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Original Article

Expression and role of defense components in *Bacillus subtilis* treated rice plants against *Xanthomonas oryzae* pv. *oryzae*

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Abstract

Bacterial leaf blight (BLB) of rice has a high epidemic potential and usually causes severe damage. This research was conducted to assess the efficacy and characterize the mechanism of the systemic resistance of rice plants induced by the *Bacillus subtilis* strain CaSUT007 to BLB. The results revealed 30% reduction in the severity of BLB in the treated rice plants, and real-time PCR measurements indicated a significant 1.1–1.2-fold increase in their concentrations of the defense genes of phenylalanine ammonia-lyase (PAL) and ascorbate peroxidase (APX). In addition, Fourier transformed infrared spectroscopy characterization of the biochemical changes in the rice leaves indicated alterations to the lignins, pectins, and amide I vibrations - these lead to the generation of defense barriers and the reinforcement of cell walls against *Xanthomonas* infection and invasion, thereby contributing to disease reduction. Phylogenetic trees of *pal* and *apx* revealed a significant number of polytomies among these two gene families. Moreover, analysis of the active sites of the protein PAL and APX showed one serine rotamer and a single mutation-sensitive glutamic acid residue in the region of the binding site/pocket. The possible interactions of PAL and APX with other proteins revealed insight into the defense mechanism: APX6 interacts directly with MDAR5, MDRA3, DHAR1, and other important defense proteins, while PAL has direct interactions with 4CL4, 4CLL9, and 4CL3, among other defense proteins. Therefore, treatment with the *B. subtilis* strain CaSUT007 promoted faster, stronger and more intense responses in rice plants against BLB.

Keywords: *Bacillus subtilis*, Defense genes, Leaf blight, Protein interaction

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Introduction

The management of plant diseases is essential in ensuring that quantity and quality in food production meet the annually increasing demand for food security. Various types of disease management approaches can be adopted to promote resistance to or protection against diseases (He et al., 2021). The emergence of several new requirements in modern crop production in recent years has led to a change in the concerns and attitudes of both farmers and consumers toward chemical-based pesticides and produce safety. As suggested by agricultural scientists, biological controls using beneficial microorganisms constitute a sustainable approach to combating various plant diseases and an alternative solution to the use of synthetic agrochemicals, which has lead to an increase in the development of biopesticides. Several beneficial microorganisms, such as *Agrobacterium*, *Bacillus*, *Burkholderia*, *Trichoderma*, and *Pseudomonas*, have been utilized in this regard to characterize the biotic stress responses of host plants for controlling various diseases. These beneficial microorganisms also act as crutial promoters of plant growth and development (Rêgo et al., 2018; Gupta et al., 2021; Iula et al., 2021; Salifu et al., 2022). Iula et al. (2021) indicated that, in addition to the promotion of plant growth, treatment with *Trichoderma* could increase the activity of the phenylpropanoid biosynthetic pathway and lead to the accumulation of cytokinins, auxins, and jasmonic acid. Rice plants treated with *Bacillus pyrrocinia* or *Pseudomonas fluorescens* have displayed increases in biomass (88%), root biomass (67%), length (40%), leaf area (3%), and water use efficiency (63%) compared with controls (Rêgo et al., 2018). Shan et al. (2013) indicated that *B. methylotrophicus* displayed 89.9% biocontrol efficiency toward rice blast and colonized rice tissues after 10 days of its application (leaf tissues > stem tissues > root tissues). In another study, *Bacillus subtilis* HA1 culture filtrate helped increase the development of shoots and roots in tomato plants and enhanced the total phenolic (27%) and flavonoids (50%) contents (El-Gendi et al., 2022). Amongst beneficial microorganisms, agents based on the *Bacillus* genus have been the most useful for and applicable to biological controls and plant biomass production (Shafi et al., 2017). The *Bacillus* genus comprise aerobic gram-positive bacteria with a motile nature, and the optimal *in vitro* temperature and pH for their growth is 20–25 °C and 6.0–8.0, respectively (Sidorova et al., 2020). *Bacillus* species can produce bioactive compounds with a wide range of inhibitory activities toward phytopathogen and induce resistance mechanisms in host plants – although these require further elucidation - leading to a reduction in the severity of plant diseases. According to Yildirim et al. (2021), *Bacillus subtilis* Bs1 and *Bacillus mojavensis* ApBm strains can prime bell pepper seeds, enhancing germination and the emergence of bell pepper seedlings. The reduction in diseases achieved by using *Bacillus* spp. results from multiple modes of resistance activity within the host plants (Bathke et al., 2022; Choub et al., 2022). Specifically, plants consistently display innate and inducible defense characteristics, along with the expression of defense genes, a synthesis of defense-related carbohydrates, lipids, and proteins. The inducible defense reactions of host plants primed or enhanced by beneficial *Bacillus* species against pathogenic infections are more intense and occur more rapidly (Nie et al., 2017; Thumanu et al., 2017). Bacterial leaf blight (BLB) and its causal agent, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), present a significant problem for rice cultivation (Buddhachat et al., 2021; Khan et al., 2022); notably, BLB is destructive and difficult to control due to vascular invasion of rice plants. Sharma et al. (2017) reported that annual yield losses of 10–50% in moderate conditions and up to 100% in favorable ones due to BLB have been recorded in African and Asian countries. The method of induced resistance offers a promising alternative to bactericides for controlling BLB and plays an essential role in the integrated management of rice diseases.

