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Global Metabolic Changes by *Bacillus* Cyclic Lipopeptide Extracts on Stress Responses of Para Rubber Leaf

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Abstract

Changing environmental conditions can generate abiotic stress, such as the scarcity of water and exposure to chemicals. This includes biotic stress like *Phytophthora palmivora* infection, which causes leaf fall disease and inhibits the growth rate of para rubber seedlings, resulting in economic loss. To prevent abiotic and biotic stresses, biocontrol agents such as cyclic lipopeptides (CLPs) from *Bacillus* spp. have been introduced to reduce the use of chemically synthesized fungicides and fertilizers. This study aimed to use *Bacillus* CLP extracts as a biological agent to stimulate the plant growth system in para rubber seedlings under stress conditions compared with the exogenous plant hormone (salicylic acid, SA). CLP extracts obtained from *B. subtilis* PTKU12 and exogenous SA were applied to the leaves of para rubber seedlings. The extracted metabolites from each treatment were analyzed by untargeted metabolomics for metabolite identification and metabolic networks under stress responses. In both treatments, 1,702 and 979 metabolites were detected in the positive and negative ion modes of electrospray ionization, respectively. The differential analysis revealed that the accumulation of up-regulated metabolites in the treatment of CLP extracts was higher than in the exogenous SA treatment, belonging to 56 metabolic pathways. The analysis of metabolic pathways indicated that CLP extracts employed alanine, aspartate, and glutamate metabolisms for stress responses leading to plant growth promotion. These findings revealed that the metabolic network for plant growth promotion induced by *Bacillus* CLP extracts could be considered a protective option for para rubber plantations.

Keywords:

Bacillus subtilis;
Cyclic Lipopeptide Extracts;
Exogenous Salicylic Acid;
Para Rubber;
Phytophthora palmivora.

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1- Introduction

Para rubber trees (*Hevea brasiliensis*) play a key role in the global rubber industry. Para rubber trees can produce natural rubber, which provides polymers for the strategic raw materials used in more than 40,000 products worldwide. The trees grow primarily in humid tropical lowlands enveloped by dense tropical rainforests at an altitude of below 400 meters [1]. Agriculturists in Southeast Asia also pay attention to para rubber plantations in terms of income and employment [2]. Similarly, numerous microorganisms, especially fungal pathogens, can grow rapidly in humid tropical

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environments, which makes them key factors in decreasing the growth rates of plantations due to fungal diseases, resulting in economic loss. One of the most common fungal diseases in para rubber trees is leaf fall disease, which is caused by fungi in the genus *Neopestalotiopsis* [3] and fungal-like organisms in the genus *Phytophthora* [4]. Previous works have published that *Phytophthora palmivora* is a fungal-like pathogen causing leaf fall disease in para rubber trees and seedlings [5, 6], resulting in biotic stress conditions. Not only can biotic stress, such as from fungal infection, significantly impact plant growth during the plant development period, but so can abiotic stress, such as drought [7]. Therefore, many gardeners have used chemically synthesized fungicides and fertilizers that affect the environment and human health when handling the problems generated by biotic and abiotic stresses during the period of plantation.

To avoid the use of chemically synthesized fungicides and fertilizers in biotic and abiotic stress management, potent agents with environmentally friendly properties are required. The application of phytohormones like salicylic acid (SA) has been introduced for plant growth promotion during stresses [8, 9] as a biological approach. Previous work reported that exogenous SA could enhance the growth rate of sugar beet under fomesafen stress by increasing the photochemical efficiency [10]. During biotic stress, exogenous SA demonstrated the capacity to reduce disease severity and stimulate the enzyme activity of catalase, peroxidase, and phenylalanine ammonia-lyase, which were related to plant immunity in the leaves of para rubber seedlings challenged by *P. palmivora* [9]. Not only have phytohormones been used to manipulate abiotic and biotic stresses, but biological approaches such as plant-growth-promoting rhizobacteria (PGPR) are also used as an alternative approach for plant management [11]. PGPR are rhizosphere bacteria and play an important role in promoting plant growth via various mechanisms such as the induction of systemic resistance, expression of antifungal activity, suppression of pathogens, increase of plant enzymatic activity, and enhancement of nutrient absorption [12].

Bacillus subtilis is one kind of PGPR that has the ability to activate host plant resistance to biotic agents, such as fungal pathogens [13]. *B. subtilis* has been reported to be antagonistic to fungal pathogens such as *Penicillium digitatum* [14] and *Microsporum canis* [15]. A previous study demonstrated that *B. subtilis* NCIB 3610 isolated from the soil of mango orchards decreased the growth of *Pestalotiopsis mangiferae*, *Botryodiplodia theobromae*, and *Macrophoma mangiferae* by 57%, 61%, and 58%, respectively [16]. *B. subtilis* also showed the potential to control the growth of *P. palmivora* in cacao [17]. In addition, *B. subtilis* has been reported to improve abiotic stress, such as drought tolerance, in agriculture systems by enhancing plant nutrition and chlorophyll concentrations and reducing the negative effects of drought conditions [18]. During salt stress, *B. subtilis* showed the capacity to improve root and shoot growth as well as enhance the accumulation of oleic, linoleic, and linolenic acids in plant leaves [19]. Furthermore, *B. subtilis* displayed the ability to increase the content of proline, glycine, betaine, and choline, including the synthesis of photosynthetic pigments and carbohydrates, resulting in seed germination and plant growth under the condition of salt stress [20].

According to the plant immunity induction properties of *B. subtilis* during stress responses, it might produce some compounds with antifungal activity and properties of defensive induction, resulting in plant growth promotion. *B. subtilis* has the ability to produce cyclic lipopeptides (CLPs), which are secondary metabolites consisting of three main family compounds, including fengycins, iturins, and surfactins. CLPs are biosynthesized through a peptide ring mechanism by nonribosomal peptide synthetases. Lipoinitiation is driven to connect a fatty acid chain to the peptide ring [21, 22]. Fengycin can control the growth of fungal pathogens by binding to the sterols and phospholipids of the fungal cell membrane, causing leakage of the cell membrane. Besides, iturin A can induce the expression of pathogenesis-related protein 1 and plant defensing 1.2, which are proteins involved in plant defense [23]. Fengycin and surfactin have been reported to enhance the expression of *glucanase* and *peroxidase* genes in mandarin fruit [24], while fengycin A and iturin A demonstrated the potency of plant photosynthesis involved in plant growth promotion [25]. In addition, *Bacillus* CLPs have been reported to have the ability to increase seed germination and plant growth promotion in chili peppers, lettuce, tomatoes, and peas [26].

Exogenous SA and *Bacillus* CLPs could be used to manipulate stress conditions during the plantation period for plant growth promotion. Understanding the metabolic response to stress induced by the application of *Bacillus* CLP extracts on plant growth promotion when compared with exogenous SA remains ambiguous. To clarify the metabolic changes in plants responding to stress, metabolomics is a powerful technique to profile the metabolites in metabolic pathways [27, 28]. A previous study revealed the induction of glycine, serine, and threonine metabolism by the application of *Bacillus* CLPs responding to stress in mandarin fruit using a metabolomic approach [29]. Moreover, the metabolomic approach also presented the ability to profile the metabolic changes triggered by endophytic bacteria responding to plant growth promotion [30]. Thus, metabolomics is a suitable technology to explore up- and down-regulated metabolites in many metabolic pathways and could be used to indicate the comparative effects between *Bacillus* CLPs and exogenous SA on the induction of metabolic changes in response to stress in para rubber seedling leaves.

This study extracted *Bacillus* CLP extracts for testing the antifungal activity against *P. palmivora in vitro* to support *Bacillus* CLP extracts as an alternative agent for reducing the biotic stress factor, which affects plant growth promotion. To provide *Bacillus* CLP extracts as a potential agent of plant growth promotion *in vivo*, *Bacillus* CLP extracts were used to explore stress responses at a metabolic level compared with exogenous plant hormones in the leaves of para

rubber seedlings. This study aimed to evaluate the effect of *Bacillus* CLP extracts compared with exogenous SA on the metabolic response to stress in the leaves of para rubber seedlings for plant growth promotion. To better understand the *Bacillus* CLP extracts' action on plant growth promotion, the metabolic changes induced by different agents applied to the leaves of para rubber seedlings were analyzed using untargeted metabolomics. This study describes the rationale and background of the work in the introduction section. The research methodology, sample preparation, and analytical methods are exhibited in the Materials and Methods section. The experimental results and data interpretation are shown in the results and discussion section. The summary of this study is presented in the conclusion section, which is followed by the declaration section.

2- Materials and Methods

2-1- Research Methodology

The research methodology in Figure 1 provides an overview of the experiments.

