Research Article

Isolation and characterization of bacteria associated with sunscald-affected Capsicum annuum L.

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Abstract:

Bell pepper is a crop with high economic value and its productivity in India is low due to numerous biotic and abiotic diseases, managed through various chemical and biological treatments. Present work includes isolation and characterization of selected bacterial species from pepper and monitoring their growth in presence of representative chemical and biocontrol agents. Fifteen isolates from different parts of sunscald-affected capsicum were microbially characterized, of which three isolates forming yellow mucoid colonies were selected, so as to include pathogenic bacteria if any. These isolates, identified as Rosenbergiella epipactidis, Acinetobacter sp. and Bacillus aquimaris based on partial 16S rDNA sequencing, did not show significant pathogenicity on capsicum fruit when tested in an in vivo infection assay; suggesting their neutral or beneficial nature. While CuSO4 could drastically inhibit the growth of all the isolates in an in vitro plate assay, two biocontrol isolates, P4 and FS2 showed varied degree of effect on the growth of capsicum-associated isolates. Overall, the study reports yet unknown occurrence of R. epipactidis as capsicum-associated bacteria, perhaps endophyte, and develops an insight over probable impact of chemical and biocontrol agents used in pepper disease management on the abundance and diversity of inherent bacteria.

Keywords: Capsicum annuum L., Rosenbergiella epipactidis, Bacillus aquimaris, abundance and diversity of inherent bacteria in capsicum

INTRODUCTION

Bell pepper (*Capsicum annuum* L.), a member of *Solanaceae* family, is one of the most economically important crops across the world. In India, it is commercially cultivated in Karnataka, Tamil Nadu, Himachal Pradesh, Uttar Pradesh as well as in Rajasthan, at temperatures less than 30 °C (Jadon et al., 2016; Negi et al., 2018). Bell peppers have high nutritional value being rich source of vitamins A, B6 and C, calcium, and folic acid and have high urban consumption. Besides, the crop also finds pharmaceutical and cosmetic applications (Manoj Kumar et al., 2008). Therefore, despite lesser favourable natural climatic conditions in India, bell pepper is increasingly cultivated in greenhouses and naturally ventilated polyhouses under milder climatic conditions across Goa, Maharashtra and other regions including Gujarat. Under open field cultivation conditions, capsicum yields are up to 20-40t/ha, whereas in a greenhouse the yield could mount up to 100-120t/ha. However, major limitation to productivity of this crop is frequent occurrence of various biotic and abiotic diseases.

Fruit-associated microbiota plays a significant role in maintaining plant health. While numerous fungal pathogens, both surface-associated and endophytic,

are well-known pathogens of bell pepper, affecting stem, leaves and fruits of pepper plant (Jadon et al., 2016; Mmbaga et al., 2018), few bacterial pathogens like *Xanthomonas campestris*, *Ralstonia solanacearum* and *Clavibacter michiganensis* are also identified (Huang et al., 2017). On the other hand, several bacterial endophytes are also associated beneficially with pepper, mainly including members of genus *Pseudomonas, Bacillus, Burkholderia, Paenibacillus, Pandoraea, Enterobacter, Rhizobium* and *Serratia* to name a few (Paul et al., 2013; Irabor and Mmbaga, 2017). These endophytic bacteria present in varying abundance in pepper plants, either prevent the colonization and spread of pathogenic microbes by acting as biocontrol agents or could confer resistance against abiotic stress (Sziderics et al., 2007).

Commonly, the microbial diseases of pepper plant are controlled by interventions involving use of various chemicals mainly including the copper-based compounds (Rather et al., 2012). More eco-friendly management of capsicum diseases is increasingly preferred using biocontrol agents which include *Bacillus*, *Pseudomonas*, *Paenibacillus Flavobacterium* and *Trichoderma* spp (King and Parke, 1993; Manoj Kumar et al., 2008; Chandra et al., 2010; Seggara et al., 2013; Torres et al., 2016). While both these approaches could reduce the disease incidences, they could significantly affect the inherently associated microbiota.