A decrease in the size of lesions, or disease reduction, results in defense components during the defense reactions to pathogenic infections. The system of defense components consists of many layers, including those of defense genes (such as *pal* and *apx*), enzymes (such as PAL and APX), tissues (or the histopathological layer involving the superoxide anion and the hypersensitive response), and organic molecules (defense carbohydrates, lipids, and proteins). Defense gene expression has been investigated in several research studies on systemic resistance after applying beneficial *Bacillus* species. Wang et al. (2016) showed that treating tobacco plants with a type of *Bacillus* protein elicitor could activate the defense gene *pal* against *Botrytis cinerea* and *Tobacco mosaic virus*. Further, the *B. subtilis*

strain CBR05 can increase the activation of the defense genes *pal* and *β–1,3–glucanase* approximately three and two fold, respectively, resulting in resistance against tomato bacterial spot disease (Chandrasekaran et al., 2017).

Further, many systematic research studies have been conducted to further investigate the differences in defense carbohydrates, lipids, and proteins between treated and un-treated host plants underlying disease resistance mechanisms. Spectroscopy-based techniques could provide better sample throughput with reliable results. Fourier transformed infrared (FTIR) spectroscopy is a valuable analytical technique for detecting biochemical changes in cell defense compositions (Wang et al., 2018); it is inexpensive, non-destructive, and relatively less time-consuming than other methods, providing precise and unique biochemical information based on spectral data. FTIR spectroscopic measurements have revealed an increase in the lipids, lignin, pectin, and polysaccharides contents in chilli tissues pre-treated with *B. subtilis* compared with un-treated case. Moreover, the conversion of α–helices (peak at 1650 cm^{-1}) to β–sheets (peak at 1600 cm^{-1}) in the mesophyll tissues of the treated samples was been detected by FTIR spectroscopy (Thumanu et al., 2017). In another study, after the application of the abiotic elicitor SA, FTIR spectroscopic analysis revealed a change in the lignin, and pectin content in the rice leaf tissues as well as a high level of amide I β–sheet structures in the treated rice plants (Le Thanh et al., 2017). FTIR microspectroscopy has further indicated that the polysaccharides group gives rise to high spectral values in rice plants treated with SA (Thepbandit et al., 2021). Pectins, lignins, protein, and cellulose FTIR bands at 1650, 1735, and 1114 cm^{-1} have also been observed in rice plants treated with commercial BIG® (chitooligosaccharides) (Siriwong et al., 2021). Considering previous literature, FTIR spectroscopic studies for the detection of biochemical alterations in rice leaf cells after treatment with the biotic elicitor *Bacillus* can generally be considered novel.

