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Exploitation of 16s rRNA Gene as a Tool for Identification of *Pseudomonas syringae* pv. *tagetis*, the Causal Agent of Bacterial Leaf Spot Disease of French Marigold

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A B S T R A C T

The aim of this study was to isolate, identify *Pseudomonas syringae* pv. *tagetis* (*Pst*) from seeds of French marigold (*Tagetes patula*). A total of 25 bacterial pathogens were isolated and tested for basic biochemical and host tests. Results of basic biochemical and host tests primarily indicated the presence of *Pseudomonas syringae* pv. *tagetis*. The 16S rRNA gene sequencing was performed on PCR amplified product with 8F and 1492R primers. The two isolates Tp-03 and Tp-09 showing 99.34% and 99.20%, sequence homology with *Pst* strain LF2012 which was isolated from seeds of French marigold.

Introduction

India is widely recognized for its abundance of medicinal plants, which serve as a valuable source of pharmaceuticals for the management and treatment of various ailments. According to Soule (1994), experts have referred to a fragrant annual plant belonging to the Asteraceae family as Tagetes. Nevertheless, different taxonomic groups within the family Asteraceae that possess inflorescences characterized by golden or yellow capitula are commonly referred to as "marigolds." The genus Tagetes consists of approximately 33 species, of which only five species have been officially introduced into Indian gardens. These species include *Tagetes erecta* L. (commonly known as Aztec or African Marigold), *Tagetes minuta* L. (also known as *Tagetes glandulifera* Schrank), *Tagetes patula* L. (referred to as French Marigold), *Tagetes lucida* Cav. (known for its sweet scent), and *Tagetes tenuifolia* Cav. (commonly called Striped Marigold) (Rydberg, 1915). The marigold plant's predominantly yellow or orange blossoms have been traditionally employed in India for culinary purposes, as well as in the preparation of herbal infusions and medicinal treatments. Various products such as tea, elixirs, balms, and creams can be derived from both fresh and dried plant materials.

Marigolds possess several attributes that contribute to their significant value and extensive cultivation as ornamental plants across the globe. Marigolds have gained popularity among gardeners due to their cultivation convenience, adaptability to diverse

environmental and technical conditions, and consistent production of flowers throughout the year (Patyka *et al.,* 2019).

In addition, marigolds have gained popularity among both amateur and commercial flower growers due to their diverse range of utilities, short production period, wide variety of attractive color palettes, shapes, sizes, and plant habits. Marigolds have extensive applications in industries and medicine, as well as serving as natural sources for dyes, insecticides, and herbicides (Camele, *et al.,* 2019; Laosinwattana *et al.,* 2018).

The germination process, whereby a plant emerges from a seed, represents a pivotal stage in the life cycle of a plant. The primary objective of seeds within agricultural systems is to initiate the commencement of a fresh crop cycle. Seeds are frequently produced on a large-scale for commercial purposes, undergoing extensive manipulation and treatment, and subsequently sown in a standardized fashion across vast geographical regions. Nevertheless, within ecosystems, seeds serve multiple purposes beyond their role in initiating the life cycle and facilitating species reproduction. The aforementioned functions encompass the facilitation of dispersion, adaptation to, and persistence in novel habitats (El-Hefny, *et al.,* 2000). During the process of seed germination, both seeds and seedlings are particularly vulnerable to various factors that can lead to their death. These factors include dehydration, granivores, as well as infections caused by fungal and bacterial pathogens that are present in the seeds themselves (Goszczynska, 2000). Following the process of germination, seedlings persistently encounter threats to their establishment posed by pathogens. Consequently, the progression from seed to seedling represents a critical stage in the life cycle of plants, holding significant importance in both natural and agricultural contexts. The reason for this is that the seed-to-seedling stage represents one of the initial phases in the life cycle of a plant (Bhalodia *et al.,* 2011).

