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EVALUATION OF PLANT GROWTH PROMOTING POTENTIAL AND BIOCONTROL ACTIVITIES OF *BACILLUS SP. STRAIN CA2*, A RHIZOBACTERIA FROM *CHAKHAO AMUBI*

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ABSTRACT

The experiments were conducted during 2019-22 in Microbial Biotechnological Research Laboratory (MBRL), Department of Biochemistry, Manipur University, India. In the present study, 65 rhizospheric bacterial isolates were obtained from the rhizospheric soil of *Chakhao Amubi* (CA) rice (black rice, Manipur). These 65 isolates screened, 17 were found to exhibit antifungal activities against one or more test pathogens *viz. Rhizoctonia solani (MTCC4633)*, *Curvularia oryzae (MTCC287)*, *Fusarium oxysporum (MTCC2605)*, *Pyricularia oryzae (MTCC1477)* and *Aspergillus niger (MTCC1344)*. All 65 rhizospheric isolates were further subjected to plant growth promoting (PGP) assays *viz.* ammonia, indole acetic acid (IAA), siderophore, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production and phosphate solubilization. Thirty-three (33) rhizobacterial isolates showed good PGP activities. One isolate (CA2) exhibited potent antifungal (*R. solani*-73.75%, *F. oxysporum*- 71.25%, *C. oryzae*-92.5%, and *A. niger*-78.75%) and promising PGP activities (IAA-62.91 µg ml^{-1}, siderophore-74.37 % and phosphate- 200 µg ml^{-1}) was selected for seedling vigor tests on *Chakhao Amubi* Rice. CA2 augmented the germination rate and exhibited a higher vigor index (627.53) over the control (164.47) and also showed significant increase of plant growth under greenhouse conditions. The rhizobacterial isolate has been characterized as a *Bacillus sp. strain CA2* (Accession No. OM868071). Strain CA2 holds promise for development as a bioinoculant for black rice.

(Key words: Rhizospheric bacteria, *Chakhao Amubi* rice, plant growth promoting (PGP), vigor index, rice seedlings, *Bacillus sp*.)

INTRODUCTION

India is the second most populous country in the world (Coad and Tamvada, 2012) and also affluent in natural resources. Inspite its rich natural resources our country is facing shortage of essential commodities such as staple food to support its huge population. The farmers therefore depend on agrochemicals to increase their agricultural yield to meet the demand of ever increasing population. Excessive use of agrochemicals deteriorates soil value and also affects helpful PGP soil microorganisms (Dewangan and Anwar, 2021). So there is a need to search more economical and friendlier means of increasing agricultural yield thereby expanding the horizon of bio fertilizers based agricultural practices.

Rhizobacteria are associated with the soils near the roots and intimately interact with the exudates of the roots (Kennedy, 1998). Plant growth promoting rhizobacteria (PGPR) are among the beneficial soil microorganisms found in the rhizospheric region of the plant. PGPR are diverse and plays significant roles in growth and development of plants. PGPR protect the plant from the attack of pathogenic microorganisms acting as biocontrol agents. In other way, PGPR can increase the growth of the plant by providing phytohormones like IAA and nutrients such as nitrogen, phosphorus, etc. Siderophore, an iron chelator is also produced by PGPR and help in chelating the traces amount of iron available in the soil further unavailing iron to pathogenic microorganisms making them death due to iron starvation (Chandrashaker *et al.*, 2014; Bala and Khanna, 2022). ACC deaminase is an enzyme secreted by PGPR which can degrade ethylene precursor delaying senescence thereby promoting plant growth (Murali *et al.*, 2021).

Chakhao, the endemic rice of Manipur is rich in essential amino acids, minerals, and carotene and has been recently proposed as a nutraceutical supplement for the treatment of some common dreaded diseases like cancer, diabetes, heart disease, and Alzheimer's disease (Wang *et al.*, 2007). As the yield is very poor (about 2.5 tons/hectare), Chakhao is grown in very limited acreage by farmers in Manipur only for ceremonial and cultural purposes. Chakhao production can be enhanced by promoting the use of bacterial isolates possessing biocontrol and PGP activities (Bele *et al.*, 2022; Maulina *et al.*, 2022). PGPR can also act as potent biocontrol agents against pathogenic fungi thereby promoting the growth of the plant. Extensive research is done worldwide on plant growth promoting bacteria (PGPB) and PGPR for developing bio-fertilizers, as they are less harmful and friendlier to the environment and human health.

