EFFECTS OF NITROGEN LEVELS ON THE EXPRESSION OF *OSSWEET* GENES AND THE RESISTANCE TO *XANTHOMONAS ORYZAE* PV. *ORYZAE* IN BACTERIAL BLIGHT SUSCEPTIBLE AND RESISTANT RICE CULTIVARS

WACHIRAYA KAMHUN

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Agricultural Biotechnology
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By Wachiraya Kamhun

has been approved by the Graduate School as partial fulfillment of the requirements

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Academic Paper M.S. Thesis in Agricultural Biotechnology, Naresuan University, 2023

Keywords

ABSTRACT

Two prominent Thai rice varieties, namely Phitsanulok 2 (PSL2) and RD47, have historically been extensively cultivated in the lower northern region of Thailand. Unfortunately, their susceptibility to bacterial blight (BB) disease caused by Xanthomonas oryzae pv. oryzae (*Xoo*) has led to significant yield losses in this area. The spread and severity of BB disease are also influenced by farm management practices. This research aimed to investigate the impact of varying nitrogen fertilizer levels on both the severity of BB disease and the expression of the Sugars Will Eventually be Exported Transporters 11 (*SWEET11*) and *SWEET14* genes in the rice cultivars RD47, PSL2, and IRBB21.

Rice plants were subjected to inoculation with *Xoo* isolate *Xoo*16PK002, which was obtained from the Phitsanulok province. A direct correlation between BB disease severity and increasing nitrogen concentrations, ranging from 16 kg/rai to 36 kg/rai, was observed. Fourteen days after infection (dai), the BB-resistant cultivar IRBB21 exhibited the highest resistance against *Xoo*16PK002, while the PSL2 and RD47 cultivars proved to be completely susceptible.

Differential expressions of *SWEET11* and *SWEET14* were detected in rice plants subjected to varying nitrogen levels during the early stages of plant development. Notably, the expression of *SWEET11* displayed a substantial increase at 48 and 72 hours post-infection (hpi), while the expression of *SWEET14* remained undetectable. These expression patterns suggest that *Xoo*16PK002 harbors the TAL effector PthXo1, which specifically targets the Effector Binding Elements (EBEs) of *SWEET11* but not those of *SWEET14*, within the indica rice cultivars RD47, PSL2, and IRBB21.

ACKNOWLEDGEMENTS

First and foremost, I would like to give my greatest thank you to my advisor,

Assistant Professor Dr. Tepsuda Rungrat, co-advisor, Associate Professor Dr. Kumrop Ratanasut and Assistant Professor Dr. Thanita Boonsrangsom for their guidance, valuable advice, and insight throughout the length of the study.

I would like to give my appreciation to Assistant Professor Dr. Kawee Sujipuli and Assistant Professor Vaiphot Kanjoo for all their comments on the thesis.

I, especially, want to thank my family for their endless love, understanding and support. I thank my friends for their friendship and encouragement throughout the period of the study.

This work was partially supported by grants from Naresuan University, Thailand and a postgraduate grant (R2565B007) from National Research Council of Thailand.

Finally, I would like to express my sincere thanks to all others involved not being mentioned for their supports enabling me to complete this thesis.

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CHAPTER I

INTRODUCTION

Background of the Study

Rice (*Oryza sativa* L.) is an important economic crop for both domestic consumption and export to the world market. From the Office of Agricultural Economics data in the year 2018/2019, in the northern region of Thailand, the cultivated area is 13.81 million rai, the yield is 7.84 million tons of paddy, an average yield of 577 kg/rai (Office of Agricultural Economics, 2019), which is considered to be a relatively low yield. Due to the potential of the species invasion of diseases and pests of rice, improper fertilizer use for rice varieties. Disease attack such as Rice Blast Disease, Bacterial Blight disease. One disease that has an epidemic in the lower northern of Thailand. Resulting in fewer products is Bacterial Blight disease. Bacterial Blight is a widespread vascular rice disease caused by *Xanthomonas oryzae* pathovar *oryzae*. It causes of seedlings yellowing and drying of leaves leading to yield loss. The infestation of the pathogen will result in a 20-50% reduction in rice yields (Ou, 1972). The decrease in rice yields can be up to 80% when the epidemic is severe (Perumalsamy et al., 2010). Over application of nitrogen fertilizer will exacerbate disease incidence, which could result in more leaf growth that was over succulent and more susceptible to certain diseases (Reddy, 1979). In 2016, the incidence of leaf blight disease was quite high when using increased nitrogen fertilizer rates (Sharma, 2016). The low amount of nitrogen fertilizer (4 kg/rai) had the lowest effect on the severity of the disease, while it decreases yield (Chaudhary et al., 2009).

Signaling pathway triggered by *Xoo* in rice. Molecular studies have found that *Xoo* injects TALEs into the rice cell. TALEs directly bind to specific DNA sequences of rice, named EBEs, these are located on either strand in promoter regions and bidirectionally drive expression of host target genes, bacteria use TAL effectors that bind to the promoter regions of susceptibility (S) genes.

Nitrogen is an essential mineral nutrient for normal plant growth. Excessive use of N fertilizers could result in more leaf growth that was over succulent and more

susceptible to certain diseases. In 2001 It was found that BB disease incidence considerably with increasing rate of N fertilizer.

Molecular studies have shown susceptibility to leaf margin pathogens occurring in rice depending on the specific factor of *Xoo*, known as the Transcription Activator-Like (TAL) effector (Hutin et al., 2015) which induces rice effector-binding element (EBE) of susceptibility gene expression (Boch et al., 2009; Moscou and Bogdanove, 2009). Several TAL effectors play a key role in the severity of bacterial infections (Boch & Bonas, 2014). The severity of *Xoo* infection largely depends on the individual TAL effector (White and Yang, 2009), which has different EBE specificity. Two rice *Xoo* susceptibility genes, *OsSWEET11* and *OsSWEET14*, were activated by the TAL effectors of several *Xoo* strains (Chu et al., 2006; Yang et al., 2006; Antony et al., 2010; Chen et al., 2012). The severity of *Xoo* depends on the activation of the SWEET genes (Yang et al., 2006; Antony et al., 2010; Liu et al., 2011; Streubel et al., 2012; Li et al., 2013). Breeding rice susceptible to blight through regulating the expression of SWEET genes, especially *OsSWEET11* and *OsSWEET14*, in combination with appropriate amount of nitrogen fertilizer is one approach to develop and improve the resistance characteristics of blight disease in susceptible rice varieties.

The study of the correlation between the expression of *OsSWEET* genes and the amount of nitrogen fertilizers on the severity of blight disease would provide insight in the nitrogen-based on BB susceptibility for the development of the BB susceptible cultivars through manipulation of *OsSWEET11* and *OsSWEET14* genes.

Objectives

1. To analyze the expression of *OsSWEET11* and *OsSWEET14* in susceptible rice (RD47 and PSL2) and resistant (IRBB21), responding to different nitrogen levels in the *Xoo*16PK002 isolate.

2. To study the effect of nitrogen fertilizers on virulent of bacterial blight disease.

Scope of research

1. Study and analyze the expression of *OsSWEET1* and *OsSWEET14* genes with different nitrogen fertilizer content in rice varieties RD47, PSL2 and IRBB21.

2. Study the interaction of nitrogen fertilizer levels at 12 kg/rai, 24 kg/rai and 36 kg/rai. with sucrose content in rice leaves in varieties RD47, PSL2 and IRBB21.

3 . To study the severity of blight incidence related to nitrogen fertilizer content. and sucrose levels in rice leaves.

Terminology

1. **EBE** Effector-Binding Elements. It is a specific region on the promoter of the *OsSWEET* gene that is TAL-specific. When activated by TAL, it induces gene transcription.

2. ***OsSWEET*.** It is *Oryza sativa* Sugars Will eventually be Exported Transporters, a group of genes responsible for the transport of sugar in rice. and is a weak gene (susceptibility (S) genes) against blight

3. **TAL** refers to the Transcription Activator-Like (TAL) effector protein that *Xoo* releases into rice cells by binding to Effector-Binding Elements (EBE) to stimulate the transcription of the *OsSWEET* gene that promotes *Xoo* ability to invade plants.

4. **RD47** refers to Indica rice cultivar RD47 that is not photosensitive. High yield, average 793 kg per rai. good physical seed quality the seeds are long and slender, the belly is small, the color quality is good to very good. good stability It was somewhat more resistant to brown planthopper than RD41 and somewhat more resistant to blast disease than Phitsanulok2. Susceptible to bacterial blight for planting in irrigated fields in the lower northern region.

5. **PSL2** means Phitsanulok 2 Indica rice. not sensitive to light Productivity is high, about 807 kg per rai. and stable in yielding brown planthopper resistance white-backed planthopper and green leafhoppers good paint quality seed belly can be grown in all regions in irrigation areas, susceptible to bacterial blight.

6. **IRBB21** refers to indica non-glutinous rice developed from wild rice *O. longistaminata* and *O. sativa* cultivar IR24. IRBB21 is a standard rice variety with *Xa21* resistance gene.

7. **Gene expression**. It is the process by which the genetic code, the nucleotide sequence of a gene, is used to direct protein synthesis and produce the structures of the cell. Genes that code for amino acid sequences are known as “structural genes” The process of gene expression involves two main stages: Transcription, the production of messenger RNA (mRNA) by the enzyme RNA polymerase, and the processing of the resulting mRNA molecule; and Translation, the use of mRNA to direct protein synthesis, and the subsequent post-translational processing of the protein molecule. (Genetics for Higher Education, University of Leicester, http://wwzxaw2.le.ac.uk) gel faster than large ones. When a gel is stained with a DNA-binding dye, the DNA fragments can be seen as bands, each representing a group of same-sized DNA fragments (Biotechnology, Khan Academy, www.khanacademy.org).

8. **Real Time- Quantitative Polymerase Chain Reaction (qRT-PCR)**. It is a major development of PCR technology that enables reliable detection and measurement of products generated during each cycle of PCR process. It monitors the amplification of a targeted DNA molecule during the PCR, i.e., in real-time, and not at its end, as in conventional PCR. Real-time PCR can be used quantitatively (quantitative real-time PCR), and semi-quantitatively, i.e., above/below a certain amount of DNA molecules (semi quantitative real-time PCR). Two common methods for the detection of PCR products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence (https://www.ncbi.nlm.nih.gov).

Hypothesis of the Study

1. Rice varieties RD47 PSL2 and IRBB21 have the same *OsSWEET11* and *OsSWEET14* genes, but their response to *Xoo* is different between susceptible (RD47, PSL2) and resistant (IRBB21).

