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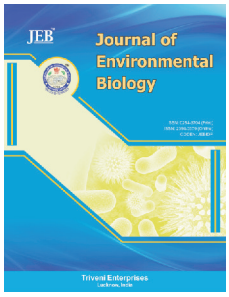

Bioefficacy of antimicrobial peptide biosynthesis-gene-linked antagonistic *Lysinibacillus sphaericus* strains for management of bacterial plant diseases in Andaman and Nicobar Islands, India

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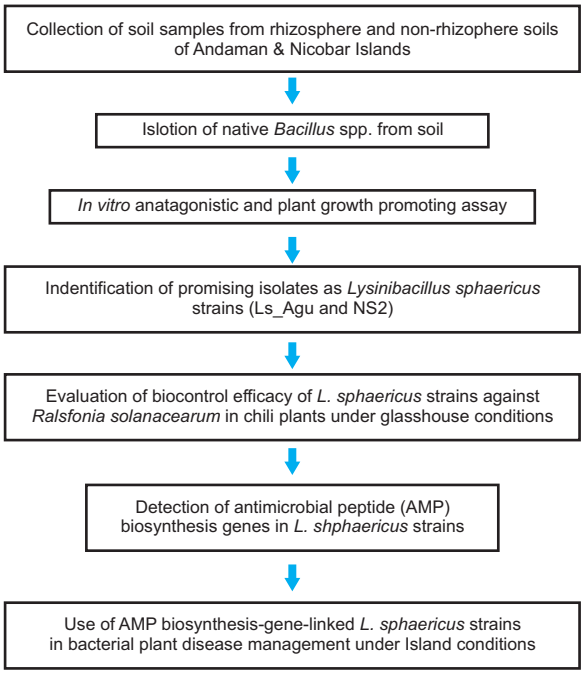
Abstract

Aim : The present study aimed to investigate the bioefficacy of antimicrobial peptide-gene-linked antagonistic *Lysinibacillus sphaericus* strains isolated from Andaman and Nicobar Islands, India for controlling diseases in plants.

Methodology : *Bacillus* spp. isolated from different niches of Andaman & Nicobar Islands were characterized for antimicrobial and PGP- traits. The identity of potential isolates was confirmed by 16SrRNA gene sequence analysis. Biocontrol potential of selected isolates against chili bacterial wilt pathogen was tested in glasshouse conditions. The antimicrobial peptide biosynthesis genes responsible for antagonistic activities of *Lysinibacillus sphaericus* strains were analysed by PCR based methods.

Results : Out of fifty two isolates tested, two *Lysinibacillus sphaericus* strains (Ls_Agu and NS2) collected from Nicobar sea sand and chili crop rhizosphere of South Andaman Islands were found good in *in-vitro* antagonistic potential against important plant bacterial pathogens: *Ralstonia solanacearum* (solanaceous bacterial wilt) and *Xanthomonas oryzae* pv. *oryzae* (rice bacterial blight) using an agar diffusion assay. In glasshouse study, both the strains Ls_Agu and NS2 showed significant biocontrol efficacy (88.9% and 66.7%) against *Ralstonia solanacearum*, which causes bacterial wilt disease in chili. The antimicrobial potential of these strains were further ascertained by presence of three AMP biosynthesis genes (surfactin, bacilysin and fengycin) in both the strains through PCR amplification.

Interpretation : The overall results revealed the antimicrobial peptide-gene-linked strains Ls_Agu and NS2 of *L. sphaericus* have ability to control bacterial plant disease under Islands conditions and the same strains could be utilized for popularization on Islands for management of the bacterial diseases.



Introduction

The members of bacterial genus *Bacillus* comes under phylum Firmicutes are the most characterized group among bacterial community and among which *Lysinibacillus* is one such member having Gram-positive, rod-shaped, round and spore forming nature in the family Bacillaceae. *Lysinibacillus* is commonly found in soil, plants and animals (Hayat *et al.,* 2013) and around 23 species of industrial purpose have been reported worldwide till date. Few of the *Lysinibacillus* spp. have been reported for their nitrogen fixing ability (Reghuvaran *et al.,* 2012), plant growth promoting activities (Vendan *et al.,* 2010) and biological control against fungal pathogens (Ahmad and Khan, 2015). But originally the *Lysinibacillus sphaericus* were earlier reported for its potential of utilizing as larvicidal agents against mosquito (Wirth *et al.,* 2013) having insecticidal toxin that controls mosquito growth. The management of bacterial plant diseases is very important worldwide. Non availability of suitable chemicals, high cost of antibiotics and emergence of multi drug resistant bacteria have made the management of phyto-bacterial diseases highly challenging and complex. Recent reports have highlighted the successful utilization of native antagonistic bacterial species against important plant diseases in many regions of the world.

