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Characterization of *Trichoderma* isolates and assessment of antagonistic potential against *Fusarium oxysporum* f. sp. *cumini*

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Abstract

Cumin wilt caused by *Fusarium oxysporum* f. sp. *cumini* is one of the most destructive diseases responsible for causing even up to 60 per cent yield losses in cumin belt of India. Due to the soil inhabiting and seed borne nature with aggressive sporulation ability of the pathogen, sustainable and effective management of this disease using cultural practices and chemical methods is tedious. However, the uses of resistant varieties as well as novel biocontrol agents offer more economic and environmental friendly method of management which can be integrated with regulated chemical methods to achieve maximum disease suppression. Therefore, in the present study *Trichoderma spp*. isolated from banana rhizosphere of wilt suppressive and salt affected soils of Uttar Pradesh were characterized using morphological and molecular methods. The isolates were evaluated for their antagonistic potential against the pathogen *F. oxysporum* f. sp. *cumini* through dual culture assay. Out of 21 *Trichoderma* isolates screened, three isolates *viz*., CSR-T-2, CSR-T-3 and CSR-T-4 showed significant inhibition of *F. oxysporum* f. sp. *cumini* with 62.65, 79.85 and 84.31 per cent inhibition, respectively. The three promising isolates were characterized morphologically on the basis of their colony characters on different culture media as well as microconidia size, setae, colour, hyphae, chlamydospores *etc.* The molecular identification for confirmation of. sp.cies status of these isolates were done by sequencing ribosomal RNA using ITS1 and ITS4 universal primers. The 3 isolates *viz*., CSR-T-2, CSR-T-3 and CSR-T-4 were identified as *T. koningiopsis* (KJ812401), *T. reesei* (MH997668) and *T. asperellum* (MN227242), respectively. In the present study the isolate CSR-T-4 identified as *T. asperellum* was found to be best in inhibiting the mycelia growth of cumin wilt pathogen under *in-vitro* conditions and thus can be further exploited for the biological management of cumin wilt under field conditions in form of bioformulation.

Key words: *Fusarium oxysporum* f. sp. *cumini*, Cumin, *Trichoderma*, suppressive soils, antagonism

Introduction

Trichoderma, an ascomycete soil inhabiting fungi of family *Hypocreaceae* with *Hypocrea* as its teleomorphic form, was first described by Persoon (1794). With the increasing concerns for environmental and human health due to the unbridled use of chemicals in agriculture, the biological control of the plant diseases have become a popular field of research. Among a long list of different fungi and bacteria as potential biocontrol agents (BCA), *Trichoderma* is the most widely used and researched fungal BCA due to its tremendous biocontrol potential against some of the most stubborn and deadliest soil borne fungal pathogenic genera like *Fusarium, Rhizoctonia*, *Sclerotinia*, *Pythium* and *Phytophthora*. For the first time, the biocontrol potential of *T. lignorum* was demonstrated against *Rhizoctonia solani* by Weindling in (1934). *Trichoderma* is an ubiquitous soil inhabiting fungus currently accommodating more than 260 species (Plessis *et al.,* 2018) which are adaptive to diverse ecological conditions (Zeilinger *et al.,* 2016). The biocontrol potential of *Trichoderma* is attributed to its ability to secrete cell wall degrading enzymes such as chitinases, β-1,3-glucanases and proteases *etc.* and then ultimately penetration of the target organism called mycoparasitism (Woo and Lorito, 2007), secrete antimicrobial secondary metabolites like gliotoxin, viridin, gliovirin, and trichoviridin (Sivasithamparam and Ghisalberti, 1998) as well as competition with the target pathogens for space and nutrients (Benitez *et al.,* 2004; Verma *et al.,* 2007), and ultimately modification of host plant's rhizosphere microbiome diversity by inhibiting phytopathogenic fungal growth (Harman *et al.,* 2004). In addition to these biocontrol mechanisms, the colonization of host plant roots by *Trichoderma* spp. has also been reported to boost the plant growth by alleviating the host plant's defense mechanism (Yedidia *et al.,* 2003).