Hence, it is of utmost importance to understand the characteristics and consequences of microbial interactions occurring prior to and during the susceptible phases of plant growth. These interactions play a pivotal role in shaping the patterns of plant population and community dynamics within natural environments, as well as determining the outcomes of crop production in agricultural systems (Balouiri, *et al.,* 2016).

During a field survey conducted in the Alwar district of Rajasthan, samples of seeds from various tehsils were collected. It was observed that the French marigold seeds were naturally infected with *Pseudomonas syringae* pv. *tagetis*, which is known to cause Bacterial leaf spot. The isolation of this pathogen was primarily carried out from diseased plant parts, particularly the seeds. *Pseudomonas syringae* pv. *tagetis* is the causative agent responsible for the occurrence of bacterial leaf spot in marigold plants. This disease, characterized by its seed transmission capability, poses a significant threat to the early stages of marigold growth, resulting in severe devastation. The first documented sighting of the phenomenon occurred in the United States in 1978, specifically in a field located in Wisconsin (Styer *et al.,* 1980). Subsequently, it was observed in greenhouses in North Carolina during the period spanning from 1983 to 1984.

One of the initial indications of this disease is the emergence of melanized lesions on the cotyledons of young plants. Additional symptoms encompass the presence of necrotic lesions on foliage encompassed by irregular chlorotic tissues, chlorotic and distorted growth at the apex, and in certain cases, the ailment may ultimately result in the demise of the affected botanical specimen. Shane and Baumer (1984) conducted research which revealed that the pathogen accountable for bacterial leaf spot on marigolds is also implicated in the development of diseases affecting zinnia, common ragweed, sunflowers, and Jerusalem artichokes. The management of leaf spot infection in marigolds can be effectively achieved by utilizing seeds that are free from the bacteria responsible for the disease, as these bacteria are transmitted through the seeds. Identifying and

effectively managing *Pseudomonas syringae* pv. *tagetis* poses challenges due to the absence of its metabolic profile and pathogenicity information in the currently accessible database (Mir *et al.,* 2019).

The most frequently employed detection methods rely on visual inspection, growth patterns, serological properties, and bioassays. However, these approaches exhibit limitations in terms of sensitivity, efficiency, and specificity, and are also characterized by increased labor and time requirements. Currently, polymerase chain reaction (PCR) based gene sequencing has emerged as a highly effective method for the detection of bacterial pathogens in seeds and other plant materials (Guilbaud *et al.,* 2016).

Pseudomonas syringae exhibits a diverse array of pathovars, each with distinct host specificities. However, there is currently limited understanding of the pathovar of French marigold. Therefore, the purpose of the present study was to isolate and identify bacterial pathogens from diseased French marigold seeds cultivated in Rajasthan. This was achieved through the utilization of biochemical and molecular characteristics.

Materials and Methods

Isolation and purification of the pathogen

The present study was conducted in Department of Botany, RR college, Alwar, Rajasthan India. Approximately 39 suspected samples were collected from 5 sites of Alwar district. The seeds of French marigold were surface sterilized by immersing in 90% (v/v) ethanol for approximately one minute and followed by 1% (v/v) NaOCl for 5 min and subsequently washed with sterile distilled water. Surface sterilized seeds were transferred on nutrient agar (NA) medium plate and incubated for 2-3 days at 28±2°C. The bacterial colonies developed under the seeds were chosen and a diluted suspension was streaked on semi selective King's B (KB) medium.

Colonies showing typical *Pst* characteristics were subjected for identification by using various biochemical and molecular methods.

Pathogenicity test (Host test)

On the basis of primary biochemical characteristics selective isolates were used to study their virulence on French marigold and on other hosts like *Sunflower*, *Jerusalem-artichoke*, *Willow-leaf sunflower* and *Common ragweed*. Leaves of marigold and other hosts were artificially inoculated by puncturing and injecting a drop of bacterial suspension with a concentration of 1×10^7 CFU/mL whereas sterile tap water was inoculated as a control (Young, 2010; Peix *et al.,* 2018). These leaves were incubated for 7 days with alternate daylight/darkness cycles at 25±2°C and observed daily for pathogenicity and development of symptoms (Schaad and Kendrick, 1975; Saettler *et al.,* 1989).

