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Aetiology and molecular characterization of the pathogens associated with soft rot disease of *Aloe vera* (L.) Burm. f.

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ABSTRACT

Soft rot is a devastating disease of aloe and the infected plants developed symptoms of rotting tissues from collar region upwards. Aloe plants artificially inoculated with a *Dickeya* spp. (reported earlier as the causal agent) at the root zone but did not develop the disease symptoms, eliminating the possibility of pathogen to entering through roots. Besides, a *Fusarium* spp. was repeatedly isolated from collar regions of the naturally infected plants. Further, it was established that lesions produced due to fungal infection predisposed the subsequent infection of the bacterial pathogen. Sequencing results and phylogenetic analysis based on three partial genes of bacteria (*dnaX, icdA* and *mdh*) and fungus (*ITS, TEF-1α* and *RPB-2*) confirmed the identity of pathogens as *Dickeya zeae* and *Fusarium falciforme*, respectively. An artificial inoculation technique was developed for quick screening of aloe germplasm for resistance of bacterium. Among 40 accessions screened, none was found resistant, however, *F. falciforme* failed to produce lesion on two accessions (Guj4 and Raj3), consequently making them resistant to soft rot disease upon combined inoculation with both the pathogens. Besides the genetic constituent, rapid rotting was observed at 35 °C but not at and below the 15 °C temperature. *In planta,* the bacterium concentration increased gradually with the rise of incubation temperature between 15 and 35 °C. The present study suggests possible management aspects of the problem through (i) exploiting host resistance and (ii) escaping post–harvest decay by storing and transporting aloe leaves at temperatures ≤ 15 °C and (iii) avoidance of water stagnation in field.

1. Introduction

Aloe vera (L.) Burm. f. (syn. *A. barbadensis* Mill.) is a succulent xerophyte, suitable for growing under warm, dry tropical and subtropical climates around the world. The genus *Aloe* of the family *Liliaceae* is comprised of about 400 species (Hęś et al., 2019). In addition to the pharmaceuticals and cosmetics, it is also widely used in food industries as containing more than 200 active compounds and antioxidants (Embuscado, 2015). Aloe leaf juice is used for treating burns, suppurative wounds and alleviating pain occurring due to injuries (Varaei et al., 2017; Maan et al., 2018; Hekmatpou et al., 2019). Aloe gel having moisturising properties therefore, has become an indispensable component of various skin care products (NCCAM, 2012; Radha and Laxmipriya, 2015). The cathartic properties of aloe attributed by presence of anthraquinones, including hydroxyanthracene derivatives, aloinA, aloin B, isobarbaloin and aloe emodin (Hamman, 2008). Awareness about the natural source of antioxidants for health care, nutritional value of food, extensive use in cosmetics and pharmaceuticals, increased the demand of aloe or products containing aloe. Large-scale cultivation of aloe practised in Barbados, in north of the USA and the Caribbean islands to meet demand of the western world. In India, organised cultivation of aloe (*A. barbadensis* and *A. chinensis*)

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catching up and being cultivated in certain pockets of Gujarat, Rajasthan, Madhya Pradesh, Uttar Pradesh and gradually expending toward the southern part.

Soft rot disease of aloe caused by *Pectobacterium chrysanthemi* (*Dickeya* spp.) is a potential problem for the aloe growers in India (Mandal and Maiti, 2005). The disease incidence ranged from 10% to 26% in the field conditions (Saran et al., 2019). The aloe plant gets infected at any stage, from transplanting to harvesting stage to soft rot disease. *Dickeya* spp. and *Pectobacterium* spp., placed in the family *Pectobacteriaceae,* cause soft rot diseases on many of the plant species. These bacteria have wide host range and habitat in verity of environmental niches such as soil, water and on plant surface without producing any symptoms (Shi and Cooksey, 2009; Toth et al., 2021). The pectolytic bacteria classified under the genera *Dickeya* and *Pectobacterium,* not only produce pectinases, but also produced other cell wall degrading enzymes (de Laatet al., 1994; Curland et al., 2021). Symptom development on host determined by various factors including, host susceptibility, virulence factors and favourable environmental conditions. Once the bacterium enters inside the host tissue by active or passive mode, soft rotting of tissues are the first major visible symptoms. Natural injuries incited by mechanical damage during agricultural operations, fungal infection, insect feeding, nematode infestation and any other means, provided passive entry to the bacterium into host. *Dickeya* usually initiate soft rot at natural openings (*e.g.*, lenticels) and/or in wounds (Reverchon et al., 2016). deLaat et al., in 1994 opined that infection possibly started from the roots, those later degenerated. Once the soft rot disease infection started, it is very difficult to be managed. The aetiology of disease provides an important insight in to formulating the management strategies against any plant disease, but the pathogenesis of aloe soft rot is unknown so far.

Considering the wide adaptability of the crop, economic importance of disease and research gaps the present study was devised to understand the aetiology of the disease and characterization of associated pathogens. Besides that, attempts were also made to identify the resistance source of aloe against soft rot disease. It will lead to manage the disease with low-cost technologies without application of chemical pesticides, such as genetic resistance, good agricultural practices and drainage facility.

2. Materials and methods

2.1. Field observations and plant material

The experiments were carried out at the research farm of ICAR–Directorate of Medicinal and Aromatic Plants Research (DMAPR), Anand, India, located at 22° 35' N, 72° 55' E at an altitude of about 45.1 m above mean sea level. The maximum and minimum temperature ranged between 44.0 °C and 12.7 °C during the experimentation. A susceptible clone of *A. barbadensis* (IC112527) was used in this study, unless mentioned. Healthy and young suckers were randomly collected from the research plot by uprooting, washed 3–4 times under running tap water and rooted for 20 days in trays containing sterile moist sand. The rooted plants were transplanted into 2 L plastic pots containing autoclaved garden soil and kept in a green house till first new leaf emerged (approx. 30 days). No additional light arrangement, fertiliser or pesticide application was provided. Plants were watered on alternate days. Temperature varied between 31 and 42 °C during the study period.