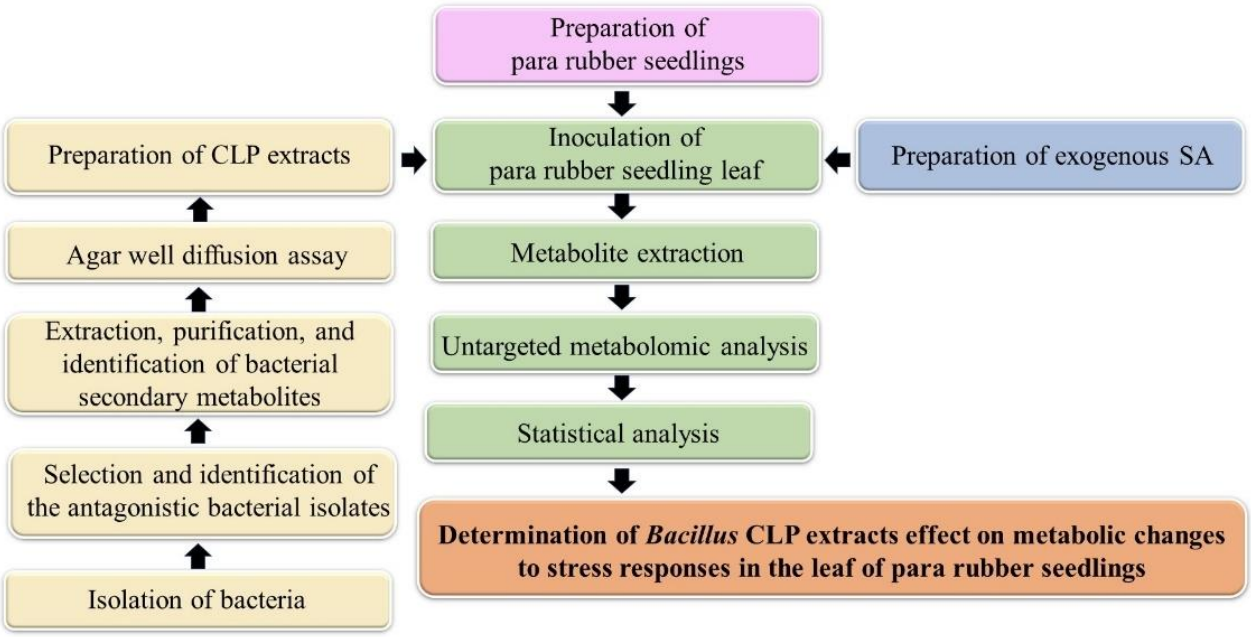


Figure 1. Flowchart of the research methodology

2-2- Para Rubber Seedlings and Fungal Pathogen

The samples used in this study comprised 45-day-old para rubber seedlings (*H. brasiliensis*) cloned RRIM600 under an organically controlled plantation from para rubber orchards located in Chanthaburi Province, Thailand. The para rubber seedlings were kept in a climate-controlled room at room temperature (28 °C) under 12 h/12 h of light/dark photoperiod [9] for 3 days without watering to generate the stress condition before starting the experiment.

P. palmivora was provided by the Nanomaterials Chemistry Research Unit, Department of Chemistry, Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University, Nakhon Si Thammarat, Thailand, with the cooperation of the Songkhla Rubber Research Center, Songkhla, Thailand. P. palmivora was verified for mycology by microscopic examination. Fungal isolates with the P. palmivora microscopic appearance (heterothallic with amphigynous antheridia and spherical oogonia, papillate caducous-sporangia, varying in shape from ovoid-ellipsoid to obpyriform) were selected as fungal-like pathogens. The fungal mycelia were routinely maintained on potato dextrose agar (PDA) at room temperature for five days before the testing of dual culture and agar-well diffusion assays.

2-3- Isolation of the Bacteria

Soil samples were collected from rubber plantations located in the Patthawi Sub-district, Makham district, Chanthaburi Province, Thailand to isolate the *Bacillus* bacteria. The isolation of the *Bacillus* bacteria was conducted following the previous method [31]. Briefly, each soil sample of 5 g was suspended in 95 mL of sterile distilled water and shaken strenuously. To demolish all vegetative microbial cells, the soil samples were incubated in a rotary water bath shaker at 100 rpm and 80 °C for 30 min. The 10-fold serial dilutions (10^{-3}, 10^{-4}, and 10^{-5}) of the soil samples were spread onto nutrient agar and then incubated at 37 °C for 24 h. All single colonies were subcultures on plates of nutrient agar. The characterization of the Gram staining, cell shape, and presence of the spores was performed. The bacteria with Gram-positive rods with spores were selected for the *Bacillus* spp. Isolates.

2-4- Selection and Identification of the Antagonistic Bacterial Isolates

A dual culture assay was performed following the previous study [31] to determine the antagonistic properties of *Bacillus* spp. on the growth of *P. palmivora*. Briefly, a 0.1 cm agar plug of *P. palmivora* culture grown on a PDA plate was incubated in the center of a fresh PDA plate at room temperature for 48 h. The pellet of each bacterial sample was prepared from the bacterial culture, which was shaken at 250 rpm and 30 °C for 24 h by centrifuging at 8,000 rpm for 15 min. The pellet was streaked on a PDA plate 1 cm away from the fungal plug and incubated at room temperature for 48 h. The calculation for the percentage of hyphal growth inhibition was carried out by using the formula from a previous study [32]. Three replicates were performed for each *Bacillus* isolate.

The *Bacillus* isolates with antagonistic activity were identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) by the Office of Scientific Instrument and Testing, Prince of Songkla University, Songkhla, Thailand. Moreover, Sanger sequencing was also performed on the polymerase chain reaction products of extracted bacterial DNA using universal 16S rRNA bacterial primers; 27F (forward: 5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (reverse: 5'-GGTTACCTTGTTACGACTT-3') by the Apical Scientific, Selangor, Malaysia for identification. To confirm the species of antagonistic bacteria, the sequences were submitted to nucleotide BLAST against the National Center for Biotechnology Information (NCBI) database. The *Bacillus* isolate that showed the highest antagonistic activity was selected for extracting and purifying the CLPs.

2-5- Extraction, Purification, and Identification of Bacterial Secondary Metabolites

The selected *Bacillus* strain was used to extract the CLPs, which were secondary metabolites. The preparation of the CLP extracts was performed according to previous studies [24, 33]. Briefly, the selected *Bacillus* strain was cultured in a Luria-Bertani broth at 200 rpm and 37 °C for 72 h in an incubator shaker. Centrifugation was performed at 10,000 rpm for 15 min to collect the supernatant. The pH of the supernatant was adjusted to 2.0 with 6 N hydrochloric acid and then centrifuged at 10000 rpm and 4 °C for 20 min to collect the pellet. Pellets were extracted twice with 80% of ethanol, dried by rotary evaporation at 65 °C, and redissolved in 80% of ethanol, respectively, to prepare the CLP extracts. The preparation of the *Bacillus* fengycin, iturin A, and surfactin was conducted from the CLP extract solution according to a previous study [34]. Briefly, fengycin, iturin A, and surfactin were purified by preparative thin-layer chromatography (PTLC) using a chloroform-methanol-water (65:25:4 v/v) solution as a mobile phase. The CLP bands on the PTLC plate were recovered from silica gel by ethanol extraction. Solid phase extraction (SPE) was carried out to increase the purity of the fengycin, iturin A, and surfactin using a solution of step gradients at 25% to 80% of acetonitrile in 0.1% of trifluoroacetic acid as a mobile phase. To identify each CLP, MALDI-TOF MS was performed by the National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathum Thani, Thailand to verify the molecular mass of the fengycin, iturin A, and surfactin compared to their commercial standards (Sigma-Aldrich, USA) as detailed in previously published articles [15, 34].

2-6- Agar Well Diffusion Assay

The antifungal properties of the CLP extracts and individual CLPs on the inhibition of the growth of *P. palmivora* were determined using the agar well diffusion assay following the method described in previous studies [15, 35]. Briefly, a 0.1 cm diameter of the actively growing mycelial plug earned from 5-day-old *P. palmivora* [36] was placed on the center of a PDA plate and incubated at room temperature for 48 h before starting the experiment. Two cavities with a diameter of 0.5 cm were created approximately 1 cm away from the edge of the growing mycelium on the PDA plate. Concentrations of the CLP extracts, fengycin, iturin A, and surfactin in 80% of ethanol at 10 mg/mL, 1.5 mg/mL, 1 mg/mL, and 3 mg/mL, respectively, were added to the cavities. The solution of 80% of ethanol was used as a control. The radial extension of the *P. palmivora* mycelium was observed at 24 h, 48 h, and 72 h after treatment. The inhibition percentage of the CLP extracts and individual CLPs was calculated according to a previous study [32]. The concentration of each agent used in the agar well diffusion assay was determined using the value of half-maximal effective concentration (EC_{50}) in the preliminary experiment according to the methods in previous reports [15, 37].