In silico analysis or bioinformatics is a very powerful tool for analyzing the data related to systemic resistance mechanism against plant diseases (Papathoti et al., 2020). Analyzing the protein-protein interactions is crucial for a better understanding of the function of biological systems involved in disease resistance (Valente et al., 2013). In rice plants, *in silico* analysis has been used to successfully predict the resistance genes that encode receptor-like cytoplasmic kinases involved in *Magnaporthe oryzae* infection (Zheng et al., 2021). Furthermore, in rapeseed seeds, *in silico* analysis has been employed to predict the 3D structure of seed storage proteins with high binding energy to bacterial enzymes and demonstrating antimicrobial activity (Rahman et al., 2020). In addition, Papathoti et al. (2020) used the *in silico* approach to detect the role of SA in *Fusarium* control, showing that SA could interfere with the SKP1-CUL1-F-box complex, which plays a crucial role in protein-like interactions via hydrogen bonding. Finally, *in silico* research on induced resistance in *Bacillus*-treated rice plants presents a novel area of study.

The research objectives are to evaluate the reduction of BLB severity and understand the resistance responses of rice plants from the early gene expression (*pal* and *apx*); biochemical alteration of defense carbohydrates, lipids, and proteins; and prediction of the protein interactions of PAL and APX in rice plants after treatment with *B. subtilis* strain CaSUT007.

Material and Methods

Rice materials, pathogen inoculum, and beneficial *Bacillus*

Rice seeds of a susceptible cultivar KDML105; a virulent *Xoo* strain, SUT–122; and a beneficial strain, CaSUT007, of *B. subtilis* (*Bacillus*) were obtained from the Bio pesticide Laboratory, Institute of Agricultural Technology, Suranaree University of Technology, Thailand, and applied in this research.

Xoo and beneficial *Bacillus* were grown in nutrient broth (Merck, Germany) at 26 ± 2°C for 48 h, with shaking at a constant speed of 150 rpm for the log phase of bacterial growth. The bacterial suspensions were centrifuged at 10,000 ×g for 20 min and the precipitate was resuspended in sterile distilled water, adjusting the density to 10^8 CFU mL^{-1}. The optical density of the suspension was measured using a DeNovix DS–11 Spectrophotometer (DeNovix, USA) at a wavelength of 600 nm.

Rice cultivation, induction treatment, and pathogen inoculation

A total of 100 g of rice seeds was soaked in a solution of 95% ethanol (v/v) in a glass beaker for 2 min to surface-sterile them and then washed twice with sterile distilled water and soaked in distilled water overnight. Subsequently, the water was drained, and the seeds were mixed with the freshly

prepared *Bacillus* suspension (Sha et al., 2016), pre-incubated on wet Whatman filter paper for 24 h. The seeds were then planted in pots containing soil with added organic fertilizer. The rice pots were placed in a net house with a natural regime and relative humidity of approximately 65–80%. The foliage of the *Bacillus*-treated rice plants was further sprayed three times with a fresh *Bacillus* suspension at 15-day intervals. The *Bacillus*-non treated rice seeds and plants were treated similarly but with distilled water. After growing the rice plants for 50 days, six fully matured leaves per pot were selected and inoculated with a prepared suspension of *Xoo*, using the leaf-tip-cutting technique (Kim et al., 2016). Thereafter, the plants were incubated under conditions favoring *Xoo* infection namely a relative humidity of 95% at 25 ± 2°C for 24 h. After treatment with the pathogen, the rice plants were transferred into a net house.

Assessment of disease reduction in *Bacillus* treated rice plants

The experiment involved a completely randomized design (CRD) with two treatments including the *Bacillus*-treated and *Bacillus*-non treated samples, five replications, and six mature leaves of two rice plants for each replication. The experiment was repeated three times. After the *Xoo* inoculation, the lesion length from the inoculated tip was recorded to assess the progression of BLB. The disease severity of BLB (DSBLB) was recorded three times at 7-day intervals using the following disease scale: 0 – no symptom at leaf cut position; 1 – discoloration at leaf cut position; 2 – BLB lesion length less than 15 mm; 3 – BLB lesion length less than ¼ leaf length; 4 – BLB lesion length at ¼-½ leaf length; 5 – BLB lesion length more than ½ leaf length; 6 – BLB lesion covers most area of the leaf; and 7 – dead leaf (Ezuka and Horino, 1974). The reduction percentage (RP) of DSBLB was calculated according to the equation: $RP = [(DSBLB_{Bacillus\text{-non treated}} - DSBLB_{Bacillus\text{-treated}})/DSBLB_{Bacillus\text{-non treated}}] \times 100\%$.