2.2. Isolation of pathogens

2.2.1. Fungal isolates

Freshly, soft rot infected (naturally) plants of aloe were collected from different locations (Table 1). Tissues samples from collar region and roots of the infected plants were collected for isolation of the associated pathogens. The associated fungus was isolated from the fresh and naturally infected plants tissues following the procedure described by Meena and Roy (2020) and for single microspore isolation standard method was followed (Choi et al., 1999; Meena and Roy, 2020).

Table 1
Details of the isolated and characterized pathogens used in the study.

Pathogen	Location and year of collection	Target gene	GenBank accession #
Dickeya zeae IMI 389157	Boriavi, Anand, Gujarat, 2018	*dnaX*	MH790975.1
		icdA	MH790976.1
		mdh	MH790977.1
Dickeya zeae BAG–20	Boriavi, Anand, Gujarat, 2020	*dnaX*	MW660849.1
		icdA	MW6608451.1
		mdh	MW6608453.1
Dickeya zeae LAG–20	Lambhvel, Anand, Gujarat, 2020	*icdA*	MW6608452.1
		mdh	MW6608455.1
Dickeya zeae KNG–20	Kevadiya, Narmada, Gujarat, 2020	*dnaX*	MW660850.1
		mdh	MW6608454.1
Dickeya zeae BNG–21	Bhumeliya, Narmada, Gujarat, 2021	*dnaX*	OL848049.1
		icdA	OL848050.1
		mdh	OL848051.1
Fusarium falciforme AVRP–18	Boriavi, Anand, Gujarat, 2018	ITS	MK911728.1
		TEF-1α	MK968889.1
		RPB-2	MK968890.1
Fusarium falciforme BAG–20	Boriavi, Anand, Gujarat, 2018	*TEF-1α*	MW691193.1
		RPB-2	MW691192.1
Fusarium falciforme LAG–20	Lambhvel, Anand, Gujarat, 2020	ITS	MW699612.1
		TEF-1α	MW691195.1
		RPB-2	MW691194.1
Fusarium falciforme KNG–20	Kevadiya, Narmada, Gujarat, 2020	ITS	MW659167.1
		TEF-1α	MW691197.1
		RPB-2	MW691196.1

2.2.2. Bacterial strains

The strains of bacterial pathogen were also isolated from the fresh and naturally infected aloe leaves and purified through repeated subculturing, from the samples collected from different locations (Table 1). The most aggressive strain of bacterium (IMI 389157) was used for the *in planta* experiments following the methods described earlier (Mandal andMaiti, 2005).

2.3. Establishment of pathogenicity

2.3.1. Fungal isolates

Virulence of the fungal isolates was established by inoculating host plants at the collar region (detailed below). The aseptically grown plants were inoculated using 0.5 cm diameter fungal plugs from a 7-days old culture growth. The fungal plugs placed along with the plant surface at any of the three places: i) inside leaf whorl, ii) at collar region, and iii) along the roots (below pseudo stem). Whereas, the control plants received sterile PDA plugs. Those plants were uprooted between 4 to 15 days after inoculation to observe and record the changes. The pathogen was re–isolated away from the inoculated foci and associated fungus was compared morphologically with the parent culture.

2.3.2. Bacterial strains

Pathogenicity of all the isolated bacterial strains (Table 1), was established by infiltrating the detached aloe leaves (Mandal and Maiti, 2005). The rooted plantlets of aloe were grown in small plastic boxes (70 mm × 50 mm) hydroponically, containing 1x Hoagland solution. The plants were placed on laboratory benches (28–35 °C) receiving diffused sunlight. After acclimatization for 10 days, each plant was inoculated either at i.) leaf, ii.) collar region or iii.) root. The leaves were inoculated by puncturing with a sterile needle and pipetting 20 μl of bacterial suspension ($A_{600} = 0.5 \pm 0.2$). The wounds were wrapped with adhesive cellophane tape to reduce desiccation. To inoculate at the collar region, the plants were punctured horizontally to a depth of ~5 mm with a sterile needle and these were placed in the growing medium so that the wounded areas remained submerged. The growing medium was supplemented with bacterial culture suspension having $A_{600} = 0.5$

± 0.2 using a spectrophotometer. In the third group, few root tips were clipped and inoculated by growing in Hoagland solution fortified with bacterial cells of $A_{600} = 0.5 \pm 0.2$, to understand the possible mode of entry through roots. For each group, appropriate controls were maintained, where the plants were wounded similarly but did not receive any bacterial cells. The experiment was conducted following the randomized complete block design (RCBD), where each treatment had four replications and the experiment repeated twice. The plants were incubated at room temperature and monitored for 10 days post inoculation. The pathogen was re–isolated from the symptomatic plants for comparison.

2.3.3. Inoculation of both the Pathogens

The plants grown in sterile garden soil were divided in to two groups. Plants of the 1stgroup were inoculated with the aggressive fungal isolate at any of the three places (as described above) and plants of the 2nd group were kept without any fungal inoculation. Successively, ten days later, half of those fungal inoculated plants of 1st group were exposed to the bacterial pathogen by flooding soil with a suspension of test bacterium cells consisting $A_{600} = 0.5 \pm 0.2$. Similarly, half of the plants of 2nd group (without fungal inoculation) were exposed to the bacterial pathogen and the remaining were kept control (absolute control), where neither fungal nor bacterial pathogens were inoculated. Hence, there were four independent treatments, each was replicated thrice and each replication comprised of two plants. The experiment was repeated twice and was conducted following RCBD.

2.4. PCR assay and sequencing

The fungal pathogen grown on potato dextrose broth (PDB) at 25 ± 1 °C for 5 d. Fungal mycelium was harvested and washed with distilled water. Genomic DNA was isolated from the mycelia using the Nucleo–pore® gDNA fungal/bacterial mini kit (Genetix Biotech Asia Pvt. Ltd, India) following the manufacturer's instructions. Three genes, internal transcribed spacer (ITS) of rDNA, RNA polymerase II (*RPB 2*) and translation elongation factor (*TEF-1α*) were amplified in the PCR assay following the references mentioned in Table 2.

