K2 a Newly Isolated Strain of *Bacillus amyloliquefaciens* Regulates Responsive Proteins for Its Survival and Promotes Plant Growth of Rice Seedlings against Bacterial Leaf Blight and Salt Stresses

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ABSTRACT

Soil salinity limits the growth and productivity of crop plants worldwide including Thailand. Plant growth promoting rhizobacteria (PGPR) can elicit plant tolerance against both biotic and abiotic (adverse-environmental effects) stresses that may become an alternative process in crop management system. In this study, a new PGPR strain is hypothesized whether it can adapt to high salt concentrations and promote seedling growth of rice against *Xanthomonas oryzae* pv. *oryzae* (Xoo), causal organism of bacterial leaf blight, under salt stress that might correlate with its biosynthesis of stress-responsive proteins production. Forty eight bacteria were isolated from different salt -soil lands, and strain K2 (obtained from mangrove plant rhizosphere at Bangkrachao, Samut Prakan) was selected for further investigation based on its superior in biocontrol activity and survival in nutrient broth (NB) plus 12% NaCl which indicated that K2 was a halophilic bacterium. Strain K2 was identified using physiological and biochemical properties, 16s rRNA and *gyrB* nucleotide sequencing analysis revealed that K2 was placed in *Bacillus amyloliquefaciens.* Culture filtrates of strain K2 grown in NB with or without 5%NaCl for 24 h were subjected to proteomic analysis. Strain K2 and its culture filtrates exhibited a good performance in Xoo suppression shown by plate assay, and increased growth enhancement of rice seedlings (root length and shoot height) under both normal and salt-stress conditions. Of the shotgun proteomic LC-MS/MS identified proteins, the specific proteins were up-regulated in K2 – salinity grown cells. A key role for proteins predominantly expressed in above analysis was likely mediating plant health tolerated to Xoo and salt stresses. Those involving proteins included the iron scavenging and transport, defense mechanism, the synthesis and transport of compatible solutes, protein expression in protecting K2 survival and adaptation, biocontrol activity, plant growth promotion, and induced salt tolerance was discussed.

Keywords: halophilic bacteria, phylogenetic analysis, *Xanthomonas oryzae* pv. *oryzae*, high-throughput proteome analysis

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INTRODUCTION

Impact of biotic and abiotic stressors has become a great concern to the stability of plant growth development worldwide (FAO and ITPS, 2015). *Xanthomonas oryzae pv. oryzae* (Xoo) caused bacterial blight disease (BB) and soil with high salt concentration are a serious threat to rice production in Northeast regions of Thailand (Sangwanna *et al.,* 2018; Kheoruenromne, 2007). BB could be severe and caused economic losses up to 74% depending on susceptible of cultivars, growth stage, growning season, and the planting location. Except for Xoo-attacking, rice has been classified as a salt-sensitive crop that is affected severely at early seedling stage (Sangwanna *et al.,* 2018). A tolerance level of salt concentration limiting rice growth and development is estimated as 6.9 to 8 dSM-1 or about 0.25 to 0.5% salt (Van Genuchten and Gupta, 1993). In this respect, decreased development of plant growth with increase in salt concentration in saline soli directly affective rice productivity (Todaka *et al.,* 2012) and that 56% yield losses in sensitive cultivar was recorded (Van Genuchten and Gupta, 1993). However, most rice cultivars grown in Thailand have the ability to resist 12 dSM-1 or 0.75% NaCl (Saengsanga *et al.,* 2017), where 0.3 m.ha salt soil spreading to 29% total area of paddy field emerged in the Northest region (Kheoruenromne, 2007). Management of these biotic(BB) and abiotic (salt stress) agents with multi-stress conditions of rice production system would complicate in enhancing an entirely optimum yield.

Biological control through the use of antagonistic bacteria as a biological control agent (BCA) or plant growth promoting rhizobacteria (PGPR) may be a promising alternative to accomplish a successful control of both BB and salt-stress conditions (Prathuangwong *et al.,* 2012; Egamberdieva *et al.,* 2019). Several studies have been conducted to investigate beneficial effects of BCA and PGPR on phytopathogenic suppression which come from their ability to produce multiple antimicrobial metabolites, and induced systemic resistance (ISR) through the manipulation of physical and biological properties of host plants resulted in stimulating of plant defense tolerant to biotic and abiotic stresses (Prathuangwong and Buensanteai. 2007; Egamberdieva *et al.,* 2019). Few reports have focused on manipulate saline soil in agriculture, PGPR may adapt them in high salt concentrations and increase soil fertility by releasing plant nutrient which eventually promote plant growth. (Egamberdieva *et al.,* 2019). Screening for the possibility of PGPR that enhance stress resistance and benefit plants thus, would contribute to a future aspect of approaching plant growth promotion against disease and extreme-saline pressure. As part of PGPR facilitate plant tolerance to salinity, the moderately halophilic bacteria, which are well adapted to live and thrive in high-salt environments, have a potential to promote growth and alleviate saline stress effects via regulating physiological and molecular processes in plants (Amoozegar *et al.,* 2009). Beneficial effect of PGPR under salt stress has been

related to induce plant tolerance against many other adverse-environmental stresses (Dodd and Perez- Alfocea, 2012) which indicates that one selective strain may possibly possess and the mechanisms which contribute to biocontrol activity.

Some species of *Bacillus* had developed several strategies to survive in salinity environment during osmotic up- and down-shifts (Canovas *et al.,* 2000). The majority of moderately halophilic *Bacillus* responds to an external salinity by accumulation of low-molecular weight organic compounds as compatible solutes (Zaprasis *et al.,* 2012) such as amino acids, ectoines, betaines, and sugars (Canovas *et al.,* 2000). Most of those relevant compounds changing in bacterial gene expression under adverse-environmental stresses that were detected in culture filtrates of bacterial grown cells suggested that the individual or coordinated compounds markedly play a role in protecting their survival (Yoshida *et al.,* 2001). The mechanisms involved in the protein/gene expression process are still limited in experimental works and needed to furthers elucidate. This study presents the potential of new strain *Bacillus amyloliquefaciens* K2 which was isolated from rhizophere soil of mangrove plant for improving plant growth and controlling BB disease under salinity condition. Furthermore, we describe the molecular mechanisms of saline adaptation in *B. amyloliquefaciens* K2 based on high-throughput proteome analysis.

MATERIAL AND MATHODS

Bacterial collection and isolation

Soil samples were randomly collected from healthy plant rhizospheres of mangrove, fern, bamboo, banana, betel palm, and cork tree grown in the area of high salt concentrated soil in 6 sub districts in Bangkrachao district, Samut Prakan province, Thailand in 2016. Soil suspension was prepared by mixing 5 g. of soil in 20 ml of sterile distilled water. It was then spreaded on semi-selective medium (hypersaline ATCC; 5%NaCl, 2%$MgSO_4.7H_2O$, 0.3%Na-citrate, 0.1%yeast extract, 0.5% tryptone, and 0.05%K_2HPO_4). Then cultured plates were incubated for 48 h at room temperature. After 48 h incubation, 48-isolates were selected and pre-screened by paper disc diffusion methods against *Xanthomonas oryzae* pv. *oryzae* (Xoo) the causal organism of bacterial blight of rice (BB), Pratuangwong *et al.* (2009). Bacterial strains that were capable of inhibiting Xoo were selected for further tests.

