ACC deaminase-producing plant growth-promoting bacteria protect *Vigna radiata* against salt and boron stress.

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Abstract
Environmental stresses, such as drought, flooding, salinization of soil with salt and heavy metals, are important factors that limit crop productivity. Soil salinization is a serious threat to plant growth and crop productivity. Boron toxicity poses an upcoming threat in agricultural zones where excessive irrigation water is used. Plant growth-promoting (PGP) bacteria are considered an inventive, practical, and environmentally beneficial way to counteract salt and boron stress. This study aimed to identify PGP bacteria (PGPB) capable of producing PGP substances under salt and boron stress. A total of 41 isolates were selected for PGP traits screening. Amongst 41 isolates, SON2-F, TKD-B6, and BVB-1 exhibited various plant growth-promoting activities, including aminocyclopropane-1-carboxylate deaminase (ACCD) activity. These three PGPBs were further screened for salt, boron, and thermotolerance. Under salt stress, PGPB were tolerant to 8% NaCl, and all three isolates tolerated boron concentrations up to 25%. Molecular identification revealed the following PGPB: SON2-F as *Citrobacter arsenatis,* TKD-B6 as *Enterobacter cloacae,* and BVB-1 as *Enterobacter roggenkampii.* The present study is the first to report *Citrobacter arsenatis* as a promising bioinoculant to enhance the plant growth in *Vigna radiata* in stressed soil due to salt and boron.

Keywords: Plant growth-promoting bacteria (PGPB), aminocyclopropane-1- carboxylate deaminase (ACCD), *Citrobacter arsenatis,* saline stress, boron toxicity.

1. **INTRODUCTION:** Land resource management is critical because it supports all life forms on the planet. Humans' basic needs for food, clothing, and places to live are met by the agricultural industry. Fertile fields are necessary components of successful agricultural operations. Many countries worldwide are currently experiencing land degradation in various forms and degrees due to the increased demand for food, fodder, and fuel, as well as heavy industrial activity. According to the Food and Agriculture Organization of the United Nations (FAO), by 2050, worldwide agricultural production will have to be quadrupled to meet global human population needs (Izzeddine Zakarya Zerrouk, 2019). Competing demands have resulted in the severe depletion of natural resources, leading to a short supply of agricultural land. Desertification, soil erosion, waterlogging, and salinization are all causes of land degradation. Individually, land degradation due to salinization has affected the productivity of approximately 1000 million hectares (M-ha) of agricultural land. In India, salt-affected soils

cover 6.73 million hectares, with 56 percent sodic and 44 percent saline land (DK Sharma, 2016). According to estimates, abiotic stress due to salinity is expected to damage 30% of the crop yield worldwide by 2025 (Sankalp Misra, 2017). Intensive agriculture, owing to excessive water consumption and indiscriminate use of chemical fertilizers, has resulted in the conversion of productive fields to barren lands around the world, including India.

One of the main reasons for the change from productive lands to infertile lands is the excessive accumulation of salts in the soil. Nitrogen (N), phosphorus (P), and potassium (K) deficiencies are common in salt-affected soils (Anshu Kumar, 2020). However, the high pH of sodic soils reduces the bioavailability of micronutrients, including Fe, Al, Zn, Mn, and Cu. Plants' ability to survive is harmed by high ion concentrations, particularly Na^+ and Cl^-, which reduce the ability of plants to absorb water, negatively impacting plant cells and growth (Sevda Amini, 2016). Boron (B) released into the environment through both natural and anthropogenic sources poses additional stress on plants. Mining waste, waste from processing industries, B fertilizer application, and the use of wastewater for irrigation are examples of human-caused sources of B released into the environment (Shiv Bolan H. W., 2023). For plants to survive, boron is a necessary element that is mostly absorbed in the form of boric acid. The range for boron deficiency and toxicity is very narrow, and agricultural plant yield is mostly negatively affected by low boron availability, which can be addressed by the use of B fertilizers. However, B toxicity is quite critical, where numerous methods can be used to mitigate soil boron toxicity; however, these methods are expensive, time-consuming, and frequently have only temporary results. (Brdar-Jokanovic, 2020). Although there is evidence of B insufficiency in agricultural soils, B toxicity can prevent plant growth in soils found in dry and semiarid locations (Shiv Bolan H. W.-J., 2023). Microbes producing ACCD enzyme can offer variable degrees of resistance and tolerance in their host plants to various biotic and abiotic stresses. As a result, the bacteria are capable of stimulating plant growth in hostile environments. Scientific research has demonstrated the ability of PGPB with high ACCD activity to colonize, protect and boost crop productivity in the presence of a variety of stressors. Therefore, studies on PGPB with high ACCD activity that can colonize plants and boost their productivity in real-world soil conditions are required. ACC deaminase characteristics and regulatory information currently available are primarily for enzymes isolated from Pseudomonas sources (Rajnish P. Singh, 2015). Alternative resources must be investigated to learn to better understand this enzyme and its effective application in the field. Traditional breeding has resulted in the development of salt-resistant plants. Modern genetic engineering has shown promise, but its acceptability and long-term viability remain unclear (Glick V. P., 2001). The actions of plant growth-promoting bacteria (PGPB) include restoring hormonal and nutritional balance, solubilizing nutrients such as phosphorus, zinc and potassium, producing phytohormones such as indole-3-acetic acid and 1-aminocyclopropane-

1-carboxylate deaminase (ACCD), and protecting against phytopathogens by synthesizing siderophore and hydrogen cyanide (Ji, et al., 2020). Through enhanced nutrient uptake, improved hydration, improved root and shoot growth, and increased chlorophyll content, PGPB can enhance the resistance of plants to a range of illnesses. Mung beans, also referred to as green grams (*Vigna radiata L.*), are an important pulse crop in India. It is an ancient and well-known leguminous crop in Asia (K.Geetha, 2014). Globally, the growth and productivity of *Vigna radiata* (mung bean) is drastically affected due to salinity, where more than 60 % of the yield loss of mung bean is reported even with a 50 mM concentration of NaCl (Shreya Deasi, 2022). Boron individually affects germination, root growth, and chlorophyll content of the Mung beans drastically when the concentration is beyond 10 ppm. Mung beans are less tolerant to B toxicity (Ammarah Hasnain, 2011). To the best of our knowledge, studies investigating ACCD possessing PGPR protecting against the combined stress of saline and boron with green gram have not yet been reported.