For preparation of the bacteral DNA template, cells from the 48 h growth were lysed in Triton X-100/ sodium–azide buffer and the cell free supernatant was directly used as template in the PCR assay (Abolmaaty et al., 2000). Three central metabolism genes *viz., dnaX* (DNA polymerase III), *icdA* (isocitrate dehydrogenase) and *mdh* (malate dehydrogenase) were amplified from the bacterial DNA with the PCR (Table 2).

2.5. In-silico sequence analysis

The amplicons retrieved with PCR assays were separated in 1.2% agarose gel, purified using QIAquick® PCR & Gel Cleanup Kit (Qiagen) and sequenced bi–directionally, availing commercial services (Eurofins Genomics India Pvt. Ltd., India). The received sequence contigs primarily processed with BLASTn analysis for putative identification of the pathogen, followed by sequences assembled. Multiple sequence alignment was performed using the cluster W algorithm and sequences were edited manually in Bioedit 7.1.3 software, where required. The phylogenetic tree was constructed with MEGA X (Kumar et al., 2018) using the assembled sequences of bacterial and fungal species sequenced in present study, and those of closely related species were retrieved from NCBI GenBank. Maximum likelihood method and best-fitted model chosen with 1000 bootstrap replication (Tamura and Nei, 1993) for analysis. Branches with less than 60% bootstrap value were collapsed.

The bacterial strains were identified based on the BLAST analysis and phylogenetic tree using the partial sequences of central metabolism genes *viz.*, *dnaX*, *icdA* and *mdh* from the present study and including the sequences of closely related type species of the genera *Dickeya* and *Pectobacterium*. Similarly, the *Fusarium* isolates identified with the default settings of the polyphasic identification process in *Fusarium* Multilocus Sequencing typing (MLTS) database (http://fusarium.mycobank.org). In MLTS database partial sequences of *ITS*, *TEF-1α* and *RPB-2* genes of four isolates from aloe were used for species determination, whereas other 32 fusarium variants were used for phylogenetic analysis.

Table 2
List of PCR Primers used in the present study to characterize *Dickeya zeae* and *Fusarium falciforme*.

Target gene	Primer	Sequence 5′ to 3′	Reference
dnaX	DNAX-F	TATCAGGTYCTTGCCCGTAAGTGG	Slawiak et al. (2009)
	DNAX-R	TCGACATCCARCGCYTTGAGATG	
mdh	MDH-F	CCCAGCTTCCTTCAGGTTCAGA	Ma et al. (2007)
	MDH-R	CTGCATTCTGAATACGTTTGGTCA	
icdA	ICD F	GGTGGTATCCGTTCTCTGAACG	Ma et al. (2007)
	ICD R	TAGTCGCCGTTCAGGTTCATACA	
ITS	ITS1-F	TCCGTAGGTGAACCTGCGG	White et al. (1990)
	ITS4-R	TCCTCCGCTTATTGATATGC	
EF-1α	EF-1α F	ATGGGTAAGGAAGACAAGAC	O'Donnell et al. (2010)
	EF-1α R	GGAAGTACCAGTGATCATGTT	
RPB-2	RPB-2 F	GGGGWGAYCAGAAGAAGGC	O'Donnell et al. (2010)
	RPB-2R	CCCATRGCTTGYTTRCCCAT	

2.6. Inoculation assay for quick screening of germ plasms

Full grown aloe leaves (5–9th leaves from the top) were used to standardise the rapid screening technique in an experiment, following RCBD. The leaves were washed with distilled water and excess water was wiped with tissue paper and punctured at the middle of the lamina with a sterile cork borer to make a well. The bacterial suspension ($A_{600} = 0.5 \pm 0.2$, 20 µl) was pipetted into the well, sealed with clear cellophane tape and incubated at different temperatures (15, 20, 25, 30 and 35 °C) for 48 h. Control leaves received sterile distilled water in place of the bacterium suspension. Diameter of the zone of tissue maceration was measured at 24 h intervals. Four replications (each consisting of two leaves) were maintained. To ensure the reproducibility and authenticity of the results, experiment was repeated twice. Similarly, for fungal isolate, an individual detached leaf was inoculated by placing a 0.5 cm diameter plug of 7 days old culture along the plant surface, mycelia faced the plant epidermis. Control plants received sterile PDA plugs. The agar blocks were sealed with transparent tape to prevent desiccation. The leaves were incubated at 25 ± 2 °C and monitored till 10 days post inoculation.

2.7. Effect of different temperatures

2.7.1. Fungal isolate

The fungal isolate AVRP-18 was inoculated on sterile PDA plates and incubated at five different temperatures, varying between 15 °C and 35 °C (15, 20, 25, 30 and 35 °C). The fungal colony diameter was measured after 4 days of inoculation. The experiment was conducted following RCBD with three replications and repeated twice.

2.7.2. Bacterial strains

Nutrient broth (NB) and 2% aloe leaf extract (LE, 2 g chopped aloe leaf rind boiled in water for 30 min, decoction was collected and final volume was made up to 100 ml) were inoculated with the bacterium isolate and also incubated at five different temperatures from 15° to 35°C, at an interval of 5 °C. After 24 and 48 h of inoculation, bacterial growth was vortexed and turbidity was measured in a spectrophotometer (Biomate 3, Thermo Electron Corporation, Madison, USA) at A_{600} nm. The experiment conducted following RCBD with three replications for each treatment and repeated twice.

Further, *in planta* bacterial growth was also measured. Mature leaves

were infiltrated with 200 μl bacterial suspension (~10^8 cells/ml) using a hypodermic syringe and incubated at the aforementioned temperatures. Leaf tissues from the inoculation site were collected after 24 and 48 h, weighed and crushed in 1 ml sterile water using a mortar and pestle. Bacteria concentration in this mixture was estimated by dilution plating method and expressed as colony forming units per g (CFU/g) of leaf tissue.