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There is a need for a survey of PGPB to enhance local production. The present study aimed to investigate the biocontrol and bio- fertilizing potential of the rhizospheric isolates from *Chakhao Amubi* rice.

MATERIALS AND METHODS

Soil sampling and bacterial isolation

All the experiments discussed here were conducted during 2019-22 in Microbial Biotechnological Research Laboratory (MBRL), Department of Biochemistry, Manipur University, India.

Rhizospheric soil of *Chakhao Amubi* grown in Leihaokhong, Thoubal (93.93°E, 24.70°N) was collected for the isolation of associated bacteria. Rhizospheric bacteria were isolated using 3 different media i.e., Starch Casein Nitrate Agar (SCNA), Nutrient Agar (NA) and Gauze's Medium No. 1 (GM1). 1 g of the soil sample was weighed and added to 99 ml of sterile distilled water (SDW). The sample was thoroughly vortexed (150 rpm, 30°C, and 10 min). Serial dilutions for the soil suspension were then performed (10^{-2} to 10^{-6}). The aliquots of various dilutions from 10^{-2} to 10^{-6} were spread plated on agar plates. The isolation plates were incubated for 3-4 days at 30°C. Morphologically distinct bacterial colonies were subcultured till pure cultures were obtained (Krieg *et al.,* 1984).

Biocontrol assay

Biocontrol assay of the rhizospheric bacteria were carried out by dual culture method (Trivedi *et al.,* 2008; and Khamna *et al.,* 2009). The inhibition zones are due to diffusible compounds by the bacterial isolate in the biocontrol assay. After the full growth of fungal mycelia on the control plates, the inhibition in the remaining plates was measured. The percentage of mycelial growth inhibitions was calculated using the formula:

$$[(R-r)/R] \times 100$$

Where, R represents the radial growth of the test pathogen in the control plates (measured in mm), and r is the radial growth of the test pathogen in the test plates.

Plant growth-promoting (PGP) traits screening and estimation

Indole Acetic Acid (IAA) production test

For qualitative test, IAA production was determined by inoculating bacterial isolates in a Nutrient Broth (NB) medium containing 2 mg ml^{-1} of L-tryptophan, incubating at 150 rpm and 30ºC for 5 days (Bano and Musarrat, 2003). The fully grown culture broth was centrifuged (10,000 rpm, 10 min) and 1 ml of the supernatant was mixed with 2 ml of Salkowski's reagent. A pink colour indicates a positive test for IAA production.

For the quantitative assay, 5 ml aliquot was withdrawn periodically from each culture flask at 24 hours intervals and centrifuged at 10,000 rpm for 10 min. 1 ml of the supernatant was mixed with 2 ml of Salkowski's reagent and incubated at 30°C for 20 min. The absorbance was measured at 530 nm and the amount of IAA produced was calculated by comparing it with the standard IAA curve. The amount of IAA produced was also further compared with their corresponding dry cell masses (Bano and Musarrat, 2003 with some modifications).

Phosphate solubilization test

Spot inoculation on NBRIP-BP (National Botanical Research Institute's Phosphate Growth Medium) was carried out to check the phosphate solubilization by bacterial isolates. A halo zone around the bacterial colony after 4 days of incubation at 30°C indicated a positive test (Mehta and Nautiyal, 2001).

Quantitative estimation of phosphate solubilization was done by inoculating in 100 ml of NBRIP medium (pH 7). It was then incubated in a shaker incubator (150 rpm, 30°C). 5 ml aliquot withdrawn periodically at 24 hours intervals was centrifuged (10,000 rpm, 10 min) and the supernatant was analysed for pH and Phosphate concentration (Kapri and Tewari, 2010). KH_2PO_4 was used as the standard (Fiske and Subbarow, 1925).