2. Nitrogen fertilizer levels influenced the severity of blight and *OsSWEET* gene expression.

CHAPTER II

LITERATURE REVIEW

Important rice cultivars in lower Northern Thailand

Rice is the major staple food and uses over half of the arable land and labor force in Thailand. Thailand has exported rice worldwide and was ranked by the US Department of Agriculture (USDA) to be the world's No. 2 rice exporter (7.5 million tons) behind India in 2019-20. Rice exports in the first half of 2021 (from January 1 to June 23) were valued at US$1.382 billion. In the first two months of the year, India was the top exporter with 72,203 tons, followed by Pakistan 19,575 tons, Vietnam 13,978 tons, Myanmar 10,899 tons, while Thailand was in the fifth place with 6,059 tons. Rice production in Thailand is on track to recover from 2019-20 when output was severely affected by adverse weather conditions and pest outbreaks (Thai Rice Exporters Association: The Nation Thailand, https://www.nationthailand.com/business). The most popular Thai rice cultivars include fragrant rice like jasmine rice such as jungwad and pathumthani, non-fragrant rice like PSL2 and BOAC cultivars, and glutinous rice like Niaw Ubon and Niaw San-pah-tawng (Ricepedia: The online authority in rice, http://ricepedia.org/thailand). The rice cultivars RD47 and Phitsanulok 2 (PSL2) have been one of popular cultivars grown in lower Northern Thailand. (Bureau of Rice Research and Development: Rice Department Thailand, http://www.brrd.in.th/rkb). These cultivars are rather resistant to brown plant hopper (BPH) and blast disease but susceptible to the bacterial blight (BB) disease (Bureau of Rice Research and Development: Rice Department Thailand, http://www.brrd.in.th/rkb). Rice cultivar RD47 was developed by Phitsanulok Rice Research Center (PSLRRC). The plant height is about 90-100 centimeters. The harvest time lies around 104-107 days after sowing. It has high yield at the maximum 793 kg per rai with good seed quality. The PSL2 cultivar was derived from the three-line cross, at PSLRRC, Thailand. The characteristics include the following: The plant height of the PSL2 cultivar is about 114 centimeters and the harvest time lies around 119 days after sowing. It also has high yield at the maximum 807 kg per rai and good seed quality. Both

cultivars are photoperiod insensitive. (Development: Rice Department, Thailand, 2564). Improvement of BB resistance in these two rice cultivars through plant breeding has been managed by using various BB resistance genes (*Xa*). More than 30 BB resistance genes have been reported (Chen et al., 2008; Kumar et al., 2011). Among these resistance genes, *Xa21* was identified (Song et al., 1995) and widely exploited in rice breeding programs (Singh et al., 2001, Perez et al., 2008). *Xa21*, originally derived from wild African rice *Oryza longistaminata*, is a dominant gene which confers a broad-spectrum resistance to many *Xoo* strains (Khush et al., 1989). *Xa21* was introgressed into BB susceptible O. sativa variety IR24, so called. IRBB21, a BB-resistant near-isogenic line of the BB susceptible rice line IR24. During a 5-year period (1998 to 2002) of testing at the Central Rice Research Institute, Cuttack, India, IRBB21 had been consistently resistant to the disease in both wet and dry seasons in the BB trap nursery where the effectiveness of different resistance genes under natural disease condition was evaluated (Sirisha et al., 2004). Rice cultivar IRBB21 has been globally used as a *Xa21* donor parent for BB resistance improvement in many rice cultivars via backcross breeding. Introgression of the *Xa21* gene from the IRBB21 cultivar into the cultivars RD47 and PSL2 using marker assisted selection has been carrying out to generate BB resistant RD47 and PSL2 isogenic lines through backcross breeding at Naresuan university (Suachowna et al., 2016; Sagun et al., 2019). BB disease in rice by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most serious diseases in rice. BB disease can be more severe in susceptible rice varieties under high nitrogen fertilization. The earlier the disease occurs, the higher the yield loss. Yield loss due to BB disease can be up to 70% in susceptible varieties grown in environments favorable to the disease. On seedlings, infected leaves turn grayish green and roll up. Vera Cruz (2000) cited that among rice diseases, BB disease is one of the costliest and can damage as much as 60–70% of the plant resulting in crop failure, especially when disease strikes at the seedling stage (IRRI Knowledge Bank). As the disease progresses, the leaves turn yellow to straw-colored and wilt, leading whole seedlings to dry up and die. On mature plants, lesions usually develop as water-soaked to yellow-orange stripes on leaf blades or leaf tips or on mechanically injured parts of leaves. Lesions have a wavy margin and progress toward the leaf base. On new lesions, bacterial ooze resembling a milky dew drop can be observed when weather is damp such as early in the morning and rainy

season. The bacterial ooze later on dries up and becomes small yellowish beads underneath the leaf. Lesions on severely infected leaves may extend to the leaf sheath. Use of a resistant variety is the simplest and the most cost-effective management for diseases. (IRRI Knowledge Bank, www.irri.org)

BB pathogen Xanthomonas oryzae pv. oryzae

Xoo is diverse, with distinct phylogenetic clades comprising *Xoo* from Asia, *Xoo* from Africa, and X. *oryzae* pv. *oryzicola* (Xoc) from Asia and Africa (Triplett et al., 2011; Hajri et al., 2012). *Xoo* has a large biodiversity with various levels of virulence and new mutations, leading to new effectors, continuously appear. More than 30 races of *Xoo* has been reported to occur. The difference strains have not been clearly defined with specific reactions being assigned to each rice cultivar (Ercolini et al., 2007). *Xoo* pathogenicity depends on a specific class of virulence factors, called transcription activator-like effectors (TALEs), which resemble eukaryotic transcriptional activators (Hutin et al., 2015). At the translocation into the plant cell and import in the nucleus, TALEs bind to specific promoter elements, called effector binding elements (EBEs) which are located on either strand in promoter regions and bidirectionally drive expression of host target genes (Scholze et al., 2011; Streubel et al., 2017; Wang et al., 2017). This recognition triggers transcription of the targeted gene, whose function often determines the outcome of the interaction. Rice resistance to Xoo often relies on executor genes distinct from classical resistance genes, whose transcriptional activation by TALEs triggers immunity, leading to dominant resistance (Zhang et al., 2015). Several TALEs have been found to be essential virulence factors of *Xoo* in BB susceptibility by inducing host susceptible genes and after that promoting pathogen infection and disease development (Yang et al., 2004; Yang et al., 2006, Antony et al., 2010 and Yu et al., 2011). TALEs are thought to be the most important virulence factor of Xanthomonas bacteria (Boch et al., 2010). Virulence of most *Xoo* strains mainly relied on the presence of individual TALEs and the ability to induce specific host susceptibility genes (White et al., 2009). Molecular Mechanism of Xa21-Mediated BB Resistance in Rice

At present, only nine *Xa* genes including *Xa1, Xa3/Xa26, xa5, Xa10 xa13, Xa21, Xa23, xa25, and Xa27* have been characterized at molecular level and these

encode various types of proteins (Vikal et al., 2017). Several studies reported that the molecular mechanism of BB resistance in rice was largely different from the mechanism of blast resistance. Most of the characterized BB resistance genes encode proteins different from the most common R protein, nucleotide-binding site-leucine rich repeat (NBS-LRR) protein (Liu et al., 2010). Except for *Xa21* and *Xa26* which encode for similar receptor-like proteins, the products of the other *Xa* genes are unique and not found in other plant species (Dai et al., 2007). This suggests that the molecular mechanism of rice-*Xoo* system is more complicated and a unique pathosystem to study the interactions between hosts and pathogens. The products of the nine characterized BB resistance genes were classified into six different classes of proteins and thus may give a wide scenario of understanding at molecular level (Vikal and Bhatia, 2017). The *Xa21* was the first characterized rice BB resistance gene and was one of the most intensively studied genes at molecular level (Song et al., 1995). This gene confers a race-specific resistance to *Xoo*, and is the most widespread BB resistance gene in the rice cultivated area, thereby providing broad-spectrum resistance. *Xa21* encodes the receptor kinase-like protein consisting of a putative extracellular domain with LRR, a single pass transmembrane domain, and an intracellular domain with serine/threonine kinase. The receptor domain LRR has hypothetical function in pathogen recognition, while the kinase domain functions in subsequent signal transduction. This character is unique from other cloned plant resistance genes (Wang et al., 1998). The *Xa21*-mediated resistance is not expressed in the early developmental stages but gradually increases from the seedling stage to later stages, with 100% resistance at the adult stage (Century et al., 1999). The gradual increase of the *Xa21* expression during rice development is associated with development controlled *Xa21*-mediated resistance (Zhao et al., 2009). Ectopic expression of Xa21 can generate rice plants with a high level of resistance to *Xoo* at both seedling and adult stages (Park et al., 2010; Zhao et al., 2009). Activation of *XA21*-mediated immunity required the specific *Xoo* protein, named *RaxX*, which is highly conserved in many plants pathogenic *Xanthomonas* species. Therefore, *Xoo* strains that lack *RaxX*, or carry mutations in the single *RaxX* tyrosine residue (Y41), are able to evade XA21-mediated immunity. Y41 of *RaxX* is sulfated by the prokaryotic tyrosine sulfotransferase *RaxST*. Sulfated, but not non-sulfated, *RaxX* triggers hallmarks of the plant immune response in an *XA21*-dependent

manner. A sulfated, 21–amino acid synthetic *RaxX* peptide (*RaxX21-sY*) is sufficient for this activity. *Xoo* field isolates carrying an alternate *raxX* allele could overcome *XA21*-mediated immunity. It seems that coevolutionary interactions between rice host and *Xoo* pathogen contribute to diversification of *RaxX*. *RaxX* is considered as a pathogen-associated molecular pattern, and thus, Xa21 can be classified both as a plant pattern recognition receptor (PRR) and an R protein (Pruitt et al., 2015).

Sugar transporters (SWEET family) in plant

The recently identified SWEETs constitute a family of sugar transporters (Chenet al.,2010). On average, 20 SWEET genes have been reported to be present in several higher plant species (Chen LQ et al., 2015). Five phylogenetically related members (*OsSWEET11*-15) of the 22 SWEET genes in rice (*OsSWEET*) support the virulence of *Xoo* (Nin o-Liu et al., 2006; Li et al., 2013; Schornack et al., 2013; Streubel et al., 2013). *SWEET11*, *SWEET13* and *SWEET14*, which belong to the SWEET family clade III, are targeted by several TALEs (Antony et al., 2010; Yang et al., 2006; Zhou et al., 2015). A systematic analysis of rice SWEET paralogs revealed that all, and only, clade-III members can play as susceptibility genes (Streubel et al., 2013). As clade III SWEETs encode sugar transporters which mediate glucose and sucrose export, induction of SWEET gene by TALEs could trigger sugar release to the apoplast, providing a nutrient source to pathogen growth (Chen et al., 2014; Chen et al., 2015; Cohn et al., 2014). Chen et al. (2012) reported that *At SWEET11* and *At SWEET12*, two sucrose transporters from *Arabidopsis thaliana* which are members of the SWEET family, played an important role in sucrose efflux from parenchyma to the apoplastic interface before sucrose was loaded into the sieve element/companion cell (SE/CC) complex of phloem in the small veins of Arabidopsis leaves. Chen et al. (2015) further reported *AtSWEET11*, working as a sugar efflux transporter in the nucellar epidermis, together with *AtSWEET12* and *AtSWEET15*, was involved in Arabidopsis seed development.

The *SWEET11* gene in rice

OsSWEET11 (also designated as *Os8N3* and *Xa13*), a homolog of Arabidopsis At *SWEET11*, was first characterized as a pathogen-responsive allele in rice (Yang et al., 2006; Antony et al., 2010; Chen et al., 2010; Yuan et al., 2010). Previous studies have shown that SWEETs play diverse functions in plant development and production, and in the senescence of leaves (Quirino et al., 1999), sugar loading in phloem (Chen et al., 2012), nectar production (Lin et al.,2014), pollen viability (Yang et al., 2006) and grain filling (Ma et al.,2017). It was also found to be essential for pollen development in rice cultivar Zhonghua 11 and Kitake (Chu et al., 2006; Yang et al., 2006).

The expression of *OsSWEET11* was very weak in leaves of rice cultivars IRBB13 and IR24 under normal growth conditions (Chu et al. 2006). *OsSWEET11* and *OsSWEET15* are highly expressed in the rice caryopsis and localize to the nucellus and have essential roles in rice grain filling (Yang etal., 2018). *OsSWEET11* is known to be a target of the type III effector secreted from *Xoo* strain PXO99A and can be highly induced on PXO99A infection and negatively related to resistance to BB (Yang et al., 2006). Yang (2006) stated that rice plants deficient in bacterial mediated *OsSWEET11* induction, or natural genetic variation (xa13), were resistant to *Xoo* strain *PXO99A*, which depends on the major TALE gene *pthXo1* for full virulence. At the same time, both silenced and xa13-containing plants were not generally resistant to bacterial infection. Infectivity by *Xoo* strain *PXO86* was unaffected by either silencing or *xa13*, and *PXO86* did not cause *OsSWEET11* induction in normal or xa13-containing plants. Meanwhile, *Xa13* expression in *PXO99*-challenged IR24 plants increased by six folds at 8 hours postinoculation (hpi) and 47 folds at 72 hpi compared with the non-treatment control.