Interestingly, a commonly encountered climatic disease, sunscald caused by persistent exposure of pepper fruit to sunlight and heat (Borkar and Yumlembam, 2016), is likely to influence the abundance and nature of both beneficial and pathogenic bacteria associated with the fruit. Hypothetically, sunscald-damaged tissue with wrinkled surface becomes more prone to bacterial infections and at the same time the hardened sunscald-affected area could show enrichment of endophytic bacteria more tolerant to abiotic stresses. The present work involves isolating and identifying selected bacteria from sunscald-affected capsicum fruits, *in vivo* assessment of their pathogenicity for capsicum and testing the effects of chemical and biocontrol agents on their growth.

MATERIALS AND METHODS

Sampling of capsicum fruits

Surface properties of the fruits with reference to standard manuals available for management of capsicum crop, were used to sample sunscald-affected fruits from greenhouse facility in Ajarpura Village, Anand district, Gujarat. Healthy fruits from the same cultivation system were obtained for use as controls in experiments.

Isolation of bacteria and their preliminary microbial characterization

Within 24 h of sampling the diseased capsicum fruits, approximately 0.5 x 0.5 cm of piece was cut from infected fruit, stem and peduncle using sterilized knife and was mounted on nutrient agar and potato dextrose agar slants prepared earlier. Infected tissue from three different sites on a single fruit were used as microbial source and the slants were then incubated at 30 °C for 48 h to ensure optimum isolation of distinct microbial species. In parallel, similarly derived infected tissue was suspended in 1 ml sterile normal saline pre-dispensed in sterilized centrifuge tubes. The tubes were mildly vortexed for 1 minute, centrifuged at 8,000 rpm for 5 minutes and resultant supernatant was subsequently serially diluted. A volume of 100 µl of 10^{-1}, 10^{-2} and 10^{-3} dilutions were spread on the nutrient agar plates which were subsequently incubated overnight at 30 °C. Distinct bacterial colonies obtained were selected based on visual analysis of colony characteristic and were subjected to further microscopic analysis based on Gram staining, endospore staining and cell shapes by standard procedures. Selected individual colonies were maintained as a pure culture for further study.

Extraction of genomic DNA from isolates

Single colony was inoculated into 3 ml nutrient broth and incubated at 30°C for overnight under shaking conditions (150 rpm). A 1.5 ml of overnight grown culture was harvested by centrifugation of 8,000 rpm for 5 minutes at room temperature. After discarding the supernatant, pellet was washed thrice with 1 ml of SET buffer (pH=8.0) and finally resuspended in 100 µl of the same with addition of 50 µl of lysozyme (10 mg/ml) and mixed properly. After subsequent addition of 30 µl of RNase A (2 mg/ml), the volume was made upto 500 µl with SET buffer. Subsequently, 50 µl of Proteinase K (10 mg/ml) and 50 µl of 10% SDS were added and contents were incubated at 37 °C for 1 h. Followed by this, 100 µl of 5M NaCl and 80 µl of cetyltrimethylammonium bromide solution were added and incubated for 10 min at 55°C. The mixture was extracted with equal volume of Tris-saturated phenol and Chloroform:Isoamyl alcohol (24:1) and centrifuged at 10,000 rpm for 10 min at 4 °C. Aqueous phase was transferred in fresh sterile centrifuge tubes, and again was extracted with equal volume of chloroform followed by centrifugation at 10,000 rpm for 10 min at 4 °C. The resultant aqueous

phase was transferred to fresh sterile tube and genomic DNA was precipitated overnight by adding 0.8 volume of isopropanol. Precipitated DNA was pelleted by centrifugation at 10,000 rpm for 5 min at 4 °C, washed twice with 70% ethanol, air-dried and was dissolved in 50 µl TE buffer. Isolation of genomic DNA was confirmed using agarose gel electrophoresis.

Molecular characterization of selected isolates

16S rRNA gene was amplified using universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3'), in PCR reactions performed in 20 µl of volume containing 20 picomoles of each primer, 0.2 mM dNTPs each, 30 ng of template genomic DNA, 1U of Taq DNA polymerase and 1X PCR buffer (used as per manufacturer's instructions). Amplifications were achieved in Biorad thermocycler with following program: initial denaturation at 94 °C for 3 minutes followed by 35 cycles each consisting of denaturation at 94 °C for 30 s, primer annealing temperature at 58 °C for 30 s, primer extension at 72 °C for 1 min 30 s and a final extension of 72 °C for 10 min. PCR amplicons of desired size (~1500 bp size) were confirmed on 1 % agarose gel. Partial DNA sequence of the confirmed PCR product was obtained as outsourced service (1st Base, Singapore) and were subjected to BLAST analysis for identification of the isolates (http://www.ncbi.nlm.nih.gov).