Survey on expression of defense-related genes (*pal* and *apx*) in *Bacillus*-treated rice plants

The experiment was conducted using a CRD, with *Bacillus*-treated and *Bacillu* -non treated samples, five replications, six mature rice leaves per replication. After the pathogen inoculation, rice leaf samples were collected at three observation time points (0, 12 and 24 hours after inoculation [HAI]), immediately frozen in liquid nitrogen, and then stored at –80 °C in a refrigerator. Later, 100 mg of the rice samples was ground with a sterile chilled pestle and mortar to extract RNA by using the QIAGEN RNeasy® Plant Mini Kit (QIAGEN, MD, USA). The obtained RNA was then evaluated using a DeNovix DS–11 spectrophotometer (DeNovix, USA). For each sample, 100 ng of RNA was measured and used in a reverse transcription PCR (RT-PCR) experiment to survey the expression of the defense-related genes *pal* and *apx*. The specific oligonucleotide primers used in the present research have been described by Peng et al. (2011), with an internal *actin* gene. The primer sequences included *pal* (F5'-AGTACTTGACCGGGGAGAAGA-3' and F5'-GGCATCGTAACTTCCAAAGAAC-3'), *apx* (F5'-AAATACTGGAGCCTCATGGAGA-3' and F5'-AGTTCTATGCTTTGACCCTTGG-3'), and *actin-1* (F5'-GGCACCACACCTTCTACAATGAG-3' and R5'-ACACCATCACCAGAGTCAAGCA-3'). In an RT-PCR cycle, 50 µL of the reaction mixture was prepared with the QIAGEN® OneStep RT-PCR Kit, following the protocol of the manufacturer (QIAGEN, MD, USA), using a BioRad MJ Mini Personal Thermal Cycle (Singapore). Subsequently, 2.5 µL of the RT-PCR product at was gently mixed with 0.5 µL of 6X DNA Loading Buffer (Gene DireX's Novel Juice, USA) and loaded into one well of a 1.0% (w/v) agarose gel (UltraPure™ Agarose, Invitrogen, Spain). Electrophoresis was performed in 1X TBE buffer at 100 V for 20 min, using an electrophoresis system (BioActive Co., Japan). The bands on the electrophoresis gel were visualized under UV light using a GelDoc-It2 Imager (Ultra-Violet Products Ltd., UK). This experiment was technically performed in triplicate (repeating the measurements for each sample three times), and bands were quantified using Image J software.

Characterization of biochemical changes in *Bacillus*-treated rice leaves

The experiment was conducted using a CRD with *Bacillus*-treated and *Bacillus*-non treated samples, five replications, and one leaf per replication. The measurements were performed in triplicate. Leaf samples were collected 14 days after inoculation (DAI) and their biochemical alterations were characterized by FTIR, following the methods described by Le Thanh et al. (2017) and Thumanu et al. (2017). The mean of the five replications was then used to generate graphs, using the Origin 6.0 software (Origin Lab Corporation, USA).

In silico analysis of protein PAL and APX

ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to translate and analyze the open reading frames (ORF). The SMART program (http://smart.embl-heidelberg.de/) and the National Center for Biotechnology Information (NCBI) Conserved Domains Database (CDD; http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) were used to find the conserved domains of PAL and APX. The software tools of the ExPASy software (http://us.expasy.org/tools) were utilized to predict the basic chemical and physical features of these proteins, and the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) was applied to estimate the cleavage sites of the signal peptides. The TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used to predict the transmembrane helices in PAL and APX, and the subcellular localization of the proteins were predicted using the PSORT program. The secondary structures of the proteins were analyzed using GOR IV software (https://npsa-prabi.ibcp.fr/cgi-bin/npsa automat.pl?page=/NPSA/npsa gor4.html), and their 3D models were predicted using SWISSMODEL software (http://swissmodel.expasy.org/).