Siderophore production test

For qualitative test, SCNA (without iron) amended with CAS-substrate (chrome azurol S) were inoculated with 6 mm agar plugs of bacterial isolate and incubated at 30°C for 7 days. The formation of an orange colour halo surrounding the colony was considered a positive result for siderophore production (You *et al*., 2005 with some modifications).

Quantitative estimation of siderophore production was done on five different iron deficient liquid media: Starch casein nutrient broth (SCNB), Casamino acid medium (CAA), Nutrient broth (NB), Succinic acid medium (SM) and Bharbhiaya and Rao medium (BR) by CAS-shuttle assay (Sayyed *et al.,* 2005). 5 ml aliquots were withdrawn periodically at 24 hours intervals and centrifuged (10,000 rpm, 10 min). An equal volume of CAS reagent was added to the supernatant. Absorbance at 630 nm was noted. A reference made of 1 ml uninoculated broth and 1 ml CAS reagent was used. The amount of siderophore produced (percentage siderophore units) was calculated by using the formula:

$$\text{Percentage siderophore units} = [(Ar-As)/Ar] \times 100$$

Where, Ar represents the absorbance of reference and As represents the absorbance of the sample at 630 nm.

Ammonia production test

For qualitative test, the bacterial isolates were inoculated in 10ml of peptone water and incubated in a shaker incubator (150 rpm, 30°C) for 4 days. To each test tube, 0.5 ml of Nessler reagent was then added. A colour change of brown to yellow indicated ammonia production by the bacterial isolates (Cappucino and Sherman, 1992).

1-Aminocyclopropane-1-Carboxylic Acid (ACC) deaminase production test

Nitrogen-free Dworkin and Foster's (1958) salts minimal agar medium (DF) supplemented with 2 g of

$(NH_4)_2SO_4$ as a sole nitrogen source was used for screening ACC deaminase production. Isolates were inoculated on the media and then incubated at 30^{0}C for 4 days. Bacterial growth indicated a positive test for ACC deaminase production.

Seedling vigor index test and evaluation of plant growth promoting potential under greenhouse conditions

Chakhao Amubi rice seed germination by the isolate was examined by seedling vigor index test (Maulina *et al.*, 2022).

[Vigor index = Per cent germination × Seedling length]

(Where seedling length= (mean shoot length + mean root length)

Chakhao Amubi rice growth promotion under greenhouse conditions was performed following the protocol of Bele *et al.* (2022) with some modifications. Dried sandy loam soil was distributed into 18 pots. The 18 pots were used in triplicates for the different treatments *viz.,* control, CA2, control with RS, CA2 with RS, control with FO, CA2 with FO and were arranged them in randomized block design. The pots were kept moist by watering them every 2 days. The number of seedlings was reduced to 5 per pot after a week. After 15 days, the sclerotia of fungal test pathogens were introduced to the pots for challenging conditions. 60 days later the different growth parameters were measured.

Statistical analysis

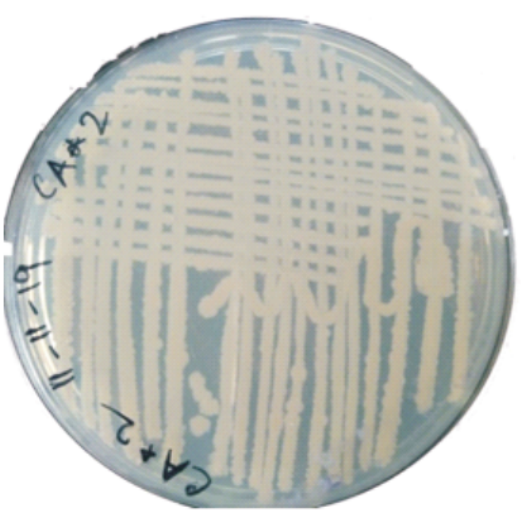
All the data were subjected to one-way analysis of variance (ANOVA) followed by independent t-test at pd"0.05 using the SPSS software.