Trichoderma genus accommodates about 260 species many of which are included based on the DNA sequence data (Samuels *et al.,* 2012; Jaklitsch *et al.,* 2013; Chaverri *et al.,* 2015). The different *Trichoderma* spp. are difficult to distinguish morphologically as there are paucity of morphological characters of taxonomic significance (Samuels *et al.,* 1998) and existence of morphologically cryptic species (two or more species considered as same species due to the morphological similarities) in *Trichoderma* (Kullnig *et al.,* 2001; Rai *et al.,* 2016). The *in vitro* antagonistic potential of *Trichoderma* spp. against *Fusarium* spp. was recognized long way back (Whipps and Lumsden, 2001). Since then different *Trichoderma* species have been exploited and recruited for the successful and sustainable management of diseases like maize stalk rot caused by *F. graminearum* (Li *et al.,* 2016), tomato wilt caused by *F. oxysporum* f. sp. *lycopersici*,

(Ghazalibiglar *et al.,* 2016); fusarium wilt in common bean incited by *F. oxysporum* f. sp. *phaseoli* (Carvalho *et al.,* 2014), panama wilt in banana caused by *F. oxysporum* f. sp. *cubense* (Bubici *et al.,* 2019). In cumin (*Cuminum cyminum* L.), wilt caused by *F. oxysporum* f. sp. *cumini* (Foc) is attributed to be the most destructive disease responsible for 0-96 per cent yield losses (http://krishikosh.egranth.ac.in/handle/1/5810043544). The transmission of cumin wilt takes place through soil or seed borne pathogen' propagules (Deepak and Patni, 2004). There are associated risk of deteriorating environmental, soil and human health with haphazard use of chemicals, thus it is advisable to use effective and virulent BCAs either alone or in combination with fungicides to achieve satisfactory and sustainable disease management. Thus in the present study, the *in vitro* antagonism of three *Trichoderma* isolates *viz*., CSR-T-2, CSR-T-3 and CSR-T-4 against the cumin wilt pathogen *F. oxysporum* f. sp. *cumini* was studied. Subsequently, the three isolates were characterized morphologically as well as their species were identified based on the ribosomal RNA gene sequencing.

Materials and methods

Collection of infected cumin samples and isolation of *F. oxysporum* f. sp. *cumini*: Infected parts of cumin plant showing disease symptoms were obtained from KVK Banasthali Vidyapith, Jaipur- Rajasthan which lies between latitude (26° 19' 13.08'' N) and longitude (75° 53' 9.24'' E). Parts of plants with symptoms of Fusarium wilt infection were surface sterilized by immersion in 0.3 % sodium hypochlorite for 10 minutes, and then in 70 % ethanol and later rinsed thoroughly with sterile distilled water. The small sections were transferred to potato dextrose agar (PDA) medium in petri plates and incubated at 26 ± 2 °C for seven days. The characteristic growth of the fungus with morphological characters of micro conidia and macro conidia and chlamydospores were observed. Pure cultures were maintained on PDA slants and stored at 4 °C in the refrigerator. To ensure the isolated pathogen as cause of fusarium wilt in cumin, Koch postulates were proved in cumin plants planted in pots containing sterilized soil inoculated with the pathogen (10^6 spores/mL). Un-inoculated plants were kept as control. The plants were observed for the development of symptoms.

Sample collection and isolation of *Trichoderma* species: The composite soil samples were collected from the banana rhizosphere of salt affected and Fusarium suppressive soil. Different *Trichoderma* species were isolated using soil dilution method on potato dextrose agar medium using dilutions 10^{-3} to 10^{-5} and the plates were incubated at 28±2 °C and observed at frequent intervals for the development of colonies. Three days old colonies morphologically similar to *Trichoderma* were picked up and purified by single hyphal tip method on fresh PDA plates. The green coloured colonies were identified by comparing with taxonomic key described by Barnett *et al.* (1972) and the cultures were stored in the refrigerator at 4 °C.