***In vivo* infection assay**

The infection assay was set up using a method used by Fieira et al. (2013) with necessary modifications. Single colony of the selected isolate was inoculated in 3 ml Luria broth and culture was allowed to grow overnight under shaking conditions (30 °C, 150 rpm). Resultant cells were harvested by centrifugation at 8,000 rpm for 2 minutes, washed with 1 ml of sterile normal saline and finally resuspended in the same to prepare the active inoculum. Three microlitres of this inoculum was mixed with 10 µl of 0.4 % agar and the total volume was spotted on a healthy capsicum fruit, surface sterilized using ethanol. The inoculated fruits were incubated at 30 °C and infection was monitored over a period of 6 days, with exposure of sunlight on 5th and 6th days.

***In vitro* antimicrobial assay**

Ability of Cu^{2+} as well as laboratory isolates *Pseudomonas aeruginosa* P4 (P4) and FS2 to inhibit the growth of isolated bacteria was tested by modified dual culture plate assay. Cells harvested from 3 ml overnight Luria broth-grown P4 and FS2 cultures were subsequently washed twice by and re-suspended in 1 ml normal saline aseptically, to prepare inoculum. Similarly prepared cell suspension of the pepper-derived bacterial isolate was diluted 1:1000 times and 0.1 ml of it was spread uniformly on nutrient agar medium. Subsequently, 3 µl of freshly prepared P4 and FS2 inoculum was spotted in two quadrants of the plate. Five microliters of 0.1 mM $CuSO_4$ was spotted in third quadrant as chemical agent while 3 µl of freshly prepared *E. coli* DH5α inoculum was spotted in the fourth quadrant as negative control. Formation of clear zone of inhibition around the zone of bacterial growth after 72 h incubation at 30 °C was taken as a measure of antimicrobial ability.

RESULTS

Preliminary disease analysis of capsicum fruit, isolation and characterization of bacteria

Capsicum fruits with a typical phenotype of white patch with wrinkled surface and edges, depending on the extent of disease, were obtained from local polyhouse plantations of capsicum. Visual analysis of the infected area could classify the disease as sunscald (Fig. 1A) (Borkar and Yumlembam, 2016). Tissues including affected patch, peduncle and stem of the diseased fruit were used to isolate bacteria. Fungal isolates obtained were not carried forward in the study.

Figure 1: Selected bacterial isolates obtained from sunscald-affected capsicum (A) Suncald-affected capsicum fruit used as source of isolation (B) Three distinct yellow and mucoid colonies selected for further studies

Fifteen visually distinct colonies from across all tissues and isolation conditions were considered for further study (Table 1). Six of these isolates were Gram positive and endospore positive while remaining 9 isolates were Gram negative and endospore negative. Most isolates showed short rods and cocci cell types. After comparing the preliminary colony characteristics of all 15 colonies, 3 distinct isolates I-1, I-3 and I-9 were selected for further analysis (Fig. 1B). These isolates were selected on the basis of pale to bright yellow colour and mucoid colony features displayed by commonly known bacterial pathogens of capsicum, so as to possibly include both pathogenic and beneficial bacteria.

Table 1: Characterization of bacterial isolates obtained from sunscald-affected capsicum

Isolate [1]	Gram reaction	Endospore staining	Cell shape
I-1	-ve	-ve	Short rods
I-2	-ve	-ve	Cocci
I-3	-ve	-ve	Short rods
I-4	-ve	-ve	Cocci
I-5	+ve	+ve	Rods
I-6	+ve	+ve	Rods
I-7	-ve	-ve	Short rods
I-8	+ve	+ve	Short rods
I-9	+ve	+ve	Short rods
I-10	+ve	+ve	Short rods
I-11	+ve	+ve	Rods
I-12	-ve	-ve	Cocci
I-13	-ve	-ve	Cocci
I-14	-ve	-ve	Short rods
I-15	-ve	-ve	Short rods