2.8. Screening of aloe genotypes against the soft rot disease

Following the detached leaf inoculation method, the available aloe accessions were screened for soft rot disease resistance. Three mature leaves from different accessions (Supplementary Table S1) were inoculated. Progress of tissue maceration at 24 h intervals was recorded to ascertain the host reaction. *In planta* inoculation assay was also performed to ascertain the differences in host reactions. Five plants (considering each as a replication) from each aloe accessions were grown in plastic pots and inoculated successively with purified fungal isolate followed by the bacterium strain, as described earlier. Inoculated plants were kept under surveillance daily for the next 10 days to observe the soft rot symptoms development. The genotype was rated as susceptible, if soft rot symptoms appeared in any of the five plants.

2.9. Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the statistical package MSTAT-C version 1.4 (Crop and Soil Science Division, Michigan State University, USA) following RCBD model. Means were compared with least significant differences (LSD) ($P = 0.05$).

3. Results and discussion

3.1. Field observations and symptoms

Dickeya and *Pectobacterium* cause devastating soft rot diseases in many vegetable, ornamental and fruit crops (Adeolu et al., 2016). The present study was conducted to understand the aetiology to develop an economically viable management technology. The soft rot disease symptoms were mostly prevalent during summer (April-June) and after first rain (June-July). During summer months, symptoms of soft rot appeared followed by application of irrigation or rain and the disease incidence was recorded at 10–26% under the field conditions. Contrary to the earlier hypothesis that the soft rot disease infection initiates through the root (de Laat et al., 1994), it was observed that infected plants had an intact root system similar to healthy ones; moreover, neither fungal nor bacterial pathogens were isolated from these roots. Hence, it necessitated an in-depth investigation of the infection process. Usually rotting started from the basal leaves and within one day, all the leaves became symptomatic. Rotten leaves drooped down and as the disease progressed some of the leaves bulged due to gas formation (Fig. 1a). Later, the affected leaves disintegrated and leaked to release a slimy mass emitting foul smell (Fig. 1b). Upon uprooting the healthy as well as diseased plants, it was observed that both were having intact and healthy root systems (Fig. 2a). The bacterium belongs to the genus *Dickeya* usually initiate soft rot at natural openings (*e.g.*, lenticels) and/or in wounds (Reverchon et al., 2016). Careful observations of the infected aloe plants revealed no involvement of injuries caused by insects or, agricultural operations *etc.*, however, an irregular brown spot was invariably noticed on pseudo–stem at the collar region of the infected plants (Fig. 2b). The lesion gradually enlarged and the centre of spots turned brownish black with a reddish margin. Splitting of pseudo–stem revealed the extent of damage caused due to infection and tissues beneath the surface of the spot were also disintegrated (Fig. 2c). As severity of the disease (noted by number of affected leaf and extent of leaf rotting) progressed, more extensive damage was observed on the subsurface layer of the affected pseudo–stems (Fig. 2d). In advanced stages of the disease development, the whole basal portion of pseudo–stem was rotten leaving only coarse fibres (Fig. 2e).

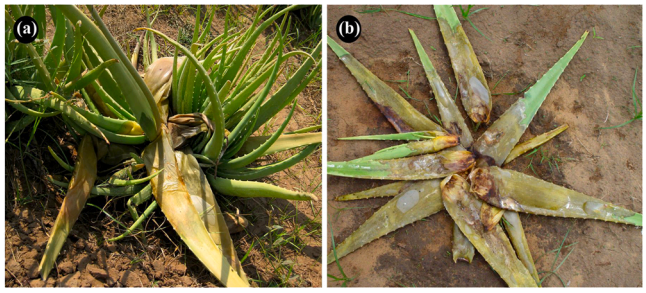

Fig. 1. (a) Leaf rot symptoms under field conditions. Please note the bulging of leaves due to gas formation. (b) Advanced stage of the disease showing leaking leaves.

3.2. Aetiology and pathogenesis of soft rot

In previous studies, it was reported that *F. solani* and *F. oxysporiums* were responsible for basal rot of aloe (Ji et al., 2007; Ayodele and Ilondu, 2008), whereas, Dubey and Pandey reported that *Sclerotium rolfsii* as the causal agent of collar– and root–rot of aloe (Dubey and Pandey, 2009). Later on, it was also reported that a destructive leaf rot disease caused by *F. oxysporum* on *Aloe barbadensis* (Kawuri et al., 2012). Recently, it was reported that *Fusarium* spp. cause leaf spot on several *Aloe* spp. (Avasthi et al., 2018) and *F. xylarioides* was identified as the causal agent of root and stem of aloe in China (Zhu et al., 2021). In the present study, plants inoculated with the fungus isolate (AVRP-18) at collar region developed irregular, reddish spots at the site of inoculation within four days. Splitting of pseudo–stem through such spots revealed sub-epidermal lesion similar to that of a natural infection. The re-isolated fungus from such tissues yielded a colony resembling the original one, establishing the Koch's postulates. However, none of the plants developed typical soft rot symptoms till the end of the experiment. Moreover, none of the plants from other three groups (inoculated at leaf whorl, at root and control) developed any visible change. On the other hand, in bacterial bioassay typical symptoms of soft rot disease were developed on the hydroponically grown plants when inoculated by the bacterium isolate at the collar and above. However, none of the root inoculated and control plants developed soft rot symptoms. Interestingly, roots remained normal and intact in all the plants irrespective of leaf symptoms.