However, *PXO99A* inoculation did not induce the expression of *xa13* confirming that a low expression level of this gene is the basis of xa13-mediated resistance (Chu et al., 2006). These observations are consistent with the hypothesis that *OsSWEET11* is a host gene for strain-specific susceptibility to BB disease of rice.

The *SWEET14* gene in rice

OsSWEET14 (previously named Os11N3) stands out as an interesting example of convergent evolution because it is targeted by unrelated TALEs from multiple, phylogenetically distinct *Xoo* strains, including: *AvrXa7* from strain *PXO86* (Philippines), PthXo3 from strain *PXO61* (Philippines), Tal5 from strain MAI1 (Mali) and TalC from strain BAI3 (Burkina Faso) (Antony et al., 2010; Chu et al., 2006; Streubel et al., 2013; Yu et al., 2011; Zhou et al., 2015). While *OsSWEET14* is targeted by four different TALEs, i.e., *AvrXa7, PthXo3, TalC* or *Tal5* (Streubel et al., 2013; Yang et al., 2006; Zhou et al., 2015; Yu et al., 2011), *AvrXa7, PthXo3, PthXo1, PthXo2* and *PthXo2*-like TALEs are present in Asian strains (Oliva et al., 2019). *TalC* and *Tal5* have only been isolated from African strains, and *TalC* exists in all the African *Xoo* strains sequenced, while Tal5 is present in half of the strains (Oliva et al., 2019). *OsSWEET14* is the target of all sequenced African *Xoo* strains and most Asian *Xoo* strains. Since *Xoo* activates *OsSWEET14* by binding TALEs to the specific EBEs in the promoter region, great efforts were invested into generating resistant rice plants by genetic editing of the promoter region of *OsSWEET14* or by identifying natural EBE-mutant alleles in germplasm reservoir for resistant rice breeding (Pavan et al., 2010; Li et al., 2012). Interestingly, EBEs recognized by four *TALEs, AvrXa7, PthXo3, TalC* and *Tal5*, were found to overlap or to be in a close vicinity (Hutin et al., 2015). In particular, *TalC* directly activates *SWEET14* through recognition of a DNA box located upstream from the EBEs for *AvrXa7*, *PthXo3* and *Tal5* (Yu et al., 2011). Engineering mutations within the *AvrXa7* EBE in the *OsSWEET14* promoter resulted in disease resistance against an Asian *Xoo* strain carrying the *AvrXa7* effector (Li et al., 2012). In addition, a naturally occurring deletion encompassing the *AvrXa7* and Tal5 EBEs in *O. barthii* wild rice species was recently shown to confer broad-spectrum resistance to BB disease (Hutin et al., 2015).

OsSWEET14 knock-out mutants are retarded in growth and their seeds are smaller than those from wild-type plants (Antony et al., 2010). Zeng et al. (2020) stated that *OsSWEET14* had the highest expression level in the stem, the enhanced plant height might be due to the lower efficiency of sugar transportation in the stem, which needs to be studied further. In addition, *OsSWEET14* may not be responsible for reproductive development since its expression in the anther, palea, lemma and pistil were relatively

low. These imply the enhancement of plant height needs to be considered if *OsSWEET14* knockout mutants are targeted to confer BB resistance.

CHAPTER III

MATERIALS AND METHODS

Plant materials and growth conditions

Rice (*Oryza sativa* L. ssp. indica) cultivars RD47 PSL2 and IRBB21 provided by Phitsanulok Rice Research Center, Thailand, were used in this research. The experimental rice plants were grown in the greenhouse under natural light and temperature. The water-soaked seeds for 24 hours were covered by moist tissue paper until germination. Water every morning and evening to maintain moisture When the rice seeds germinated with buds with small shoots and roots, transplanted into seedling trays 1 seed per 1 well. When rice plants were 14 days, transplanted into pots measuring 10×8 inches, 1 plant per pot containing soil approximately 5 kg per pot for fertilizing, divided into 2 times, each time equally, which is the first time before planting the black in the pot. The second fertilizer application was when rice was 42 days old. When rice was 49 days old, the ability to cause leaf margin disease was assessed in greenhouse conditions.

Xanthomonas oryzae pv. *oryzae* (*Xoo*) inoculum preparation and inoculation

Testing for bacterial blight resistance in rice populations by using *Xanthomonas oryzae* pv. *oryzae* isolate *Xoo*16PK002 that courtesy of Center of Excellence in Research for Agricultural biotechnology, Naresuan University, Phitsanulok was inoculated on the rice plant by clipping method (Kauffman et al.,1973). Bacteria for inoculation were prepared by growing on nutrient agar (NA) medium (beef extract 3 g/L, peptone 5 g/L and agar 15 g/L), and it was incubated at 28 ± 2 ˚C for 72 hr until the germ grows into a single colony. A single colony of *Xanthomonas oryzae* pv. *oryzae* was further sub-cultured on NA medium at 28 ± 2 ˚C for 72 hr and suspended with sterilized water, the inoculum suspensions containing about 10^8 cfu/ml (OD600 = 0.2) which was confirmed by measurement using a spectrophotometer (BOECO MODELS S-200 VIS & S-220 UV/VIS, Hamburg, Germany) with A_{600} OD. This sub-culture was used to inoculate the tested plants grown in the greenhouse. All plants were inoculated

with *Xoo*16PK002 at the tillering stage by clipping 2-3 cm from the tip of five youngest fully expanded leaves.

Greenhouse experiment

Greenhouse experiment was conducted using factorial in RCBD (randomized complete block design) with three replicates consisted of three different levels of fertilizer (N-P-K): 12-6-12 (N12), 24-6-12 (N24) and 36-6-12 (N36) kg/rai, and three rice cultivars: RD47, PSL2 and IRBB21. Surface-sterilized seeds were soaked in water at 37 °C for 24 hr. Germinated seedlings were transferred to a seedling tray. At the 14 day after sowing, seedlings were transferred into plastic pots which contained 5 kg of farm soil with one plant per pot. The fertilization was divided into two parts equally. The fertilizers were applied to the plants at 30 and 60 d after transplanting. All plants were inoculated with at the tillering stage by clipping 2-3 cm from the tip of five youngest fully expanded leaves.

Evaluation of pathogenicity of *Xanthomonas oryzae* pv. *oryzae*

Pathogenicity was assessed from cut marks formed on the rice leaves at 7, 14, 21 day after inoculation, Plants were characterized as resistant or susceptible base on under greenhouse condition, lesion length on cut leaves were measured and scored for BB resistance according to IRRI Standard Evaluation System (Table1)

Table 1 IRRI Standard Evaluation System for BB resistance in the greenhouse infection test (IRRI, 1996).

Lesion Length (cm)	Level of BB resistance
0-5	resistant (R)
>5-10	moderately resistant (MR)
>10-15	moderately susceptible (MS)
>15	susceptible (S)

Analysis of sucrose content

The youngest fully expanded leaves of an individual plant were used to observe sucrose content by weighing 0.1 g fresh, crushed with liquid nitrogen. Then, one ml of distilled water was added, and the samples were incubated at 65 °C for 20 min. The samples were centrifuged at 14,000 rpm for 5 min. The extracted translucent was added with a megazyme sucrose/d-glucose assay kit (Megazyme, Ireland), divided into 2 parts: part A and part B. The absorbance was measured at a wavelength of 510 nm using a spectrophotometer (BOECO MODELS S-200 VIS & S-220 UV/VIS, Hamburg, Germany), and the sucrose content was calculated using the following formula:

$$\text{g/L of sample solution} = (\Delta B - \Delta A) * F * \text{Dilution} * 0.0095 \quad \text{____(A)}$$

ΔB is absorbance of free D-glucose plus D-glucose from sucrose).

ΔA is absorbance of free D-glucose

F is factor to convert from absorbance to µg for 100 µg D-glucose (= 100/ absorbance for 100 µg D-glucose).

Dilution is dilution of the original sample solution

$$\text{Sucrose} = \frac{(A)}{\text{weight}_{\text{sample}}\ (\text{g/L sample solution})} \quad (\text{mg/g})$$

RNA extraction

Total RNA was extracted from each 100 mg leaf sample using total RNA Extraction Kit (Plant) (RBC Bioscience., Taiwan) following the manufacturer's instructions. Each RNA sample was treated with DNase I (Thermo Fisher Scientific, Waltham, MA) to remove possible gDNA contaminants according to the manufacturer's instructions. Total RNA was quantified using Nano drop 2000 (Thermo Fisher Scientific, Waltham, MA) and the RNA integrity was assessed by agarose gel electrophoresis.

cDNA synthesis

The first strand cDNA was synthesized from 500 ng total RNA using first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA) One microliters of cDNA and 0.5 μM each primer was used in 20 μl of PCR reaction following the manufacturer's recommendation.

Reverse Transcription PCR (RT-PCR) Analysis

PCR was used GoTaq® GreenMaster Mix (Promega, Madison WI).The primer sets Sw11gc2-RT used for detection of *SWEET*11 transcripts and primer set Sw14RT used for detection of *SWEET*14 use OnePCR™ Plus (GeneDireX, Inc, Taiwan)The endothelial differentiation factor (*Edf*) gene was used as a reference gene. Amplification of *Edf* was performed using primers based on Wang et al. (2016). The reaction mixture for *Edf* was amplified for the denaturation step of 15 s at 94°C followed by 27 cycles of 30 s at 94°C, 30s at 60°C and 40 s at 72°C, and ending with a final elongation step of 5 min at 72°C. RT-PCR products were detected by 1.2% agarose TAE gel electrophoresis (100V for 45 min) and visualized under the Gel documentation system transilluminator.

The primers used to RT-PCR *SWEET11* and *SWEET14* gene fragments from RD47, PSL2 and IRBB21.

Gene *SWEET11* primer pairs is as follows: Sw11gc2-For (RT) 5'-AGTCGACGGGAGGGTACAGCT-3' and Sw11RT-Rev New 5'-TTCGGGTACATGACGTAGGG-3' expected size 472bp PCR 1 cycle: 94°C, 5 min; 40 cycles: 94°C, 15 s; 60°C, 15 s; 72°C, 45 s;1 cycle: 72°C, 5 min

Gene *SWEET14* primer pairs is as follows: Sw14RT-for 5'-CTACCTGGCCCCACTGCCG-3' and Sw14RT-Rev 5'-GTGCGCACCACCAGCCT-3' expected size 405bp PCR 1 cycle: 94°C, 3.30 min; 40 cycles: 94°C, 15 s; 72°C, 45 s 1 cycle: 72°C, 5 min

Quantitative Real Time RT-PCR (RT-qPCR) Analysis

Analysis of RT-qPCR was carried out using SYBR Green Master Mix (Quantabio, USA) and 12.5 µl of PCR reaction containing 1 µl of cDNA and 0.3 µM of primer Sw11RT3-For 5'-CGCCCCTCTCTCCATCATCT-3' Sw11RT2b-rev 5'-CCGCCCACGTTCGGGTACAT-3'expected size 156bp

Expression levels of all the genes of interest under different conditions were assayed through real time RT-qPCR performed on an Eco 48 Real Time PCR System (PCR Max; United Kingdom) through Eco™ Study Software. The following conditions were set based on primers specified above:

Total volume of reaction: 12.5 µl

UDG Incubation: 50°C for 2 min

Polymerase Activation: 95°C for 10 min

3 Step-PCR Conditions

Denaturation: 95°C for 15 s

Annealing: 60°C for 30 s

Extension: 72°C for 30 s

Melting Curve

95°C for 15 s - 55°C for 15 s - 95°C for 15 s

For qPCR reactions, the Perfecta SYBR Green Master Mix (Quanta Biosciences, USA) was used. Gene expression levels were analyzed using delta-delta ct method relative to *Edf* as internal controls.

