Evaluation of the bioformulation of potent native strains of *Trichoderma* spp. against the foot rot/gummosis of Kinnow mandarin

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Abstract

Background: Foot rot/gummosis of citrus, caused by *Phytophthora* spp., is the most important disease of citrus, responsible for the yield losses of 10 to 30% in fruit production in major citrus growing countries. Considering the burden of disease, the present study was undertaken to evaluate different native potent strains of *Trichoderma* spp. against the foot rot/gummosis of citrus.

Results: Based on molecular characteristics, i.e., ITS ribosomal DNA sequence analysis, 3 isolates of *Phytophthora nicotianae* var. *parasitica* and 7 isolates of *Trichoderma* (6 isolates of *T. asperellum* and one of *T. harzianum*) were identified. *Trichoderma* isolates and Metalaxyl-M + Mancozeb were used to assess antagonistic activity against the foot rot/gummosis under field conditions. *T. asperellum* T20 mutant reduced gummosis lesion size (95.67%), improved feeder root density (0.0236 cm^3), increased number (990.16), and yield of fruits (168.83 kg).

Conclusions: The talc-based bioformulation of T20 mutant (*T. asperellum*) strain, used as a soil treatment, was able to manage effectively the gummosis/foot rot of citrus and also exhibited the highest yield.

Keywords: Citrus foot rot/gummosis, Kinnow, *Phytophthora nicotianae* var. *parasitica*, T20 mutant *Trichoderma asperellum*

Background

Citrus is one of the most economic groups of horticulture crops worldwide and the second-largest fruit crop grown in over more than 50 countries commercially under different agro-climatic conditions (Naqvi 2003). The Kinnow (*Citrus reticulata* Blanco) is the hybrid of 2 important cultivars—"King" (*C. nobilis*) × "Willow leaf" (*C. deliciosa*)—and is cultivated on a very large scale in Indian and Pakistan Punjab. In Punjab, it is mostly grown in Fazilka, Ferozpur, Hoshiarpur, Muktsar, Bathinda, and Faridkot districts. The area under Kinnow cultivation in 2018–2019 was 55.5 thousand ha with a yield of 237 q/ha and production of 1312.4 thousand tons of fruit in Punjab (Anonymous 2019).

Foot rot/gummosis in citrus, caused by *Phytophthora* spp., is a serious and common disease and is responsible for significant economic losses. It attacks citrus species in nursery beds causing damping-off to fibrous root rot, crown rot, premature leaf fall, foot rot, and gummosis in mature orchards and infects almost every part of citrus plants (Shekari et al. 2012). Traditionally, foot rot/gummosis of citrus has been managed by chemical treatment but chemical control causes an imbalance in the microbial community, destroys the activity of beneficial microorganisms, causes chemical environmental pollution, toxicity in foods, and development of resistance in the pathogens.

In view of organic farming and sustainable agriculture, there is a need to investigate for successful biological control, which could, therefore, be a better choice for managing the disease (Gade and Lad 2018). Among the biocontrol agents, *Trichoderma* is the most exploited soil

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fungi due to its versatility, adaptability, and ease of handling in addition to its control mechanisms (Mukherjee et al. 2013). *Trichoderma* spp. have been focused on their antagonistic and mycoparasitic ability to reduce the disease incidence caused by phytopathogens (El-Sharka-wya et al. 2018). They control the soil-borne pathogens through secretion of lytic enzymes (glucanases and cellulase), antimicrobial metabolites, competition, and induction of defense responses in target plants thereby stimulating the plant growth and enhancing crop productivity (Sallam et al. 2019).

Considering the catastrophe of the disease, the present investigations aimed to (a) isolate and characterize *Trichoderma* spp. associated with rhizospheric soil of Kinnow and (b) evaluate the biofungicidal formulated from potential potent native strains of *Trichoderma* spp. against the foot rot/gummosis of Kinnow to manage the disease effectively.

Methods
The present study was carried out at Kinnow mandarin orchards grown in the Abohar region (Punjab) over a period of 2 years (2016/17 and 2017/18).

Sample collection and isolation of *Phytophthora*
Soil samples were collected from Kinnow mandarin growing areas of Abohar region of Punjab state (India), which were affected by *Phytophthora*. Soil sampling was done up to the depth of 20 cm from gummosis affected Kinnow trees. The rhizospheric soil samples were collected from the 4 different directions around the infected trees and were mixed thoroughly to make a single sample. Along with the soil samples, the feeder roots were also collected for further study.

Three isolates (P1, P2, and P3) of *Phytophthora* spp. were isolated from the naturally infected plant materials by using the modified leaf-disk baiting technique of (Grimm and Alexander 1973). Fibrous roots of infected Kinnow plants along with their rhizospheric soil were placed in the 500 ml glass beakers and the volume was made up to 500 ml by adding sterile distilled water. Kinnow plant fibrous roots and soil suspension were allowed to settle down. Six to eight leaf piece sections (10 mm^2) of rough lemon (*C. jambhiri*) were floated on the surface of the water in the beaker. Beakers were covered with black carbon paper and incubated for 4 days in a dark chamber at 20 °C. Leaf sections were then removed and plated on CMA-PARPH medium. The CMA-PARPH medium consisted of antibiotic ingredients and corn meal agar as a selective medium. The antibiotic ingredients used were hymexazol (0.08 g), rifampicin (0.012 g), ampicillin (0.25 g), pimaricin (0.01 g), and pentachloronitrobenzene (0.01 g). These were mixed in l00 ml of DMSO (dimethyl sulfoxide) and

subsequently added to 1 l of autoclaved CMA medium. Furthermore, the plates were incubated in the darkness at 20 °C for 7 days.

