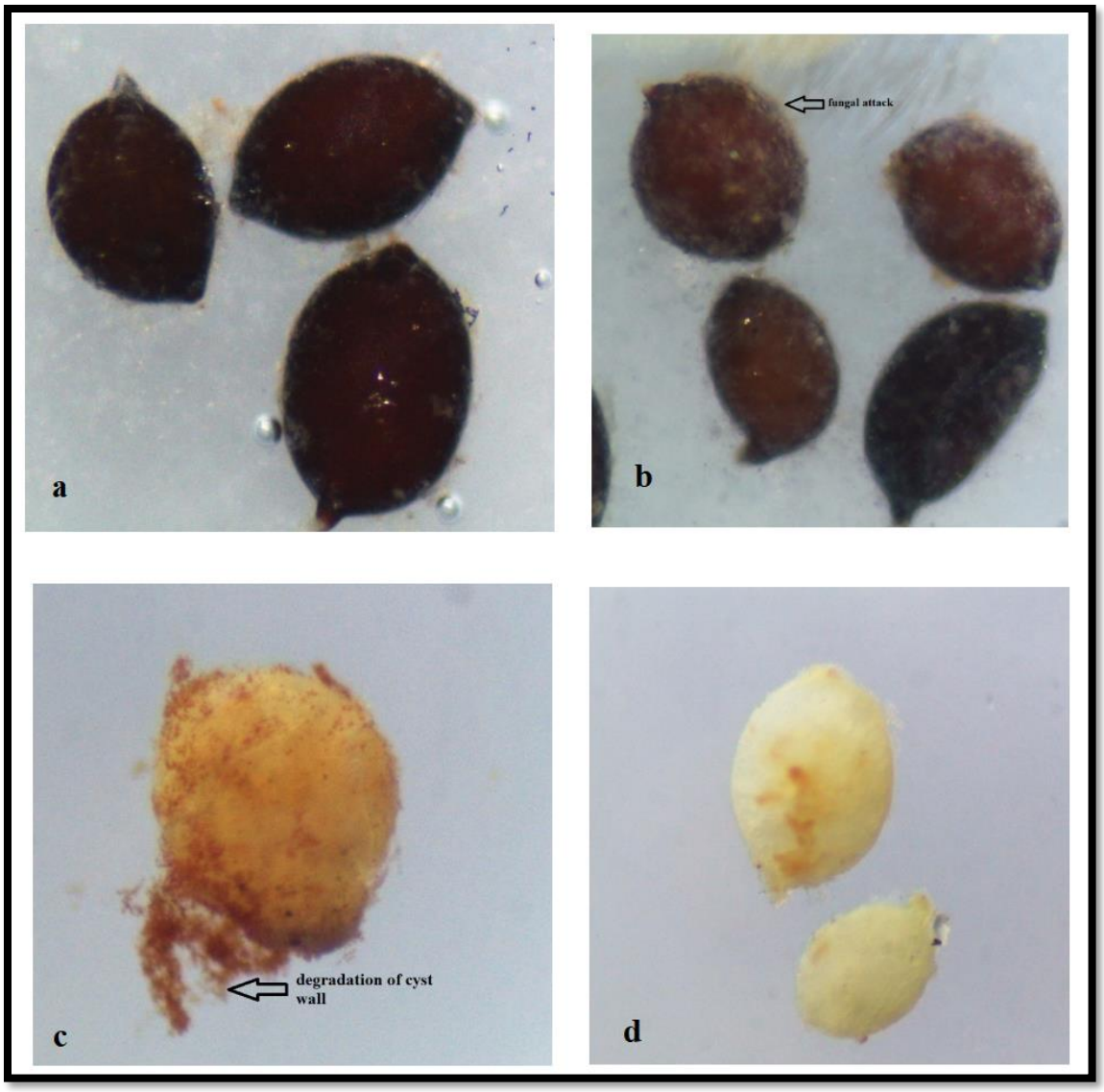


Figure 41: Graphical representation of the effect of different concentrations of *Verticillium lecanii* on the percentage of leftover cysts *Heterodera avenae* at different time intervals, i.e., 24, 48, 72, 96, 120, 144 and 168hours after inoculation (HAI).