The current study was conducted to identify microorganisms that have ACCD activity to promote plant growth under salt and boron stress. Three agroclimatic zones in Maharashtra were selected for the collection of soil samples that may harbor diverse microflora that are likely to promote plant growth under salt and boron stress.

2. MATERIALS AND METHODS

2.1 Soil Sampling and Isolation

Soil samples from salty lands located in the Kolhapur, Pune, and North Konkan agroclimatic zones of Maharashtra were collected in sterile zip-lock bags, sealed, and brought to the laboratory. Samples collected were rhizospheric and bulk soils from 10-15 cm depth.
Soil samples were diluted to 10^{-3} in sterile saline and inoculated in sterile media supplemented with 200 mM NaCl and 20 mM H_3BO_3. Nutrient broth, Norris nitrogen-free broth, Ashby's broth, and Soil extract broth were used. The inoculated flasks were incubated at 30 °C and 120 rpm until growth occurred. A loopful of enriched broth was streaked on sterile solid medium plates containing 200 mM NaCl and 20 mM H_3BO_3. Isolates were selected based on visible differences in colony characteristics. The selected isolates were preserved on Nutrient agar slants and stored at 4 °C.

2.2 Phenotypic characterization of the isolates

The phenotypic characteristics of the isolates were determined using routine microbiological methods, such as Gram staining and catalase and oxidase tests.

2.3 Primary screening of isolates for IAA production and ACC deaminase activity. Plant growth-promoting (PGP) activities of isolates were determined using the respective media in the presence of salt and boron (80 mM of NaCl + 8 mM H_3BO_3).

2.3.1 Indole 3-Acetic acid (IAA) production:

The cultures were inoculated in YMD broth and incubated for 48 h. The colorimetric assay, which uses Salkowski reagent (0.5M ferric chloride in sulfuric acid), was used to screen the isolates' ability to produce IAA. Following incubation, the cultures were centrifuged at 8,000 rpm for 5 minutes. One millilitre of the clear supernatant and one millilitre of Salkowski's reagent were added. The intensity of the pink color developed was measured at 530 nm, and the unknown concentration was determined using a standard graph of IAA with known concentrations ranging from 1 to 10 µg/ml. (Federica Brunoni, 2019).

2.3.2 ACC deaminase production: Utilizing modified techniques from Penrose and Glick (2003) and Honma and Shimomura (1978), isolates were screened for ACC deaminase production. This technique measures the quantity of α-keto-butyrate generated as a result of ACC hydrolysis by the enzyme ACC deaminase.

2.4 Sequencing of the 16S ribosomal RNA gene for identification

Isolates showing the highest IAA and ACC deaminase activities were selected for identification. The National Centre for Microbial Resource (NCMR), Pune (India), sequencing facility was used to identify the isolates. The standard phenol/chloroform extraction procedure (Sambrook J, 1989) was used to isolate genomic DNA, and the 16S rRNA was amplified by PCR using universal primers 16F27 and 16R1492. The amplified 16S rRNA gene PCR product was purified using PEG-NaCl precipitation and then sequenced on an ABI 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Additionally, internal primers were used to perform sequencing from both ends, ensuring that each site was read at least twice. The Lasergene package was used for assembly, and the NCBI database was used for identification.

2.5 Secondary screening and characterization of isolates for PGP activities.

2.5.1 Nitrogen (N_2) fixation:

Screening of selected 3 isolates for N_2-fixing activity was performed using Ashby's mannitol nitrogen-free agar medium and Norris glucose nitrogen-free agar medium. After spot inoculation of bacterial suspension, plates were incubated for 24-48 h at 28 °C. The visible growth of isolates at the spot of inoculation was considered to be positive for N_2 fixing activity.

2.5.2 Phosphate solubilization

Selected isolates were screened for their ability to solubilize phosphate on Pikovskaya agar plates. Five microliters of each culture suspension were spot inoculated onto Pikovskaya agar plates (Akhilesh Kumar, 2020). Incubated for 24-48 hours at 28 °C. After incubation, a clear zone around

the colony indicated phosphate solubilization. The solubilization index was calculated using Equation 1 (Joseph Ezra John, 2023).

$$\text{Solubilization index} = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}} \quad \ldots..(1)$$

2.5.3 Potash solubilization

Potash solubilization ability was screened using Aleksandrow medium plates. Five microliters of the culture suspension were spot-inoculated on Aleksandrow agar plates. Incubated for 24-48 h at 28 °C. After incubation, a clear zone around the colony indicated phosphate solubilization. (Mahendra Vikram Singh RAJAWAT, 2016).

2.5.4 Zinc solubilization

Zinc solubilization activity was detected using mineral salt medium plates containing zinc oxide (Praveen Kumar Goteti, 2013). Five microliters of the culture suspension were spot inoculated onto zinc oxide medium plates in marked sectors. Incubated for 24-48 hours at 28 °C (Praveen Kumar Goteti, 2013). After incubation, a clear zone around the colony indicated phosphate solubilization.

2.5.5 Siderophore production:

Isolates were tested for siderophore synthesis on the Chrome-azurol S agar medium. Equivalent sectors of Chrome Azur S agar plates were prepared, streaked with isolates, and incubated for 24 - 48 h at 28 °C (Praveen Kumar Goteti, 2013). The formation of orange halos surrounding the colonies was identified as an indication of siderophore-producing capability.

2.5.6 Chitinase production:

Colloidal chitin agar plates were used to determine chitinase production (Shahla Pashapour, 2016). Spot inoculation of bacterial suspension was performed on colloidal chitin agar plates containing 1% colloidal chitin, then incubated for 24-48 h at 28 °C. Post-incubation zone of hydrolysis around the colonies was recorded by the addition of Gram's iodine to the plates.

