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ORIGINAL ARTICLE

Salt tolerant *Methylobacterium mesophilicum* showed viable colonization abilities in the plant rhizosphere

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Abstract The source of infection has always been considered as an important factor in epidemiology and mostly linked to environmental source such as surface water, soil, plants and also animals. The activity of the opportunistic pathogens associated with plant root, their adaptation and survival under hostile environmental condition is poorly understood. In this study the salt tolerance ability of *Methylobacterium mesophilicum* and its colonization in the root and shoot of plants under severe drought and salt stress conditions were investigated. The colonization of plant by *M. mesophilicum* was investigated in a gnotobiotic sand system, and their survival in pots with saline soil. Bacterial strain was found to colonize rhizosphere of cucumber, tomato and paprika grown under normal and salt stress condition and reached up to 6.4×10^4 and 2.6×10^4 CFU/g root. The strain was resistant to Gentamicin, Ampicillin, Amoxicillin plus Clavulanic acid, Cefotaxime, neomycin, penicillin and was also tolerant to salinity stress (up to 6% NaCl). These abilities play important roles in enabling persistent colonization of the plant surface by *M. mesophilicum* strains. In conclusion, this study provides background information on the behaviour of opportunistic pathogen *M. mesophilicum* on plants and their survival in harsh environmental conditions.

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1. Introduction

The incidence of microbes with a potential risk to human health in the soil, plant and water resources has been reported previously (Berg et al., 2005; Park et al., 2005; Egamberdieva et al., 2008; Lai et al., 2011). The pink-pigmented, and

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facultative methylotrophic bacteria *Methylobacterium* are commonly found within the natural environment such as soils, plants, lake sediments and also in association with humans (Corpe and Rheem, 1989; Green, 2001; Omer et al., 2004). Several species of *Methylobacterium* have been reported as causing infections in humans, such as persistent bacteremia in a child with lymphoma (Fernandez et al., 1997; Lai et al., 2011), bloodstream infections (Lai et al., 2011), peritonitis, and pneumonia (Sanders et al., 2000; Kovaleva et al., 2014).

The frequent persistence of *Methylobacterium* genus in the clinical surroundings has been explained by their ability to tolerate stress factors and antimicrobials (Yano et al., 2013; Kovaleva et al., 2014). The source of infection has always been considered as an important factor in epidemiology and mostly linked to environmental source such as surface water, soil, plants and also animals (Lederberg, 1997). There are several reports on the *Methylobacterium* spp. isolated from various natural environments i.e., plant leaves, roots (Green, 2001; Omer et al., 2004), and nodules (Sy et al., 2001). Ivanova et al. (2001) found *Methylobacterium* spp. on plant phyllosphere and in plant rhizosphere. The endophytic *Methylobacterium mesophilicum* was isolated from citrus plants (Gai et al., 2009), *Methylobacterium nodulans* from the rhizosphere of crotalaria podocarpa and *Methylobacterium oryzae* from rice stem and leaf (Yim et al., 2010). The improved plant growth, yield, and nodulation of leguminous plants by *Methylobacterium* species have been reported by several authors (Sy et al., 2001; Radha et al., 2009). However, there are few studies available on the biology of the opportunistic pathogens associated with plant root, their adaptation and survival under hostile environmental conditions. In the present study we investigated the tolerance of *M. mesophilicum* strain to salinity stress and their colonization abilities in the rhizosphere and phyllosphere of cucumber, tomato and paprika grown under severe drought and salt stress conditions.

2. Material and methods

2.1. Bacterial strain and growth characters

The strain *M. mesophilicum* previously isolated from the root of wheat was obtained from the National Culture Collection of Uzbekistan. The strain was grown on ammonia mineral salt (AMS) medium which contains methanol as a sole carbon source at 28 °C (Whittenbury et al., 1970).

2.2. Characterization of strain

Carbon utilization of bacterial strain was tested in nutrient broth containing 1% of D-glucose, D-sucrose, D-Xylose, L-Arabinose, Fructose, D-mannitol, maltose, glycerol, and methanol (Cowan, 1974). The method of Castric (1975) was used to determine the HCN production by bacterial strain, lipase activity was investigated by Howe and Ward (1976) using Tween lipase indicator assay, and protease activity by methods of Brown and Foster (1970). The production of glucanase was determined using glucan substrate lichenan (Walsh et al., 1995), whereas cellulase activity using substrate carboxymethylcellulose (Hankin and Anagnostakis, 1977). The salt tolerance of bacterial strain was tested in Luria–Bertani (LB) medium supplemented with 0–6% of NaCl.

2.3. Antibiotic resistance patterns

Antibiotic resistance of *M. mesophilicum* strain against the antibiotics of human and veterinary significance was analysed using a modified Kirby–Bauer disc-diffusion method (Bauer et al., 1966).