2-7- Inoculation of Para Rubber Seedling Leaves

SA (product number: S7401) used as the exogenous plant hormone was purchased from Sigma-Aldrich, USA. The 20 mL solutions of CLP extracts (10 mg/mL), SA (5 mM) [9], and 80% ethanol (control) were prepared and each was sprayed onto the leaves of para rubber seedlings without physical injuries or infections. Subsequently, the treated leaves were individually covered with plastic bags to maintain the humidity and incubated for 72 h in a climate-controlled room. Each treatment was carried out in triplicate, and each replicate consisted of 5 plants. Six leaves were detached from two petioles in the same layer of the seedling stem for metabolite extraction.

2-8- Metabolite Extraction

The treated leaves in each replicate of the same treatment was pooled and ground to a fine powder in liquid nitrogen. Metabolite extraction was performed according to the previous study [29]. Briefly, 15 mg of fine powder was weighed, and then 1 mL of a mixture of methanol/water/chloroform (2.5:1:1 v/v/v) was added and incubated at 40 °C for 5 min.

After centrifugation, 950 µL of supernatant was transferred to a new tube. Water (400 µL) was added to the supernatant and centrifuged. The polar phase solution of individual treatment was filtered using syringe filters (0.22 µm, Ligand Scientific, Thailand) for untargeted metabolomic analysis.

2-9- Untargeted Metabolomic Analysis

Liquid chromatography (LC-MS) grade water, acetonitrile, and formic acid were purchased from Fisher Scientific, USA. To monitor the potential contamination of sample processing, a procedural blank sample of water was analyzed. Quality control (QC) samples were prepared by mixing equal volumes of extracted metabolite solutions from all treatments for monitoring the stability of the acquisition system. Metabolomics profiling was performed using an ultraperformance liquid chromatography (UPLC) system (Vanquish Flex UHPLC, Thermo Scientific, Germering, Germany) consisting of a binary pump with built-in degasser (Binary Pump F), a column compartment (Column Compartment H), and an autosampler (Split Sampler FT) coupled with an Orbitrap Exploris 120 system equipped with a heated electrospray ionization (H-ESI) source and a hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). The UHPLC column was a Hypersil Gold C-18 column (2.1×150 mm, 1.9 µm) (Thermo Fisher Scientific, USA). Before conducting the analysis, the system and mass calibration were executed for both positive and negative ionization using a Thermo Scientific Pierce FlexMix Calibration Solution (Thermo Fisher Scientific, USA).

For each metabolite solution, 5 µL was injected into the UPLC system. LC Solvent A was 0.1% (v/v) formic acid in 5% (v/v) acetonitrile, and Solvent B was 0.1% (v/v) formic acid in acetonitrile. The gradient elution profile was as follows: t = 0 min, 20% B; t = 3.5 min, 35% B; t = 18 min, 65% B; t = 21 min, 95% B; t = 21.5 min, 95% B; t = 23 min, 98% B; t = 24 min, 98% B; t = 25.5 min, 20% B; t = 36.6 min, 20% B [38]. The flow rate was 180 µL/min. The samples were randomly injected in no particular order. Ten QC samples were run before the samples to equilibrate the detection system. Furthermore, the QC sample was also injected every ten sample runs during analysis [29]. The parameter sets for MS analysis were carried out according to previous work [39]. Briefly, positive and negative ion modes of electrospray ionization were done with a range of mass-to-charge ratio (*m/z*) from 70–1050. A full MS scan at 60,000 resolutions followed by data-dependent MS^2 at 15,000 resolutions were performed, respectively. The collision energies of 20, 40, and 60 eV were set to acquire the MS^2 spectrometry data. Metabolite identification was done using Compound Discoverer software version 3.3 (Thermo Fisher Scientific, USA) against the mzCloud (https://www.mzcloud.org) and ChemSpider compound (http://www.chemspider.com) databases for positively identified metabolites. The identification of putatively identified metabolites was performed via MyCompoundID MS software (https://mcid.chem.ualberta.ca) against the Human Metabolome Database (HMDB) library and the Evidence-based Metabolome Library (EML) database [40].

2-10- Statistical Analysis

The agar well diffusion assay data sets were analyzed for significant differences by ANOVA. Differences with $p < 0.05$ were judged to be significant. Tukey's range test was used to determine the significant differences in the mean values, which were reported as mean ± standard error (n = 3). Compound Discoverer software (Thermo Fisher Scientific, USA) was used to analyze the raw data for differential analysis via a volcano plot. Principal component analysis (PCA) was conducted using ggpubr and mixomics packages in the R program and MetaboAnalyst 5.0 was used to perform pathway analysis [41].

3- Results and Discussion

The scarcity of water and exposure to chemicals or pathogens in the environment generate stress in plants. To adapt to changing surroundings, plants have to activate a suite of molecular and cellular processes to respond to stresses. PGPR, root-associated bacteria, play an important role in plant growth promotion and plant defensive induction by employing the stimulation of metabolites and proteins in various pathways. *B. subtilis*, a member of PGPR, shows a positive effect on plants by modulating the stress responses via the activation of induced systemic resistance triggered by CLPs [42]. The effect of CLP extracts obtained from *B. subtilis* PTKU12 on plant growth promotion under stress conditions in the leaves of para rubber seedlings was investigated and compared with exogenous plant hormone treatment.

3-1- Isolation and Identification of Antagonistic Bacillus Strains

After the process of bacterial screening, there were 90 bacterial isolates (PTKU1 to PTKU90) with Gram-positive rods with spores, which were selected from the soil samples. Each *Bacillus* isolate was tested for the ability to control the growth of *P. palmivora* using the dual culture assay. Among the 90 bacterial isolates, eight isolates displayed higher than 70% efficacy for fungal pathogen growth inhibition (Table 1). The bacterial isolate PTKU12 showed the greatest antifungal activity to suppress the radial extension of the *P. palmivora* mycelium at 89.73% ± 2.81. The identification result of the eight antagonistic bacterial isolates by MALDI-TOF MS revealed that there were five bacterial species including *B. megaterium*, *B. subtilis*, *Fictibacillus macauensis*, *B. mojavensis*, and *B. amyloliquefaciens*, which played

a role in the antagonistic activity (Supplementary Table S1). Then, the bacterial isolate PTKU12 was identified using the Sanger sequencing technique. The analysis of forward and reverse sequences obtained from bacterial isolate PTKU12 by blasting against the NCBI database presented that bacterial isolate PTKU12 was identified as *B. subtilis*, which showed the best match to *B. subtilis* strain ANctcri3 (accession no: HQ286641.1) with percent identification of 99.31% in the NCBI database (Supplementary Table S2).

Table 1. Inhibition of bacterial isolates on the growth of Phytophthora palmivora by a dual culture assay with a value of inhibition higher than 70%

Name of bacterial isolate	%inhibition ± S.D.
Bacterial isolate PTKU12	89.73 ± 2.81
Bacterial isolate PTKU73	84.14 ± 6.24
Bacterial isolate PTKU57	83.78 ± 9.22
Bacterial isolate PTKU20	82.52 ± 3.43
Bacterial isolate PTKU3	82.16 ± 7.49
Bacterial isolate PTKU80	81.21 ± 6.82
Bacterial isolate PTKU43	75.85 ± 26.27
Bacterial isolate PTKU40	70.80 ± 4.68

The screening of *Bacillus* bacteria was performed to select the PGPR with antagonistic activity to the *P. palmivora* pathogen that causes biotic stress in para rubber seedlings. In a dual culture assay, the bacterial isolate PTKU12 with the highest antagonistic manner was identified as *B. subtilis*. The antagonistic potential of *B. subtilis* in this study displayed the same manner as in previous studies that reported the antagonistic properties of *B. subtilis* against *P. palmivora* generating black pod rot disease on cacao [43]. Moreover, *B. subtilis* LKM-BL isolated from the tissues of healthy cacao trees presented the highest antifungal activity to hinder the growth of the pathogenic fungus *P. palmivora* using the dual culture method [44]. It could be suggested that *B. subtilis* PTKU12 could be considered an antagonistic bacterium, which could control the growth of *P. palmivora* for plant growth promotion. Moreover, *B. subtilis* could produce secondary metabolites; such as fengycins, iturins, and surfactins, which are CLPs with antifungal properties [15, 24, 31, 34].