Prediction of protein–protein interaction network of PAL and APX

The rice genes *pal* and *apx* were scored through transcriptomics data and genome-wide expression. The interactions of proteins with PAL and APX were investigated using a protein–protein interaction network built with the stringApp Cytoscape plug-in. In Cytoscape, the network was further characterized using NetworkAnalyzer software.

Statistical analysis

Data from the experiments were analyzed and subjected to an analysis of variance (ANOVA) test using the Statistical Package for the Social Sciences (SPSS), version 20 (IBM Corporation, USA). Mean significant values were determined by Duncan's multiple range test or t-test at p = 0.05.

Results

Disease reduction in *Bacillus*-treated rice plants

Seed and foliar application of the elicitor from fresh *Bacillus* effectively reduced BLB in the foliage of the rice cultivar KDML105, confirming the efficiency of the induced resistance. The results indicate that at 7, 14, and 21 DAI, the *Bacillus*-treated plants exhibited low BLB severity (8.1, 23.8, and 27.1%, respectively) under highly disease-conducive environmental conditions compared with the *Bacillus*-non treated plants (10.8, 33.3, and 39.1%, respectively). Ultimately, a 30% reduction in BLB severity was observed in the *Bacillus*-treated rice plants at 21 DAI (Table 1).

Table-1: BLB severity and its percentage decrease in rice leaves treated with *B. subtilis* strain CaSUT007

Treatments	Severity of BLB[1/] (%)			Percentage decrease of BLB severity (%)		
	Days after *Xoo* inoculation			Days after *Xoo* inoculation		
	7	14	21	7	14	21
Bacillus-treated	8.1 ± 4.0[b]	23.8 ± 4.5[b]	27.1 ± 4.6[b]	24.7	28.6	30.5
Bacillus-non treated	10.8 ± 1.1[a]	33.3 ± 4.1[a]	39.1 ± 8.0[a]			
Significance	*	*	*			
Coefficient of Variation (%)	16.3	5.3	4.1			

[1/] Mean ± SE (standard error) followed by the same letter ("a" or "b") indicates no significant difference according to t-test at p = 0.05.

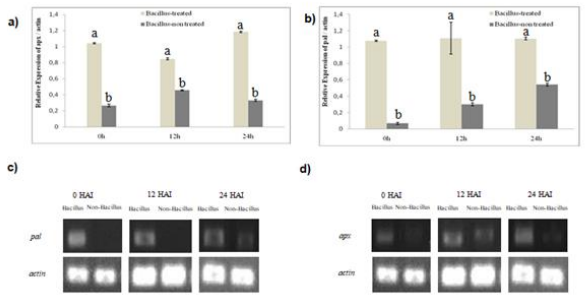

Figure-1. The relative expression of the (a, c) ascorbate peroxidase (*apx*) and (b, d) phenylalanine ammonia-lyase (*pal*) genes in the *Bacillus*-treated and *Bacillus*-non treated rice plants compared with the *actin* control gene at 0, 12, and 24 HAI.

The expression of *pal* and *apx* genes in *Bacillus*-treated rice plants

The effectiveness of the treatment with the *Bacillus* elicitor in protecting rice plants against BLB was characterized by inducing defense genes. In the challenge inoculation with *Xoo*, the *Bacillus*-treated rice plants displayed a significant 1.1–fold increase in *apx* activity at 0 HAI and a maximum expression (1.2–fold increase) at 24 HAI, while the *apx* activity

of the *Bacillus*-non treated control showed a 0.3–0.5 fold decrease within 0–24 HAI (Figs. 1a and 1c). Regarding the expression of the *pal* gene, the *Bacillus*-treated rice plants exhibited a significant 1.1–fold increase in activity, which was maintained up to 24 HAI. On the other hand, at 24 HAI, the *pal* activity of the *Bacillus*-non treated control showed 0.6–fold decrease (Fig. 1b and 1d).