Screening of antagonistic potential of *Trichoderma* strains against *Fusarium oxysporum* f. sp. *cumini*: The antagonistic activity of *Trichoderma* spp. was screened *in vitro* against *Fusarium oxysporum* f. sp. *cumini*, by dual culture plate technique as described by Cherkupally *et al.* (2017). *Trichoderma* isolates and a pathogen species to be tested were cultured separately on PDA for 7 days. After 7 days, 5 mm mycelial plugs (taken from the edge of fungal colonies) of each species to be tested were transferred to PDA plates using cork borer. The mycelial plug of *Trichoderma* species and pathogens was placed equidistant from the periphery so that they would get equal opportunity for their growth and the growth was monitored at every 24 hours to calculate inhibition percentage After the incubation period, the radial growth of Foc in control, as well as in treatment plate was measured and the per cent inhibition was calculated using the formula (Vincent 1947):

$$L = \frac{(C - T)}{C} \times 100$$

Where,

L = Per cent inhibition of radial growth of pathogen (%)
C = Radial growth of the pathogen (cm) in control
T = Radial growth of the pathogen (cm) in treatment

Morphological and molecular characterization of *Trichoderma* spp.: The characteristics of *Trichoderma* spp. like colony appearance and sporulation pattern were examined by culturing on five media *viz*., Potato Dextrose Agar (PDA), Rose Bengal Agar (RBA), Nutrient Agar (NA), Corn Meal Agar (CMA) and Czapek Agar (CZA) at 28 ± 2 °C for 5 days. For observing colony characteristics and growth rate, mycelial bit was taken from the actively growing margin of 5 days old culture, grown on PDA. A 7 mm mycelial disc was placed at the center of all petri dishes. The plates were kept for incubation at 28 ± 2 °C. Radial growth was measured at 24 h intervals until colony covered the whole petri dish. The microscopic examination and measurements of conidiophores and microconidia were also made.

For molecular identification of the three *Trichoderma* isolates, the pure culture of same were inoculated in potato dextrose broth containing chloramphenicol at 25-30 ppm final concentration and incubated for 7 days at 28 ± 2 °C. The mycelium mats of all the three isolates were collected and dried under aseptic conditions. The dried mycelium was crushed into fine powder using liquid nitrogen in sterilized pestle mortar. Total fungal DNA was isolated using HiPurA™ Fungal DNA Purification Kit (Himedia) following the manufacturers' instructions. The DNA was subjected to PCR amplification using ITS1 and ITS 4 universal primers. The amplification was performed in with initial denaturation of 94 °C for 3 min followed by 35 cycles of 94 °C for 15 sec, 52 °C for 40 sec and 72 °C for 1 min with a final extension at 72 °C for 5 min. The PCR product checked in 1.5 % agarose gel stained with ethidium bromide and sequenced using the custom services of Xcelris Labs Limited, Ahmedabad, India. The sequence was annotated by BLAST analysis and phylogenetic tree was constructed using MEGA Version X

Results

***In-vitro* antagonistic efficacy of the *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *cumini*:** Twenty one *Trichoderma* spp. were isolated and were subjected to *in-vitro* antagonistic assays. Out of twenty one, three *Trichoderma* isolates *viz*., CSR-T-3 (*T. reesei*), CSR-T-2 (*T. koningiopsis*) and CSR-T-4 (*T. asperellum*) showed significant antifungal activity against *Fusarium oxysporum* f. sp. *cumini*. These three fungal antagonists showed a significant increase in the inhibition percentage between 48 hours and 120 hours. Among these three, isolate CSR-T-4