In vitro susceptibilities of *M. mesophilicum* to Ampicillin (AMP, 10 μg), Amoxicillin/Clavulanic acid (AMC/20 + 10 μg), Penicillin (PEN, 10 μg), Chloramphenicol (CLR/30 μg), Cefotaxime (CTX/30), Tetracycline (TET/30 μg), Streptomycin (STR/100 μg), Erythromycin (ERY/15 μg), Neomycin (NEOM/100 μg), and Gentamicin (GEN/10 μg) were determined using the Neo-Sensitab (Rosco Diagnostica A/S) antibiotic discs. The Mueller–Hinton broth (Difco Laboratories, Detroit, MI) used to culture bacteria for 24 h and 100 μl of cell suspension plated on agar plate. The antibiotic discs (6 mm diameter) were placed on the agar surface. Antibiotic effectiveness against bacteria was determined after two days of incubation at 28 °C by measuring the zones of inhibition around the discs. The resistance to antibiotics was analysed according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2001).

2.4. Plant associated traits

The antagonistic potential of experimental bacterial strain was carried out using the triple layer agar method (Herr, 1959) against some plant pathogenic fungi (*Fusarium oxysporum* Schlecht. ex Fr., *F. solani* [Mart.] Sacc., *Gaeumannomyces graminis* var. tritici, *Pythium ultimum* var. ultimum, *Alternaria alternata* [Fr.] Keissler, *Botrytis cinerea* Pers). The production of indole 3-acetic acid (IAA) was determined spectrophotometrically according to the method of Bano and Musarrat (2003).

2.5. Colonization in the rhizosphere

The colonization abilities of bacterial strain in the rhizosphere of cucumber (*Cucumis sativus*), tomato (*Solanum lycopersicum*) and paprika (*Capsicum annuum*) were studied in gnotobiotic sand tubes (25 mm in diameter, 200 mm in length) as described by Simons et al. (1996), containing sand and vermiculite (1:1) soaked with diluted nitrogen-free Jensen nutrient solution supplemented with 100 mM NaCl. The seeds of cucumber, tomato and paprika were sterilized and treated with the overnight cultured bacterial strain (10^8 cells/ml) suspension and were planted into sterile glass tubes, one seed per tube with ten replicates. The seedlings were grown in a climate controlled plant growth chamber with a 16-h light period at 22 °C and an 8-h dark period at 16 °C. After 15 days, 1 cm root tip was cut, vortexed and diluted suspension (10^{-3} and 10^{-4}) was spread on agar plates. After three days bacterial colonies were counted and colony forming units (CFU per 1 cm of root tip) were enumerated.

2.6. Survival in the plant and soil

The bacterial strain of *M. mesophilicum* plated on LB agar medium amended with 200 μg/ml rifampicin for obtaining antibiotic resistance mutants. The sterilized seeds of tomato, cucumber and paprika were inoculated with bacterial

suspension (10^8 CFU ml^{-1}) and sown in pots filled with saline soil. Plants were grown under greenhouse conditions for 2 months. Then, plants were harvested and root and shoots were separated. One gram root and macerated shoot were shaken with 9 ml PBS (20 mM sodium phosphate, 150 mM NaCl) with cycloheximide (100 μg/ml, Sigma, St. Louis, USA) for 30 min and plated on LB agar supplemented with 200 μg/ml rifampicin. The plates were incubated for three days and CFU of rifampicin resistant mutants were enumerated (Egamberdiyeva and Hoflich, 2002).

2.7. Statistical analyzes

Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 2007 and mean comparisons were conducted using a least significant difference (LSD) test ($P = 0.05$). The standard deviations (SD) were also calculated.

3. Results

M. mesophilicum strain was able to utilize several carbon sources such as D-glucose, D-sucrose, D-Xylose, L-Arabinose, D-mannitol, glycerol, and methanol. The maltose and fructose were not utilized as a carbon source by bacterial strain.

The strain *M. mesophilicum* can withstand NaCl concentrations up to 6% (Fig. 1), and showed resistance to higher temperature up to 40 °C. The strain was negative for lipase, protease, glucanase, and cellulase activities and did not produce HCN. The strain was resistant to Gentamicin, Ampicillin, Amoxicillin plus Clavulanic acid, Cefotaxime, neomycin, penicillin, but not to erythromycin, chloramphenicol, tetracycline, and streptomycin.

The strain did not show antagonistic activity towards the phytopathogenic fungi *F. oxysporum*, *F. solani*, *G. graminis pv. tritici*, *P. ultimum*, *A. alternata* and *B. cinerea*. The IAA production of bacterial strain showed that *M. mesophilicum* produces IAA in media contained up to 4% NaCl and tryptophan addition induced auxin production (Fig. 2).