[1] Isolates I-1, I-2, I-3, I-6, I-7, I-8 are isolated from fruit rot on nutrient agar plates, I-4, I-5, I-10, I-11 and I-12 are isolated from fruit rot on nutrient agar slants, I-9 is from rotten peduncle on agar plate, I-13 and I-14 are from stem rot on nutrient agar plate, I-15 is from healthy fruit on agar plate; +ve -positive, -ve –negative

Molecular identification of the selected isolates

Genomic DNA from these isolates was isolated and subjected to amplification of 16S rDNA gene as ~1500 bp amplicon. Partial DNA sequence analysis using BLAST tool revealed maximum identity of I-1, I-3 and I-9 to *Rosenbergiella epipactidis, Acinetobacter sp.* and *Bacillus aquimaris,* respectively (Table 2). Of these, isolate I-1 with similar extent of query cover but relatively lesser Max. Score of BLAST output, also showed a significant homology to *Phaseolibacter flectens*, a probable plant pathogen.

Table 2: BLAST analysis-based molecular identification of selected isolates

Isolate	Identified	Query length	% Identity
I-1	*Rosenbergiella epipactidis/ Phaseolibacter flectens*	1270 (97%)/ 1270 (98%)	97% / 96%
I-3	*Acinetobacter sp.*	1167	99%
I-9	*Bacillus aquimaris*	1077	96%

***In vivo* evaluation of efficacy of selected isolates as bacterial pathogen for capsicum**

Pathogenicity of isolates I-1, I-3 and I-9 for capsicum was monitored using an *in vivo* infection assay as described in 'Materials and Methods' section. Figure 2 depicts that none of the isolates could show any infection phenotype till 96 h, until and unless exposed to sunlight. From fifth day onward, gradual development of disease phenotype on healthy fruit at the site of inoculation was observed which invigorated upon further exposure to sun on sixth day.

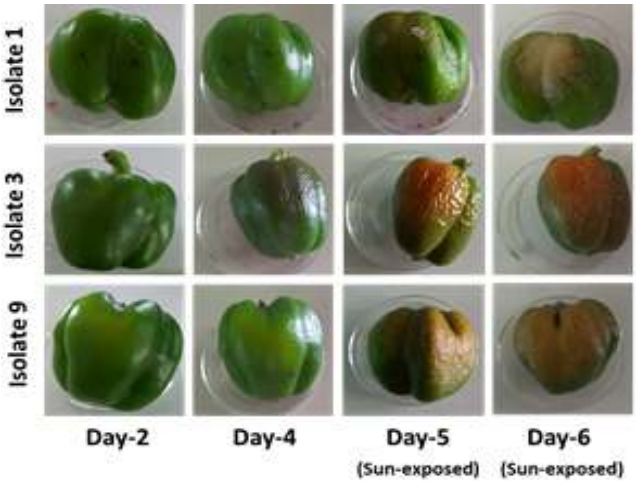

Figure 2: *In vivo* assay to monitor efficacy of selected isolates to infect capsicum.

Advancement of disease phenotype as monitored up to 6 days with last two days of sunlight exposure post inoculation

Effect of chemical and biological disease management agents on growth of selected isolates

All the three pepper-derived isolates were subjected to *in vitro* plate assay to monitor effects of representative chemical agent $CuSO_4$ as well as biological agents P4 and FS2 (rhizospheric lab isolates) on their growth. $CuSO_4$ could inhibit the growth of all the three tested isolates I-1, I-3 and I-9 to drastic extents, with I-9 showing maximum growth inhibition. Biocontrol isolate P4 could significantly inhibit growth of I-1 and I-9 but not I-3 while FS2 failed to inhibit growth of any of the three isolates (Fig. 3). Negative control *E. coli* DH5α showed no antibiosis of isolates.