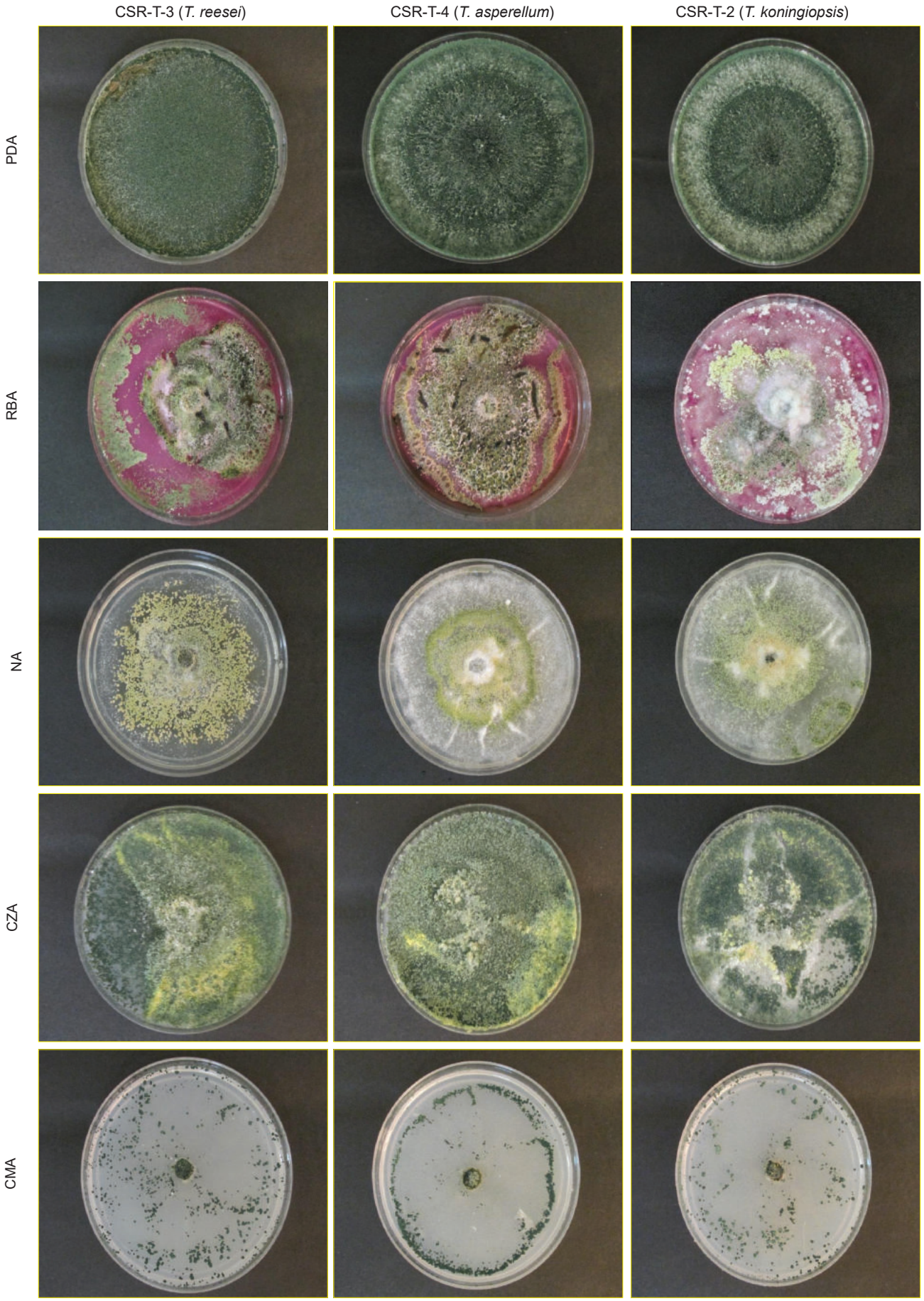

Fig. 1. Variations in colony morphology of different *Trichoderma* isolates grown on different media

Table 1. Description of morphological characteristics of three different isolates of *Trichoderma* spp.

