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ISOLATION AND CHARACTERISATION OF ENDOPHYTIC BACTERIA FROM *ELEUSINE CORACANA* FROM THE FIELDS OF DARBHANGA

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ABSTRACT Endophytic bacteria hold an assurance for better plant growth and crop production.But the potential of endophytic bacteria has not been studied with reference to the coarse grain so far.Finger millet is a rain-fed crop which is highly nutritious and can be seen as a mean to ensure food security and agricultural sustainability in the long run.Endophtyic bacteria were isolated from roots,stems and leaves of this crop collected from different areas of the district Darbhanga.The samples were surface sterilised and isolated by culturing on NA medium.The endophytic bacterial isolates were then analysed morphologically using Gram's staining and at molecular level using PCR amplification.It was found that 52.17% (24) and 47.83% (22) were Gram positive and Gram negative respectively. Out of total endophytic bacterial isolates, 7 isolates were detected as Pseudomonas sp .Thus,it can be concluded that endophytic bacteria hold an assurance for better plant growth and crop production.

KEYWORDS : Endophytic bacteria,finger millet,isolation

INTRODUCTION

Finger millet (Eleusine coracana) is a staple food for drought prone areas of the world.It is popularly known as ragi,madua,nagli and kapai in vernacular languages in different parts of the country. These are highly nutritious,non-glutinous and non acid forming cereals.They are considered most digestible and least allergenic grains available.They release lesser percentage of glucose over longer period of time which lowers the risk of diabetes.

Nutritional quality of Finger millet

Finger millet is the richest source of calcium i.e. 300-350mg/100 gm of grain.The millet protein has well balanced amino acid profile and is a good source of methionine,cystine and lycine.These essential amino acids can provide special benefit to those who depend on plant food for their protein requirement.

The major proportion of the carbohydrate content of millet is in the form of non starchy polysaccharides and dietary fibres which help in prevention of constipation,lowering of blood cholesterol and slow release of glucose to the blood stream during digestion.

Finger millets are also rich in important vitamins like thiamine, riboflavin,folic acid and niacin.Lower incidence of cardiovascular diseases,duodenal ulcer and hyperglycemia (diabetes) are reported in people who cosume millets regularly.

Table 1:Nutrient composition of finger millet(per 100 g)

Food grain	Protein(g)	Carbohydrates(g)	Fat (g)	Fibre(g)	Mineral(g)	Calcium (mg)	Phosphorous(mg)	Iron (mg)
Finger millet	7.3	72	1.3	3.6	2.7	344	283	3.9

Role of Finger millet in food security and sustainable agriculture

National food security could be effective when food and fodder needs of the region will be met in the region itself. Finger millet grow well in dry areas as rain fed crops.Consumption of millet will encourage the farmers in dry land areas to grow such crops that are best suited to those regions.Moreover, this is a step towards sustainable agriculture.As this kind of sustainable cropping practices will be encouraged by introducing diversity in our diets.In this way we are not only respecting nature's biodiversity but also encouraging farmers to not change the regional cropping pattern by growing wheat and rice everywhere

MATERIALS AND METHODS

Collection of samples

For the isolation endophytic bacteria,healthy leaves,stems and roots of *Eleusine coracana* were collected from randomly selected healthy wild and cultivated plants from the fields in the different blocks of Darbhanga.Three samples were collected from each of the three blocks which are Kusheshwar sthan(W),Biraul and Alinagar,Thus,nine healthy plants were carefully removed and were placed in clean sterile plastic bags immediately after collection and then brought to the laboratory for further experimental purpose.

Surface sterilisation

The healthy plants after being washed under slow running tap water were separated into stems,roots and leaves.stems and roots were cut into sections 2-3 cm long.Sub-samples were prepared from each sample for isolation of endophytes.The samples were disinfected by immersing the samples in 70% ethanol for 1-3 minutes and 4% aqueous solution of sodium hypochlorite for 1.5 minute,again rinsed with 70% ethanol and finally rinsed with sterile distilled water(SDW) 4-5 times in the laminar air flow cabinet.