The colonization of *M. mesophilicum* in the rhizosphere of cucumber, tomato and paprika was also tested in the gnotobiotic sand system under growth cabinet conditions. The bacterial strain was able to colonize in the rhizosphere of plants grown under both normal and salinated conditions (100 mM NaCl). The salinity inhibited their colonization ability in the rhizosphere up to 50%. The root colonization in the rhizosphere was $64.1 - 24.2 \times 10^3$ (CFU per 1 cm of root tip) for tomato, $35.8 - 14.9 \times 10^3$ (CFU per 1 cm of root tip) for cucumber, and $15.8 - 5.6 \times 10^3$ (CFU per 1 cm of root tip) for paprika grown under normal and salinated condition respectively (Fig. 3a–c).

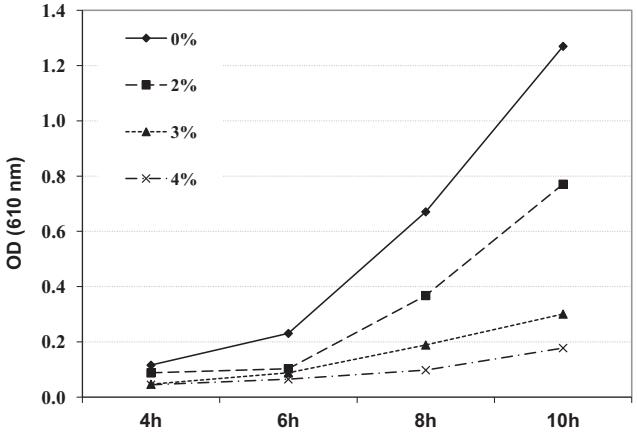

Figure 1 Survival of *Methylobacterium mesophilicum* after 10 h of incubation at various NaCl concentrations (0–6%).

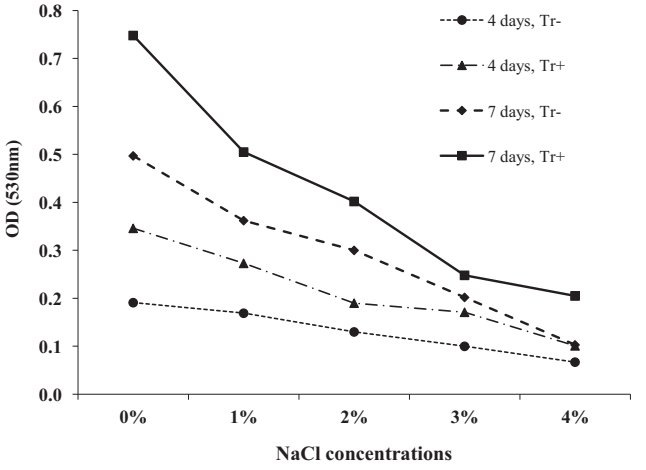

Figure 2 The production of IAA by *Methylobacterium mesophilicum* under various NaCl conditions (Tr + bacteria grown in medium with tryptophan, and Tr − without tryptophan).

Rifampicin-resistant isolates derived from *M. mesophilicum* were tested for their ability to colonize cucumber, tomato and paprika roots, leaves and soil during the vegetation time up to 2 months after sowing. *M. mesophilicum* was able to establish a population in the soil of the root zone of all tested plants grown under salt affected soil. The level of colonization varied between the plants, where the colonization of introduced bacterial strain was lower in the rhizosphere and phyllosphere of paprika (Table 1).

4. Discussion

The plants are colonized by various bacterial species including beneficial, parasitic, and pathogenic bacteria such as *Arthrobacter*, *Bacillus*, *Enterobacter*, *Mycobacterium*, *Pseudomonas*, *Stenotrophomonas*, and *Staphylococcus* (Sessitsch et al., 2004; Egamberdiyeva, 2005; Egamberdieva, 2012; Egamberdieva et al., 2008). Plants are also attractive host for opportunistic human pathogenic bacteria (Berg et al., 2010). We showed here that the opportunistic pathogen *M. mesophilicum* is able to colonize rhizosphere of cucumber, tomato and paprika grown in both normal and salinity conditions. Poonguzhali et al. (2008) reported that *Methylobacterium suomiense* CBMB120 was able to colonize in the rhizoplane and in the phylloplane of tomato. In other report *Methylobacterium* sp. strain NPFM-SB3, isolated from *Sesbania rostrata* stem nodules was able to colonize in the rhizosphere of rice (Senthilkumar et al., 2009).

