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Original Research Article

Evaluation of Antimicrobial and Antibiotic Sensitivity of Chilli Root Endophytic Bacteria for Eco friendly Biofertilizer

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A B S T R A C T

Microbial endophytes are promising source for the production of distinct metabolites with potential pharmacological activities Total three endophytic bacteria CER5, CER6 and CER11 were isolated from root of *Capsicum annum* (chilli) through surface sterilization method and were screened for biological activity against clinical pathogens. Putative isolate CER5 was identified as *Klebsiella oxytoca* AVSCE5 (KM104324)by colony characteristics, morphological, biochemical and molecular characterization. The antibiotic sensitivity of endophytic bacteria was determined by disc diffusion method against a number of antibiotics. *Klebsiella oxytoca* AVSCE5 (KM104324), chilli root endophyte showed strong microbial activity against pathogenic bacteria. It is also proved that the isolate lost the multi drug resistant property due to its habituation inside the healthy tissue of host plant.

Keywords

Antimicrobial and antibiotic sensitivity, chilli root

Introduction

Majority of plants including medicinal plants and vegetable crops are the important part of the human society to combat diseases. In developing country like India, medicinal plants are the important source for people to fight against various diseases and disorders. Various plant species are explored by ethnic societies for their medicinal applications. Various parts of plant are traditionally used by ethnic societies for their preliminary health treatment like fruit, leaves, root, and stem bark in the form of infusions, decoctions, concoctions (Khan *et al.*, 2012). Recently, focus on plant research has increased all over the world for the production of secondary metabolites. In search for new drugs or secondary metabolites, microbial endophytes are promising source for production of effective and unique metabolites with potential pharmacological activity (Aly *et al.*, 2010). Endophytic bacteria are those bacteria which invade and colonize within the living plant tissues, without causing any apparent harmful effects (Bacon and White, 2000; Wilson, 1995). In order to colonize the plant and for competition from other microorganisms they produce antimicrobial compounds and many enzymes (Lima *et al.*, 2005). A variety of antibiotics (Pleban *et al.*, 1997), enzymes, anticancerous (Stierle *et al.*, 1993), anti-inflammatory (Trischman *et al.*, 1994), antifungal (Korzybsky *et al.*, 1978) and biological control (Hallmann *et*

al., 1997) agents have been isolated from endophytic microorganisms. Approximately, 30,000 plant species are present on earth (Strobel and Daisy, 2003), but only a few plant associated endophytic microorganisms have been studied thoroughly. Therefore, there is a need to explore or discover novel and interesting endophytic microorganisms capable to producing natural products and bioactive secondary metabolites (Strobel *et al.,* 2004).

Several important attributes and medicinal properties of plants may be due to endophytes residing in it. Numerous studies have also demonstrated that endophytes synthesize bioactive compounds which help to promote plant growth and increase resistance in plant against their pathogens (Rosenblueth and Martines-Romero, 2006; Ryan *et al.,* 2008).

Ether extract and alcoholic extract of fruit of Embilica officinalis has been reported to have strong antimicrobial property while fruit extract of *F. jangomas* showed good activity against *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Escherichia coli* (Srivastava *et al.,* 2010).

With this background the present research work has been focused to evaluate antibiotic susceptible or antibiotic resistant properties of endophytic bacteria isolated from chilli root (*Capsicum annum* US 341).

Materials and Methods

Collection of plant sample

For isolation of endophytic bacteria, Capsicum annum US341 from LAM, Guntur was selected. Roots, were carefully cut out, tagged and stored in plastic bags at 4^{o}C. Within 5-6 hours, these plant materials were used for the isolation of endophytes.

Isolation of endophytic bacteria

The collected plants were first briefly washed under running tap water and the stems, leaves were cut into small pieces. These small pieces were rinsed in sterile distilled water and after that surface sterilization was performed by treating first with 70% ethanol for 30 sec and then with 0.1% mercuric chloride solution for 2min. The disinfected parts of plants were rinsed again with distilled water and drained. After that, they were cut longitudinally with a sterile scalpel, and after removing the outer layer of plant part inner exposed parts of plant were placed on autoclaved nutrient agar (Hung and Annapurna, 2004). Surface disinfected intact plant parts were also placed on nutrient agar as control. All plates were incubated at 30oC for several days. Bacterial colonies around the cut pieces were selected as endophytic bacteria.

Screening of antibacterial activity and antifungal activity

Clinical pathogens gram positive *(Streptococcus* sp, *Staphylococcus aureus* MTCC-737) and gram negative bacteria (*Pseudomonas* sp., *E. coli* MTCC-443) and *Colletotrichum gleosporoides* were used to evaluate the antimicrobial activity of endophytic bacteria. All pathogenic bacteria were obtained from Department of Microbiology, ANU, Guntur. Screening of endophytic bacteria was performed in two steps i.e. primary screening and secondary screening.