Out of several mechanisms, the production of antimicrobial peptides (AMPs) was found to be very important bio-control mechanism of plant pathogen in several *Bacillus* species (Gonzalez-Sanchez *et al.,* 2010). *Bacillus* species contain as many as 24 diverse AMP genes that allow the biosynthesis of antibiotics like iturin, bacilysin, bacillomycin, fengycin, surfactin, mersacidin, ericin, subtilin, subtilosin and mycosubtilin (Chung *et al..* 2008). The presence of a greater number of AMP genes has been correlated with the efficiency of antagonist in controlling plant pathogens (Mora *et al.,* 2011). Among *Bacillus* community, *B. subtilis/amyloliquefaciens* are primarily well known for the presence of antimicrobial peptide (AMPs) biosynthetic genes and their byproducts which are advocated to be directly involved in antimicrobial activities against plant pathogens (Athukorala *et al.,* 2009) and thus have been recommended as a potential biocontrol agents. Meager studies on biocontrol activity of *Lysinibacillus* against plant bacterial pathogens and studies on its AMP biosynthesis genes. In this study, native strains of *Lysinibacillus sphaericus* isolated from Andaman and Nicobar Islands were evaluated for their *in-vitro* antagonistic potential against two important bacterial pathogens *Ralstonia solanacearum* (solanaceous bacterial wilt pathogen) and *Xanthomonas oryzae* pv. *oryzae*: (rice bacterial blight pathogen) followed by detection of antimicrobial peptide (AMPs) biosynthesis genes in two strains.

Materials and Methods

Isolation of bacteria : A total of 52 *Bacillus* like bacteria were isolated from rhizosphere of crops and non-rhizosphere soils collected from different locations of Andaman and Nicobar Islands using semi-selective isolation procedure (Travers *et al.,* 1987). The two bacterial isolates which showed consistent plant growth promoting (PGP) and *in-vitro* antagonistic activities were identified using 16S rRNA gene sequencing.

Evaluation of *in-vitro* antagonistic potential and plant growth promoting traits : The antagonistic activity of these two potential strains were tested *in-vitro* against the highly virulent bacterial pathogen strains *R. solanacearum* strain CRs_Sg1 and *X. oryzae* strain ANBB_16 collected from culture collections of ICAR-CIARI, Port Blair using agar well diffusion assay on King's medium Briefly, in agar diffusion test, 48 hr old grown *R. solanacearum* or *X. oryzae* pv. *oryzae* strains were mixed in King's medium B (0.25 µl ml^{-1} at 10^8CFU ml^{-1}) separately. 20 µl of the exponential growth phase of antagonistic culture (approximately 3.5 x 10^8 CFU ml^{-1}) was added in 7 mm wells in the plates and sterile water was used as negative control in the experiment. The experiment was repeated thrice and the antagonistic ability was recorded as inhibition zone around the well (radius in mm) after 48hr. The plant growth promoting traits like siderophore production (Schwyn and Neilands, 1987), indole acetic acid (Gordon and Weber, 1951) and phosphate solubilization (Nautiyal, 1999) were evaluated *in vitro* as mentioned.

Detection of antimicrobial peptide (AMPs) biosynthesis genes : To study the presence of AMP biosynthesis genes, the genomic DNA was extracted from antagonistic *Lysinibacillus sphaericus* strains by standard procedures (Sambrook and Russell, 2001). A set of sixteen primers were used for PCR screening. PCR amplifications were carried out in 50 µl reaction mixtures at C1000TM Thermal Cycler (Bio-Rod) with the following cycle conditions: initial denaturation at 95°C for 15min; 40 cycles of 95°C for 1 min, 55°C or 52°C for 1min, and 72°C extension for 1.5 min; and a final extension at 72°C for 7 min. A total of 5 µl of each amplification reaction was analyzed by electrophoresis using 1.5% agarose gel, followed by ethidium bromide staining and visualized in Gel-documentation system (Bio-Rad).

Evaluation of antagonist potential against *R. solanacearum* : The *in-planta* bio-control efficacy of both the strains was tested against chili bacterial wilt pathogen *Ralstonia solanacearum* in glass house condition as per Ramesh *et al.,* (2012) with slight modifications. The virulent pathogen strain CRs_Sg1 was collected from culture collection center, Plant Pathology Laboratory, CIARI, Port Blair. Thirty day old chili plants (*Capsicum annuum*) were transferred to 15 cm diameter plastic pots containing sterilized potting mixture of 2:1:1 (Red soil: FYM: Sand) and maintained in glass house conditions. After acclimatization for one week period, freshly prepared bio-control bacterial suspension of 50 ml (10^8cfu ml^{-1}) was poured into the soil at the base of each plant. One week after inoculation of bio control, a 20 ml suspension of *R. solanacearum* strain (5×10^7cfu ml^{-1}) was poured into the soil to give a final bacterial concentration

of 10^6cfu g^{-1} d.wt. In total, four treatments were followed (Two treatments with antagonistic bacteria + pathogenic bacteria, one treatment of pathogenic bacteria alone and another with normal water). All the treated plants were incubated at 28˚-32˚C and at 80-90% RH under glasshouse conditions for wilt development up to 30 days. Three replications were maintained per treatment with nine chili plants in three pots/ treatment. Wilt incidence was recorded at weekly intervals up to one month and wilting percentage was calculated at the end of the experiment by the formula given by Guo *et al.* (2004).The disease incidence and bio-control efficiency was also calculated.