Biochemical changes in *Bacillus*-treated rice leaves

The Fig. 2 shows the original and second-derivative average FTIR spectra of the biochemical substances in the KDML 105 rice leaves within the two ranges of 3000–2800 and 1800–900 cm^{-1}. Fig. 2c presents the second-derivative spectra of the *Bacillus*-treated and *Bacillus*-non treated rice leaves in the range of 3000–2800 cm^{-1}. The peaks at 2920 and 2851 cm^{-1}, both assigned to C–H stretching vibration, reveal the different characterizations of the spectra of the *Bacillus*-treated and *Bacillus*-non treated rice leaves in Fig. 2d. The biochemical changes in the *Bacillus*-treated leaves led to significant vibrational peaks in the FTIR spectrum at 1319, 1105 and 991 cm^{-1} compared to the spectrum of the *Bacillus*-non-treated control. These C–O stretching bands correspond to the C-O bonds in hemicelluloses and lignins, at 1319 cm^{-1}; the C–O–C bonds in glycosides, at 1105 cm^{-1}; and the C–O bonds in sucrose, at 991 cm^{-1}. In addition, the α–helix structure (1647 cm^{-1}) of the amide structure within the *Bacillus*-treated leaves was transformed into a β–sheet one (1634 cm^{-1}). However, the band of the α–helix structure is more intense within the *Bacillus*-non treated control (Fig. 2d).

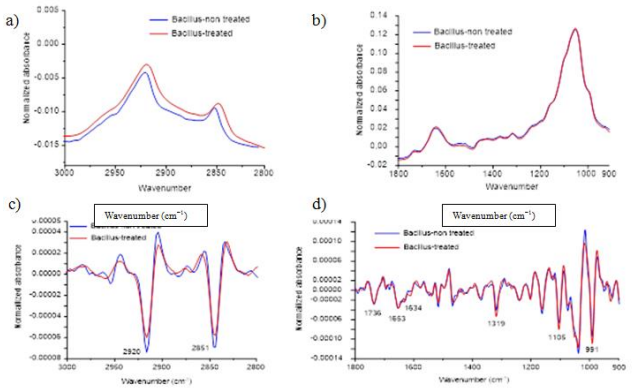

Figure-2. The (a, b) representative original and (c, d) second-derivative average FTIR spectra of the treated and non-treated rice leaves at 14 DAI in the range of (a, c) 3000–2800 cm^{-1} and (b, d) 1800–900 cm^{-1}

Prediction of protein structure of PAL and APX

The protein structure of PAL had a high confidence level and a model quality score of 0.76. The target coverage of the protein structure model was calculated as 92%, the root mean square deviation as 0.48, and the template modeling score as 0.74. The ligand-binding site residue with the highest number of contacts was predicted to be tryptophan for PAL (Fig. 3).

The structure of the l-PAX protein was the best predicted, with the highest confidence level. The structure had a maximum target coverage of 85%, a root mean square deviation of 0.55, and a template modeling score of 0.85. The most likely ligands at the binding site were predicted to be cadmium ions. Among the ligand-binding residues for the APX protein were tryptophan, glycine and glutamic acid (Fig. 3).

The findings related to the PAL and APX active sites indicate that the binding pocket region had a rotamer (serine) and a mutation-sensitive glutamic acid residue. The catalytic residues in APX were predicted to be glutamate and aspartate.

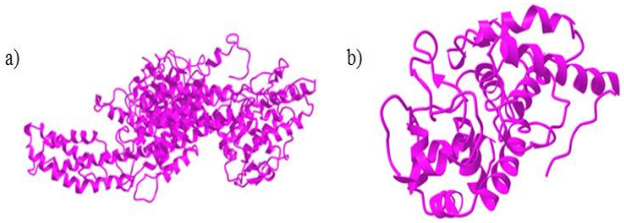

Figure-3. The protein structures of PAL (a) and APX (b) structure

Protein interactions of PAL and APX

The study revealed the possible interactions of PAL and APX with other proteins, indicating their roles in the defense mechanism. PAL has direct interaction with 4CL4, 4CLL9, 4CL3, and other important defense proteins, while APX6 interacts directly with MDAR5, MDRA3, DHAR1, and other proteins involved in the defense mechanism (Fig. 4).