Yield and agronomic performance in the greenhouse

In the period spanning January to December 2020, a greenhouse study involved the cultivation of four carefully chosen BC3F3 lines—namely, IRBB21 and RD47. The planting arrangement followed a complete randomized design (CRD), with each plot occupying dimensions of 25.4 cm x 22.68 cm. Several key parameters were meticulously documented, encompassing plant height, tillers per hill, panicles per hill, days to flowering (DTF), days to harvesting (DTH), panicle length, filled grains per panicle, 100-grain weight (g), and grain yield per hill (g).

Statistical Analysis

The data from all conditions were used to obtain the mean and SD. The SEs of the means were also calculated and were presented in the graphs as error bars. Data were analyzed using one-way analysis of variance. Duncan's multiple range test at the 95% confidence level ($p < 0.05$) was used to compare the difference between treatments. Statistical analysis was performed using the R software (R Core Team, 2021).

CHAPTER IV

RESULTS AND DISCUSSION

Xoo infection tests on rice cultivars RD47, PSL2 and IRBB21

Sixty-day old plants were artificially inoculated by the clipping method with *Xoo*16PK002 isolated from Phitsanulok province, Thailand. The lesion length (LL) was measured at 7, 14 and 21 day after inoculation (dai) and the resistance levels were scored according to the IRRI standards (Table 2 and 3). The LL was less than 1 cm in all plants treated with sterile water (Mock) (Figure 1). For *Xoo*16PK002 inoculated plants, IRBB21 showed higher resistance (< 5 cm LL) than RD47 and PLS2. RD47 and PSL2 cultivars exhibited complete susceptibility (> 15 cm LL) from 7 dai at nitrogen level 36 kg/rai (Figure 3).

Table 2 Lesion length (LL) measurement (cm) on leaves of rice cultivars RD47, PSL2 and IRBB21 treated with sterile distilled water at 7, 14 and 21 days after Mock inoculation (dai).

Cultivars	Nitrogen level	7dai	14dai	21dai
RD47	N12	0±0.00	0.03±0.00^{c}	0.13±0.00^{b}
	N24	0±0.00	0.1±0.00^{bc}	0.14±0.01^{b}
	N36	0±0.00	0.1±0.00^{bc}	0.17±0.00^{b}
PSL2	N12	0±0.00	0.17±0.00^{ab}	0.28±0.01^{ab}
	N24	0±0.00	0.23±0.08^{a}	0.45±0.18^{a}
	N36	0±0.00	0.14±0.01^{ab}	0.26±0.01^{ab}
IRBB21	N12	0±0.00	0.18±0.01^{ab}	0.28±0.01^{ab}
	N24	0±0.00	0.18±0.01^{ab}	0.28±0.01^{ab}
	N36	0±0.00	0.16±0.01^{ab}	0.28±0.01^{ab}
P value	Cultivars	ns	<0.001	0.005
	Nitrogen level	ns	0.16	0.413
	Cultivars*Nitrogen level	ns	0.303	0.384
CV%			35.1	40.99

Significant difference of mean among treatment (P<0.001).

Means followed by the same letter do not differ significantly (P<0.05)

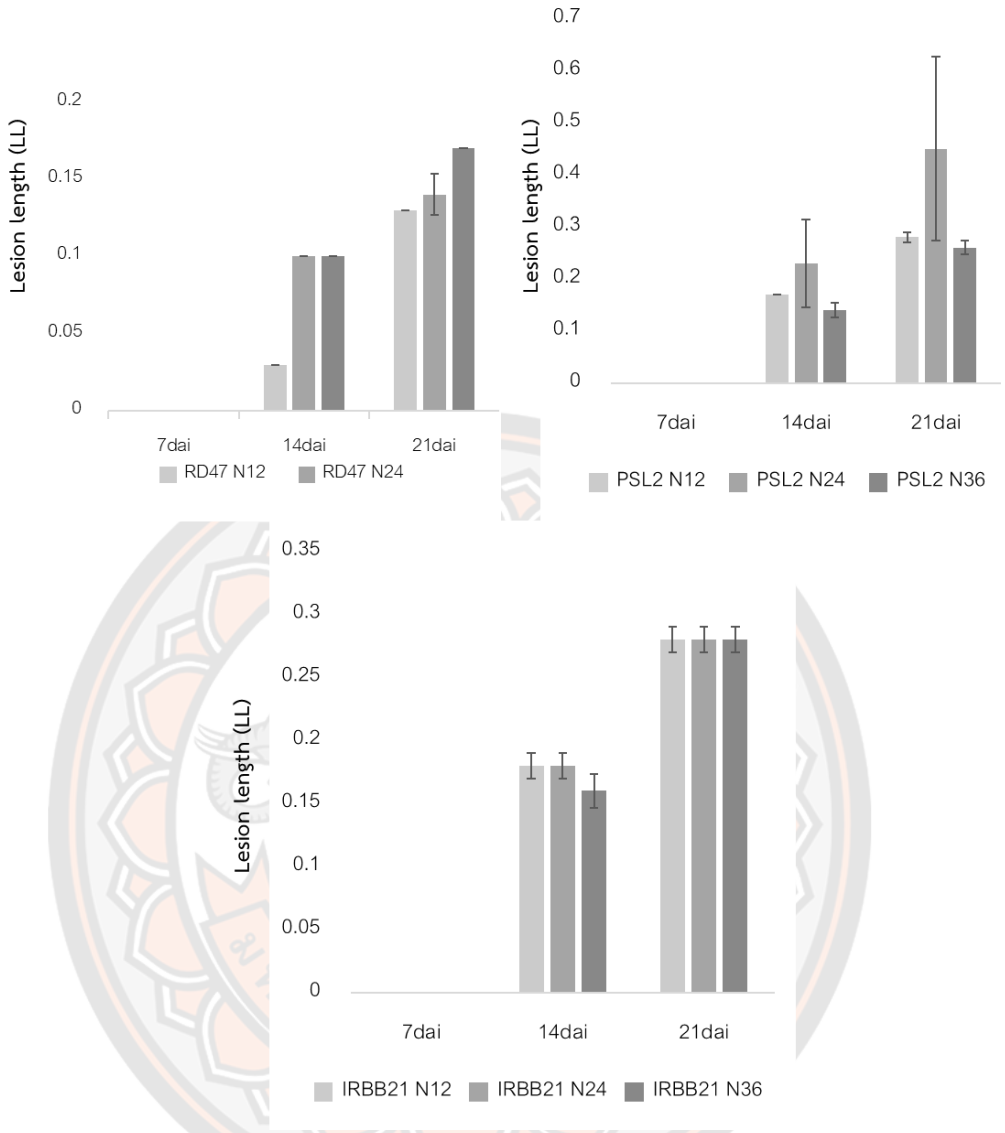

Figure 1 Lesion length (LL) measurement (cm) on leaves of rice cultivars RD47, PSL2 and IRBB21 treated with sterile distilled water at 7, 14 and 21 days after Mock inoculation.

Table 3 Lesion length (LL) measurement (cm) on leaves of rice cultivars RD47, PSL2 and IRBB21 inoculated with *Xoo*16PK002 at 7, 14 and 21 days after *Xoo* inoculation (dai).

Cultivars	Nitrogen level	7dai	14dai	21dai
RD47	N12	14.86±0.57^{a}	46.28±0.34^{a}	52.60±0.66^{a}
	N24	15.19±0.14^{a}	45.55±0.31^{a}	52.67±0.60^{a}
	N36	15.79±0.20^{a}	45.99±0.38^{a}	52.93±0.22^{a}
PSL2	N12	10.33±0.26^{b}	24.71±0.36^{d}	39.55±1.76^{c}
	N24	10.40±0.54^{b}	28.03±0.38^{c}	48.86±0.25^{b}
	N36	15.15±0.34^{a}	37.26±1.13^{b}	50.00±0.35^{b}
IRBB21	N12	10.07±0.19^{b}	14.67±0.75^{g}	17.91±0.49^{e}
	N24	10.91±0.19^{b}	16.40±0.20^{f}	19.52±0.24^{e}
	N36	11.06±0.31^{b}	18.09±0.51^{e}	22.77±0.71^{d}
P value	Cultivars	<0.001	<0.001	<0.001
	Nitrogen level	<0.001	<0.001	<0.001
	Cultivars Nitrogen level	<0.001	<0.001	<0.001
CV%		4.64	3.13	3.24

Data are presented as the mean ± standard error of lesion lengths of 5 leaves per treatments. Means followed by the same letter are not significantly different according to DMRT at p = 0.05.

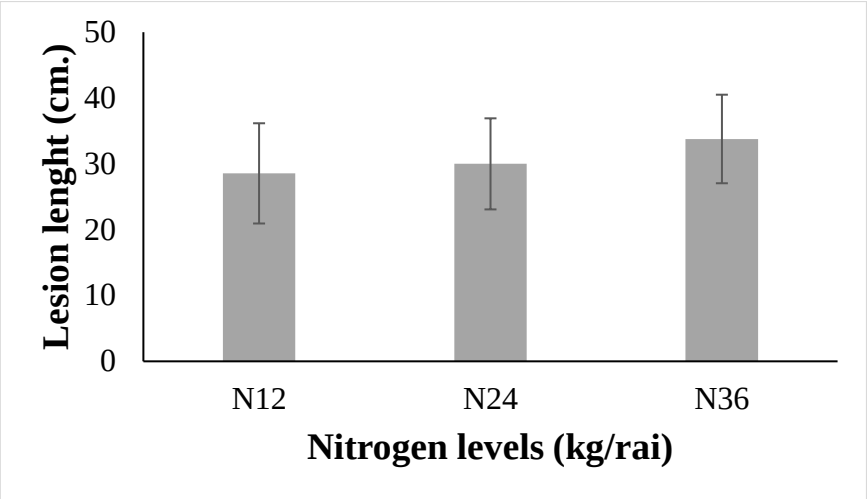

Figure 2 Lesion length (LL) measurement (cm.) on leaves of rice cultivars RD47, PSL2 and IRBB21 inoculated with *Xoo*16PK002 at 14 dai after *Xoo* inoculation at different nitrogen levels (kg/rai).

Table 4 Reaction of rice cultivars against *Xanthomonas oryzae* pv. *oryzae* in the three different nitrogen levels at 7, 14, 21 day after *Xoo* inoculation (dai).

Cultivars	Nitrogen level	Reaction against N level		
		7dai	14dai	21dai
RD47	N12	MS	S	S
	N24	S	S	S
	N36	S	S	S
PSL2	N12	MS	S	S
	N24	MS	S	S
	N36	S	S	S
IRBB21	N12	MS	MS	S
	N24	MS	MS	S
	N36	MS	MS	S