a
b
fungal attack
c
degradation of cyst wall
d

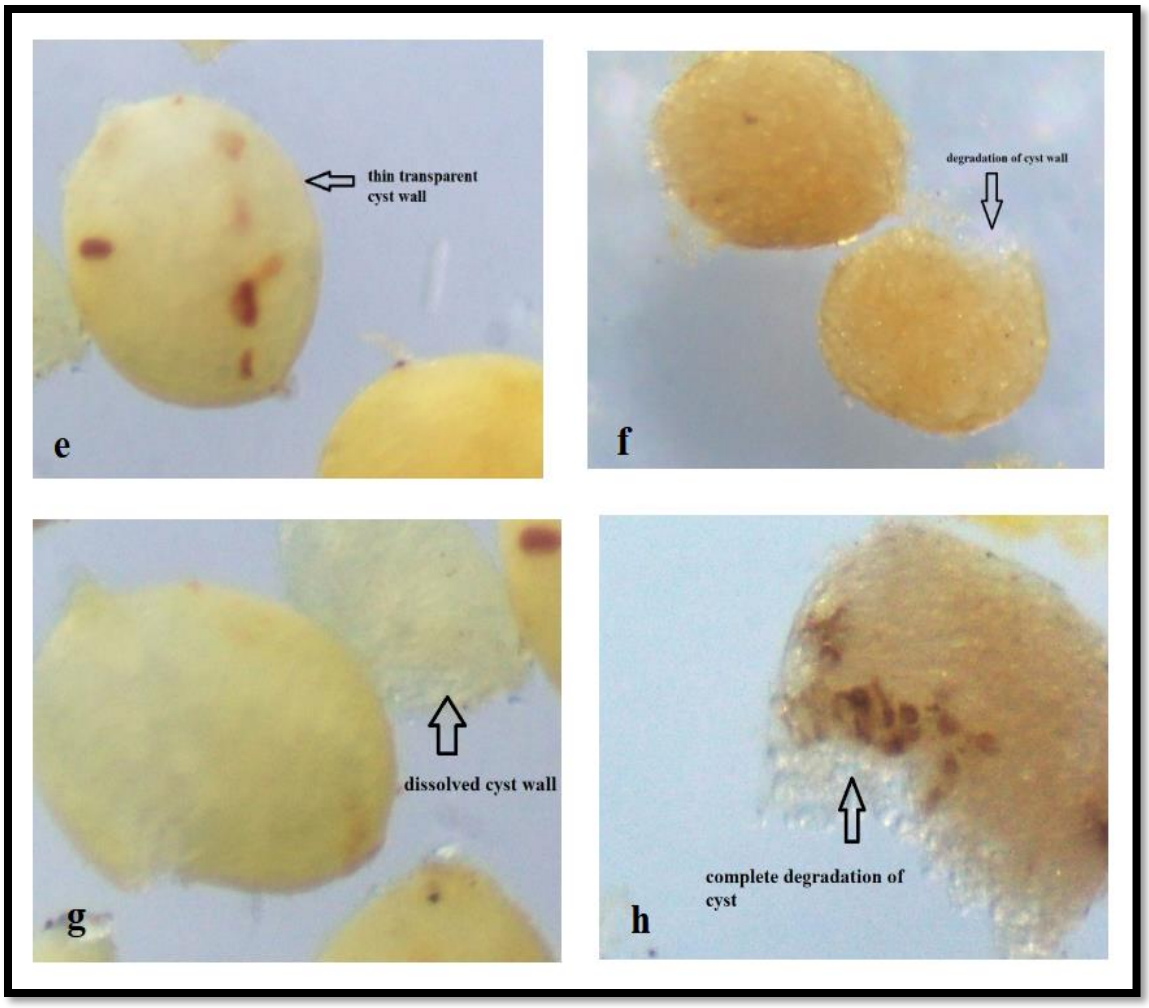


Figure 42: Effect of 30% concentrations of *V. lecanii* (T3) against the cyst of *Heterodera avenae* at different time intervals. **a.** attack of fungus on cyst at 8 HAI; **b.** colonization of fungus on cyst at 24 HAI; **c.** disintegration of cyst wall at 48 HAI; **d.** disintegration of cyst wall at 72 HAI; **e.** thin cyst wall at 96 HAI; **f.** degradation of cysts wall at 120HAI; **g**. the disintegration of cyst wall along with the debris of cyst body content at 144 HAI; **h.** complete degradation of cyst at168 HAI.