2.6 Determination of stress tolerance of selected isolates:

2.6.1 Screening of isolates for salt tolerance:

Initial screening of isolates for salt tolerance was performed by observing their growth in Luria Bertani broth supplemented with 5,10,15,20, and 30 % concentrations of NaCl. Incubation was done for 24-48 h at 28 °C. Further screening of isolates for salt tolerance was done by using Luria Bertani broth supplemented with various concentrations of NaCl (6,7,8,9, and 10%). Visible growth in the form of turbidity in the broth was recorded as positive for salt tolerance. (Rajnish P. Singh, 2015)

2.6.2 Screening of isolates for Boron tolerance:

Initial screening of isolates for boron tolerance was performed by using Luria-Bertani broth supplemented with various concentrations of H_3BO_3 (25,50,100,200,300, 400, and 500 mM). Further screening of isolates for Boron tolerance was performed by observing their growth in Luria-Bertani broth supplemented with various concentrations of H_3BO_3 (25,50, and 75 mM). Visible growth in the form of turbidity in the broth was recorded as positive for Boron tolerance (Fujiwara, 2010).

2.6.3 Screening of isolates for temperature tolerance:

Isolates were screened for temperature tolerance by observing their growth in Luria-Bertani broth incubated at different temperatures (35,40,42.5, and 45 °C). Visible growth in the form of turbidity in the broth was recorded as positive for temperature tolerance.

2.7 Gnotobiotic assay with Green gram (*Vigna radiata*) seeds:

2.7.1 Seed germination assay with *Vigna radiata* (Mung Sindhu NVL 605) without stress:

The selected seeds were first surface sterilized with 1% NaOCl for 90 s, then in 70% ethanol for 30 s, followed by rinsing in sterile distilled water twice, and finally air dried in a laminar air flow environment. (Kay Thi Oo, 2020). Bacterial cells were extracted from a culture that had been incubated for 24 h, and the resulting bacterial inoculum was diluted with sterile distilled water to achieve 0.05 McFarland's standard absorbance at 580 nm. After two hours of soaking in a bacterial suspension, the seeds were coated with the culture. Three replicates of the experiment were conducted, and the outcomes were compared with those of control seedlings that received water treatment rather than a bacterial isolate. A minimum of 45 seeds, treated and untreated with bacteria, were placed in a Petri dish containing sterile 0.8 % agar prepared in Hoagland's solution (Gianmaria Oliva, 2023)and were incubated for 8 days under light and dark conditions. The percentage germination (PG) of the seeds was calculated on day 4. The seedlings' root and shoot lengths (RL, SL) were recorded to calculate the vigor index (VI). Using the formula VI = (RL + SL) × PG for each seedling, VI was calculated (Aref A. Abdul-Baki, 1973).

2.7.2 Seed germination assay with *Vigna radiata* with salt and boron stress:

The initial process was similar to the previous assay, but bacteria-treated and untreated seeds were placed per Petri dish containing sterile 0.8 % agar prepared in Hoagland's solution amended with 40 mM NaCl and 7.5 mM H_3BO_3. All plates were incubated for eight days under light and dark conditions, and growth parameters were recorded as per the previous assay.

2.8 Statistical Analysis:

Every seed germination experiment was performed in duplicate with appropriate controls. Minitab Software Ver. 17 was used to statistically analyze the collected data. Tukey Pairwise Comparisons at the $p<0.05$ level of significance were used to compare the means using the one-way ANOVA test.

3. RESULTS AND DISCUSSION

3.1 Sample Collection and Isolation:

Arid and Semi-arid zone agriculture is massively irrigated with contaminated water, making the soil saline to sodic and leading to infertility. Saline soil is often associated with excessive Boron contamination, which negatively affects crop production (Deshmukh, 2015). In this study, three agro-climatic zones (Koli V. P., 2013) Concerning the salinity problem were selected for sampling. Rhizospheric and bulk soils from the salty lands of three agroclimatic zones of Maharashtra were collected from Kolhapur, Konkan, and Baramati. These zones are selected because reports are available that show the presence of salt-affected lands in those areas (S.S.P., 2013) (K.D. Patil, 2016) . Soil samples from these areas may give us indigenous phyto-beneficial bacteria for native crops that may not benefit from other well-established PGPB (Sankalp Misra, 2017). A total of 41 bacterial morphotypes were isolated and screened on different media supplemented with salt and boron.

3.2 Phenotypic characterization of the isolates:

The isolates exhibited a range of cell morphologies, from small to medium to long rods. Eleven out of 41 isolates were Gram-positive. Furthermore, all isolates tested positive for catalase because this enzyme hydrolyses hydrogen peroxide, which is a known reactive oxygen species and protects cells from oxidative damage.

3.3 Primary screening of isolates for IAA production and ACCD activity.

Data related to plant growth-promoting traits are shown in Table 1.

3.3.1 Indole 3-Acetic acid (IAA) production:

A delicate equilibrium of the levels of this hormone must always be maintained for plants to flourish. Every plant has an internal IAA pool that is generated, separate from the production of soil bacteria. Screening for IAA production is primary because this trait has greater potential for PGP and colonization properties (Hassan Etesami, 2015)**.** According to previous reports, IAA and ACC deaminase probably cooperate to promote plant growth, particularly root elongation. (Bernard R. Glick, 2007). Consequently, it was determined that rhizobacteria's IAA production characteristics and ACCD activity were essential for stimulating plant growth (Glick B. R., 2014). A total of 21 out of 41 isolates were found to produce IAA, with BVB-1 at the highest, followed by TKD-B6. The amounts of IAA produced by these isolates were 16 µg/ml and 10 µg/ml, respectively (Table 1).

3.3.2 ACC deaminase production:

In higher plants, ACC is the immediate precursor of ethylene production as a hormone; however, under various biotic and abiotic stresses, ethylene is a stress hormone causing deleterious effects on plants. One of the major mechanisms by which PGPB helps plants resist environmental stresses is the production of ACC deaminase enzyme, which hydrolyzes ACC to α-keto-butyrate and ammonia (Glick B. R., 2014). Ten out of 21 IAA-producing isolates were found to be ACC deaminase positive.

Based on the quantitative production of IAA ability, three isolates were selected for further identification, PGP characterization, and seed germination assays.