Molecular identification of *Phytophthora* isolates
DNA extraction of *Phytophthora* isolates
Mycelial cultures of 3 isolates were grown in corn meal (CM) broth for 7 days and incubated at 20 °C. DNA of *Phytophthora* was extracted, using cetyl trimethyl ammonium bromide (CTAB), as described by Doyle and Doyle (1990). In the extraction buffer, 1% polyvinylpyr-rolidone and 0.2% β-mercaptoethanol were used. DNA samples were evaluated both qualitatively and quantitatively by 0.8% agarose gel electrophoresis.

PCR amplification *Phytophthora* isolates
Extracted genomic DNA of *Phytophthora* isolates were amplified by PCR with genus-specific primers (Ph2 5-"ATACTGTGGGGACGAAAGTC"-3 and ITS4 5-"TCCTCCGCTTATTGATATGC"-3) (White et al. 1990). The PCR-amplification reactions were performed in a 11-µl mixture containing 0.8 µl MgCl$_2$, 2.0 µl PCR buffer, 1.5 µl dNTPs mix, 1.0 µl of each primer, 4.0 µl of template DNA, 0.4 µl of Taq polymerase, and 0.3 µl of sterile water. The PCR reaction was incubated in a programmable thermal cycler starting with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s; annealing at 50 °C for 30 s; extension of the DNA strands at 72 °C for 1 min; and a final extension step at 72 °C for 10 min. Similarly, species-specific primers, Pn5 5-"GAACAATGCAACTTATTGGACGTTT"-3 and Pn6 5-"AACCGAAGCTGCCACCCTAC"-3 were used. PCR reaction was started with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s; annealing at 55 °C or 30 sec; extension at 72 °C for 1 min; and final extension at 72 °C for 10 min (Ippolito et al. 2002). The amplified products of the PCR (11 µl) were subjected to 1.5% agarose gel electrophoresis in 0.5X TBE buffer with the 100 bp and 50 bp ladder. The gel was visualized under UV light and the photographs were captured in the Gene Genius Gel Documentation system.

Trichoderma culture
Sample collection from healthy Kinnow orchards
Rhizospheric soil samples were collected by spade from healthy Kinnow orchards from different regions nearby Abohar (Punjab). These samples were then brought to the laboratory and stored at 2–4 °C to reduce their microbial activity.

Isolation of *Trichoderma*
A selective isolation method was employed to obtain highly effective antagonist fungus of the *Trichoderma* genera. The dilution plate method described by Hassan

and El-Awady (2011) was used for the isolation of 25 isolates of *Trichoderma*. In this method, 10^{-5} serial dilutions of each soil sample were prepared in sterilized distilled water. Aliquots of each soil suspension (0.5 ml) were transferred by triplicate to *Trichoderma* selected media (TSM) (Elad et al. 1981). The media plates were incubated at 25 ± 1 °C for 96 h. Furthermore, potato dextrose agar (PDA) media was used for purification and maintenance of the cultures and were preserved at 4 °C.

Molecular characterization of *Trichoderma* isolates
DNA extraction of *Trichoderma* isolates
The genomic DNA of 7 different isolates of *Trichoderma* (parent) was extracted from 7 days old mycelia growth on potato dextrose broth (PDB), using the CTAB, as described by Doyle and Doyle (1990). In the extraction buffer, 1% polyvinylpyrrolidone and 0.2% β-mercaptoethanol were used. DNA samples were evaluated both qualitatively and quantitatively by using 0.8% agarose gel electrophoresis. For this study, the internal transcribed spacer (ITS) regions of the rDNA repeat from the 3′end of the 18S and the 5′end of the 18S gene were amplified by using primers i.e. ITS-1(5-"TCTGTAGGTGAACCTGCGG"-3) and ITS-4(5-"TCCTCCGCTTATTGATATGC"-3) which were synthesized based on conserved regions of the eukaryotic rRNA gene (White et al. 1990).

PCR amplification
The PCR-amplifications were performed in a 11 μl mixture containing 0.8 μl $MgCl_2$, 1.5 μl dNTPs mix, 1.0 μl of each primer, 4.0 μl of template DNA, 0.4 μl of Taq polymerase and 0.3 μl of sterile water. For ITS amplification, cycle parameters included an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min; primer annealing at 62 °C for 2 min; primer extension at 72 °C for 3 min; and a final extension at 72 °C for 5 min (Hermosa et al. 2000). The amplified products of the PCR (11 μl) were subjected to 1.5% Agarose Gel Electrophoresis in 0.5X TBE buffer at 5 V/cm for 60 min with 100 bp ladder. The gel was visualized under UV light and the photographs were captured in the Gene Genius Gel Documentation system.

Purification and nucleotide sequencing for identification of PCR products
The amplified PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Inc.) as per the manufacturer protocol. Sequencing of ITS region of purified products was done, using both forward and reverse primers at SciGenom Labs (P) Ltd. Cochin (Kerala), India.

DNA sequences analysis
The sequences were extracted by using CHROMAS Lite 2.1.1 and these reads were aligned using DNA Baser assembler v.5.15.0 to generate a consensus sequence. The sequences were further subjected to Basic Local Alignment Search Tool (BLAST) software for comparison with the sequences of *Trichoderma*, previously submitted at National Centre for Biotechnology Information (NCBI) database (Druzhinina et al. 2005).