16s rRNA tool for identification

Isolation of the genomic DNA

The genomic DNA of the biochemically identified Pst strains was extracted by CTAB method reported by Sambrook and Russell (2001). The 5 ml of fresh bacterial suspensions were subjected to centrifugation at 10,000 rpm for 5 min and obtained pellet was further suspended in TE buffer (567 µl, 10 mM Tris, 1 mM EDTA) by gentle mixing on vortex. Subsequently SDS (30 µl, 10%) and proteinase-K (3 µl, 20 mg/ml) were added to above suspension and incubated at 37°C for 1 hour in a water bath. After that sequentially 100 µl volume of 5M sodium chloride and 80 µl volume of CTAB/NaCl (10% CTAB dissolved in 0.7M NaCl) were mixed to the above preparation. Successively phenol, chloroform and isoamyl alcohol (25:24:1) were mixed to it properly, and then centrifuged at room temperature for 1-15 min at 10000 rpm. The supernatant was collected in a fresh micro-centrifuge vial and mixed with 300 µl isopropanol and 50 µl sodium acetate. The subsequent blend was again centrifuged at RT (room temperature) for 10 min at 10000 rpm. Obtained supernatant was

cleansed with 70% ethanol (chilled) and agitated and again centrifuged at 4°C for 4 min at 10000 rpm. Now the pellet obtained contains DNA, was dried and suspended in 50 µl TE buffer and kept at 4°C until further use.

Amplification and sequencing of 16S rDNA

It is observed that the 16S rDNA and other housekeeping gene sequences used as an easy marker tool to analyse taxonomy of bacterium of interest (Chaturvedi *et al.*, 2018). The 16S rDNA of selective isolates were amplified using the forward and reverse primers F8 (5′-AGTTTGATCCTGGCTCAG-3′) and R1492 (5′-ACCTTGTTACGACTT-3′) as given in literature (Edward *et al.*, 1989; Stackebrandt and Liesack, 1993) in Veriti® 96 well Thermal Cycler (Model No. 9902) by outsources (Xcelris labs ltd, Ahmedabad, Gujarat, India). The final reaction volume for each sample was 25 µl containing 10X buffer template DNA (with 50 ng), primer (0.25 µM of each), 200 µM of each dNTPs, 0.7U Taq DNA polymerase and 1.5 mM $MgCl_2$ for amplification.

The PCR cycling was started with initial denaturation at 96°C for 2 min followed by 30 cycles involving of 94°C for 30 s (denaturation), 60°C for 1 min (annealing) and 72°C for 1 min (amplification); and final extension at 72°C for 10 min (final amplification). PCR products along with 2µl of DNA ladder (500bp) were electrophoresed using 1% agarose gels (with ethidium bromide) at 50 Volt for 1 h and bands were observed under UV light.

The amplified 16S rRNA gene sequences of two isolates TP-03 and TP-09 were sent for sequencing by using BigDye Terminator v3.1 Cycle Sequencing Kit by outsource (Xcelris Labs Ltd., Ahmedabad, Gujarat, India). Obtained sequences were analysed, aligned and corrected by using GeneTool Lite and BioEdit software and subsequently the corrected sequences were submitted to NCBI to get accession number.

Phylogenetic analysis of 16S rRNA gene sequences

The experimental sequences were analysed by using BLAST tool to find out similarities of these sequences with other sequences and reference sequences in database. Sequences from current study and similar reference sequences were aligned by ClustalW and the phylogenetic tree was constructed by using MEGA VI (Kumar *et al.*, 2016) with the maximum likelihood method based on a GTR+G+I model (Tavare, 1986).