3.4 Sequencing of 16S ribosomal RNA gene for Molecular identification:

The BLAST analysis in the NCBI database showed that the 16S ribosomal RNA genomic sequence of the SON2-F, TKD-B6, and BVB-1 were 99.52% and 99.36% 99.93 % related to *Citrobacter arsenatis* (Acc. No. PQ097197) and *Enterobacter cloacae* (Acc. No. PQ097301), and *Enterobacter roggenkampii* (Acc. No. PQ097306), respectively. However, many studies also reported on the genera within the family Enterobacteriaceae as members of PGPB, including *Citrobacter*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Kluyvera*, *Pantoea*, and *Serratia* (Chaitanya Kumar Jha, 2011). *Enterobacter cloacae* is a bacterium that has demonstrated promise in the production of hormones that promote plant development and numerous molecular-level bioactive compounds (Xiaobo Wang Z. W., 2023). Many reports are available on *Enterobacter cloacae* as a PGP bacterium. Genetic studies on *Enterobacter cloacae* exhibit multiple plant growth-promoting traits under abiotic stress conditions as an endophyte (Pavithra Ramakrishnan, 2023) (Xiaobo Wang Z. W., 2023).In case of the Citrobacter genus, many strains of *Citrobacter freundii and Citrobacter braakii* are reported for plant growth-promoting activities (S Denaya, 2021) (Ayomide Emmanuel Fadiji, 2023). However, there are no reports on *Citrobacter arsenatis* as a PGPB. *Citrobacter arsenatis* was first reported as a new species in 2021 and was isolated from freshwater sediment in China (Hanlin Wang, 2021). To the best of our knowledge, its plant growth-promoting activity has not yet been reported. *Enterobacter roggenkampii* is a reported plant growth-promoting endophyte. The molecular basis of proteomic and genomic approaches to *Enterobacter roggenkampii* has been reported for its potential to promote plant growth under biotic and abiotic stresses (Dao Jun Guo R. K.-P., 2020) (Dao Jun Guo D.-P. L., 2021). The phylogenetic relation of the isolates is shown in Figs. 1, 2, and 3.