Preparation of the sample

Surface disinfected tissue was aseptically homogenised using sterile pestle and mortar.A small amount of SDW was added and the tissue was ground until fully homogenised.The homogenised tissue was then serially diluted to 10^{-2},$10^{-3}$and 10^{-4} gradient dilution by adding SDW.

Media for isolating endophytic bacteria

Endophytic bacteria were isolated and cultured in Nutrient Agar culture media containing 3 g beef extract,5 g peptone,5 g NaCl,15 g agar and 1000 ml SDW with pH 7.0-7.2 and in Pseudomonas Agar (For Fluorescein) which contained Casein enzyme hydrolysate 10.0 g/ L,Protease peptone10.0 g/ L,K_2HPO_4 1.5 g/ L, $MgSO_4.7H_2O$ 1.5 g/ L, Agar 15.0 g/ L and SDW 1000 mL with pH 7.0-7.2.

Isolation,purification and subculture of endophytic media

For inoculation 0.1 ml of the aliquot was spread on the NA medium .The inoculations were done in triplicates separately for each sample of root,stem and leaf extract.The plates were then sealed using parafilm tape and were incubated 28°C for 48-72.The total number of colonies was counted after the colonies appeared and expressed as cfu(colony forming unit).

Table 2: Isolates of endophytic bacteria from different tissues of finger millet

Sources	Tissue	Endophytic bacteria
Biraul	Root(7)	BIRR1,BIRR2,BIRR3,BIRR4,BIRR5, BIRR6,BIRR7
	Stem(4)	BIRS1,BIRS2,BIRS3,BIRS4
	Leaf(5)	BIRL1,BIRL2,BIRL3,BIRL4,BIRL5
	Total=16	
Kusheshwar Sthan	Root(6)	KUSR1,KUSR2,KUSR3,KUSR4,KUSR5,KUSR6
	Stem(5)	KUSS1,KUSS2,KUSS3,KUSS4,KUSS5
	Leaf(4)	KUSL1,KUSL2,KUSL3,KUSL4
	Total=15	

Alinagar	Root(6)	ALIR1,ALIR2,ALIR3,ALIR4,ALIR5, ALIR6
	Stem(4)	ALIS1,ALIS2,ALIS3,ALIS4
	Leaf(5)	ALIL1,ALIL2,ALIL3,ALIL4,ALIL5
	Total=15	
	Grand total=46	

BIRR:Biraul root KUSR:Kusheshwar sthan root ALIR:Alinagar root
BIRS:Biraul stem KUSS:Kusheshwar sthan stem ALIS:Alinagar stem
BIRL:Biraul leaf KUSL:Kusheshwar sthan leaf ALIL:Alinagar leaf

MORPHOLOGICAL CHARACTERISATION

A) Gram Staining Tests: The gram staining was carried out using standard staining procedure and technique.

B) Shape:This was observed under light microscope for each colony.A loopful of the culture was taken for microscopic examination of the bacteria which is isolated for identification and characterisation.

Molecular characterization of endophytic bacteria by using PCR amplification

A)Isolation of genomic DNA from bacterial endophytes

Selected endophytic bacterial isolates were grown in 10 ml of Luria broth medium at 28°C for 24 hours. Bacterial suspension was transferred to 15ml Falcon tube and centrifuged at 8000 rpm for 10 min to form pellet.The supernatant was discarded and the pellet was resuspended in 650µl of extraction buffer by pipetting up and down repeatedly.The DNA samples were incubated at 65°C for 30 min. 100µl of Potassium acetate was added to precipitate cellular proteins.700-800µl of chloroform/isoamylalcohol was added, mixed thoroughly and centrifuged at 10000 rpm for 4 to 5 min. The aqueous phase containing the DNA was transferred to a new 2ml microcentrifuge tube. To this 0.6 ml of chilled isopropanol (600µl) was added and mixed gently till the appearance of white thread like structure. The tubes were again centrifuged at 10000 rpm for 5 minutes. The supernatant was discarded and pellets were washed with 70% ethanol. DNA pellet was air dried for about 2 hours. The pellet was dissolved in 100µl of TE buffer and stored at 4°C.

