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Sonal Kumar
Department of Plant Pathology, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

RKS Tiwari
Plant Pathology, BTC College of Agriculture and Research Station, Indira Gandhi Krishi Vishwavidyalaya, Bilaspur, Chhattisgarh, India

VK Nirmalkar
Plant Pathology, BTC College of Agriculture and Research Station, Indira Gandhi Krishi Vishwavidyalaya, Bilaspur, Chhattisgarh, India

PK Keshri
Soil Science, BTC College of Agriculture and Research Station, Indira Gandhi Krishi Vishwavidyalaya, Bilaspur, Chhattisgarh, India

Jayant Sahu
Department of Plant Pathology, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

Corresponding Author:
Sonal Kumar
Department of Plant Pathology, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

Evaluation of novel bacterial isolates of Chhattisgarh for salinity tolerance

Sonal Kumar, RKS Tiwari, VK Nirmalkar, PK Keshri and Jayant Sahu

Abstract
Bacillus spp. is an important member of biological agent which generally used as bio control agents against number of foliar, seed borne and soil borne diseases of cereal, pulses and vegetables. Excess of salt in some parts of Indian soil creates problem of using bio agents due to detrimental effect of salt on bacterial growth and development. Application of bio agents in salinity affected area requires tolerance towards salinity. In present investigation different novel isolates of *Bacillus* spp. found effective against *Rhizoctonia solani* and *Sclerotium rolfsii in vitro* were tested for their tolerance towards different NaCl concentration (salinity level). Isolates were grown in different NaCl concentrations (salinity level) i.e. 5%, 10%, 15% and 20%. Growth and development of bacteria were recorded in terms of optical density (OD) and colony forming units (CFUs/ml). Higher growth of isolates at salinity level 5 per cent indicated the possibility of using such isolates in saline soil for plant growth promotion and suppression of diseases. Salt tolerance towards 20% NaCl concentration recorded (OD) in isolate *B. velezensis* (BI 36) (0.042*), B. subtilis* (BI 68) (0.042) and *B. subtilis* (BI 72) (0.042) followed by BI 44 (0.034) and *B. velezensis* (BI 43) (0.032). At 20% NaCl concentration, maximum CFUs were observed in isolate *B. velezensis* (BI 36) (5.8 × 10^6 CFUs/ml) followed by *B. subtilis* (BI 72) (5.7 × 10^6 CFUs/ml), *B. subtilis* (BI 68) (5.6 × 10^6 CFUs/ml) and *B. tequilensis* (BI 22) (4.8 × 10^6 CFUs/ml), which indicates salt tolerant characteristics of these isolates.

Keywords: *Bacillus* spp., bio agent, optical density, CFUs, salinity

Introduction
The genus *Bacillus* was classified in the order Eubacteriales and family Bacillaceae. *Bacillus* is a Gram positive, motile, aerobic, rod shaped. It is almost everywhere naturally occurring saprophytic bacterium commonly recovered from different sources i.e. soil, water, air and decomposing plant material. Characteristics *B. subtilis* colony is traditionally circular, wrinkled surface with ragged edges, coloured cream to white. *Bacillus* has an ability to form a tough protective specialized structure called endospore, which may be cylindrical, ellipsoidal or spherical and located centrally, subterminal or terminal allowing the organism to tolerate extreme environmental conditions (Alexander, 1977) [1]. Jadhav *et al*. (2010) [3] studied that salt tolerance of *Bacillus* isolates at 1-15% NaCl concentrations. The results indicated that *B. subtilis* were able to grow at 7% NaCl. Furthermore, *B. subtilis* subsp. *subtilis* NCIB 3610T and *B. sonorensis* NRRL B-23154T were able to grow at high salt concentrations of 10% (1.75M) and 15% (2.75M), respectively. Satapute *et al*. (2012) [10] studied salt tolerance of *B. subtilis* strain AS-4 on different concentration of NaCl at 10% and 15%. At 10% NaCl concentration optical density (660nm) 0.35, 0.55, 0.70, 0.70, 0.55 and 0.45 was observed at different time interval (5, 10, 15, 20, 25 and 30 hours). Furthermore, 0.08, 0.30, 0.40, 0.61, 0.70 and 0.70 optical density was reported at 15% NaCl on same time period. The strain were able grow in high NaCl concentration i.e. 10% and 15%, respectively. However, upward growth was observed during the first 25 hours of incubation and then growth decreased.

