RESEARCH ARTICLE

ISOLATION AND FUNCTIONAL CHARACTERIZATION OF ENDOPHYTIC BACTERIA ISOLATED FROM MICROPROPAGATED SUGARCANE PLANTLETS

M. Manju[1] and V. Jayakumar[2*]

Abstract

Twelve endophytic bacteria were isolated from surface sterilized micropropagated sugarcane plantlets of variety Co 86032. While most of the colonies were white, a few produced yellow, orange and red colour colonies. Among the bacteria isolated, five were Gram negative bacilli, four were Gram positive bacilli and three were Gram positive cocci. When the 12 bacteria were tested for their functional properties in vitro, two showed phosphate solubilization property, 11 were positive for siderophore production and all 12 produced indole acetic acid (IAA). The isolate ESTS 10 showed strong phosphate solubilization and siderophore production property and also produced IAA to a level of 106 µg/ml. None of the isolated bacteria produced HCN under in vitro condition; however, all of them showed enhanced IAA production in the range of 82-322 µg/ml in the presence of tryptophan within 48 h of inoculation. This enhanced IAA production along with siderophore production property may help the plant in promoting shoot and root growth/biomass when these endophytes are reintroduced in to the plant system, especially at acclimatization stage of micropropagated plantlets.

Key words : Sugarcane, endophytic bacteria, plant growth promotion, phosphate solubilization, siderophore, IAA

Introduction

Bacterial endophytes are consistently reported in the root, stem, leaf, fruit, seed and tuber tissues of a wide range of agricultural, horticultural and forest plants but they represent a largely untapped resource for the discovery of novel traits. This microbial community could play an important role in agriculture by conferring advantages to the plant by producing antibiotics (Strobel and Daisy 2003), phytohormones (Lee et al. 2004) and siderophores (Rashid et al. 2012), promoting phosphate solubilization (Dawwam et al. 2013) biological nitrogen fixation (Baldani et al. 1986), and increasing resistance to pathogens (Reiter et al. 2002) and parasites (Hallmann et al. 1997). These beneficial effects of endophytes promote crop growth with consequent reflection in yield (Ryan et al. 2008). In sugarcane, endophyte research is focused on nitrogen fixing diazotrophic bacteria and the commonly reported endophytic bacteria include *Gluconacetobacter diazotrophicus* (Döbereiner et al. 1988), *Herbaspirillum seropedicae*, *H. rubrisubalbicans* (Baldani et al. 1986) and *Acetobacter diazotrophicus* (Dong et al. 1994). It was reported that production of sugarcane could increase by up to 35% due to inoculation of diazotrophic endophytes (Boddey et al. 2003). Few

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attempts on endophytes other than diazotrophs in the crop showed concomitant existence of multiple genera of endophytic bacteria with huge potential of plant growth promoting properties (Mendes et al. 2007; Fávaro et al. 2012; Quecine et al. 2012; Beneduzi et al. 2013; Wei et al. 2014). However, presence of such endophytic bacteria in micropropagated sugarcane plantlets is not yet known. Micropropagated sugarcane plants are produced for various reasons in which the process of explant disinfection may eliminate many plant growth promoting endophytic bacteria (Oliveira et al. 2002). Nevertheless, the chances of occurrence of a few endophytes that are closely associated with the crop cannot be ruled out. In the present study, an attempt was made to isolate the endophytic bacteria associated with tissue cultured sugarcane plantlets and assess their plant growth promoting properties.

Materials and methods

Plant material and sample preparation

Micropropagated healthy sugarcane plantlets of variety Co 86032, ready to be planted in polyethylene bags for acclimatization, were selected for the isolation of endophytic bacteria. The root and shoot portions of plants were separated using a sterile knife, washed individually with sterile water and cut into pieces. The cut roots and shoots were washed separately with 70% ethanol for 3 min followed by sterile water and the process was repeated three times. The samples were collected in a sterile screw capped vial (lysing matrix A tube of tissue lyser) and 1 ml of sterile water was added. The final wash solutions of root and shoot were collected separately in sterile tubes. The surface sterilized samples were homogenized in Fast Prep-24™5G sample preparation system for 1 min, centrifuged at 5000 rpm for 10 min and the supernatants were used for isolation of bacteria.