Screening for biocontrol activity and plant growth promotion

Based on pre-screening result, 9 isolates were chosen for futher study which was to test against various phytopathogens including Xoo, *X. axonopodis* pv. *glycines* (Xag), and *X. campestris* pv. *campestris* (Xcc) caused BB, soybean bacterial pustule, and black rot of crucifers respectively using paper disc diffusion method (Pratuangwong *et al.,* 2009). These 9 isolates were also tested on their antagonistic properties against BB under salt stress condition using

the same above method (NA medium plus with 0.3, 0.6, 0.9, and 1.2% NaCl). The bacterial antagonistic strains including *Bacillus amyloliquefaciens* HS105, *B. amyloliquefaciens* KPS46, and *Pseudomonas fluorescens* SP007s from culture collection laboratory at Department of Plant Pathology, Kasetsart University, Bangkok were used as comparison in this study and all tests.

Determination for plant growth promotion under salt stress conditions was conducted. The germinated seeds of rice cv. KDML105 were prepared by soaking seeds with sterilized distilled water and incubated at 24 h room temperature, then soaked with 5% Clorox for 5 min and subsequently rinsed with sterilized distilled water. The germinated seeds were treated with 0.2 OD_{600} nm (10^8 CFUmL-1) of each of the 9 selected strains using 1 ml of bacterial suspensions per 3 g of germinated seeds. They were then grown on water agar (WA) which had 0, 0.3, 0.6, 0.9, and 1.2% NaCl concentration. Seeds treated with each of 3- bacterial antagonistic strains from culture collection laboratory were also included in the experiment. All treatments were incubated at room temperature (28-32°C) for 14 days.

Assessment of salt tolerance

A new strain that showed greater inhibition of pathogens in previous tests was selected to further use for salt adaptability. The selected strain was then a targeting and assessed for growth and salt tolerance compared to the 3-collective strains. Bacterial cells were inoculated into nutrient broth supplemented with a series of increasing salt concentration (0, 2, 4, 6, 8, and 12% of NaCl) and incubated at room temperature and shaken at 250 rpm. Bacterial growth was monitored using plate count followed the procedures described previously (Prathuangwong *et al.,* 2009). The highest concentration of salt with no significant effect on the growth pattern of the bacteria was taken as their tolerance level.

Morphological and biochemical characterization

The colony morphology of all newly isolated strains was determined after 24 h and 72 h incubation on NA agar medium. Physiological and biochemical characteristics of 9 newly strains were assessed which followed the procedures by Bergey's Manual of Determinative Bacteriology 9th edition (Holt *et al.,* 1994). Pigmentation, gram staining, endospore staining, motility, and urease test were done as standard protocol (Cappuccino and Sherman, 2004). The catalase activity was determined and starch hydrolysis was tested to detect an enzyme alpha-amylase. Acid production from carbohydrates utilizations of glucose, lactose, sucrose were detected by the visible change in color from red to yellow (Arahal *et al.,* 2007) compared with the 7 collective strains. The best strain, which showed capacity for suppressed various diseases and enhanced plant growth under salt stress, would be using molecular analysis selected for further genotypic identification procedure.

Molecular identification and phylogenetic tree analysis

Genomic DNA of K2 was extracted from bacterial cells cultured in LB at 24 h with CTAB method followed by Ausubel *et al.* (1987). The 16s rRNA and gryB (gyrase subunit B) genes were used for sequence analysis (Singh *et al.,* 2013). The primers specific to *Bacillus* sp. 16S rRNA (6F 5' GGAGAG TTAGATCTTGGCT-CAG 3' and 1510R 5'AGTCG TAACAACGT AACCCTG CAGCAG 3') and *gyrB* genes (gyrBF 5' GAAGTCATCATGAC CGTTCTGCA 3' and gyrBR 5'AGC AGGGTACGGATGTGCGAG-CC 3') were used to amplify the DNA target of K2 using PCR base technique following standard protocol (Thermo Fisher Scientific Inc.). Then, PCR products were purified and sequencing at Macrogen Company, Korea. The nucleotide sequences obtained from 16S rRNA and gyrB regions of K2 were compared to reference sequences retrieved from GenBank database using Blastx (Wang *et al.,* 2007). The sequence alignment of K2 with other bacterial species was constructed by MEGA 6.0 (Tamura *et al.,* 2013) with evolutionary distances computed using the neighbor-joining model to build the phylogenetic tree. Validity of branches in the resulting tree was evaluated by UPGMA phylogeny bootstrap re-sampling support of the data sets with 1,000 replications.

Effect of K2 and culture filtrates in vitro and in vivo

Culture filtrate extraction

The K2 strain was grown at room temperature in NB media with and without added 5%NaCl. Cells were harvested at 24 h, and 72 h after incubation. The cell pellet and supernatant were separated by centrifugation at 10,000 x g for 10 min, 4°C. The cell pellet was washed three times in sterile water, suspended in sterile distilled water and the optical density of suspension was adjusted to 0.2 OD_{600} nm. The supernatant was filtrated through a 0.2 nm nitrocellulose filter (Minisart® syringe filters, Germany). The cell suspension and supernatant retained for further testing at -20°C until use.

***In vitro* experiment**

The K2 culture filtrate was test for production of antibacterial metabolites and growth inhibition of Xoo using disc diffusion method described above. The agar plates were inoculated with Xoo suspension which was then spreaded throughout the agar surface A hole with a diameter of 6 mm was punched aseptically with a sterile cork borer to render the well, and a volume 20 µl of K2 filtrates was introduced into the well, then incubated under room temperature. The tests were compared with culture filtrates of 3-collective strains (SP007s, HS105, KPS46), K2 cells suspended in dH_2O, and K2 grown cells with its NB medium culture. Diffusing in the agar medium would be interpreted as an antimicrobial activity based on the appearance of inhibition zone around the agar well.

Plant inoculation

Two rice genotypes, moderate and salt tolerance KDML105 and Pokkali cultivars (Reddy *et al.,* 2017) were used in this experiment. Rice seeds were treated at 1% (w/v) with culture filtrates of K2 (treatment; CF), K2 cell suspension (K2), K2 grown cells

with its NB medium culture (Cul), and 1mM glycine betaine (GB; the osmoprotective compound), and distilled water was used as control (dH_2O). Germinated seeds were grown in the plate containsing water agar (WA) medium with and without 0.9% NaCl, and incubated under artificial light illumination of 16-h/8-h (day/night) at room temperature. At 3, 5, 7, 9, 10, and 14 days after planting, seedlings were measured for shoot height, and root lengths. The experiment was designed in CRD with 3 replications.