Materials and Methods
Different novel isolates of *Bacillus* spp. found effective against *Rhizoctonia solani* and *Sclerotium rolfsii* under *in vitro* conditions were tested for their salt tolerance. In this experiment the effect of salt on growth of bacterial isolates were studied *in vitro* with different NaCl concentration in nutrient broth medium.
Ten ml of nutrient broth medium amended with various concentrations of NaCl *viz.*, 5%, 10%, 15% and 20% were taken in 30 ml capacity of test tubes for each isolates and autoclaved. Initial population of all isolates was 1×10^8/ml was used in present study. Inoculated tube with

amended of different NaCl concentration were incubated at 28±1 °C for 96 hour and unamended NaCl tubes served as control. The optical density (OD600nm) (Kumar *et al*., 2019) [6, 7] was recorded using spectrophotometer. CFUs were also counted using serial dilution technique.

For enumeration 0.1 ml /100 µl diluted bacterial suspensions of 10^{-8} dilution was used by pour plate method using Kenknight and Munaeir's medium (Killani *et al*., 2011) [5]. Plates were used in triplicate and incubated at 28±1 °C. After 96 hours of incubation bacterial colonies were counted.

Table 1: Effect of different NaCl concentrations on optical density (600nm) of isolates of *Bacillus* spp.

Isolates	Optical density (600nm)				
	Control	5%	10%	15%	20%
B. subtilis (BI 28)	1.266	0.728	0.367	0.062	0.022
B. subtilis (BI 49)	1.076	0.733	0.321	0.052	0.021
B. subtilis (BI 68)	1.346	0.976	0.632	0.183	0.042
B. subtilis (BI 72)	1.224	0.758	0.532	0.062	0.042
B. tequilensis (BI 22)	1.318	0.826	0.554	0.088	0.029
B. tequilensis (BI 31)	1.131	0.927	0.408	0.072	0.028
B. tequilensis (BI 44)	0.938	0.620	0.432	0.052	0.034
B. tequilensis (BI 77)	1.080	0.923	0.664	0.143	0.025
B. velezensis (BI 35)	1.124	0.832	0.643	0.123	0.032
B. velezensis (BI 36)	1.105	0.831	0.623	0.137	0.042
B. velezensis (BI 43)	1.295	0.825	0.523	0.063	0.032
B. *velezensis* (BI 78)	1.011	0.800	0.754	0.153	0.026