Isolation of endophytic bacteria and characterization

To isolate the endophytic bacteria, Nutrient Agar (NA), Tryptic soy agar (TSA) and semi-solid minimal media with various carbon sources were used. The minimal media was composed of magnesium chloride 0.5 g, ferric chloride 2 µg, sodium molybdate 2 µg, potassium hydrogen phosphate 0.6 g, potassium dihydrogen phosphate 0.4 g, carbon source 5%, yeast extract 100 mg and agar 1.6 g per liter. Minimal media were prepared using sucrose, xylose, citric acid and starch flour as carbon sources. From the homogenized root or shoot material, 50 µl of supernatant was transferred to a sterile Petri plate, molten media of NA and TSA were added over the sample, mixed gently and allowed to solidify. To 10 ml of each semi-solid minimal media prepared in test tubes, 50 µl of supernatant was added separately. Besides, a set of control was plated with the final wash solution of plant material in order to rule out the presence of any bacteria from the surface of plant. The plates and tubes were maintained at 28ºC and observed for bacterial growth till 7 days after inoculation (DAI). The bacteria of each morphotype observed on plate were isolated and pure cultured in Petri plate on NA medium. The isolated bacteria were phenotypically characterized by observing cultural and morphological characters, viz. size, shape, margin, elevation, consistency, opacity and pigmentation. The bacteria were characterized further for Gram's staining reaction.

Assessing functional properties

Phosphate solubilization

The bacteria were individually streaked on Pikovskaya agar medium (Pikovskaya 1948) containing tri-calcium phosphate [Ca_3 (PO_4)$_2$] and incubated at 28ºC for 7 days. Development of a

clear zone at inoculation site on the culture plates, i.e. opaque medium turning transparent, was noticed as an index of phosphate solubilization. The culture plates were recorded for appearance of clear halo around the colonies for positive results and they were grouped based on diameter of the halo, i.e. <1 mm as weak, 1 - 2 mm as moderate and >2 mm as strong phosphate solubilizing reaction.

Siderophore production

This was performed by the method described by Schwyn and Neilands (1987) which involved the use of Chrome Azurol S (CAS) containing indicator plates. For 1 l medium, 60.5 mg CAS was dissolved in 50 ml of water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.H_2O$, 10 mM HCl). This solution was slowly added to 72.9 mg HDTMA dissolved in 40 ml water and the resultant solution was autoclaved. The nutrient agar medium was amended with CAS dye (media appear blue-green), plated, bacteria were streaked on the medium and incubated at 28ºC for 7 days. Formation of halo zone and change in colour of the medium indicated siderophore production and they were grouped based on diameter of the halo, i.e. <5 mm as weak, 5 - 10 mm as moderate and >10 mm as strong reaction.

Indole acetic acid production

Production of indole acetic acid (IAA) by bacteria was investigated using the method of Sarwar and Kremer (1995). The endophytic bacteria were inoculated in LB broth amended with tryptophan (50 µg/ml) and incubated for 48h at 200 rpm. After incubation, cells were removed by centrifugation at 10000 rpm for 15 min, 2 ml supernatant was taken and 2-3 drops of ortho-phosphoric acid was added. To this filtrate, 4 ml of Salkowski's reagent (1 ml of 0.5M $FeCl_3$ in 50 ml 35% $HClO_4$) was added and the samples were incubated for 30 min at room temperature. The optical density of the samples was determined spectrophotometrically at 530nm and the readings were plotted against standard curve of IAA.