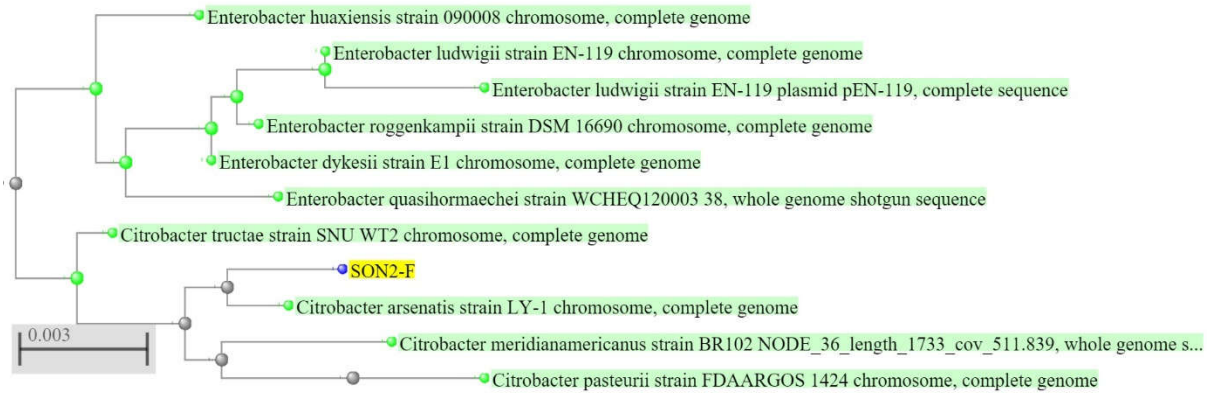


Fig. -1: Phylogenetic tree of SON2-F

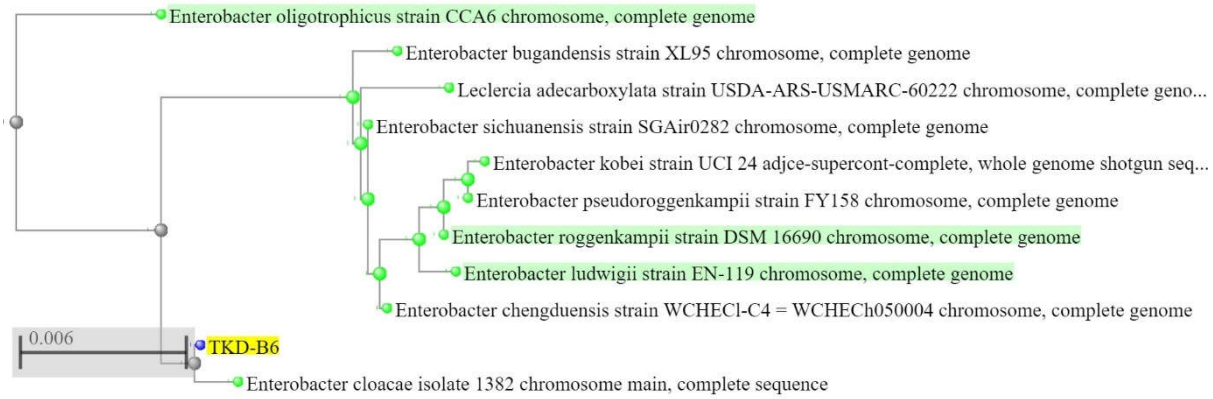


Fig. -2: Phylogenetic tree of TKD-B6

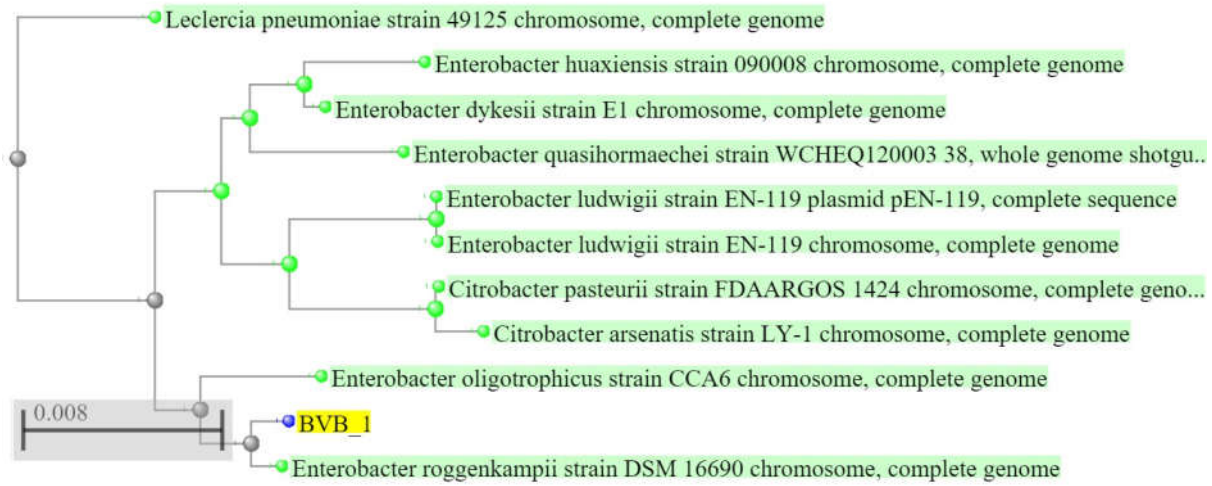


Fig. -3: Phylogenetic tree of BVB-1

3.5 Secondary screening and characterization of isolates for plant growth-promoting activities.

3.5.1 Nitrogen (N_2) fixation:

Three selected isolates could fix nitrogen on both media, i.e., Ashby's N_2-free as and Norris N_2-free medium. Nitrogen-fixing *Enterobacter spp*. have been isolated from various plants. Biological N_2 fixation (BNF) provides nitrogen, the nutrient that limits plant growth the most, and is a crucial characteristic of relevant Enterobacter spp. that function as plant growth enhancers. One strain of *Enterobacter roggenkampii* is reported to enhance sugarcane production in soils having low Nitrogen content (Guo D-J, 2023).

3.5.2 Phosphate-solubilizing ability:

Phosphate-solubilizing ability was detected in all three isolates. The phosphate solubilization indices of *Citrobacter arsenatis, Enterobacter cloacae,* and *Enterobacter roggenkampii* were 1.6, 1.7, and 1.75, respectively (Table 1), showing similar activity. As Enterobacter spp. can solubilize inorganic phosphate, they promote plant development. This capacity is most likely caused by the release of phosphatases and organic acids, which promote the solubilization of inorganic phosphates and make it

easier for plant roots to absorb phosphorus, thereby promoting plant growth and production. Effective P solubilizers include the majority of the members of the Enterobacteriaceae family. (Bhagya Iyer, 2017).

3.5.3 Potash solubilizing ability:

The third main macronutrient vital for plant growth is potassium (K). The majority of the potassium in the soil is found in the form of minerals such as silicates and insoluble rocks. Soluble potassium concentrations in the soil are typically quite low because insoluble in K-bearing minerals (Priyanka Parmar, 2013). The potash-solubilizing ability was found to be highest in *Enterobacter roggenkampii* with a hydrolysis index of 3 (Table 1).

3.5.4 Zinc solubilizing ability:

Zinc (Zn) is an important component of healthy plant growth. When inorganic zinc (Zn) is added to the soil, a significant amount changes into an insoluble form. Zinc-solubilizing bacteria (ZSB) are intriguing substitutes for Zn supplementation because they can convert insoluble Zn into forms that are available to plants (Murad Ali, 2023). Among the three isolates, only *Enterobacter roggenkampii* was able to solubilize Zn.

3.5.5 Siderophore production:

Stomatal conductance, transpiration rates, photosynthesis, and vegetative growth of plants are negatively affected by salt stress, which also contributes to iron shortage. Salinity-tolerant siderophore-producing PGPB may be a useful non-transgenic strategy for recovering salinity-affected soils for farming (Anjney Sharma, 2023). Although all three isolates under study can produce siderophores, the present study is the first report of *Citrobacter arsenatis* for siderophore production, which helps the process of ferric iron uptake from the environment to sustain crops under salt stress.

3.5.6 Chitinase production:

The second most common natural polymer, chitin, is a component of the fungal cell wall that is broken down by endo- and exo-chitinases. For chitinase production, the bacterial genera most well-known for this trait were *Aeromonas, Bacillus, Serratia, Streptomyces, and Vibrio.* (Bhagya Iyer, 2017). Among the three isolates, only *Enterobacter cloacae* showed chitinase production that may offer protection to the plants from fungal pathogens or pests and serve as biocontrol agents.

3.6 Screening of isolates for stress tolerance:

3.6.1 Screening of isolates for salt tolerance:

The three isolates were not able to grow above 8% NaCl concentration, suggesting that these isolates have a mild halophilic nature. Most of the crops cannot tolerate salt stress above 2% of NaCl concentration in the soil (Sowmyalakshmi Subramanian, 2016). The selected isolates may play an important role in mitigating salt stress on crops. Data related to the screening of isolates for Salt Tolerance are shown in Table 2.

3.6.2 Screening of isolates for Boron tolerance:

The selected isolates were able to grow up to 75 mM concentration of H_3BO_3. Although the highest concentration tolerated by any terrestrial plant is 25 mM H_3BO_3 (Rajendra Prasad, 2014). Therefore, the isolates showed promising potential to function under higher levels of boron stress. The data related to the screening of isolates for boron tolerance are shown in Table 3.

3.6.3 Screening of isolates for temperature tolerance:

The selected isolates were able to grow up to 40 °C except for *Enterobacter roggenkampii,* which could grow up to 35 °C; therefore, the selected isolates can survive in soil as bioinoculants for plants under moderate temperature stress. The data for thermotolerance of isolates is shown in Table 4.