3-2-Antifungal Properties of CLPs on the Growth Inhibition of P. palmivora

An Agar well diffusion assay was carried out to confirm the mode of action of CLPs on the growth inhibition of *P. palmivora*. Concentrations of the CLP extracts, fengycin, iturin A, and surfactin at 10 mg/mL, 1.5 mg/mL, 1 mg/mL, and 3 mg/mL, respectively, were applied to the *P. palmivora in vitro* following EC_{50} values in the preliminary experiment. The radial extension of the mycelium growth was measured at 24 h, 48 h, and 72 h to determine the inhibition percentage. In Figure 2, the potential of fengycin, iturin A, and surfactin on the inhibition of the *P. palmivora* growth was decreased from 24 h to 72 h when compared with the control. While the antifungal action of the CLP extracts was increased from 24 h to 48 h, it was decreased from 48 h to 72 h. In Table 2, the CLP extracts, fengycin, iturin A, and surfactin demonstrated inhibition at 60.03% ± 3.03, 64.26% ± 1.27, 71.95% ± 2.78, and 57.10% ± 1.56, respectively, at 24 h after treatment. The efficacy of the CLP extracts, fengycin, iturin A, and surfactin could hinder fungal mycelial growth with the inhibition of 72.49% ± 3.39, 55.48% ± 4.34, 66.23% ± 1.93, and 43.37% ± 2.50 at 48 h after treatment, respectively (Table 2). The inhibition of 61.76% ± 3.68, 52.19% ± 3.95, 50.93% ± 2.77, and 33.04% ± 2.30 was investigated in the applications of the CLP extracts, fengycin, iturin A, and surfactin, respectively, at 72 h after treatment (Table 2 and Figure 3).

Table 2. Inhibition percentage of each treatment on the growth of *Phytophthora palmivora*. The standard errors of the mean value for the three trials are shown. The same letter above shows that there was no significant difference between them at $p<0.05$ according to Tukey's range test.

Treatments	Time after treatment (h)		
	24	48	72
	% inhibition ± S.D.	% inhibition ± S.D.	% inhibition ± S.D.
CLPs extracts	60.03 ± 3.03^{ab}	72.49 ± 3.39^{c}	61.76 ± 3.68^{c}
Fengycin	64.26 ± 1.27^{b}	55.48 ± 4.34^{b}	52.19 ± 3.95^{b}
Iturin A	71.95 ± 2.78^{c}	66.23 ± 1.93^{c}	50.93 ± 2.77^{b}
Surfactin	57.10 ± 1.56^{a}	43.37 ± 2.50^{a}	33.04 ± 2.30^{a}

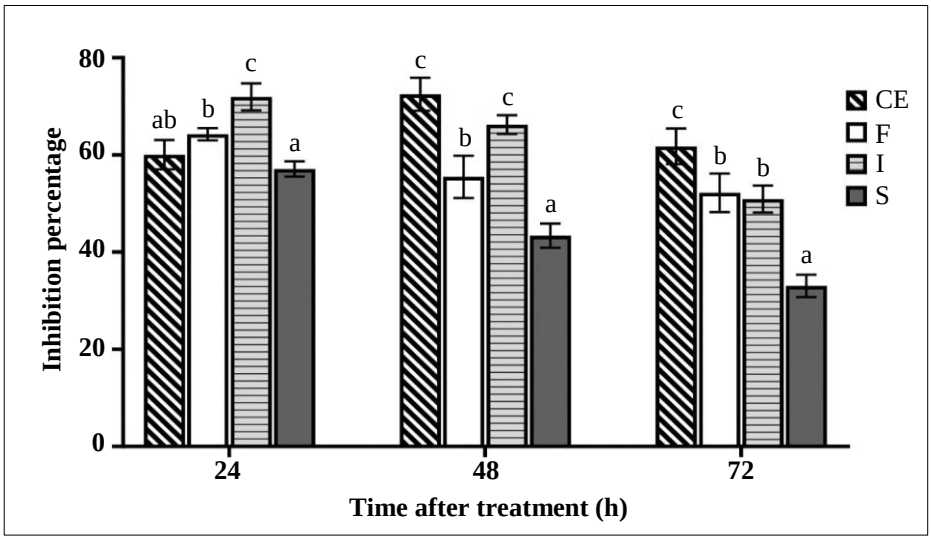


Figure 2. Inhibitory effect of each treatment on the growth of *Phytophthora palmivora*. The vertical bars represent the standard errors of the mean value for the three trials; the columns with the same letter above show no significant difference between them at $p < 0.05$ according to Tukey's range test. Abbreviations: CE: cyclic lipopeptide extracts (10 mg/mL); F: fengycin (1.5 mg/mL); I: iturin A (1 mg/mL); S: surfactin (3 mg/mL).

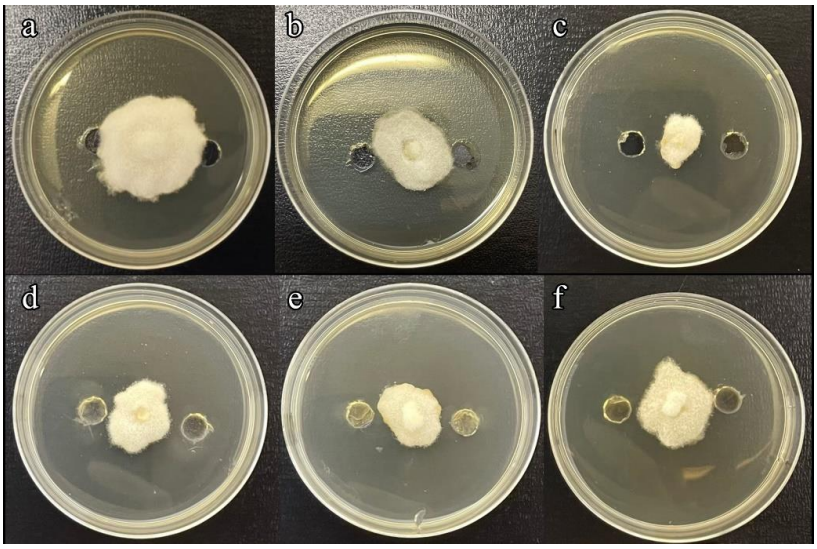


Figure 3. Antifungal efficacy of each treatment on the growth of Phytophthora palmivora at 72 hr by the agar well diffusion assay. *Phytophthora palmivora* (a), 80% ethanol (control) (b), 10 mg/mL of cyclic lipopeptide extracts (c), 1.5 mg/mL of fengycin (d), 1 mg/mL of iturin A (e), and 3 mg/mL of surfactin (F).

To verify the action of the CLP extracts and individual CLPs obtained from *B. subtilis* PTKU12 on the inhibition of the growth of *P. palmivora*, an agar well diffusion assay was conducted. According to the statistical analysis, iturin A presented the strongest inhibition (71.95% ± 2.78) to control the growth of *P. palmivora* with a statistically significant difference at 24 h after treatment. While the CLP extracts demonstrated the greatest act of fungal control at 48 h after treatment (inhibition of 72.49% ± 3.39), they showed no statistically significant difference when compared with the iturin A treatment (inhibition of 66.23% ± 1.93). However, the CLP extracts exhibited inhibition at 61.76% ± 3.68, which was the highest antifungal potential to manipulate the growth of *P. palmivora* with a statistically significant difference at 72 h after treatment. Applications of the *Bacillus* CLPs presented an inhibitory effect to suppress the extension of the *P. palmivora* colony, which was unable to spread on the plate. It could be indicated that the *Bacillus* CLPs obtained from *B. subtilis* PTKU12 limited the growth of *P. palmivora* to an abnormal form when compared to the control. This result was similar to a previous study that exhibited the efficacy of secondary metabolites, such as iturin, which was extracted from *B. subtilis* strain ME488 on the suppression of the *Phytophthora* fungus growth in pepper [45].

In the terms of inhibition, the CLP extracts exhibited the greatest antifungal efficacy to control the growth of *P. palmivora* at 48 h and 72 h after treatment. The CLP extracts could overcome the growth of the fungal pathogen because CLP extracts contained a mixture of secondary metabolites, such as fengycin, iturin A, and surfactin [14, 15, 24]. Each secondary metabolite might function synergistically, resulting in better antifungal activity. However, the synergistic action of the individual CLPs should be studied in a future experiment. The *Bacillus* secondary metabolites revealed activity in the control of the growth of fungal pathogens in the previous reports [46-50]. The CLPs could generate the

leakage of the fungal cellular contents and suppress the fungal branch formation and growth [51, 52] as an antifungal action. This study revealed the activity of the CLP extracts obtained from *B. subtilis* PTKU12 to reduce the mycelial growth rate of *P. palmivora*. It could be suggested that the *in vitro Bacillus* CLP extracts, which had an antifungal behavior to suppress the growth of *P. palmivora* causing stress conditions in para rubber trees, could be used as a potential agent to support plant growth promotion. To determine the properties of plant growth promotion for *Bacillus* CLP extracts *in vivo*, it was applied to the leaves of para rubber seedlings under stress conditions compared with exogenous SA.