Results and Discussion

A total of 52 *Bacillus* like isolates were obtained from rhizosphere and non-rhizosphere soil samples of Andaman and Nicobar Islands, India (Data not shown). All the isolates were screened for *in-vitro* antagonistic and PGP traits and subsequently two potential isolates were selected for further *in-planta* and PCR analysis. The genetic identity of two isolates was confirmed as *Lysinibacillus sphaericus* using 16S rRNA gene sequence analysis (GenBank accession KP864632 and KJ013550).

The *in-vitro* studies using agar diffusion results revealed that both the *Lysinibacillus sphaericus* isolates were having high potential of antagonism against both the plant bacterial pathogens tested. For solanaceous bacterial wilt pathogen *R. solanacearum*, Ls_Agu and NS2 showed inhibition zone of 10.06 mm and 9.33 mm whereas for rice bacterial blight pathogen *X. oryzae*pv. *oryzae*, the zone of inhibition was15.17 mm and 12.68 mm, respectively (Fig. 1). Among PGP traits tested, both the isolates were found to be good siderophore producers, whereas in contrast no P solubilization and IAA productions were noted. This study is in the line of work described by Hakizimana *et al.* (2011), wherein *Lysinibacillus fusiformis* was identified as a potential biocontrol agent as it showed biocontrol activity against *Phytophthora cinnamomi*. Also the strain of *Lysinibacillus fusiformis* was reported to be positive for most of the plant growth promoting traits, indicating their role in growth promotion of ginseng (Vendan *et al.,* 2010). In the present study, for the first time siderophore production and *in vitro* antagonistic potential in *L. sphaerecus* was recorded.

Earlier the role of AMP genes in conferring better antagonistic activity was reported in many *Bacillus* spp. by several studies (Chung *et al.,* 2008, Kim *et al.,* 2010 and Shanthiyaa *et al.,* 2015). The antimicrobial peptide genes (AMPs) which encompass cyclic lipopeptides such as bacillomycin, fengycin, iturin and surfactin etc., play a vital role in antagonistic efficacy of genus *Bacillus* which has been again linked to the presence of the AMP biosynthetic genes such as *bmyB, fenD, ituC* and *srfA*. However, no reports available related to presence of AMP biosynthesis genes in *Lysinibacillus* strains and their role in imparting antibacterial antagonism earlier. In the present study the PCR analysis for AMP biosynthesis genes revealed the presence of three AMP biosynthesis genes viz., surfactin, bacilysin and fengycininin in the Ls_Agu and NS2 strains (Table 1). The strain

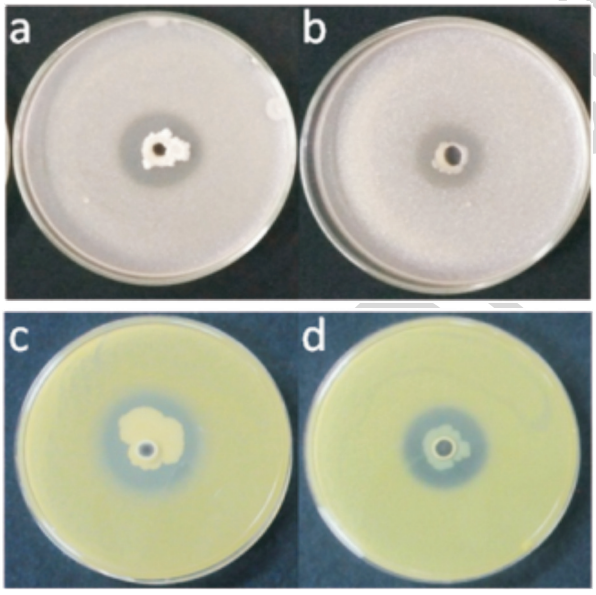


Fig. 1 : *In-vitro* antagonistic activity of *Lysininibacillus* spp. against *R. solanacearum* strain CRs_Sg1and *X. oryzae* pv. oryzae strain ANBB_16

Legends :
a& b - Ls_Agu and NS2 strain against *R. solanacearum* strain *CRs_Sg1*
c& d - Ls_Agu and NS2 strain against *X. oryzae strain ANBB_16*