Results and Discussion

Isolation and purification of the pathogen

In total 39 bacterial strains were isolated and purified on NA medium from French marigold seeds collected from fields. Colonies from all the isolates were convex, whitish, mucoid, raised, smooth and glistering colonies and able to produce characteristic green fluorescent pigment in the KB medium. This fluorescence principally indicated these colonies to be *Pseudomans* spp on NA medium.

Basic Biochemical and Host tests

Isolated strains were negative for Gram's staining and positive for KOH solubility and catalase activity which indicated that isolated bacterial strains were rod shaped gram negative. Isolated colonies were producing a yellow – green to blue fluorescent pigment on iron deficient King's B (KB) medium, indicated possible bacterial group may be fluorescent pseudomonas spp. (Mohan and Schaad, 1987). Pathogenicity of pathogen was tested on leaves of *French Marigold* and on other hosts like *Sunflower, Jerusalem artichoke, Willow-leaf sunflower* and *Common ragweed* and they showed development of disease symptoms such as necrosis and chlorosis of the leaves which were observed after incubation of 4-7 day. In previous studies, similar responses of biochemical and hypersensitivity reactions of *Pseudomonas syringae* pathovars have been observed in other plants

(Mougou and Boughalleb-M'hamdi, 2018; Khezri and Mohammadi, 2018). Also, pathogenicity test in different members of marigold family were showing green sunken lesions after 3 days of filtration, confirmed *Pst* infection. Jangir *et al.,* (2018) also performed these biochemical tests to identify infection of *Ralstonia* sp. in soyabean plant.

Molecular characterization of isolates

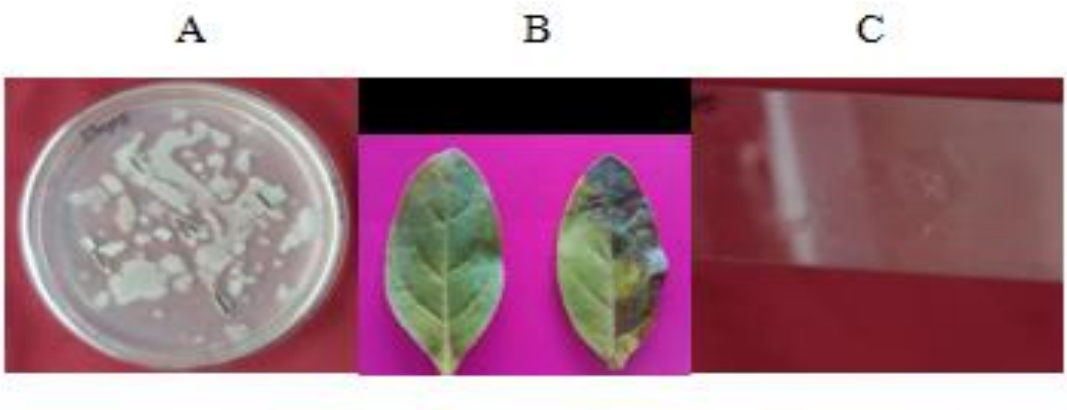
Although 25 isolates were identified as *Pst* in biochemical characteristic analysis but only two of them (Tp-03 and Tp-09) which were more vigorous in pathogenicity host test were taken for molecular characterization and bio-control studies. After isolating the genomic DNA form strains TP-03 and TP-09, both of these strains were subjected to a molecular characterization process. The 16S rDNA sequence was adequately amplified and observed on the gel for a subset of the strains (Fig 5) In addition, these amplified sequences were successfully sequenced by an outsourcing.