3-3- Submetabolome Profiling and Metabolite Identification

After the inoculation of para rubber seedlings by CLP extracts and exogenous SA, the extraction of metabolites and untargeted metabolomic analysis was performed to evaluate the action of CLP extracts on metabolic changes compared with exogenous SA in the leaves of para rubber seedlings under stress conditions. A total of 2,618 metabolites were detected in the duplicate analysis of leaf samples from all treatments (1,702 and 979 metabolites in positive and negative ion modes of electrospray ionization, respectively). It should be noted that the same metabolite was filtered for one metabolite, resulting in a decreased number of detected metabolites. For metabolite identification, 4 databases (mzCloud, ChemSpider compound, HMDB, and EML) were used. There were 22 metabolites which were matched by *m/z* and retention time to both mzCloud and ChemSpider compound databases, while 32 and 520 metabolites were matched by *m/z* and retention time to mzCloud and ChemSpider compound databases, respectively (Supplementary Tables S3-4). The detected metabolites that were mass-matched to mzCloud or ChemSpider compound databases were considered positively identified metabolites. In the HMDB library and the EML library, the *m/z* of detected metabolites were mass-matched to 2,269 and 50,490 metabolites, respectively, which were considered putatively identified metabolites (Supplementary Tables S5-6). The metabolome composition of the leaf samples emerged as very intricate and the positively identified metabolites, coupled with many more putatively identified metabolites, covered a wide range of metabolic pathways, which will be discussed in the section on pathway analysis.

3-4- Plant Metabolome Profiles and Comparison

From the data set for the LC–MS/MS runs in both positive and negative ion modes of electrospray ionization, statistical tools were operated to analyze the metabolome differences in the samples of CLP extracts and exogenous SA treatments compared with control samples. PCA presented distinct separations from the tested samples using different groups of substances. The metabolome data could be separated into three major groups (Figure 4) plus the QC group (Supplementary Figure S1). The clustering of QC runs determined the repeatability of the analysis. In Figure 4, samples from disparate treatments are labeled with distinct colors. The treatments of CLP extracts, exogenous SA, and control were labeled with light blue, light green, and light orange, respectively. Although the metabolome data of each treatment in the PCA plot were not separated from each other, there were some distinct patterns used to separate the metabolome data from tested treatments. A tight clustering of the CLP extract samples is shown in the PCA plot. Some metabolome data of the exogenous SA group was also clustered together with CLP extract samples. However, there were some metabolome data in exogenous SA treatment also clustered together with the control group shown in the PCA plot. The oval shapes of exogenous SA and control treatments were close to each other, while the oval shape of the CLP extract group stepped away from the control group. It could be suggested that some metabolome data in CLP extracts treatment might be in the same manner with exogenous SA treatment. At the same time, most of the metabolome data in CLP extracts treatment revealed a different manner from exogenous SA treatment.

To determine the metabolic changes, differential analysis of different treatments was performed using binary comparisons. The resulting volcano plots indicated the metabolic changes of the leaf samples after being exposed to CLP extracts and exogenous SA compared with the control group (Figure 5). The effect of the control group was rebated using binary comparisons of the submetabolome in the treated treatment versus the control in each ion mode of electrospray ionization. Any change of more than 1-fold and a *p*-value of below 0.05 in metabolite concentration was considered to be significant. The volcano plot analysis of the CLP extracts group versus control in both positive and negative ion modes of electrospray ionization (Figure 5-aand 5-b) designates that 895 metabolites out of 2,618 were down-regulated (i.e., lower metabolite concentration in the treated samples than that of the control) and 312 metabolites were up-regulated (i.e., higher metabolite concentration in the treated samples). In the exogenous SA treatment versus control (Figures 5-c and 5-d), 131 metabolites out of 2,618 were found to be down-regulated and 44 metabolites were up-regulated. According to the metabolite with significant changes in the two binary comparisons, CLP extracts treatment presented the highest number of up-regulated metabolites (49 metabolites). Exogenous SA treatment showed a lower number of metabolite changes in both up-regulated metabolites (30 metabolites) and down-regulated metabolites (43 metabolites). It could be suggested that CLP extracts could induce metabolic changes better than exogenous SA in the leaves of para rubber seedlings during stress conditions. Because of a greater induction of metabolic changes by CLP extracts, the pathway analysis in this study only discussed the action of CLP extracts on triggering metabolic changes to stress responses in the leaves of para rubber seedlings.

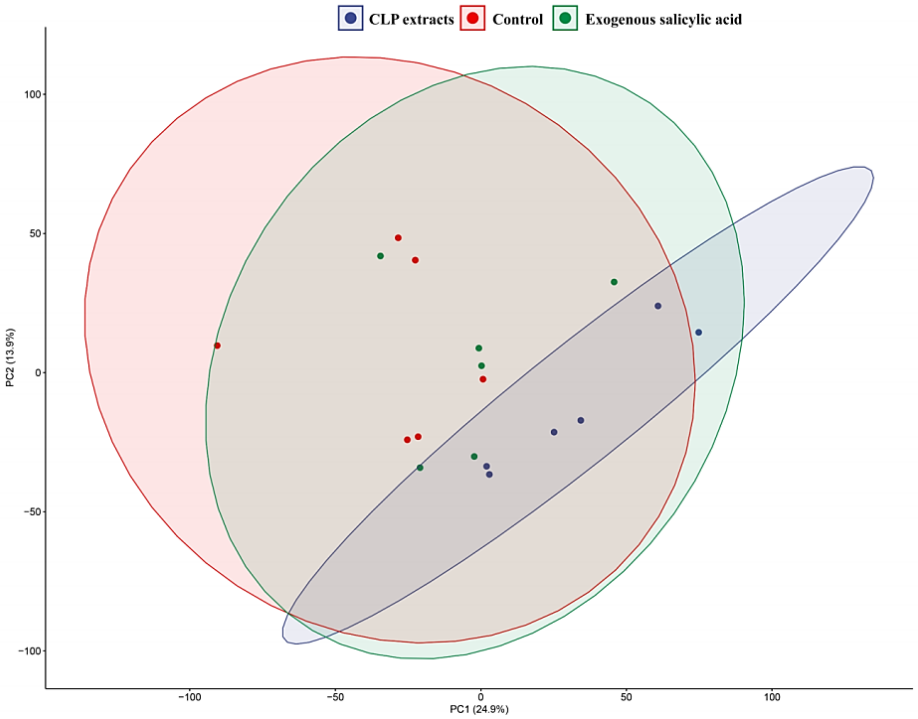


Figure 4. Principal component analysis plot of the metabolomic data obtained from different groups of samples. CLP extracts, group of cyclic lipopeptide extracts; Control, group of ethanol; Salicylic acid, group of exogenous salicylic acid.