Preparation of bioformulation of *Trichoderma* spp.
The most effective 7 isolates of *Trichoderma*, i.e., parents and mutants, were formulated in talc powder. The talc powder (carrier) was autoclaved for 30 min at 15 psi at 121 °C. The selected strains of *Trichoderma* spp. were grown as broth culture in the selective media for the preparation of formulation. Mycelial mat along with broth was homogenized and 600 ml of this mixture was added to 1 kg of talc powder under aseptic conditions and dried. One percent carboxy methylcellulose (CMC) was added to the mixture as an adhesive (Aulakh et al. 2017).

Orchard treatments
A field experiment was laid out for the management of foot rot/gummosis on *Kinnow* mandarin (13-year-old *Phytophthora* infected orchard) at Abohar region (30° 9′ 0′′ North and 74° 11′ 0′′ East) of Punjab state (India) in randomized block design (RBD) during 2016–2017 and 2017–2018. Kinnow trees, which were grafted on rough lemon (*Citrus jambhiri*) rootstocks, planted in 1-acre block were used for this experiment.

Kinnow trees showing typical symptoms of *Phytophthora* infection, i.e., profuse oozing/bleeding of gum were chosen for the experiment. In each experiment, there were 3 repetitions per treatment in RBD. *Trichoderma* isolates and chemicals (Metalaxyl-M + Mancozeb) were used as soil treatment as well as applied as trunk paste and paint, respectively. The abovementioned treatments were done twice a year, i.e., during the months of February and July for the subsequent 2 years as described:

Seven native potent isolates of *Trichoderma* (parent and mutant) (Tables 1) and 2 standard checks (T. *viride* and T. *harzianum*) were used for the preparation of talc-based bioformulation.

a) *Trichoderma* talc-based bioformulation was applied as soil treatment as well as trunk paste treatment. The protocol is described as follows:
Soil treatment—100 g talc-based bioformulation of *Trichoderma* was mixed in 2.5 kg of FYM (Farm Yard Manure) and was kept for 7 days under shade for priming. The mixture was then applied to the feeder root zone (basin area) of infected trees.

Table 1 Reference sequences retrieved for phylogenetic analysis from GenBank

Isolate name	Genus	Species	Gene	Base pair	GenBank accession numbers
T2	*Trichoderma*	*asperellum*	ITS	606	MK210562.1
T3	*Trichoderma*	*asperellum*	ITS	602	MK210429.1
T4	*Trichoderma*	*asperellum*	ITS	607	MK210428.1
T16	*Trichoderma*	*asperellum*	ITS	602	MK211208.1
T20	*Trichoderma*	*asperellum*	ITS	616	MK210235.1
T21	*Trichoderma*	*asperellum*	ITS	600	MK209012.1
T25	*Trichoderma*	*harzianum*	ITS	619	MK209008.1

Trunk paste treatment—A thick paste was prepared by adding sterilized water to talc-based bioformulation of *Trichoderma* and was applied to lesions on infected tree bark.

b) Standard chemical (Metalaxyl-M + Mancozeb) drenching near feeder root zone area (25 g in 10 l of water) and applied as trunk paint (2 g in 100 ml of linseed oil) on the lesion on the bark.

c) Untreated infected plants were maintained as control (Singh et al. 2015).

Disease assessment and growth parameters evaluation
Under the study field conditions, native strains of *Trichoderma* established themselves in the rhizospheric soil and root system of *Phytophthora* infected Kinnow trees, leading to an increase in feeder root density and reduction in the lesion size, thereby reducing the incidence of foot rot/gummosis and increasing the number and yield of fruits.

The percent recovery of the infected area was assessed by noticing the variations in gummosis lesion size (cm^2) (Bairwa et al. 2015). Observations were recorded before and after the time of application for each treatment individually. The formula used is as follows:

Percent recovery lesion size = (initial value – final value)/initial value × 100

Feeder roots density was computed by using the water displacement method. (Harrington et al. 1994; Yesmin 2019). The number and fruit yield per plant were also recorded.

Data analysis
The whole data was in triplicates and was represented as mean ± standard deviation. The variations in the results obtained concerning gummosis lesion size (arcsine transformation), feeder root, and plant growth parameters (number of fruits and weight) were analyzed by using computer software CPCS 1. The results were analyzed by using one-way analysis of variance (ANOVA) with SPSS software version 22. The treatment means were separated by Duncan's multiple range test (DMRT) and were determined by the magnitude of F value ($p \leq 0.05$). Correlations were observed between various parameters by using SPSS software version 22.

Results
Isolation and molecular characterization of *Phytophthora*
Three isolates (P1, P2, and P3) of *Phytophthora* were identified by isolation strategy and were identified up to genus and species level by using specific primers (Table 2). Genus-specific primers viz. Ph2 and ITS4 hybridized for all isolates of *Phytophthora* gave a single band of 700 bp and species-specific primers viz. Pn5B and Pn6 gave a single band of 120 bp (Fig. 1). Results obtained from molecular characterization confirmed that P1, P2, and P3 isolates of *Phytophthora* were *P. parasitica* var. *nicotianae*.