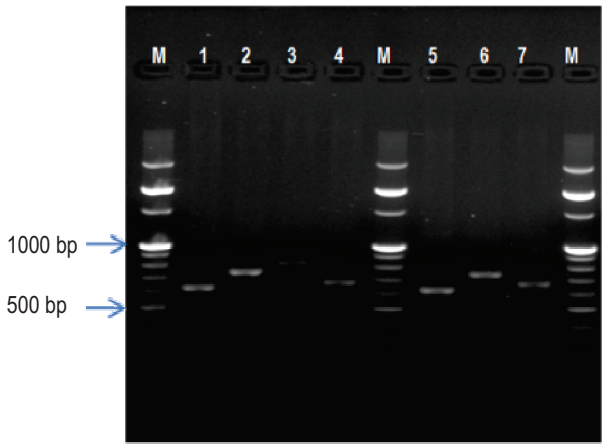


Fig. 2 : Amplification of AMP biosynthesis genes in *L. sphaericus* strains. Lane M is a 100-bp ladder. Lane 1 to 4 is Ls_Agu and Land 5 to 7 is NS2. Lanes 1 and 5 - surfactin; Lanes 2, 3 and 6 - bacilysin and Lanes 4 and 7 - fengycin

Table 1 : Detection of antibiotic biosynthesis genes in *Lysinibacillus sphaericus* strains

Antibiotics	Genes symbol	Primers	Sequences	PCR product (bp)	*L. sphaericus* size (Ls_Agu)	*L. sphaericus* Ns2
Surfactin	*srfA*	SRFA-F1	5'-AGAGCACATTGAGCGTTACAAA	626	Yes	Yes
		SRFA-R1	5'-CAGCATCTCGTTCAACTTTCAC			
Bacilysin	*bac*D	BACD-F1	5'-AAAAACAGTATTGGTYATCGCTGA	749	Yes	Yes
		BACD-R1	5'-CCATGATGCCTTCKATRCTGAT			
	*bac*AB	BACAB-F1	5'-CTTCTCCAAGGGGTGAACAG	815	Yes	No
		BACAB-R1	5'-TGTAGGTTTCACCGGCTTTC			
Fengycin	*fen*B	FENB-F1	5'-CCTGGAGAAAGAATATACCGTACCY	670	Yes	Yes
		FENB-R1	5'-GCTGGTTCAGTTKGATCACAT			

Table 2 : Antagonistic efficacy of *Lysinibacillus sphaericus* strains (Ls_Agu and Ns2) against *Ralstonia solanacearum* strain CRs_Sg1 under glasshouse conditions after 30 days of growth period

Treatments	Antagonistic efficacy (%)	
	Wilt incidence	Biocontrol efficacy
Ls_Agu + pathogen	16.67	83.33
Ns2 + pathogen	33.33	66.67
Pathogen alone (control)	100.0	-
Normal water	0	-

Ls_Agu amplified with four primer sets whereas strain NS2 with three primer pairs (Fig. 2). This might also be one of the strong reasons for the effective antagonistic efficacy of both the strains against *Ralstonia solanacearum* and *Xanthomonas oryzae* pv. *oryzae*.

The *in-planta* glass house studies (Table 2) revealed the antagonistic strains Ls_Agu (16.67%) and NS2 (33.33%) showed significant reduction in disease incidence at 30 days after bacterial inoculation when compared to pathogen inoculated treatments (100%). The bio-control efficiency of Ls_Agu and NS2 was 83.33% and 66.7%, respectively, when compared to water control (0%) in greenhouse. Though the biocontrol potential of endophytic *Lysinibacillus* spp. against fungal plant pathogens have been reported earlier (Hakizimana *et al.*, 2011), the antibacterial antagonism potential is being reported for the first time in this study. Earlier, the antagonistic potential against multiple plant pathogens using bacilli was reported by many researchers. Chung *et al.* (2008) reported that *Bacillus subtilis* strain ME488 could suppress the growth of 39 out of 42 plant pathogens tested using *in-vitro* antagonistic assay. Recently, Sakthivel *et al.* (2017) reported that *Bacillus subtilis* strain Bs_Ane from Neil Island, India showed better antagonistic potential (growth inhibition) against three phytopathogens, *Ralstonia solanacearum*, *Xanthomonas oryzae* and *Colletotrichum gloeosporioides.*

This study indicated that the *Lysinibacillus sphaericus* strains Ls_Agu and NS2 identified from Andaman & Nicobar Islands with broad spectrum antagonistic traits would be a potential source for utilizing them in successful eco-friendly management of plant diseases in the Islands and to the best of our knowledge this is the first study on antibacterial efficacy of *Lysinibacillus* strains possessing antimicrobial peptide biosynthesis genes.

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