Table 1: Plant growth-promoting traits screening results

Sr. no.	Isolate	IAA production (ug/ml)	ACCD production	Phosphate solubilization	N_2 Fixation (Norris Medium)	N_2 Fixation (Ashbys Medium)	Potash solubilization	Zinc solubilization	Siderophore production	Chitinase production
1	*Citrobacter arsenatis* (SON2-F)	**5**	+	+ (1.6)*	+	+	+(2.0)*	-	+	-
2	*Enterobacter cloacae* (TKD-B6)	10	+	+ (1.7)*	+	+	+(1.66)*	-	+	+(1.83)*
3	*Enterobacter roggenkampii* (BVB-1)	16	+	+ (1.75)*	+	+	+(3.0)*	+ (3.5)*	+	-

**Values in bracket indicates solubilization index, positive (+), negative (-)*

Table 2: Screening of isolates for salt tolerance

Sr. no.	Treatment	NaCl concentration (%)					
	Isolate	**5**	**6**	**7**	**8**	**9**	**10**
1	*Citrobacter arsenatis* (SON2-F)	+	+	+	+	-	-
2	*Enterobacter cloacae* (TKD-B6)	+	+	+	+	-	-
3	*Enterobacter roggenkampii* (BVB-1)	+	+	+	+	-	-

Growth (+), No Growth (-)

Table 3: Screening of isolates for Boron tolerance

Sr. no.	Treatment	H3BO3 concentration (mM)					
	Isolate	25	50	75	100	200	300
1	*Citrobacter arsenatis* (SON2-F)	+	+	+	-	-	-
2	*Enterobacter cloacae* (TKD-B6)	+	+	+	-	-	-
3	*Enterobacter roggenkampii* (BVB-1)	+	+	+	-	-	-

Growth (+), No Growth (-)

Table 4: Screening of isolates for temperature tolerance

Sr. no.	Treatment	Temperature (degrees Celsius)			
	Isolate	35	40	42.5	45
1	*Citrobacter arsenatis* (SON2-F)	+	+	-	-
2	*Enterobacter cloacae* (TKD-B6)	+	+	-	-
3	*Enterobacter roggenkampii* (BVB-1)	+	-	-	-

Growth (+), No Growth (-)

3.7 Gnotobiotic assay with *Vigna radiata* seeds:

Three isolates were identified as multifarious PGPB and screened on a growth promotion basis by bioassay, *Citrobacter arsenatis*, *Enterobacter cloacae,* and *Enterobacter roggenkampii*. These three isolates were tested in a laboratory with highly inhibiting conditions of salt and boron (40mM NaCl + 7.5mM H_3BO_3) for *Vigna radiata* (Mung Sindhu NVL 605). Although endophytic PGPB have been reported based on genomic and proteomic studies, no reports have been found about seed germination trials on *Enterobacter roggenkampii.* Only *Enterobacter cloacae* has been reported for its PGP activities in wheat, Chickpea, Rice, etc. (Rajnish P. Singh, 2015) (Bhagya Iyer, 2017) (Swati Pattnaik, 2020).

3.7.1 Seed germination assay with *Vigna radiata* without stress:

The selected 3 isolates were tested individually to promote plant growth during *Vigna radiata* germination under normal salt and boron conditions. Total seed germination, highest lateral root formation, and Vigor index of green gram were observed in all three isolates, but significantly with *Enterobacter cloacae* compared with the control. In terms of average root length, *all three isolates* were found to be the best for green gram, which had the highest average with *Enterobacter cloacae*.

Shoot length was high in green gram with *Enterobacter cloacae* and *Enterobacter roggenkampii,* but it was not statistically significant when compared with the control. All three isolates significantly increased the vigor index of green gram, showing the highest with *Enterobacter quasirogenkampii.* Data on all the growth parameters measured are shown in Table 5. The growth comparison among the treatments is shown in Figs. 4 and 5.

Table 5: Effects of isolates on green Gram seed germination without the stress of salt and boron.

Parameters/ Treatments	Lateral Root Number	Root mean length (cm)	Shoot Mean length (cm)	Vigor Index (cm.%)	Percent germination (%)
Control	12.72^{c} (±3.74)	4.773^{b}(±1.32)	17.75^{b}(±4.04)	2252.3^{b} (±469.6)	100
***Citrobacter arsenatis* (SON2-F)**	18.056^{b} (±4.13)	7.859^{a} (±1.54)	17.059^{b} (±2.90)	2491.8^{a} (±396.5)	100
***Enterobacter cloacae* (TKD-B6)**	**20.489^{a} (±4.59)**	**8.069^{a} (±1.73)**	**18.262^{a} (±2.15)**	**2633.1^{a} (±309.2)**	100
***Enterobacter roggenkampii* (BVB-1)**	17.211^{b} (±4.14)	7.52^{a} (±1.62)	17.99^{b} (±1.93)	2551^{a} (±294)	100

Standard errors are indicated by values in brackets, and the parameters were recorded eight days after treatment.
Tukey's test indicates that values superscribed with the same letter do not significantly differ ($p < 0.05$).

3.7.2 Effect of PGPB isolates on germination of *Vigna radiata* seeds under stressed conditions of salt and boron (40 mM NaCl and 7.5 mM H_3BO_3)

The experimental outcomes also showed that even under the stress of extreme salt and boron, the growth of the test plants greatly improved upon inoculation with the selected isolate (Table 6). The growth comparison among the treatments is shown in Fig. 6. No significant effect was observed on lateral root formation. Boron and salt stress may have severely affected the formation of lateral roots. Although the germination percentage was low compared to the control, significant differences in the mean root and shoot length, as well as the vigor index of *Vigna radiata* seeds, were noted only with *Citrobacter arsenatis*. Among the treatments, *Citrobacter arsenatis* showed the highest root length, shoot length, and vigor index. The lengths of uninoculated plant shoots and roots drastically decreased during salt stress, yet they significantly increased when treated with *Citrobacter arsenatis*. The reason for the longer roots of plants is probably that these inoculants made higher auxin concentrations available as IAA. Plants have been reported to be protected against stresses such as chromium stress when siderophores are produced in association with IAA. (Mani Rajkumar, 2005)

The B concentration in the shoots of mustard (*Brassica campestris*) increased as the Zn application increased, indicating a complex interaction between B and Zn. According to one report, zinc application causes a drop in shoot B content in 25 distinct wheat cultivars; however, each cultivar exhibits a varied reaction (Muhammad Nasim, 2015).

Table 6: Effects of isolates on *Vigna radiata* seed germination under stress of salt (40 mM) and Boron (7.5 mM).