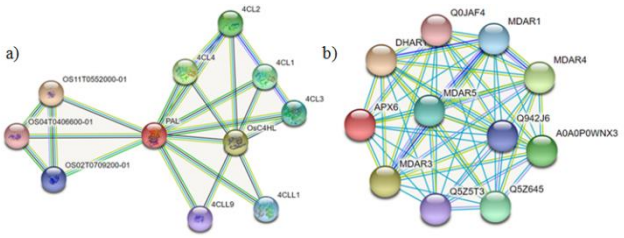

Figure-4. Protein interactions of PAL (a) and (APX (b)

Gene ontology terms for PAL showed a motif association with pollen exine formation and the phenylpropanoid, organic cyclic compound, and cellular aromatic compound metabolic processes. The molecular function includes 4–coumarate–CoA ligase activity, CoA–ligase activity, acid-thiol ligase activity, ATP binding, and catalytic activity of the *pal* genes.

Gene ontology analysis of APX revealed another motif association with L–ascorbic acid synthesis, response to cadmium ion, cellular oxidant detoxification, hydrogen peroxide catabolic process and protein glutathionylation.

The molecular function includes ion binding, oxidoreductase activity, flavin adenine dinucleotide binding, antioxidant activity, monodehydroascorbate reductase activity, glutathione dehydrogenase (ascorbate) activity, heterocyclic compound binding, glutathione transferase activity and organic cyclic compound binding of the *apx* gene.

Discussion

Rice plants have an immune defense system with resistance against a wide range of phytopathogens, including *Xoo*. In addition, this defense system can be activated, or enhanced to lead to stronger and faster defense reactions. The two popular and commonly researched types of resistance induction are induced systemic resistance (ISR) and systemic acquired resistance. From these, ISR can be elicited by beneficial microorganisms, such as *Bacillus*, living on the rhizosphere and phyllosphere, leading to an array of defense mechanisms against infection and the invasion of bacterial pathogens (Kumar et al., 2012). In the present studies, rice plants treated with a fresh suspension of *B. subtilis* strain CaSUT007 showed systemic resistance against BLB, with disease reduction at approximately 30%. The results are in line with the studies of Neher et al. (2009), Ji et al. (2014), Shakeel et al. (2015), and Ahmad et al. (2019) on cucumbers, cantaloupes, rice, *Arabidopsis*, and peanuts. Neher et al. (2009) reported that the anthracnose severity in cucumber and cantaloupe reduced by 41 and 24%, respectively, after treatments with *Bacillus* spp. According to Ji et al. (2014), seed soaking with an endophytic *Bacillus* species significantly improved the height, dry weight, and disease resistance of rice plants. Previous reports have also stated that *B. cereus* can activate the mechanism of induced resistance in *Arabidopsis* plants against *Botrytis* leaf spot, leading to smaller necrotic lesions and fewer yellowing symptoms (Nie et al., 2017). Beneficial rhizobacteria strains of *Bacillus* sp. and *B. cereus* have also been economically advantageous in managing the rice pathogens *Pyricularia oryzae* and *Fusarium moniliforme*, suppressing them by approximately 22–29% and producing different biocontrol determinants under *in vitro* conditions (Shakeel et al., 2015). Beris et al. (2018) showed that a SA signaling pathway was activated due to foliar and soil amendment applications of *B. amyloliquefaciens*, leading to elevated responses to an invasion of *Potato virus Y* and *Tomato spotted wilt virus*. Further, Ahmad et al. (2019) showed that in peanuts, *B. subtilis* could be applied as a bioagent in controlling several soil borne diseases including *Rhizoctonia solani* and *Slerotium rolfsii*. The results of the present research indicate that ISR brought about by *Bacillus* may give rise to various defense properties, which could originate from genetic, physiological, biochemical, or structural changes within the rice plants during the process of *Xoo* attack. These biotic stress responses of rice plants are addressed in the following discussion.