Previous studies suggested that simultaneous and sequential inoculations of potato by *Verticillium dahlia* and soft rot causing *Pectobacterium* spp., often produce additive and synergistic effects on infection (Rahimian and Mitchell, 1984). It also increased the severity of potato early dying disease. Co–inoculation increased *in planta* population of *Pectobacterium* compared to a plant receiving the bacterium alone (Dung et al., 2014). The results corroborate and established that in case of the sterile soil culture experiment, symptoms appeared only on those plants, which were sequentially inoculated with the fungal isolate at the collar region followed by flooding with the isolated bacterium strain suspension. Moreover, when inoculated separately, neither bacterium strains nor fungal isolates were compatible enough to develop any symptoms in the plants. Symptoms of soft roton the basal leaves appeared four days after post inoculation by bacterium, only on those plant which were previously inoculated with the fungal inoculum. Almost all leaves of the inoculated plants, starting from the base, were rotten. Splitting pseudo–stems of these plants revealed extensive internal tissue disintegration. It was also observed that plants inoculated only with fungal inoculant at the collar region developed lesions at the site of inoculation without any leaf rotting symptoms. And at the end of experimentation plants showed no damage to roots in any of the

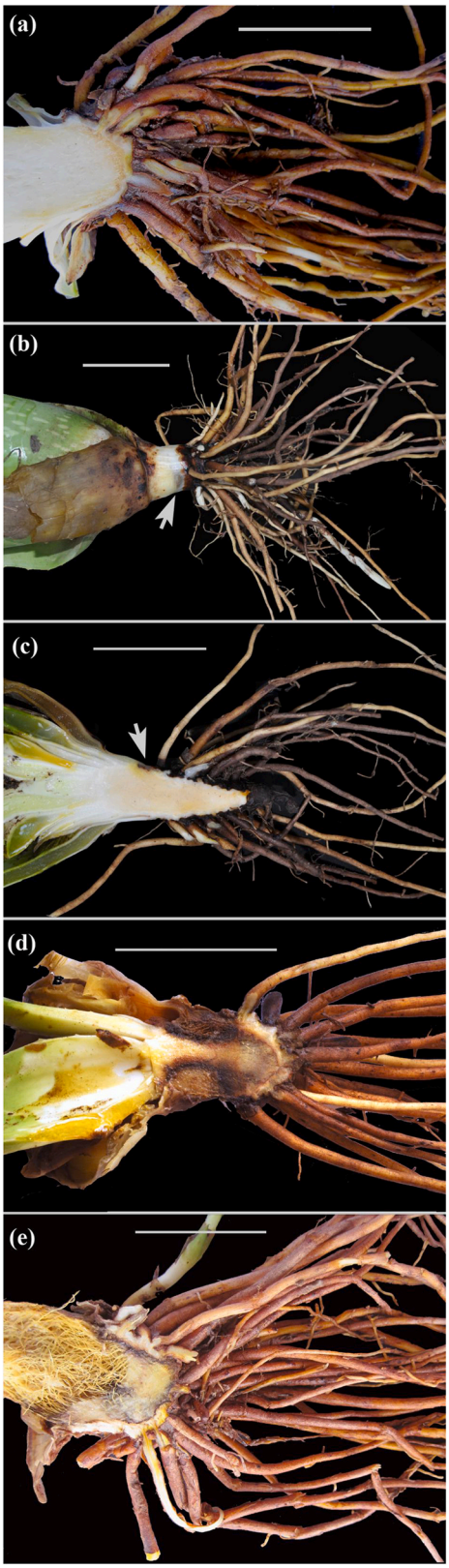

Fig. 2. Disease symptoms at the collar region of field–grown aloe plants; (a) Split pseudo–stem from a healthy plant. (b) The initial stage of infection. (c) Split pseudo–stem of the plant shown at (b). (d) Advanced stage of the disease development with penetrating lesion and (e) a pseudo–stem is left with coarse fibres. Please note dark lesions at arrows and healthy root systems of all the plants.

treatments.

Classically, *Fusarium* was thought to be a soil inhabiting opportunistic plant pathogen, mostly infecting the plants through roots (Booth, 1971). However, all the the four isolates of *F. falciforme* (Table 1), showed specificity for collar tissues of *A. barbadensis* (was not isolated from roots of the infected plants). Similar tissue specificity was observed for *D. zeae* as well. Artificial inoculation with thebacterial suspension at different plant sites revealed that infection at the pseudo-stem or leaf was essential for the reproduction of soft rot symptoms in aloe. Soft rot bacteria mostly depend on host injuries caused during agricultural operations and insect feeding for entering into host tissues (Pérombelon, 2002; Toth et al., 2011). Czajkowski et al. (2010) observed that soil borne *Dickeya* colonised potato root tissues irrespective of root damage. Natural openings that occurred during lateral root formation indicated for pathogen entry. However, in this study, *D. zeae* strain IMI 389157 failed to produce disease symptoms, when inoculated through root tissues. This agreed with Rahimian and Mitchell's (1984) findings where soft rot causing *P. carotovorum* sub sp. *carotovorum* failed to colonise potato stem when introduced through clipped roots. Poor survival of *D. zeae* in partial anaerobic conditions of Hoagland solution might be a reason for the failure of disease establishment. However, this strain was earlier found to be facultative anaerobic in nature (Mandal and Maiti, 2005). Moreover, at the end of experimentation, the growing medium containing the bacterium cells wes introduced into fresh aloe leaves and soft rot symptoms were reproduced (data not shown). Thus, it can be safely claimed that under field conditions, soft rot of aloe is caused by a sequential infection of *F. falciforme* and *D. zeae*. It was also established that infection by *F. falciforme* resulted in the development of necrotic spots at the collar region of *A. barbadensis* and the host was predisposed to soft rot causing *D. zeae* bacterium.

However, it was also observed that the associated *F. falciforme* species did not produce extensive rotting, rather initiated collar infection; thereby opening an avenue for infection by the *D. zeae*.

3.3. Morphological and molecular characterization

Identification and characterization of soft rot disease associated pathogens was also an objective of the study. The associated fungus, which was repeatedly isolated from the freshly infected plants,initially produced a whitish colony on PDA, which later turned pinkish brown and appeared orange from the reverse side of the plate (Fig. S1). The isolated fungus produced hyaline, slightly curved, multi–celled and 2–4 septate conidia of $23 \pm 4 \times 5 \pm 2.5$ μm size. Microconidia were single–celled or, one septate, cylindrical or, pointed at ends, straight or, slightly curved, measuring $12.5 \pm 1.5 \times 5 \pm 1$ μm and was tentatively identified as *Fusarium* sp. Interestingly, no fungal colony was isolated from the roots of the infected plants; while white bacterial colonies appeared on NA plates. However, when tested by the developed detached leaf assay, these bacterial isolates were found to be non–pathogenic on aloe. Identification of *Fusarium* species based on morphological features alone encountered several discrepancies (Wang et al., 2011; O'Donnell et al., 2015), therefore it was further characterized by partial sequencing of the *ITS*, *TEF-1α* and *RPB2* genes. The assembled sequences submitted to the NCBI GenBank and accession numbers were assigned (Table 1). The comparative analysis of these three gene sequences (*ITS*, *TEF-1α* and *RPB-2*) with the sequences of *Fusarium* MLST database, revealed that all the four isolates AVRP–18, BAG-20, LAG-20 and KNG-20 belong to *Fusarium falciforme*.