Molecular characterization

Genomic DNA extraction and PCR amplification of 16S rDNA sequence were performed. The PCR product obtained was sent to Agri Genome Labs Pvt. Ltd. (Kerala, India) for sequencing. BLASTn tool was utilized for bacterial identification by comparing those sequences deposited in GenBank (www.ncbi.nlm.nih.gov). The bacterial sequence was subjected to BLAST alignment analysis using the NCBI GenBank database to obtain the accession number.

RESULTS AND DISCUSSION

A total of 65 rhizospheric bacterial isolates were obtained from the rhizospheric soil of *Chakhao Amubi.* All the 65 rhizospheric isolates were screened for antifungal activities against five (5) rice fungal pathogens. Out of the 65 isolates, 17 were shown antagonistic activities against one or more pathogens whereas CA2 was found to show antifungal activities against four (4) of the five (5) rice fungal pathogens (*R. solani*-73.75%, *F. oxysporum*-71.25%, *C. oryzae*-92.5%, and *A. niger* 78.75%) as shown in Fig. 1-2 and Table 1.



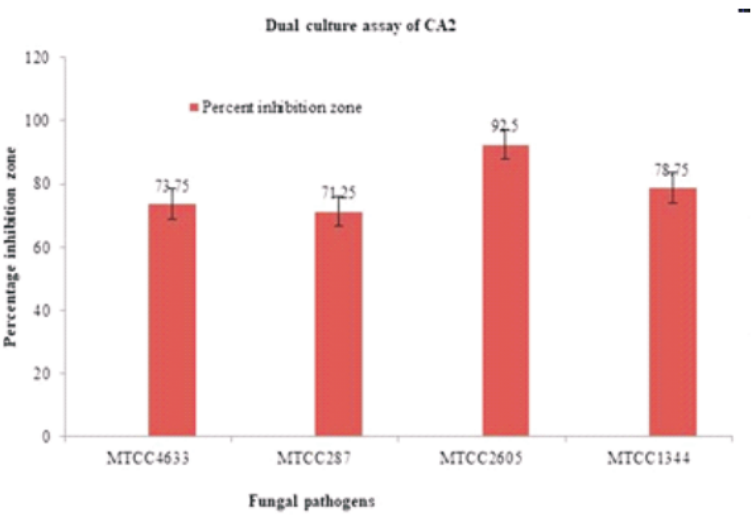


Fig. 1. The rhizospheric isolate CA2. Fig.2. Graphical representation of per cent inhibition by CA2 against the pathogens

Table 1. Results of the dual culture assay of the isolated bacteria

Sl. No.	Bacterial isolates	*Rhizoctonia solani* (RS)	*Curvularia oryzae* (CO)	*Fusarium oxysporum* (FO)	*Pyricularia oryzae* (PO)	*Aspergillus niger* (AN)
		Percentage mycelial inhibition				
1.	CA1	78.75	67.5	-	-	-
2.	**CA2**	**73.75**	**71.25**	**87.5**	-	**78.75**
3.	CA5	67	-	-	-	-
4.	CA7	-	-	47.5	-	-
5.	CA8	-	59.25	63.25	-	-
6.	CA9	57	-	63.5	-	-
7.	CA11	-	72.5	67.5	-	-
8.	CA14	-	54	-	-	-
9.	CA17	60.5	-	55	-	-
10.	CA21	-	-	-	52	60
11.	CA26	-	-	79	-	-
12.	CA27	-	-	-	-	81.25
13.	CA33	-	68.5	-	-	-
14.	CA45	54.5	58.25	-	-	-
15.	CA51	-	67.5	60.5	-	-
16.	CA52	-	58.5	-	55.5	-
17.	CA60	-	73.75	-	75.5	65

Plant growth promoting traits such as ammonia production, IAA production, phosphate solubilization, siderophore production and ACC deaminase production were also analyzed for all the 65 isolates. Out of the 65 isolates, 23 were found positive for ammonia production, 11 for IAA production, 8 for phosphate solubilization, 4 for siderophore production and 17 for ACC deaminase production. Among the positive isolates, CA2 showed positive results for all the PGP traits screened. Therefore, CA2 was shortlisted for further quantitative assays. The quantitative assay for IAA production by CA2 showed the highest amount of IAA production on the 10th day of incubation i.e. 62.9 µg ml^{-1} corresponding to a dry cell mass of 0.019 g (Fig. 3). The rhizospheric isolate CA2 could solubilize phosphate up to 200 µg ml^{-1} corresponding to a pH of 2.69 on the 3rd day of incubation (Fig. 4). CA2 could produce the highest siderophore units in SCNB media (74.37%) on the 6th day of incubation (Fig. 5).