B) Quantification of genomic DNA by Gel electrophoresis

The quality and quantity of the genomic DNA was checked on agarose gel electrophoresis.The 1X TBE buffer was prepared by diluting 50X TBE buffer.100ml of 1X TBE buffer was added to 0.8% agarose by melting in a microwave oven.2 µl ethidium bromide was added after the agarose gel cooled.The solution was poured into a casting tray containing a sample comb and allowed to solidify at room temperature.The gel on the tray was placed horizontally into the electrophoresis chamber and the top of the gel was flooded with fresh running buffer (TBE) to cover the gel to depth of about 1 mm. The DNA sample with dye was sucked into the pipette and was gently expelled into the well. The lid and power leads were placed on the apparatus, 100 V current was applied and flowing of current was confirmed by observing the bubbles coming out from the electrodes and viewing the gel in UV transilluminator (Giongo et al 2010).

C) Polymerase Chain Reaction (PCR) amplification of 16S rRNA ITS gene by genus specific primers

16S r RNA gene
8F 5'-AGTTGATCCTGGCTCAG -3'
1492R 5'-ACC TTG TTA CGA CTT-3'

PCR mixture (15µl) contained 3.5 µl Taq polymerase buffer, 1.0 µl MgCl2, 1.0 µl of forward primer, 1.0 µl of reverse primer, 0.3 µl Taq polymerase, 2.0 µl of template DNA, 0.5µl dNTP and 5.7 µl distilled autoclaved water was added to make final volume to 15 µl. PCR was carried out in a thermocycler with an initial denaturation for 5 min at 95°C, then 35 cycles of 15 sec at 95°C, 15 sec at 52°C, 1.30 min at 72°C and a final extension for 5 min at 72°C and 4°C for storage (Sacchi et al 2002).

RESULTS AND DISCUSSION

Morphological characteristics of endophytic bacterial isolates of finger millet

On the basis of morphological studies (Table 2), 23 endophytic bacteria which were grown on nutrient agar medium produced medium-sized, irregular-shaped, off-white and rough colonies and thus, were tentatively placed in genera *Bacillus sp* (Plate 1).Moreover, 13 isolates which produced round-shaped and raised colonies with entire margins, mucoid and cream in colour were tentatively placed as *Klebsiella sp*. 10 isolates cultured on Pseudomonas agar medium produced medium-sized, round-shaped and flat colonies with smooth margin and cream colour and tentatively belonged to genera *Pseudomonas sp* (Plate 2). Further, few isolates also produced a fluorescent green pigment on King's B medium.

Table 3: Morphological characteristics of endophytic bacterial isolates of finger millet

Characteristics	Morphology of endophytic bacterial isolates		
Cell shape	Rod	Rod	Rod
Elevation	Flat	Raised	Umbonate
Texture	Mucoid	Mucoid	Mucoid
Margin	Regular	Regular	Irregular
Colour	Yellow	Cream	Cream
Size	Medium	Small	Medium
Motility			
Consistency	Rough	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque
Colony shape	Round	Round	Polymorphic
Pigmentation	Fluorescent green pigment was produced by some isolates	None	None
Bacterial isolates (Tentatively)	*Pseudomonas sp.*	*Klebsiella sp.*	*Bacillus sp.*

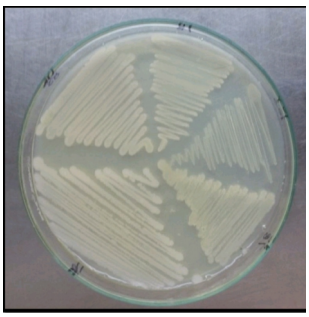

Fig1: Diversity of endophytic bacteria isolated from the samples of finger millet streaked on Nutrient agar medium