Strain K2 increases inducible protein expression under salt stress

Bacterial adaptability and biological properties associated with PGPR commonly produce stress respons proteins including antimicrobial compounds for developing their biocontrol activity. Strain K2 was expected to produced different protein compounds to protect its survival and plant health from salt stress and pathogen attack. These compounds would become key to its utilization to the full benefit. The cell-free supernatants from the strain K2 was obtained as described above (culture filtrate extraction) and subjected to separate and identify the involved protein compounds.

Protein extraction and digestion

Total proteins were extracted from K2 cultured at 2 conditions (normal and salt stress) in NB medium (NB + 5% NaCl for salt stress condition). To identify the changed proteins in exoproteome, it was extracted from K2 culture filtrates where cellular proteome extracted from cell suspension at 24 h cultures using same separated method described above (Li *et al.,* 2020). The 5 ml of each cell suspension and culture filtrates was precipitated with 0.5% sodium dodecylsulfate (SDS) and the pellet was collected by centrifugation at 12,000 rpm for 10 min. The proteins were fractured by infusing in liquid nitrogen 5 min, dissolved in 0.5% sodium dodecylsulfate (SDS) and stored at -20°C until use. The protein concentration was measured according to Lowry method using Bovine Serum Albumin (BSA) as standard protein (Li *et al.,* 2020).

Dissolved the proteins in sample buffer that contained 100 mM ammonium bicarbonate to a final protein concentration of approximately 5 g/L. Added 10 mM Dithiothreitol (DTT), incubated the dissolved protein sample at room temperature for 1 h and then added 20 mM Iodoleacetamide (IAA), incubated for 1h in the dark at room temperature. After the incubation period the sample were allowed to cool to room temperature. Added the appropriate volume of MS grade trypsin (1 g/L stock) such that the ratio of trypsin: protein in the sample was between 1:20 and 1:100 (w/w). Incubated the digestion reaction at 37°C for 3 h. Terminated the digestion reaction by added formic acid to a final concentration of 5% (v/v) and centrifuged at 12,000 rpm for 5 min. The clear supernatant (the peptide solutions) was then transferred to a new tube and stored at -20°C until use.

LC-MS/MS analysis

The peptide solutions were analyzed using an HCTultra PTM Discovery System

(Bruker Daltonics Ltd., U.K.) coupled to an UltiMate 3000 LC System (Dionex Ltd., U.K on a nanocolumn (PepSwift monolithic column 100 m i.d.× 50 mm). The effluent samples were electro-spayed into a mass spectrometer for LC–MS/MS analysis of peptides and then generated the spectral data for future protein quantitation and identification against database search (Tunsagool *et al.,* 2019).

Protein quantitation and identification

Protein quantitation and identification were achieved by Differential Analysis software (DeCyderMS, GE Healthcare) (Li *et al.,* 2020). The analyzed LC-MS/MS data from DeCyderMS was then sent to database searching though the Mascot software (Matrix Science, London, UK). Protein identification was performed by searching against the *Bacillus amyloliquefaciens* non-redundant subset database of National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/). Data normalization and quantification of the changes in protein abundance between the control and treated samples were performed and visualized using Jvenn program. The list comparison of identified proteins were displayed in Venn diagrams (Tunsagool *et al.,* 2019).

Data analysis

The experimental testing was designed in Completely Randomized Design (CRD). The analysis of variance were performed using Least Significant Difference (LSD) at $P < 0.05$ by Statistix version 8. (Analytical Software, Tallahassee, USA.).

RESULTS AND DISCUSSION

Bacterial isolation and screening for biocontrol activity and plant growth promotion

In total, 48 bacterial isolates were able to grow in semi-selective media (hypersaline ATCC), suggesting that these isolates were salt tolerant or halophilic bacteria (data not shown). Out of 48 salt tolerant isolates, 9 strains could inhibit all tested pathogens, *X. oryzae* pv. *oryzae* (Xoo), *X. axonopodis* pv. *glycines* (Xag), and *X. campestris* pv. *campestris* (Xcc) when evaluated by paper disc diffusion method under normal conditions (Figure 1, Table 1). The strain K2, a novel salt tolerance bacterial strain in this study, had similar abilities to inhibit the three bacterial plant pathogens when compared with BCA strains; *B. amyloliquefaciens* HS105, *B. amyloliquefaciens* KPS46, and *P. fluorescens* SP007s. Under high salt stress conditions, the strain K2 showed a clear inhibitory halos to inhibit the growth of Xoo, whereas the inhibitory activity of other comparative BCA strains were very limited (Table 1). This suggested that the K2 was able to upregulated gene expression and show antimicrobial activity against Xoo under salt stress conditions. This experiment also indicates that K2 might produce bioactive molecules, such as antimicrobial peptides and biosurfactants to protect its own survival under high salt stress conditions (Egamberdieva *et al.,* 2019).

For plant growth promoting evaluated under normal conditions, the growth of KDML105 rice seedlings treated with the strain K2 was similar to those 3-comparative strains. Under salt stress conditions, the

growth of rice affected by high salt concentration were observed when grown in the media supplemented with 0.9 % NaCl. When concentration level was increase to 1.2% NaCl, no seed germination was found. Our results showed that the strain K2 significantly enhanced growth of rice seedlings grown under salt stress (0.9% NaCl) when compared with non-treated control and comparative strains (Table 1). Several works reported that in vitro experiments PGPR has been losing ability to promot plant growth with increasing salinity (Upadhyay *et al.,* 2009). These results suggested that the presence of NaCl may be a trigger for salt tolerant bacteria K2 to induce factors that facilitate the growth of rice under salt stress conditions (Ventosa *et al.,* 1998).

Growth of the strain K2 with different salt concentrations

The effect of NaCl concentration on the growth of K2 strain was studied to understand its survival in NB supplemented with different concentrations of NaCl (1-12% w/v). The result showed that K2 was able to grow in NA supplemented with 12% NaCl, while the comparative strains HS105, KPS46, and SP007s could survive in only 6%NaCl. At the concentration of 2% NaCl, the comparative strains were found to be affected but not for K2. K2 growth rate started decreasing at 6% NaCl and still be survived throughout its lag phase growth of 12% NaCl concentrations (Figure 2). Moderately halophilic or halotolerant bacteria are defined as those prokaryotes that grow best in media containing 0.5 – 2.5 M NaCl or 2.92 – 14.6% NaCl (Canovas *et al.,* 2000). The genus *Bacillus* that also belongs to moderately halophilic group is generally found in soil and competed with other organisms within the stress environment due to its capability to form extremely resistant spores and produce metabolites that have antagonistic effects on other microorganisms (Amoozegar *et al.,* 2009). Result of this study proved that *B. amyloliquefaciens* K2 was a halophilic bacteria.