We have also determined salt stress tolerance of *M. mesophilicum* and its survival after inoculation onto plants. The

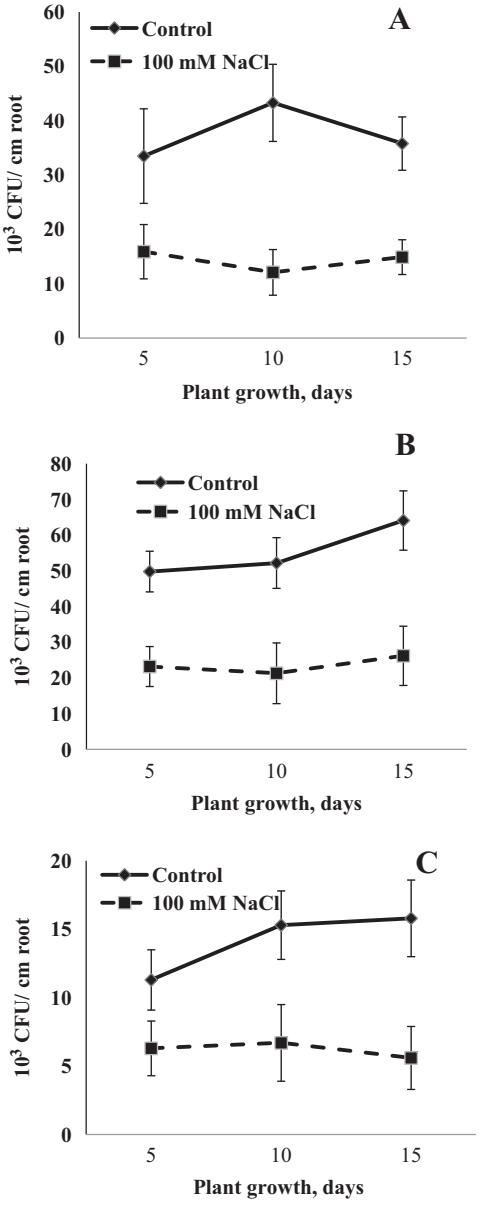

Figure 3 (A–C) The root tip colonization ability of *Methylobacterium mesophilicum* in a gnotobiotic sand system under normal and salt stress condition (plants were grown for 15 days under gnotobiotic sand system, (A) cucumber; (B) tomato; (C) paprika, error bars show SD from the mean).

strains *M. mesophilicum* demonstrated a high tolerance to salt stress up to 6%, and survive in soil, root and leaves regardless of the plant type. The rhizosphere of tomato and cucumber was more colonized by introduced strain compared to paprika grown in saline soil. The stress tolerance of bacterial strains has been shown to play an important role in adaptation of strains to the ecological stressed factors (McArthur and Tuckfield, 2000; Kummerer, 2004). According to Bouma and Lenski (1988) bacterial resistances to antibiotics are one of the key factors regulating tolerance to harsh environmental conditions. The plant roots, which are rich in nutrients also attract pathogens (Roberts et al., 2000; Ji and Wilson, 2002), where they may enrich and compete with the indigenous microflora for available carbon sources (Gilbert et al., 1993; Jablasone et al., 2005). We found that *M. mesophilicum* strain utilizes various carbon sources such as glucose, sucrose, xylose, arabinose, mannitol, glycerol, and methanol. The strain showed multidrug resistance to Gentamicin, Ampicillin, Amoxicillin plus Clavulanic acid, Cefotaxime, Neomycin, Penicillin. The persistence of antibiotic resistance bacteria in ground water, soil, and plants is a growing public health concern, because of possible further increase in their incidence rate into the environments (McKeon et al., 1995).

Table 1 Colonization of *Methylobacterium mesophilicum* in the soil of the root zone, rhizosphere and phyllosphere of plants (pot experiments with salinated soil, values in 10^3 CFU/g soil, root, leaves and they represent the mean of six plants per treatment). Numbers in the same line marked with the same letter do not show significant differences ($P < 0.05$).

Plants	Soil	Rhizosphere	Phyllosphere
Cucumber	182.5 a	1460.0 b	48.0 a
Tomato	265.0 b	2130.0 c	110.0 b
Paprika	124.4 a	987.0 a	24.0 a

The opportunistic pathogenic bacteria can function also as a plant beneficial microbe, through their production of biological active compounds. *M. mesophilicum* was shown to produce IAA and several fungal cell wall degrading enzymes, which might explain the capacity of this strain to stimulate plant growth and protect them from various fungal pathogens. Some *Methylobacterium* isolates are able to synthesize plant growth regulators like zeatins, cytokinins and auxins (Ivanova et al., 2000,2001; Lidstrom and Chistoserdova, 2002).

5. Conclusion

This study provides background information on the behaviour of opportunistic pathogen *M. mesophilicum* on plants and their survival in harsh environmental conditions. The salt tolerance and antibiotic resistance abilities of the strain allow competing with the indigenous microbes and persisting in the rhizosphere and phyllosphere of plants.

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