Parameters/ Treatments	Lateral Root Number	Root mean length (cm)	Shoot Mean length (cm)	Vigor Index (cm.%)	Percent germination (%)
Control	0^{a}(±0.00)	1.755^{b} (±0.55)	2.02^{b}(±0.74)	357.5^{b}(±103.0)	100
***Citrobacter arsenatis* (SON2-F)**	0^{a}(±0.00)	**2.015^{a} (±0.43)**	**2.459^{a}(±1.40)**	**410.9^{a}(±154.7)**	92.22
***Enterobacter cloacae* (TKD-B6)**	0.0455^{a}(± 0.426)	1.5387^{c} (±0.46)	2.085^{ab} (±1.11)	351.0^{b}(±130.9)	97.77
***Enterobacter roggenkampii* (BVB-1)**	0^{a}(±0.00)	1.23^{d}(±0.46)	1.4925^{c} (±0.63)	266.9^{c} (±97.4)	98.88

Standard errors are indicated by values in brackets, and parameters were recorded eight days after treatment.
Tukey's test indicates that values superscribed with the same letter do not significantly differ ($p < 0.05$).

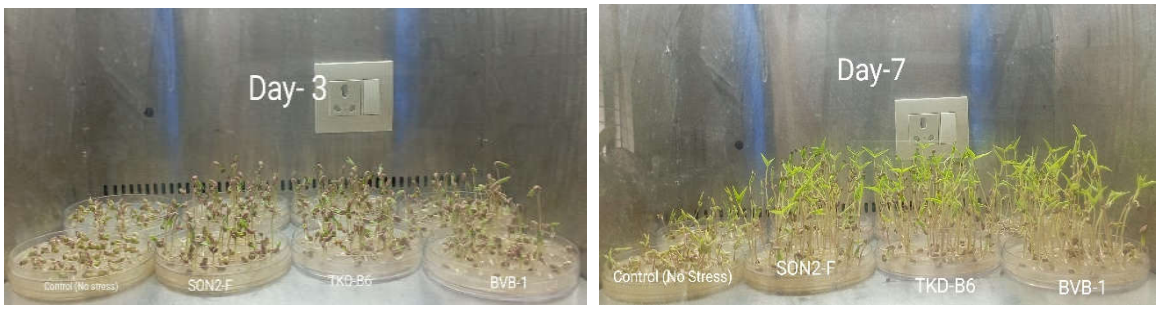
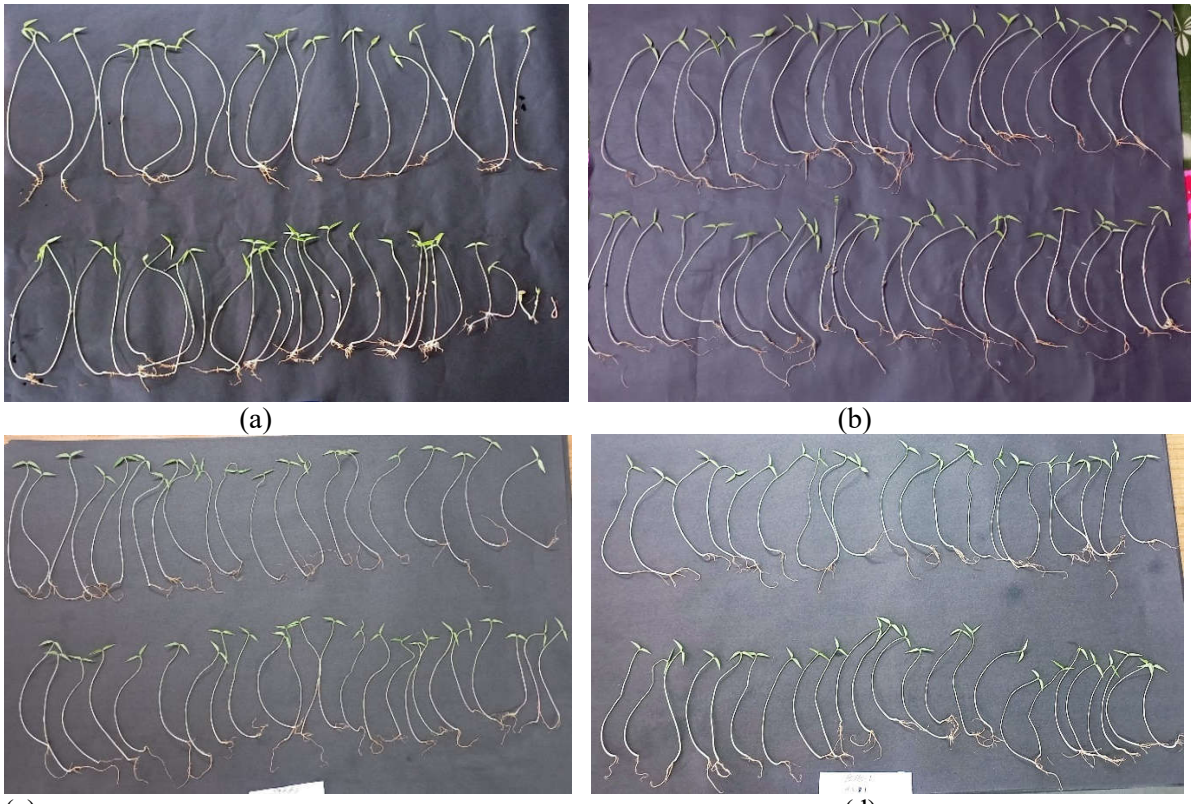