HCN production

Production of HCN by the isolates was detected by the method of Bakker and Schippers (1987). The bacteria were streaked on NA medium in Petri plate, closed with a lid containing Whatman No. 1 filter paper soaked in 0.5% picric acid and 2% sodium carbonate, sealed with parafilm and incubated at 28ºC for 4 days. Change of filter paper colour from yellow to orange and then to dark brown indicated positive reaction and the absence indicated negative reaction.

Results and discussion

Isolation of endophytic bacteria and characterization

The present study was aimed at isolating culturable endophytic bacteria from micropropagated sugarcane plantlets and assessing their functional properties. A total of 12 endophytic bacteria, 10 from shoot and two from root were isolated and these appear to belong to seven tentative genera (Table 1). No bacterial colony was identified in the final wash solution plated on media, which ruled out the presence of any phyllosphere residing bacteria. Maximum number of bacteria could be isolated on NA medium whereas no endophyte could be isolated from any of the minimal media. Although the association of several endophytic bacteria with sugarcane crop was reported in earlier studies (Dong et al. 1994; Gillis et al. 1989; Viswanathan et al. 2003), our study appears to document the first time isolation of endophytes from micropropagated sugarcane plantlets. The isolated endophytic bacteria varied in their morphological properties with few exceptions. While most of the colonies were white,

Table 1. Endophytes isolated from micropropagated sugarcane plantlets and their characters

Isolate	Plant part used for isolation	Media used for isolation	Gram's staining reaction	Shape	Colony morphology	Genus
ESTR 1	Root	Tryptic soy agar	Gram Positive	Rod	White, opaque, flat with undulate elevation, irregular, wet look	*Bacillus* sp.
ESTR 2	Root	Tryptic soy agar	Gram Positive	Rod	Milky white, opaque, raised with lobate elevation, irregular, dried colony	*Bacillus* sp.
ESTS 3	Shoot	Nutrient agar	Gram Positive	Cocci	Light orange, opaque, raised with entire margin, regular, mucoid	*Micrococcus* sp.
ESTS 4	Shoot	Nutrient agar	Gram Negative	Rod	Pale yellow, opaque, raised with undulate elevation, irregular, mucoid	*Enterobacter* sp.
ESTS 5	Shoot	Nutrient agar	Gram Positive	Rod	Pale yellow, opaque, raised with entire margin, regular, mucoid	*Pseudomonas* sp.
ESTS 6	Shoot	Nutrient agar	Gram Positive	Cocci	Yellow, opaque, raised with entire margin, pin head colonies, regular, mucoid	*Micrococcus* sp.
ESTS 7	Shoot	Nutrient agar	Gram Negative	Rod	Creamy white, opaque, raised with entire margin, regular, mucoid	*Gluconacetobacter* sp.
ESTS 8	Shoot	Nutrient agar	Gram Negative	Rod	Creamy white, opaque, raised with entire margin, irregular, dried	*Pantoea* sp.
ESTS 9	Shoot	Nutrient agar	Gram Negative	Rod	Creamy white, opaque, raised with entire margin, regular, mucoid	*Gluconacetobacter* sp.
ESTS 10	Shoot	Nutrient agar	Gram Positive	Cocci	Pale yellow, opaque, flat with undulate elevation, irregular, mucoid	Not known
ESTS 11	Shoot	Tryptic soy agar	Gram Positive	Rod	Pale yellow, opaque, flat with undulate elevation, irregular, dried	*Bacillus* sp.
ESTS 12	Shoot	Nutrient agar	Gram Negative	Rod	Red, opaque, raised with umbonate elevation, regular, mucoid	*Serratia* sp.

few produced yellow, orange and red colour colonies. Among the colonies, five were Gram negative bacilli, four were Gram positive bacilli and only three were Gram positive cocci. While the most common bacteria reported to have been isolated from sugarcane is diazotrophic bacteria such as *Acetobacter diazotropicus, Herbaspirillum* and *Azospirillum* spp. (Sevilla et al. 2001), in the present study, many non-diazotrophic bacteria were isolated from tissue cultured plantlets of sugarcane.