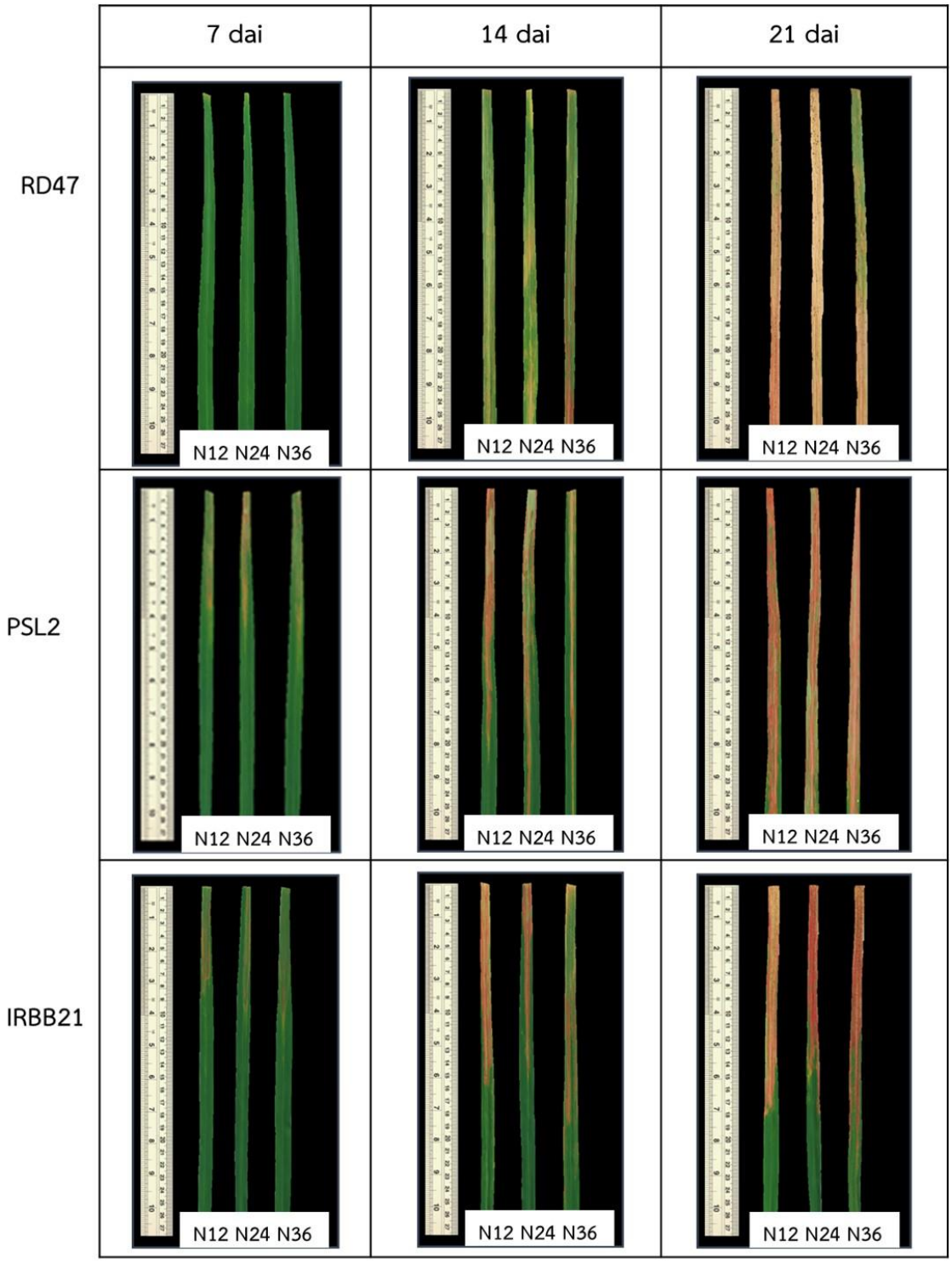

Figure 3 Severity of bacterial blight disease on three rice cultivars at 7, 14, 21 days after inoculation with *Xanthomonas oryzae* pv. *oryzae*. Levels of lesion length on rice leaves indicating resistant (R), moderate resistant (MR) and susceptible (S).

Analysis of sucrose content

An analysis of sucrose content in rice leaves revealed that the different levels of nitrogen fertilizers influenced sucrose levels of all plants (Figures 4). Increasing the nitrogen fertilizer rates results in an increase in sucrose content of control plant. At 14 d after inoculation, the sucrose content was decreased compared to the experiment in which the leaves were cut with distilled water (Mock). The leaves that were treated with sterile distilled water remain higher sucrose levels than the inoculated leaves. These phenomena are the same in all rice cultivars. The results indicated that the BB pathogen grows and invades the leaves rapidly when there is a high sucrose concentration in the leaves. As after 14 d of infestation of *Xoo*, the, the level of sucrose in the leaves is significantly reduced. This mean that pathogen used sucrose as a source of nutrient for growth of itself, resulting disease spread rapidly in plant with high nitrogen level applied. This is in line with research showing that sucrose is produced in the mesophyll cells, and it is taken in the phloem cells for transport throughout the plant. Sucrose may be released from cells in higher amounts, thereby making it easier for pathogens to invade (Chen et al., 2012). Nitrogen is derived through a sugar transport mechanism in plants (Wu et al., 2018), where low levels of sucrose contribute to low disease susceptibility (Singh et al., 2021). For growth and production, nitrogen plays a key role in promoting growth and stimulate the plants caused increasing tillering, thus affecting the number of spikes per area. It also increases the number of seeds per spike (Dobermann and Fairhursf, 2000). This result corresponds to Yang et al, (2018) reporting that *OsSWEET11* and *OsSWEET15* are necessary for sugar efflux from the maternal nuclear epidermis as well as efflux from the ovular vascular trace to the apoplasm and also may contribute to sucrose influx into the aleurone. The expression of *OsSWEET14* was detected in vascular tissues, including the stem, leaf sheath, leaf blade and root. The disruption of *OsSWEET14* led to increased plant height without a reduction in yield (Zeng et al., 2020). Over-fertilization of nitrogen fertilizers increases the incidence of disease. Therefore, considering the yield and cost of fertilizing, it should not be applied more than what is necessary.

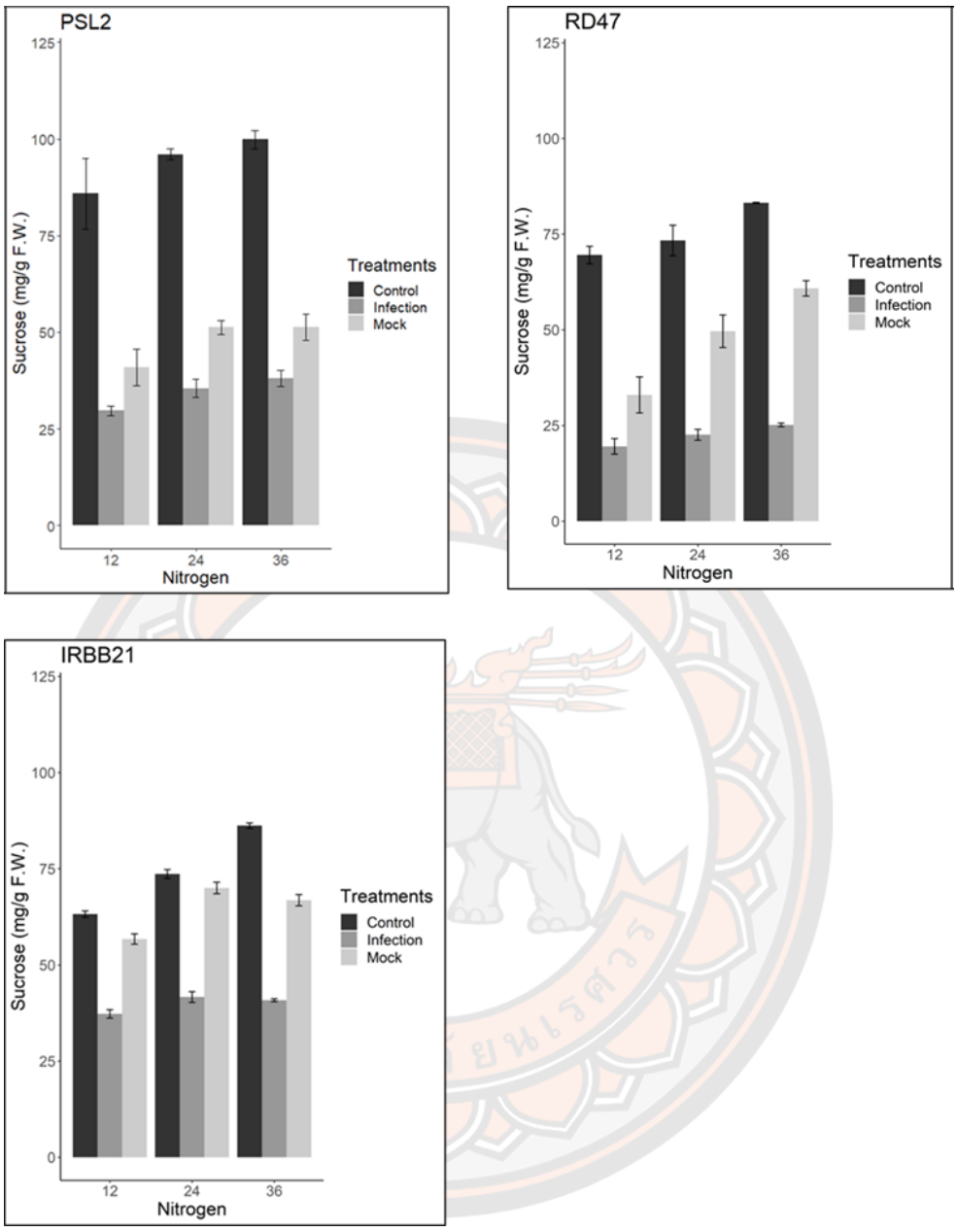

Figure 4 Sucrose content in the rice leaves of all rice cultivars under different nitrogen concentrations. Black color, control; gray color, *Xanthomonas oryzae* pv. *oryzae* infection leaf; white color, mock. Each data point represents the mean ±SE from three plants. Evaluation of yield performance of rice cultivars RD47, PSL2 and IRBB21 Based on agronomic performance,

Analysis of Agronomic traits

Three rice cultivars were evaluated during the monsoon season in 2021 with three different nitrogen levels (12, 24 and 36 kg/rai) application. Yield components observed in the experiment included plant height, tiller per hill, panicle per hill, flowering date, harvesting date, panicle length, filled grain per panicle, 100-grain weight, and grain yield per hill (Table 5 and 6). In non-infected plants (mock inoculation), there are non-significant different in plant height among all three nitrogen levels, but differ among rice cultivars ranged from (92 - 105 cm). All three cultivars produced the highest productive tiller per hill at 36 kg N/rai, similarity to the panicle per hill. It has been reported that the productive hiller is the important trait to determine grain yield (Yuan, 2017). The difference in nitrogen fertilizer levels has no affection in flowering date and harvesting date, however the difference was due to the plant genetic itself. Total filled grain number per panicle ranged from 104-126 in RD47, 106-155 in PSL2, and 151-186 in IRBB21. The nitrogen level at 12 kg/rai was presented to produce the highest total filled grain per panicle, 100-grain weight and grain yield per hill in all three rice cultivars. The grain yield per hill of plant applied with 12, 24 and 36 kg N/rai ranged from 39, 39 and 37 g in RD47, 36, 33 and 31 g in PSL2, and 49, 51 and 46 g in IRBB21. This study demonstrated that several important yield components traits are highly responsive to the nitrogen level at 12 kg/rai.

Under *Xoo* inoculation, plant height, number of tiller per hill and number of panicle per hill are obviously lower than non-infected plants in all three cultivars. The flowering date and harvesting date are shorter than the non-infected plants. After *Xoo* inoculation, plants were severely susceptible to BB, resulting in panicle length, filled grain per panicle, 100-grain weight, and grain yield per hill could not be observed.

Table 5 Agronomic traits and yield of three cultivars (Mock) in monsoon season of 2021.