The phylogenetic analysis showed that sequenced isolates of *Fusarium* spp. constituted a complex of morphologically ambiguous species; presently known as *F. solani* species complex (FSSC, Chehri et al., 2015; O'Donnell et al., 2015). A total of 55 diagnosable species were arranged in three clades, *i.e.*, clades 1, 2 and 3. Most of the agriculturally important *Fusarium* species were accommodated in clade 3. The concatenated sequences of *ITS*, *TEF-1α* and *RPB-2* genes from 32 fusaria belonging to FSSC, one outgroup member (*F. staphyleae* NRRL 22316)

and concatenated sequences of three genes of four isolate from this study were aligned. Further, by using the aligned concatenated sequences of *ITS*, *TEF-1α* and *RPB-2* genes phylogenetic tree was constructed which revealed that all four isolates infecting aloe, formed a group with the clade represented by *F. falciforme* NRRL 43441 with an overall bootstrap value of 81%. The fusaria infecting aloe, hence, confirmed as members of clade 3 of FSSC (Fig. 3). The *F. falciforme* (FSSC 3–4) was one of the most haplotype–diverse species of this clade (Chehri et al., 2015). *Fusarium* MLTS database as well as the phylogenetic analysis showed that the sequenced isolate on aloe shared a position with *F. falciforme*.

The taxonomic positions of enteric plant pathogenic bacteria, earlier grouped under *Erwinia*, had undergone several changes leading to the establishment of two new genera, *Pectobacterium* and *Dickeya* (Samson et al., 2005; Zaczek-Moczydłowska et al., 2019). *Dickeya* species are listed among the top 10 most important phytopathogens due to high economic consequences (Mansfield et al., 2012; Adeolu et al., 2016). At present genus *Dickeya* contains 12 recognized species and two subspecies while *Pectobacterium* encompasses 18 species (Curland et al., 2021; Toth et al., 2021). Multi-locus Sequence Analysis (MLSA), as a taxonomic relevant tool for species delineation was widely used in grouping of bacteria (Young et al., 2008; van der Wolf et al., 2014; Zhang et al., 2016; Dees et al., 2017). These studies differed in the number and type of target genes considered for MLSA. Young and Park (2007) used four (*atpD*, *carA*, *recA* and 16 S rDNA) concatenated sequences, while van der Wolf et al. (2014) considered 10 (IGS, *dnaX*, *recA*, *dnaN*, *fusA*, *gapA*, *purA*, *rplB*, *rpoS*, *gyrB*) and Dees et al. (2017) employed three (*dnaX*, *icdA*, *mdh*) genes for MLSA based phylogeny. Interestingly, even with the use of a limited number of genes involved in central metabolism (Dees