Isolation and molecular characterization of *Trichoderma*
By employing the isolation strategy, a total of 25 isolates of *Trichoderma* spp. were obtained from the Kinnow orchards, out of which 7 potent isolates were selected on basis of in vitro and biochemical analysis. All the 7 most potent strains of *Trichoderma* (Parent) were identified, using ITS Primers (ITS1 and ITS4). The ITS primers amplified a region of nearly 600 bp from genomic DNA of *Trichoderma* isolates. Out of 7 isolates of *Trichoderma*, 6 (T2, T3, T4, T20, T16, and T21) showed 100% similarity with *Trichoderma asperellum* and one isolate (T25) showed a resemblance with *Trichoderma harzianum* (Fig. 2). All sequences obtained in this study were submitted to NCBI GenBank and their accession numbers were obtained (Table 1).

The dendrogram was generated by unweight pair-group methods with arithmetic mean (UPGMA), using BIO Profil 1D image software and NTSYSpc software (Fig. 3). Based on the results obtained, all 7 isolates were grouped into 2 main clusters, one cluster representing *T. asperellum* and the other *T. harzianum*. Furthermore, the *T. asperellum* cluster was sub-grouped into two: the first subgroup with 3 isolates and the second one also with 3 isolates.

Table 2 *Phytophthora* genus and species specific primers sequence

Primer name	Sequence (5′-3′)	Direction	Fragment size (bp)	Specificity
Ph2	ATACTGTGGGGACGAAAGTC	Forward	700	At genus level
ITS4	TCCTCCGCTTATTGATATGC	Reverse		
Pn5B	GAACAATGCAACTTATTGGACGTTT	Forward	120	At species level
Pn6	AACCGAAGCTGCCACCCTAC	Reverse		

Effect of application of *Trichoderma* isolates on feeder roots density and gummosis lesion size of *Kinnow* mandarin under field conditions

For the maintenance of the feeder roots and lesion size recovery of foot rot/gummosis, 7 different isolates of *Trichoderma* (parent and mutant) and standard checks (*Trichodema viride*, *T. harzianum*, and Metalaxyl-M+ Mancozeb) were used in the experiment. With the establishment of abovementioned isolates of *Trichoderma* spp. in the root system of Kinnow plants over a period of 2 years, the treated plants showed significant improvement in feeder roots and lesion size recovery. In the treatment, T20 mutant showed the maximum volume of feeder roots ($0.0236 \, cm^3$), followed by the T16 mutant ($0.0211 \, cm^3$) over the control ($0.003 \, cm^3$) (Table 3). These results were also testified by using Duncan's test and found to be statistically significant at 5% level ($p \leq 0.05$). Maximum percent recovery of gummosis lesion size was noticed in treatment with T20 mutant (95.67%), followed by treatment withT16 (82.80 %) over the control (− 39.91%) (Table 4).

Effect of application of *Trichoderma* isolates on the number of fruits and yield under field conditions

Among all the isolates, the maximum number of fruits and weight of fruits per tree were noticed in the T20 mutant isolate (990.16), followed by the T16 mutant (931.17) as compared to the control (544.17) (Table 5).

In the case of yield, a similar trend was recorded as above, i.e., maximum in T20 mutant (168.83 kg/plant), followed by T16 mutant (158.78 kg/plant) as compared to control (92.63 kg/plant). The same was testified using Duncan's test, and results were found to be statistically significant in both the number of fruits and yield of Kinnow per plant under field conditions.

Correlation analysis

Pearson's coefficient of correlation was used to study the relationship between different parameters (Table 6). Correlation analysis in the study showed that feeder root density significantly ($p \leq 0.05$) correlated with recovery of lesion size (0.9245). An increase in the number of fruits per plant was noticed with an increase in feeder root density (0.4462) and with an increase in recovery of lesion size (0.3261).

Similar trend was also observed in the weight of fruits per plant which showed a significant ($p \leq 0.05$) correlation with feeder root density (0.8149), recovery of lesion size (0.7171), and number of fruits per plant (0.7082).

Discussion

The results of the above study clearly showed that the biocontrol agents like *T. asperellum* and *T. harzianum* recovered from Kinnow rhizospheric soil reduced the incidence of foot rot/gummosis and improved plant growth parameters in Kinnow under field conditions.
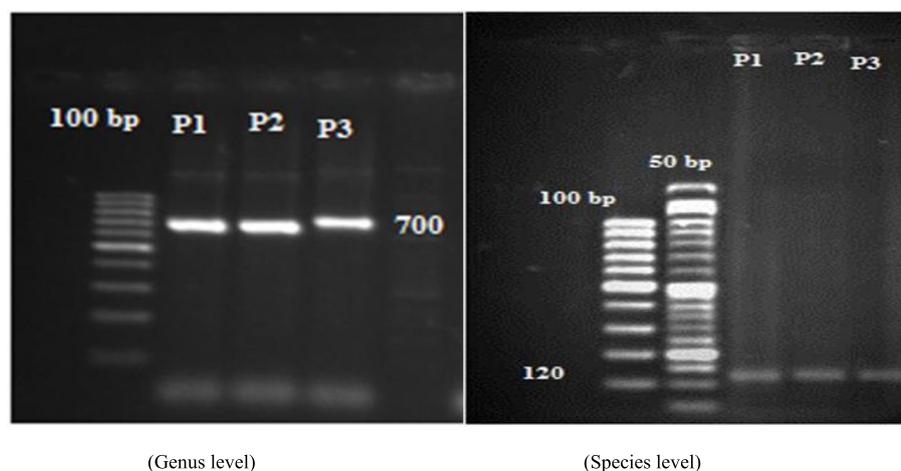
Fig. 1 DNA profile generated by *Phytophthora* genus and species by using specific primers, M= 100 bp and 50 bp