Primary Screening

Determination of antibacterial activity through primary screening was done by perpendicular streak method (Oskay *et al.,* 2004). Nutrient agar plates were prepared in which isolated endophytic bacteria were

streaked in the centre and these plates were incubated at 37^{o}C for two days. Later the pathogens (*Staphylococcus aureus, Staphylococcus aureus MTCC-737, Escherichia coli, Pseudomonas sp., Proteus vulgaris, Klebsiella sp., E. coli* MTCC-443) were single streaked perpendicular to endophytic strain without touching it and again incubated at 37^{o}C for 24 hours. Antagonism was measured by measuring the inhibition zone.

Secondary Screening

Secondary screening of endophytic bacterial strains was done by agar well diffusion method (Pandey *et al.,* 2004) using Ethyl acetate for extraction. 100 µl of pathogenic microorganisms in nutrient broth (absorbance 0.1 at 660 nm) was spread on Nutrient Agar (NA) plates. A well of 8 mm diameter was bored with the help of sterile cork borer and 150 µl of solvent extract was loaded into wells. One well without solvent extract was kept for control. These plates were incubated at 37°C for 24 h.

Antibiotic susceptibility test of pathogenic bacteria

The antibiotic sensitivity of endophytic bacteria was determined by disc diffusion method against a number of antibiotics (Norfloxacin-10 µg.disk-1, Cefotaxime-30 µg.disk-1, Ceftriaxone-30 µg.disk-1, Ciprofloxacin-5 µg.disk-1, Ofloxacin-5 µg.disk-1) with different concentrations, and pathogenic bacteria like *E.coli, Bacillus subtilis, Streptococcus, Staphyloccus, Proteus.*

Morphological and Biochemical Characterization

Standard morphological and biochemical tests were performed for the identification of endophytic bacteria. They were characterized by gram staining, endospore formation and biochemical tests like citrate utilization, oxidase, nitrate reduction, catalase, MR and VP test. They were identified according to Bergey's Manual of Systemic Bacteriology.

Results and Discussion

Total three endophytic bacteria CER5, CER6 and CER11 were isolated through surface sterilization method (Hung and Annapurna, 2004) from root of *Capsicum annum. (plate 1)*

Antimicrobial activity of endophytic bacteria was studied against Gram positive (*Staphylococcus, Streptococcus)* and Gram negative (*Escherichia coli, Pseudomonas*) and *Collitotrichum gleosporoides.* All the three isolates showed antagonism against *Colletotrichum* (Table1), CER-5 inhibited both Gram positive and Gram negative pathogenic bacteria used in the study, while CER-6 and CER-11 were unable to inhibit bacterial pathogens(table1)

Identification of chilli root endophytic bacteria

Three selected endophytes CER5, CER6 and CER11 were identified by colony characteristics, morphological, biochemical and molecular characterization. (Table2, Table3 and Table4),

According to morphological and biochemical characterization of all three bacterial strains, they were found to be Gram negative. Gram negative isolates exhibited negative results for endospore test and were found as the only isolates showing antimicrobial activity against all pathogenic bacteria tested. CER-5, CER-6 were classified as *Klebsiella* sp. according to their

physiological, endospore and biochemical tests (Goryluk, 2009). CER-5 strain was able to grow in presence of 6% NaCl, 0.1% phenol and 0.01% sodium azide. While CER-6 was found to be able to grow in the presence 8% NaCl, 0.1% phenol and 0.15% sodium azide. This shows that these two strains can even grow in such harsh conditions. Growth of CER-6 in presence of 0.15% sodium azide is noticeable although the growth was very less.

During biochemical characterization, CER-5 showed negative results for oxidase, positive results for nitrate reduction, and positive results for H_2s production, catalase and indole production test, and VP test citrate utilization test while negative results for MR test. In contrast to the above strain, cer-11 showed negative results only for indole production test, MR test, VP test when performed. Based on the characteristics CER5 identified as *Klebsiella* sp, CER6 as *Klebsiella* sp and CER11 *Agrobacterium*. As CER5 showed both antifungal and antimicrobial activity, CER5 was subjected to molecular characterization by 16s rRNA partial gene sequencing and identified as *Klebsiella oxytoca* AVSCE5 (KM104324).