Functional properties of endophytic bacteria

The importance of plant associated bacteria in promoting plant growth is well recognized (Germida et al. 1998). The mechanisms of plant growth stimulation by associative bacteria are most probably related to greater mobilization of nutrients and phytohormone production (Lifshitz et al. 1987). Phosphorous (P) is one of the most important plant nutrients and a large portion of inorganic phosphate applied to soil as fertilizer is rapidly immobilized (Rodriguez and Fraga 1999). Bacteria possess the capacity to solubilize immobilized mineral phosphates (Goldstein 1986) and it was also reported as a common trait among endophytes isolated from crops (Hardoim et al. 2012). In our study, the bacteria ESTS3 possessed moderate P solubilization ability whereas another one, i.e. ESTS10 showed very strong phosphate solubilization activity (Table 2). The Gram positive endophyte ESTS10 solubilized the entire P in 90 mm plate in 7 DAI, which made the full media transparent (Fig. 1). Bacterial genera like *Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium* and *Serratia* are reported as the most significant phosphate solubilizing bacteria (Bhattacharya and Jha 2012). Siderophores are low molecular weight

Table 2. In vitro functional properties of endophytic bacteria isolated from micropropagated sugarcane plantlets

Isolate	Phosphate solubilization	Siderophore production	IAA production (µg/ml)	HCN Production
ESTR 1	-	-	88.9	-
ESTR 2	-	+	123.9	-
ESTS 3	++	++	82.8	-
ESTS 4	-	++	140.3	-
ESTS 5	-	+	122.1	-
ESTS 6	-	+	108.9	-
ESTS 7	-	++	89.9	-
ESTS 8	-	+	167.8	-
ESTS 9	-	+	134.9	-
ESTS 10	+++	+++	106.7	-
ESTS 11	-	+	169.9	-
ESTS 12	-	+	322.1	-

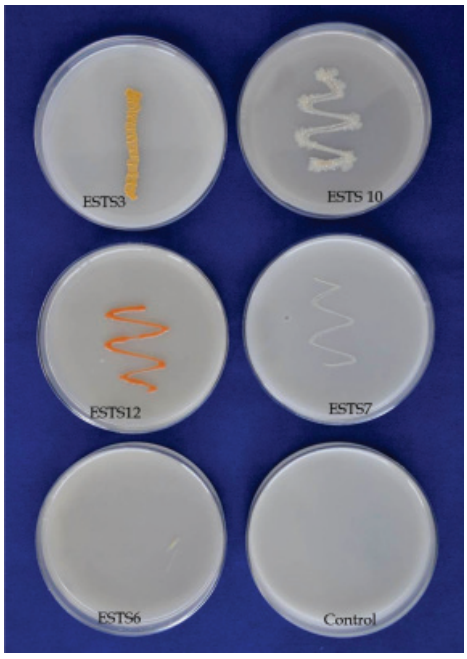

Fig 1. Phosphate solubilization by endophytic bacteria isolated from micropropagated sugarcane plantlets

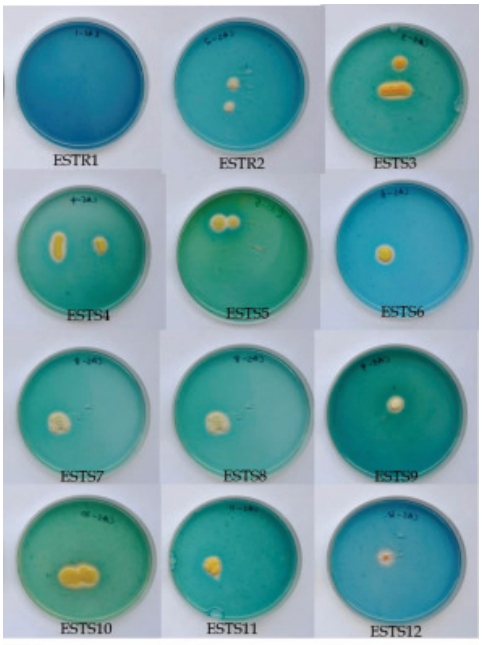

Fig 2. Siderophore production by endophytic bacteria isolated from micropropagated sugarcane plantlets