To control the populations of cyst nematodes, the efficiency of *Verticillium lecanii* (*V. lecanii*) (ITCC 7084) was evaluated in the present study. Different treatments viz., (Treatment) T1-10%, T2-20%, T3-30% and T4-0% (control) cell suspension of 1×105 CFUs / ml of *V. lecanii* were given to the cysts of *C. estonica*, *H. avenae* and *G. rostochiensis*. A fungal attack on the cyst wall was observed for *C. estonica* at 8 and 24 HAI (hours after inoculation), but no change in the cyst wall was observed. Univariate Tukey test indicates that significant differences were observed at 72 HAI in a number of cysts left ($F = 253.808$, $df = 3, 20$, $P < 0.001$). The observations were terminated after 72 h, as all the cysts were disintegrated by *V. lecanii* at Treatment 3 (30% cell suspension). The percentage of left-over cysts of *C. estonica* after the treatment with *V lecanii* shows the maximal effect is with 30% suspension (0%) followed by 20% suspension (6.89%) and 10% suspension (13.44%) compared to untreated control (100% normal cysts) (Table 31). This evaluation indicates that percentages of leftover cysts were found inversely proportional to the concentration of cell suspension of *V. lecanii* and the duration of the exposure.

For *G. rostochiensis* fungal colonization noticed up to 96 HAI, but no change in the cyst's wall was observed. However, no significant difference was observed at 144 HAI ($P=0.413$) in the number of cysts left. A significant difference in the number of cysts left was noted at 264 HAI ($F=143.667$, $df= 3,20$, $P< 0.001$). Univariate Tukey test indicates that in Treatment 3 at 264 HAI, there is a significant difference in the number of cysts left compared to Treatment 1, Treatment 2 and Treatment 4. At 264 HAI the observations were terminated, as the entire cyst was disintegrated at Treatment 3. At 264 HAI percentage of left-over cysts of *G. rostochiensis* after the treatment with *V. lecanii* shows maximal effect is with 30% suspension (3.34%) followed by 20% suspension (80%) and 10% suspension (93.34%) compared to untreated control (100% normal cysts) (Table 32). The percentages of leftover cysts were found inversely proportional to the concentration of cell suspension of *V. lecanii* and the duration of the exposure.

No change was observed for *H. avenae* cysts up to 48 HAI on the cyst's wall. Only fungal colonization was observed on the wall of the cyst. No significant difference was observed at 48 and 72 HAI, (*P=0.413*) (*P=0.089*) in the number of cysts left in each treatment. Univariate Tukey test indicates that at 168 HAI considerable difference in the number of cysts left was noted (*F= 201.852, df = 3,20, P< 0.001*). The observations were terminated at 168 HAI, as the entire cyst was disintegrated from the higher concentration (Treatment 3). At 168 HAI percentage of leftover cysts was noted in 30% *V. lecanii* suspension (3.34%), 20% *V. lecanii* suspension (73.34%) and 10% *V. lecanii* suspension (96.67%) compared to untreated control (100% normal cysts) (Table 33). This study indicates that percentages of leftover cysts were found inversely proportional to the concentration of cell suspension of *V. lecanii* and the duration of the exposure. The present study suggests that *V. lecanii* effectively disintegrated the cysts *C. estonica*, *G. rostochiensis* and *H. avenae*. The complete disintegration of *G. rostochiensis* cysts occurred at 264 HAI in whole, disintegration *C. estonica* cyst occurred at 72 HAI and *H. avenae* entire cysts were disintegrated at 168 HAI.

In this study, we found the efficiency of *V. lecanii* isolate (ITCC 7084) against the cysts of *C. estonica*, *H. avenae* and *G. rostochiensis* under laboratory conditions (25±1^{o}C). Hsiao *et al*. (1992) discussed that for the growth and development of *V. lecanii* high humidity is required, and a temperature 15^{o}C - 25^{o}C is required for colony growth, spore germination, and sporulation of the fungus. Alavo *et al*. (2015) reported that *V. lecanii* penetrates the host body through an external cuticle. *V. lecanii* has three important life phases: adhesion and germination of the fungal spores, cuticle penetration by a germ tube, and fungal growth and development inside the host, which results in the death of the host organism. It develops spores and fungal hyphae under optimal environmental conditions (Quinlan, 1988). Caryol *et al*. (1989) studied the nematicidal properties of nematophagous fungus *Paecilomyces lilacinus* culture filtrates reveal that *Heterodera* spp. and *Meloidogyne* spp. were entirely paralysed by the culture filtrates. Our results are in agreement with work done on parasitism of *V. lecanii* against several plant-parasitic nematodes (PPNs) (Hänssler and Hermanns, 1981; Hänssler, 1990; Uziel and Sikora, 1992;