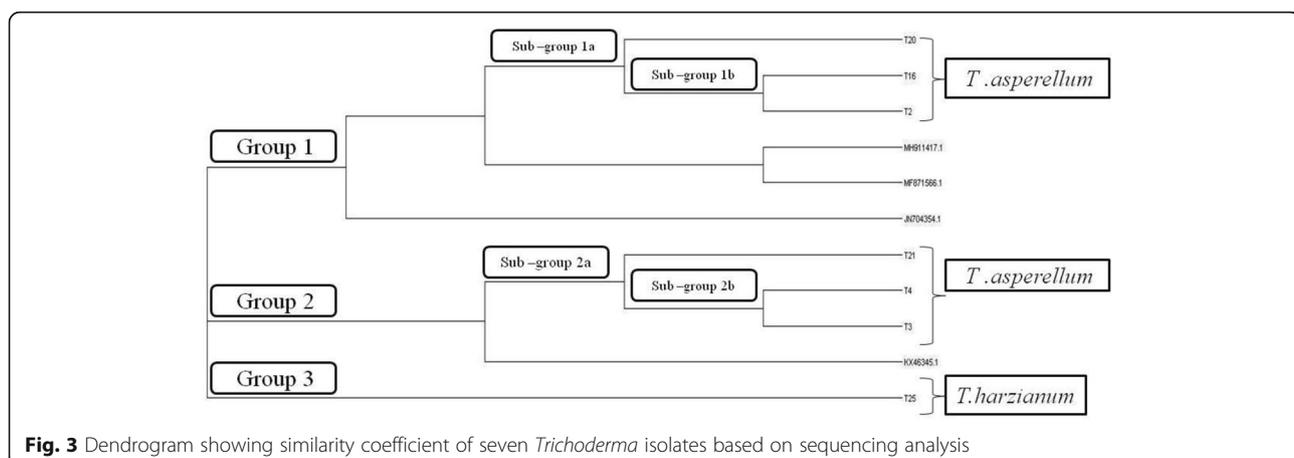**Fig. 2** DNA profile generated by ITS primers with *Trichoderma* isolates M= 100 bp marker
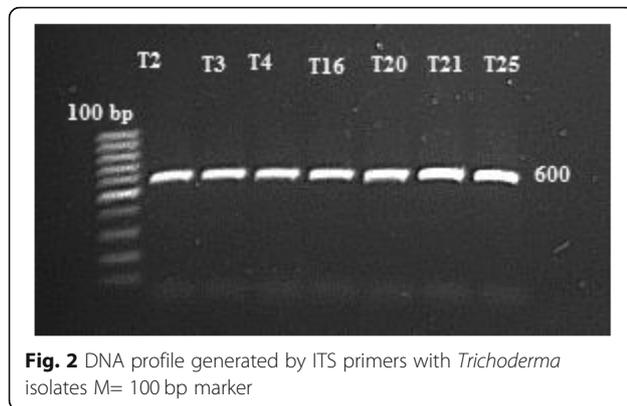
Genus *Trichoderma* is used as biological control agent worldwide for the control of soil-borne pathogens, exhibits different modes of action such as growth regulation, competition by space and nutrients, decreases infection sites, and reduces the damage by pathogens that affect the development of the roots (Adnan et al. 2019).

In the present study, *Trichoderma* species, such as *T. asperellum* and *T. harzianum* were identified based on molecular approaches and have been frequently isolated from the rhizosphere worldwide (Jang et al. 2018). Molecular studies using the ITS region were able to classify each strain within the corresponding species. The results obtained with the studied isolation strategy proposed to agree with Baker and Cook's (1974) hypothesis that the isolation of potential antagonists against a specific pathogen by searching healthy plants in sites favorable to the development of the pathogen could prove to be effective. More recently, Andrade-Hoyos et al. (2020) isolated and molecularly characterized ten endophytic *Trichoderma* spp. strains against *P. cinnamomi* by use of internal transcribed spacer region and found minimum disease incidence in *T. asperellum* (T-AS2 and T-AS7) inoculated avocado seedlings. Ahmed (2015) used

universal primers (ITS) for amplification of 18S rRNA gene fragment and identification of strain *T. asperellum*. Similarly, in the present study, ITS primers (ITS1&4) were used to identify the *Trichoderma* spp.

The native strain of *Trichoderma* significantly decreased foot root/gummosis caused by *P. nicotianae* var. *parasitica* in Kinnow trees. This was supported by increasing percentage recovery of lesion size and feeder root density observed after *Trichoderma* bioformulation treatments than the untreated control. Since, fruit yield is the function of total fruit number and fruit weight, therefore higher fruit yield under *Trichoderma* application may be attributed to increased feeder root density and reduced lesion size.

That is why native strains of *Trichoderma* could promote the use of biological control agents over chemical fungicides. The results obtained in this study are concordant by numerous works demonstrating the preventive effectiveness of native strains of *Trichoderma* spp. against different species of *Phytophthora*. Mohamed et al. (2020) concluded that *T. asperellum* reduced the disease severity up to 10.7–26.5%, and up to 26.6–36.6% under greenhouse and field conditions, respectively, by suppression of the pathogen as well as induction of plant systemic resistance. Similarly, Sanchez et al. (2019) found out that the regional strain of *T. harzianum* 1367 controlled the rot area caused by *P. cactorum* by 97%, with the lowest average lesion area (0.11 cm^2). Likewise, Promwee et al. (2017) concluded that indigenous strains of *T. harzianum* isolated from rhizosphere soil of rubber trees controlled *P. palmivora* better than the commercial *Trichoderma* strain, which could be due to environmental adaptation of the indigenous strains. Iuliana et al. (2017) showed that the inoculation of the *T. asperellum* T36 significantly reduced the percentage of dead plant seedlings and can be used as a biological alternative to pesticides for the control of *P. parasitica* in pepper seedlings. Our observations were also coincident with the