Cultivars	Nitrogen level (kg/rai)	Height (cm)	Tiller per hill	Panicle per hill	DTF	DTH	Panicle length	Filled grain per panicle	100 grain weight (g)	Grain yield per hill (g)
RD47	N12	93.77±2.09^{bc}	18.33±0.33^{e}	14.67±0.33^{e}	90.67±0.33^{c}	122±0.00^{c}	27.87±0.28^{a}	126.67±7.51^{d}	3.23±0.04^{b}	39.86±1.10^{c}
	N24	98.57±0.98^{a-c}	29.67±1.20^{bc}	17.67±0.33^{c-e}	91.33±0.33^{c}	122±0.00^{c}	25.83±0.41^{bc}	123.33±6.23^{d}	3.18±0.04^{b}	39.74±1.25^{c}
	N36	98.33±2.83^{a-c}	39.33±1.67^{a}	28.33±1.76^{a}	91±0.00^{c}	122±0.00^{c}	28.27±0.41^{a}	104.33±2.40^{e}	3.20±0.01^{b}	37.58±0.77^{cd}
PSL2	N12	101.4±4.06^{ab}	17.67±1.76^{e}	15±2.08^{de}	83.33±1.20^{d}	124±0.00^{a}	24±0.40^{d}	155.67±7.06^{bc}	3.48±0.04^{a}	36.03±0.83^{d}
	N24	100.4±1.35^{a-c}	21.67±1.20^{d}	18.33±1.33^{b-d}	84±1.53^{d}	124±0.00^{a}	26.13±0.09^{b}	106.33±2.96^{e}	3.48±0.01^{a}	33.16±1.36^{e}
	N36	105.57±1.06^{a}	30.33±0.33^{bc}	18.67±0.33^{bc}	82±0.00^{d}	124±0.00^{a}	27.40±0.35^{a}	127.67±4.81^{d}	3.49±0.01^{a}	31.82±0.48^{e}
IRBB21	N12	92.53±0.38^{c}	29.33±0.83^{bc}	10.67±0.88^{f}	94.67±0.33^{a}	123±0.00^{b}	24.67±0.23^{cd}	186.67±4.63^{a}	3.39±0.02^{a}	49.76±0.45^{ab}
	N24	94.07±3.01^{bc}	30.33±0.33^{bc}	17±0.58^{c-e}	92±0.00^{bc}	123±0.00^{b}	25.07±0.57^{bc}	170.33±4.33^{a}	3.14±0.04^{b}	51.86±1.06^{a}
	N36	95.9±3.37^{bc}	30.33±0.33^{bc}	21.±670.33^{b}	93.67±0.33^{ab}	123±0.00^{b}	25.07±0.35^{b-d}	151.67±3.76^{c}	3.19±0.03^{b}	46.29±0.47^{b}
P value	Cultivars	0.002	<0.001	≤0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	N level	0.596	<0.001	<0.001	0.493	0.291	<0.001	<0.001	0.004	0.045
Cultivars*N level		0.037	0.004	<0.001	0.058	0.03	<0.001	≤0.001	<0.001	0.004
CV%		4.31	7.44	10.53	1.33	<0.001	2.42	6.38	1.65	3.93

Data are presented as the mean ± standard error of 5 plants per treatments. Means followed by the same letter are not significantly different according to DMRT at p = 0.05.

Table 6 Agronomic traits and yield of three cultivars (Infection) in monsoon season of 2021.

Cultivars	Nitrogen level (kg/rai)	Height (cm)	Tiller per hill	Panicle per hill	DTF	DTH	Panicle length (cm)	Filled grain per panicle	100 grain weight (g)	Grain yield per hill (g)
RD47	N12	88.1±1.54^{e}	17.33±0.33^{d}	7.33±0.88^{cd}	89.67±1.20^{bc}	122.00±0^{c}	0±0	0±0	0±0	0±0
	N24	96.7±1.54^{ab}	17.33±0.88^{d}	7.33±0.33^{cd}	89.67±0.88^{bc}	122.00±0^{c}	0±0	0±0	0±0	0±0
	N36	94.13±1.23^{bc}	20.33±0.33^{c}	9.33±0.88^{bc}	88.67±0.33^{c}	122.00±0^{c}	0±0	0±0	0±0	0±0
PSL2	N12	92.37±1.26^{cd}	16.33±0.88^{d}	6.33±0.33^{d}	83.67±0.67^{d}	124.00±0^{a}	0±0	0±0	0±0	0±0
	N24	98.47±0.55^{a}	21.67±1.20^{bc}	18.33±1.33^{a}	83.33±0.33^{d}	124.00±0^{a}	0±0	0±0	0±0	0±0
	N36	95.53±0.29^{a-c}	21.67±0.88^{bc}	8±0.58^{cd}	85.33±0.88^{d}	124.00±0^{a}	0±0	0±0	0±0	0±0
IRBB21	N12	94.27±1.11^{bc}	23.33±0.88^{ab}	9.67±0.67^{bc}	95.33±0.33^{a}	123.00±0^{b}	0±0	0±0	0±0	0±0
	N24	92.47±1.34^{cd}	24±0.58^{ab}	11.33±0.33^{b}	91.67±0.67^{b}	123.00±0^{b}	0±0	0±0	0±0	0±0
	N36	89.4±0.67^{de}	25.33±0.33^{a}	11±0.58^{b}	90.33±0.33^{bc}	123.00±0^{b}	0±0	0±0	0±0	0±0
P value	Cultivars	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001			
	N level	<0.001	<0.001	<0.001	0.036	0.0291	<0.001			
Cultivars* N level		<0.001	0.019	<0.001	0.002	0.031	0.329			
CV%		2.11	6.34	12.81	1.36	0	2.82			

Data are presented as the mean ± standard error of 5 plants per treatments. Means followed by the same letter are not significantly different according to DMRT at p = 0.05.

Expression analysis of *SWEET11* in rice inoculated with the *Xoo*16PK002 applied with different nitrogen levels

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was performed to evaluate the expression of *SWEET11* in the 49 -day old plants of rice cultivars RD47, PSL2, IRBB21 applied with nitrogen fertilizers at 12, 24 and 36 kg/rai (Figure 5-7). After being inoculated with *Xoo*16PK002, the *SWEET11* expression was undetectable at 0 hours after inoculation (hpi). The expression of *SWEET11* in the RD47, PSL2 and IRBB21 plants applied with 12, 24 and 36 kg/rai was abundantly increased at 48 and 72 hpi. This indicates that *SWEET11* of experimental indica rice is turned off or very low expressed at this stage. Ma et al. (2017) also reported very low expression of *SWEET11* in leaf blade of rice at the 6-week-old stage. *OsSWEET11* is expressed abundantly in developing rice panicle (Chu et al., 2006) and caryopsis (Ma et al., 2017). *SWEET11* responds to *Xoo*16PK002. The expression of *SWEET11* was virtually induced as early as 24 hpi in BB susceptible rice cultivar IR24 (Chu et al., 2006; Zaka et al., 2018) while numerously induction of *SWEET11* was detected at 72 hpi (Chu et al., 2006). The *SWEET11* gene of rice cultivar Nipponbare is induced in a transcription activator-like (TAL) effector-dependent manner that the specific TAL effector, PthXo1 from *Xoo* activate *SWEET11* through recognition of TAL effector binding elements (EBEs) located at the promoter region of *SWEET11* (Yang et al., 2006). The TAL effector of *Xoo* strain SK2-3 from Thailand was also identified as PthXo1 which was like that of *Xoo* strains from Phillipines (*PXO99A* and *PXO79*), Napal (*NXO260*) and India (*IX-280*) (Oliva et al., 2019). It is possible that *Xoo*16PK002 carry the TAL effector PthXo1 which recognizes the EBEs of *SWEET11* in indica rice cultivars RD47, PSL2 and IRBB21. The EBE of *SWEET11* would be the target of genome editing for mutation to prevent the recognition of the TAL effectors from *Xoo*16PK002 for the Induction of the *SWEET11* expression. The EBE sequence editing is the alternative approach from the use of the BB resistance gene, such as *Xa21*, to improve the BB resistance in rice cultivars RD47 and PSL2.

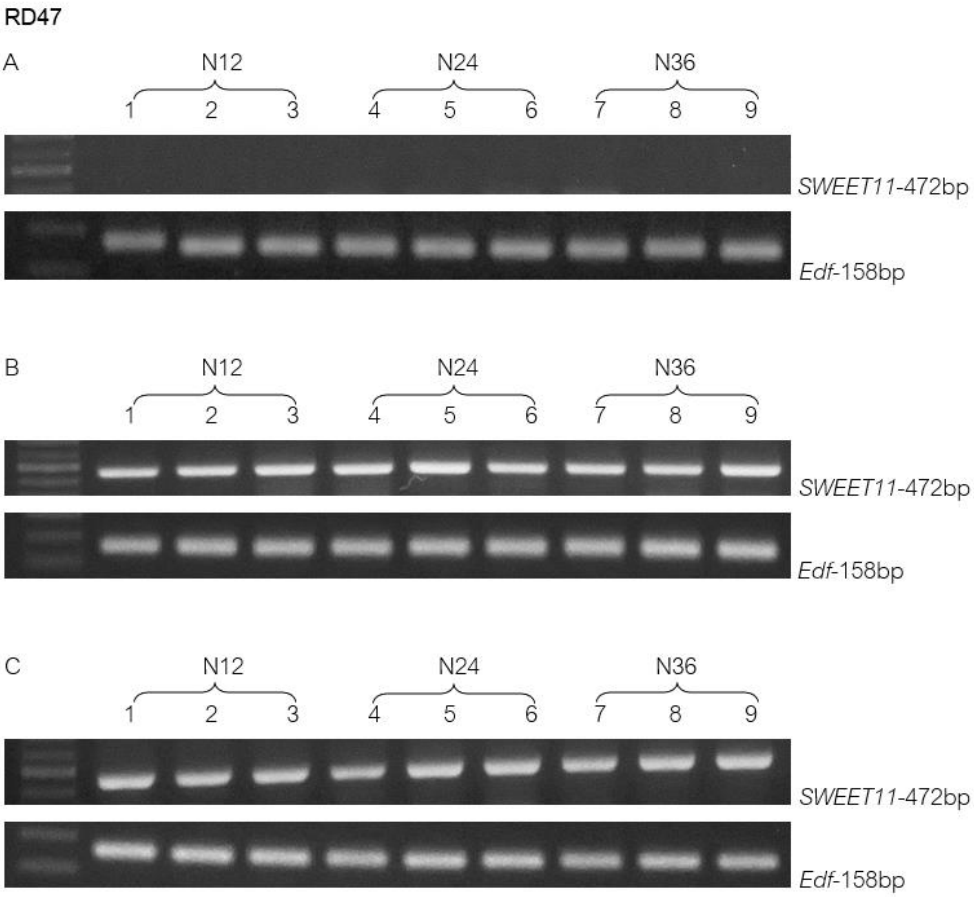

Figure 5 The RT-PCR products of *OsSWEET11*. The expression of *OsSWEET11* in *Xoo*16PK002 infected RD47 plants at 0 (A), 48 (B) and 72 (C) hours after inoculation (HAI). Lane 1, 2 and 3 are three biological replicates.

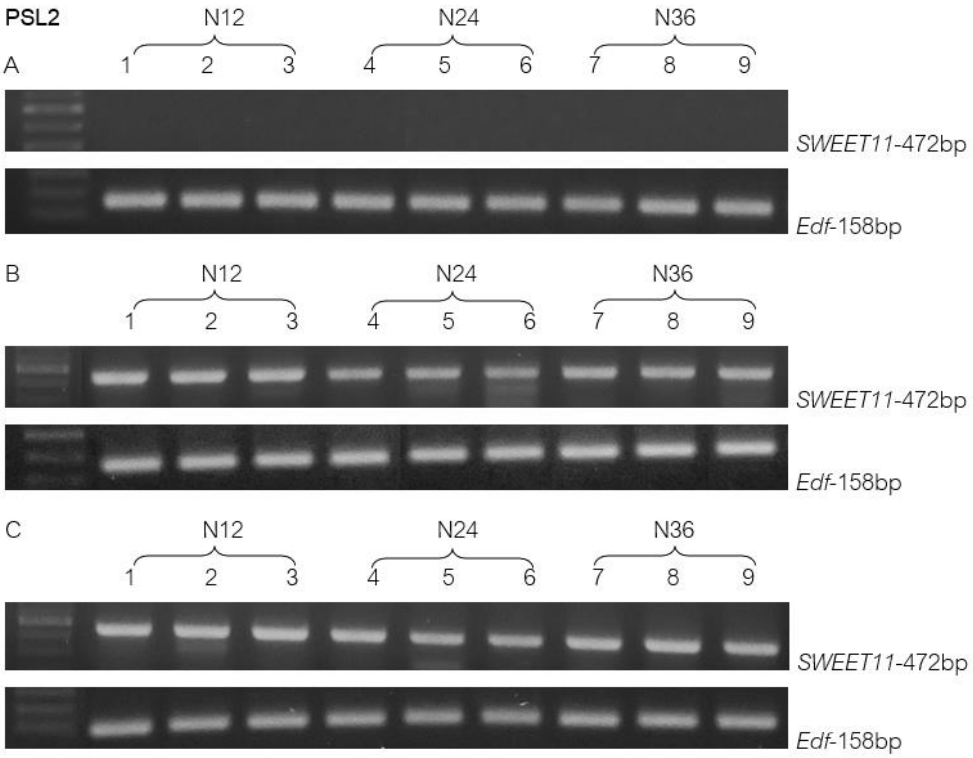

Figure 6 The RT-PCR products of *OsSWEET11*. The expression of *OsSWEET11* in *Xoo*16PK002 infected PLS2 plants at 0 (A), 48 (B) and 72 (C) hours after inoculation (HAI). Lane 1, 2 and 3 are three biological replicates.