Isolate	Colony growth rate (cm/day)	Colony colour	Reverse colour	Colony edge	Mycelial form	Conidiation	Conidiophore branching	Conidial colour	Chlamydospores
CSR-T-2	7 cm in 4 days	Dirty green	Dull green	Smooth	Floccose	Ring like zones	Regular	Green	Observed
CSR-T-3	7 cm in 4 days	Dark green	Light green	Smooth	Arachnoids	Flat	Densely branched and regular	Green	Not observed
CSR-T-4	7 cm in 3 days	Green	Light green	Smooth	Arachnoids	Ring like zones	Branched and regular	Green	Not observed

showed the highest inhibition percentage of 84.31 % (Fig. 2) at 120 hours after inoculation, whereas CSR-T-2 showed the lowest inhibition values (62.65 %). The inhibition percentage of CSR-T-3 was 79.85 %. Based on the significant antagonism shown by these three *Trichoderma* isolates, these were subjected to morphological as well as molecular characterization.

Categorization of *Trichoderma* isolates based on radial growth and cultural characteristics: The maximum radial growth was recorded in isolates CSR-T-4 at 5 days after inoculation (7.9 cm). Isolate CSR-T-3 recorded growth of 7.50 cm while minimum growth rate was observed at CSR-T-2 (6.90 cm). The colony characteristics of these three isolates were observed on PDA, RBA, NA, CMA and CZA. On PDA, CSR-T-3 produced dark green colored colony (Fig. 1). The isolates CSR-T-2 and CSR-T-4 produced a dense dark green colored colony with a concentric ring in the periphery, however this ring was more pronounced in case of CSR-T-2. On RBA, all the three isolates produced uneven irregular dense growth. CSR-T-3 and CSR-T-4 produced darker green colonies while yellowish green colored colonies were observed in case of CSR-T-2. On NA, CSR-T-3 produced yellowish green colony with uniform conidiation. CSR-T-4 produced colony with yellowish green colour in the center and sparse white mycelia growth in the periphery. Similarly, CSR-T-2 produced sparse white mycelia growth in the periphery with green conidiation scattered all over the plate but less dense in the periphery. On CDA, all the three isolates fully covered the plate with uneven conidiation. CSR-T-3 and CSR-T-4 produced yellow to green colored condition unevenly distributed while white and green colored conidiation was obtained in case of CSR-T2. On CMA, there was very sparse white growth with dispersed green conidiation all over the plate in case of CSR-T-3 while in CSR-T-4, sparse white growth with green conidiation in form of ring at the periphery was observed. Very less conidiation unevenly distributed all over the surface was obtained in case of CSR-T-2. The three different species of *Trichoderma* exhibited different growth rates on six media at same temperature of 28 °C. CSR-T-4 grew faster on all media followed by CSR-T-3 and then by CSR-T-2.

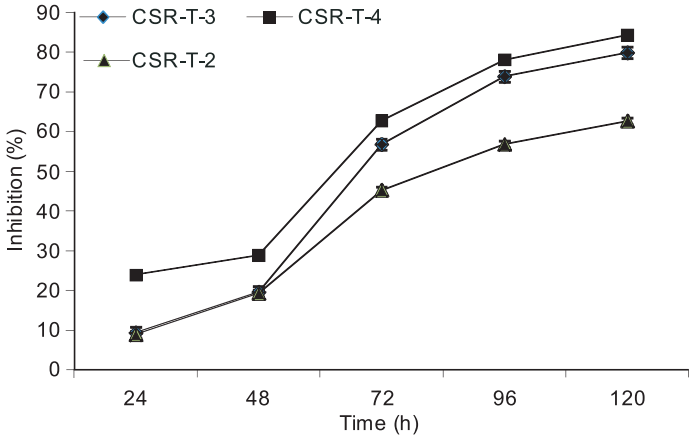

Fig. 2. Inhibitory effect of *Trichoderma* isolates against pathogenic isolate of cumin (*Fusarium oxysporum* f. sp. cumini).