Fig. 3 Dendrogram showing similarity coefficient of seven *Trichoderma* isolates based on sequencing analysis

Table 3 Effect of application of Trichoderma isolates on feeder root density of Kinnow mandarin under field conditions

Isolates	Feeder root density (cm^3)						
	February–July 2016 (mean ± SD)[1]	July–February 2016–2017 (mean ± SD)[1]	Pooled mean 2016–2017 (mean ± SD)[1]	February–July 2017 (mean ± SD)[1]	February–July 2017–2018 (mean ± SD)[1]	Pooled mean 2017–2018 (mean ± SD)[1]	Pooled mean (Februray 2016 to July 2018) (mean ± SD)[1]
T2 Parent	0.0064±0.0007^h	0.0083±0.0008^{hi}	0.0073±0.0012^{ghi}	0.0109±0.0026^{ij}	0.0156±0.0007^h	0.0132±0.0031^{hi}	0.0103±0.0040^{defg}
T2 Mutant	0.0077±0.0006^g	0.0104±0.0008^g	0.0090±0.0016^{fgh}	0.0167±0.0002^f	0.0222±0.0023^{ef}	0.0195±0.0033^{ef}	0.0143±0.0065^{bcdef}
T3 Parent	0.0068±0.0011^{gh}	0.0089±0.0005^h	0.0079±0.0014^{efgh}	0.0128±0.0003^{hi}	0.0163±0.0019^{cdef}	0.0145±0.0023^h	0.0112±0.0042^{cdef}
T3 Mutant	0.0080±0.0008^g	0.0126±0.0007^e	0.0103±0.0026^{def}	0.0170±0.0007^f	0.0234±0.0025^f	0.0202±0.0038^{def}	0.0152±0.0065^{bcdef}
T4 Parent	0.0117±0.0008^{de}	0.0112±0.0007^{fg}	0.0114±0.0007^{cde}	0.0156±0.0007^{fg}	0.0214±0.0005^{bcd}	0.0185±0.0032^{fg}	0.0150±0.0047^{bcdef}
T4 Mutant	0.0139±0.0004^c	0.0167±0.0005^c	0.0153±0.0016^b	0.0218±0.0011^{bc}	0.0256±0.0007^{def}	0.0237±0.0022^{bc}	0.0195±0.0052^{abc}
T16 Parent	0.0106±0.0005^{ef}	0.0118±0.0009^{ef}	0.0112±0.0009^{cde}	0.0176±0.0012^{ef}	0.0231±0.0022^{def}	0.0203±0.0034^{def}	0.0158±0.0057^{abcde}
T16 Mutant	0.0151±0.0011^b	0.0184±0.0004^b	0.0168±0.0019^{ab}	0.0236±0.0021^b	0.0273±0.0013^b	0.0254±0.0026^b	0.0211±0.0054^{ab}
T20 Parent	0.0113±0.0012^{def}	0.0126±0.0000^e	0.0120±0.0010^{cd}	0.0205±0.0003^{cd}	0.0257±0.0003^{bc}	0.0231±0.0029^{bcd}	0.0175±0.0068^{abcd}
T20 Mutant	0.0164±0.0003^a	0.0204±0.0002^a	0.0184±0.0022^a	0.0275±0.0004^a	0.0299±0.0006^a	0.0287±0.0014^a	0.0236±0.0062^a
T21 Parent	0.0056±0.0003^{ij}	0.0073±0.0004^{ij}	0.0065±0.0010^{hi}	0.0090±0.0002^{jk}	0.0122±0.0003^i	0.0106±0.0018^{ij}	0.0085±0.0028^{efg}
T21 Mutant	0.0063±0.0002^h	0.0084±0.0005^{hi}	0.0073±0.0012^{fgh}	0.0144±0.0004^{gh}	0.0173±0.0008^{gh}	0.0159±0.0017^{gh}	0.0116±0.0051^{cdef}
T25 Parent	0.0078±0.0007^g	0.0116±0.0004^{ef}	0.0097±0.0021^{efg}	0.0137±0.0010^{de}	0.0183±0.0030^g	0.0160±0.0032^{gh}	0.0128±0.0044^{cdef}
T25 Mutant	0.0102±0.0005^f	0.0144±0.0004^d	0.0123±0.0023^c	0.0192±0.0017^{gh}	0.0247±0.0009^{cde}	0.0220±0.0032^{cde}	0.0171±0.0062^{abcd}
T.viride*	0.0120±0.0004d	0.0115±0.0006^{ef}	0.0118±0.0005^{cd}	0.0135±0.0014^{gh}	0.0162±0.0007^{gh}	0.0149±0.0018^h	0.0133±0.0021^{bcdef}
T.harzianum*	0.0050±0.0000j	0.0063±0.0007^j	0.0057±0.0008^{ij}	0.0079±0.0001^k	0.0099±0.0003^j	0.0089±0.0011^i	0.0073±0.0021^{fg}
Metalaxyl-I-M+Mancozeb*	0.0146±0.0008bc	0.0163±0.0012^c	0.0155±0.0013^b	0.0198±0.0012^{cd}	0.0229±0.0004^{ef}	0.0214±0.0019^{cdef}	0.0184±0.0037^{abcd}
Control	0.0047±0.0008j	0.0043±0.0007^k	0.0045±0.0007^j	0.0026±0.0004^m	0.0017±0.0004^k	0.0021±0.0006^k	0.0033±0.0014^g
CD ($p \leq 0.05$)	0.001	0.001	0.001	0.002	0.002	0.002	0.003