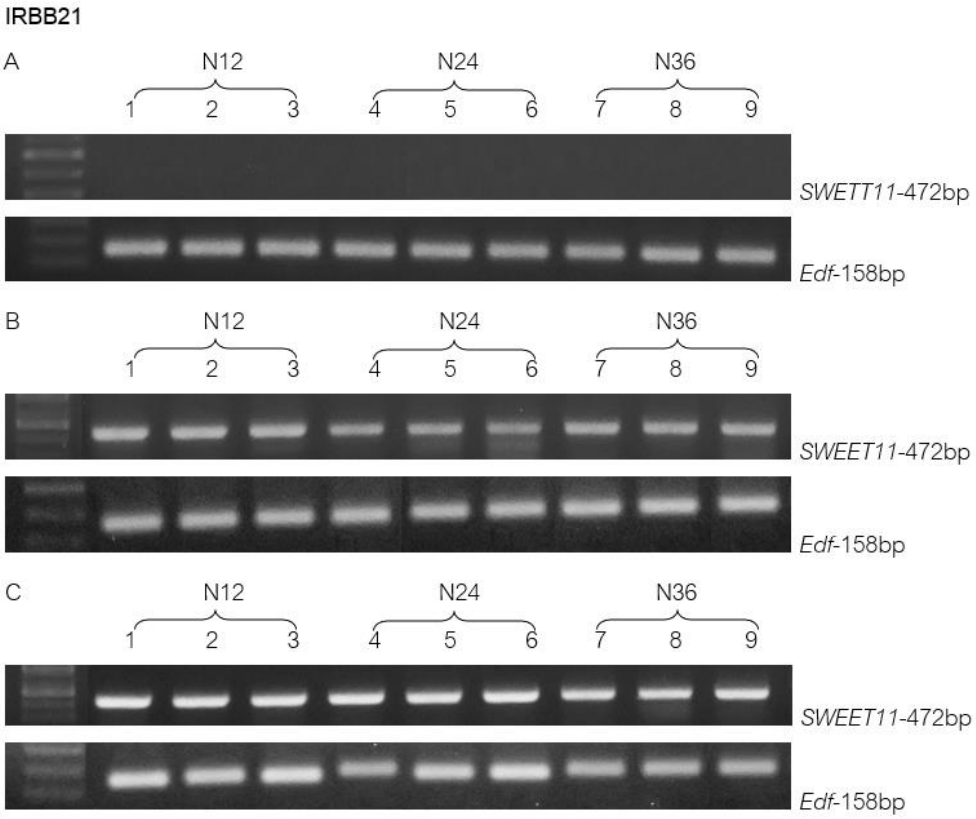

Figure 7 The RT-PCR products of *OsSWEET11*. The expression of *OsSWEET11* in *Xoo*16PK002 infected IRBB21 plants at 0 (A), 48 (B) and 72 (C) hours after inoculation (HAI). Lane 1, 2 and 3 are three biological replicates.

Expression analysis of *SWEET14* in rice inoculated with the *Xoo*16PK002

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was performed to evaluate the expression of *SWEET14* in the 49-day old seedlings of rice cultivars RD47, PSL2, IRBB21 after being inoculated with *Xoo*16PK002 the *SWEET14* expression was detectable from 0 hpi and 48 and 72 hpi in all experimental (Figure 8-10). Zeng et al. (2020) demonstrated that *OsSWEET14* was highly expressed in vegetative tissues including the leaf blade. *OsSWEET14* is a rice susceptibility gene that has been previously reported as a target of the major virulence TAL effectors including AvrXa7, PthXo3 and TalC from Asian *Xoo* strains *PXO86* and *JXO1A* and *BAI3* from the African *Xoo* stains (Chu et al., 2006; Antony et al., 2010; Yu et al., 2011). The result implies that *Xoo*16PK002 do not carry any virulent TAL effectors which can recognize the EBEs of *SWEET14* in indica rice cultivars RD47, PSL2 and IRBB21. Therefore, *SWEET14* is not the major target of *Xoo*16PK002 enabling compatibility on the experimental indica rice

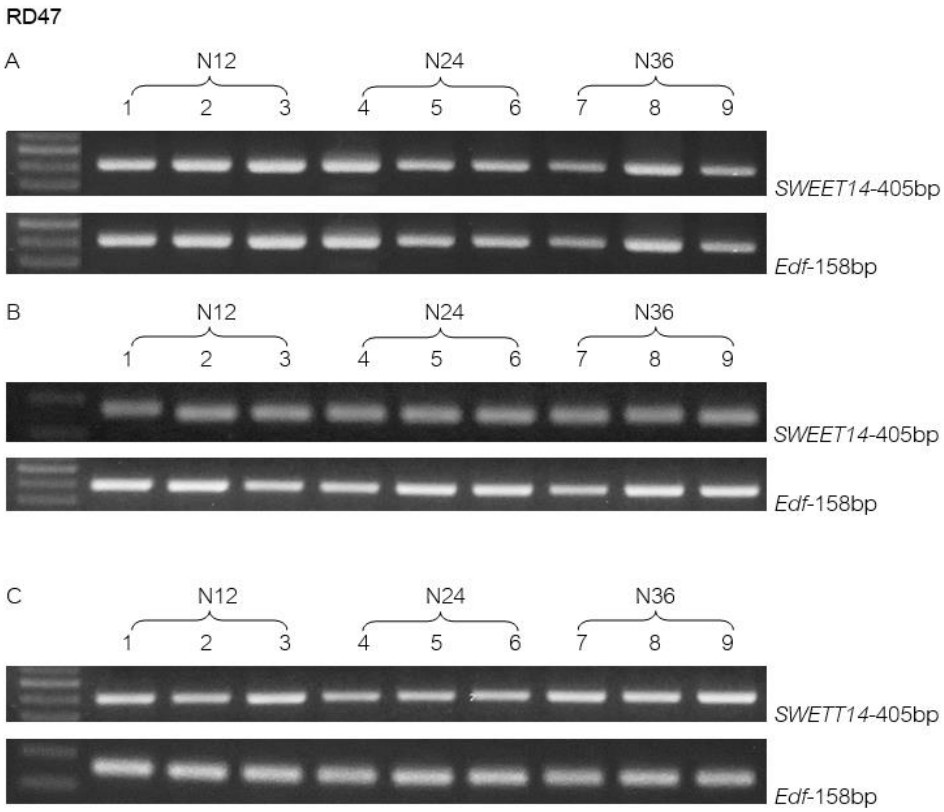

Figure 8 The RT-PCR products of *OsSWEET14*. The expression of *OsSWEET14* in *Xoo*16PK002 infected RD47 plants at 0 (A), 48 (B) and 72 (C) hours after inoculation (HAI). Lane 1, 2 and 3 are three biological replicates.

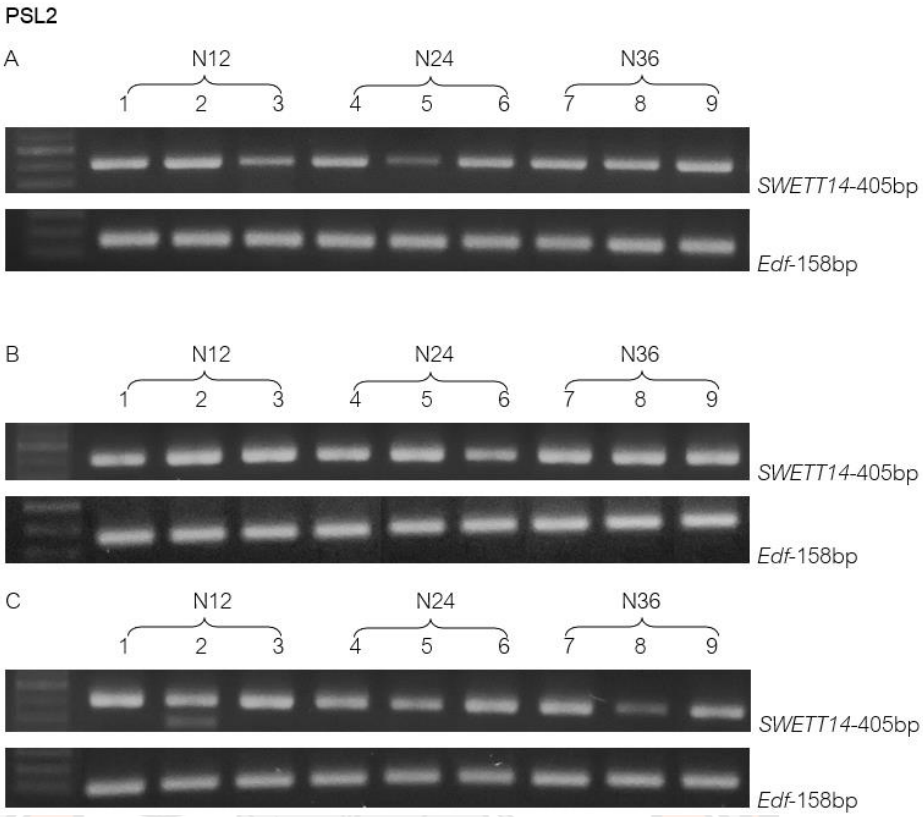

Figure 9 The RT-PCR products of *OsSWEET14*. The expression of *OsSWEET14* in *Xoo*16PK002 infected PSL2 plants at 0 (A), 48 (B) and 72 (C) hours after inoculation (HAI). Lane 1, 2 and 3 are three biological replicates.

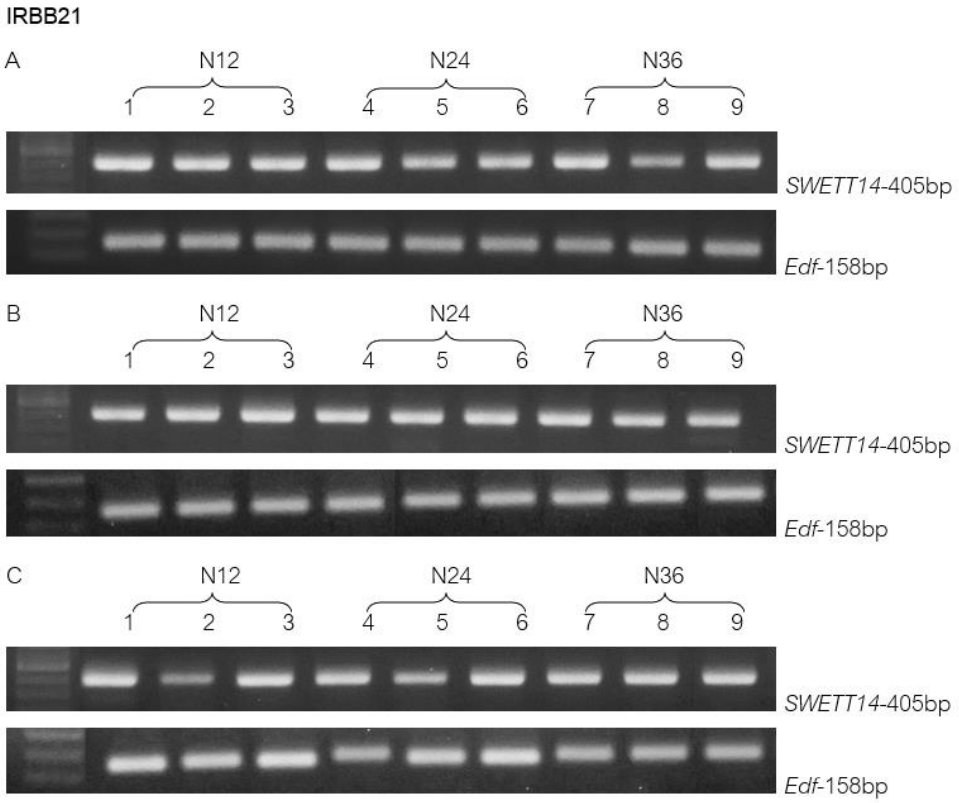

Figure 10 The RT-PCR products of *OsSWEET14*. The expression of *OsSWEET14* in *Xoo*16PK002 infected IRBB21 plants at 0 (A), 48 (B) and 72 (C) hours after inoculation (HAI). Lane 1, 2 and 3 are three biological replicates.