Meyer, 1998; Meyer, 1999; Shinya *et al*., 2008; Hussain *et al*., 2018). In some reports, wild and mutant strains of *V. lecanii* were effective under greenhouse conditions against PPNs (Reddy *et al*., 1996). Shinya *et al*. (2007) used four hybrid strains of *V. lecanii* (AaF23, AaF42, AaF80, AaF103) to suppress soybean cyst nematode populations in the greenhouse. Their study concludes that *V. lecanii* releases some compounds that effectively inhibit the hatch of embryonated eggs of *H. glycines*. The study conducted by Shinya *et al*. (2007); (2008) agrees with our study in which *V. lecanii* effectively damaged the cysts of *C. estonica, H. avenae* and *G. rostochiensis*. In this study, *V. lecanii* filtrates tend to be more effective against *C. estonica* and *H. avenae* than *G. rostochiensis*. Our research found a significant decrease in the percentage of normal cysts remaining in 30% spore suspensions (Treatment 3) compared to the control.

The eco-friendly approach is the use of antagonists instead of toxic chemicals to suppress plant-parasiticnematode. *In vitro* assay is a preliminary investigation to check the potential of *V. lecanii* against cysts nematodes. *V. lecanii* showed significant damage to the eggs and adult females of *M. incognita* (Eapen *et al*., 2005). The study also indicates that *V. lecanii* showed effective parasitism against plant-parasitic nematode *Meloidogyne* spp. This supports the present study. Hänssler and Hermanns, (1981) determined the ability of *V. lecanii* against the cysts of *H. schachtii*. According to them, within 48 hours after inoculation, the entire surface of *H. schachtii* cysts was covered with fungal mycelium. They concluded that *V. lecanii* produces a lytic enzyme, which helps it penetrate the surface of the cysts. Their work dramatically supports our study, and according to our results, within 48 hours *V. lecanii* infection, hyphae spread on the entire cyst of *C. estonica, H. avenae* and *G. rostochiensis*.

Lecanicillium spp. is one of the important nematophagous fungi and has the potential to act as a biopesticide against plant-parasitic nematodes, Gan *et al*. (2007). They demonstrated that some *Lecanicillium* spp. efficiently infects the eggs of *M. incognita*. According to the report, immature eggs are more susceptible to fungal attack than mature eggs enclosing juveniles (Chen and Chen, 2003; Kim and Riggs, 1991). According to

Meyer *et al*. (1998) *in-vitro* analysis, *H. glycines* seemed to improve the growth of the fungus on the young roots. Their results proved that instead of the presence of a nutrient medium, nematodes act as an additional nutrient source for fungal growth. Our *in-vitro* analysis agrees with the study, wherein cyst inoculum act as an additional nutrient source for fungus growth which ultimately leads to the degeneration of the cyst. In tissue culture studies, Meyer *et al*. (1998) concluded that the presence of *H. glycines* in the host plant's roots might increase the association of fungus with the roots. *V. lecanii* have a wide-ranging host range, such as insects, plant-parasitic nematodes and phytopathogenic fungi (Goettel *et al*., 2008; Meyer *et al*., 1990); this may indicate that multiple strains of *V. lecanii* can control the multiple pest difficulties. By using r-DNA sequencing analysis recently, the genus *Verticillium* was renamed into a new genus known as *Lecanicillium*, and all the insect pathogens were placed in this genus (Jams and Zare, 2001). *V. lecanii* provided significant results in *in-vitro* studies and showed efficient results in pot trials (Shinya *et al*., 2008a). Shinya *et al*. (2008a) concluded that a hybrid strain of *V. lecanii*, AaF42 reduces 93% *H. glycines* egg density in pot trials. Meyer *et al*. (1997) investigated the pathogenicity of *V. lecanii* against *H. glycines* in small field trials. They concluded that the combination of syringic acid, vanillic acid and *V. lecanii* could efficiently reduce the population of *H. glycines* in soybean fields.

Clarke, (1968) investigated the composition of the cyst wall of *G. rostochiensis*. He stated that the cyst wall contains 72% of protein, and amino acids, namely proline, glycine and alanine, were found to be abundant. Lipid composition (2%) was detected in the cyst wall of *G. rostochiensis*. This indicates that the cyst wall is mainly composed of protein content. According to the investigations of Clarke, (1968) cyst wall primarily consists of protein and lipid. Based on our observations and earlier studies, it can be hypothesized that *V. lecanii* can produce some potential metabolites that can cause lysis of the cell wall of cysts and the eggs inside the cyst. Hasan *et al*. (2013) investigation confirmed the production of extracellular enzymes, i.e., amylase, protease and lipase in *V. lecanii*. Their results conclude that proteolytic activity was highest at pH 7 and 9. Amylolytic activity was maximum at pH 3, whereas lipolytic activity was maximum at pH 7. Hasan *et al*.