NaCl had an effect of shortening period of lag phase growth (0-9 h) in K2 strain at 6% NaCl which began at 0-12 h and going to rapid growth of exponential phase at 12-30 h (Figure 3). Roebler *et al.* (2003) reported that period of lag phase was the time taken for adjustments of the bacterial cells to new conditions, no cell division occurs, population size doesn't increase in which individual organisms grow in size and for exponential phase, cells begin to divide and generation time reaches a constant minimum, most rapid growth, numbers of cells produce over the cells dying, cells are at highest metabolic activity, and most susceptible to adverse environmental factors at this stage. In this test strain K2 entered to exponential phase faster when stimulated with salt, indicating that K2 was a good-adaptive bacterium which increased the amount of cell number within 9 h and up to 30 h. The adaptation of the bacterial cells to salt stress and osmotic challenges apparently have many facets. The mechanisms responsible for salt stress tolerance and growth rate

were ranged from induction of stress tolerance proteins (Upadhyay *et al.,* 2009). To better characterize the mechanism of saline adaptation of strain K2, the proteome analysis was further conducted using a mass spectrometry (MS)–based high-throughput proteomics.

Morphological and biochemical characterization

K2 showed cellular morphology pertaining typical of spore-forming Gram-positive bacteria. Colony on NA showed opaque white and glossy, irregular form, undulate margin, raised elevation and appears mucoid (Supplementary Figure 1). This strain was positive for catalase, urease, starch hydrolysis and acid production from glucose and sucrose tests, and the motility, whereas negative for lactose growth. The morphological and biochemical characterization results of K2 correlated with previously *Bacillus* group reported by Priest *et al.*(1988). Based on these properties, the strain K2 was closely related with comparative strains *B. amyloliquefaciens*

Table 1 Efficacy of bacterial antagonists against phytopathogenic bacteria and on plant growth promotion under plate assay at 2-conditions[1/].

Antagonistic bacteria	Growth inhibition[2/]				Promoted plant growth[3/](cm)			
	Normal			Salt	Shoot height		Root length	
	Xoo	Xcc	Xag	Xoo+1.2%	Normal	Salt	Normal	Salt
K2	0.82b	0.70d	0.68b	0.79a	8.8a	4.93a	6.0a	3.52a
K7	0.48g	0.40h	0.38f	0.40d	7.96bc	3.42de	5.38bc	2.78de
Ph1	0.62e	0.51f	0.57d	0.55c	7.98bc	4.08b	5.36bc	3.2abc
KH1	0.22k	0.40h	0.38f	0.40d	7.5e	3.34de	5.56b	3.08cd
KH 5	0.56f	0.60e	0.48e	0.40d	8.1b	3.50de	5.56b	2.84de
KH11	0.71d	0.70d	0.56d	0.70b	8.04b	3.98bc	5.54b	3.32ab
Fe2	0.24j	0.28j	0.28h	0.20f	7.74d	3.28ef	5.06d	2.66e
M4	0.31i	0.30i	0.20i	0.21f	7.5e	3.68cd	5.07d	3.06cd
L1	0.37h	0.48g	0.30g	0.18g	7.42ef	3.45de	5.08d	2.98de
HS105	0.82b	0.73c	0.68b	0.32e	8.84a	3.79bc	6.12a	0.73f
KPS46	0.81b	0.74bc	0.65c	0.32e	8.86a	3.53de	6.18a	0.52fg
SP007s	0.86a	0.75b	0.68b	0.29e	8.88a	2.92f	6.22a	0.27g
Kan	0.89a	0.78a	0.70a	0.71b	-	-	-	-
dH2O	0.0l	0.0k	0.0j	0.0h	7.28g	2.29g	4.61e	0.19g
CV(%)	0.12	0.24	0.51	0.50	0.08	0.85	0.26	0.27
LSD	0.09	0.161	0.44	0.15	2.23	2.16	2.23	2.23

[1/]Means in the same columns followed by a common letter are not significantly different at the 5 % level by LSD.

[2/] Plate assay on NA medium with (Salt) or without (Normal) 1.2% NaCl.

[3/]Ability to promote plant growth of rice seedlings under normal and salt (0.9% NaCl) conditions.

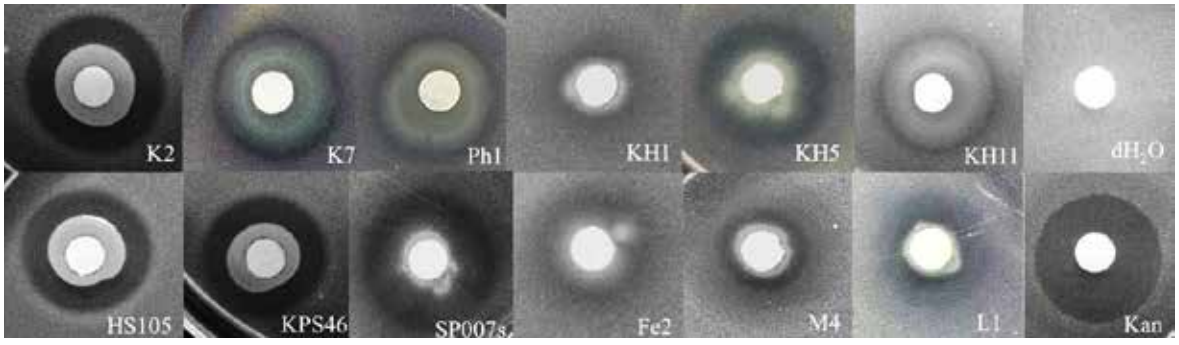


Figure 1 Inhibition effect of 9 strains including K2 and the 3-comparative strains (*Bacillus amyloliquefaciens* HS10, *B. amyloliquefaciens* KPS46 and *Pseudomonas fluorescens* SP007s) against plant pathogenic *Xanthomonas oryzae* pv.*oryzae* grown spreading on NA surface compared with 50 ppm kanamycin (Kan) and sterile distilled water (dH2O), that served as negative and positive controls, respectively

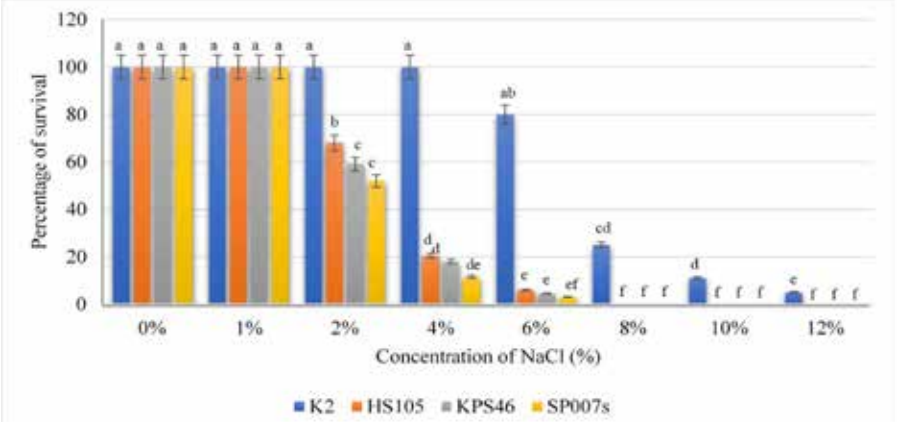


Figure 2 Effect of NaCl with different concentrations on bacterial survival of the strain K2 compared with *Bacillus amyloliquefaciens* HS105, B. *amyloliquefaciens* KPS46, and *Pseudomonas fluorescens* SP007s

HS105 and KPS46, and *B. subtilis* BN111 (culture collection laboratory, KU) (Supplementary Table 1). A group of genus *Bacillus* could not be reliably identified into species-specific level, which using only traditional importance in basic and applied microbiology of phenotypic traits, due to those *Bacillus* members (particularly *B. subtilis* and *B. amyloliquefaciens*) were highly closed relating in taxonomical position (Holt *et al.,* 1994).Thus, the species-specific strain *Bacillus* K2 has been identified and distinguished from those *Bacillus* spp. clusters using the molecular-approached analysis under the next experiment.