Micro morphological characteristics: While examining the five days old culture of CSR-T-3, CSR-T-4 and CSR-T-2 grown on PDA, the following micro morphological differences were observed (Table 1). The conidia of CSR-T-3 were globose, conidiophores were densely branched and verticillate. Phialides were divergent, cylindrical in shape and slightly inflated. The conidia of CSR-T-4 were subglobose to obovoid in shape. The branching of conidiophores were frequent in verticillate manner. Phialides were convergent and ampulliform or flask shaped. CSR-T-2 produced globose to subglobose conidia, branched conidiophores with branching at less than 90°. Phialides were slightly lageniform (flask) in shape and divergent.

Molecular characterization of *Trichoderma* species: The total DNA of three *Trichoderma* isolates was subjected to PCR amplication using ITS1 and ITS4 primers for their species identification. An amplification product of ~600 bp was observed in all the three isolates which were sequenced using custom services of Xcelris Labs Limited. The annotated sequences thus obtained were submitted at NCBI database vide accession numbers KJ812401 (CSR-T-2), MH997668 (CSR-T-3) and MN227242 (CSR-T-4). The three isolates *viz*., CSR-T-2, CSR-T-3 and CSR-T-4 were identified as *T. koningiopsis*, *T. reesei* and *T. asperellum,* respectively. A phylogenetic tree through neighbor joining method was constructed using the present sequences as well as other *Trichoderma* species obtained from NCBI database through MEGA software (Fig. 3).

Discussion

In India, cumin is largely grown in India in more than five lakh hectare with the production of three tones. The crop suffers due to several diseases which negatively influence the yield. Wilt is one of the most destructive disease of the cumin crop (Khare *et al.,* 2014) which results in yield loss up to 60 % in Rajasthan and 25 % have been reported from North Gujarat. Recent reports revealed that the cumin varieties *viz*., JC-2000-21 and JC-2000-22 which were considered tolerant to wilt has become susceptible (Talaviya *et al.,* 2017). Thus, currently biocontrol agents seems to be only sustainable measure to cope with the soil-borne diseases in general and fusarium wilt in particular. With this objective, 3 out of 21 *Trichoderma* spp. that were isolated from the banana rhizosphere of salt affected and wilt