T2, T3, T4, T16, T20 and T21 = T. asperllum, T25 = T. harzianum
*Standard check
[1]Mean ± SD (standard deviation); values labeled with different letters are significantly different from the control level by Duncan test at 95.0% confidence

Table 4 Relative efficacy of *Trichoderma* parent and mutant isolates on percent recovery of lesion size

Isolates	Recovery of lesion size (%)		
	2016–2017	2017–2018	Total percent recovery
T2 Parent	17.82 (24.94)	26.46 (30.27)	44.28 (41.60)
T2 Mutant	24.83 (29.30)	27.17 (31.35)	52.00 (46.11)
T3 Parent	18.07 (24.46)	27.30 (31.23)	45.37 (42.31)
T3 Mutant	25.84 (29.70)	30.13 (33.08)	55.97 (48.63)
T4 Parent	25.24 (30.10)	30.41 (33.25)	55.65 (48.30)
T4 Mutant	30.97 (33.76)	40.29 (39.30)	71.26 (57.75)
T16 Parent	31.45 (34.04)	34.36 (35.70)	65.82 (54.28)
T16 Mutant	36.71 37.21	46.10 (42.67)	82.80 (68.44)
T20 Parent	36.18 (36.95)	43.51 (41.21)	79.69 (63.37)
T20 Mutant	44.45 (41.79)	51.38 (45.77)	95.67 (75.81)
T21 Parent	18.16 (23.62)	21.63 (27.46)	39.79 (38.87)
T21 Mutant	24.07 (28.73)	26.71 (31.35)	50.79 (45.43)
T25 Parent	20.77 (26.93)	27.97 (31.84)	48.74 (44.26)
T25 Mutant	26.87 (31.01)	32.85 (34.74)	59.71 (50.68)
*T.viride**	27.29 (41.47)	29.31 (31.98)	56.60 (48.86)
*T.harzianum**	17.98 (25.07)	20.07 (26.59)	38.05 (38.04)
Metalaxyl-M+Mancozeb*	35.44 (36.43)	37.35 (37.63)	72.80 (58.78)
Control	(-)16.50 (23.64)	(-)23.41 (23.40)	(-)39.91 (39.11)
CD ($p \le 0.05$)	10.29	8.90	11.66

T2, T3, T4, T16, T20 and T21 = *T. asperellum*, T25 = *T. harzianum*
*Standard checks
Figures in parenthesis is arc sine transformation

research of Singh et al. (2015) where the soil application of *T. harzianum* along with Bordeaux paste and copper oxychloride (0.3%) spray improved percent recovery of gummosis lesion (54.70), fruit yield (58.5 kg/plant) and canopy volume (16.9 m³). The work of Melo et al. (2015) also supported that *Trichoderma* spp. isolates exhibited potential for use as biological control agents, both in vitro (by the production of antimicrobial compounds) and in the alfalfa seedling bioassay against *P. nicotianae* by affecting the production of sporangia and mycelia. Bairwa et al. (2015) also concluded that bioagents based treatment of stem painting with Bordeaux paste, followed by application of *Trichoderma* + *Pseudomonas fluorescens* with the carrier material FYM, significantly recovered lesion size (22.82%), reduced feeder root rot index (24.39%), increased fruit yield (212.84 kg/tree) and the number of fruits (1084 fruits/tree). Gade and Koche (2012) used the combination of Metalaxyl, Neem cake, Fosetyl-Al, *Trichoderma* spp., and *P. fluorescens.* They recorded a significant level of inhibition in population density, the intensity of root rot, and gummosis in Nagpur mandarin.

The capacity of isolates of *Trichoderma* sp. to promote growth in plants has been well documented (Sanchez et al. 2019). In the present experiment, significant differences were observed from the control without antagonist in the number of fruits and yield, the biggest difference being from *T. asperellum* T20 Mutant. This result would indicate that the effects of *Trichoderma* on the growth and vigor of the trees depend on the native rhizospheric *Trichoderma* isolates used and not on the species. Sukhada et al. (2011) proved that plants pre-inoculated with *G. mosseae* + *T. harzianum*, provided the best results when infected with *P. parasitica* var. *nicotianae*, with increased plant height, girth, and yield and also reduced disease severity over control without an antagonist. The work of Kean et al. (2010) also supported that *Chaetomium* and *Trichoderma* gave significantly disease control equal to the chemical fungicide (metalaxyl) when compared to the control.

The above facts depict that with the use of bioformulation of T20 mutant (*T. asperellum*) in Kinnow mandarin orchard, the disease could be managed effectively under field conditions. It led to an increase in the feeder root density that enhanced the recovery of lesion size and resulted in the highest fruit yield among various treatments.