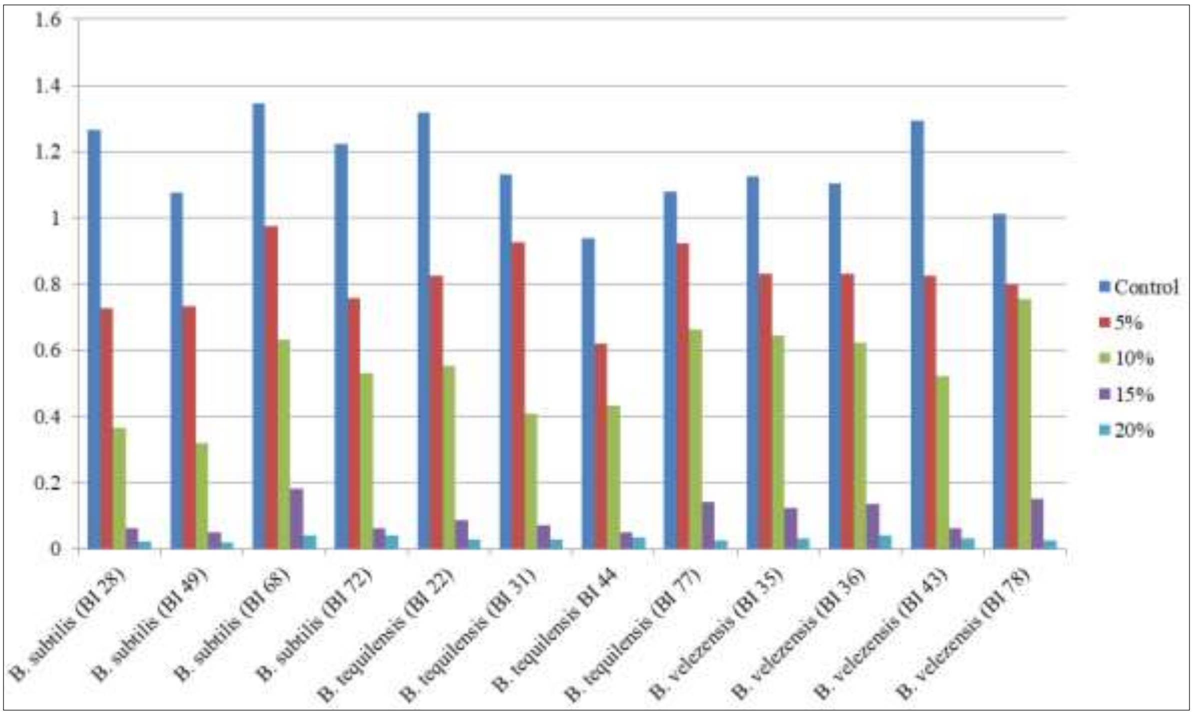

Fig 1: Effects of different NaCl concentrations on optical density (600nm) of isolates of *Bacillus* spp.

Table 2: Effect of different NaCl concentrations on colony forming units (CFUs) of isolates of *Bacillus* spp.

Isolates	Colony Forming Units (CFUs/ml)				
	Control	5%	10%	15%	20%
B. subtilis (BI 28)	4.23×10^{10}	3.20×10^{9}	2.03×10^{8}	1.46×10^{7}	4.6×10^{6}
B. subtilis (BI 49)	4.21×10^{9}	3.36×10^{9}	1.86×10^{8}	1.33×10^{7}	3.1×10^{6}
B. subtilis (BI 68)	5.52×10^{10}	5.66×10^{9}	3.53×10^{8}	2.78×10^{7}	5.7×10^{6}
B. subtilis (BI 72)	2.26×10^{10}	3.53×10^{9}	2.56×10^{8}	1.50×10^{7}	5.6×10^{6}
B. tequilensis (BI 22)	5.18×10^{10}	3.65×10^{9}	3.16×10^{8}	1.62×10^{7}	4.7×10^{6}
B. tequilensis (BI 31)	1.70×10^{10}	5.24×10^{9}	2.26×10^{8}	1.48×10^{7}	3.5×10^{6}
B. tequilensis (BI 44)	2.24×10^{9}	4.06×10^{8}	3.66×10^{8}	1.50×10^{7}	4.8×10^{6}
B. tequilensis (BI 77)	4.93×10^{9}	4.86×10^{9}	3.69×10^{8}	2.53×10^{7}	2.7×10^{6}
B. velezensis (BI 35)	1.60×10^{10}	4.06×10^{9}	3.46×10^{8}	2.46×10^{7}	5.2×10^{6}
B. velezensis (BI 36)	1.50×10^{10}	3.80×10^{9}	3.30×10^{8}	2.63×10^{7}	5.8×10^{6}
B. velezensis (BI 43)	4.66×10^{10}	4.60×10^{9}	2.16×10^{8}	1.54×10^{7}	3.3×10^{6}
B. velezensis (BI 78)	3.10×10^{9}	3.06×10^{8}	3.86×10^{8}	1.67×10^{7}	2.8×10^{6}