(2013) stated that *V. lecanii* produce extracellular enzyme protease, amylase and lipase. Proteolytic and lipolytic activity was maximum at pH 7. These two investigations supported the present study that *V. lecanii* may secrete protease and lipase; this may degrade the cyst wall of *C. estonica*, *G. rostochiensis* and *H. avenae*. This indicates that *V. lecanii* produce nematode cyst wall degrading enzymes. The present study demonstrated the biocontrol potential of *V. lecanii* against the cyst of *C. estonica, G. rostochiensis and H. avenae*. The cysts disintegrated in 30% spore suspensions (Treatment 3) of *V. lecanii*. In earlier studies, the same results of disintegration of cysts and cysts and eggs of *H. schachtii* within 48 hours (Hänssler and Hermanns, 1981) and 60 hours (Hänssler, 1990), respectively, were reported. Some other nematophagous fungus, such as *Nematophthora gynophila*, *V. chlamydosporium* prevents cyst formation and parasitizes adult females of *H. avenae* (Kerry *et al*., 1984: Kerry and Crump, 1980). The use of fungal and bacterial microorganisms to control the cysts nematode population is an efficient strategy. The use of fungus to control the cyst's nematode supports our findings. Some researchers proved earlier that using nonpathogenic species of the genus *Verticillium* to prevent the cyst's nematode population is one of the feasible strategies.

So future studies should focus on identifying, testing, and formulating these enzymes/metabolites produced by *V. lecanii* against PPNs. In addition to this, the strain can be evaluated in field trials against different PPNs; also, its ability of fast act against cyst nematodes can be exploited, especially in the organic farming system, where nematodes constitute a major limiting factor and one of the difficult to manage pests of the crop plants. The present study concluded that *V. lecanii* showed a dose and exposure time-dependent activity against cysts populations of *C. estonica*. Therefore, it can be a very powerful bio-rational solution for managing the nematodes in the Himalayan Biosphere, a biodiversity hotspot of the world.

Chapter-7

Conclusions

Chapter-7

Conclusions

The survey was conducted in the agricultural fields of Himachal Pradesh during November 2018 to December 2021; 10 districts, namely Kangra, Mandi, Kullu, Chamba, Shimla, Una, Hamirpur, Bilaspur, Solan and Sirmour, were covered to detect the occurrence of cysts nematodes. Soil samples were collected from various agriculture farms and private agricultural fields. Many physiological variations within the genera have been reported earlier. Therefore, morphological and morphometrical characteristics of isolated cysts nematodes were studied, and sequences of 18S small subunit-(ribosomal) rRNA and Internal Transcribed Spacers (ITS)-rDNA were studied for molecular characterization. Morphological and morphometrical studies revealed that the cysts of the district Una population were dark brown to moderately brown and lemon-shaped with distinct neck and vulval cone. The cysts of district Mandi, Chamba and Shimla populations were light brown to dark brown and lemon-shaped with distinct neck and vulval cone. The cysts of district Mandi, Chamba, Kangra, Shimla and Kullu populations were pale brown to golden brown and spheroid or globose in shape. Among all cyst populations, the cysts population of district Una were much bigger in size (701.7 ± 23.22) than the cysts of district Mandi, Chamba and Shimla populations (560.09 ± 65.92) and cysts of district Mandi, Chamba, Kangra, Shimla and Kullu populations (609.8±63.06 µm). Morphological and morphometrical characteristics of Una population matched with genus *Heterodera*.

Amplified ITS-rDNA resulted sequence (OM049243.1) showed 99.27% similarity with KC736872.1 *Heterodera avenae* population from Morocco. Morphological and morphometrical characteristics of cysts collected from district Mandi, Chamba and Shimla populations matched with genus *Cactodera*. Amplified ITS-rDNA resulted sequence (MN658364.1, MW821356.1 and MW821355.1) showed 96.12% similarity with *C. estonica* KC771888.1, 96% similarity with HM560730.1, 95.89% similarity with JN684904.1 from China. Morphological and morphometrical cysts populations of district Mandi, Chamba, Kangra, Kullu matched with genus *Globodera.* Amplified 18S small

subunit- rRNA gene resulted in sequence (MZ508280.1, MZ518783.1, MZ508279.1 and MW577347.1) showed 99.05% similarity with KJ636272.1 *Globodera rostochiensis* population from the Netherlands, 99.05% similarity with MZ613180.1 *Globodera rostochiensis* population from China. Morphological and molecular studies of collected cysts revealed the occurrence of three cyst nematodes genera, namely *Heterodera*, *Cactodera* and *Globodera*. *H. avenae* has been isolated from the wheat field, whereas *G. rostochiensis* and *C. estonica* have been isolated from potato fields.