Relative expression of *SWEET11* in *Xoo*-infected RD47, PSL2 and IRBB21 applied with different nitrogen levels

When examining the *SWEET11* expression in rice cultivars RD47, PSL2, and IRBB21, using 49-day old seedlings and subjecting them to varying nitrogen fertilizer levels (12, 24, and 36 kg/rai), it was observed that the *SWEET11* expression remained consistently low at 0 hours, 24 hours, and 72 hours across all three rice varieties, RD47, PSL2, and IRBB21, during Mock inoculation. The expression levels exhibited remarkable similarity (Figure 11)

Following Xoo-inoculation, the analysis of *SWEET11* gene expression in RD47 and PSL2 rice varieties indicated its absence at 0 hours. Expression became evident at 24 hours and then doubled by the 72-hour mark, with a pronounced increase particularly in the presence of 12 kg nitrogen/rai. In contrast, the IRBB21 rice variety displayed lower *SWEET11* gene expression compared to RD47 and PSL2 varieties, signifying a noteworthy reduction in expression levels relative to these initial two strains. (Figure 12)

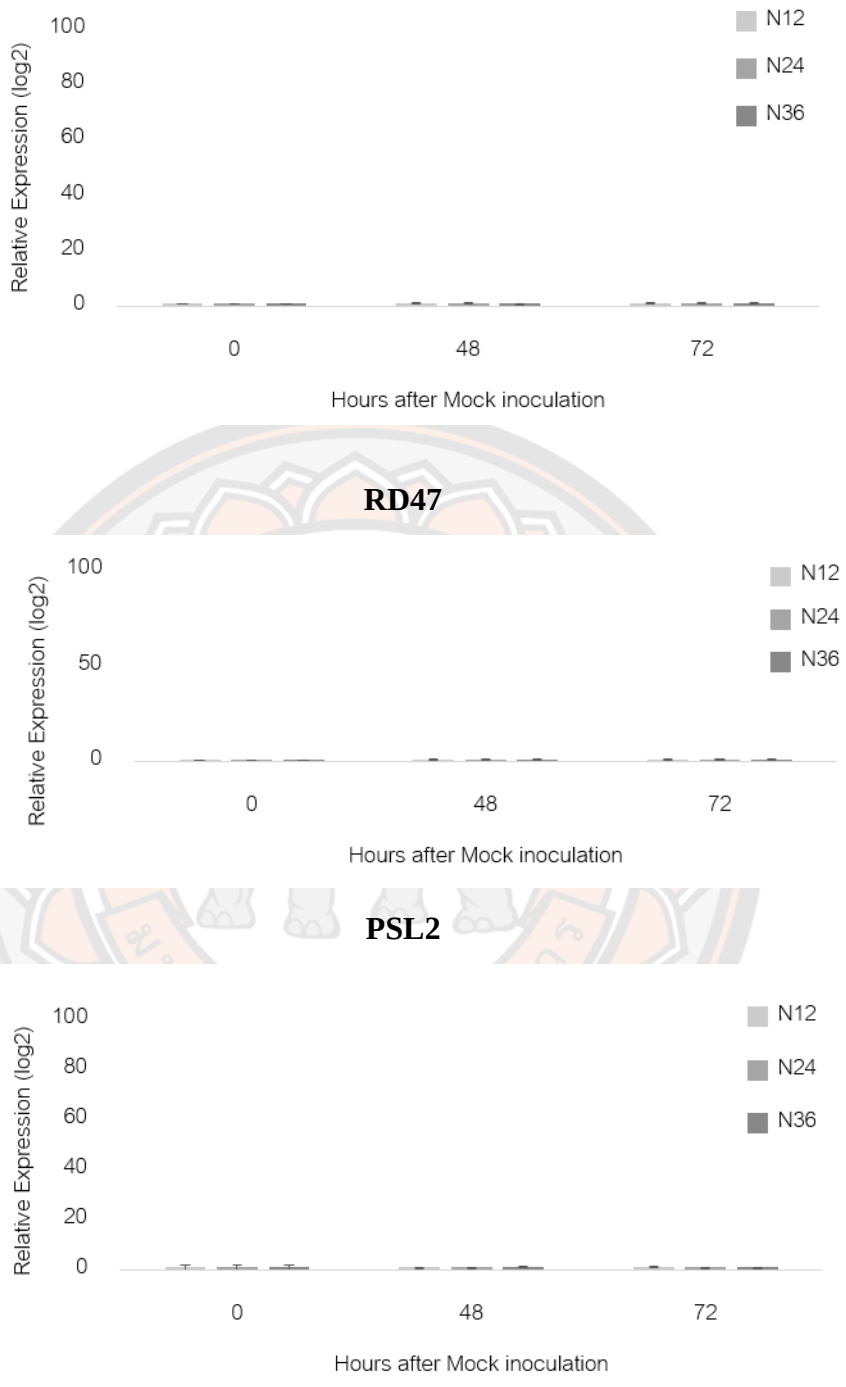

Figure 11 The relative expression of *SWEET11* in Mock-inoculated RD47, PSL2, IRBB21 applied with nitrogen fertilizer at 12, 24 and 36 kg/rai. Data was collected at 0, 48,72 hours after inoculation. Expression analysis was normalized with *Edf*.

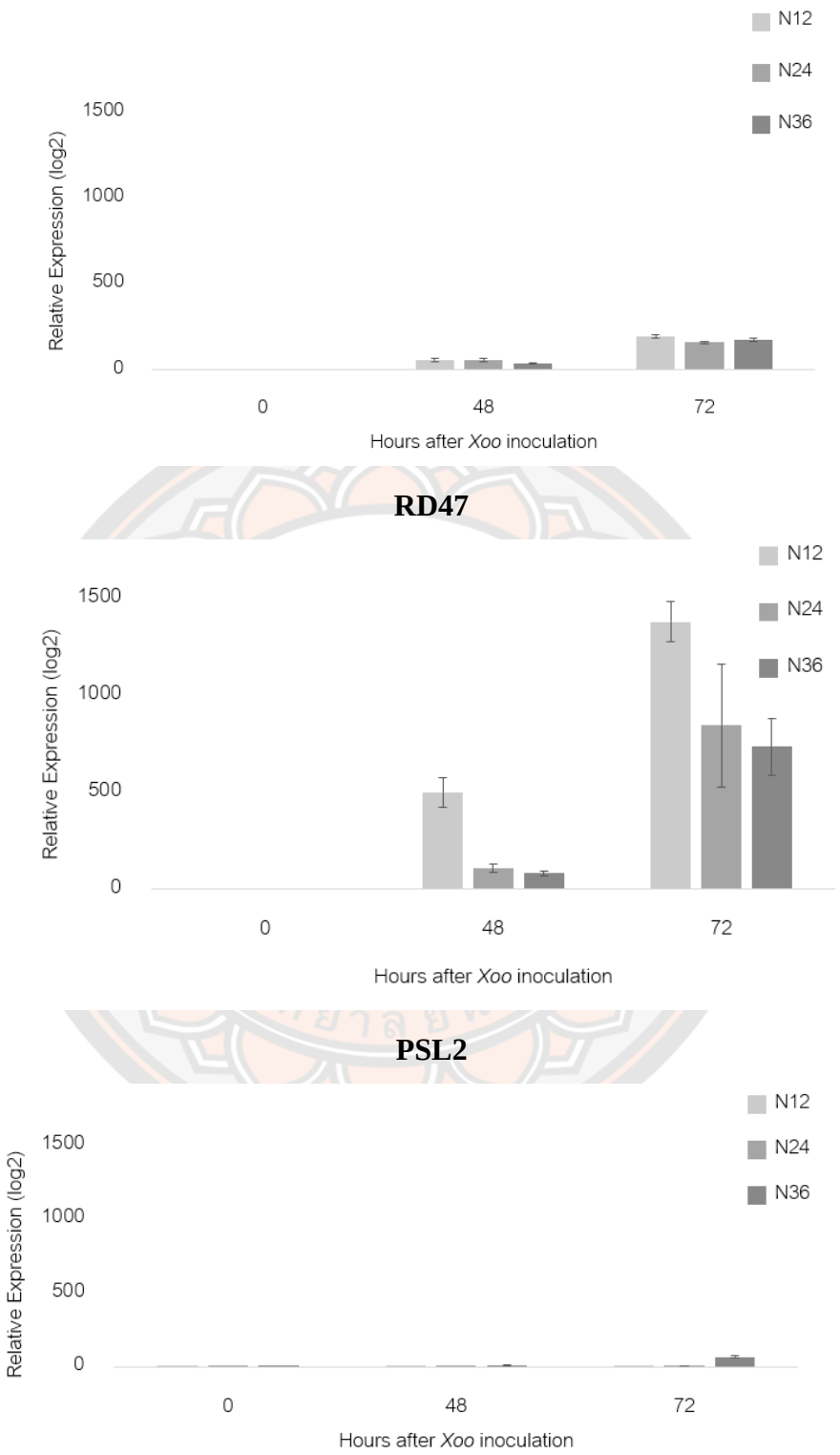

Figure 12 The relative expression of *SWEET11* in *Xoo*-inoculated RD47, PSL2, IRBB21 applied with nitrogen fertilizer at 12, 24 and 36 kg/rai. Data was collected at 0, 48,72 hours after inoculation. Expression analysis was normalized with *Edf*.

CHAPTER V

SUMMARY, CONCLUSION, AND RECOMMENDATION

This study investigated the impact of different nitrogen fertilizer levels on the resistance to BB disease and the expression of *SWEET11* and *SWEET14* in three rice cultivars: RD47, PSL2, and IRBB21, following infection *with Xanthomonas oryzae* pv. *oryzae* isolate *Xoo*16PK002. The assessment of BB resistance revealed that IRBB21 demonstrated resistance to BB disease, whereas RD47 and PSL2 exhibited susceptibility to *Xoo*16PK002, with the greatest lesion length observed at the nitrogen fertilizer level of 36 kg/rai. The findings revealed a direct correlation between the lesions length and increasing nitrogen fertilizer concentrations. This relationship is linked to higher sucrose content in rice leaves, which in turn can intensify the severity of BB disease. Consequently, to enhance BB disease resistance in rice plants, the recommended nitrogen fertilizer application rate is 12 kg/rai, leading to a reduced occurrence of BB disease.

The expression of *SWEET11* was not detected at 0 hours post-inoculation (hpi). However, in RD47, PSL2, and IRBB21 plants subjected to nitrogen fertilizer levels of 12, 24, and 36 kg/rai, the expression of *SWEET11* substantially increased at 48 and 72 hpi. Meanwhile, *SWEET14* expression was detectable at 0 hpi and also at 48 and 72 hpi across all experimental conditions.

The lesion length increased proportionally as nitrogen concentrations were elevated. This trend corresponded with higher sucrose levels detected in rice leaves when a more substantial amount of nitrogen fertilizer was administered. The rise in sucrose within plant cells has the potential to exacerbate the severity of BB disease, as sucrose serves as a fundamental component for plant growth and plays a vital role in the transportation of sucrose through a cluster of sucrose transport genes (*OsSWEET* genes), it also becomes associated with susceptibility to BB disease.

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