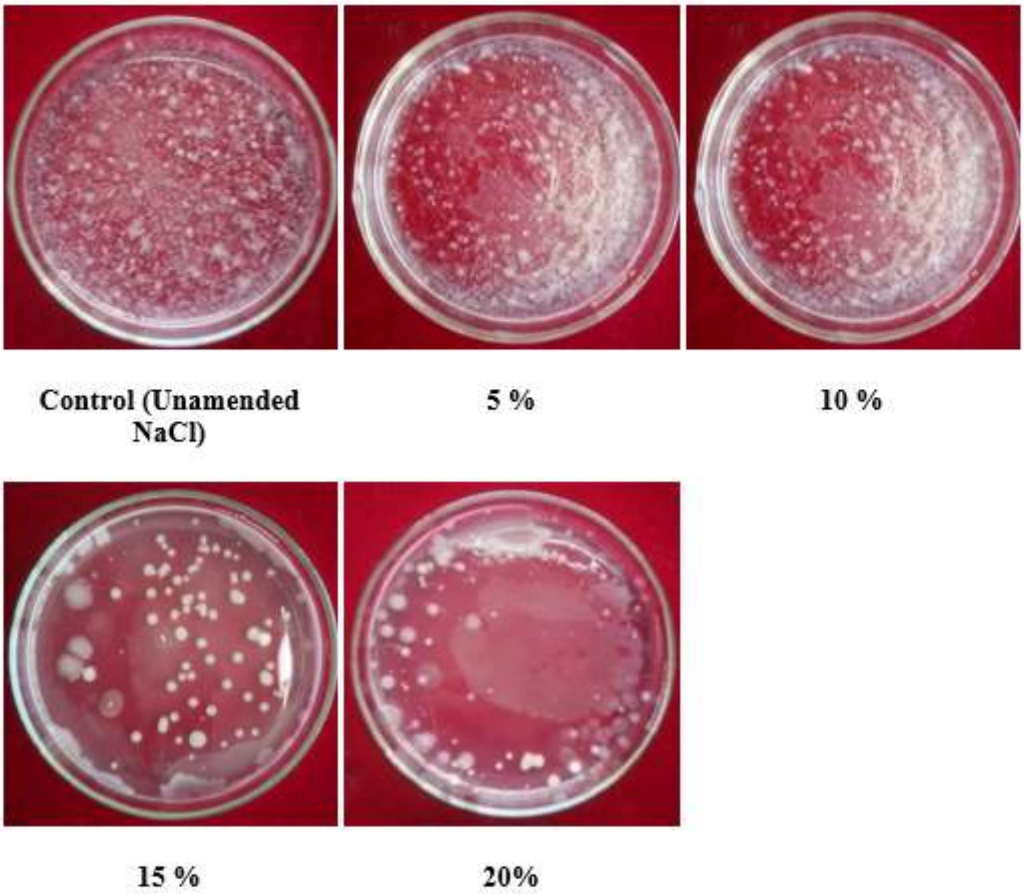

Fig 2: Effects of different concentration of NaCl on colony forming units (CFUs) of *Bacillus* spp.

Results and Discussion

The effects of different salt concentrations of NaCl (5%, 10%, 15% and 20%) and unamended NaCl (control) on the growth of isolates was studied and data are presented in the Tabular form. Data depicted in the Table 1, Fig. 1 revealed that the growth of different isolates at various NaCl concentrations. However, optical density of control (unamended NaCl) was higher than treatments amended with NaCl, which indicate that salinity decreases the growth of bacteria. There was negative trend between increase in salinity level and optical density of different isolates (OD- 0.620 to 0.973 at 5% NaCl, 0.321 to 0.754 at 10% NaCl, 0.052 to 0.153 at 15% NaCl, 0.021 to 0.042 at 20% NaCl). 15 and 20 per cent salinity were found detrimental for growth of most of isolates (OD - 0.021 to 0.183). Growths of some of the isolates were found satisfactory at 5 per cent salinity level. Isolates *B. subtilis* (BI 68) (0.976) was found to be least affected at salinity level 5 per cent followed by *B. tequilensis* (BI 31) (0.927), *B. tequilensis* (BI 44) (0.923) and *B. velezensis* (BI 35) (0.832). Higher growth of isolates at salinity level 5 per cent indicated the possibility of using such isolates in saline soil for plant growth promotion and suppression of diseases. At maximum salinity level i.e. 20% maximum OD recorded in isolate *B. velezensis* (BI 36) (0.042), *B. subtilis* (BI 68) (0.042) and *B. subtilis* (BI 72) (0.042) followed by BI 44 (0.034) and *B. velezensis* (BI 43) (0.032), whereas lowest were recorded in isolate in *B. subtilis* (BI 49) (0.021). Salinity tolerant isolate *B. velezensis* (BI 36) can be employed in an area where soil is affected by salt.