Table 5 Effect of application of *Trichoderma* isolates on number of fruits and yield under field conditions

Isolates	Number of fruits per plant			Weight of fruits per plant (kg)		
	2016–2017 (mean ± SD)[1]	2017–2018 (mean ± SD) [1]	Pooled mean (mean ± SD)[1]	2016–2017 (mean ± SD)[1]	2017–2018 (mean ± SD) [1]	Pooled mean (mean ± SD)[1]
T2 Parent	787.67±2.52[g]	796.67±5.77[h]	792.17±6.34[g]	130.75±0.42[g]	139.42±1.01[h]	135.08±4.80[fgh]
T2 Mutant	826.67±5.28[e]	840.00±10.00[f]	833.33±13.66[ef]	137.23±2.54[e]	147.00±1.75[f]	142.11±5.70[def]
T3 Parent	808.67±3.21[f]	810.00±10.00[gh]	809.33±6.68[gf]	134.24±0.53[f]	141.75±1.75[gh]	137.99±4.27[efg]
T3 Mutant	853.33±5.77[d]	856.67±11.55[e]	855.00±8.37[de]	141.65±0.96[d]	149.92±2.02[e]	145.79±4.74[cde]
T4 Parent	812.33±2.52[f]	816.67±11.55[g]	814.50±7.84[fg]	134.85±0.42[f]	142.92±2.02[g]	138.88±4.61[efg]
T4 Mutant	884.33±5.77[c]	890.00±10.00[cd]	887.17±7.94[c]	146.80±0.96[c]	155.75±1.75[cd]	151.27±5.06[c]
T16 Parent	850.00±10.00[d]	858.33±2.89[e]	854.17±8.01[de]	141.10±1.66[d]	150.21±0.51[e]	145.65±5.11[cde]
T16 Mutant	926.67±2.89[b]	935.67±4.04[b]	931.17±5.85[b]	153.83±0.48[b]	163.74±0.71[b]	158.78±5.46[b]
T20 Parent	882.00±7.21[c]	885.00±8.66[cd]	883.50±7.31[c]	146.41±1.20[c]	154.88±1.52[cd]	150.64±4.79[c]
T20 Mutant	988.67±2.31[a]	991.67±2.89[a]	990.17±2.86[a]	164.12±0.38[a]	173.54±0.51[a]	168.83±5.18[a]
T21 Parent	750.00±10.00[h]	765.67±5.13[i]	757.83±11.14[h]	124.50±1.66[h]	133.99±0.90[i]	138.55±5.33[h]
T21 Mutant	805.00±18.03[f]	820.00±10.00[g]	812.50±15.41[fg]	133.63±2.99[f]	143.50±1.75[g]	149.25±5.83[efg]
T25 Parent	808.67±3.21[f]	816.33±10.97[g]	812.50±8.36[fg]	134.24±0.53[f]	142.86±1.92[g]	129.25±4.89[efg]
T25 Mutant	874.00±5.29[c]	876.67±5.77[d]	875.33±5.16[cd]	145.08±0.88[c]	153.42±1.01[d]	138.57±4.64[cd]
*T.viride**	881.67±2.89[c]	895.00±5.00[c]	888.33±8.16[c]	146.36±0.48[c]	156.63±0.88[c]	151.49±5.66[c]
*T.harzianum**	763.33±15.28[h]	773.33±5.77i	768.33±11.69[h]	126.71±2.54[h]	135.33±1.01[i]	131.02±5.03[gh]
Metalaxyl M+Mancozeb*	760.33±4.51[h]	883.33±15.28[cd]	821.83±68.12[f]	126.22±0.75[h]	154.58±2.67[cd]	140.40±15.64[ef]
Control	578.33±11.50[i]	510.00±10.00[j]	544.17±38.65[i]	96.00±1.91[i]	89.25±1.75[j]	92.63±4.05[i]
CD (*p* ≤ 0.05)	13.98	14.26	23.06	2.32	2.55	4.25

T2, T3, T4, T16, T20 and T21 = *T. asperllum*, T25 = *T. harzianum*
*Standard Check
[1]Mean ± SD (standard deviation); values labeled with different letters are significantly different from the control level by Duncan test at 95.0% confidence

Table 6 Correlations (Pearson) between different parameters under field conditions

Parameters	Feeder root density (cm³)	Recovery of lesion size (%)	Number of fruits per plant	Weight of fruits per plant (kg)
Feeder root density (cm³)	1			
Recovery of lesion size (%)	0.9245	1		
Number of fruits per plant	0.4462	0.3261	1	
Weight of fruits per plant (kg)	0.8149	0.7171	0.7082	1

Significant at *P* ≤ 0.05

Conclusion

The outcome of the present study strengthens the eco-friendly approach of using the talc-based bioformulation of T20 *Trichoderma asperellum*, as soil application and trunk paste for management of foot rot/gummosis of citrus. By this approach, the use of Metalaxyl-M + Mancozeb can be avoided or reduced, thus reducing the chances of the development of resistant strains and non-target inhibition of beneficial microflora.

Abbreviations
CMA: Corn meal agar; CM: Corn meal; PARPH: Pimaricin, ampicillin, rifampicin pentachloronitrobenzene, and hymexazol; CTAB: Cetyl trimethyl ammonium bromide; TBE: Tris borate EDTA; TSM: Trichoderma selected media; PDA: Potato dextrose agar; PDB: Potato dextrose broth; ITS: Internal transcribed spacer; BLAST: Basic Local Alignment Search Tool; CMC: Carboxy methylcellulose; DMSO: Dimethyl sulfoxide

Authors' contributions
The study was designed by all authors. AKC performed the molecular analysis. Interpretation of the data, manuscript writing, and reference settings were done by the first author AKC. Errors and grammatical mistakes in the manuscript were removed and edited by all authors (AKC, NS, and DS). Then, the final manuscript was read and approved by all authors. Author on reasonable request.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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