Molecular identification and phylogenetic analysis

Molecular identification of the strain K2 was done by analysis of 16S rRNA and gyrase B (*gyr*B) gene sequences. On the basis of comparison of 16S rRNA and *gyr*B sequences with the NCBI database, the bacterial strain

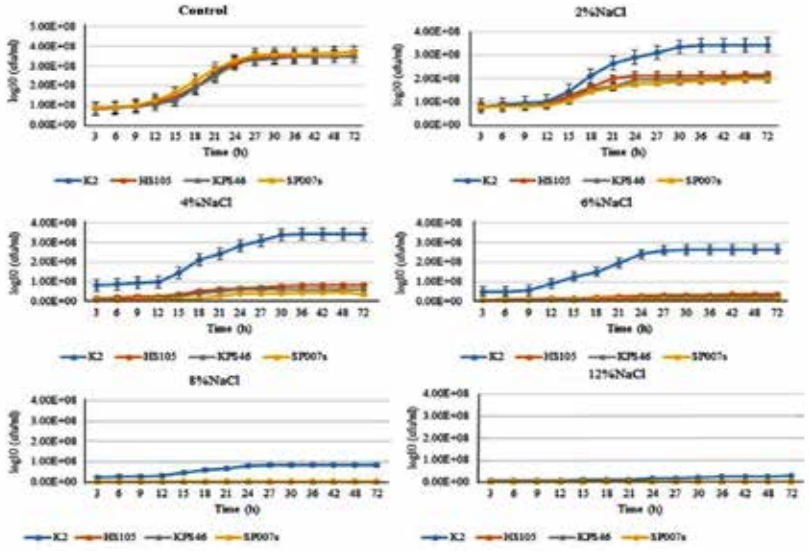


Figure 3 The growth rate of the strain K2 compared with *Bacillus amyloliquefaciens* HS105, *B. amyloliquefaciens* KPS46, and *Pseudomonas fluorescens* SP007s grown in different NaCl concentration media

K2 showed maximum similarity 99% with *Bacillus amyloliquefaciens* ML376 (accession no. KC692189) and 99% with *B. amyloliquefaciens* LL3 and DSM7 respectively.

The phylogenetic analysis based on 16S rRNA revealed that *B. amyloliquefaciens* K2 closely related to the type strains of *B. amyloliquefaciens* XH7, TA208, J5518, LL3, TUL308, and NBRC15535 (88% bootstrap values from NJ phylogeny based on 1,000 replicates) (Figure 4A). Given that *gyr*B gene sequences have been used in phylogenetic studies of the *Bacillus anthracis,* -cereus, –thuringiensis, - subtilis, and amyloliquefaciens group (Wang *et al.,* 2007), phylogenetic analysis based on *gyr*B gene was also carried out to yield highly relatedness values. The results showed that *B. amyloliquefaciens* K2 was clustered together with the members of *B. amyloliquefaciens* and closely related to the PGPR type strains of *B. amyloliquefaciens* LL3 and DSM7 (Figure 4B). The phylogenetic analysis confirmed that the strain K2 was *Bacillus amyloliquefaciens,* and the general characteristics and features of K2 strain was in agreement with the *B. amyloliquefaciens* type strain shown in Supplementary Table 2. *Bacillus amyloliquefaciens* is one of the largest species of PGPR with multiple phytobeneficial traits. Some *B. amyloliquefaciens* such as NBRISN13 strain has been reported for having salt stress tolerance and plant growth promotion properties. (Chauhan *et al.,* 2019).

Effect of K2 and culture ltrates

The effect of culture filtrates of strain K2 and of 3-comparative strains on the inhibition of Xoo growth was shown in Table 2. In the agar plate assay, the inhibition zone against Xoo growth obtained from the culture filtrate of K2 grown in NB medium with or without NaCl (K2/CF and K2/CF+NaCl of 3.06 and 2.84 mm inhibition) was similar to that obtained from the culture filtrates of the

comparative strains grown under normal conditions (HS105, KPS46, SP007s of 2.98, 3.05, 3.11 mm inhibition respectively). Results indicated that K2 grown under salt stress condition was able to release some substances into the culture filtrates and had effect on inhibiting the growth of Xoo (Yoshida *et al.,* 2001).

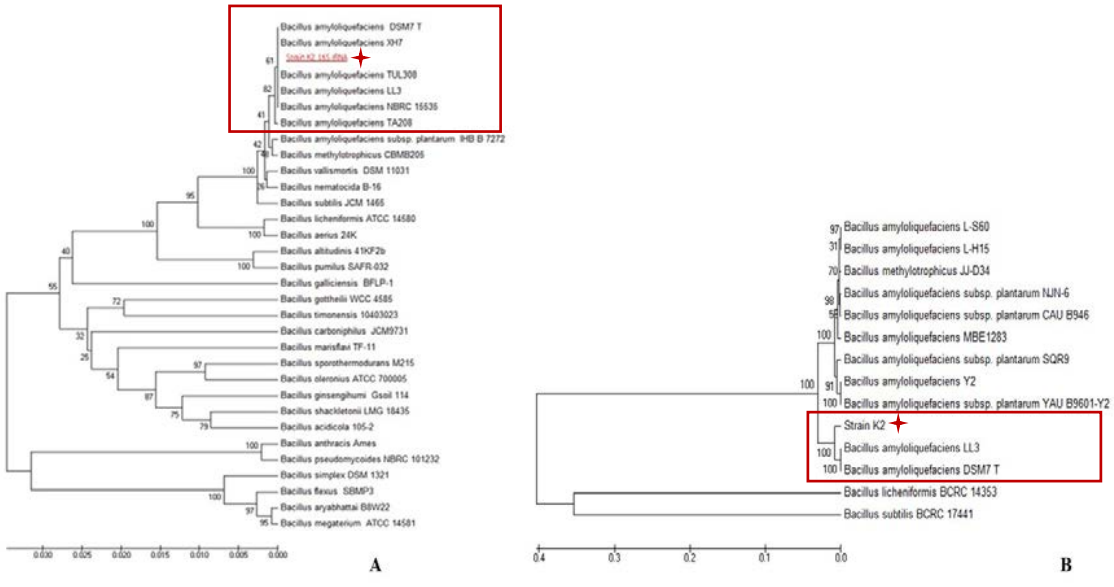
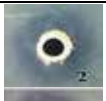
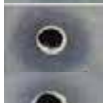