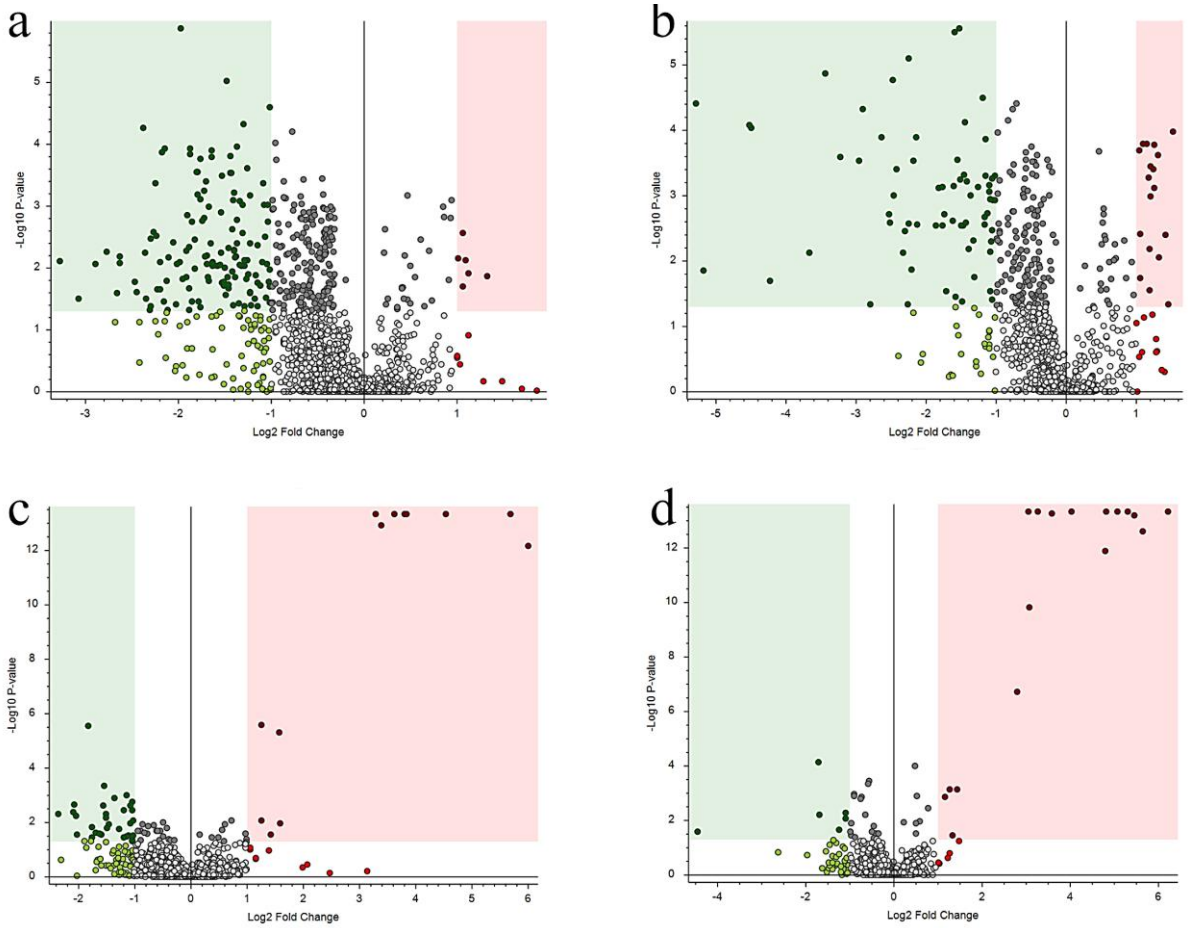
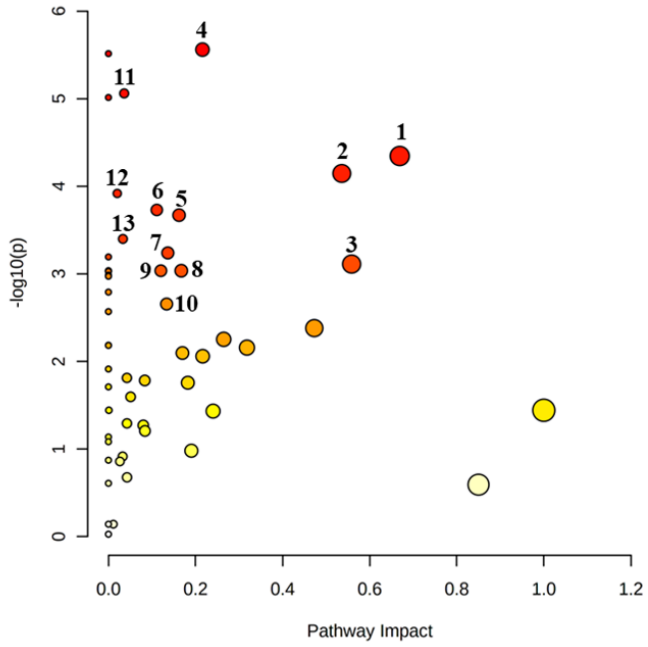


Figure 5. Volcano plots of binary comparisons of metabolites in treatment vs. control. (a) CLP extract treatment group vs. ethanol group in positive ion mode of electrospray ionization. (b) CLP extract treatment group vs. ethanol group in negative ion mode of electrospray ionization. (c) Exogenous plant hormone treatment group vs. ethanol group in positive ion mode of electrospray ionization. (d) Exogenous plant hormone treatment group vs. ethanol group in negative ion mode of electrospray ionization. The significant metabolites are shown in red or green with fold change >1 and *p*-value <0.05.

3-5- Metabolite Regulation in Plant Growth Promotion to Stress Responses

CLP extracts obtained from *B. subtilis* PTKU12 triggered the greatest number of metabolite changes in plant stress responses (Figure 5), suggesting that CLP extracts are better than exogenous SA for eliciting the system of plant growth promotion. The positively identified metabolites were submitted to MetaboAnalyst 5.0 software based on the database of *Arabidopsis thaliana* since no database of the para rubber plant was available. The positively identified metabolites were matched to 56 metabolic pathways, as shown in Figure 6. Each pathway consisted of an impact and a *p*-value according to the number of 'hits' recorded and the significant factors of the detected metabolites. The metabolism of alanine, aspartate and glutamate, which plays an important role in stress response, was found to have the greatest pathway impact and statistical significance located in the top right corner. Among 56 pathways, positively identified metabolites not only were matched to the metabolism of alanine, aspartate and glutamate but also were matched to several pathways such as glycine, serine and threonine metabolism, glyoxylate and dicarboxylate metabolism, cysteine and methionine metabolism, lysine biosynthesis, aminoacyl-tRNA biosynthesis, butanoate metabolism, pyruvate metabolism, glycolysis/gluconeogenesis, glutathione metabolism, carbon fixation in photosynthetic organisms, nicotinate and nicotinamide metabolism, and sulfur metabolism during stress responses following pathway impact and statistical significance.



1	Alanine, aspartate and glutamate metabolism
2	Glycine, serine and threonine metabolism
3	Glyoxylate and dicarboxylate metabolism
4	Cysteine and methionine metabolism
5	Lysine biosynthesis
6	Aminoacyl-tRNA biosynthesis
7	Butanoate metabolism
8	Pyruvate metabolism
9	Glycolysis/Gluconeogenesis
10	Glutathione metabolism
11	Carbon fixation in photosynthetic organisms
12	Nicotinate and nicotinamide metabolism
13	Sulfur metabolism

Figure 6. Overview of metabolic pathway analysis relating to stress responses

Figures 7 and 8 show the metabolites in alanine, aspartate and glutamate metabolism and glycine, serine and threonine metabolism, respectively. The metabolites yet to be identified are shown in light blue boxes. The positively identified metabolites, which matched both mzCloud and ChemSpider compound databases, are highlighted in dark green, while the positively identified metabolites, which matched the mzCloud or ChemSpider compound database are labeled with light green. The putatively identified metabolites are highlighted in orange. The individual positively or putatively identified metabolites in the pathway are displayed via box plot to present the significant level changes compared to the control group.

L-glutamate, gamma-aminobutyric acid, succinic acid, L-aspartic acid, L-alanine, pyruvic acid, and alpha-ketoglutaric acid are highlighted in green color, while L-arginosuccinic acid and L-glutamine are labeled in orange for alanine, aspartate and glutamate metabolism (Figure 7). Noticeably, pyruvic acid plays an important role in alanine, aspartate and glutamate metabolism as well as glycine, serine and threonine metabolism, which is considered a key metabolite to link both pathways. In Figure 8, L-aspartic acid, 3-phosphonooxypyruvic acid, pyruvic acid, L-serine, glycine, L-threonine, 2-oxobutyric acid, choline, and glyoxylate are highlighted in green, while L-tryptophan is labeled with orange in glycine, serine and threonine metabolism. Moreover, glyoxylate can be found in both glycine, serine and threonine metabolism and glyoxylate and dicarboxylate metabolism. Thus, glyoxylate was a key metabolite linking both pathways. According to the box plot, most metabolites are either a precursor or substrate to produce the following metabolites in the pathway. Therefore, metabolite concentration in the CLP extract treatment showed a lower level than those found in the control group (Figures 7 and 8), indicating the higher activity of metabolism induction responding to stress for plant growth promotion.