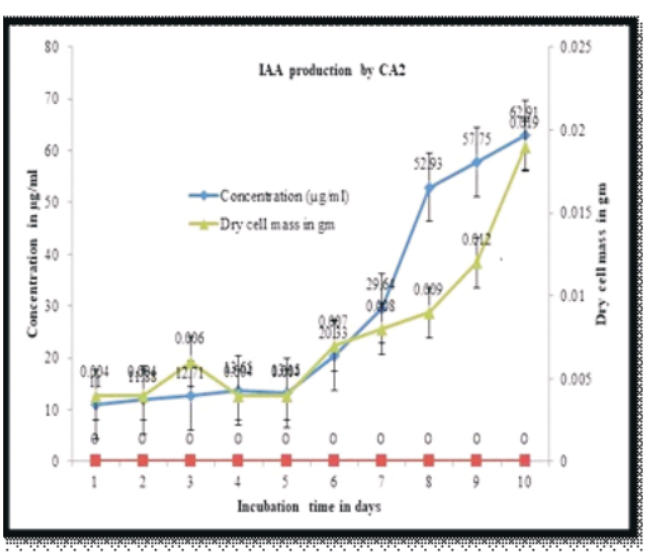


Fig.3. Quantitative assay of IAA production by CA2

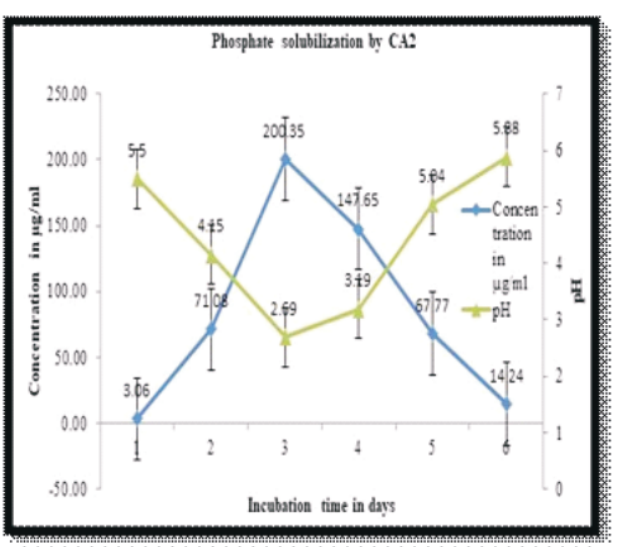


Fig. 4.Quantitative assay of phosphate solubilization by CA2

The most promising isolate CA2 was further screened for *in vitro* seed germination test. The strain showed higher vigor index (627.53) relative to the control (164.47) and data are shown in Table 2. Assessment of plant growth parameters under greenhouse conditions for the different treatments was observed and the results were significant as shown in Fig. 6 (a-b) and Table 3.