Consensus sequence of 1501 bp (TP-03) and 1495 bp (TP-09) of 16S rDNA were produced by establishing contiguous alignments with the use of GeneTool software. Based on the similarities between the two sequences and the type strain, BLAST identified both of the sequences corresponded to the species *Pseudomonas syringae* pv. *tagetis*. Both (Tp-03 and Tp-09)16SrDNA sequences were repaired and then submitted to the NCBI Gene Bank under the accession numbers OR016447 and OR016448 respectively (NCBI FASTA file given below). In the phylogenetic study of 16S rDNA sequences, both of the sequenced strains of bacteria grouped together in the same clade as the type strains. In addition, TP-03 and TP-09 revealed sequence similarities with strain *Pst* strain LF2012 16S ribosomal RNA gene, partial sequence GenBank: KP796138.1, which was isolated from Bacterial leaf spot in marigold plants caused by *Pseudomonas syringae* pv. *tagetis* in, at a level of 99.34% and 99.20%, respectively. It is reported earlier also that the best possible method to identify *Pss* relies on the amplification and sequencing of the 16S rRNA gene (O'Brien *et al.*, 2011). In another study, PCR and sequencing technique was used to identify *Pss* in diseased parts of wheat and barley plants (Guilbaud *et al.,* 2016; Yoshioka *et al.,* 2020). In studies on other pathovars also, 16S rRNA gene sequencing has been proved to be a tool of excellence for their identification on various crops (Verma *et al.,* 2016; Chaturvedi *et al.*, 2018).

Phylogenetic analysis of 16S rDNA sequence

Given that all isolates induced disease symptoms such as necrosis and chlorosis in the host plants, it can be inferred that all isolates exhibited a high degree of pathogenicity. Out of the 25 isolates that underwent biochemical characteristic analysis, only two isolates (TP-03 and TP-09) were chosen for further molecular characterization and bio-control activity due to their robustness and rapidity in the pathogenicity host test. These isolates were found to be positive for *Pst*. Following the extraction of genomic DNA from strains TP-03 and TP-09, a molecular characterization procedure was conducted on both strains. The amplification of the 16S rDNA sequence was successfully achieved and visualised on the gel for a specific group of strains, as depicted in Figure 2. Furthermore, the amplified sequences denoted as TP-03 and TP-09 were effectively subjected to sequencing through an outsourcing process, resulting in the generation of consensus sequences spanning 1495 bp (TP-03) and 1501 bp (TP-09) of the 16S rDNA. The contiguous alignments were established with the aid of GeneTool software. The genomic resemblances and type strain prompted BLAST to classify both entities as members of the *Pseudomonas syringae* pv. *tagetis* species. The 16S rDNA sequences of TP-03 and TP-09 were rectified and subsequently deposited in the NCBI Gene Bank with the accession numbers OR016447 and OR016448, correspondingly.

Fig.1 Results of colony morphology, KOH solubility test and host test



A: White, glistening and mucoid colonies of *Pseudomonas syringae* pv. *tagetis* on NA
B: Positive hypersensitive host reaction
C: Bubbles indicating KOH solubility test

Fig.2 1.2% Agarose gel indicating single 1500 bp of 16S rDNA amplicon (A: Agarose gel electrophoresis of isolated bacterial genomic DNA; Lane 1: DNA ladder of 33kb; Lane 2-4: DNA isolated with approx 33kb in size, B: PCR product of 16s rRNA gene amplification; Lane 1: DNA ladder of 1kb; Lane 2-7: *P. syringae* pv. *tagetis* isolates)