For the management of cyst nematodes, the *in-vitro* effect of nematophagous fungi *Verticillium lecanii* on the cysts of *C. estonica, G. rostochiensis,* and *H. avenae* has been studied. The impact of different treatments *viz*., T1-10%, T2-20%, and T3-30% cell suspension of *V. lecanii* isolate was observed against cysts of *C. estonica, H. avenae and G. rostochiensis* at 8, 24, 48, and 72 hours after inoculation (HAI). The results revealed that *in-vitro* effect of Treatment 3-30% cell suspension of *V. lecanii* at 72 Hours After Inoculation, significant differences were noted in the number of cysts left for *C. estonica*. whereas no significant differences were observed in the number of cysts left after 8 and 24 HAI in each treatment compared to untreated control. For *G. rostochiensis in-vitro* effect of Treatment3-30% cell suspension of *V. lecanii* at 216 and 240 HAI significant difference in the number of cysts left was noted in Treatment 3-30% cell suspension compared to the untreated control. At 144 HAI, no significant difference was observed in the number of cysts left in each treatment. For *H. avenae in-vitro* effect of Treatment 3-30% cell suspension of *V. lecanii* at 168 HAI considerable difference in the number of cysts left was noted. At 48 and 72 HAI, no significant difference was observed in the number of cysts left in each treatment. The present study concluded that *V. lecanii* showed a dose and exposure time-dependent activity against cysts populations of *C. estonica, G. rostochiensis* and *H. avenae. In-vitro* studies conclude that 30% *V. lecanii* (T3) showed effective against the cysts of neamtodes. This survey confirmed the occurrence of *C. estonica, G. rostochiensis and H. avenae* in Himachal Pradesh. The present study concludes that among three genus cysts nematode, *G. rostochiensis* showed the highest occurrence in the soil. The present study revealed the occurrence of the genus *Cactodera* in districts Mandi,

Chamba and Shimla, Himachal Pradesh. This work firstly reported the occurrence of *C. estonica* from Himachal Pradesh. This study gives us knowledge about the distribution and identification of various cysts' nematodes in Himachal Pradesh. This information is valuable for designing multiple effective strategies to prevent the spread of cyst nematodes into new and unaffected agricultural areas.

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Appendices

Appendices1 :Abbreviation table

PPN	Plant-parasitic nematodes
J2	Second stage juvenile
J3	Third stage juvenile
J4	Fourth stage juvenile
Fig	Figure
PCN	Potato cyst nematode
CCN	Cereal cyst nematode
PGPR	Plant growth promoting rhizobacteria
mi RNA	Micro RNA
DCL-1	Dicer- like 1
P.D	Population Density
T1	Treatment 1
T2	Treatment 2
T3	Treatment 3
T4	Treatment 4
HAI	Hours after inoculation
NaOH	Sodium hypochlorite
KCl	Potassium chloride
HCl	Hydrogen chloride
$MgCl_2$	Magnesium chloride
DTT	Dithiothreitol
rpm	Revolution per minute
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
SPSS	Statistical Package for the Social Sciences
KVK	Krishi Vigyan Kendra

ICAR	Indian Council of Agriculture Research
CPRI	Central Potato Research Institute
HPWHUN	Himachal Pradesh Wheat Field Una
HPMD	Himachal Pradesh Mandi
HPCH	Himachal Pradesh Chamba
HPSH	Himachal Pradesh Shimla
HPPOMD	Himachal Pradesh Potato Mandi
HPPOCH	Himachal Pradesh Potato Chamba
HPPOPL	Himachal Pradesh Potato Palampur
HPPOSH	Himachal Pradesh Potato Shimla
HPPOKL	Himachal Pradesh Potato Kullu
ITS	Internal transcribed spacer
NCBI	National Center for Biotechnology information
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
CFU	Colony forming unit
ITCC	Indian Type Culture Collection