Antibiotic Susceptibility of Chilli root endophytic bacteria

So far literature revealed that majority if the *Klebsiella* strains isolated from medical devices and hospital environment showed antibiotic resistant against broad spectrum of antibiotics. *Klebsiella* organisms are resistant to multiple antibiotics. This is thought to be a plasmid-mediated property. β-lactamaces are constitutive are usually produced at low levels and provide resistance against ampicillin, amoxillin, ticarcillin. *Klebsiella pneumonia* carbapenemases (KPC; Ambler class A beta lactamaces) confer broad resistant and are associated with a higher mortality rate (>50%). Many isolates are a single sequence type, ST258. Susceptibility is limited to gentamycin, tigecycline, and colistin. As the CER5 is identified as *Klebsiella oxytosa* in order to rule out the hazardous antibiotic resistance property of the strain and antimicrobial activity were evaluated by secondary screening method using ethyl acetate extract.

Antibiotic susceptibility test of pathogenic bacteria

In order to check the efficacy of antimicrobial activity of compounds produced by CER-5, CER-6, CER-11 isolates, a comparative study with respect to several known and available antibiotics was performed against CER5,CER6 and CER11as shown in the Table5, and normal soil microorganisms such as *Pseudomonas aureginosa* and *E.Coli* (Plaie2) and compared with clinical *Klebsiella.* Results revealed that endophytic *Klebsiella* CER5 is sensitive to antibiotics and not showed any antimicrobial activity against rhizospheric microorganisms.

Table.1 Anti-fungal activity and anti-bacterial activity

Sl.no	Isolate name	*Colletotrichum*	*E.coli*	*Peudomonas*	*Streptococcus*	*Staphyloccus*
1	CRE-5	+	+	+	+	+
2	CER-6	+	-	_	-	-
3	CER-11	+	-	_	-	-

Table.2 Colony characteristics of chilli endophytic root

Sl.no	Isolate name	Colony characters in agar media	Surface growth	Clouding	Sedimentation
1	CER-5	White slimy raised colonies	No	Heavy	No
2	CER-6	White slimy raised colonies	No	Heavy	No
3	CER-11	White slimy raised colonies	No	Slight	No

Table.3 Biochemical characteristics of chilli endophytic root

Sl. no	Isolate name	Oxidase test	Nitrate reduction test	H_2s production	Catalase	Indole production	MR test	VP test	Citrate utilization
1	CER-5	-	+	+	+	+	-	+	+
2	CER-6	-	+	+	+	+	-	+	+
3	CER-11	+	+	+	+	-	-	-	+

Table.4 Morphological characteristics of chilli endophytic root

Sl. no	Isolate name	Gram stain	Cell shape	Endospore	Motility	Tentative identification
1	CER-5	_	Rod	-	-	*Klebsiella* sp.
2	CER-6	_	Rod	-	-	*Klebsiella* sp.
3	CER-11	_	Rod	-	+	*Agrobacterium* sp.