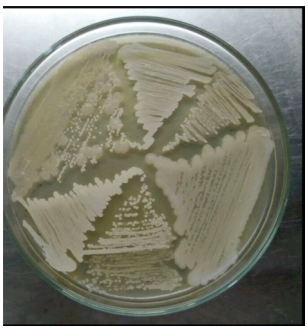

Fig2: Diversity of endophytic bacteria isolated from the sample of finger millet streaked on Pseudomonas agar medium

Molecular characterization of potential endophytic bacteria on the basis of 16 S rRNA gene ITS (internal transcribed spacer) marker Promising native endophytic bacterial isolates were characterized on the basis of 16S rRNA gene ITS marker according to Zhao et al (2011). Genetic diversity of endophytic bacterial isolates was assessed by using two genus specific primers (*Pseudomonas sp.* and *Klebsiella sp.*).Two primers amplified the specific segment of DNA with different size of band for detection of specific genera with genus specific primers from genomic diversity of endophytic bacterial isolates. The two oligonucleotide sequence (8F and 1492R) of 16S rRNA universal ITS marker was amplified by PCR. Further, all the amplified PCR product produced a single band with approximately 1100bp in length in all the endophytic bacterial isolates. For detection of *Pseudomonas sp*. P 11F and P 11R genus specific primers were used. All the amplified PCR product of endophytic bacterial isolates produced a single band with approximately 150 base pair in length and difference among them was not visible in 1.5% agarose gel.

Out of 11 endophytic bacterial isolates, 7 isolates (BIRR3, BIRL3, KUSR2,KUSR5,KUSS3,ALIS2 andALIL3) were detected as *Pseudomonas sp* .

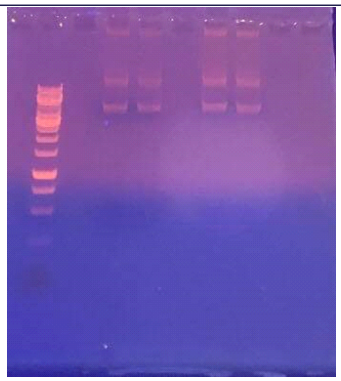

Fig: PCR amplification of 16S rRNA gene with specific primer of endophytic bacterial isolates

CONCLUSION

Among the beneficial plant associated bacteria, bacterial endophytes are really important. It has been suggested through different studies that endophytic bacteria hold great promise to enhance agricultural productivity and sustainability. This has been reported in several studies that bacterial endophytes improve the growth and health of various agricultural crops. Endophytic bacteria are believed to promote the growth of their host plant and also fitness through different direct and indirect mechanism.Bacterial endophytes may strongly influence the performance, growth and stress tolerance of the plants in which they inhabit.

Moreover, on the basis of morphological studies, 23 endophytic bacteria which were grown on nutrient agar medium produced medium-sized, irregular-shaped, off-white and rough colonies and thus, were tentatively placed in genera *Bacillus sp*.Moreover, 13 isolates which produced round-shaped and raised colonies with entire margins, mucoid and cream in colour were tentatively placed as *Klebsiella sp*. 10 isolates cultured on Pseudomonas agar medium produced medium-sized, round-shaped and flat colonies with smooth margin and cream colour and tentatively belonged to genera *Pseudomonas sp*. On the basis of Gram's reaction, out of 46 bacterial endophytes, 52.17% (24) and 47.83% (22) were Gram positive and Gram negative respectively.

Promising native endophytic bacterial isolates were characterized on the basis of 16S rRNA gene ITS marker according to Zhao et al (2011).7 isolates(BIRR3,BIRL3,KUSR2,KUSR5,KUSS3,ALIS2 andALIL3) were detected as *Pseudomonas sp* .

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