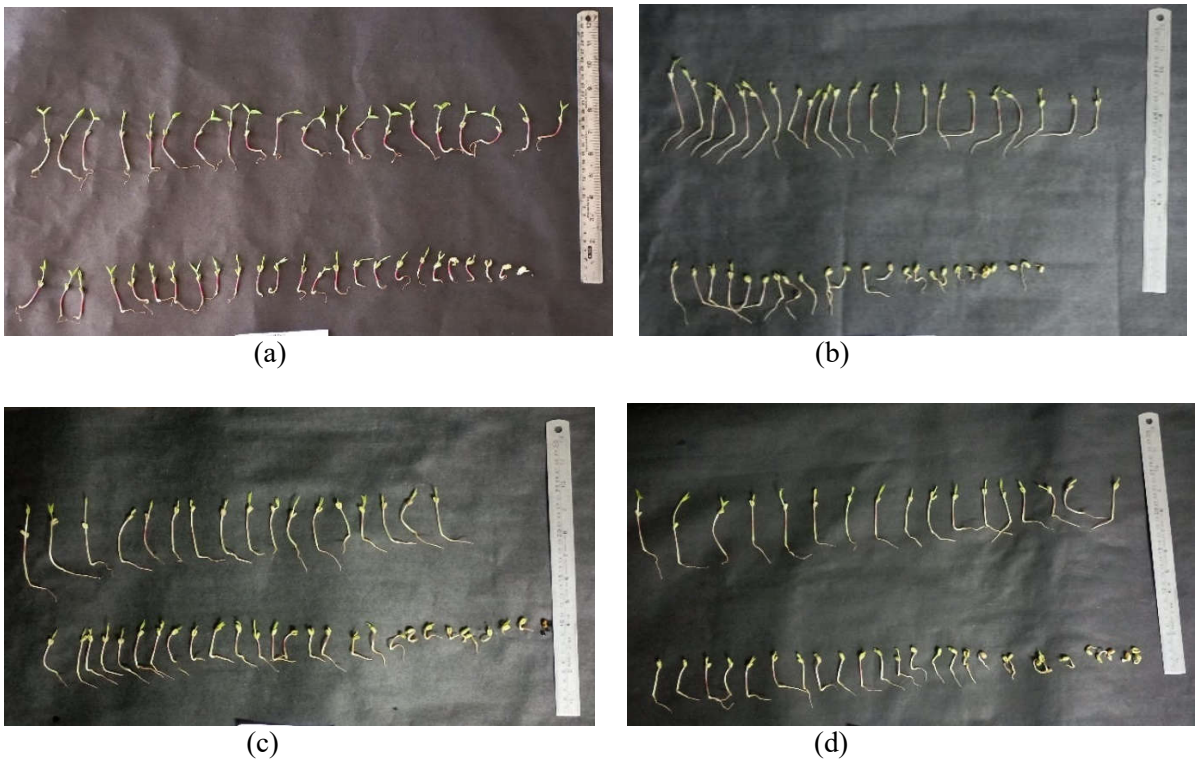
Fig. -4: Seed germination trial with selected isolates using Green gram (*Vigna radiata*) variety **Mung Sindhu NVL 605** variety.



(a) (b)

(c) (d)

Fig. 5: a. Control without the stress of salt and boron, b. SON_2-F treated seedlings grown under normal conditions, c. TKD-B6 treated seedlings grown under normal conditions, d. BVB-1-treated seedlings were grown under normal conditions.



(a) (b)

(c) (d)

Fig. 6: a. Control with stress 40mm NaCl + 7.5 mM H_3BO_3, b. SON_2-F treated seedlings grown under stressed conditions of salt and Boron, c. TKD-B6 treated seedlings grown under stressed conditions of salt and Boron, d. BVB-1 treated seedlings grown under stressed conditions of salt and Boron

4. Conclusion

Salt, drought, pH, temperature, organic pollutants, heavy metals, waterlogging, phytopathogens, and other environmental stressors reduce agricultural productivity. Most crop plants experience a growth loss of more than 50% as a result of these stresses. Therefore, food production must quadruple globally to feed everyone on the planet. While great work has been done to understand how saline affects plants, there has not been much success in sustainably managing productivity losses. Irrigation water may occasionally contain harmful ions, including nitrate (NO_3), fluoride (F), and boron (B), which can endanger crops and soils. The major source of B toxicity in soils is the continuous use of salt-containing irrigation water along with B. In certain situations, the prevention of toxicity by leaching with high-quality water, gypsum amendments, or growing crops that are tolerant of B toxicity may not always be achievable. The toxicity and sensitivity of different plant species to B deficiency vary. Thus, in India's intensive cropping systems, timely and appropriate B fertilization is essential for crop sustainability. The enhancement of agricultural output in saline soils can be achieved by utilizing salt-tolerant plant growth-promoting bacteria (ST-PGPB). These ST-PGPBs support the establishment of salt-tolerant plants with several defence mechanisms against salinity, such as reactive oxygen species (ROS), efflux systems, secondary metabolite production, and the creation and storage of suitable solutes to balance external osmotic pressure. Research on ST-PGPB also shows how highly productive it may be in restoring and improving agroecosystems that are experiencing salinity-related issues. Ethylene stress harms plant productivity. The application of bacteria that promote plant growth as a productive tool for agriculture has recently garnered considerable interest. These bacteria can reduce the negative consequences of ethylene production under stressed conditions by synthesizing ACC deaminase. Several studies have demonstrated the efficiency of ACC deaminase-producing bacteria in both biotic and abiotic environments. Therefore, removing ACC deaminase-producing bacteria from stressful settings and the rhizosphere of resistant plants is a practical strategy for improving plant development under environmental stressors.

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The present findings indicate that the three selected isolates, *Citrobacter arsenatis, Enterobacter cloacae,* and *Enterobacter roggenkampii,* which live in conditions of salt, boron, and temperature that are extreme or harmful to many crops, can produce phytohormones, ACC deaminase, and bioactive substances that are beneficial to crops. Under normal salt and boron conditions, all these isolates are beneficial in *Vigna radiata* as a plant model. However, *Citrobacter arsenatis* might be a useful candidate for the growth of plants under the abiotic stress of salt and boron in sustainable agriculture. The application of such green biotechnology will sustainably benefit agroecosystems.

Ethical statement:

As this manuscript does not involve research on humans or animals, nor does it include vulnerable populations, an ethical statement is not applicable.

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Data Availability Statement:
Citrobacter arsenatis (Acc. No. PQ097197) and *Enterobacter cloacae* (Acc. No. PQ097301), *Enterobacter roggenkampii* (Acc. No. PQ097306). Please find below the link to access this data.
https://www.ncbi.nlm.nih.gov/gene/?term
All raw data of the experiments will be available with the following link.
https://figshare.com/s/657b014d0e489320d4d0
https://figshare.com/s/1243ae9da3bb428ecfde

Author contributions:
Vishal Landge: Conceptualization, Methodology, and Software. **Vishal Landge**: Data curation, writing- original draft preparation. **Dr. Niranjan Patil**: Visualization, Investigation. **Dr. Niranjan Patil***:* Supervision. **Dr. Niranjan Patil**: Software, Validation. **Dr. Niranjan Patil:** Writing-Reviewing and Editing.
All authors have read and approved their contributions.

Disclosure of interest:
We have no conflicts of interest to disclose.

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