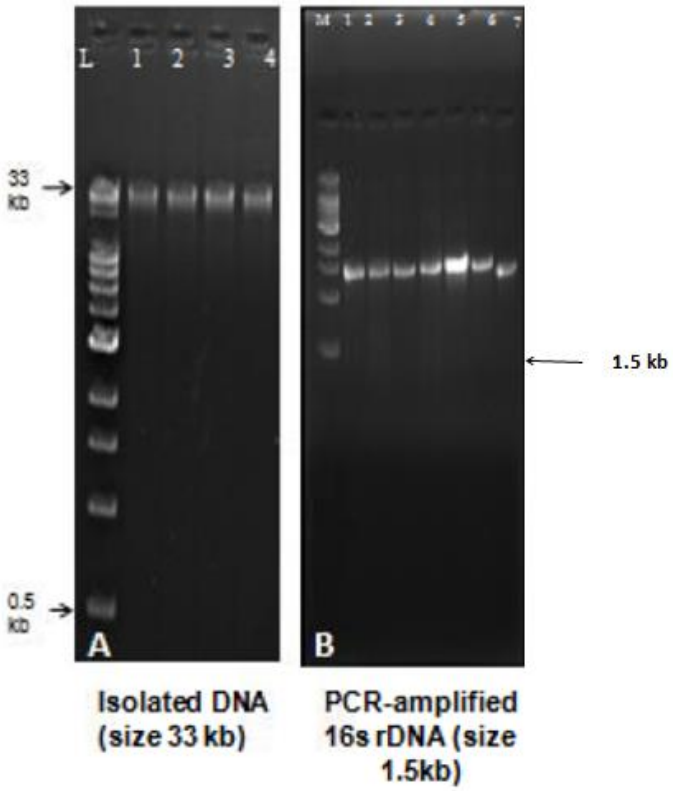
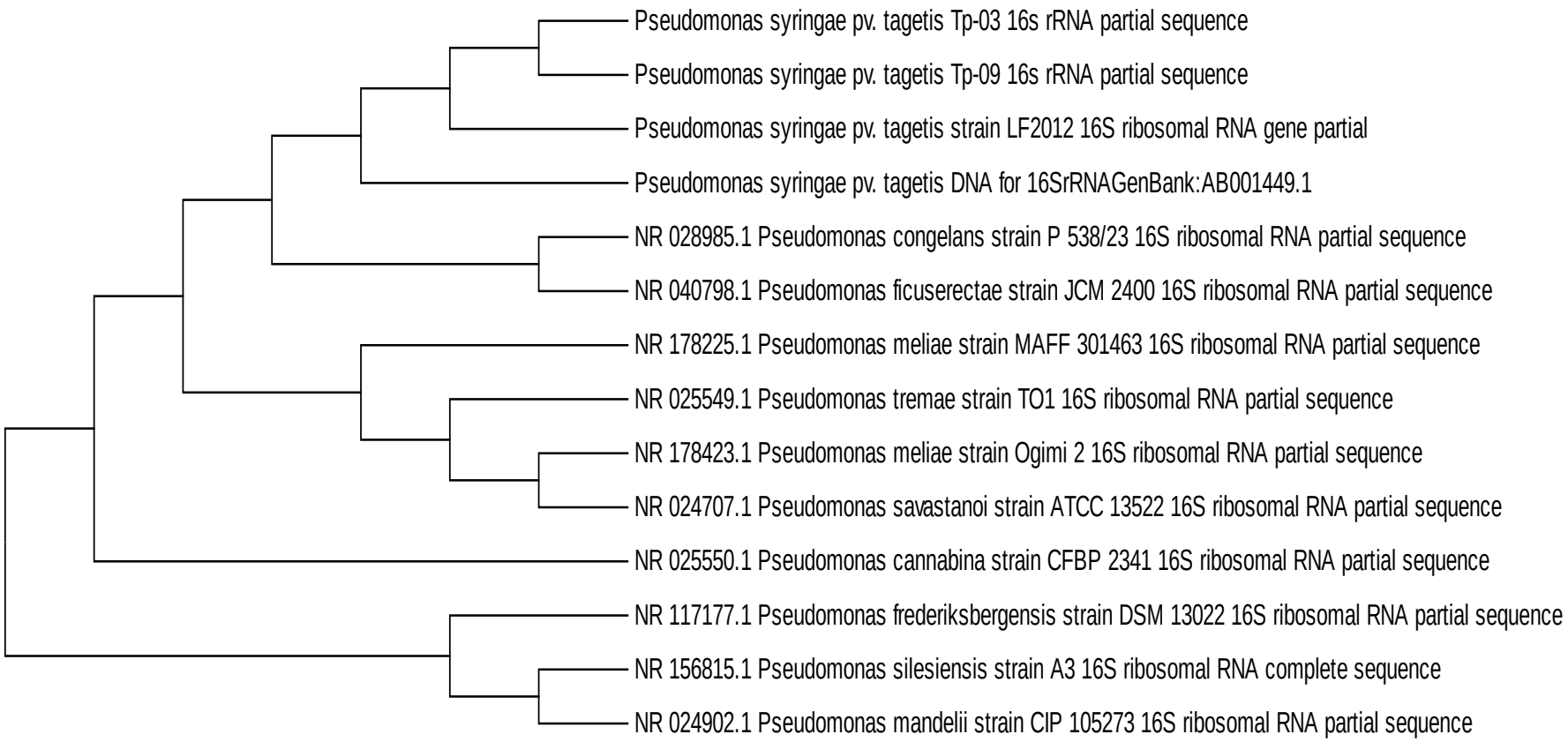