Figure 4 Phylogenetic analysis of the partial 16S rRNA (A) and *gyrB* (B) sequences of *Bacillus* sp. comparisons with Mega6, bootstrap values from UPGMA phylogeny based on 1,000 replicates. Strain K2 with asterisk and its highly closed relating strains are in bold

Table 2 Effect of culture filtrates of *Bacillus amyloliquefaciens* K2 on the inhibition of *Xanthomonas oryzae* pv. *oryzae* determined by agar diffusion method[1/]

Treatment[2/]	Xoo inhibition zone (mm)[3/]		Treatment[2/]	Xoo inhibition zone (mm)[3/]	
K2	3.04a		HS105/CF	2.98a	
K2/CF	3.06a		KPS46/CF	3.05a	
K2/CF+NaCl	2.84ab		SP007s/CF	3.11a	
K2/Cul	3.00a		dH_2O	0c	
C.V(%)	3.56				

[1/] Means in the same columns followed by a common letter are not significantly different at the 5 % by LSD

[2/] K2 = cell suspension (cells harvested at 24h culture), K2/CF = culture filtrate detected from K2-72h NB culture, K2/CF+NaCl=culture filtrate detected from K2-72h NB with 5%NaCl, K2/Cul = K2 grown cells with its NB culture, dH2O = distilled water. Culture *filtrates* of 3-comparative strains detected as the same methods at normal growth condition were also included in the experiment.

[3/] Diameter of inhibition zone is averaged from 3 replications

The culture filtrates from salt stress of K2 cells was used to promote rice seedling growth under salt stress conditions. They were able to increase root length and shoot height, although in a lesser extent comparing with K2 cell suspension (K2) indicated that the secreted proteins from K2 had efficacy to promote plant growth under salt stress. Growth of rice seedlings treated with K2 grown cells together with its NB liquid culture was not effective on promoting plant growth under both normal and salt conditions (Table 3). This result might indicate the toxic substances accumulated in NB grown cell cultures at lag phase condition used in the test that affected and decreased bacterial survival and activity. PGPR strain K2-culture filtrates had the highest ability to express multiple features to help plant toleranting to different stressness and promote plant growth at the same time. It possesses and expresses all main mechanisms of biocontrol activity and induces systemic resistance of plant compared its alone culture filtrate treatment (Prathuangwong and Buensanteai, 2007).We have to note that growth of rice seedlings KDML105 (root lengths and shoot height; Table 3) was greater enhanced by K2 strain inoculation than that of Pokkali under salt stress experiments. This result was in agreement with those reports that almost PGPR would better stimulated enhancement of plant health in sensitive than the resistant plant cultivars (Li *et al.,* 2020). Also, based on the evidence of plant-susceptible vs resistant responding to K2-inoculated seedlings of rice, we precisely confirmed the genetic background of KDML105 and Pokkali as moderately-tolerant and tolerant cultivars to high NaCl concentration levels respectively. This experiment showed K2 strain (K2 grown cells and culture filtrates) was able to promote rice seedling growth under salt stress. Thus, K2 was considered as a novel Salt-Tolerant Plant Growth Promoting Rhizobacteria (ST-PGPR). This study suggested that K2 might produce various types of phytohormones, such as auxins, gibberellins, cytokinins, secondary compounds (such as exopolysaccharides and osmolytes proline, trehalose, and glycine betaines), regulate plant defense systems, and activate plant's antioxidative enzymes under salt stress conditions (Tunsagool *et al.,* 2019).

Strain K2 increases inducible protein expression under salt stress conditions

Proteomic analysis of salt-responsive proteins in the cells of *B. amyloliquefaciens* K2 revealed that 5,830 proteins were identified in normal conditions, whereas 5,759 proteins were from K2 grown in NB supplemented with 5% NaCl. Analysis of specific proteins which expressed in the tested conditions showed that 2,397 proteins were only detected in salt stress, 2,326 proteins found in normal conditions, and 3,433 proteins were common in both conditions (Figure 5A). Under salt-stress, the identified proteins in cells of K2 were distributed in different biological process functions includeding carbohydrate transport and metabolism, signal transduction, and defense mechanisms (Figure 5B).

Table 3 Effect of *Bacillus amyloliquefaciens* K2 and its culture filtrates on growth enhancement of two- rice seedling cultivars (KDML105 and Pokkali) grown in normal and salt stress conditions[1/]

Treatment[2/]	Root length (cm)				Shoot height (cm)			
	KDML105		Pokkali		KDML105		Pokkali	
	N	S	N	S	N	S	N	S
K2	6.71bc	4.47c	6.72a	4.76ab	19.74a	13.74a	20.33a	14.07b
K2+NaCl	7.19a	4.95b	7.10a	5.13ab	19.88a	14.50a	20.01ab	14.92a
K2/CF	6.27cd	3.74d	6.08c	4.06cd	18.32b	9.76b	18.67c	11.87c
K2/CF+NaCl	6.95ab	4.06d	6.23b	5.18a	19.54a	8.58b	19.63b	13.78b
K2/Cul	6.04d	3.90d	6.21b	4.22c	17.2cd	9.08b	17.91d	11.96c
K2/Cul+NaCl	5.84d	4.39c	6.05c	4.59b	16.63d	8.00b	16.33e	13.53b
dH2O	5.93d	1.24e	6.0c	2.85d	17.86b	3.26c	16.71e	10.21d
GB	6.17cd	5.68a	6.06c	5.55a	17.27c	15.16a	18.21cd	15.52a
C.V(%)	17.35	17.93	13.36	17.01	6.01	16.72	6.40	10.71
LSD	0.754	0.649	0.643	0.581	0.643	0.612	0.568	0.612

[1/] Means in the same columns followed by a common letter are not significantly different at the 5 % level by LSD.

[2/] K2 = cell suspension (cells harvested at 24h culture), K2+NaCl = cell suspension (cells harvested at 24h NB with 5%NaCl culture), K2/CF = culture filtrate detected from K2-72h NB culture, K2/CF+NaCl=culture filtrate detected from K2-72h NB with 5%NaCl, K2/Cul = K2 grown cells with its NB culture, K2/Cul+NaCl = K2 grown cells with its NB culture with 5%NaCl, dH2O = distilled water, GB = glycine betaine. Seedlings growth conditions included; N = seedlings grown in WA without 0.9% added NaCl and S = seedlings grown in WA with 0.9%NaCl. 3/Growth development of root lengths and shoot height are averaged from 3 replications

In this study, the highest detected specific protein in K2 cells under salt stress condition was cysteine desulfurase, which is involved in Fe–S cluster biogenesis (Table 4). Both cysteine desulfurase and ferri-bacillibactin esterase are involved in iron scavenging, suggesting that K2 experienced iron limitation in salt stress. Iron is an essential nutrient for both bacterial metabolisms and plant growth. Bacilysin synthetase, *bac*D, which is involved in antibiotic bacilysin biosynthesis was also highly increased in the cells and in culture filtrate of K2 grown under salt stress suggesting that K2 was able to produce and secrete more the bacilysin when stressness was elevated (Table 4). Other secondary metabolites included surfactin and macrolactin were expressed at similar level in both normal and salt stress. Whereas bacillaene was not expressed under high NaCl concentration (Supplementary Table 3). In this study, only K2 strain was able to inhibit Xoo under salt stress compared to those 3-comparative strains. As already shown, K2 was obviously up regulated production of bacilycin in high NaCl concentration, and its culture filtrates comprising bacilysin showed highest improvement of Xoo suppression and plant growth enhancement. Thus, it could be postulated that salt induced the production of

bacilysin and this antibiotic might be the key antimicrobial compound of K2 under salt stress condition.