Amongst different strains of *B. tequilensis*, tolerance towards 20% NaCl concentration recorded in isolates BI 44 (0.034) followed by BI 22 (0.029), respectively. Whereas, among different strains of *B. subtilis* isolates, tolerance towards 20% NaCl concentration recorded in isolate BI 68 (0.042) and BI 72 (0.042), respectively. Whereas in *B. velezensis*, tolerance towards 20% NaCl concentration recorded in isolate BI 36 (0.042) and BI 35 (0.032). Jadhav *et al*. (2010) [3] reported that *B. subtilis* was found to be able to tolerate pH ranges from 5.0 to 10, while maximum growth at pH 7.0-7.5.

Effects of different salinity level on Colony Forming Units (CFUs)

Colony forming unites (CFUs/ml) were also recorded at different level of salinity and varied with different salinity level with lowest CFUs at maximum salinity level (20%). Data presented in the Table 2, Fig. 2 depicted that maximum CFUs recorded at lowest salinity (5% NaCl) being highest in *B. subtilis* (BI 68) (5.66×10^9 CFUs/ml), followed by *B. tequilensis* (BI 31) (5.24×10^9 CFUs/ml), *B. tequilensis* (BI 44) (4.86×10^9 CFUs/ml), *B. velezensis* (BI 43) (4.60×10^9 CFUs/ml) and *B. velezensis* (BI 35) (4.06×10^9 CFUs/ml). At 20% NaCl concentration, maximum CFUs were observed in isolate *B. velezensis* (BI 36) (5.8×10^6 CFUs/ml) followed by *B. subtilis* (BI 72) (5.7×10^6 CFUs/ml), *B. subtilis* (BI 68) (5.6×10^6 CFUs/ml) and *B. tequilensis* (BI 22) (4.8×10^6 CFUs/ml), which indicates salt tolerant characteristics of these isolates.

Same isolates were evaluated for plant growth promotion in chickpea by Sonal *et al*. (2023) [12] and they found enhaced growth parameters in chickpea So this findings indicate the possibility of using such isolates in saline soil for plant growth promotion and suppression of diseases in saline soil.

Amongst different strains of *B. tequilensis*, tolerance towards 20% NaCl concentration observed in isolates BI 44 (4.8×10^6 CFUs/ml) and BI 22 (4.7×10^6 CFUs/ml). Whereas, among the strains of *B. subtilis*, tolerance towards 20% NaCl concentration observed in isolate BI 68 (5.7×10^6 CFUs/ml) and BI 72 (5.6×10^6 CFUs/ml). However, among different strains of *B. velezensis*, tolerance towards 20% NaCl concentration observed in isolate BI 36 (5.8×10^6 CFUs/ml) followed by BI 35 (5.2×10^6 CFUs/ml).

These results were in support with the findings of Patel *et al*., (2017) [9] who reported that isolates of *Bacillus* spp. were able to grow in 15 g NaCl (w/v), whereas *Bacillus subtilis* S65 isolate were able to grow in the presence of 14 g NaCl (w/v). Jamali *et al*., (2019) [4] reported *Bacillus subtilis* strain RH5 tolerance to salt up to 12% of NaCl concentration. Another isolates i.e. *B. velezensis* CPA1–1, had multiple antibiotic-related genes and able to grow at broad range of salinity (5% - 60%) (Li *et al*. 2020) [8]. Damodaran *et al*. (2019) [2] reported that among the different strain of *Bacillus* spp. salt tolerance observed in *Bacillus tequilensis* at varying concentration of NaCl (0.5, 5.0, 7.5, 10.0%).

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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