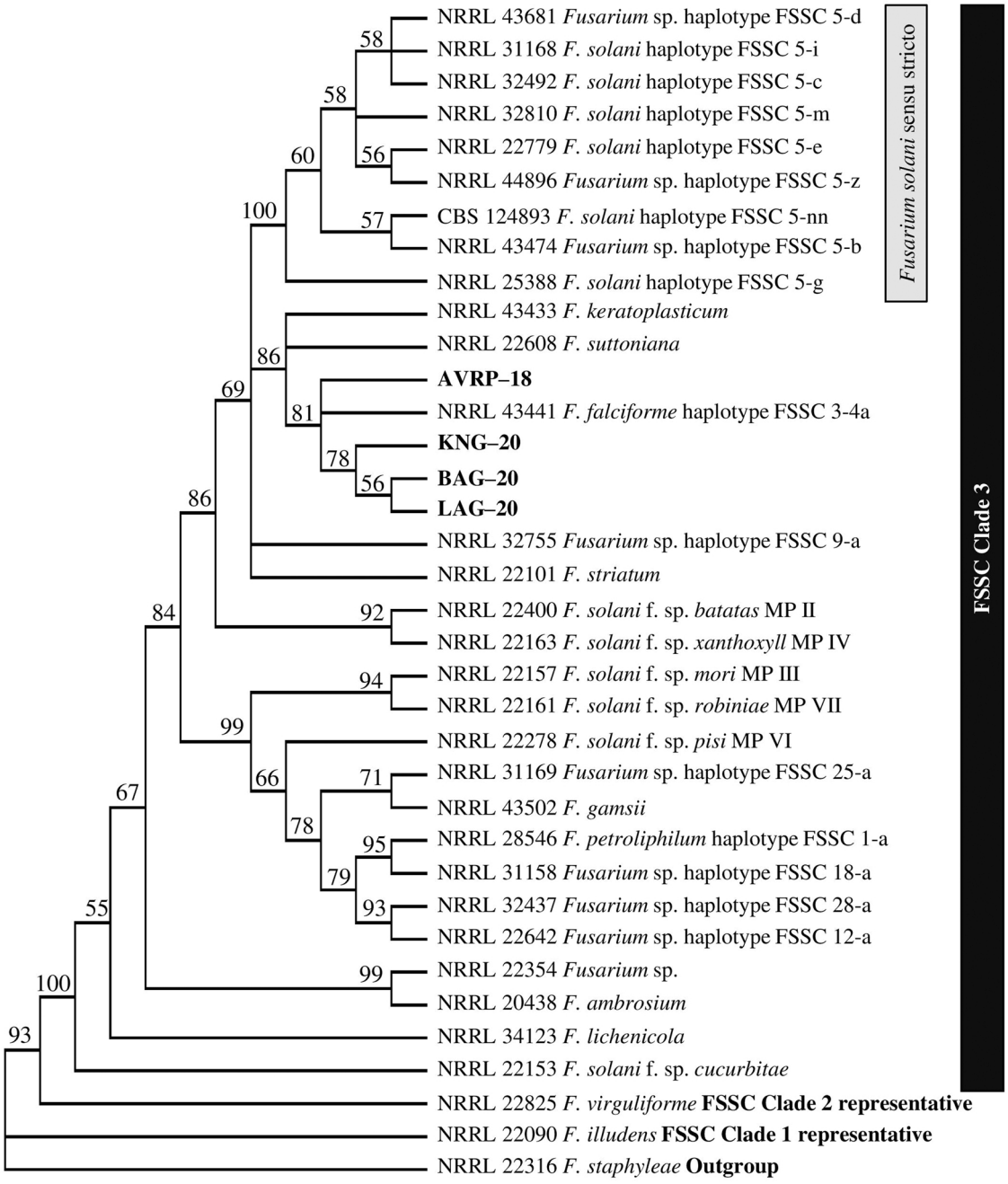

Fig. 3. Phylogenetic tree of fusaria infecting aloe (indicated by bold faced) and other *Fusarium* spp. based on Maximum Likelihood method. Bootstrap values are shown next to the branches. Branches with < 50% bootstrap values are collapsed.

et al., 2017), MLSA produced similar clustering patterns akin to those obtained by Zhang et al., (2016). A single gene (*recA*) based phylogenetic tree was also published recently (Zaczek-Moczydłowska et al., 2019). Considering the previous studies, in the presents study three genes *viz.*, *dnaX*, *icdA* and *mdh* from aloe infecting bacterial strains were sequenced and consensus sequences were submitted to NCBI GenBank, and accession numbers assigned as described (Table 1). The phylogenetic analysis performed using the concatenated *dnaX*, *icdA* and *mdh* sequences of the five test bacterium strains (IMI 389157, BAG–20, KNG–20, LAG–20 and BNG-21) with references to their close relatives.

All the sequenced strains of bacteria were grouped in the same clade with the *D. zeae* type strain NCPPB 2538 with bootstrap value of 94% (Fig. 4). All other species of the *Dickeya* and *Pectobacterium* were accommodated in clearly distinguishable clades and the test bacterium strains were identified as a member of *D. zeae*.

Moreover, the phylogenetic trees revealed that the genetic variability within the fungal and bacterial pathogens involved in inciting soft rot disease on aloe was quite high. Even two *F. falciforme* isolates, AVRP–18 and BAG–20 originating from the same location were also genetically varied greatly.

3.4. Aetiology at different temperatures

Consequent upon the varying incubation temperatures, differential lesion development was recorded on leaves inoculated with *D. zeae* IMI 389157. the rate of tissue maceration was measured in terms of the diameter of the soft rot zone, which increased with the rise in incubation temperature up to 35 °C (Fig. S2a). However, incubation at 15 °C or below, the bacterium failed to induce soft rot symptoms on inoculated aloe leaves. During 48 h of incubation at 20 °C lesion diameter increased marginally, while a significant increase in the zone of maceration was observed between 25 and 35 °C. Lower temperature (below 20 °C) produced inconsistent results, which is evident from the higher standard deviation at the 48 h data point. Such variations between replications could not be obliterated even with several repetitions. Nevertheless, incubation at 35 °C produced consistent results and almost the entire leaf was rotten within two days. This treatment resulted in significantly ($p = 0.05$) the highest lesion diameter. the degree of maceration was also high at 35 °C as the leaf became very soft with leakage of slimy mass. Degradation also resulted in eruptions on the epidermal layer due to gas formation. Hence, incubation at 35 °C was selected to screen genotypes for resistance against soft rot bacterium. However, leaves inoculated with *Fusarium* isolate AVRP–18 develop neither any symptoms nor any perceptible change in comparison to the control.

Incubation temperature affected the *in vitro* and *in planta* growth rate of *D. zeae* IMI 389157 (Fig. S2b, c). Both the liquid media (NB and LE) supported bacterial growth at all the temperatures tested. Differential growth rates were found at varying temperatures in NB and a similar trend was followed in LE after 24 h of inoculation. After 48 h of incubation at 20, 25 and 35 °C there was no significant difference in the produced turbidity, however, minimum turbidity was observed at 15 °C, while the maximum was recorded at 30 °C. *In planta* growth patterns of the bacterial isolates were significantly influenced by the varying temperature. The bacteria concentrations in leaf tissue increased with the increase in incubation temperature up to 35 °C temperature (Fig. S2c). The lowest CFU (8.8×10^4 and 3.3×10^5 at 24 and 48 h after inoculation, respectively) was observed at 15 °C, which multiplied to 157.1 and 283.9 times after 24 and 48 h after incubation at 35 °C, respectively. Incubation temperatures significantly influenced soft rot symptoms development and 35 °C was the most favourable, while at 15 °C no external manifestation was discerned. *In vitro* growth study suggested that failure of symptoms development at low temperature was not due to lack of survival and multiplication by *D. zeae* at this temperature. Also, the trend of *in vitro* bacterial multiplication rate and tissue maceration were not matched (at an incubation temperature 35 °C). Further, it was also noted that there was no correlation between *in vitro* productions of extracellular pectin degrading enzymes by the bacteria at different temperatures and symptoms development (data not shown) indicating the existence of complex host-bacteria interaction. However, *in planta* concentration of bacterium increased gradually with the rise of incubation temperature from 15° to 35°C. Accordingly, the zone of tissue maceration and *in vivo* bacterium concentration was significantly correlated (r = 0.96 and 0.95, at 24 h and 48 h after inoculation, respectively). Pathogenesis of soft rot causing bacteria was influenced