In addition, proteins related to sodium efflux (Na+/H+ antiporter NhaC protein), potassium uptake, ion transport, oxidative stress (SOS response-associated proteins, glutathione peroxidase, superoxide dismutase) and the synthesis and transport of compatible solutes (proline dehydrogenase, betaine-aldehyde dehydrogenase) were increased under salt stress conditions (Supplementary Table 3). Therefore, sodium ion efflux and hydrogen and potassium ion uptake help maintain osmotic balance of K2 under salt stress. Several molecular approaches to saline adaptation have been discovered in bacteria, including the accumulation of compatible solutes (Chen and Murata, 2011), potassium uptake, and sodium effluxion (Waditee *et al.,* 2005). We assume that K2 is like other bacteria in coping with unfavorable osmotic conditions, it rapidly taken up compatible solutes that can be accumulated to high levels in its cells. In this study, we found that both proline and betaine were highly accumulated in K2, suggesting that these compounds could serve as osmo-protective compounds of this new strain (Supplementary Table 3).

The proteins that involved in cell cycle control and cell division, co-enzyme transport and metabolism, and cell motility were reduced in K2 cells grown in salt stress conditions (Figure 5B). Cell motility and energy production also play critical roles in halotolerance of other *Bacillus* sp. (Amoozegar *et al.,* 2009). Flagellum-related genes have been found to be down-regulated in highly saline environment, suggesting that decreased cell motility allows more energy to be available for osmoprotection (Waditee *et al.,* 2005). This indicates that the growth and motility of K2 strain is impaired to allow more energy for its cell survival under high salt stress conditions (Figure 3).

Secreted proteins of K2 under salt stress conditions were determined using shotgun proteomic analysis. In total, 11 proteins were detected in culture filtrated of K2 grown under salt stress and 14 proteins were detected in normal condition (Table 4, Supplementary Table 4). Production of secondary metabolites, bacilysin synthetase that highly expressed in the cells of K2 was also detected in K2 cultures filtrated under salt stress tests, indicating that K2 strain was able to secrete bacilysin into the environment under salt stress conditions. Surfactin was also detected as secreted protein in salt stress but its quantity was lower than that in normal conditions. Several strains of *B. amyloliquefaciens* have been described to produce surfactin and reported on the antimicrobial activity which exposed to an outstanding surface active property in that the attachment is the important step to innitiate a benefit of successful plant-microbe interaction (Wu *et al.,* 2014). Our study showed an evidence that K2 secreted both bacilysin and surfactin under salt stress, this could explain the ability of this bacterium to inhibit bacterial pathogens and promote plant growth under

Table 4 Top 5 of expression proteins in *Bacillus amyloliquefaciens* K2 cells and its secretion proteins induced by salt stress

Protein family (gene)	Sequence	Gene function	Protein expression[1/]		Accession Number[2/]
			Control	Salt	
Expression proteins in K2 cells induced by salt stress					
Ferri-bacillibactin esterase BesA (BesA)	ALRFCLNPGGPHTPYSPS	Catalyzes the hydrolysis of the trilactone cycle of ferri-BB complex	17.0577	25.6328	A0A4V7TTK2
Bacilysin synthetase (bacD)	DLGGCPPHMFYKSAAEK	Catalyzes the ligation of L-alanine and L-anticapsin to produce the bacilysin antibiotic	18.0463	24.2381	A0A2H4RAP8
Iron complex transport system ATP-binding protein (yvr)	DGRALPK	ATPase activity	14.089	24.169	I2CA17
Putative carbamoyl-phosphate synthase large chain (carB)	AIGESTIHFLREEVAEPRLAPFAH QYEEFYQDTK	Carbamoyl-phosphate synthase (glutamine -hydrolyzing), metal ion binding	19.2108	23.6993	I2C240
Cysteine desulfurase (FVD42__19410)	GEELMHLLVEKAVGPVSFNIIHP ASNEAPTNIVVGDRTCR	Biosynthesis of Fe–S clusters, involved in cellular iron homeostasis and in the biosynthesis of selenoproteinss.	0	23.6959	A0A5C8IKH6
Secreted protein expression from K2 culture induced by salt stress					
Bacilysin D synthase (bacD)	QISEAVDNSNSK	Amino acid modication, biosynthetic process	20.7984	20.7728	Q8KWS8
Surfactin synthetase C (srfC)	INDEEIFDFACK	Amino acid modication, biosynthetic process	20.3543	14.8177	Q70KJ4
YuxL (yuxL)	DYTLSQINVPEYEDK	Protein metabolism	19.1806	12.3555	A0A4V7TSM9
DNA topoisomerase 4 subunit A (parC)	AYHTMER	chromosome segregation	20.2029	18.6215	A0A5C8IXT3
L-aspartate oxidase (nadB)	DVAVGAR	NAD biosynthetic process	21.0477	19.4948	A0A5C8IPK2

[1/]Log2 ratio from proteomics data. Control and salt = K2 cells grown in NB with (Salt) and without (Control) 5%NaCl respectively. 2/Protein accession number from UniprotKB.