Fig.3 Phylogenetic tree of 16S rDNA gene sequences of two bacterial strains (in bold) isolated from *T. patula* seeds collected from Alwar, Rajasthan together with related type strains of *P. syringae tagetis*. The tree was constructed by means of a maximum likelihood (ML) method using Mega 6 software.



As per prior research conducted by O'Brien *et al.,* (2011), the most efficacious method for detecting *Pseudomonas syringae* pathovars is through the amplification and sequencing of the 16S rRNA gene. The dynamic nature of *P. syringae* genomes is apparent, as they frequently engage in horizontal gene transfer, a mechanism for gene exchange, as noted by Baltrus *et al.,* (2014). Ravindran (2015) has demonstrated that there exists a conserved set of core genes that are essential for fundamental housekeeping functions in both *P. syringae* and plants. Previous studies have corroborated the notion that identification of the *Pseudomonas syringae* populace can be achieved through the utilisation of PCR and sequencing techniques (Kawaguchi, 2017).

In a separate study, Guilbaud *et al.,* (2016) and Yoshioka *et al.,* (2020) employed the polymerase chain reaction (PCR) and sequencing techniques to identify the presence of *Pss* in affected regions of wheat and barley crops. Previous studies have shown that the utilisation of 16S rRNA gene sequencing is a proficient approach in the identification of various pathovars of *Pseudomonas syringae* across multiple crops. This has been demonstrated by Verma *et al.,* (2016) and Chaturvedi *et al.,* (2018).

Further, in our study, the reference sequence of Pst strain LF2012 16S ribosomal RNA gene, partial sequence GenBank: KP796138.1, which was isolated from Bacterial leaf spot in marigold plants caused by *Pseudomonas syringae* pv. *tagetis*, showed sequence homology at a level of 99.34% and 99.20%, respectively for TP-03 and TP-09.

Thus *Pseudomonas syringae* pv. *tagetis* was isolated from seeds of French marigold (*Tagetis patula*) and identified on the basis of its 16s rDNA sequence and other phenotypical characteristics from Alwar district of Rajasthan state. Earlier also, genomic fingerprinting (GF) including PCR and sequencing has been employed for detecting the Pst population (Kawaguchi, 2017).

This study established that *Pseudomonas syringae* pv. *tagetis* (Pst) is a seed borne pathogen in French marigold which is a major food crop of human beings during winters in torpical regions. Our study indicated that strains of *Pst* were highly diverse in their host range. The 16S DNA phylogenic studies confirmed that 2 strains of isolates TP-03 and TP-09 have 99.34% and 99.20%, similarity with type strains of Pst.

The above studies provide detail information about the isolation, characterization, and identification of pathogen associated with diseased French marigold seeds. Further research is desirable to evaluate different solvents for the extraction efficiency of presence of more anti-microbial metabolites, interpretation of chemical structures and test their properties against this pathogen.

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