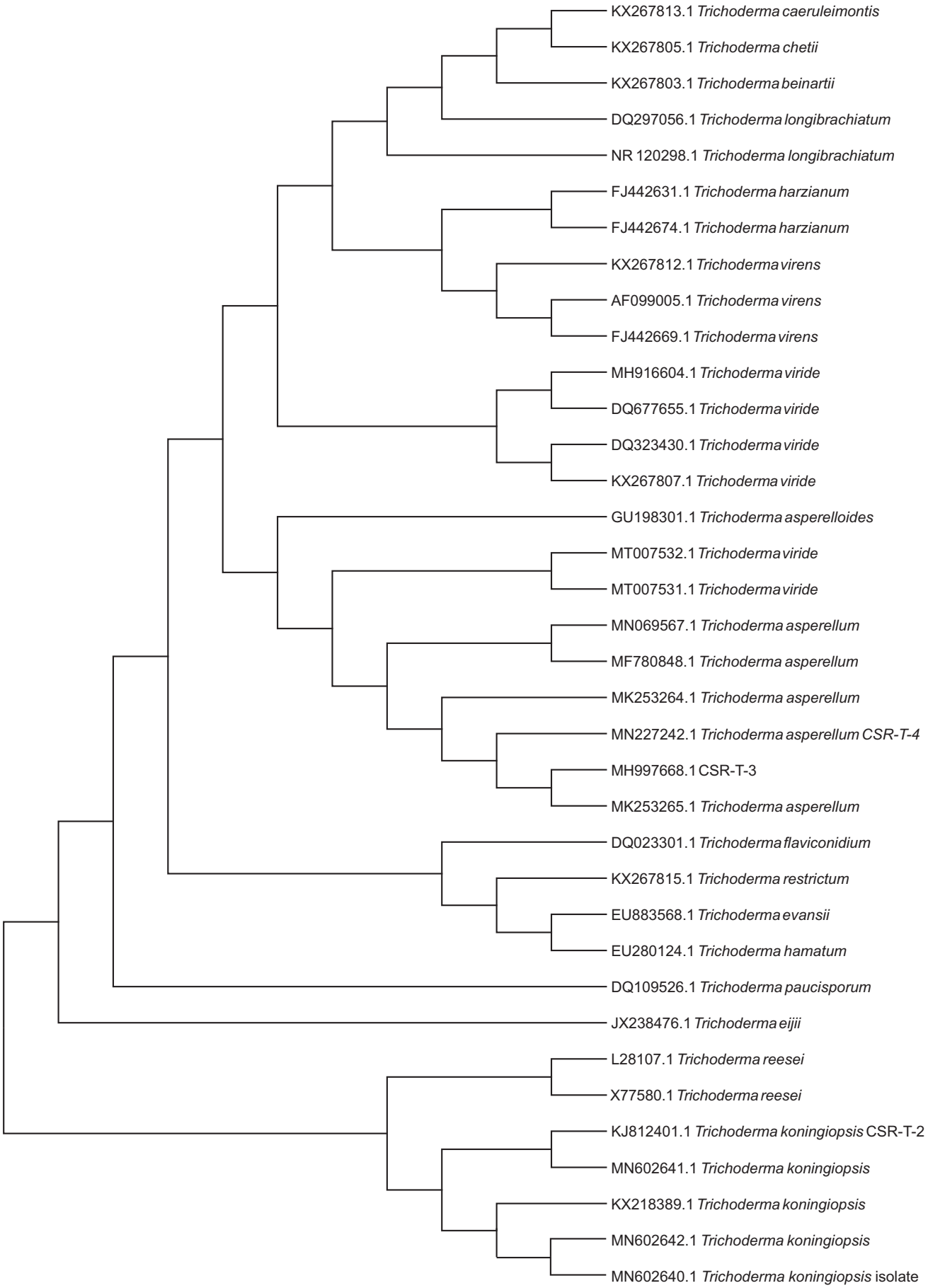

Fig. 3. 18S rRNA gene based phylogenetic relationship of different *Trichoderma* spp. Tree was constructed by neighbour-joining method using MEGA X software

suppressive soils of Uttar Pradesh under abiotic and biotic stress (Damodaran *et al.,* 2019) were characterized on the basis of their cultural, micro-morphological features and molecular techniques. Screening of the antagonistic potential of isolated *Trichoderma* isolates against *F. oxysporum* f. sp. *cumini* were conducted and 3 *Trichoderma* isolates showed strong antagonistic potential which inhibited >80 % mycelial growth of cumin wilt pathogen. Out of three fungal antagonists studied for their efficacy, *T. asperellum* showed maximum extent of inhibition followed by *T. reesei* and *T. koningiopsis*. The microscopic images taken from the point of interaction between the biocontrol agent and pathogen demonstrated the parasitism of *F. oxysporum* f. s*p. cumini* hyphae by *Trichoderma* species. Antagonist hyphae were observed to be growing towards the pathogen hyphae and coiled it completely. The biocontrol agents were observed to produce knob like structure to derive their nutrition called as haustoria. These haustorial knob like structures with penetration pegs, penetrate the host and finally dissolve the protoplasm and shrink the hyphae which may lead to lysis (Weindling 1932). Antagonism by *Trichoderma* spp. against a range of soil borne plant pathogens has been reported earlier (Papavizas, 1985; Elad *et al.,* 1982).

Observations on the growth and colonization of the test pathogens in dual culture screening by the antagonistic isolates proved that different species of *Trichoderma* have variation in their ability to inhibit the growth of the pathogen *F. oxysporum* f. sp. *cumini*. Moreover, often a biocontrol agent inhibiting the pathogen effectively in laboratory conditions fail to replicate similar effective results in the field due to the complexity of the environment under *in vivo* conditions. Therefore, it is necessary to exploit the best performing isolate *T. asperellum* in the management of fusarium wilt in cumin under field conditions.

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