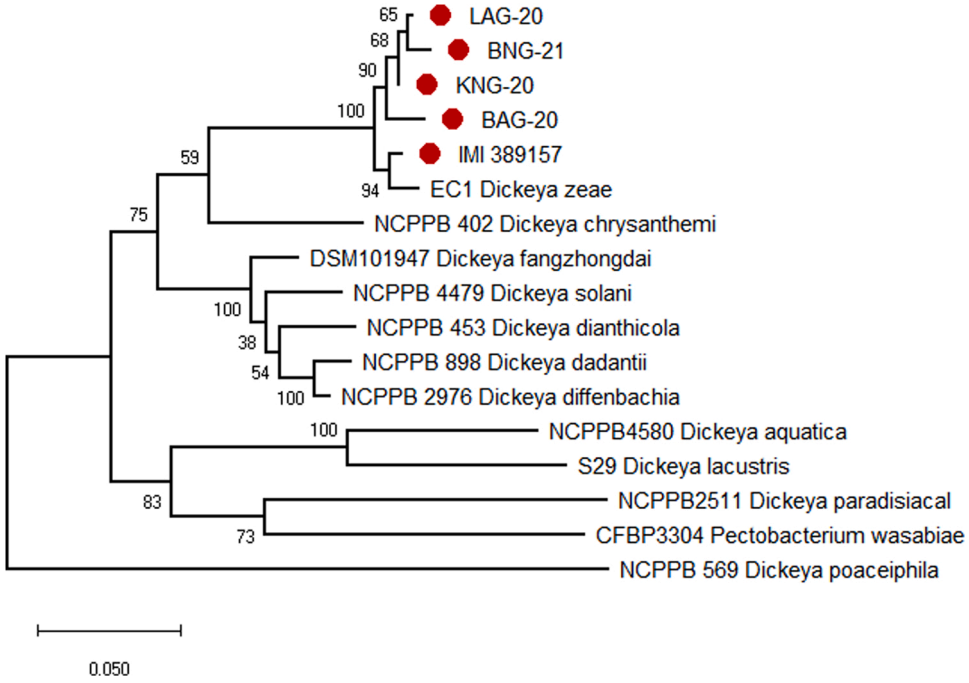

Fig. 4. Molecular phylogenetic tree of four bacteria isolates causing soft rot of aloe, other *Dickeya* spp. and *Pectobacterium* spp. by Maximum Likelihood method. The bootstrap consensus tree is inferred from 1000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches.

by several environmental conditions including temperature and humidity. (Farrar et al., 2000; Hasegawa et al., 2005). Absence of tissue maceration by soft rot bacteria at 15 °C also was observed in carrot (Farrar et al., 2000). However, further studies would be necessary to decipher molecular mechanism underlying differential pathogenicity at varying temperatures. Nevertheless, the present study indicates that in absence of genetic resistance in *A. barbadensis*, practical management of the disease can be achieved by targeting either of the pathogens, *F. falciforme* or, *D. zeae*. The present work also suggests that a storage temperature of 15 °C or lower can be effective to preserve, store, and transport aloe leaves without affecting their quality due to bacterial decay.

Mycelium growth of the fungal pathogen was significantly ($p = 0.05$) influenced by the incubation temperatures (Fig. S2d). Radial growth showed an increasing trend with enhanced temperature up to 30 °C, whereas, 35 °C adversely affected the growth. Four days of post inoculation, the smallest colony diameter (1.4 cm) was recorded at an incubation temperature of 15 °C while the largest colony (4.9 cm diameter) was produced at 30 °C. However, the mycelial growth was reduced by 11.5% at 35 °C.

3.5. Screening of aloe germplasm against soft rot disease

Exploitation of the genetic resistance is one of the most economical and environment friendly approach of crop protection. However, standardization of precise screening methods to evaluate a large number of genotypes (nullifying environmental influence) is a prerequisite to distinguish between 'true resistance' and 'escape'. "Detached leaf inoculation assay" for any disease has the advantage to screen a large number of germplasm and chemicals against pathogens with minimum resources. The most aggressive isolates (data not shown) of the pathogens were used to determine the resistance status of the aloe accessions against soft rot disease. The developed "detached leaf inoculation assay" described here was found easy and quick for screening the available germplasm of aloe against soft rot disease. The results presented (Table S1), revealed that none of the genotypes had genetic resistance against *D. zeae* IMI389157. Also, there was no significant difference in the zone of tissue maceration among the genotypes (data not shown). Hence, it was concluded that all the available aloe accessions screened (Table S1) were equally susceptible to *D. zeae* IMI 389157.

The combined inoculation with *Fusarium* isolate AVRP–18 and *D. zeae* strain IMI389157 produced differential reactions among the inoculated aloe genotypes. Over a period of time, the method was found to be reliable and produced reproducible results. Soft rot disease symptoms were produced on all the genotypes of *A. barbadensis* and rated as susceptible, but the accessions belonging to *A. chinensis* and *A. perryi* species did not produce soft rot symptoms till the end of experimentation. Among the 40 accessions belonging to 3 *Aloe* spp., none was found genetically resistant to *D. zeae*. Interestingly, two accessions belonging to *A. chinensis* and *A. perryi* did not develop soft rot symptoms even with co-inoculation of *F. falciforme* and *D. zeae.* The resistant status of these two aloe accessions against the other three isolates of *F. falciforme* (BAG–20, LAG–20 and KNG–20) were confirmed through artificial inoculation (data not shown). This opens up the possibility of developing future cultivars to combat this disease.

The present study suggests the possibility of management of the problem through (i) exploiting host resistance (ii) escaping post–harvest decay by storing and transporting aloe leaves at temperatures ≤ 15 °C and (iii) avoidance of water stagnation in the field.

4. Conclusions

Soft rot disease of aloe caused by sequential infection of *F. falciforme,* followed by *D. zeae* , and developed rotting of tissues from collar upwards. The identity of the associated pathogens was established based on the morphology and molecular tools by partial sequencing of three genes of both, bacteria (*dnaX, icdA*and *mdh*) as well as fungi (*ITS, TEF-1α* and *RPB-2*). The developed artificial inoculation technique will be useful for the quick screening of aloe germplasm for resistance. Temperature is a crucial factor in soft rot symptoms development as rapid rotting was observed at 35 °C but not at 15 °C.

Declaration of Competing Interest

The authors declare that they do not have any known competing financial interests or personal conflicts that could have appeared to influence the work reported in this manuscript and research paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jarmap.2023.100492.

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