The colonization of rice plants by a benign and eco-friendly biological agent such as *B. subtilis*, using the natural process of bio-priming leads to broad-spectrum defense activities in the treated plants (El-Borollosy and Oraby, 2012). Among the numerous types of microbial determinants, the O-antigen side chains of lipopolysaccharides and cyclic lipopeptides (LPs) in the outer membrane in *Bacillus* act as biochemical signal molecules, resulting in the production of different defense compounds and significant ISR elicitors. In plants, the manner of receiving external signals from *Bacillus* and transducing, integrating these specific signals into their target genes may involve a multi-step phosphorelay pathway (Huo et al., 2020). The induced signals could activate defense or stress-responsive genes including *pal* and *apx*. The present work revealed an increased expression of *pal* and *apx* in the *Bacillus*-treated rice leaves at the three observation time points of 0, 12, and 24 HAI. Several similar research studies have supported the enhanced expression of *apx* and *pal* in the induced resistance in plants. In the current study, the expression of *pal* in rice plants showed a 1.1–fold increase, lower than the *pal* gene activity (two fold increase) in tomato plants treated with *B. subtilis* strain CBR05, as reported by

Chandrasekaran et al. (2017). The production of both *pal* and *apx* sharply increased in pigeon peas after treatment with *B. cereus* strain BS03, proving that ISR limits the severity of *Fusarium* wilt (Dutta et al., 2008). Latha et al. (2009) showed that tomato seed treatment and foliar sprays with *B. subtilis* induced the high activity of defense enzymes including PAL and APX, and increased disease resistance, leading to the control of *Alternaria* blight. In another study, *B. amyloliquefaciens* LJ02FB was found to stimulate cucumber seedlings to elevate the production of *pal* and *apx* compared with the un-treated control (Li et al., 2015).

Most previous studies have evaluated the efficacy of endophytic *Bacillus* and detected the expression of several pathogen-defense genes or enzymes (Sha et al., 2016). However, the mechanism of ISR regarding the changes in defense carbohydrates, lipids, and proteins within bacteria-treated rice plants is not correctly understood. The present research is the first to characterize the biochemical changes in the cells of *Bacillus*-treated rice plants inoculated with *Xoo*, using FTIR spectroscopy. In Raj et al. (2012) study on pearl millet plants, treatment with *B. pumilus* strain INR7 importantly increased lignin, callose, and H_2O_2 deposition, leading to resistance to downy mildew. The present research yielded similar results to those of previous studies by Thumanu et al. (2017), on the biotic elicitor *Bacillus*, and Le Thanh et al. (2017), on the abiotic elicitor SA.

The target proteins PAL and APX were further studied with regard to their interactions with other proteins to achieve a better understanding of the molecular processes underlying systemic resistance. The organic compound binding to APX molecules could play an important role in the biochemical changes of proteins and carbohydrates in rice plants treated with the elicitor *Bacillus*. Jiang et al. (2016) found that the APX OsAPX8 on rice thylakoid membranes was responsible for in charge of the resistance/tolerance to BLB in rice plants. The second target protein in this study, PAL, showed evidence of being linked to the defense responses of rice plants. An important biological process of PAL is organic cyclic compound metabolism process and the CoA–ligase activity of PAL molecules supports the biochemical defense changes within *Bacillus*-treated rice leaves. According to Xu et al. (2020), 4–coumarate–CoA ligase helps in the cold acclimation of tobacco plants (abiotic stress in plants).

Conclusions

In this research, the mechanism of systemic resistance in rice plants induced by *Bacillus* was investigated with regard to the expression of the defense genes *pal* and *apx*, and the biochemical changes in proteins and carbohydrates. *In silico* analysis indicated the possible interactions of PAL and APX with other proteins playing a role in the defense mechanism. Extensive research needs to be conducted regarding the field assessment; a bio formulation of the *Bacillus* elicitor could then be developed as a natural, eco-friendly, biocompatible, and cost-effective product.

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