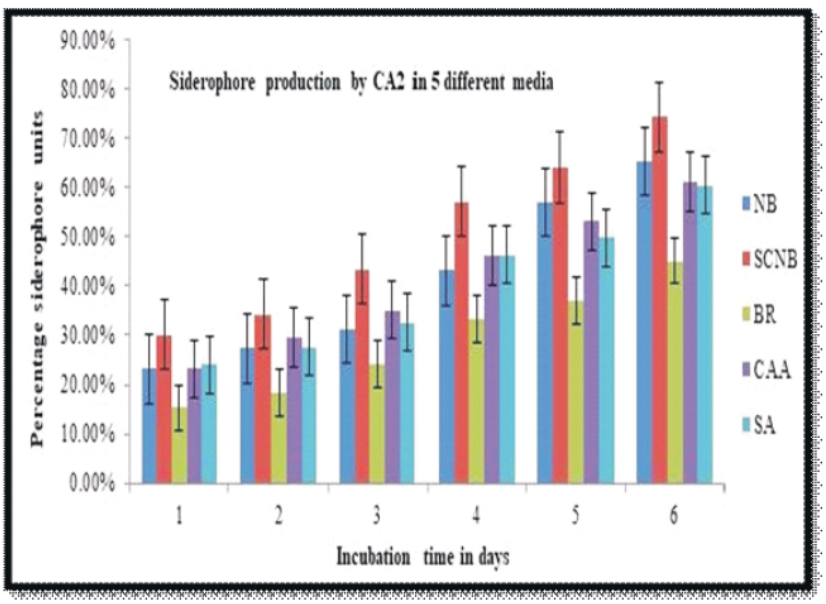


Fig. 5.Quantitative assay of siderophore production by CA2 in five different media.

Table 2. *In vitro* seed germination test of control and CA2 treated sterilized *Chakhao Amubi*rice seeds

Treatments	Fresh weight (g)	Dry weight (g)	Root length (cm)	Shoot length (cm)	% seed germination	Seedling length	Vigor index
Control	0.07±0.01a	0.02±0.00b	1.42±0.78c	0.95±0.53e	69.4±2g	2.37±1.27i	164.47
CA2	0.07±0.00a	0.03±0.00b	4.29±0.77d	3.38±0.53f	80.66±9.90h	7.78±1.28j	627.53

*Values with different alphabet within a column are significant at Pd"0.05.

Table 3.*Chakhao Amubi* rice plant growth promotion by CA2 in pathogen free and pathogen challenged greenhouse conditions

Treatments	Plant height (cm)		Fresh matter (g)			Dry matter (g)			No.of tillers
	Shoot	Root	Shoot	Root	Fresh plant matter	Shoot	Root	Dry plant matter	
Control	61.8±0.53b	10±0.3b	27.2±2.00a	7±0.32b	34.2±2.32a	9.8±0.50b	3.7±0.31b	13.5±0.81a	2.6±0.19b
CA2	81.8±0.07a	16.3±0.61c	51.8±0.08b	18.8±0.43a	70.6±0.52b	22±0.63a	6.2±0.23a	28.2±0.86b	5.3±0.27a
Control with RS	49.2±0.76c	8.4±0.41d	17.2±0.27c	6±0.26c	23.2±0.53c	7.6±0.15c	2.6±0.19c	10.2±0.34c	2.4±0.25bc
CA2 with RS	77.3±0.67e	14.7±0.65a	37.2±0.53d	15.4±0.48d	62.6±1.01d	16.3±0.33d	6.2±0.21a	22.5±0.54d	5.2±0.31a
Control with FO	47.4±0.4cd	9.2±0.27de	15.8±0.27e	5.8±0.31e	21.6±0.58ce	7.2±0.14e	2.4±0.27d	9.6±0.41ce	2.6±0.34bd
CA2 with FO	61.2±0.51ef	12.8±0.47a	38.4±0.64f	14.2±0.28f	52.6±0.92df	16.9±0.26f	5.4±0.41a	22.3±0.67df	4.2±0.23a