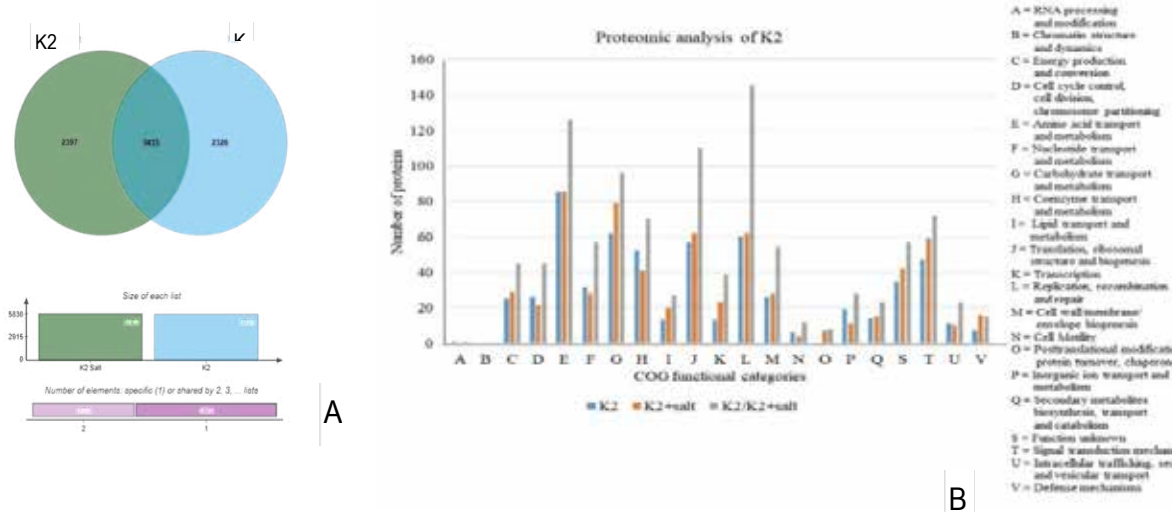


Figure 5 Proteomic analysis of *Bacillus amyloliquefaciens* K2 strain. A = Venn diagram showing numbers of detected protein expression commonly found in K2 cells grown in NB media with (K2 Salt) or without (K2) 5%NaCl. B = COG functional categories of expression proteins identified from K2 strain included; K2 = expressed proteins of K2 cells grown in NB, K2+salt = expressed proteins of K2 grown in NB with 5%NaCl, and K2/K2+salt = common expressed proteins of K2 in both normal and salt stress conditions (NB media with and without 5%NaCl)

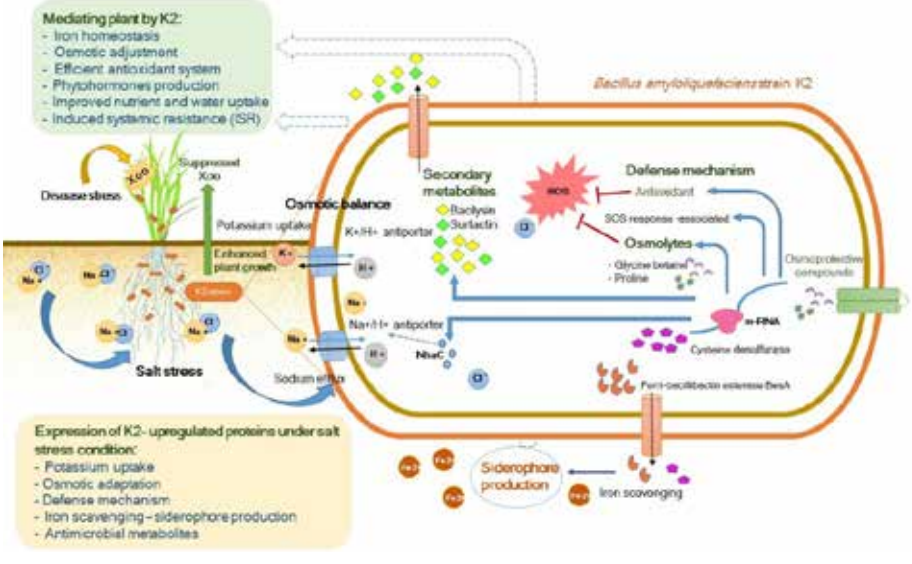


Figure 6 Schematic diagram summarizing a key role of proteins predominantly expressed in strain K2 that likely to mediate plant health against *Xanthomonas oryzae* pv. *oryzae* (Xoo) and salt stresses

high salt environment. In conclusion, this proteomic approach provides an overview and improvement of biocontrol mechanisms and cell adaptation to salt stress in a novel strain *B. amyloquefaciens* K2.

The data allowed us to summarize the adaptation mechanisms of K2 strain under salt stress and its molecular mechanisms to improve plant growth through the detection of specific proteins by analysis of the exoproteome and cellular proteome of this novel strain K2 which was constituted into model of protective proteins-induced salt tolerance (Figure 6). General stress proteins such as protein related to sodium efflux, potassium uptake, iron scavenging and transport help maintain bacterial osmotic balance, and proteins associated with protection against oxidative stress were produced by K2 under salt stress. These proteomes probably ensure proper functioning of other proteins under this condition. In response to increased salinity, the bacterial cell begin to uptake compatible solutes such as glycine betaine and proline betaine that increases their concentration in the cytoplasm to counteract the outflow of water under salt stress. This scenario shows the versatility of responses that a halotolerant organism can display and allow to understand how its efficient responses to environmental fluctuations and lead to an adaptive advantage for survival in those habitats. We found that strain K2 produced and secreted larger amounts of the antibiotic bacilysin and surfactin which probably involved in antimicrobial secondary metabolites to disease (BB) suppression. Obviously, a key role for proteins predominantly expressed in K2 is likely mediating plant health tolerated to BB disease and salt stresses through induced expression of iron homeostasis, osmotic adjustment and osmoprotectant compounds synthesis, up-regulation of efficient antioxidant system and phytohormones, improved nutrient and water uptake, and ISR in plant (Rubiano-Labrador *et al.,* 2015; Yang *et al.,* 2009).

CONCLUSION

The present study was initiated to isolate and characterize based adaptive-antagonistic bacteria with purpose to stimulate plant growth and suppress diseases in rice grown under salt stress. The targeting *B. amyloliquefaciens* strain K2 was highly-closed relating to PGPR type strains *B. amyloliquefaciens* LL3, DSM7, XH7, and TA208 confirming that the two-gene regions i.e. 16S rRNA and gyrB could be an efficient alternative target for identification and taxonomic analysis of *B. amyloliquefaciens* group members. Strain K2 thoroughly promoted plant growth enhancement of rice seedlings against biotic and abiotic stresses under normal and adverse environment in that high NaCl concentrations obviously repressed the development of plant seedlings. Rice seedlings cv. KDML105 the moderately salt tolerance however, was better response to K2 inoculation than Pokkali (cv. tolerance) confirming their apparent genetic backgroud in this test system.

Biocontrol activity in vitro and in vivo experiments showed the capability of K2-culture filtrates was increased plant growth promotion and suppressing pathogenic Xoo to a lesser extent than K2-grown cell suspension. These evidence demonstrated

the strain K2 produced essential and stress-responsive proteins to protect bacterial cells and promote plant growth development. The protective process in which K2 could be a well adaptive as halophilic bacterium habitant and synthesize a group of proteomic compounds mediated plant salt stress, Xoo, and K2 interaction (as a 4-way-interaction) is summarized in schematic diagram shown in Figure 6. However, additional studies on identification of K2-up-and down-regulated genes and host-plant gene expression by key proteins identified in this work should be confirmed further by mutagenesis analysis using protein-defective gene mutant, compared to K2 wildtype.

Links;

Supplementary Table 1-4

https://drive.google.com/le/d/15aA__26Syb6H1YpqQxIlHp9SRnsIWk0Rg/view?usp=sharing

Supplementary Figure 1-2

https://drive.google.com/le/d/1sxmE6XNX4R3LeCHjC1Vpc7-JcXplVGQt/view?usp=sharing

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