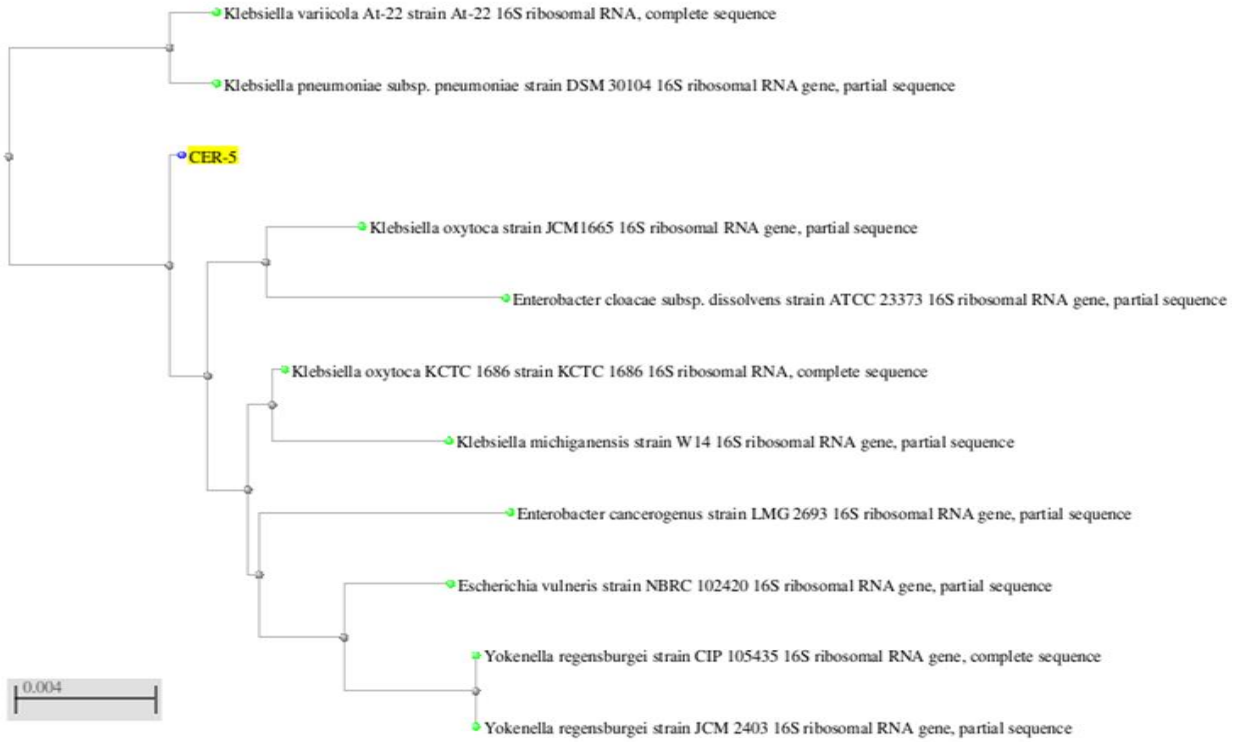
Table.5 Antibiotic Susceptibility test

Antibiotic disc	CER5	CER6i	CER11	Clinical Klebsiella as standard
Norfloxacin-10 µg.disk-1	Sensitive	Sensitive	Sensitive	Resistant
Cefotaxime-30 µg.disk-1	Sensitive	Sensitive	Sensitive	Resistant
Ceftriaxone-30 µg.disk-1	Sensitive	Sensitive	Sensitive	Resistant
Ciprofloxacin-5 µg.disk-1	Sensitive	Sensitive	Sensitive	Resistant
Ofloxacin-5 µg.disk-1	Sensitive	Sensitive	Sensitive	Resistant

Plate.1 Isolates of chilli endophytic root

CER5 CER6 CER11

Phylogenetic tree of CER5 (Klebsiella oxytosa AVSCE7 - KM 104324)



Pathogenic microorganisms now a day become resistant to the effective antibiotics (Bisht *et al.,* 2009). Hence, discovery of novel and effective antibiotics are necessary (O'Donnell *et al.,* 2010). In the recent time endophytes have emerged as novel source of antibiotics. In this study, endophytes from various parts of three different plants have been isolated. Till now there is no report about the isolation of endophytes from *Klebsiella*. This is for the first time endophytes showing antimicrobial activity are being reported from *Klebsiella.* This study evidenced that *Klebsiella* can be the potential but under exploited resources for endophytes. Bacterial endophytes isolated from Capsicum annum have shown promising inhibition activity against pathogenic bacteria (both Gram positive and Gram negative) used in the study. Shibumon *et al.,* (2010) reported about the isolation of 2, 3 Dihydroxy Benzoic acid as phenolic antimicrobial compound from acetonic fruit extract of Flacourtia inermis roxb which showed strong antimicrobial activity against multi drug resistant bacteria (Shibhumon and Benny, 2010). It could be possible that such antimicrobial activity in the plant extracts may be due to endophytes inhabiting in it. Further there is also report about the chloroform fraction prepared from roots of F. jangomas and F. separia showing strong antimicrobial activity against pathogenic bacteria (Sarker *et al.,* 2011). Medicinal and endemic plants should be studied for endophytes as parts of these plants are expected to harbor rare and interesting endophytes for the production of natural and potential bioactive compounds (Strobel and Daisy, 2003)

As per our literature survey, antimicrobial compound from endophytic bacteria isolated from Capsicum annum has not been earlier reported. A total of 3 isolates of bacterial endophytes were isolated from Capsicum

annum. Out of these, one isolate CER-5, showed broad spectrum of antimicrobial activity against both Gram positive and Gram negative pathogenic bacteria used in the study. Bacillus sp., B. licheniformis, Paenibacillus sp., B. pumilus and B. subtilis bacterial endophytes have been isolated from medicinal plants and are reported to produce antibiotics (Madigan *et al.,* 2005). In another report, bacterial endophytes were isolated from different parts of Plectranthus tenuiflorus (medicinal plant in Saudi Arabia) and among all endophytes, Bacillus sp. exhibited the strong antimicrobial activity against human pathogenic bacteria tested (*Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Streptococcus agalactiae, Proteus mirabilis and Candida albicans)* (Deeb *et al.,* 2012).

The bacterial endophytes isolated from *Capsicum annum* (isolates CER-5,) in this study have shown promising antimicrobial activity against human pathogenic bacteria. The further study may lead to the development of potential antibiotic drug which is the need of the day. In present study endophytic *Klebsiella* isolated root showed strong microbial activity against pathogenic bacteria. It is also proved that it lost the multi drug resistant property due to its habituation inside the healthy tissue of host plant. It is a promising observation stating that this *Klebsiella oxytosa* can be used as an ecofriendly biofertilizer in vegetable crops. Further investigation need to be carried out β-lactamase producing plasmids in this strai

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