*Values with different alphabet within a column are significant at Pd"0.05

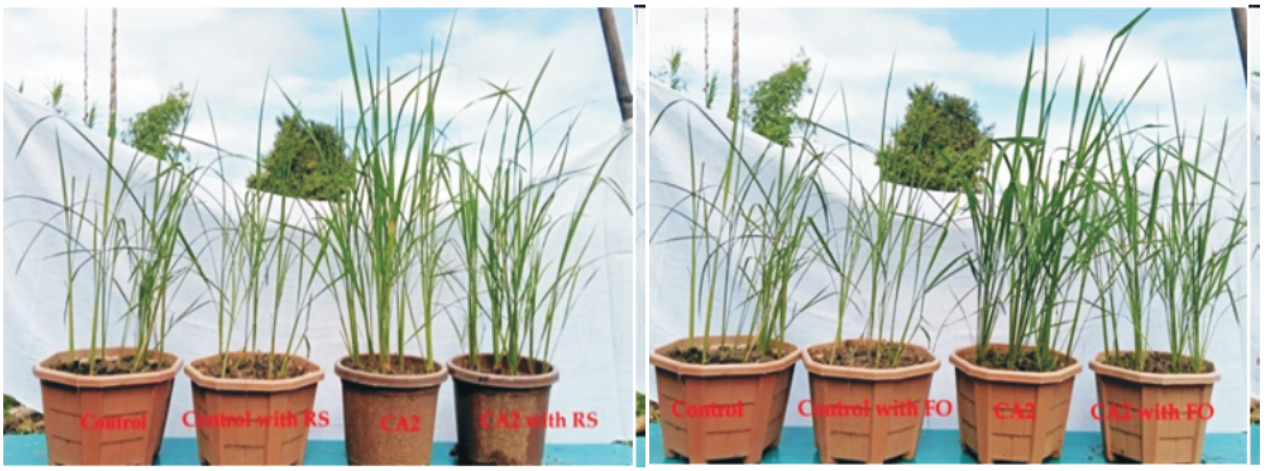


Fig.6. *Chakhao Amubi* rice growth promotion under greenhouse conditions. Different treatments- (a) Control, CA2, control and CA2 treated with RS and (b) Control, CA2, control and CA2 treated with FO

The multi-trait PGP strain CA2 was chosen for molecular characterization. After analyzing the sequence obtained, CA2 had the highest similarity index with *Bacillus sp. CCMMB1014*. CA2 has now been designated as *Bacillus sp. strain CA2* (OM868071).

Biocontrol activities such as antagonistic effects against the phytopathogenic fungi and siderophore production (74.37%) shown by the strain CA2 were significant. Similar findings of biocontrol activities shown by rhizobacteria were reported by Reverchon *et al.* (2019). IAA is a plant growth hormone and so the IAA produced by rhizospheric bacteria can increase the growth of the plant directly. The strain CA2 produced IAA (62.91 µg ml^{-1}) which was considerable when compared with the rhizobacteria reported by Restu *et al.* (2019). Phosphate is a macronutrient of the plants and most of the phosphate present in the soil is in compound form and so unavailable to the plants. Rhizospheric bacteria can solubilize the phosphate compounds making it available to the plants. The strain CA2 can solubilize phosphate up to 200 µg ml^{-1} which was higher than phosphate solubilizing index observed by some workers (Narula *et al*., 2002). The strain CA2 augmented *Chakhao Amubi* rice seed germination showing higher vigor index (627.53) over the control (164.47) (Maulina *et al*., 2022). *Chakhao Amubi* rice plant growth promotion under greenhouse conditions by the strain CA2 in both pathogen free and pathogen challenged conditions were observed. There were assessment of plant growth parameters after 60 days of plantation and showed percentage increase in growth significantly for the CA2 treated rice plants over their respective controls. The percentage increase in growth of growth parameters such as shoot length (32.36%), root length (63%), fresh matter (106.43%), dry matter (108.88%) and number of tillers (103.84%t) can be observed for the CA2 treated rice plants over the untreated control plants. The growth parameters are also significantly increased in pathological conditions of CA2 treated over untreated rice plants. The strain CA2 enhanced seed sprouting and plant growth due to the production of ammonia, phytohormones and antifungal metabolites such as siderophore and antifungal phenolic compounds (Shobha and Kumudini, 2012; and Khan *et al*., 2020).

The present study was aimed to isolate and screened for antifungal and PGP traits analysis of the rhizobacterial isolates from *Chakhao Amubi*. From the study, we observed that PGPR possessed various PGP traits and biocontrol activities. One of the most promising and potent isolate, *Bacillus sp. Strain CA2* could produce IAA, siderophore and solubilized phosphate in significant amount and also possessed antagonistic activity against phytopathogenic fungi. The study demonstrated the plant growth promoting potential of the strain CA2 under controlled conditions. The ability of plant growth promotion by the strain CA2 could be because of biocontrol activities and PGP traits shown by the bacterium. Thus it could be concluded that the strain CA2 must be exploited as biological fertilizer for sustainable agriculture of Chakhao rice.

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