iron chelating compounds usually classified by the ligands used to chelate the ferric iron. The major groups of siderophores include the catecholates (phenolates), hydroxamates and carboxylates (Ali and Vidhale 2013). The endophytic bacteria were reported to produce siderophores at various levels (Rashid et al. 2012) and in sugarcane ecosystem most of the associated bacteria, i.e. both endophyte and rhizosphere bacteria, were already reported to produce siderophores (Beneduzi et al. 2013). In our study, 11 out of the 12 isolated endophytic bacteria showed positive reaction for siderophore production and among these, one endophyte, namely ESTS10 showed strong chelating property and three viz. ESTS3, ESTS4 and ESTS7 produced siderophore at moderate level (Fig. 2).

IAA, a member of phytohormones, is generally considered to be the most important native auxin. The endophytic bacteria produce low to moderate level of IAA (Uma Maheshwari et al. 2013; Szilagyi-Zecchin et al. 2014) and, normally, the bacterial isolates from the rhizosphere are more efficient auxin producers (Sarwar and Kremer 1995). The rhizosphere and endophytic bacteria isolated from sugarcane were already reported to produce IAA ranging from 0.1 to 264 µg/ml with 16 isolates producing >100 µg/ml in 2 days after incubation (Beneduzi et al. 2013). In our experiment also, all 12 endophytic bacteria were identified as potential IAA producers, i.e. a production level of >82 µg/ml IAA. Among them, ESTS12 produced exceptionally high level of 322 µg/ml IAA within 2 days of inoculation, which is apparently a very high level of IAA production by endophytic bacteria. Mirza et al. (2001) reported production of 688 µg/ml of IAA by *Enterobacter* sp., an endophyte isolated from sugarcane, but only 7 days after inoculation. Increase in IAA production with the age of bacterial cultures has already been reported (Gonzalez-Lopez et al. 1986). The secondary metabolite cyanide is produced by several microorganisms (Knowles 1976) and is common in rhizosphere inhabiting

microorganisms of crops (Bakker and Schippers 1987). In our experiment, none of the isolates produced hydrogen cyanide and similar results were reported by Mendes et al. (2007) when they isolated endophytic bacteria from sugarcane. Overall, 11 out of 12 bacteria isolated possessed a combination of siderophore and IAA producing ability and two possessed a combination of three properties, viz. P solubilization, siderophore and IAA producing ability. Among them, ESTS10 was identified as the best isolate with strong P solubilization, siderophore and IAA producing ability. Presence of plant growth promoting (PGP) properties in vitro can be taken as an important criterion in the selection of endophytes for their field application. Dias et al. (2009) found correlation between the in vitro data of phosphate solubilization and auxin production with PGP observed in the greenhouse. They reported that endophytic bacteria isolated from micropropagated strawberry plantlets showed enhanced phosphate solubilization and IAA production in vitro and their application in acclimatization stage of strawberry seedlings promoted shoot and root growth. In field condition also, when these endophytes were reintroduced in to the plant system they were reported to promote plant growth in many crops through P solubilization (Verma et al. 2001). It was reported that application of higher IAA producing endophytic bacteria to micropropagated sugarcane plantlets increased shoot biomass (70%), root biomass (55%) and root surface area (Mirza et al. 2001). These earlier findings indicate that the IAA and siderophore positive bacteria isolated in our study may play a role in shoot and root production and subsequent growth of micropropagated sugarcane plantlets. Further studies are needed to utilize these endophytic bacteria possessing PGP properties through application to sugarcane seedlings at acclimatization stage and planting materials for improving plant growth and yield.

Acknowledgement

The authors are thankful to the Director, ICAR-Sugarcane Breeding Institute, Coimbatore, for providing necessary facilities.

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