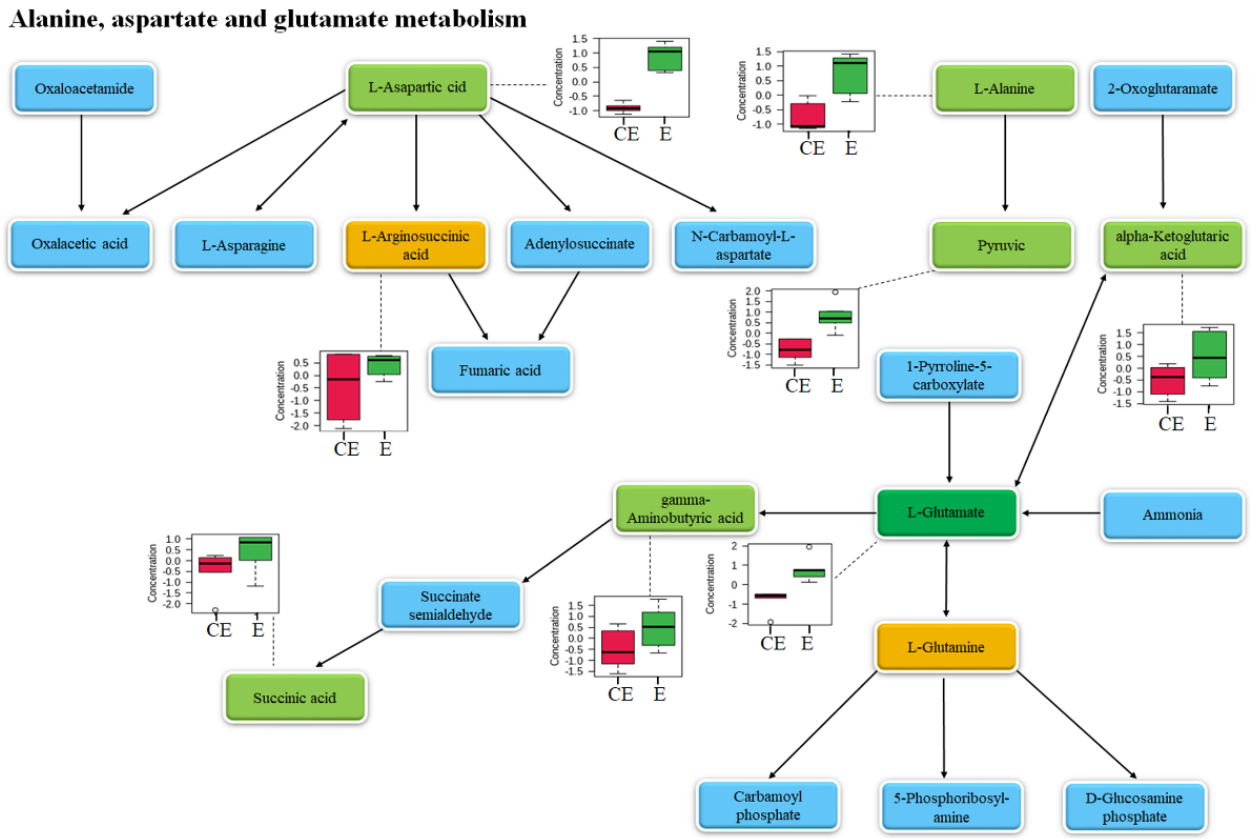


Figure 7. Metabolic pathway of alanine, aspartate and glutamate metabolism. The metabolite in the dark green box represents the positive ID that matched both the mzCloud and ChemSpider compound databases. The metabolite in the light green box represents the positive ID that matched the mzCloud or ChemSpider compound database. The orange box represents the putative ID, while the blue box represents no ID. The box plots of identified metabolites are displayed beside the corresponding metabolites. CE, cyclic lipopeptide extract group; E, ethanol.

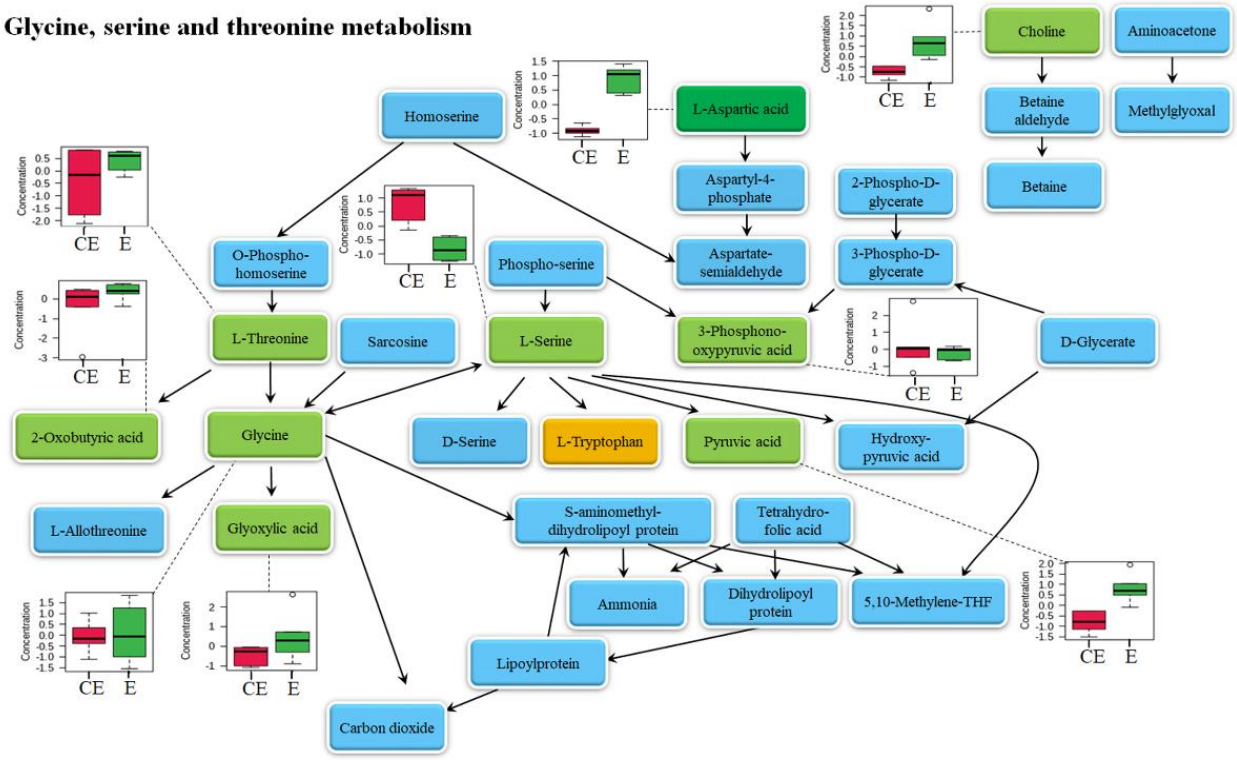


Figure 8. Metabolic pathway of glycine, serine and threonine metabolism. The metabolite in the dark green box represents the positive ID that matched both the mzCloud and ChemSpider compound databases. The metabolite in the light green box represents the positive ID that matched the mzCloud or ChemSpider compound database. The orange box represents the putative ID, while the blue box represents no ID. The box plots of identified metabolites are displayed beside the corresponding metabolites. CE, cyclic lipopeptide extract group; E, ethanol.

Validation of the effects of *Bacillus* CLP extracts on plant growth promotion was executed using a metabolomic strategy compared with exogenous SA, which is a phytohormone agent. To provide a better understanding of metabolic changes under stress conditions in plants, metabolomic analyses have been conducted, particularly in the mechanism of plant growth promotion [30, 53]. The metabolites in the categories of amino acids and derivatives, polyamines, fatty acids, and sugars and derivatives were reported to be involved in plant growth promotion induced by endophytic bacterial application [53]. During heat stress, exogenous SA displayed the ability to increase the abundance of flavonoids, polyketones, terpenoids, and polysaccharides for metabolic responses [54]. The exogenous SA treatment was also shown to affect the induction of defensive genes and proteins in para rubber seedlings against *P. palmivora* [9]. However, the manner of *Bacillus* CLP extracts on the metabolic response to stress in the leaves of para rubber seedlings for plant growth promotion mechanisms was still unclear.

In this study, metabolomic analysis was executed to compare the metabolic responses to stress by the applications of CLP extracts, exogenous SA, and ethanol (control) on metabolic changes leading to plant growth promotion. The metabolome data presented the greatest up-regulated metabolic changes in leaves treated with CLP extracts. Thus, it could be considered a potent agent to robustly induce metabolic changes responding to stress. This work found that the metabolites triggered by CLP extract treatment were related to 13 major pathways, including alanine, aspartate, and glutamate metabolism, as well as glycine, serine, and threonine metabolism. Previous work reported that the metabolism of alanine, aspartate, and glutamate and glycine, serine, and threonine metabolism were the two major pathways related to *Bacillus* CLP production [55].

Pathway analysis revealed the action of CLP extracts on the induction of alanine, aspartate, and glutamate metabolism as a major pathway with the highest impact score. There were nine identified metabolites found in this study, including L-glutamate, gamma-aminobutyric acid, succinic acid, L-aspartic acid, L-alanine, pyruvic acid, alpha-ketoglutaric acid, L-arginosuccinic acid, and L-glutamine of alanine, aspartate, and glutamate metabolism.