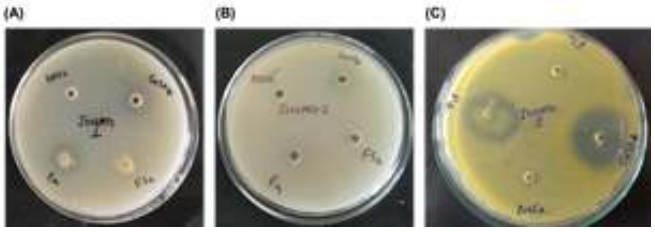

Figure 3: *In vitro* inhibition of growth of selected bacterial isolates from pepper.

In vitro antimicrobial activity of P4 and FS2 as monitored by dual culture plate assay for isolates (A) I-1 (B) I-3 and (C) I-9. $CuSO_4$ was used as chemical inhibitor of bacterial growth.

DISCUSSION

Bell pepper disease management practices involve use of various chemical-based and eco-friendly biocontrol agents. Understanding the influence of these treatments on abundance and diversity of neutral or beneficial endophytic bacteria could not only help the choice of treatment but could also reveal novel endophytes with diverse roles. The present study reports isolation of selected bacterial spp. from sunscald-affected capsicum fruit and their microbial and molecular characterization. Subsequently, effect of representative chemical and biological agents used in disease management of pepper, on growth of these isolates was also tested.

Sunscald-affected capsicum was sampled based on simple symptoms such as occurrence of a white hardened and wrinkled patch on the fleshy surface (Borkar and Yumlembam, 2016). Considering that this environmentally affected area could not only be prone to microbial infections but could also harbour exclusive endophytic population, it was used in the experiments to isolate bacterial spp. selectively. Of around 15 bacterial colonies isolated, only three I-1, I-3 and I-9 were selected for further studies. In order to ensure that bacterial pathogens were not missed out, these colonies were shortlisted based on pale-bright yellow colour and mucoid appearance, common to known bacterial pathogens of pepper i.e. *X. campestris, R. solani* and *C. michiganensis* (Huang *et al*., 2017). However, these colony characteristics could be also displayed by beneficial inherent endophytic bacteria. 16S rDNA sequencing-based analysis identified the selected isolates as *Rosenbergiella epipactidis*, *Acinetobacter spp.* and *Bacillus* spp. respectively. While *Bacillus* and *Acinetobacter,* besides several others, are commonly known endophytes of plants including capsicum (Paul et al., 2013; Irabor and Mmbaga, 2017), occurrence of *Rosenbergiella* spp. in capsicum hasn't been reported. *Rosenbergiella* spp. have been earlier isolated from floral nectar (Halpern et al., 2013; Lenaerts et al., 2014). Isolate I-1 identified as *R. epipactidis,* also showed significant identity to *Phaseolibacter flectens,* a known pathogen of beans albeit not capsicum (Aizenberg-Gershtein et al., 2016). However, *in vivo* infection assay almost ruled out this possibility of pathogenicity as evident from poorly developed infection phenotype, 4 days post inoculation with all the three isolates including I-1. The observed development of sunscald-like disease was only due to exposure to sunlight. Thus, the three isolates were most likely to be neutral or beneficial endophytes of pepper. Since such endophytes share the niche with pathogens, they often possess efficient biocontrol properties thus capably influencing the metabolism and physiology of the host plant (Amaresan et al., 2013; Paul et al., 2013). However, these beneficial effects of inherent endophytes are subject to their abundance which can be largely affected by disease management practices.

Rhizospheric biocontrol isolates P4 and FS2, respectively identified as fluorescent pseudomonads and *Enterobacteriaceae* (unpublished lab data) were used as representatives to understand their ability to affect the growth of selected isolates, in comparison with Cu^{2+} based chemical. The results overall indicated that topically applied biocontrol agents could differentially affect the growth and hence abundance, of endophytic spp of capsicum, with *Enterobacteriaceae* being relatively lesser capable of modulating the same. On the other hand, chemical pesticides which are largely Cu^{2+} -based, could cause drastic adverse effect on the endophytic bacteria.

CONCLUSIONS

Overall, this study reports otherwise unknown occurrence of *Rosenbergiella epipactidis* as capsicum-fruit associated bacteria, mostly endophytic. This study further indicated that biocontrol agents although relatively eco-friendly as compared to chemical pesticides, both these modes of disease control could modulate the abundance and diversity of inherent beneficial endophytes of capsicum; an aspect which is seldom considered before employing disease control treatments.

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