Glutamic acid was presented to play an important role in the promotion of primary callus induction, embryogenic callus formation, and callus status improvement for plant growth promotion in the halophyte *Leymus chinensis* [56]. Gamma-aminobutyric acid, or GABA, has been reported as a property to function in gated channels for plant stress tolerance [57]. Succinic acid and alpha-ketoglutaric acid, members of the tricarboxylic acid cycle coupled with pyruvic acid, are involved in energy metabolism for improved photosynthesis and plant development [58]. L-arginosuccinic acid is a derivative of L-aspartic acid, which is concerned as a central precursor for protein constituents and plays an important role in the biosynthesis of other amino acids involved in plant growth and the immune system during changes in surroundings [59]. Alanine was found to be an accumulated substance responding to stress like abiotic and biotic stresses, resulting in plant prevention due to changing environmental conditions [60]. L-glutamine is an important nitrogen source for plant growth. It was reported to have the function of providing growth for rice seedlings. Moreover, it also activates the expression of responsive genes related to the regulation of nitrogen metabolism and stress responses [61].

Furthermore, glycine, serine, and threonine metabolisms were also triggered by the treatment with CLP extracts. This was similar to an earlier study that described the action of CLP extracts on the elicitation of glycine, serine, and threonine metabolism in mandarin fruit during stress [29]. There were nine identified metabolites such as L-aspartic acid, 3-phosphonooxypyruvic acid, pyruvic acid, L-serine, glycine, L-threonine, 2-oxobutyric acid, choline, glyoxylate, and L-tryptophan found in glycine, serine, and threonine metabolism. The 3-phosphonooxypyruvic acid, a synonym of phosphohydroxypyruvic acid, was present as a property of early intermediates in photosynthetic carbon fixation [62]. L-serine acts primarily in plant metabolism and development by functioning indirectly in purine and pyrimidine biosynthesis, including in stress responses caused by abiotic and biotic conditions [63]. Glycine was described as a booster of nutrition uptake in leaves, such as nitrogen, calcium, potassium, phosphorus, iron, and zinc, which are vital elements for plant growth promotion [64]. L-threonine demonstrated the characteristic of improving abiotic stress tolerance, including plant growth and development [65]. The 2-oxobutyric acid, a synonym of 2-ketobutyrate, is a member of the threonine-degradation products. It can be converted into succinyl-CoA through propionyl-CoA catabolism to act as an intermediate in the tricarboxylic acid cycle, resulting in energy production [66]. Choline is a crucial metabolite in plants because it is a substrate in the synthesis of membrane phospholipid phosphatidylcholine and is a precursor used to produce glycine betaine, which contains strong osmoprotectant manners responding to stresses [67, 68]. Glyoxylate, which plays a vital role in the glyoxylate pathway serving energy production, was found to be accumulated in the roots of the halophyte *Mesembryanthemum crystallinum* treated by the endophytic bacteria application for plant growth promotion [69]. L-tryptophan, which is one of the essential plant growth regulators affecting physiological plant growth and development, was demonstrated to improve the vegetative and reproductive growth of crops by increasing the pod weight of chickpeas under rain-fed conditions [70]. It was indicated that those identified metabolites triggered by CLP extracts were linked as a network system to function in the leaves of para rubber seedlings, especially in alanine, aspartate, and glutamate metabolism and glycine, serine, and threonine metabolism for stress responses, leading to plant growth and development.

In this study, CLP extracts obtained from *B. subtilis* PTKU12 and exogenous SA influenced several metabolites, especially primary metabolites, to stress responses in the leaves of para rubber seedlings. Individual amino acids and their derivatives can be precursors to synthesize the accumulation of primary and secondary metabolites functioning in various pathways. The changed abundance of some amino acids could affect metabolic mechanisms such as plant growth promotion in host plants [29, 30, 53]. For example, tryptophan is a precursor to synthesizing indole-3-acetic acid, which is increased by the endophytic bacteria application in cucumber when compared with the control treatment, affecting the production of indole-3-acetic acid and resulting in an abundance of extracellular enzymes such as cellulase, lipase, and protease [30]. Thus, amino acids are one of the most changed metabolite categories in various treatments.

According to the aims of this study, *Bacillus* CLP extracts were selected as potent agents for plant growth promotion *in vivo* after a test for the antifungal activity of *Bacillus* CLP extracts was performed against *P. palmivora in vitro*. Then, *Bacillus* CLP extracts and exogenous SA were each applied to the leaves of para rubber seedlings to investigate the metabolic changes to stress responses. Metabolomics was used for the comparative analysis of metabolic changes between *Bacillus* CLP extracts and exogenous SA because metabolomics is a powerful method that can identify numerous metabolites simultaneously at the metabolic level. Untargeted metabolomics analysis presented the various metabolites triggered by *Bacillus* CLP extracts and exogenous SA. Because each metabolite plays an important role in many biological pathways, the determination of pathways that involve metabolite regulation in plant growth promotion and stress responses by CLP extracts and exogenous SA is challenging. The statistical analysis of metabolic results exhibited that *Bacillus* CLP extracts could trigger metabolite changes in stress responses better than exogenous SA in para rubber seedling leaves. This might happen because *Bacillus* CLP extracts contain the compounds fengycins, iturins, and surfactins, which might have more ability, such as synergistic action, to activate metabolic systems; exogenous SA does not contain any compounds like *Bacillus* CLP extracts. Thus, *Bacillus* CLP extracts might have more opportunities to interact with the various receptors located in the cell membrane of para rubber seedlings leaves when compared with exogenous SA. However, the signal transduction of *Bacillus* CLP extracts' action on the induction of metabolic pathways involved in plant growth promotion should be investigated and confirmed in further experiments. Pathway analysis coupled with statistical analysis revealed that *Bacillus* CLP extracts triggered the metabolism of alanine, aspartate, and glutamate as a major pathway in the leaves of para rubber seedlings to adapt both developmental and metabolic processes for prevailing stress conditions, resulting in plant growth promotion. From the results, it could be suggested that the CLP extract obtained from *B. subtilis* PTKU12 could be an alternative agent for the protection of para rubber seedlings responding to stress, leading to plant growth promotion.

4- Conclusion

Plant growth promotion by environmentally friendly agents is required under the variant environmental conditions. The present *in vitro* study exhibited the antifungal potential of the CLP extracts obtained from *B. subtilis* PTKU12. *Bacillus* CLP extracts limit the growth rate of *P. palmivora*, which causes leaf fall disease in para rubber trees, resulting in the biotic stress condition. According to CLP extracts' antifungal properties, *Bacillus* CLP extracts could be selected as an alternative agent for reducing the biotic stress factor that affects plant growth promotion. To provide more evidence for supporting *Bacillus* CLP extracts as a potential agent of plant growth promotion, *Bacillus* CLP extracts were used to investigate the stress responses at a metabolic level compared with exogenous plant hormones in the leaves of para rubber seedlings. The metabolome differences of leaves treated with CLP extracts and exogenous SA were profiled using untargeted metabolomic analysis to determine the induction properties of metabolic changes between both agents. *In vivo* studies revealed a higher effect of CLP extracts obtained from *B. subtilis* PTKU12 on metabolite changes than the exogenous plant hormone in the leaves of para rubber seedlings. During stress conditions, the positively identified metabolites in the treatment of CLP extracts were matched to the metabolism of alanine, aspartate, and glutamate as a major pathway, which played an important role in signal transductions leading to plant growth promotion. In a future study, the therapeutic effect of *Bacillus* CLPs during biotic stress, such as *P. palmivora* infection, should be investigated. Therefore, this study provided a protective approach using *Bacillus* CLP extracts from the aspect of metabolic changes as a plant growth promoter for para rubber plantations.

5- Declarations

5-1-Author Contributions

Conceptualization, P.T. and W.V.; methodology, P.T., S.R., and W.V.; software, P.T., P.K., and J.J.; validation, P.T., A.S., A.K., S.R., and W.V.; formal analysis, P.T., P.K., and J.J.; investigation, P.T. and P.K.; resources, P.K. and A.K.; data curation, P.T., P.K., and J.J.; writing—original draft preparation, P.T. and P.K.; writing—review and editing, P.T., A.S., A.K., S.R., and W.V.; visualization, P.T. and P.K.; supervision, W.V.; project administration, P.T.; funding acquisition, P.T. All authors have read and agreed to the published version of the manuscript.

5-2- Data Availability Statement

Data available in a publicly accessible repository: Sanger sequence of *B. subtilis* PTKU12 was submitted to GenBank at NCBI under submission ID: SUB11923773 (unpublished accession number: OP214768). The supplementary data presented in this study are freely available in FigShare at https://doi.org/10.6084/m9.figshare.21295479.

5-3- Funding

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5-4- Acknowledgements

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5-5- Institutional Review Board Statement

Not applicable.

5-6- Informed Consent Statement

Not applicable.

5-7- Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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